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IDENTIFICATION OF REGULATORY MECHANISMS OF GENES ENCODING CITRATE SYNTHASE IN THE YEAST Saccharomyces cerevisiae

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IDENTIFICATION OF REGULATORY MECHANISMS OF GENES ENCODING CITRATE SYNTHASE IN THE YEAST *Saccharomyces cerevisiae*

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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LIST OF ABBREVIATIONS

bp	basepair
DNA	deoxyribonucleic acid
EMS	ethylmethylsulfonate
ETS	electron transport system
*α*KGDC	alpha-ketoglutarate dehydrogenase complex
M	molar
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
RNA	ribonucleic acid
TCA	tricarboxylic acid
TE	tris-EDTA
UAS	upstream activation sequence
V	volts

Identification of regulatory mechanisms of genes encoding citrate synthase in the yeast, Saccharomyces cerevisiae

ABSTRACT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Medical College of Virginia, Virginia Commonwealth University

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The major aim of this research was to investigate the molecular mechanisms of regulation of transcription of CIT1 and CIT2, the genes encoding citrate synthase in yeast. Specifically addressed are the questions of (1) localization of cis-acting sites required for expression or regulation, (2) the roles of HAP1 and HAP2,3,4 in expression of both genes, (3) identification of other trans-acting factors involved in expression of either gene, (4) localization of cis-acting sites involved in up regulation of CIT2 in response to disruption of CIT1 or rho° status (Liao et al., 1991; Liao and Butow, 1993).

I show here that mutations in HAP2, HAP3, or HAP4 specifically prevent derepression of CIT1. Using deletions and base substitutions, derepression of

CIT1 is shown to require candidate HAP2,3,4 binding sites at -290 and -310 (distance upstream from the translational start site). Attempts at demonstrating binding of HAP2,3,4 to CIT1 upstream DNA were unsuccessful. HAP1 appeared to play an important role in lactate-derepressed, but not glucose repressed expression of CIT1. No regions were identified as being responsible for negative regulation by glucose plus glutamate. HAP2,3,4 is not required for this regulation. HAP2,3,4 also was shown to regulate CIT2 to a small degree in glucose-repressed expression and to a large degree in lactate-derepressed expression. No regions were identified as responsible for this activation.

To identify additional trans-acting factors involved in expression of CIT1 or CIT2 (e.g. activators of glucose-repressed expression), yeast containing CIT1-lacZ or CIT2-lacZ fusions were mutagenized and screened for altered expression. Seven mutants with reduced expression of a CIT1-lacZ fusion are currently under study.

Expression of CIT2 (peroxisomal citrate synthase) is not regulated by glucose, but is regulated by the rho status of the strain and by the presence or absence of a functional CIT1 gene (Liao et al., 1991; Liao and Butow, 1993). We identified a region critical for expression of CIT2 in lactate-grown cells located between -300 and -370, which is the same region Butow's laboratory has found to be important in regulation by rho.

INTRODUCTION

Saccharomyces cerevisiae

a. The organism

Saccharomyces cerevisiae, commonly referred to as baker's or brewer's yeast, is an eukaryotic, single-celled member of the Ascomycetes Family of fungi. Originally it was of interest in biochemical research because of its role in alcohol production. It has since been used extensively for genetic and biochemical studies relating to eukaryotic cell function and regulation. It is an useful organism for study because it is quickly and easily grown in large quantities, classical genetic procedures and recombinant DNA procedures are well-established, and it is nonpathogenic. It is able to grow both aerobically and anaerobically and mitochondrial function is dispensable during growth in glucose medium. Yeast have been used extensively to study mitochondrial function and respiratory growth as well as many other areas of eukaryotic cell biology (Roman 1981).

b. Genomic and Mitochondrial DNA

Yeast have 16 chromosomes and a genome size of 12.5 Mb + rDNA which typically encodes 1-2 Mb. Yeast DNA is estimated to encode 6000 - 8000 genes. Several projects are currently underway to complete detailed maps of the yeast genome and determine the DNA sequences of several yeast chromosomes, with

one chromosome (III) completed (Olson 1991, Oliver et al., 1992).

Yeast also have 75 kilobases of mitochondrial DNA (mtDNA) which encodes a few proteins necessary for electron transport and oxidative phosphorylation. These proteins complex with nuclear-encoded proteins that are imported into the mitochondria after synthesis. While the mtDNA is not absolutely required for cell viability, it is required for respiration and oxidative phosphorylation. Disruption of either mtDNA or import of nuclear-encoded proteins results in cells that are respiratory deficient (Olson, 1991).

There are several types of mutants related to mitochondria and respiratory function. Mutants designated mit- have a mitochondrial protein-encoding gene mutated (Forsburg and Guarente, 1989). Many yeast strains spontaneously produce colonies with extensive deletions or alterations of the mitochondrial genome, referred to as rho (ρ)- mutants. Complete loss of mtDNA is referred to as rho (ρ)$^{\circ}$. Both ρ^- and ρ° strains are respiratory deficient (Dujon, 1981). Nuclear respiratory-deficient mutants, petites, (pet$^-$) retain mtDNA, but have mutations in nuclear genes encoding proteins that are required for respiratory functions (Dujon, 1981). The mitochondrial genotype of the parental strains is important when crossing ρ^+ and ρ^- or ρ° strains. Crossing mutants carrying mutations in different genes does not result in wild-type progeny, but ones that are respiratory deficient (Dujon 1981).

Ethidium bromide can be used to induce ρ^- mutants by binding to the mtDNA and inducing deletions of the DNA, or if allowed to proceed further, to complete loss of the mtDNA producing ρ° mutants (Dujon, 1981). Most classical

mutagens induce ρ^- mutants with great efficiency (Dujon, 1981).

c. 2μ plasmids

In addition to chromosomal and mitochondrial DNA, most Saccharomyces cerevisiae strain also contain double-stranded DNA plasmids, including the 2-micron (2μ) circle (Broach and Volkert, 1991). This multiple-copy, 2μ extrachromosomal plasmid has made possible transformation of genetic material into yeast where it can be propagated in high copy number in the nucleus. Segments of DNA can be inserted into the 2μ circle and transformed into yeast, where they replicate in synchrony with chromosomal replication. They are present in most cells in high copy number, and are randomly dispersed between mother and daughter cell during cell division (Broach and Volkert, 1991).

d. mating types

S. cerevisiae has haploid cells of α and **a** mating types, which can mate and fuse to form a nonmating diploid. The genetic regulation involved in maintenance of **a**, α, or **a**/α types is very complicated. (Reviewed in Herskowitz, Pine, and Strathern, 1992).

When starved, the diploid will sporulate, and form four ascospores, which can be isolated by micromanipulation, propagated, and analyzed. This is very useful for determining whether a phenotype is caused by a single locus and for mapping loci (Mortimer and Schild, 1981).

Transcriptional Regulation in Yeast

Regulation of gene expression in yeast has proven to be very similar to that in higher eukaryotic systems. In general, eukaryotic RNA polymerase II-

transcribed genes require multiple transcription factors, and interactions at dispersed DNA sites; unlike prokaryotes which have a less complex regulation (Yanofsky, 1992). Verdier (1990), in an overview of yeast DNA-binding proteins, lists four basic cis-acting elements found in yeast promoters: (1) upstream activations sites (UAS) (2) silencers which mediate negative control (3) TATA boxes and (4) transcription initiation sites.

Transcription initiation requires binding of RNA polymerase II plus additional chromatographic fractions, including TFIIA-F, which are required for specific initiation and which bind in an ordered manner beginning with the binding of TFIID (and possibly TFIIA) to the TATA element (Buratowski and Sharp, 1992). Other factors can bind sequences upstream (or sometimes downstream) of the promoter and increase or decrease the rate of initiation of transcription (Buratowski and Sharp, 1992). In general expression is very low in vivo without additional upstream DNA sequences and factors. Activation of transcription requires the binding to double-stranded DNA of a protein or proteins possessing both a DNA-binding domain and an activation region (Ptashne, 1992).

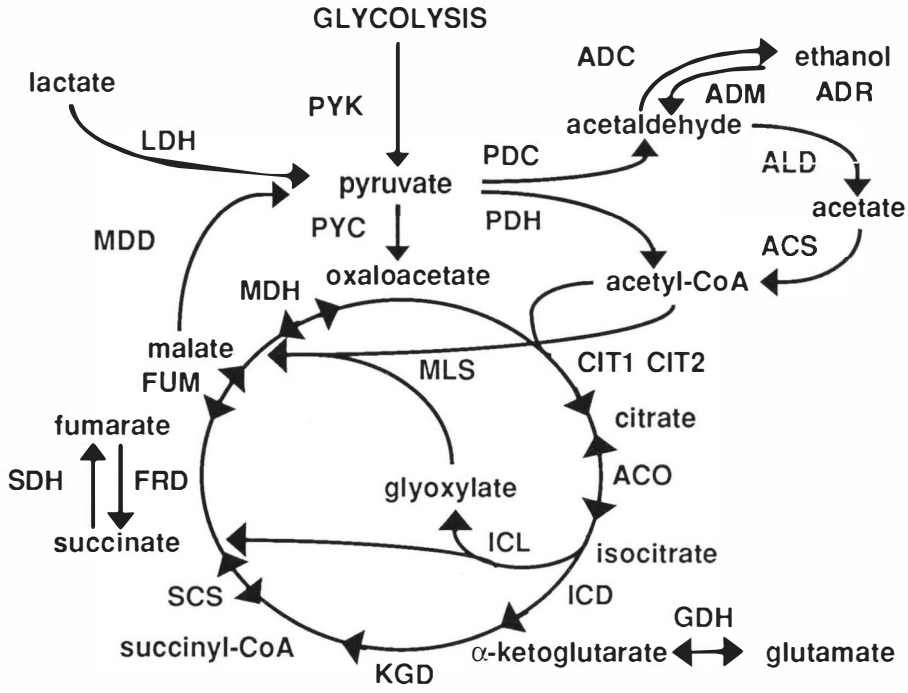
Several features of transcription have been conserved from yeast to human. The structure of RNA Polymerase II is similar. Furthermore some yeast transcriptional activators can function in mammalian cells and some mammalian activators can function in yeast. This is primarily due to conserved cis-acting elements of promoters such as a TATA box and upstream activation sites (UAS's). The latter often function similarly to higher cells enhancers, and are independent of location and orientation (Guarente, 1992).

Tricarboxylic Acid Cycle Regulation

My interest has been in the regulation of transcription of nuclear genes required for mitochondrial respiratory function. The tricarboxylic acid (TCA) cycle functions in the oxidative degradation of nonfermentable carbon sources (e.g. lactate, pyruvate, ethanol, various amino acids) to obtain energy. In addition, the TCA cycle provides intermediates for several biosynthetic pathways (e.g. synthesis of various amino acids porphyrins and purine and pyrimidine nucleotides) (Fig. 1, Fraenkel 1982). When cells are grown in minimal medium containing a fermentable sugar such as glucose, only the biosynthetic roles are required. In rich medium containing glucose, many of the biosynthetic functions are no longer required. The TCA cycle is localized in the mitochondria in eukaryotic cells along with other components of energy production, including the cytochromes of the electron transport system and the enzymes required for heme biosynthesis (Schatz and Mason, 1974; Frankel, D.G., 1981). While there has been considerable study of the regulation of nuclear genes encoding components of the latter two systems, little is known about genes encoding enzymes of the mitochondrial TCA cycle.

In eukaryotic cells, the mitochondrial TCA cycle enzymes are encoded by nuclear genes, but the levels of these enzymes often respond to demands for mitochondrial function. Several mechanisms of communication between nucleus and mitochondria have been described (Forsburg and Guarente, 1989). When Saccharomyces cerevisiae cells are grown on glucose, the levels of many enzymes and proteins of the mitochondria are reduced (Tzagaloff and Myers,

Figure 1: Yeast intermediary metabolism. Enzymes of the TCA cycle: citrate synthase (CIT), aconitase (ACO), isocitrate dehydrogenase (ICD), ***α***-ketoglutarate dehydrogenase (KGD), succinyl-CoA synthetase (SCS), fumarate reductase (FRD), fumarase (FUM), malate dehydrogenase (MDH).
Enzymes of the glyoxylate cycle:
citrate synthase (CIT), aconitase (ACO), isocitrate lyase (ICL), malate synthase (MLS), malate dehydrogenase (MDH).
Other enzymes shown:
lactate dehydrogenase (LDH), pyruvate kinase (PYK), pyruvate carboxylase (PYC), pyruvate decarboxylase (PDC), pyruvate dehydrogenase (PDH), alcohol dehydrogenase (ADC, ADR, ADM).
aldehyde dehydrogenase (ALD), acetyl-coA synthetase (ACS), glutamate dehydrogenase (GDH), malic enzyme (MDD). Intracellular localization in yeast:
mitochondrial (m), cytosolic (or perhaps peroxisomal) (c), or both (b). (Adapted from Fraenkel, 1982; with data from Duntze et al., 1969; Perlman and Mahler, 1970; Wales et al., 1980).


GLYCOLYSIS
lactate
LDH
PYK
ADC
ethanol
ADM
ADR
acetaldehyde
PDC
ALD
pyruvate
acetate
PYC
PDH
MDD
ACS
oxaloacetate
acetyl-CoA
MDH
malate
MLS
CIT1 CIT2
FUM
citrate
fumarate
SDH
FRD
glyoxylate
ACO
succinate
ICL
isocitrate
SCS
ICD
GDH
succinyl-CoA
α-ketoglutarate
glutamate
KGD

1986). When glucose becomes exhausted, S. cerevisiae cells switch to oxidation of the accumulated ethanol. The number of mitochondria increase during this process and undergo morphological changes (Pon and Schatz, 1991).

Heme, which is synthesized in the mitochondria, serves as another regulatory signal from the mitochondria to the nucleus (Forsburg and Guarente, 1989). Heme is necessary for transcription of genes encoding respiratory enzymes (Guarente and Mason, 1983), and for repression of anaerobically expressed genes (Lowry and Lieber, 1986). Heme levels reflect oxygen levels because their biosynthesis requires oxygenases. Under anaerobic conditions the cells cannot produce heme, and expression of several nuclear genes encoding heme-proteins is reduced (Forsburg and Guarente, 1989).

