Constitutive Expression of the Maltose Fermentative Enzymes in Saccharomyces carlsbergensis Is Dependent upon the Mutational Activation of a Nonessential Homolog of MAL63

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Maltose fermentation in Saccharomyces carlsbergensis is dependent upon the MAL6 locus. This complex locus is composed of the MAL61 and MAL62 genes, which encode maltose permease and maltase, respectively, and a third gene, MAL63, which codes for a trans-acting positive regulatory product. In wild-type strains, expression of the MAL61 and MAL62 mRNAs and proteins is induced by maltose and induction is dependent upon the MAL63 gene. Mutants constitutively expressing the MAL61 and MAL62 gene products have been isolated in mal63 backgrounds, and the mutations which have been analyzed map to a fourth MAL6-linked gene, MAL64. Cloning and characterization of this new gene are described in this report. The results revealed that the MAL64-C alleles present in constitutive strains encode a trans-acting positive function required for constitutive expression of the MAL61 and MAL62 gene products. In inducible strains, the MAL64 gene is dispensable, as deletion of the gene had no effect on maltose fermentation or maltose-regulated induction. MAL64 encoded transcripts of 2.0 and 1.4 kilobase pairs. While both MAL64 mRNAs were constitutively expressed in constitutive strains, they were maltose inducible in wild-type strains and induction was dependent upon the MAL63 gene. The MAL63 and MAL64 genes are at least partially structurally homologous, suggesting that they control MAL61 and MAL62 transcript accumulation by similar mechanisms.

Maltose fermentation in Saccharomyces spp. requires the presence of any one of a series of five, unlinked, dominant MAL loci (MAL1, MAL2, MAL3, MAL4, or MAL6) which confer the ability to synthesize high levels of the two maltose fermentative enzymes maltase and maltose permease (reviewed in reference 1).1 In most maltose-fermenting strains, expression of maltase and maltose permease is induced by the presence of maltose in the growth medium (7, 9, 26, 28). The MAL6 locus has been studied intensively at both the genetic (26; Chang et al., submitted for publication) and molecular (8, 19) levels and has been shown to be a complex locus composed of three genes: MAL61 encodes a component of the maltose permease (8, 13); MAL62 encodes maltase (8, 10, 15); and MAL63 encodes a trans-acting positive regulatory function required for expression of the two maltose fermentative enzymes (8; Chang, Dubin, Miller, and Needleman, submitted). Mutations within MAL63, including a deletion disruption, lead to maltose-nongerminating strains that fail to induce for the maltose fermentative enzymes and their transcripts (11, 26; Chang et al., submitted). Reversion of these mal63 mutants to maltose fermenters generally results in strains that constitutively express maltase and maltose permease, and the gene responsible for reversion/constitutivity is linked to MAL6 (25, 27). Analysis of heterozygous diploids carrying the uninducible and constitutive alleles led ten Berge and co-workers (25) to conclude that the gene responsible for constitutive expression of the maltose fermentative enzymes was an allele of the regulatory gene required for their inducible expression, that is, MAL63. We have recently reinvestigated the genetic basis behind constitutivity in two MAL6-linked constitutive strains and demonstrated that constitutive expression of maltase and maltose permease in these strains is dependent upon a trans-acting function encoded by a previously unidentified MAL6-linked gene located 2.3 centimorgans to the left of MAL63 (11). We refer to this gene as MAL64.

In this report we extend our studies of the MAL64 gene. The isolation and localization of the MAL64 gene are described, and this gene is shown to exhibit at least partial structural homology to MAL63. We further demonstrate through gene disruption experiments that, in MAL6-linked constitutive strains, the MAL64 allele MAL64-C encodes a trans-acting positive regulatory function that is required for constitutive expression of maltase and maltose permease and their respective transcripts. In contrast, the wild-type MAL64 is not essential for maltose fermentation or maltose-regulated induction in inducible strains.

