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Identification of the Upstream Activating Sequence of *MAL* and the Binding Sites for the MAL63 Activator of *Saccharomyces cerevisiae*

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Maltose fermentation in *Saccharomyces* species requires the presence of at least one of five unlinked *MAL* loci: *MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6*. Each of these loci consists of a complex of genes involved in maltose metabolism; the complex includes maltase, a maltose permease, and an activator of these genes. At the *MAL6* locus, the activator is encoded by the *MAL63* gene. While the *MAL6* locus has been the subject of numerous studies, the binding sites of the *MAL63* activator have not been determined. In this study, we used *Escherichia coli* extracts containing the MAL63 protein to define the binding sites of the MAL63 protein in the divergently transcribed *MAL61-62* promotor. When a DNA fragment containing these sites was placed upstream of a *CYC1-lacZ* gene, maltose induced β-galactosidase. These sites therefore constitute an upstream activating sequence for the *MAL* genes.

The *MAL6* complex, while sharing features with other yeast systems, presents in addition some unique problems in gene organization and regulation (11–14, 18). While the maltose system has been an area of active research for 40 years (19), the nature and regulation of the genes of *MAL6* gene complex have only recently been determined (2–4, 13, 14). Maltose utilization in *MAL6* strains of *Saccharomyces cerevisiae* is dependent on three adjacent genes: *MAL63*, encoding an activator (3), and the divergently transcribed *MAL61* and *MAL62* genes, which encode, respectively, maltose permease (2) and maltase (4). MAL63 is a member of the "zinc finger" class of regulatory proteins (8, 17), and both noninducible and constitutive *MAL63* mutations have been isolated (3; unpublished). For this large collection of *MAL63* mutants to be useful in defining the mechanism by which MAL63 activates the transcription of *MAL61* and *MAL62*, it is first necessary to determine the binding sites of MAL63.

Gel shift assays suggest that some protein in *S. cerevisiae* binds to a 387-base-pair (bp) region of the promoter, but because extracts from nonisogenic strains were used, it is not possible to determine whether this protein is MAL63 (6). Hong and Marmur have recently shown that two large deletions of the *MAL61-62* intergenic region, of 144 and 212 bp, lead to loss of maltose inducibility, indicating that an element essential for expression must lie in this region. They suggest that this element is a 34- to 36-bp inverted repeat sequence and that it serves as the *MAL63* binding site (6, 17). Using an *Escherichia coli* expression system for *MAL63*, we show instead that the MAL63 protein binds to two seemingly unrelated sites flanking this palindrome.

The *MAL63* gene was put under the control of lambda repressor cI857, and the protein was induced by a temperature shift to 41°C (details will be presented elsewhere). Figure 1 shows the results when extracts were then used in a nitrocellulose-DNA filter binding assay performed by the method of Johnston and Dover (7). Only a 320-bp *Mbo*I fragment bound, and this binding depended on the presence of the MAL63 protein in the extract.

To determine the sequences responsible for binding the MAL63 protein, we performed DNase I footprinting on this fragment (Fig. 2). The protected region was divided into two sites, sites 1 and 2, and their sequences are given in Fig. 3. The addition of maltose had no obvious effect on binding to either site 1 or site 2. The 3′ boundary of site 1 was fixed by an enhanced cleavage site that depended on MAL63 binding (top strand, Fig. 2). The 5′ boundary could not be precisely fixed, and the protected region may be slightly smaller than indicated in Fig. 3. Site 1 could also form a palindrome with 67% of the bases matching, but we do not know whether this structure plays a role in binding; the *MAL61-62* region is particularly rich in such seemingly purposeful structures. Site 1 was protected even at the lowest protein concentration tested, as indicated by the appearance of the 3′ hypersensitive site. At higher protein concentrations, a second, weaker, and unusually large binding region, site 2, became increasingly protected on both strands from DNase I cleavage. Site 2 is not a palindrome, nor did it seem to be related in sequence to site 1; however, binding sites thought to be distinct by visual inspection may turn out to have common structural features when protein-DNA contacts are examined (1, 15). In addition to these two major sites, a much weaker region of protection was seen on only the top strand between site 1 and site 2. This very weak region corresponds to the palindrome identified by Hong and Marmur (6), but it is clearly not a major binding site.