Citrate synthase

I have focused on genes encoding the TCA cycle enzyme citrate synthase. Citrate synthase catalyzes the first and rate-limiting step in the TCA cycle, the condensation of oxaloacetate and acetyl Coenzyme A to form citrate (Krebs and Lowenstein,1960; Walsh and Koshland,1985). The two genes encoding citrate synthase in Saccharomyces cerevisiae have been cloned and sequenced (Suissa, et al.,1984; Kim, et al.,1986). CIT1 encodes the major mitochondrial isozyme (Suissa et al., 1984), and CIT2 encodes a minor peroxisomal form (Kim, et al., 1986). There is evidence for and against the idea that the CIT2-encoded form is involved in the glyoxylate cycle (Kim et al., 1986; Lewin, 1990) CIT1 contains an N-terminal mitochondrial targeting sequence

(Rosenkrantz, et al., 1986), while CIT2 encodes a protein with a carboxyl-terminal tripeptide (SKL) thought to be necessary for import into peroxisomes (Lewin, et al., 1990). Deletion of this tripeptide resulted in peroxisomal citrate synthase being mistargeted to the mitochondria (Singh, et al., 1992). CIT2 encodes an N-terminal extension not found in porcine or E. coli citrate synthase, but shorter than the signal sequence found in CIT1 (Rosenkrantz, et al., 1986). The presence of the terminal tripeptide may prevent the N-terminal sequence from targeting the protein to the mitochondria (Singh, et al., 1992).

Kim, et al.(1986) have shown that simultaneous disruption of both genes essentially eliminates citrate synthase activity, and cells exhibit strong glutamate auxotrophy, decreased growth on lactate, and no growth on acetate medium. However, disruption of CIT1 alone reduces citrate synthase levels by about 95% (Kim, et al.,1986), but only produces one phenotype: an inability to grow on acetate medium. Singh, et al., (1992) found that CIT2 could not compensate for CIT1 on low energy-yielding substrates such as acetate.

Rickey and Lewin found that disruption of CIT1 severely reduced mitochondrial citrate synthase activity on both fermentative and nonfermentative carbon sources, and increased extramitochondrial activity five-fold. They were unable to determine whether the increase was due to increased enzyme level or an increase in the rate of reaction (Rickey and Lewin, 1986). Studies by Kispal, et al., found that the CIT2 protein could not replace CIT1 activity (Kispal, et al., 1988). Liao et al. has shown that disruption of CIT1 resulted in a six-fold increase in CIT2 activity and corresponding increases in the level of CIT2 mRNA

levels when cells were grown in rich medium + 2% raffinose (Liao, et al.,1991).

Glucose repression

The TCA cycle has been shown to be under glucose repression but the mechanism of this repression has not been determined. In Escherichia coli catabolite repression is under the control of cAMP and catabolite repressor protein (CRP) (Botsford, 1981). In bacteria, an inverse correlation has been shown between cAMP levels and catabolite repression. The derepression of the lac operon is a response to high cAMP concentrations in the bacterial cells, and these levels are determined by the carbon source of the growth media (Botsford, 1981). In E. coli cAMP binds to catabolite activator protein, and the complex then binds to DNA, enabling RNA polymerase to bind and/or initiate transcription of the lac operon (Botsford, 1981). In yeast a relationship between cAMP levels and catabolite repression is controversial. Many other laboratories are conducting studies in this area and I did not pursue the topic (reviewed in Broach, 1991, Gibbs and Marshall, 1989).

In yeast, glucose is preferentially fermented with derepression of a variety of genes occurring as the concentration of glucose decreases (Johnston and Carlson, 1992). Flick and Johnston (1990) have proposed a model for glucose regulation of the GAL1 promoter involving multiple regulatory mechanisms. Their model suggests that glucose is converted to an intracellular signal which acts (1) to inhibit binding of the GAL4 transcriptional activator to the UASgal (upstream activation sequence) of GAL1 and (2) to repress transcription by acting on an URSgal (upstream repressing sequence) by independent pathways

and (3) reduction in the level of the inducer of the GAL genes (Flick and Johnston, 1990).

Numerous pleiotropic regulatory genes have been isolated that affect global catabolite repression. For a summary of these genes see Johnston and Carlson 1992. HXK2 encodes hexokinase, which senses the glucose level and relays the signal through various regulatory proteins such as REG1, CID1, or GRR1, inhibiting the action of the SNF1-SNF4 heterodimer (Trumbly R.J., 1992)

SNF1 is a gene encoding a serine/threonine protein kinase required for derepression of SUC2 and some other glucose-repressible genes. SNF4 encodes

a protein complexed with Snf1 and required for maximum kinase activity (Trumbly 1992). Expression of the SNF1 gene is not glucose repressible and its product is found throughout the cell (Celenza and Carlson 1986 1989).

SSN6, isolated as a suppressor of snf1, is a negative repressor of SUC2, and appears to have a negative role in glucose repression of other genes as well (Schultz and Carlson, 1987). Snf1 is thought to prevent the repression of SUC2 by Ssn6 (Celenza and Carlson, 1987). TUP1 encodes a protein that has been found associated with Ssn6 in a protein complex. Disruption of either gene abolishes glucose repression of SUC2 (Williams, et al., 1991). Wright and Poyton (1990) have shown that both SNF1 and SSN6 are required for derepression of the glucose-repressible genes COX6 (cytochrome oxidase) and CYC1. Both cytochrome genes are also known to require the HAP2,3,4 protein

complex for derepression when grown on a nonfermentable carbon source (Wright and Poyton, 1990).

MIG1 encodes a protein that binds to SUC2, GAL1, and GAL4 promoters. Ssn6 and Tup1 lack a DNA-binding domain, and may utilize other proteins such as Mig1 for this function (Keleher, et al., 1992)

Heme-activated proteins (Hap)

In S. cerevisiae, at least some of the nuclear genes encoding components of the electron transport system (ETS) are catabolite repressed, and proteins required for this regulation have been described (Forsburg and Guarente, 1989). The nuclear gene, CYC1, encoding iso-1-cytochrome c, a heme-protein, has been shown to require normal heme levels for expression, and is catabolite repressed (Guarente, et al., 1984). Two upstream activation sequences (UAS) were identified - UAS1 and UAS2. UAS1 binds HAP1, a hemoprotein and transcriptional activator which is primarily responsive to heme levels. Heme biosynthesis is somewhat repressed by glucose (Guarente, et al., 1984, Pinkham and Keng, 1993). UAS2 is bound by a heteromeric complex composed of HAP2, HAP3, and HAP4 (Forsburg and Guarente, 1989). The HAP2,3,4 system has been shown to activate transcription of several respiratory genes when cells are grown on nonfermentable carbon sources (Forsburg and Guarente, 1989). It is believed that HAP2 and HAP3 constitute the DNA-binding domain, and HAP4 is the primary transcriptional activator (Forsburg and Guarente, 1989; Guarente 1993).

The yeast HAP2,3,4 protein complex, which is involved in transcriptional

regulation of genes involved in respiratory function, binds to an element with homology to the CCAAT box element found in many mammalian promoters (Forsburg and Guarente 1989). The human DNA-binding protein CP1 also recognizes CCAAT boxes and consists of two subunits, CP1A and CP1B, which are functionally interchangeable with HAP3 and HAP2 proteins respectively in in vitro DNA binding and transcription experiments (Becker et al. 1991; Chodosh et al. 1988)

Regulation of TCA cycle genes

It has not been determined whether the genes encoding enzymes of the TCA cycle are under the control of the proteins encoded by SNF1, SSN6, or TUP1. Recently it has been shown that HAP2,3,4 is involved in derepression of at least some of the genes encoding enzymes of the TCA cycle. Table 1 lists the genes with regions in their upstream DNA homologous to the HAP2,3,4 binding motif, UCCAATNA, (Rosenkrantz, et al., submitted). "Positive" sites are those known to respond to HAP2,3,4; "candidate" sites are ones where regulation of the gene by HAP2,3,4 has been reported, but binding of HAP2,3,4 or requirement of a particular candidate site have not been demonstrated yet. "Unknown" sites are those with a region or regions of homology to the HAP2,3,4 binding sequence, but regulation by HAP2,3,4 has not been tested. Pinkham and Keng (1992) have classified genes regulated by HAP2,3,4 into 3 categories: (1) Heme dependent, carbon source regulated (2) heme independent, carbon source regulated, and (3) heme independent, carbon source independent. The TCA cycle genes ACO1 (aconitase), CIT1 (citrate synthase), and α-KGDC genes

KGD1, KGD2, and LPD1 are category 2 genes (Pinkham and Keng 1993). All three genes encoding the α-KGDC are under catabolite repression (Roy and Dawes, 1987; Repetto and Tzagoloff, 1989 and 1990; Bowman 1992). KGD1, KGD2, and LPD1 have been shown to be dependent on HAP2 and HAP3 (Repetto and Tzagoloff, 1989, 1990; Bowman, 1992). Actual binding studies of HAP2,3,4 to the genes have not been reported for KGD1 or KGD2, however, both genes contain putative binding sites (Repetto and Tzagoloff, 1989, 1990). Binding could not be demonstrated for LPD1, leading the author to suggest that HAP2,3,4 has an indirect effect on transcription (Bowman, 1992)

Gangloff, et al. (1990) have cloned and sequenced the gene encoding aconitase, ACO1. The upstream region also contains a putative HAP2-HAP3 responsive site, but the significance of the region has not yet been determined (Gangloff, et al., 1990).

Major goals of this research were to (1) localize CIT1 and CIT2 upstream DNA sequences required for activation and regulation by glucose or glucose and glutamate (2) to determine whether HAP2,3,4 is responsible for lactate regulation of CIT1 , and which DNA sequences are required, and if HAP2,3,4 is responsible for regulation (3) to determine whether HAP2,3,4 binds CIT1 sequences in vitro.

Table 1. Known and candidate Hap2,3,4 elements.

Gene[a]		DNA sequence	Function[b]	Location[c]	Reference
CYC1 UAS2	←	gatcc ACCAAccA acgct	positive	-205	Forsburg & Guarente, 1988
UAS2up1	←	gatcc ACCAATcA acgct	positive	-205	Forsburg & Guarente, 1988
COX4	→	tgtcc ACCAATaA gatca	candidate	-610	Schneider & Guarente, 1987
					Forsburg & Guarente, 1988
COX5a	←	gtagg GCCAATcA caaac	candidate	-145	Trueblood et al., 1988
	→	atcgt tCCAATag acgtt	candidate	-170	
COX6	→	tacga GCCAATcA gggcc	positive	-285	Trawick et al., 1989 and 1992
CYT1	←	tctcc ACCAAccA aatcc	candidate	-470	Schneider & Guarente, 1991
					Oechsner et al., 1992
COR2	→	aacga ACCAATag aaggc	candidate	-185	Dorsman & Grivell, 1990
QCR8	→	tttaa GCCAATgA aaatg	candidate	-210	de Winde & Grivell, 1992
HEM1	←	ggcag ACCAATgA gcgac	candidate	-375	Keng & Guarente, 1987
HEM3	→	gtctg ACCAATaA gaaac	candidate	-315	Keng et al., 1992
SOD2	→	cctgg ACCAATaA cacag	candidate	-205	Marres et al., 1985
					J.Pinkham (personal commun.)
KGD1	←	gatgg ACCAgTtA gcttc	candidate	-400	Repetto & Tzagoloff, 1989
	→	ccttt GCCAATcA tttat	candidate	-315	
	→	acaca cCCAATgA atcta	candidate	-250	
KGD2	→	ttgca GCCAATcA aaact	candidate	-360	Repetto & Tzagoloff, 1990
	→	agtga GCCAATaA agcgg	candidate	-330	
LPD1	←	ttctc GCCAATgA gggaa	positive	-200	Bowman et al., 1992
ACN1	→	aaacc tCCAATcg ctgcc	unknown	-205	Gangloff et al., 1990
	→	tcctg ACCAATcA ataga	unknown	-290	
FUM1	←	aaaga GCCAATcA tcgaa	unknown	-280	Wu & Tzagoloff, 1987
CIT1	←	gatcc tCCAATaA cacaa	positive	-290	Suissa et al., 1984
	→	gctag ACCAAaaA tactt	positive	-310	and this work
CONSENSUS		UCCAATnA			

a) CYC1 (cytochrome c); CYT1 (cytochrome c_1); COR2 (QH2: cytochrome c oxidoreductase, subunit II); COX4, COX5a, COX6 (cytochrome oxidase subunits); QCR8 (ubiquinol cytochrome c oxidoreductase); HEM1 (δ-aminolevulinate synthase); HEM3 (porphobilinogen); KGD1, KGD2, LPD1 (subunits of α-ketoglutarate dehydrogenase); ACN1 (aconitase); FUM1 (fumarase); CIT1 (mitochondrial citrate synthase).

b) "positive"=mutational or DNA-binding evidence; "candidate"=gene regulated by HAP2,3,4 and in some cases, some promoter deletion mapping data; "unknown"=regulation by HAP2,3,4 not tested.

c) Approximate location relative to the start of transcription or translation (as in reference cited).