MATERIALS AND METHODS

Strains and growth conditions. The yeast strains used in this study are given in Table 1. Plasmids were propagated in Escherichia coli RR1. Yeast cells were grown on YEP (1% [wt/vol] yeast extract, 1% [wt/vol] peptone) plus various amounts of the specified carbon source at 30°C. Maltose fermentation is defined by growth and the production of gas 1 to 5 days following inoculation and was as described previously (10). Strain A9 (11; Chang et al., submitted) is a derivative of strain 332-5A, except it contains a deletion of the MAL63 gene and is thus an uninducible nonfermenter; the MAL63 gene was deleted in A9 with the concomitant insertion of the URA3 gene, using disruption plasmid pDM3 (5). Strain R10 was isolated as a maltose-fermenting rever-
tand of A9 and constitutively expresses both maltose fermentative enzymes (11). Strain R10u is a ura3 derivative of strain R10 and was selected by using 5-fluoro-orthic acid-containing medium as described by Boeke et al. (2). Constitutive strain C2 (27) was isolated as a maltose-fermenting revertant of the inducible strain mal6-13 (26) and is genotypically mal63-13 MAL64-C2. Constitutive strain 8-2B was derived from C2 and the derivation is presented in Dubin et al. (11). All strains used in this study carry in their background a partially functional allele of the MAL1 locus which has been shown to contain only a functional MAL12 gene encoding maltase (5, 11).

**Isolation of MAL64 DNA sequences and plasmid constructs.** DNA sequences containing the MAL64 and MAL64-C2 genes as well as flanking regions were cloned by targeted integration followed by plasmid rescue, by the method of Orr-Weaver et al. (22). Plasmids pMJC6ACla and pY6RAC (Fig. 1) were constructed with wild-type MAL6 DNA iso- lated from strain CB11 and subcloned into YIp5 that contained a deleted ClaI site. A detailed description of plasmid pY6RAC is given in Charron and Michels (6). ClaI-restricted pMJC6ACla was transformed into the inducible MAL6 strain 332-5A, integration being targeted to MAL6, and plasmid pBam11 (Fig. 1) was recovered from an appropriate transformant by plasmid rescue, using BamHI-restricted total genomic DNA (22). Similarly, ClaI-restricted pMJC6ACla and BglII-restricted pY6RAC were used to isolate plasmids pBamC2 and pB3C2 (Fig. 2), respectively, following integration at the MAL6-C2 locus in constitutive strain 8-2B and then BamHI digestion of genomic DNA isolated from reconstituted transformant. In each of the above isolations, integration at the correct genomic site was confirmed by genetic and physical analyses of the transformed strains. Plasmid pBSC2 (Fig. 2) was constructed by subcloning the 14.5-kilobase pair (kbp) BamHI-Sall fragment from pB3C2 (Fig. 2) into pBR325. Plasmids pBam11 (2μ) and pBamC2(2μ) (Fig. 1 and 2) are episomal derivatives of pBam11 and pBamC2, respectively, and were constructed by deleting their 1-kbp BamHI-Xhol fragments and replacing them with the BamHI-Xhol fragment of YEp13 (3) containing the yeast LEU2 gene and a portion of the 2μ circle. Plasmid pBamC2ΔXba(2μ) (Fig. 2) is identical to pBamC2(2μ), except it contains a deletion of two XbaI fragments (1.3 and 0.5 kbp) within the MAL64-C2 gene. Plasmids pMAL64 and pMAL64C2 contain ClaI fragments derived from pBam11 and pBamC2, respectively, subcloned into YEp5. Plasmid pMAL64C2ΔXba is identical to pMAL64C2, except it contains a deletion of the two XbaI fragments within MAL64-C2. Plasmid pDF1 contains the wild-type MAL63 gene in pLC544 (19) and was originally isolated from YEpMAL6 (12).

Disruption plasmid pDMF (Fig. 2) was constructed by restricting plasmid pBSC2 with BglII, deleting four BglII fragments, and replacing them with the 2.7-kbp BglII fragment of pCV9 containing the LEU2 gene. Disruption plasmids pDMG and pDMH (Fig. 1 and 2) were constructed by first subcloning the 2.4-kbp EcoRI fragment from pBam11 or pBamC2, respectively, into pBR325ΔB-H (a derivative of pBR325 that contains a deletion of the 346 base pairs between the BamHI and HindIII sites) followed by insertion of the 1.1-kb HindIII fragment containing the URA3 gene from YEp31 into the sole HindIII site within the yeast insert. Enzyme activity. Maltase and maltose permease activities were determined as previously described (11).

**Yeast transformations.** Yeast transformations were done by the method of Ito et al. (16), using LiAc. One-step gene disruptions were done by the method of Rothstein (24).