To determine whether the region encompassing the MAL63-binding sites was sufficient to confer maltose inducibility (i.e., whether these sites constitute the maltose gene upstream activation sequence [MAL_{UAS}]), the region between bases 7 and 136 in Fig. 3 was placed on a replicating yeast plasmid 5′ to a *CYC1-lacZ* fusion gene. β-Galactosidase activity was now under maltose control, with maltose-induced cells showing about 130-fold more activity than uninduced cells (Table 1). These results demonstrate that the inserted region is a MAL_{UAS}.

Hong and Marmur (6) point to two prominent features of the promotor region, a 34-bp inverted repeat (bases 69 to 104 in Fig. 3) and the presence of four motifs that they consider homologous (AATATTT, AAAATTT, AAAGTTT, and AAATTTT). Two large deletions, both of which retained bases 1 to 49 in and were deleted for the rest of the sequence

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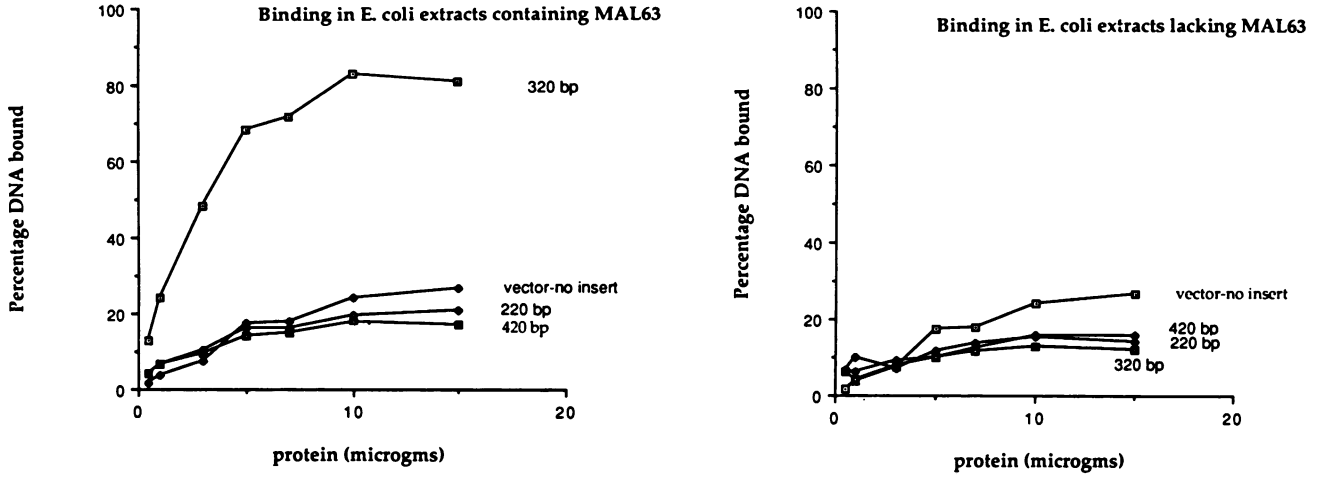

FIG. 1. Filter binding assay. The entire *MAL61-62* intergenic region contained on an *Eco*RI-*Bgl*II fragment was cloned into pUC18. From this plasmid, *Mbo*I fragments of approximately 220, 320, and 420 bp encompassing the entire promotor were cloned into the *Bam*HI site of pUC18. This plasmid was then cut with *Eco*RI, and the ends were filled in with [α-^{32}P]dATP. Crude extracts from *E. coli* containing the MAL63 expression plasmid were assayed by the nitrocellulose-DNA binding assay of Johnston and Dover (7). Reactions were performed in a 20 μl volume with end-labeled plasmid DNA (20,000 to 50,000 cpm per reaction mix) and 10 μg of sonicated salmon sperm DNA as the competitor. The experiment marked "vector—no insert" used pUC18 cut with *Eco*RI and labeled in the same way.