MATERIALS AND METHODS

Yeast strains and media

The yeast strains used in this study are described in Table 2. All cultures were grown at 30 °C on complete medium (YEP) containing 1% yeast extract (Bacto-Difco) and 2% peptone (Bacto-Difco) or on minimal medium (YMM) containing 0.67% Bacto-yeast nitrogen base without amino acids and 0.5% ammonium sulfate (United States Biochemicals) with the carbon source at 2% (Sherman et al., 1986). Under certain growth conditions 0.1% Casamino acids (Bacto-Difco) or individual amino acids (United States Biochemicals) were added at a concentration of 0.01%. Sodium glutamate (United States Biochemicals) was used at a concentration of 0.2% where indicated. Carbon sources were added at a concentration of 2% including glucose (YEPD) (United States Biochemicals), lactate (YEPL) (Mallinkrodt), galactose (YEP-gal) (Sigma), and raffinose (YEPR) (Sigma). Plates contained 2% agar (Bacto-Difco or United States Biochemicals)

TABLE 2

Yeast strains used in this study

Strain	Genotype	References
BWG1-7a	MAT**a**,ura3-52,leu2-3,112, his4-519,ade1-100	Guarente and Mason, 1983
LGW1	MAT**a**,ura3-52,leu2-3,112, his4-519,ade1-100, hap2-1	Guarente, et al., 1984
JP40	MAT**a**,ura3-52,leu2-3,112, his4-519,ade1-100,hap3-1	Hahn, et al., 1988
SLF401	MAT**a**,ura3-52,leu2-3,112, his4-519,ade1-100,hap4::LEU2	Forsburg and Guarente, 1989
PSY142	MATα,ura3-52,leu2-3,112, lys2-801	D. Botstein lab
1-7-A-L	MAT**a**,ura3-52,leu2-3,112, his4-519,ade1-100,CIT1::LEU2	Rosenkrantz, et al., 1986

MAT = mating type locus

Stock cultures

To preserve the yeast strains, yeast were grown for 2 days to stationary phase. 0.3 ml of the culture was added to a tube containing 0.7 ml of 50% glycerol, mixed, and the suspension was placed in the -70°C freezer. (To use, the culture was removed from the freezer, and the top of the contents scraped with a toothpick and the inoculum was streaked to a YEPD plate and incubated for 2 days at 30°C.. The remainder of the culture was refrozen.)

Yeast Mating strains

Matings between **a** and α strains were carried out by streaking the strains across each other on YEPD plates and incubating for 24 hours at 30°C. Cells from the intersection of the strains were restreaked to a medium selective for the

diploid cells and incubated for 48 hours.

The drop overlay method was used to determine mating type of the mutants (Spencer and Spencer, 1988). Meta and metα strains were crossed with mutants from strain BWG1-7a. This allowed for easy selection of prototrophic diploids. The strains were grown in YEPD broth cultures overnight and diluted 1:10. Two drops of the strain being tested was placed on a selective plate (SD with no amino acids added). One drop of the tester strain was placed on other drop, and one drop was placed in a different location as a control. The plates were incubated for 2-3 days at 30°C, and isolates restreaked to a fresh SD plate. Isolates were checked for the ability to sporulate to verify that they were diploid.

Sporulation

Diploid strains were grown for 2 to 3 days to stationary phase in YEPD, washed 3 times with distilled water and resuspended in 1.0 ml of sporulation medium containing 1% potassium acetate, 0.1% yeast extract, and 0.05% dextrose. Cultures were incubated on the roller drum at 30°C. for 3 to 5 days. Spores were isolated either by the Hydrophobic spore isolation method (Rockmill et al., 1991) or picking tetrads using a Cailloux Micromanipulator (Stoelting).

Hydrophobic Spore Isolation Method

After sporulating for 3 - 5 days, cells were spun down in the microcentrifuge (Beckman) for 10 seconds, supernatant was decanted and the cells were resuspended to a concentration of 5 X 10^8 cells per ml in Zymolyase,

10,000 U (ICN Biochemicals). The cells were incubated for approximately 20 minutes at 30°C., centrifuged at 14,0000 X G for 30 seconds, and washed with distilled water once. Cells were then resuspended in 100 μl of water, agitated for two minutes by Vortex mixer at maximum speed, and the supernatant discarded. After rinsing the tube several times with water, the cells were resuspended in 1.0 ml of 0.01% Nonident P-40 (Sigma), and sonicated on ice for 30 second intervals with 30 seconds rest for a total sonication time of 2 minutes using a Sonicator W225 (Heat Systems-Ultrasonics, Inc.). The cells were then plated on YEPD and incubated for 2-3 days at 30°C.

Tetrads were also dissected using a Cailloux Micromanipulator. The sporulation culture was centrifuged, washed, and resuspended in 150 ul of distilled water. 0.5 mg/ml of Zymolyase in 1 M sorbitol was added to 50 ul of the spore suspension, and digestion allowed to proceed for 10-15 minutes at 37°C. To stop the reaction, 1 ml of sterile distilled water was added, and the suspension placed on ice. A loopful of the suspension was streaked on a YEPD plate, and tetrads were picked from the streak and individual spores were deposited at discrete intervals on the plate. Plates were incubated at 30° C for 1-2 days.

Derivation of rho° strains

Rho° strains were generated by growing the wild-type strains BWG1-7a and PSY142α on YEPD medium containing 20 ug/ml of ethidium bromide for approximately 40 generations (Liao, et al., 1991). Dilutions of the culture were plated on YEPD and isolates were streaked on YEPL plates to see if

mitochondrial function had been lost. Only rho+ strains would be capable of growth on YEPL plates.

Preparation of plasmid DNA

Large scale preparations of plasmid DNA were made by Cesium Chloride-Ethidium bromide gradients (Sambrook, et al., 1989). For smaller scale preparations, Qiagen columns (Qiagen) were used. The boiled lysate method was used for E. coli minipreparations (Holmes and Quigley, 1981).

Purity and integrity of the DNA was verified by running samples on a 5% polyacrylamide gel at 119V for 1 hour and staining with ethidium bromide (Sambrook, et al., 1989).

General plasmids used in this study are shown in Table 2. pSH151, pSH152, and pSH153 were provided by Steve Hahn, and pJO71 and pJO70 were provided by Jennifer Pinkham. pCYC1, pHIS4, and pLEU2 were obtained from L. Guarente's laboratory.

Plasmid Constructions

The plasmids used for deletion constructs have been described previously (Rosenkrantz, et al., submitted), and are shown in Figure 2. Either CIT1 or CIT2 upstream sequences were used to replace the CYC1 (cytochrome c) DNA in plasmid pLG669Z, which is a shuttle vector capable of replicating in both E. coli and S. cerevisiae. Plasmid pLG669Z also contains an ampicillin resistance gene and the URA3 gene as selectible markers in E. coli and S. cerevisiae respectively, and is fused to the lacZ gene so that transcription levels can be

monitored by ß-galactosidase activity (Guarente and Ptashne, 1981). The original CIT1-lacZ construct contained bases from -805 to -10 (numbering from the translation initiation codon) of upstream CIT1 DNA fused to lacZ, and includes the TATA box and the transcription initiation region. The vector contains an EcoRV site at -211 just upstream of the TATA box (-195) and a unique BglII site

at -805 at the junction with the fragment carrying the URA3 gene. This construct is repressed in glucose medium as measured by ß-galactosidase levels, similarly to CIT1 mRNA levels (Kim et al., 1986).

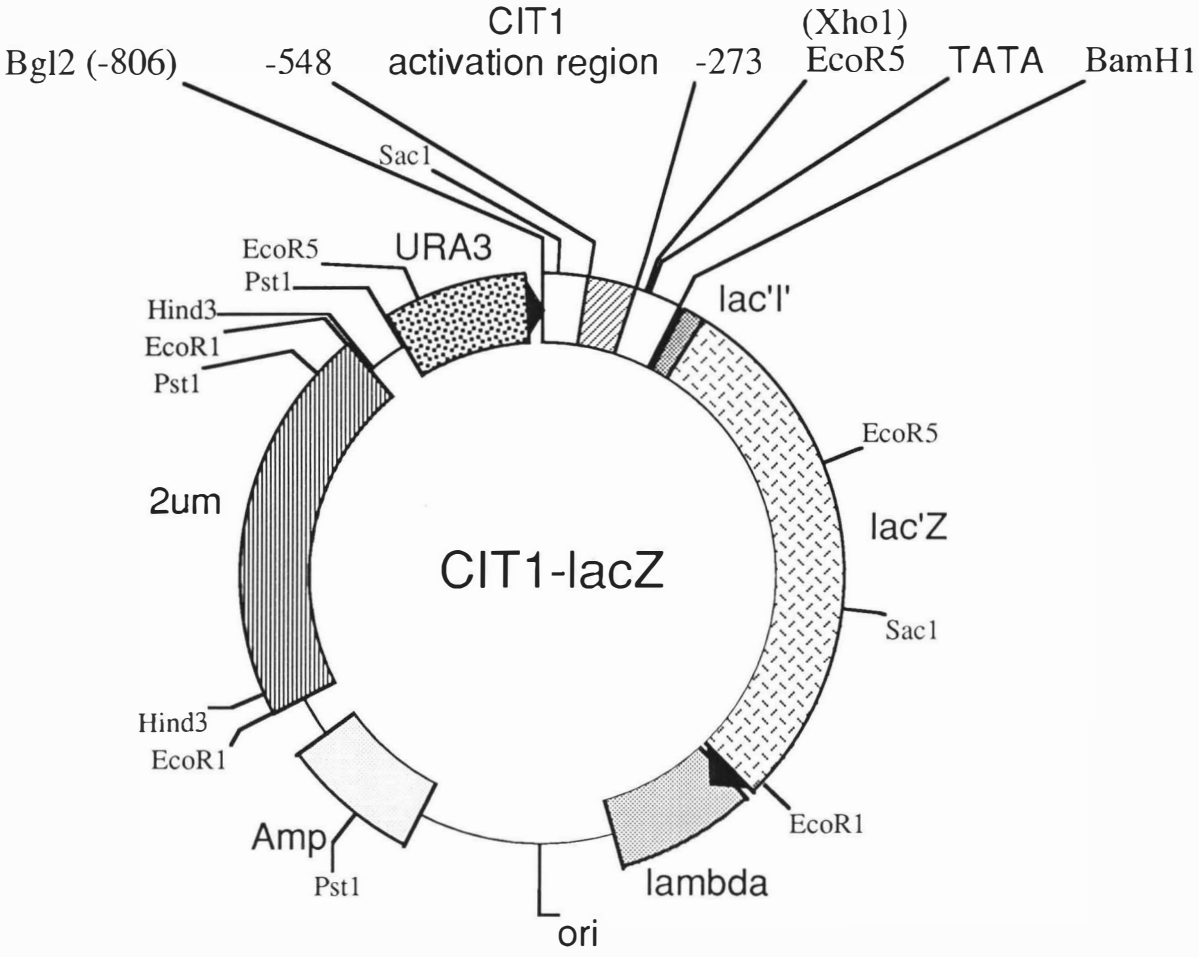
5' deletions C15, LO3, LO5, and .38 were constructed (as previously described) using Bal31 exonuclease digestion (Rosenkrantz et al., submitted). Other 5' deletions were made using polymerase chain reaction. Synthetic oligonucleotides containing a BglII site and 15-20 bases homologous to various locations upstream of CIT1 (synthesized by the Core Facility of the Medical College of Virginia, Virginia Commonwealth University) were used as primers for polymerase chain reaction amplification using Taq Polymerase (Perkin-Elmer). The 3' primer contained 19 bases homologous to the region from -147 to -166. The PCR product was used to replace the wild-type BglII to XhoI (linker at EcoRV) cassette upstream of the TATA box (-195). Constructs were verified by restriction analysis and dideoxynucleotide sequencing (Sanger)(Sequenase 2.0 kit United States Biochemical) as directed by the manufacturer. Both strands were sequenced from the BglII site to the XhoI or EcoRV site to check for misincorporation.

TABLE 3

General Plasmids used in this study

Plasmid	Description, Reference
CYC1	Iso-1-cytochrome c, lacZ fusion, Guarente, 1983
pHIS4	Histidine biosynthesis, lacZ fusion, Hinnebusch et al. 1985
pLEU2	ß-isopropylmalate dehydrogenase, lacZ fusion, Guarente et al., 1984
pSH151	HAP3 coding sequence, CYC1 promoter, lacZ fusion, Hahn et al., 1988
pSH152	HAP3 coding sequence,GAL UAS CYC1 promoter, lacZ fusion, Hahn et al., 1988
pSH153	HAP3 coding sequence, CYC1 promoter Hahn et al., 1988
pJO71	HAP2 coding sequence, GAL UAS CYC1 promoter, Olesen et al., 1987
pJO70	HAP2 coding sequence, CYC1 promoter Olesen et al., 1987

Figure 2. Plasmid used for construction of CIT1 upstream deletions (Rosenkrantz et al., submitted). CIT1 upstream sequences were used to replace the CYC1 DNA in plasmid pL669Z, a shuttle vector capable of replicating in both E. coli and Saccharomyces cerevisiae. AMP, ampicillin resistance gene of E. coli; URA3, uracil gene, selectible marker for yeast; lacZ, beta-galactosidase gene of E. coli


Bgl2 (-806)
-548
CIT1
activation region
-273
(Xho1)
EcoR5
TATA
BamH1
Sac1
EcoR5
URA3
Pst1
Hind3
EcoR1
Pst1
lac'I'
EcoR5
2um
lac'Z
CIT1-lacZ
Sac1
Hind3
EcoR1
EcoR1
Amp
Pst1
lambda
ori

3' deletions were constructed in a similar manner, using synthetic oligonucleotides (MCV Core Laboratory) containing a XhoI site and 15-20 bases homologous to various locations in CIT1 upstream DNA and used as primers for PCR. The CIT2 deletion plasmids were made in a similar way. Table 4 provides a summary of the deletions of both CIT1 and CIT2.

PCR amplification was run on a MJ Research Minicycler for 25 cycles using temperatures of 94°C for dissociation 37°C for annealing and 72°C for extension. The reaction mixture contained 100 ng of template 300 ng of each primer 10 uM of individual dNTP's (Perkin-Elmer) 10 uM $MgCl_2$ and Amplitaq polymerase (Perkin-Elmer). The primers and template were heated to 95°C for 5 minutes to denature the DNA prior to adding the remaining components to the PCR reaction tube and amplifying the DNA.

Dirty-bottle Mutagenesis

To mutagenize the regions homologous to the Hap2,3,4 binding site a PCR-based mixed or dirty-bottle mutagenesis was used (Chiang et al.,1993). Synthesized oligonucleotides complementary to the 3' end of MR4 (-273) and MR5 (-290) which contained the -290 region and the -310 region respectively were used for the mutagenesis. The 8 bases homologous to the Hap2,3,4 site were synthesized using a bottle consisting of 50% wild-type sequence and 50% of a 1:2 dilution of mixed oligonucleotides. This dilution was estimated to give an average of 1 mutation per oligonucleotide.

The oligonucleotides were used as PCR primers and were amplified under the same conditions used for the deletion constructs. The resulting product was

digested with BglII (Bethesda Research Laboratories) and XhoI (Boehringer-Mannheim) ligated to purified plasmid backbone and electroporated into E. coli DH10B cells. DNA was obtained from transformants using the Qiagen protocol and DNA sequences were determined using Sequenase 2.0.