**Gel transfer analysis of DNA and RNA.** DNA and RNA isolation and Southern and Northern (RNA) blot analyses were performed as previously described (10, 19). RNA integrity was confirmed by reprobing Northern blots with the yeast actin gene (20) or the yeast Ty1 element S13 (4).

**RESULTS**

Cloning the MAL64 and MAL64-C2 genes. We have shown that the MAL64 gene is located 2.3 centimorgans to the left of the MAL63 gene (11). To determine the functions encoded by MAL64 and its constitutive allele MAL64-C, we cloned and disrupted this chromosomal region. Nearly 12 kbp of DNA to the left of MAL63 was cloned from inducible and constitutive MAL6 strains by targeted integration of plasmid pMJC6ACla (Fig. 1) followed by plasmid rescue as described in Materials and Methods. Plasmids pBam11 and pBamC2 (Fig. 1 and 2, respectively) contain the identical 12 kbp of MAL6 DNA, except the former was isolated from the inducible MAL6 strain 332-5A (MAL64 MAL63) while the latter was isolated from the constitutive MAL6-C2 locus of constitutive strain 8-2B (MAL64-C2 mal63-13). Plasmid pB3C2 (Fig. 2) contains an even larger fragment isolated
from constitutive strain 8-2B and was similarly cloned by using plasmid pY6RAC (see Materials and Methods; Fig. 1). Restriction endonuclease mapping of these plasmids revealed few restriction site polymorphisms between the MAL6 and MAL6-C2 loci (Fig. 1 and 2). These site differences appear to represent polymorphisms among the MAL6 loci of the S. carlsbergensis strains used by ten Berge et al. (26, 27). The significance of these polymorphisms, if any, remains undetermined. However, it should be noted that loss of the EcoRI site within the MAL6 allele present at the MAL6-C2 locus may define the original mal6-C2 mutation described by ten Berge et al. (26).

**MAL64-C** encodes a positive trans-acting function required for constitutive expression of the maltose fermentative enzymes. To establish the location and function of MAL64, several disruption plasmids were constructed and used to transform inducible and constitutive MAL6 strains. The results of three disruption experiments are presented below.

Plasmid pDMF (Fig. 2) was constructed with DNA isolated from the MAL6-C2 locus of strain 8-2B and contained an 11.5-kbp deletion of the region to the left of the mutant MAL6 gene found in that strain, replacing it with the LEU2 gene. DNA sequences isolated from the constitutive locus were used to avoid the possibility of gene conversion of MAL6-C2 to MAL6 by flanking sequences during transformation. PstI-BamHI-restricted pDMF was used to transform constitutive strain 8-2B to Leu*, and nearly 50% of the transformants were maltose nonfermenters. The predicted disruption was confirmed physically for two Mal- isolates, strains 8-2BAF-5-20 and 8-2BAF-5-21, and this was further confirmed genetically in the latter strain (results not shown). Unlike the parental strain 8-2B, transformants containing this deletion no longer constitutively synthesized maltase or maltose permease and, as expected, also failed to respond to maltose (8-2BAF-5-20 and 8-2BAF-5-21, Table 2). The Northern blot analysis of RNA extracted from strain 8-2BAF-5-1 revealed that the 2.0-kilobase (kb) MAL6 homologous mRNA encoding maltose permease and the 1.9-kb MAL6 homologous mRNA encoding maltase were no longer constitutively expressed, as they are in strain 8-2B; the 2.4-kb MAL6 homologous RNA, while still detectable, also exhibited reduced levels (Fig. 3). This contrasts to a lack of effect on MAL61 and MAL62 expression when the MAL63 gene (that is, mal63-13) is deleted from strain 8-2B (strain 8-2BA63-1 in Fig. 3 of reference 11).