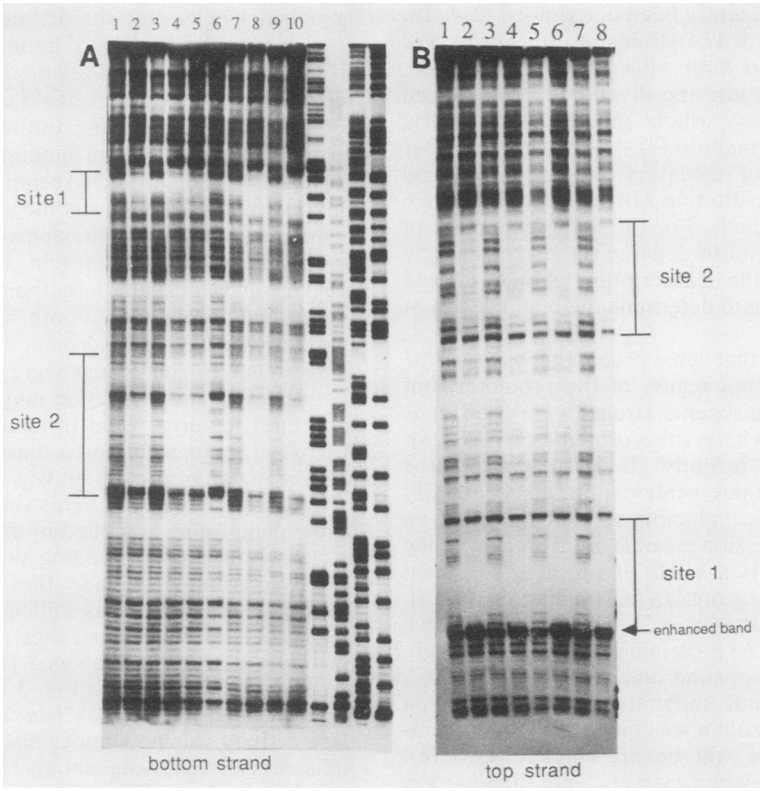

FIG. 2. DNase I footprinting. The footprinting was carried out as described before (7). One binding assay had no protein added (lane 6 in A), some had protein from *E. coli* cells containing only the vector (lanes 1, 3, 7, and 9 in A, 1, 3, 5, and 7 in B), and some had protein from *E. coli* cells containing the expression vector with the *MAL63* insert (lanes 2, 4, 5, 8, and 10 in A, 2, 4, 6, and 8 in B). The amount of protein present is indicated for each lane (in micrograms). (A) Bottom strand. Lane 1, vector, 15; lane 2, *MAL63*, 15; lane 3, vector, 20; lane 4, *MAL63*, 20; lane 5, *MAL63*, 20 (with 1% maltose); lane 6, no protein; lane 7, vector, 30; lane 8, *MAL63*, 30; lane 9, vector, 40; lane 10, *MAL63*, 40. The remaining lanes are Maxam-Gilbert (10) sequencing reactions of the footprinted fragment (G+A, A, T+C, and C, respectively). (B) Top strand. Lane 1, vector, 15; lane 2, *MAL63*, 15; lane 3, vector, 20; lane 4, *MAL63*, 20; lane 5, vector, 30; lane 6, *MAL63*, 30; lane 7, vector, 40; lane 8, *MAL63*, 40.