Mutagenesis of Saccharomyces cerevisiae

Yeast were mutagenized using the protocol of Sherman, et al. (1986) Yeast were first transformed with a CIT1 plasmid and then were grown overnight in 150 ml YEPD at 30°C on a rotary shaker at 200 rpm's to an OD_{600} of 0.5 - 1.0. Cells were spun down, washed with 5.0 ml of 10 mM sodium phosphate buffer (pH 7.0) and resuspended in 6.0 ml of 10 mM sodium phosphate buffer. The resuspension was aliquoted to 5 tubes with 1.0 ml per tube. To each tube was added 0, 10, 20, 30, or 40 ul of ethylmethylsulfonate (EMS), and the cultures were incubated on a roller drum for 60 minutes at 30°C. Five ml of 5% sodium thiosulfate was added to quench the reaction. Cells were pelleted, washed twice with 5% sodium thiosulfate, and resuspended in 5.0 ml of YEPD. The cultures were incubated overnight on the roller drum at 30° C. Dilutions of each tube were prepared in sterile distilled water and 100 ul of the dilutions spread on YEPD plates and incubated overnight at 30° C. Colonies were counted, and the concentration of EMS yielding approximately 90-95% killing was plated on minimal medium (pH 7.0) containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal), salts, vitamins, casamino acids, and 2% glucose (Guarente, 1983). Colonies that had increased or decreased expression of the CIT-lacZ fusion were darker

TABLE 4

CIT1 and CIT2 UAS Deletion Plasmids

CIT1 Plasmids with 5' deletions

Plasmid	5'terminus (3' restriction site)	Reference
C15	-806 (EcoRV)	M. Rosenkrantz
Sh4-1MR	-548 (EcoRV)	"
LO3	-457 (EcoRV)	"
LO5	-358 (EcoRV)	"
.38	-324 (EcoRV)	"

CIT1 Plasmids with 3' deletions

plasmid	Region deleted	Reference
pMR1	-361 to -211	M. Rosenkrantz
pMR2	-322 to -211	"
pMR3	-292 to -211	"
pMR4	-275 to -211	"
pMR5	-266 to -211	"
pRV12	-211, control plasmid with XhoI site	"

CIT2 Plasmids with 5' deletions

Plasmid	5' terminus (3' restriction site)	Reference
p12b	-987 (EcoRV)	M. Rosenkrantz
p1-6Sh2	-866	"
p1-12Sh2	-545	
p1-6Sh5	-426 (EcoRV)	"
p	-426 (XhoI)	"
pSh6dil2	-397	
p.65	-361 (EcoRV)	"
	-355 (EcoRV)	"
	-343	
p.60	-308 (EcoRV)	"

CIT2 Plasmids with 3' deletions

pEP18	-355	E. Pennell
pEP19	-341	"
pEP20	-332	"
pEP17	-319	"
pEP34	-315	"
pEP33	-310	"
pEP21	-304	"

or lighter blue respectively.

Transformation of Saccharomyces cerevisiae

Yeast were transformed by the lithium acetate method (Ito, 1983). Cells were grown overnight on YEPD medium to an OD_{600} of 0.5 to 1 and then transformed with 1 ug plasmid DNA obtained from either cesium chloride density gradient or Qiagen column preparations. All plasmids contained the URA3 selectible marker, therefore the cells were plated on SD-CAA-ade plates to select for uracil prototrophy.

Transformation of Escherichia coli

E. coli strain YMC9 (δlacU169 hsdR- hsdM+) were transformed by the calcium chloride method (Sambrook et al. 1989). Competent cells were prepared by the method of Davis, et al. (1981), and were frozen, thawed, and transformed as described by Morrison (1977). Transformed cells were spread on LBamp plates and incubated at 37°C overnight. Isolates were restreaked to LBamp plates for confirmation before using.

For electroporation Electromax DH10B (F-(mcrAΔ(mrr-hsdRMS-mcrBC)φ80dlacZΔM15ΔlacX74deoRrecA lendA1araD139Δ(ara,leu) 7697galUgalKλ-rpsLnyoG)(BRL) cells were used. The plasmid DNA to be used was precipitated with 95% ethanol, washed three times with 70% ethanol, and allowed to air dry. The DH10B cells were added to the precipitated DNA so that the volume electroporated was 20 ul. The cells and DNA were incubated on ice for 1 minute and then placed in the electroporation chamber (BRL E. coli pulser) and the current applied as recommended by the manufacturer using the medium

TABLE 5

Plasmids constructed with mutations in the -299 and -310 regions

Plasmids*	Template used	Mutations
WILD-TYPE, -299		ACCAAAAA
pCK57	pMR3	ACCAA**CC**A
pCK58	pMR3	ACCAA**TC**A
pCK59	pMR3	A**TT**AA**TC**A
WILD-TYPE, -310		TTATTGGA
pCK60	pMR4	T**G**ATTGG**T**
pCK61	pMR4	TT**G**TT**AA**A
pDBB1	pRV12	TTA**CC**GGA
pDBB2	pRV12	T**C**ATTAGA
pDBB3	pRV12	**C**TATT**C**GA
		Mutations -299/-310
pCK16	pMR4	ACCAA**CC**A/WT
pCK17	pMR4	ACCAA**CC**A/TTATT**AA**A
pCK18	pMR4	insertion of **TTG** at -299

*plasmids are all from this study

setting. Cells were immediately removed and placed in 1.0 ml of SOC medium (Bethesda Research Laboratories) and incubated on a roller drum at 37°C for 1 hour. Cells were then spread on LBamp plates and incubated overnight at 37° C. Isolates were restreaked to LBamp plates for confirmation before using.

Curing cells of plasmids

It was sometimes necessary to cure transformed yeast of their plasmids. Cells containing 2 u-based plasmids were grown overnight in YEPD broth, and streaked on YEPD plates for isolation of single colonies. Several isolates were restreaked to YEPD and glucose minimal plates. Cells which had lost the plasmid and its selectible marker would only be capable of growth on rich medium and not selective minimal medium.

Integrated plasmids were cured by using plates containing 5-fluororotic acid (5-FOA)(Boeke, et al., 1987). Yeast cells were grown overnight in liquid YEPD medium and an aliquot was streaked on 5-FOA plates. Cells containing the URA3 gene convert 5-FOA to a toxic compound which inhibits their growth, allowing resistant colonies (uracil auxotrophs) to grow (Boeke, et al., 1987). Plates were incubated for 4-7 days, and the resistant colonies obtained were restreaked to selective medium lacking uracil to verify the phenotype.

ß-galactosidase assays

ß-galactosidase assays were done as previously described (Guarente, 1983). Cells were grown to saturation (48-72 hours) in YEP-gal medium. An aliquot was then used to inoculate synthetic glucose or lactate medium. Cultures were grown overnight at 30°C to mid-log phase, permeablized, and

assayed for ß-galactosidase activity by measuring ONPG degradation on a Beckman Spectrophotometer at 420 nm. To calculate Miller Units of *β*-galactosidase activity, the following formula was used (Miller, 1972):

$$\frac{OD_{420} \text{ X } 1000}{OD_{600} \text{ X ml X } \Delta \text{ T (min)}}$$

Many of the strains used in this research were unable to grow or grew very slowly on nonfermentable carbon sources such as lactate. In order to assay for beta-galactosidase activity in those strains it was necessary to use a different protocol. The wild-type strain was grown on glucose minimal medium overnight and the cells were spun down and washed with sterile distilled water. The cells were then resuspended in lactate minimal medium and assayed for beta-galactosidase activity at 0, 2, 4, 6, and 24 hours to see when derepression was occurring. Figure 3 is a graph of those results. Also shown is the results for strain LGW1 (hap2-1) which cannot grow on lactate medium. Since approximately 50% of derepression of transcription had occurred by 6 hours we used this method for analyzing beta-galactosidase activity when using strains incapable of growth on lactate medium.

Extract Preparation

Yeast cell extracts were prepared as described by Arcangoli and Lesure (1985). Cells were grown overnight on minimal medium plus 2% glucose, centrifuged, and resuspended in extraction buffer (200 mM Tris-HCl pH8.0, 10 mM $MgCl_2$, 400 mM $(NH_4)_2SO_4$, 1 mM EDTA, 10% glycerol, 1ug/ml leupeptin, 1 ug/ml pepstatin, 1mM phenylmethylsulfonylfluoride (PMSF), and 7 mM ß-mercaptoethanol. The cells were disrupted by glassbead lysis using equal

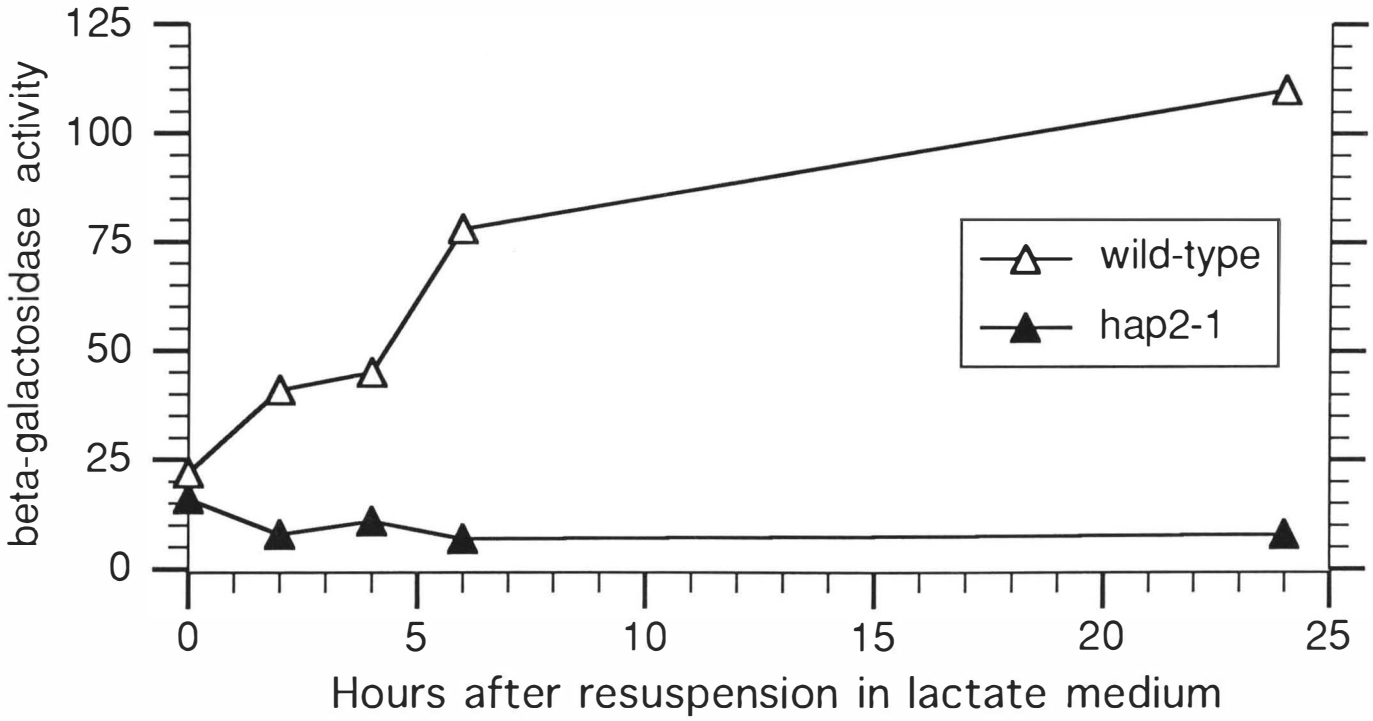
volumes of extract and glass beads. In order to break open the cells more efficiently, a small piece of glass rod was added to each tube during disruption. The extract was then centrifuged for 1 hour at 10,000 x g, and the resulting supernatant was precipitated with 40% $(NH_4)_2SO_4$. The protein was resuspended in 20mM Hepes, pH 8.0, 5 mM EDTA, pH 8.0, 1mM PMSF, 7 mM ß-mercaptoethanol, and 20% glycerol. Extracts were stored at -70° C. Extracts were also made from respiratory deficient cells by growing overnight in minimal medium plus glucose, centrifuging, and then resuspending in minimal medium plus 2% lactate for 5 hours.

Probe for DNA binding

The probe used for gel-binding experiments was a fragment of CIT1 upstream DNA that was obtained by Polymerase Chain Reaction using oligonucleotides complementary to the 3' end of the URA3 fragment and a primer homologous to 10bp of CIT1. Templates used were Qiagen preparations of the 5' deletions 3' deletions or mutants generated during this study.

100 ng of template and 300 ng of each primer was used in the reaction mixture. After denaturing the primers and template at 95°C for 5 minutes the polymerase chain reaction was run on a MJ Research Minicycler for 25 cycles using temperatures of 94°C for dissociation, 37°C for annealing, and 72°C for extension. Amplitaq polymerase (Perkin-Elmer) and 10 uM of each dNTP's (Perkin-Elmer) were used in the reaction mixture. Labelled α-^{32}P-dCTP nucleotide (specific activity 3,000Ci/mmole)was obtained from NEN/Dupont. 200 mCi ofα^{32}P-dCTP were added to a 100 ul PCR reaction. After the

Figure 3: Beta-galactosidase activity as a function of time. Cells of the wild-type strain and LGW1 (hap2-1) were grown overnight in glucose medium and then resuspended in lactate medium. Samples were taken at two hour intervals until 6 hours when 50% of derepression had occurred. A final sample was taken at 24 hours.


beta-galactosidase activity
125
100
75
50
25
0
0
5
10
15
20
25
Hours after resuspension in lactate medium
wild-type
hap2-1

completion of 25 cycles, samples were allowed to extend for 7 minutes and were stored at 4°C. The samples were extracted with chloroform and precipitated with ethanol. The DNA was then run on a 5% polyacrylamide gel at 120V for 1 hour with 100 bp size markers to separate the desired fragment from any single-stranded product or spuriously amplified fragments. The gel was stained with ethidium bromide and the correct size fragment was marked. The gel was next exposed to film for 15 minutes to verify that sufficient incorporation of radioactivity had occurred. The appropriate band was excised and electroeluted into a 10 M ammonium acetate salt cushion for 90 minutes at 150V. The DNA was precipitated with ethanol, resuspended in TE and counted in a scintillation counter to determine the incorporation of radioactivity. Counts were adjusted with buffer to approximately 10,000 counts per ul before using in the gel-binding assays.