To demonstrate that MAL64-C2 had indeed been disrupted and to further localize the gene, the following experiments were performed. Strain 8-2BAF-5-21 was transformed with plasmid pBam11(2μ), which restores nearly all of the deleted sequences with sequences derived from the wild-type MAL6 locus, or with pBamC2(2μ), a plasmid containing the same sequences only isolated from the MAL6-C2 locus (Fig. 1 and 2). Plasmid pBamC2(2μ) restored to strain 8-2BAF-5-21 the ability to ferment maltose, while pBam11(2μ) did not, suggesting that MAL64-C2 lay within the 11-kbp XhoI-EcoRI fragment. Preliminary results from
FIG. 2. MAL6-C2 locus from constitutive strain 8-2B and plasmids containing MAL6-C2 sequences. A partial restriction map of the MAL6-C2 locus, the locations and directions of transcription of the MAL61, MAL62, and mal63-13 genes, and approximate location of the MAL64-C2 gene is presented. Restriction site polymorphisms between the MAL6 and MAL6-C2 loci (Fig. 1) are indicated by (●). Plasmids pB3C2 and pBamC2 were isolated from the MAL6-C2 locus of strain 8-2B by targeted integration/plasmid rescue, using pY6RAC and pMJ66C1A (Fig. 1), respectively. The symbol • represents wild-type MAL6 sequences from pY6RAC and pMJ66C1A covalently linked to MAL6-C2 sequences as a result of cloning. Plasmids pB3C2 and pDMF were derived from pB3C2. Plasmids pBamC2(2μ) and pBamC2ΔXba(2μ) were constructed by the addition to pBamC2 of a segment of 2μ circle. Plasmid pMAL64C2 and its derivative, pMAL64C2ΔXba, contain the MAL64-C2 gene subcloned into YIp5. Disruption plasmids pDMF and pDMH were used in one-step gene disruptions following restriction at the indicated sites (†). Restriction endonuclease site abbreviations are given in the legend to Fig. 1.

subsequent gene disruption/transplacement experiments in which deletion of the Xhol-SstI fragment (Fig. 2) in strain 8-2B had no effect on the ability to ferment (data not shown) suggested that MAL64-C2 lay in a region more proximal to the MAL63 gene. The MAL64-C2 gene was first localized by the demonstration that plasmid pBamC2ΔXba(2μ) (Fig. 2), when transformed into strain 8-2BΔF-5-21, failed to restore the ability to ferment maltose. More importantly, MAL64-C2

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* Maltase activity was determined as the rate of release of p-nitrophenol from p-nitrophenol-o-D-glucopyranoside and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C. U. Noninducing growth conditions (YEP plus 2% galactose); I. Inducing growth conditions (YEP plus 2% galactose).

* Maltose permease activity is expressed as nanomoles of [14C]maltose transported in 1 minute per milligram (dry weight) of cells.

* ND, Not determined.
complementing activity was localized to a 3.4-kbp Clal fragment. Plasmids pMAL64 (constructed by using wild-type MAL6 DNA; Fig. 1) and pMAL64C2 and pMAL64C2ΔXba (constructed by using MAL6-C2 DNA; Fig. 2) were constructed and integrated into strain 8-2BAF-5-21 at the URA3 locus by restriction with NcoI to transform. Single-copy transformants were selected and examined further. Only plasmid pMAL64C2 restored the abilities to both ferment maltose and synthesize maltase constitutively (Table 2).

Disruption plasmid pDMH allows us to identify the HindIII site within the 3.4-kbp Clal fragment as lying within the MAL64 gene. Plasmid pDMH was constructed by using a 2.4-kbp EcoRI fragment derived from the cloned MAL6-C2 constitutive locus (Fig. 2) and contains an insertion of the URA3 gene in the indicated HindIII site. Transformation of strain 8-2B with EcoRI-restricted pDMH resulted in nonfermenting transformants unable to constitutively synthesize maltase (strain 8-2BΔH-1, Table 2). Transformation of strain 8-2BΔH-1 with plasmid pBamC2(2μ) restored the ability to ferment maltose, while plasmids pBam11(2μ) and pBamC2(2Xba1(2μ) did not. Clearly, the HindIII site within the 3.4-kbp Clal fragment lies within the MAL64-C2 gene.