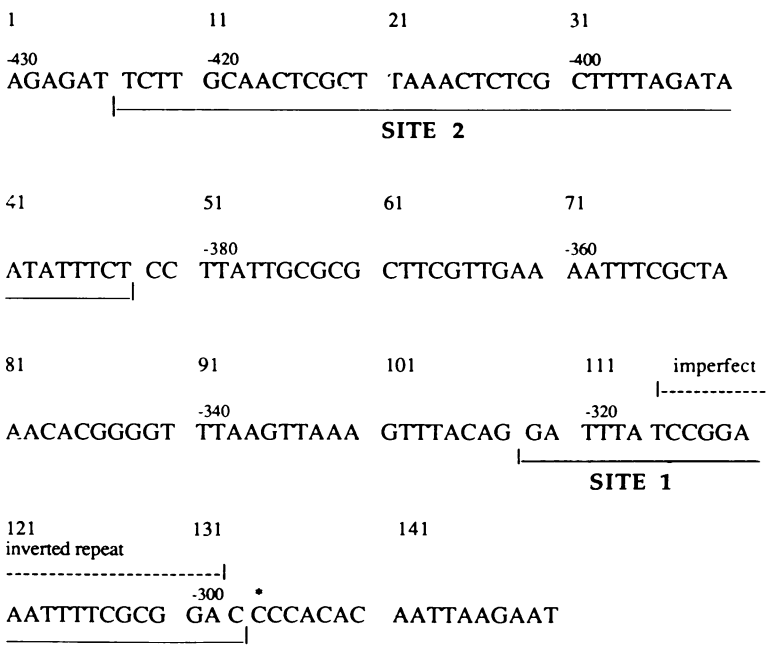

FIG. 3. We sequenced the binding sites by the Maxam and Gilbert method (10) (Fig. 2). This sequence has several additions and one deletion compared with the sequence in Hong and Marmur (6), and since we have not sequenced the entire region, the distances from the ATG are only approximate. We have therefore used the absolute coordinates to refer to particular sequences. Our sequence agrees with the results from another laboratory (C. Michels, personal communication) and is identical to that of Kopetzki et al., who sequenced the promoter region from an unrelated strain of brewer's yeast (9). The inverted repeat of Hong and Marmur (6) corresponds to bases 69 to 104.

TABLE 1. Enzyme activity[a]

Strain	Genotype	Plasmid	Medium	Activity	
				ONPGase (10^3 ΔOD_{420}/min per mg of protein)	PNPGase (nmol/min per mg of protein)
332-5A	*MAL6*	669-Z	GE	12,381	3
			MALGE	15,231	904
		670-Z	GE	35	2
			MALGE	70	1,086
		p23	GE	33	4
			MALGE	4,393	1,081
63L-1A	*MAL63::LEU2*	p23	GE	18	3
			MALGE	56	9

[a] The region encompassing bases 7 to 136 (Fig. 3) and additional *Sma*I, *Sal*I, *Bgl*II, *Xho*I, and *Kpn*I sites at the ends were synthesized by the polymerase chain reaction with GGGGTCGACAGATCTTGCAACTCGCT TAAACT as the upstream primer and GGGAGATCTGGTACCTCGAGGG GTCCGCGAAAATTTC as the downstream primer. The polymerase chain reaction product was first cloned into plasmid pUC18 and then as a *Sal*I fragment into pLGX669-Z (15) in place of the *Xho*I fragment (which contains the *CYC1* UAS) upstream of a *CYC1-lacZ* gene fusion. The resulting plasmid, p23, was sequenced to confirm the authenticity of the inserted site and then transformed into two strains isogenic except for the *MAL6* locus. Plasmid pLGX670-Z (670-Z) is pLGX669-Z (669-Z) with an *Xho*I deletion of the *CYC1* UAS (15). The cells were grown to mid-log phase on 6.7% yeast nitrogen base (Difco Laboratories) with leucine, histidine, and tryptophan and containing either 3% glycerol and 2% ethanol (GE) or 2% maltose, 3% glycerol, and 2% ethanol (MALGE) as carbon sources. The levels of β-galactosidase (ONPGase) and the α-glucosidase maltase (PNPGase) were measured in cell extracts. The full genotype of strain 332-5A is *MATa MAL64 MAL63 MAL61 MAL62 MAL12 his trp1 leu2-3,112 ura3-52*; strain 63L-1A is 332-5A with a deletion-disruption of *MAL63* (*MAL63::LEU2*) and is therefore noninducible (3).

in Fig. 3, exhibited about 17% of the normal wild-type activity. From these and other deletions which retained wild-type activity, they conclude that the inverted repeat in this region (bases 69 to 104) is implicated in the expression of *MAL62* and suggest that this repeat may be the binding site for MAL63. One difficulty with this view is that two deletions retained wild-type expression even though one half of the inverted repeat was deleted (i.e., retained only bases 1 to 86 in Fig. 3). From our studies it is possible to explain the results of their deletion analysis by assuming that maltose-regulated expression requires at least one of the two binding regions site 1 and site 2. The two deletions that showed decreased expression are deleted for both site 1 and site 2; all deletions that retained one of these sites gave full expression. However, more detailed studies with much smaller deletions in this region will be necessary to determine whether this explanation is correct. The precise role of these two sites in maltose induction also remains to be determined.

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