Gel-binding Assay

One of the most widely used methods for investigating protein and nucleic acid interaction is gel-retardation (Lane, et al., 1992). When protein binds to a DNA fragment, there is an alteration in the electrophoretic mobility of the fragment through a nondenaturing polyacrylamide or agarose gel. By using radioactively labelled DNA, the bands can be visualized by autoradiography (Garner and Revzin 1981; Lane, et al., 1992). Many parameters can effect the binding and movement of the DNA through the gel. Some of these include the salt concentration of the binding buffer, concentration of nonspecific competitor, amount of protein present, concentration of labelled DNA, temperature of running

gel, etc. These factors influence the stability of the binding complex and the migration of the complex through the gel (Revzin 1989).

The gel-binding assay was similar to that described by Arcangoli and Lescure(1985). The binding buffer contained 4% glycerol, 4mM Tris pH 8.0, 4mM $MgCl_2$, and 10 ul/ml of 10X loading dye (Sambrook et al., 1989). Gels contained 5% polyacrylamide (29 acrylamide:1 bisacrylamide) and were prerun using 1X TBE buffer (Sambrook, et al., 1989) for 30-60 minutes at 150V before loading samples.

Reaction mixtures contained binding buffer, 10,000 cpm of labelled DNA (generally 1-2 μl), 2 μg of polydI:dC (Pharmacia), and 10 μg of protein extract unless otherwise noted. Protein concentrations were estimated using the Biorad Protein Assay (Biorad Laboratories) with bovine serum albumin (Biorad Laboratories Sigma Chemical Co.) as standard. Reactions were incubated at room temperature for 25 minutes, and the samples were loaded onto a running gel with voltage applied during loading. Electrophoresis was ended when the bromphenol blue ran off the bottom (approximately 11/2 hours), and the gel was transferred onto Whatman Blotting Paper (Fisher Scientific), dried at 80° C for 30 minutes, and exposed to autoradiography film (Kodak) using Lightning Plus intensifying screens (Kodak) for 24 hours at -70°C.

RESULTS

One of the primary goals of this research was to identify regions of CIT1 upstream DNA involved in regulation of transcription. Two approaches were used: deletion and base substitution analyses.

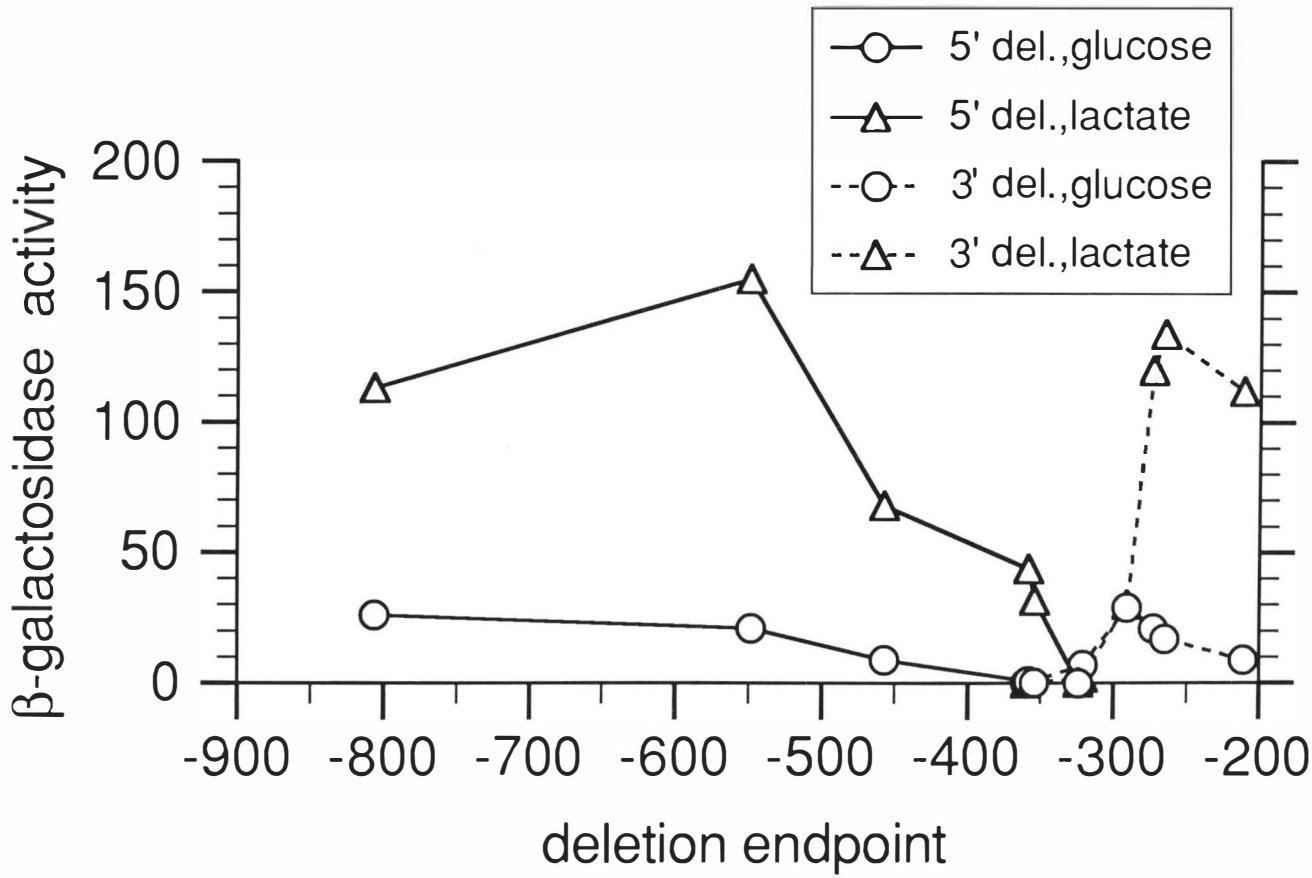
Deletion analyses of CIT1

To identify regions of importance in the DNA upstream of CIT1, 5' and 3' deletions of upstream DNA were constructed and analyzed for their effect on expression and transcriptional regulation. A CIT1-lacZ fusion plasmid containing 806 bp of upstream DNA is regulated similarly to CIT1 mRNA levels (Kim et al., 1986). Deletion of a region involved in activation of transcription should show a corresponding decrease in levels of ß-galactosidase activity. If the region contains a cis-acting element involved in silencing or negative regulation, then the ß-galactosidase activity should increase when this region is deleted.

a. Results in glucose medium using 5' deletions

The wild-type strain, BWG1-7a, was transformed with the 5' and 3' deletion constructs of CIT1 described in Materials and Methods, and the transformants were assayed for ß-galactosidase activity after growth in liquid medium. 5' deletion from -806 (measured from the translation start site) to -548 did not decrease expression of ß-galactosidase activity (derepressed activity actually increased somewhat)(Fig. 4). Deletion beyond -548 gradually reduced

Figure 4: 5' and 3' deletion mapping of CIT1 regulatory elements in a wild-type strain grown in glucose or lactate medium. ß-galactosidase activity is measured in Miller Units (Miller, 1972). Deletion endpoints are measured from the translational start site. 3' deletions were made in the -457 background and contain the same upstream DNA as that construct. All 3' deletions retain wild-type DNA downstream from -211.


5' del.,glucose
5' del.,lactate
3' del.,glucose
3' del.,lactate
β-galactosidase activity
200
150
100
50
0
-900
-800
-700
-600
-500
-400
-300
-200
deletion endpoint

activity in glucose-grown cells until -344 where all activity is gone. This could be attributed to deletion of multiple sequences essential for activation. Alternatively it is possible that this is an artifact of the deletion analysis. The movement of the URA3 gene and neighboring sequences closer to the CIT1 activation region and TATA box may possibly inhibit binding of an activator protein or the transcription initiation complex. A less intrusive method that can distinguish between these possibilities is linker scanning (McKnight, 1992) E. Pennell has made 10 bp substitutions to the complementary sequence between -548 and -457 with little effect on expression (E. Pennell, personal communication) L. Devenish has made substitutions in the region -371 to -344 where glucose repressed expression and derepressed expression is ultimately lost (Fig. 4). Substitutions from -367 to -358, or -357 to -348 reduce expression 3 to 4-fold in glucose-grown cells (Rosenkrantz, et al., submitted). The sequence of the DNA upstream of the translation start site (-195) is shown in Figure 5. The region from -367 to -348 is underlined.

b. Results in lactate medium using 5' deletions

Activity in cells transformed with the 5' deletions and grown in lactate minimal medium declined in a gradual manner similar to glucose-repressed expression. Essentially all activity was lost by -344. 10 bp substitutions between -367 and -348 had no effect on expression of CIT1 in lactate-grown cells.

c. Results in glucose and lactate medium using 3' deletions

3' deletions of DNA upstream of the TATA box also were constructed to identify

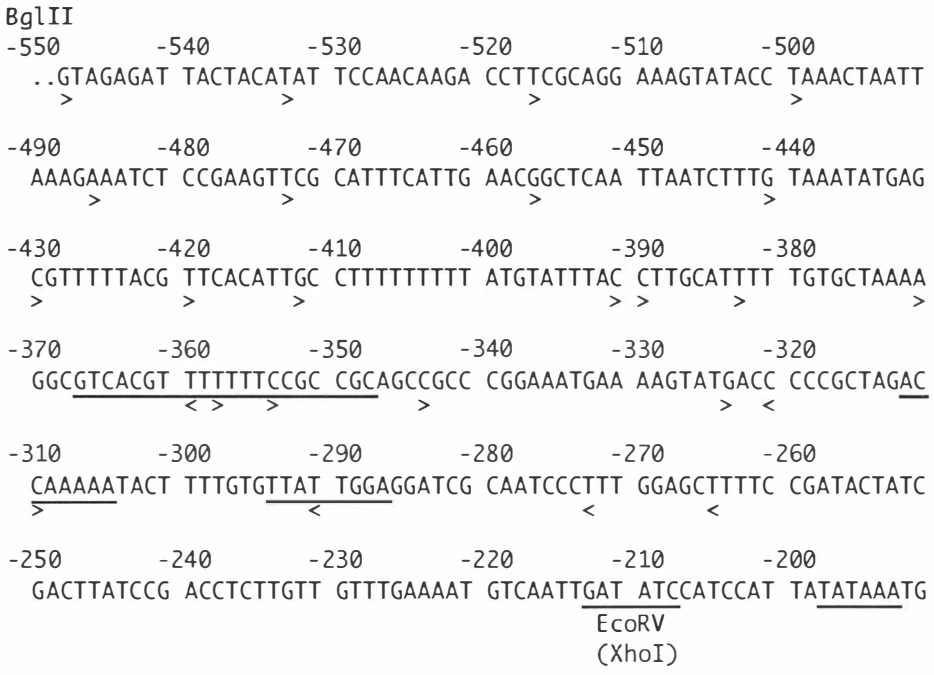
downstream regulatory element(s). Deletions from the 3' end of the -457 5' deletion construct removed bases from -211 to -265, -273, -291, -321, and -360. Removal of sequences from -211 to -265 or to -273 caused an increase in expression in cells grown in glucose or lactate minimal medium (Fig. 4, Fig. 5). However, further deletion to -291 decreased ß-galactosidase activity 4-fold when cells were grown in lactate minimal medium. The remaining level of expression was similar to that in glucose-grown cells, which was increased slightly by the deletion. This suggested that the region between -273 and -291 contains an element essential specifically for derepression of CIT1 in lactate-grown cells. Further deletion to -321 or -360 reduced activity in both glucose-grown and lactate-grown cells.

Examination of the DNA sequence between -321 and -273 revealed two regions of homology with the HAP2,3,4 binding site, ACCAATNA, one at -310 and another at -290. These sites differ by 1 bp from consensus (Fig. 4). Expression in lactate-grown cells decreased significantly when 4 bases (-287 to -290) of the -290 site were removed (-291 deletion), and removal of both sites (-321 deletion) further decreased activity in both lactate and glucose grown cells. These results suggested that the -290 candidate HAP2,3,4 site is important in derepression, and may be regulated by the HAP2,3,4 heteromeric protein complex. The role of the -310 candidate HAP2,3,4 site was unclear.

Regulation by HAP2,3,4

To determine if the upstream region of CIT1 is regulated by the HAP2,3,4 complex, the full-length construct (-806) was transformed into the isogenic

Figure 5: Upstream DNA sequence of CIT1 DNA. Areas thought to be involved in activation of transcription in glucose and lactate media and the TATA box are underlined. The 5'(>) and 3'(<) deletion constructs are indicated. (M. Rosenkrantz et al., submitted). 3' deletions were made in the -457 background, starting from a Xho1 linker placed at -211, and retain all wild-type DNA downstream of -211, and 5' of the deletion to -457.