The same region within another constitutive strain, R10, is also involved in constitutive expression of the MAL61 and MAL62 genes. Strain R10 was derived as a maltose-fermenting revertant of the uninducible mal63Δ strain A9 and constitutively expresses the two maltose fermentative enzymes (11) and their transcripts (Fig. 3). The gene responsible for constitutivity in R10 was linked to MAL6 and was shown to lie within the MAL64 allele MAL64C-R10 (11). Strain R10u is a 5-fluoro-orotic acid-derived ura3 revertant of strain R10 (see Materials and Methods) and, like strain R10, constitutively synthesizes maltase and maltose permease (Table 2) and the MAL62 and MAL61 transcripts (Fig. 3); it still contains the MAL63 deletion (results not shown). To determine whether the MAL64C-R10 allele in strain R10 was also located about 3.5 kbp to the left of MAL63 and functioned in a manner similar to the MAL64-C2 allele, this region was disrupted in strain R10u. PstI-BamHI-digested pDMF was used to transform strain R10u to Leu+, and 37% of the transformants were maltose nonfermenters. Physical analysis of two nonfermenters confirmed disruption at MAL6 (as well as the continued absence of MAL63), and disruption was further confirmed genetically for one isolate, strain R10uΔF-9-26 (results not shown). Unlike the constitutive parental strain, strain R10uΔF-9-26 was unable to constitutively synthesize maltase or maltose permease (Table 2) and, as expected, due to the absence of MAL63, this strain also failed to induce for maltase (Table 2). Northern analysis of RNA isolated from strain R10uΔF-9-26 confirmed that the maltose and maltose permease mRNAs were no longer constitutively expressed (Fig. 3). Following integrative transformation, this strain was not complemented by the wild-type MAL64 gene (plasmid pMAL64) but was complemented by the MAL64-C2 gene (pMAL64C2), which restored the ability to ferment maltose and constitutively express maltase (Table 2); as expected, plasmid pMAL64ΔXba also failed to complement. Plasmid pDMG (Fig. 1) was used to localize MAL64C-R10 to within the same 2.4-kbp EcoRI fragment as the MAL64-C2 gene. EcoRI-restricted pDMG was used to transform strain R10u to Ura+, and approximately 90% of the transformants were nonfermenters. Physical analysis of the MAL- isolates confirmed disruption of the MAL66 (results not shown). Strains containing this disruption no longer constitutively synthesized maltase or maltose permease (R10uΔG-1, Table 2). Since plasmid pDMG was constructed with wild-type MAL6-derived sequences, it is uncertain whether MAL64C-R10 was disrupted or gene converted by this transformation or both. While the definitive experiment awaits the cloning of this region from strain R10, it is almost certain that MAL64C-R10 and MAL64-C2 are alleles.

These results clearly demonstrate that the MAL64-C gene encodes a positive trans-acting product required for constitutive expression of the maltose fermentative enzymes and their transcripts in these two MAL6-linked constitutive strains. That the MAL64 gene product is not a negative regulator is further supported by the results obtained when the MAL64 gene was disrupted in the uninducible mal63-10 MAL64 strain 348-1B (Table 1); both the parental mal63-10 MAL64 strain and a transformant containing a deletion/disruption of the MAL64 gene were uninducible nonfermenters (results not shown).

MAL64 is not required for inducible maltose fermentation. In inducible strains, maltose-regulated induction of the MAL61 and MAL62 genes is dependent upon the MAL63 gene (Chang et al., submitted). Deletion of MAL63 in inducible strain 332-5A results in nonfermenters that fail to induce for the MAL61 or MAL62 transcripts (strain A9, Fig. 3) or proteins (Table 5 in reference 11). In constitutive strains, constitutive expression of the MAL61 and MAL62 gene products does not require the MAL63 gene (11) but is dependent upon an intact MAL64-C gene. We investigated the role of the wild-type MAL64 gene in inducible MAL63 MAL64 strains by disrupting this gene in inducible strain...
restricted DNA isolated from the MAL6 mal" strain 332-5A was probed with the MAL63-specific probe pH-3; three homologous fragments were detected (Fig. 4A). The strongest signal with homology to this probe is a 0.9-kbp fragment corresponding to MAL63. Strain A9 contains a deletion of MAL63 and lacks the 0.9-kbp EcoRI fragment (Fig. 4A). The two slightly weaker signals are due to cross-hybridization to 2.4- and 5.6-kbp EcoRI fragments. The 5.6-kbp fragment is unlinked to MAL6 (unpublished observations) and does not appear to derive from the mal10 locus present in this strain (M. J. Charron and C. A. Michels, submitted for publication). Its genomic location has not been determined. However, the 2.4-kbp fragment is linked to MAL6. Quite unexpectedly, DNA isolated from MAL64 disruption strains and probed with the MAL63-derived probe pH-3 revealed changes in the 2.4-kbp MAL63 homologous EcoRI fragment. For example, MAL64 deletion/disruption strain 332-5AΔF-1-5 lacked the 2.4-kbp fragment (Fig. 4A). Similar Southern analyses of other MAL64 disruption strains confirmed that the 2.4-kbp MAL63 homologous EcoRI fragment was identical to the 2.4-kbp MAL64-containing EcoRI fragment. Direct demonstration of homology between MAL64 and its flanking sequences and MAL63 and its flanking sequences was also obtained by gel transfer analysis of the cloned fragments, using probes pH-2, pH-3, pG, pE-1, pE-4, and pE-2. The results presented in Fig. 4B illustrate the extent of the homology. Two plasmids, YEPMAL6 and pBam11, which span the entire 22-kbp MAL6 region, were digested to release the EcoRI fragments in their yeast inserts and were hybridized to probes from the MAL6 region. Comparisons, particularly within a lane, give some measure of the degree of sequence homology. The results of these and other similar analyses are summarized in Fig. 1, where the regions showing homology to each of the probes are indicated as dashed lines above the restriction map of the MAL64 region. The simplest interpretation of this homology is that this region represents a tandem duplication of the MAL63 gene and flanking sequences extending into the MAL61 gene. It should be noted that no homology to the pE-1 probe was found. This indicates that some rearrangement occurred during the duplication event. From these results it is concluded that the MAL63 and MAL64 genes are structurally homologous, but the degree of homology remains undetermined.