BglII
-550 -540 -530 -520 -510 -500
..GTAGAGAT TACTACATAT TCCAACAAGA CCTTCGCAGG AAAGTATACC TAAACTAATT
-490 -480 -470 -460 -450 -440
AAAGAAATCT CCGAAGTTCG CATTTCATTG AACGGCTCAA TTAATCTTTG TAAATATGAG
-430 -420 -410 -400 -390 -380
CGTTTTTACG TTCACATTGC CTTTTTTTTT ATGTATTTAC CTTGCATTTT TGTGCTAAAA
-370 -360 -350 -340 -330 -320
GGCGTCACGT TTTTTTCCGC CGCAGCCGCC CGGAAATGAA AAGTATGACC CCCGCTAGAC
-310 -300 -290 -280 -270 -260
CAAAAATACT TTTGTGTTAT TGGAGGATCG CAATCCCTTT GGAGCTTTTC CGATACTATC
-250 -240 -230 -220 -210 -200
GACTTATCCG ACCTCTTGTT GTTTGAAAAT GTCAATTGAT ATCCATCCAT TATATAAATG
EcoRV
(XhoI)

strains LGW1 (hap2-1), JP40 (hap3-1), and SLF401 (HAP4::LEU2). To further map any effect of the HAP2,3,4 complex, the wild-type and mutant strains were transformed with the 5' and 3' deletion constructs. Similar results were obtained with all three mutant strains. Only results from LGW1 (hap2-1) strain are shown here (Fig. 6 and 7).

a. Effect of hap2-1 on expression of CIT1-lacZ fusion in glucose-grown cells

Figure 6 compares the ß-galactosidase activity of strains LGW1 (hap2-1) and BWG1-7a transformed with the 5' and 3' deletion constructs and grown in glucose medium. Deletion of a HAP2,3,4-dependent activation site should reduce expression in the wild-type strain, but not in the hap2-1 mutant. Only 3-5 units of the expression in glucose-grown cells was HAP2-dependent. This effect was not seen in the 3' deletions. The closer positioning of the TATA box in the 3' deletion constructs may compensate for the minor role of HAP2 by increasing activation by HAP2-independent mechanisms.

b. Effect of hap2-1 on expression of a CIT1-lacZ fusion in lactate-grown cells

Since strains deficient in HAP2,3,4 are respiratory deficient and cannot grow on nonfermentable carbon sources an alternative method of derepression was required. ß-galactosidase activity was measured by resuspending glucose-grown cells in lactate minimal medium for 5-6 hours, yielding partial derepression (Materials and Methods).

None of the 5' deletions strongly reduced expression of CIT1 specifically

in the wild-type strain (Figure 7). However, when the 3' deletions were tested, it was found that deletion to -291, removing half of one of the HAP2,3,4 consensus binding sites, reduced expression only in the wild-type strain. Furthermore, the remaining level of expression was similar to that in the hap2-1 strain resuspended in lactate and to that seen in both strains in glucose-grown cells (Figure 6). These facts suggest that this site is necessary for derepression of transcription of CIT1 and that it serves as a binding site for HAP2,3,4. Further deletion to -321, eliminating the other candidate HAP2,3,4 binding site at -310, resulted in a decrease in expression in both strains, and in both glucose-grown cells and lactate-resuspended cells (Fig. 6 and Fig. 7). Perhaps a site involved in HAP2,3,4-independent activation of transcription was destroyed or moved too close to the TATA box to function. In any case functionality of the -310 site requires further testing.

Effect of mutations in candidate HAP2,3,4 binding sites

Various mutations were made in both candidate HAP2,3,4 binding sites to more strictly test the functionality of either site. Figure 8 is a summary of the changes made. The pCK series of mutations were changes made by PCR with specific mutagenic primers. The pDBB group of mutants were isolated using the PCR-based dirty-bottle mutagenesis procedure described in Materials and Methods. For technical convenience, changes in the -290 site were made in the -273 3' deletion background. A 1 bp change in the UAS2 (ACCAA**C**CA of CYC1 (cytochrome c) to ACCAATCA (UAS2up1) increased expression in glucose-grown cultures 20-fold and lactate-grown cultures 2-fold (Forsburg and Guarente,

Figure 6: 5' and 3' deletion mapping of CIT1 upstream regulatory regions in wild-type and hap2-1 strains grown in glucose medium. ß-galactosidase activity is measured in Miller Units (Miller, 1972). Deletion endpoints are distance in bp from the translational start site. The location of the candidate HAP2,3,4 sites are indicated by boxes.

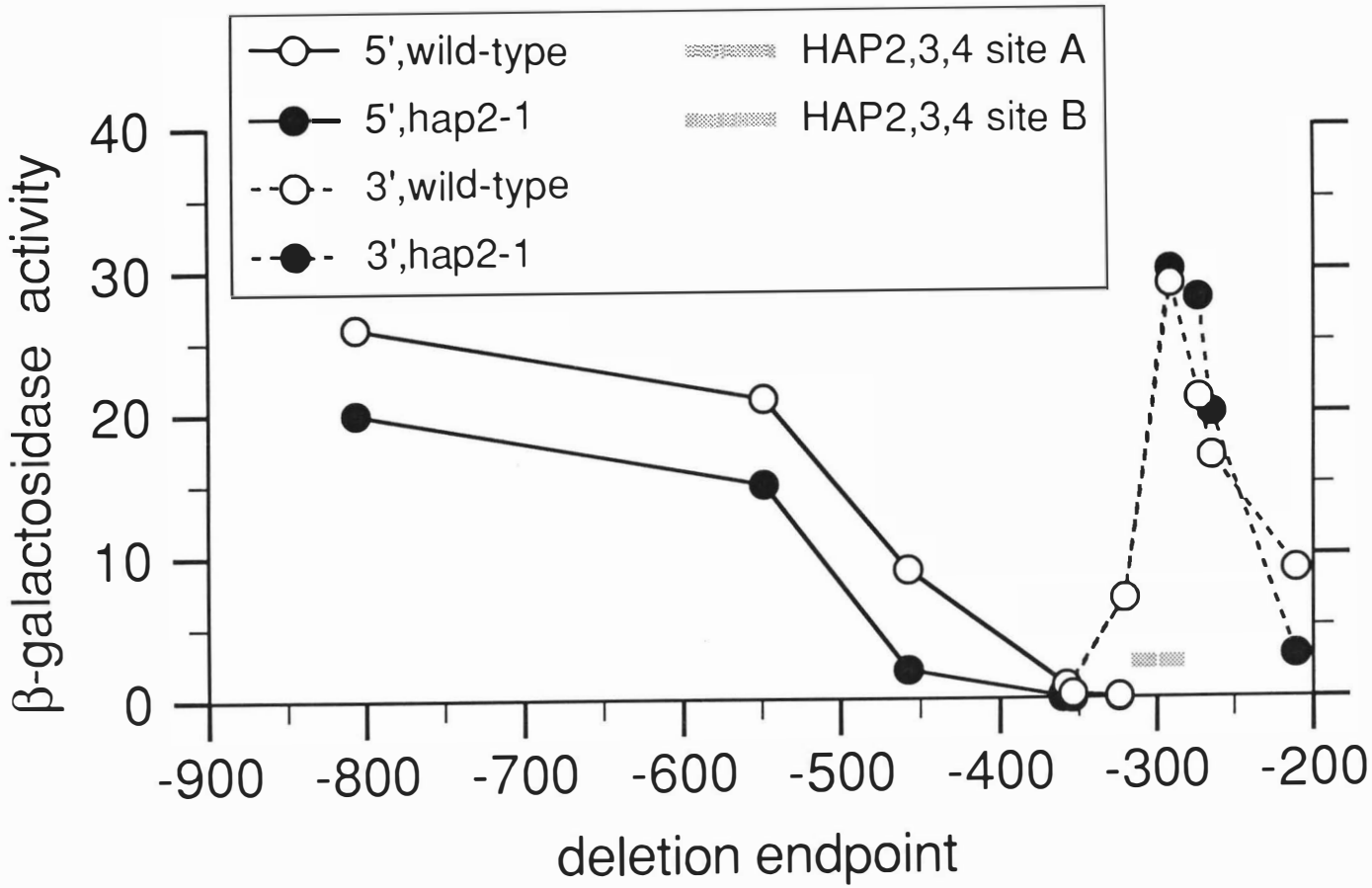
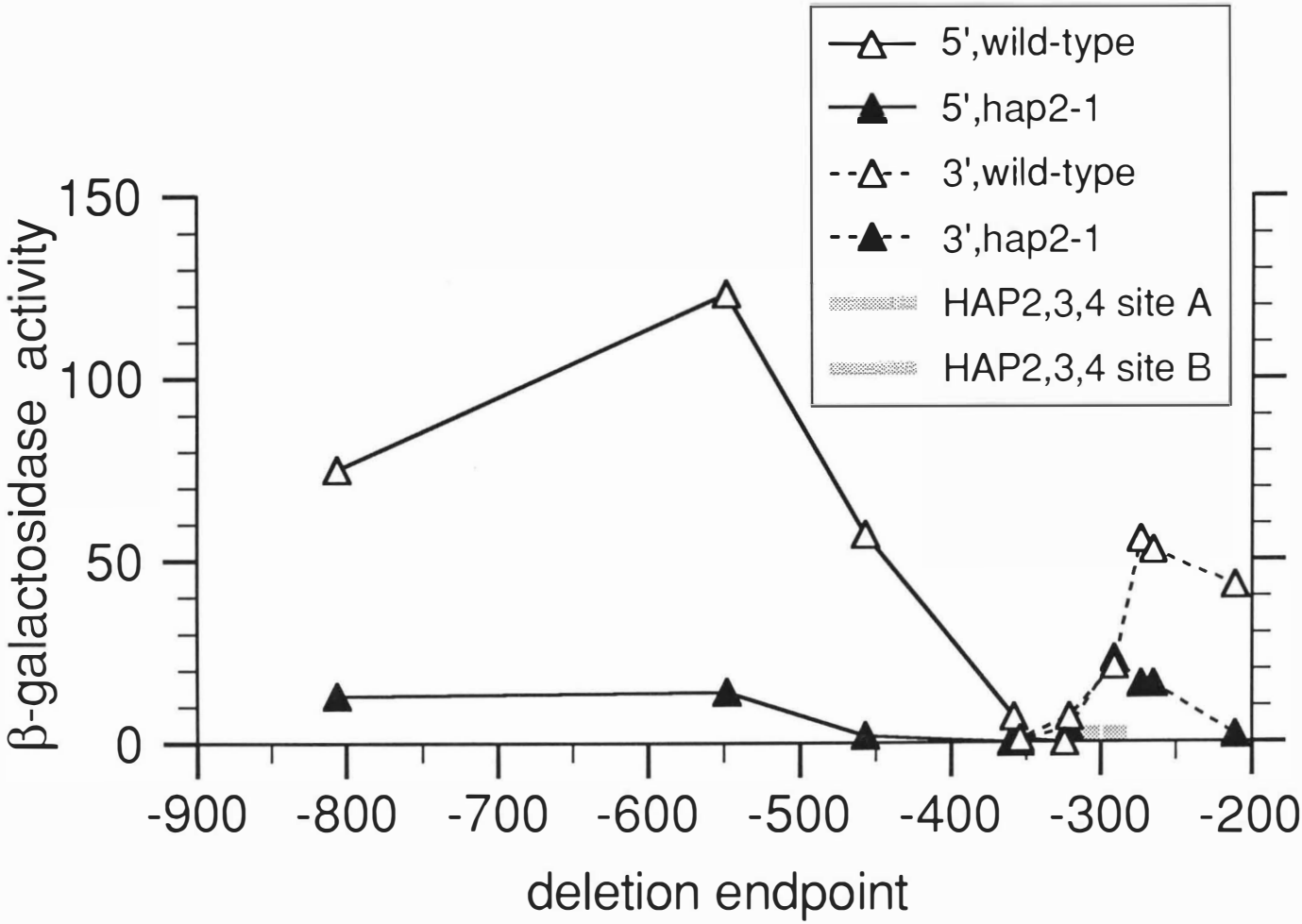

5',wild-type
5',hap2-1
3',wild-type
3',hap2-1
HAP2,3,4 site A
HAP2,3,4 site B
β-galactosidase activity
0
10
20
30
40
-900
-800
-700
-600
-500
-400
-300
-200
deletion endpoint

Figure 7: 5' and 3' deletion mapping of CIT1 upstream regulatory regions in wild-type and hap2-1 strains resuspended in lactate medium. ß-galactosidase activity is measured in Miller Units (Miller, 1972). Deletion endpoints are measured in distance in bp from the translational start site. The location of the candidate HAP2,3,4 sites are indicated by boxes.


5',wild-type
5',hap2-1
3',wild-type
3',hap2-1
HAP2,3,4 site A
HAP2,3,4 site B
β-galactosidase activity
150
100
50
0
-900
-800
-700
-600
-500
-400
-300
-200
deletion endpoint

1988). When the -290 site of CIT1 mutated to identify with the UAS2up1 sequence (pCK60), activity was increased in both glucose-grown and lactate-grown cells. The increase was not as pronounced as that seen in CYC1 when UAS2 was mutated to UAS2up1. Perhaps the native -290 site of CIT1 (TCCAATTAA) is more active than the native UAS site of CYC1 (ACCAACCA). All other mutations severely reduced derepressed expression in lactate-grown cells. This is consistent with the -290 site being involved in binding HAP2,3,4. Surprisingly, pCK61 and pDBB2 also had strongly reduced activity in glucose-grown cells, despite the fact that the hap2-1 mutation has only a small effect on CIT1 expression in glucose-grown cells (see Figure 6).

Base substitutions were also introduced into the -310 candidate site. For technical convenience these were made in the -291 background which lacks half of the -290 site, and does not derepress. Mutation of the -310 box to bring the sequence to identity with UAS2 (pCK57), a weak HAP2,3,4 site, severely decreased the activity in glucose-grown cells and lactate-grown cells. Mutations of the -310 box to match UAS2UP1 (pCK58) increased activity modestly in lactate-grown cells, but reduced activity in glucose-grown cells. Additional changes away from consensus (pCK59) prevented the increase in expression in lactate-grown cells and activity in glucose-grown cells was similar to pCK58.

To test the relative contribution of the -310 sequences, the base substitutions present in pCK57 were introduced into the -273 background, retaining the wild-type -290 site (pCK16-11). Derepression in lactate-grown cells was reduced by one-half and glucose-repressed expression was reduced 3.5 fold. Therefore, it appears that both sites activate transcription, with the -290

Figure 8: Effects of mutations of the -290 and -310 candidate HAP2,3,4 binding sites on beta-galactosidase activity. CK mutations were made by PCR using mutated oligonucleotides as primers. DBB mutations were generated using PCR-based dirty-bottle mutagenesis. For technical convenience the -290 changes were made in the -273 background, while the -310 changes were made in the
-291 background. Results are shown for the wild-type and hap2-1 strains.
Beta-galactosidase activity is measured in Miller Units (Miller, 1972).

	ß-galactosidase activity[a]					
	glucose		lactate res.		lactate	plasmid
	WT	hap2-1	WT	hap2-1	WT	
-312 -305 -294 -287 -273						
CGCTAG ACCAAAAA TACTTTTGTG TTATTGGA GGATCGCAATCCCT	21	28	56	17	115	pMR3d4
TNATTGGT						
_G_____T	38	36	113	30	141	pCK60
___CC___	24	36	30	31	35	pDBB1
_C____C_	20	24	29	22	17	pDBB3
__G__AA_	5	10	13	9	12	pCK61
__C__A__	9	16	15	15	7	pDBB2
_____CC_	6	3	24	4	59	pCK16-11
-312 -305 -291						
CGCTAG ACCAAAAA TACTTTTGTG TTAT	29	28	21	19	37	pMR3d3
ACCAATNA						
_____TC_	10	10	18	9	47	pCK58
_TT__TC_	10	22	26	18	17	pCK59
_____CC_	5	8	11	7	7	pCK57

a) Isogenic wild-type (BWG1-7a) and hap2-1 (LGW1) strains were grown in minimal medium containing 2% glucose or 2% lactate, or were resuspended from glucose medium to lactate medium for 6 hours (lactate res.). ß-galactosidase activity is expressed as the change in OD_{420} x 1000 divided by (minutes x milliliters of culture x OD_{600} of the culture).

site perhaps the more important of the two. Since the sum of the remaining derepressed expression of pCK16-11 and pCK61 (or pDBB1,2,or 3) is less than that of the wild-type (pMR3d4), it appears that activation by the two sites may be synergistic.

The close spacing of two candidate HAP2,3,4 sites is unusual. The spacing of the wild-type regions is approximately 2 helical turns, which should put the proteins on the same side of the helix, and allow them to interact. Attempts were made using PCR to add 3 bases between the two sites (generating a HpaI restriction site) which would increase the spacing by a partial turn of the helix. The addition of the HpaI site allowed rapid screening after cloning and transformation. Over 100 colonies were examined using a HpaI restriction digest. Four bands were seen in the wild-type digest. 108 minipreparations were screened, and none of these had a change in the banding pattern that would indicate that the new HpaI site had been incorporated into the sequence. The oligonucleotide used for synthesis was 42 bases and the mutation started 4 bases from the 5' end. Possibly it did not hybridize well to the complementary strand during annealing.

Gel-binding assays

Various attempts were made to determine whether the HAP2,3,4 protein complex binds to the upstream DNA of CIT1, and whether that binding requires wild-type -290 or -310 sites. Protein extracts were prepared as described in Materials and Methods from BWG1-7a cells grown in YEP-rich or minimal medium plus glucose or lactate and from BWG1-7a transformed with pJO71,

pJO70, pSH151, pSH152, and pSH153. pJO71 and pSH152 contain the GAL1,10 UAS and the CYC1 TATA box and transcriptional start site, and overproduce HAP2 and HAP3 respectively when grown in galactose medium. Plasmids pJO70, pSH151, and pSH153 contain the CYC1 promoter and when derepressed in lactate medium, produce HAP2 (pJO70) or HAP3 (pSH151, pSH153). Crude extracts were made from all cultures as described in Materials and Methods.

DNA probes were labelled with ^{32}P-dCTP using PCR as described in the Materials and Methods. The primers were complementary to the 3' region of URA3 just upstream of CIT1 DNA and a 19 bp region starting at -147; templates were plasmids bearing the -548, -457, and -359 5' deletions. The labelled fragments were gel-purified, and the DNA electroeluted and resuspended to a final concentration of 10,000 cpm/ul (0.5-1.0 ug/ml). Olesen et al. (1987 demonstrated binding of HAP2,3 to UAS2UP1 by gel mobility shift assay (GMSA). They identified the band by using extracts from hap2-1 and hap3-1 strains. They verified that the HAP2,3 complex was binding by fusing HAP2 or HAP3 to lacZ (pJO60 and pSH151) producing bifunctional fusion proteins that gave rise to bound complexes that migrated more slowly in the gel. They were unable to demonstrate binding to the native UAS2. Using similar protocols for protein preparations and gel conditions, we were unable to detect any bands which were dependent on the HAP2, HAP3, or HAP4 genes. Figure 9 shows some of the results we obtained using GMSA.

In previous work, two CIT1 complexes specific to lactate-grown cells were

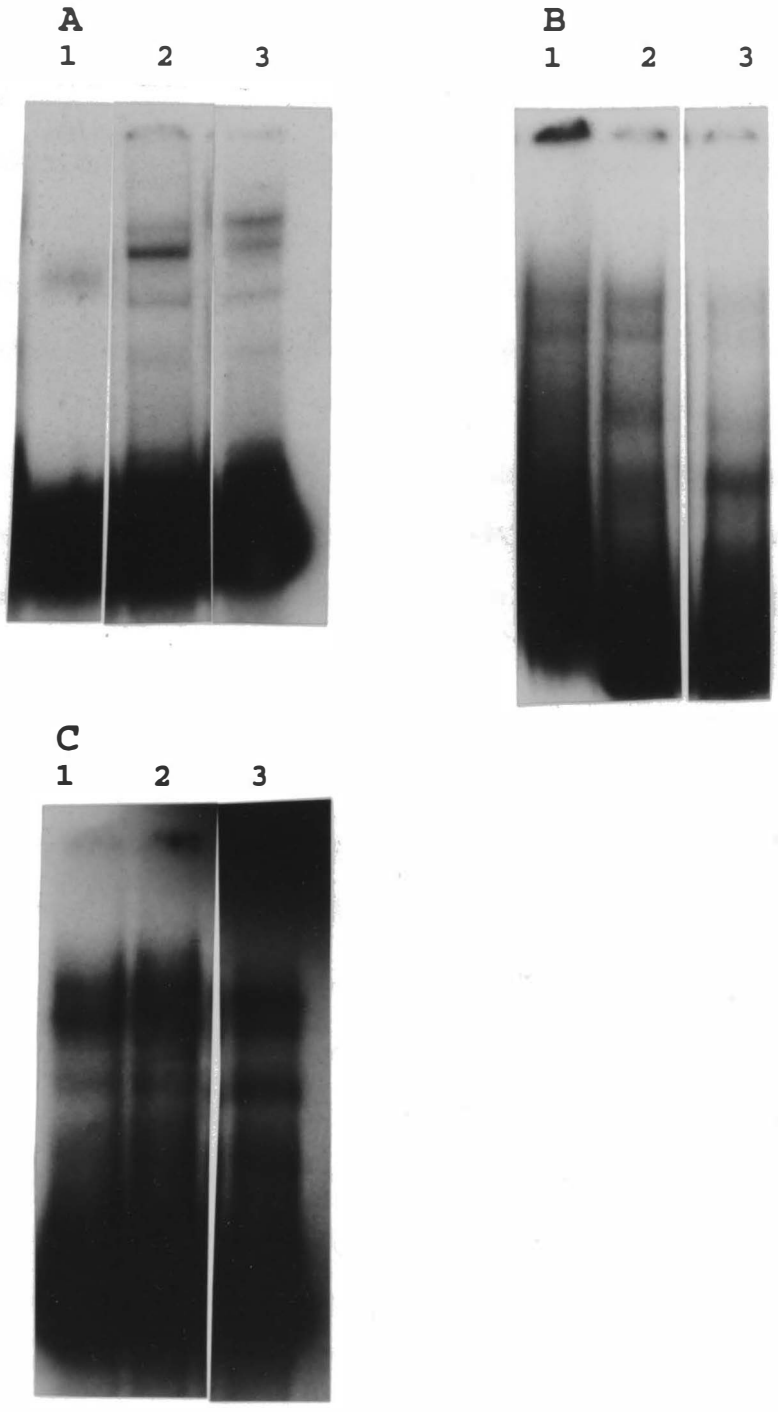
detected using crude protein extracts and a -359 to -211 DNA probe (M. Rosenkrantz, personal communication). We were unable to duplicate those results using similar techniques in this laboratory.

Since many variables can alter the binding pattern, we tried to enhance binding by changing components of the reaction mixture. First, we varied the protein concentration. The protein concentration was determined by Biorad Protein Assay (Biorad), and approximately 10 - 50 ug of protein was used. Arcangioli and Lescure (1985) used 20 ug of protein and Olesen et al. (1987) used 50 ug. The amount of nonspecific competitor (dI:dC) had to be increased to prevent the proteins from being retained in the wells when the higher protein concentrations were used. No bands specific for HAP2,3,4 were detected (data not shown).

The KCl concentration was varied from 40uM to 100uM. No differences were seen in the binding pattern using the salt concentrations mentioned (data not shown).

The next variable investigated was the nonspecific competitor. Arcangioli and Lescure (1985) and Olesen et al. (1987) used 1-5 ug of sonicated double-stranded salmon sperm DNA for their gel-binding assays. We varied the concentration from 0.5 ug to 10 ug/lane of polydI:dC, and found that 2ug gave optimal results for protein amounts in the 10-20 ug range. Boiling the dI:dC b/efore use did not enhance binding. Sonicated calf thymus DNA was also tried as a nonspecific competitor in concentrations similar to those examined for dI:dC. No significant differences were noted in banding pattern. However, dI:dC gave sharper bands and was therefore used in all following experiments (data

Figure 9: Gel Binding Assay: 5% Polyacrylamide gels were run for hours at 150V at 22°C. Protein extracts were made as described in the text. A,B, and C were separate gels. Lanes A1-3, B1, B3, and C3 contain 50 ug protein, B3, contains 20 ug, C1 and 2 contain 10 ug purified protein (generously provided by L. Guarente's laboratory).
Protein extracts used: Lanes A1 no protein, A2 HAP3 fused to beta-galactosidase (grown in lactate minimal medium), A3 wild-type (grown in lactate rich medium)
B1 HAP3 overproducer (grown in galactose minimal medium), B2 HAP2 overproducer (grown in glucose minimal medium), B3 HAP2 overproducer (grown in lactate minimal medium) C1 HAP2, HAP3 purified proteins; 2 ul protein extract; C2 HAP2, HAP3 purified protein, 3 ul protein extract C3 wild-type strain (grown in lactate rich medium)


A
1
2
3
B
1
2
3
C
1
2
3

not shown).

The time and temperature of the binding reaction were also varied. The original assays were performed by incubating the reaction mix at room temperature for 20 minutes (Olesen et al. 1987). (Incubation of the binding reaction at 4°C also had no effect.) To see if timing was a critical factor, a series of binding assays were run with the following incubation times: 1, 5,10, 20, and 30 minutes. There was no difference seen at any of those time points (data not shown).

Temperature of the electrophoresis was also examined. Running the gel at room temperature gave cleaner bands than 4°C, but the binding pattern seen was not altered. All further experiments were run at room temperature (data not shown).

We received purified HAP2 and HAP3 expressed in E. coli, and crude yeast protein extract from Y.Y. Xing and L. Guarente (Massachusetts Institute of Technology) to use in the binding assays. HAP2 and HAP3 are required for binding and can associate and bind without HAP4 (Guarente, 1992). Xing was able to demonstrate binding of HAP2,3 to UAS2up1 of CYC1 using purified HAP2 and HAP3 plus the crude protein extract (personal communication). These were used with our labelled CIT1 DNA. Also, the purified proteins were added to the overproducer extracts (i.e. HAP2 protein to the HAP3 overproducer extract and HAP3 protein to the HAP2 overproducer extract), HAP2-lacZ and HAP3-lacZ fusion protein extracts, and extracts prepared from wild-type cells grown in YEP-lactate. No bands specific for HAP2,3,4 were seen under any circumstances

(Fig 9).

The lactate-specific bands seen by M. Rosenkrantz were from 45% ammonium sulfate precipitations of cells grown in lactate minimal medium (personal communication). A range of ammonium sulfate fractions obtained from M. Rosenkrantz were tested for CIT1 binding. The protein extracts were made from cells grown in lactate-rich and lactate minimal medium. The only bands detected were enriched in the 55% ammonium sulfate fraction from cells grown in YEP-Lactate medium, and these were not lactate-specific (Fig. 11).

Changing the DNA concentration also did not change the banding pattern. The counts per lane was increased from 10,000 to 80,000 (from 1 ug DNA to 8 ug DNA) with no new bands being seen. Cold probe was added to the hot to see if increasing the concentration of DNA would detect any new bands. Using 100 fold excess of cold probe did not change the results (data not shown).

The probe in the studies done by M. Rosenkrantz was end-labelled using Klenow fragment of DNA pol1 (Sambrook, et al., 1989)(personal communication). Probes used in this study were internally labelled by PCR. However, a probe end-labelled using Klenow fragment was also used to see if additional bands could be detected in the protein extracts from the overproducer strains and the wild-type strain grown on YEP-Lactate medium. No additional bands were detected (data not shown).

The results of the gel-binding assay are inconclusive as to whether HAP2,3,4 directly binds the -310 and -290 regions, especially since I could not reproduce binding to UAS2up1 of CYC1 (data not shown). It is possible our reagents lack or contain some trace element which influences binding. Binding

of HAP2,3,4 to other regulated genes (e.g. LPD1, COX6) has also been inconclusive (Bowman, 1992; Trawick et al., 1992).

Effect of hap1-1 on regulation of CIT1

A HAP1 mutant, hap1-1 was also tested to see if HAP1 was involved in regulation of CIT1. HAP1 is a hemoprotein involved in transcriptional regulation of genes encoding respiratory proteins (Pinkham and Keng 1993). CIT1 is not regulated by heme (T. Keng personal communication). Therefore, it would seem unlikely that HAP1 would be required for activation of CIT1. However, to determine this, isogenic wild-type and hap1-1 strains were transformed with the full-length CIT1-lacZ construct and the 5' and 3' deletion constructs and assayed for ß-galactosidase activity in glucose and lactate media. There is very little difference in expression between the wild-type and hap1-1 strains grown in glucose (Fig. 10) In lactate medium, expression of the -806 CIT1-lacZ fusion was reduced 30% in the hap1-1 strain. Deletion to -291 reduced expression in the wild-type strain more than in the hap1-1 strain, indicating that these regions may be important for activation by HAP1. However, since expression was lower in the former than the latter, this data should be repeated. If a region required for activation by HAP1 cannot be localized, the effect of HAP1 may be indirect. Alternatively, the hap1-1 regulatory region of CIT1 may be located within the sequences critical for expression (approximately -457 to -273).