The MAL64 mRNAs. In an effort to characterize the MAL64 transcript(s), plasmid pZ-1 (Fig. 1) was used to probe Northern blots containing RNA isolated from various strains (Fig. 5). The wild-type MAL6 strain (MAL63 MAL64), when grown under uninducing conditions, expresses low levels of a 2.0-kb transcript. Growth in the presence of maltose results in increased levels of expression of the 2.0-kb RNA and in the appearance of a 1.4-kb RNA. When the MAL64 region is deleted (MAL63 mal64Δ), both transcripts fail to be detected. Synthesis of the 2.0- and 1.4-kb maltose-inducible transcripts is dependent upon the MAL63 gene product since no MAL64 homologous transcripts are detected in the MAL63 deletion/disruption strain (mal63Δ MAL64) grown on either galactose (not shown) or maltose. Constitutive strain R10u (mal63Δ MAL64-R10) constitutively expresses high levels of the 1.4- and 2.0-kb RNA species, and deletion of MAL64C-R10 in this strain results in loss of constitutive synthesis of both transcripts (Fig. 5). A second constitutive strain, S.2BΔ3-1 (mal63Δ MAL64-2) also constitutively expresses high levels of the 1.4- and 2.0-kb transcripts; neither transcript is detected when the MAL64C-2 gene is deleted (strain S.2BΔ3-2, Fig. 5). These results indicate that the 2.0- and 1.4-kb
transcripts are products of the *MAL64* gene. In inducible strains accumulation of *MAL64* transcripts is induced by maltose and dependent upon *MAL63*. In constitutive mutants, the *MAL64*-C transcripts are constitutively expressed, suggesting that the expression of *MAL64* is autoregulated in these strains.

Plasmid pC-1 contains the 2.7-kbp EcoRI-BglII fragment adjacent and to the right of fragment Z-1 (Fig. 1). We believe that *MAL64* extends into this fragment since pC-1 also detects a 1.4- and a 2.0-kb transcript constitutively expressed in strains carrying the constitutive mutation *MAL64*-C-R10 (results not shown).

Two *MAL63* homologous transcripts are detected in inducible *MAL6* strains, a constitutive 1.6-kb transcript and a slightly maltose-inducible 2.0-kb transcript (Fig. 6) (19). Deletion of the *MAL63* gene eliminates the 1.6-kb transcript but only reduces the level of the 2.0-kb transcript (Fig. 6) (Chang et al., submitted). (The novel 1.5-kb transcript present in strain A9 may represent a *MAL63*-URA3 hybrid transcript synthesized from the *mal63*-URA3 mutation.)

This indicates that this 2.0-kb species of transcript is, at least in part, derived from a gene other than *MAL63*. This was further supported by our finding that the *MAL13* gene of the *MAL1* locus, which is functionally and structurally homologous to the *MAL63* gene, is transcribed into a single 1.6-kb mRNA (5). The homology between *MAL63* and *MAL64* demonstrated in this report along with the finding that *MAL64* encodes a maltose-inducible 2.0-kb transcript raised the possibility that the 2.0-kb transcript detected by *MAL63*-derived probes was actually the product of the *MAL64* gene. Our results do not entirely support this hypothesis. Apparently normal levels of both the 2.0- and 1.6-kb *MAL63* homologous transcripts are present in poly(A)* RNA from strain 332-5ADF-1-5, which contains an undisrupted *MAL63* gene and a deletion of the *MAL64* gene (Fig. 6); when probed with the *MAL64*-derived probe (pZ-1), no homologous transcripts are detected (Fig. 5). (The stringency of hybridization in the Northern analyses is higher than that used for the Southern analyses.) Taken together, these results suggest that the 2.0-kb transcript is the product of some third gene having greater homology to *MAL63* than *MAL64* (perhaps encoded by the 5.6-kbp EcoRI fragment detected by pH-3) whose expression is dependent upon the *MAL63* gene product. Our results do not exclude the possibility that this 2.0-kb transcript is a mixture of transcripts encoded by *MAL63*, *MAL64*, and other homologous sequences. Final resolution of this question requires the deletion/disruption of the 5.6-kbp homologous sequence.

**DISCUSSION**

In this report, we demonstrate that *MAL64*, a *MAL6*-linked gene involved in constitutive expression of the normally inducible maltose fermentative enzymes (11, 25, 27), is located nearly 3.5 kbp to the left of the *MAL63* gene and is partially homologous to *MAL63*. The *MAL64*-C allele of this gene, present in constitutive strains, encodes a positive trans-acting function required for constitutive expression of maltase and maltose permease and their respective transcripts in the two *MAL6*-linked constitutive strains examined. Deletion of the *MAL64*-C gene in these constitutive...
Strains results in nonfermenters that no longer constitutively express the maltose fermentative enzymes and fail to constitutively accumulate their transcripts. Since these MAL6-linked constitutive mutations were originally derived from mal62 mutant strains, the mal63 mal64-c3 disruption strains also fail to respond to maltose. In contrast to the MAL64-c3 phenotype, 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 an inducible MAL6 strain elicits no phenotypic effect and the strain continues to ferment and induce normally, demonstrating that only the MAL61, MAL62, and MAL63 genes are necessary for maltose-regulated induction and fermentation. This is consistent with analyses of the four other dominant MAL loci, which demonstrate that the MAL1-MAL4 loci also contain three genes that are structurally and functionally homologous to the MAL61, MAL62, and MAL63 genes (5; M. Charron and C. Michels, manuscript in preparation).

Ten Berge et al. (25) proposed that the MAL6-linked gene responsible for constitutiveness was an allele of the positive regulatory gene required for maltose-regulated induction (26), namely, MAL63 (Chang et al., submitted). Ten Berge and co-workers reached this conclusion from their observation that mal63/mal6-C1 heterozygous diploids were generally indi-cable for maltase, and they proposed that interallelic complementation restored inducibility by the production of an oligomeric protein composed of mal63 and MAL63-C gene products (25, 27). We have shown that the MAL63 gene plays no role in MAL6-linked constitutiveness in the two constitutive strains examined (11), one of which was a derivative of constitutive strain C2 used by ten Berge et al. (27). Rather, constitutivity requires the MAL64-C gene.

The restoration of maltose inducibility in the mal63 mal64/mal63Δ MAL64-C heterozygous diploid strains described by ten Berge et al. (25, 27) cannot as 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. Transformation of the cloned MAL64 gene into the MAL64-C2 mal63Δ-13 and MAL64-C2 mal63Δ-13A strains 8-2B and 8-2BΔ3 (11) can now be used to directly examine the effects of such interactions. In this regard, it should be noted that the cloned MAL64-C2 gene is epistatic to the wild-type MAL63 gene. The MAL63 mal63Δ strain 332-5AF-1-5 transformed with plasmid pML64-C2 constitutively synthesizes maltase, while the untransformed strain is inducible (Table 2).

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 fermenta-tion is concerned. While this does not exclude the possibility that the wild-type MAL64 gene plays some unknown nones-sential function in yeast cells that is only distantly related or completely unrelated to maltose fermentation. MAL64 may instead currently serve as genetic reserve information, be-coming active only following mutation and appearance of the MAL64-C allele. Similar cryptic genes have been described in maltose responses (14). It remains to be determined whether activation of MAL64 to MAL64-C is due to mutation within regulatory or coding sequences of this gene. Sequencing the MAL64 gene and its alleles will clarify this point and reveal the extent of homology between it and MAL63. Given the structural homology between MAL63 and MAL64-C, it is likely that both products regulate the levels of the maltase and maltose permease mRNAs by a similar mechanism. MAL6-linked constitutive mutations, including MAL6-C, also confer the ability to accumulate trehalose in glucose-grown cells (21, 23). It will be interesting to determine whether the MAL6-C gene product mediates its effect on trehalose accumula-tion in a manner similar to its control of the maltose fermentative enzymes.

While the five dominant MAL loci contain three genes structurally and functionally homologous to MAL61, MAL62, and MAL63, hybridization analysis of the cloned MAL loci has revealed that not all loci contain a MAL64 homologous gene (5; Charron and Michels, in preparation). This underscores a possible unique role for the MAL64 gene, and further evidence has been obtained that is consistent with this view. In addition to MAL6-C, constitutive alleles have been isolated at the other four dominant MAL loci (17, 18, 20, 21, 29), and we have recently investigated the genetic basis for constitutivity at the MAL4 locus (6). The MAL4 locus contains three genes structurally equivalent to MAL61, MAL62, and MAL63, namely, MAL41, MAL42, and MAL43, respectively. By using a derivative of the MAL4-C constitutive strain isolated by Kahn and Eaton (17), it was demonstrated that, in contrast to MAL6-C constitutive strains (11), deletion of MAL43 (the MAL4-linked MAL63 homolog) in this constitutive MAL4 strain resulted in a maltose nonfermenter that neither constitutively nor inducibly synthesized maltase (6). Also, they clearly demonstrated that constitutivity is trans acting and that the constitutive mutation lies in the MAL43 gene. Preliminary results suggest that this is also the case in one MAL2-C constitutive strain (Charron and Michels, unpublished results). Therefore, constitutive expression of the maltose fermentative enzymes occurs by two mechanisms: (i) by mutation within the MAL-linked MAL63 homolog (which normally encodes the positive regulatory gene product required for maltose-regulated induction), as is the case at MAL4; or (ii) by mutation within a second MAL-linked MAL63 homologous gene (such as MAL64, which normally plays no essential role in inducible maltose fermentation), as in the MAL6-linked constitutive strains described here. It is therefore possible that the mutation present in other MAL6-linked constitutive mutant strains could reside within the MAL63 gene, and this possibility is currently being explored. Since not all dominant MAL loci contain a MAL-linked MAL64 homolog (Charron and Michels, in preparation), both mechanisms for constitutivity are not always available. The MAL1 locus has been cloned, and while a MAL63 homolog is present at this locus, it does not appear to contain a MAL64 homolog (5). This suggests that any MAL-linked constitutive mutations would most probably have to occur within the MAL1-linked MAL63 homolog, namely, the MAL13 gene. This analysis is consistent with the observation that an uninducible mal1 strain containing a deletion of the MAL13 gene failed to revert to a maltose fermenter (R. A. Dubin and C. A. Michels, unpublished results).

Extensive genetic analyses have indicated that maltose-regulated induction of the maltose fermentative enzymes depends upon a single MAL-linked positive regulatory gene (i.e., MAL63 at the MAL6 locus) (7, 26, 29; Chang et al., submitted). Preliminary results suggest that MAL64 defined a second regulatory component involved in maltose-regulated induction (11), we have gone on to demonstrate that this gene is only involved in constitutive expression of the two MAL-linked structural genes. Although 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 depends upon an unlinked gene, MALx (25). While MALx mal63-13 MAL64-C2 strains constitutively express maltase and maltose permease, the malx mal63-13 MAL64-C2 strains are nonfermenters and do not constitutively express the maltose fermentative enzymes. This suggests that the MALx gene product may be required for MAL64-C expression or may interact with the MAL64-C gene product, perhaps acting as an internal inducer. Despite this apparent interaction in constitutive strains, neither MALx (25) nor MAL64 plays a role in inducible maltose fermentation.

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