Regulation of CIT2 by HAP2,3,4

HAP2,3,4 appears to be a global regulator of glucose-regulated nuclear genes involved in mitochondrial respiratory function. CIT2, encoding

Figure 10: 5' and 3' deletion mapping of CIT1 upstream regulatory regions in wild-type and hap1-1 strains grown in glucose medium. ß-galactosidase is measured in Miller Units (Miller, 1972). Deletion endpoints are in distance in bp from the translational start site.

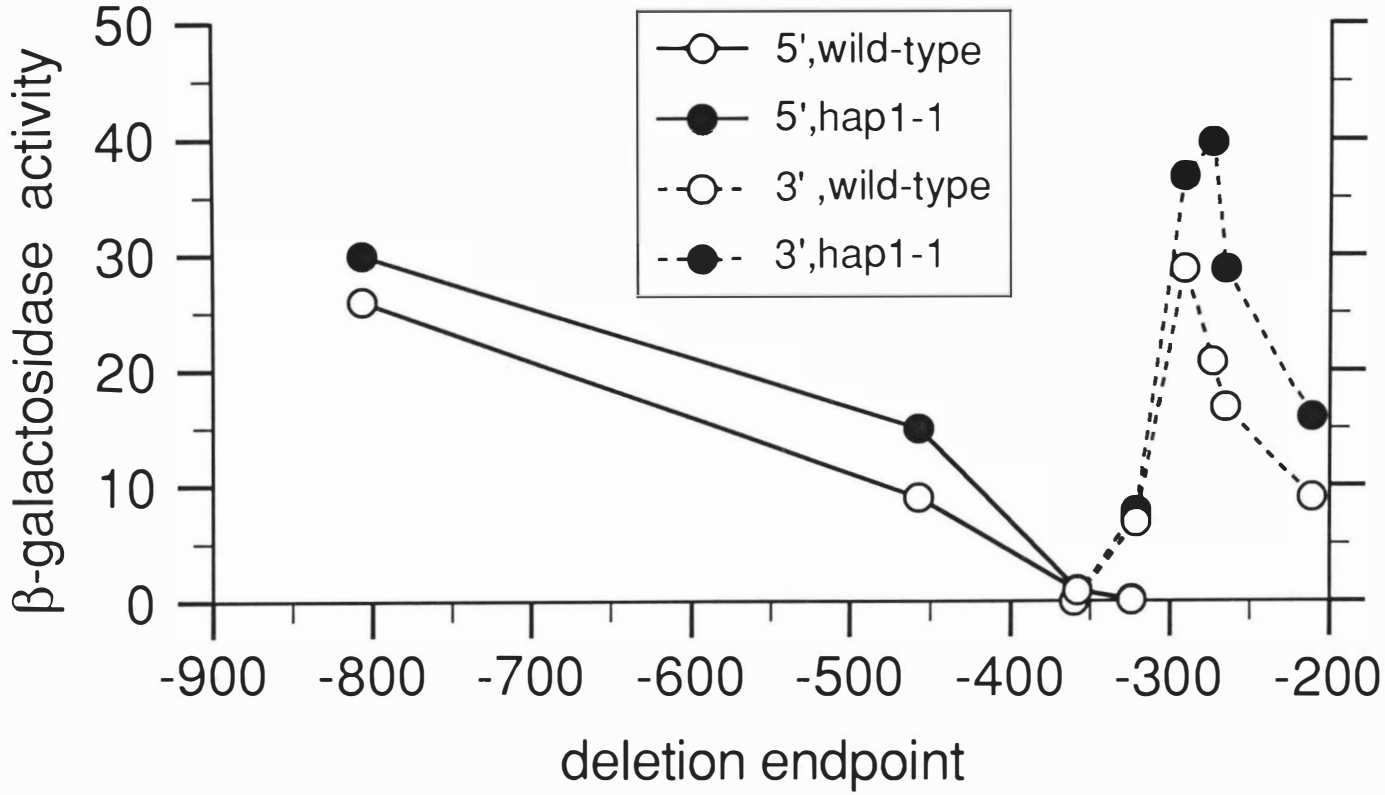
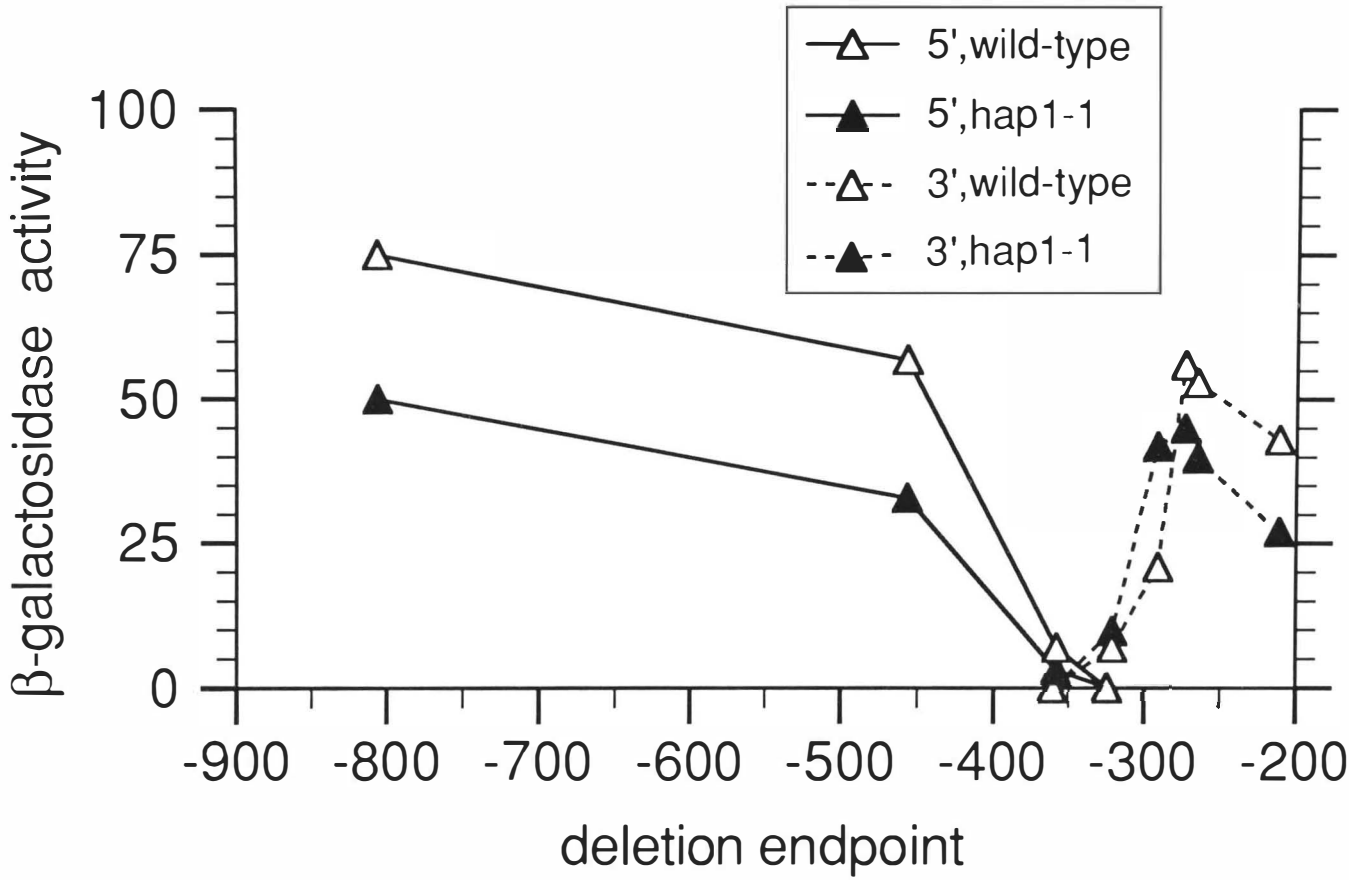

β-galactosidase activity
50
40
30
20
10
0
-900
-800
-700
-600
-500
-400
-300
-200
deletion endpoint
5',wild-type
5',hap1-1
3' ,wild-type
3',hap1-1

Figure 11: 5' and 3' deletion mapping of CIT1 upstream regulatory regions in wild-type and hap1-1 strains grown in lactate medium. ß-galactosidase is measured in Miller Units (Miller, 1972). Deletion endpoints are in distance in bp from the translational start site.


5',wild-type
5',hap1-1
3',wild-type
3',hap1-1
β-galactosidase activity
100
75
50
25
0
-900
-800
-700
-600
-500
-400
-300
-200
deletion endpoint

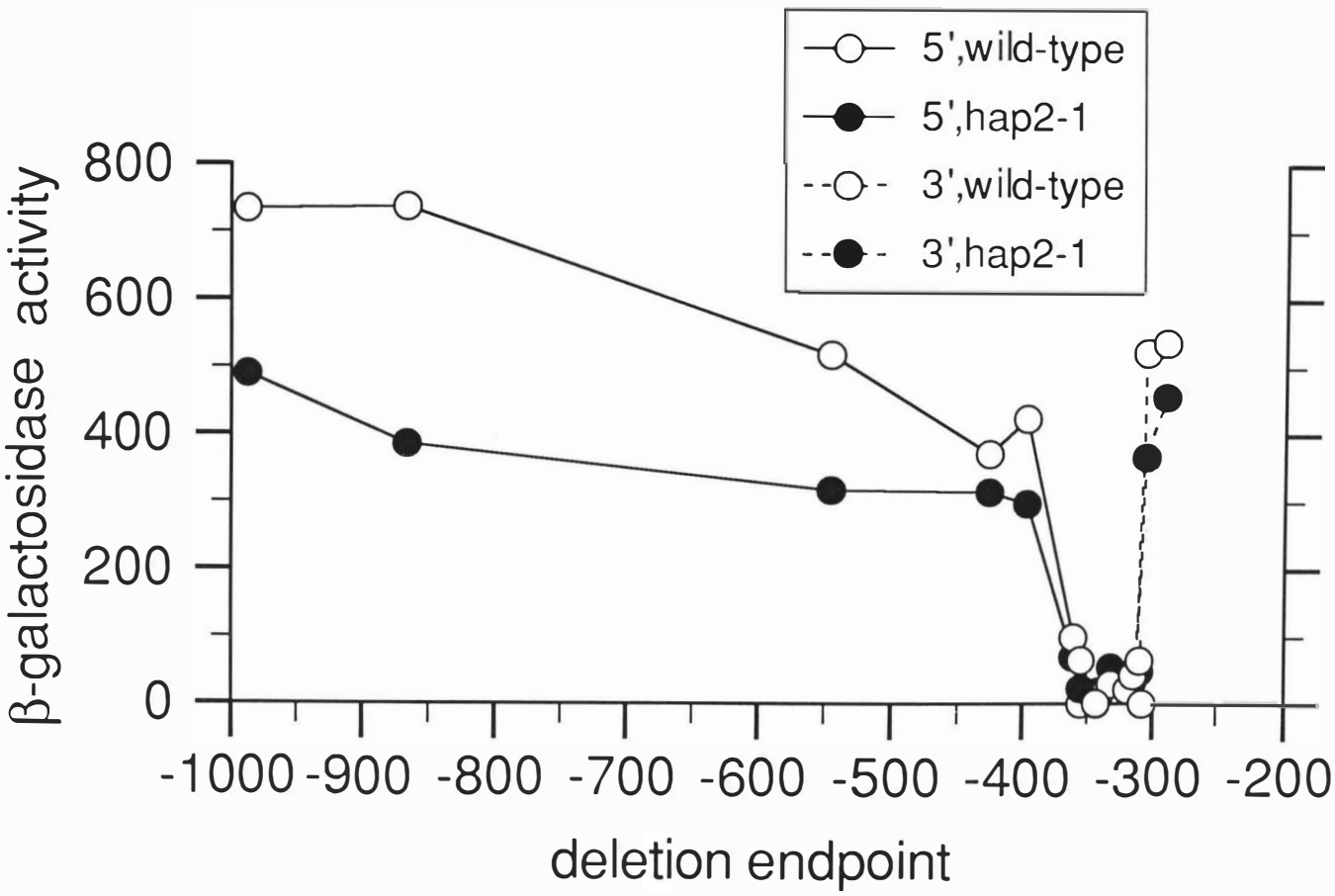
peroxisomal citrate synthase and lacking glucose repression, would presumably not be regulated by this complex. However, to determine if this was the case, the full length construct was transformed into the wild-type and hap2-1 strains, and assayed for ß-galactosidase activity in glucose medium and after resuspension in lactate medium. To map any effects, 5' and 3' deletions of CIT2 upstream DNA were also used to transform BWG1-7a and hap2-1, and assayed in a similar manner. The expression in the hap2-1 strain grown in glucose medium was about 30% lower than that seen in the wild-type strain prior to 5' deletion to -426 (Fig.12). Expression from the remaining 5' deletion constructs was similar for the two strains.

3' deletion constructs were made in the -426 background; therefore, the -289 construct has the same upstream DNA as -426, but contains a XhoI site in place of the EcoRV site seen in -426. Expression in -289 was similar to that at -426, and further deletion eliminated activity. At no point did expression in the wild-type fall to that in the hap2-1 strain.

When the transformants were grown in lactate medium, the expression of the wild-type strain was 5-fold greater than that seen in the hap2-1 strain using the full-length construct (Fig. 13). No region was identified by deletion analysis that was responsible for activation by HAP2,3,4. Also, the level of expression in the hap2-1 strain in lactate medium was lower than that seen in glucose medium. The site where activity is lost is the region Butow's laboratory has found to be responsible for retrograde regulation (Liao and Butow, 1993). This region may contain a HAP2,3,4 binding site. Alternatively, the effect of

Figure 12A: 5' and 3' deletion mapping of CIT2 upstream regulatory regions in wild-type and hap2-1 strains grown in glucose medium. ß-galactosidase is measured in Miller Units (Miller, 1972). Deletion endpoints are in distance in bp from the translational start site. Deletion constructs were made in the -426 background.

12B: Enlargement on graph 12A to show the region from -426 to -200.


5',wild-type
5',hap2-1
3',wild-type
3',hap2-1
β-galactosidase activity
800
600
400
200
0
-1000 -900 -800 -700 -600 -500 -400 -300 -200
deletion endpoint

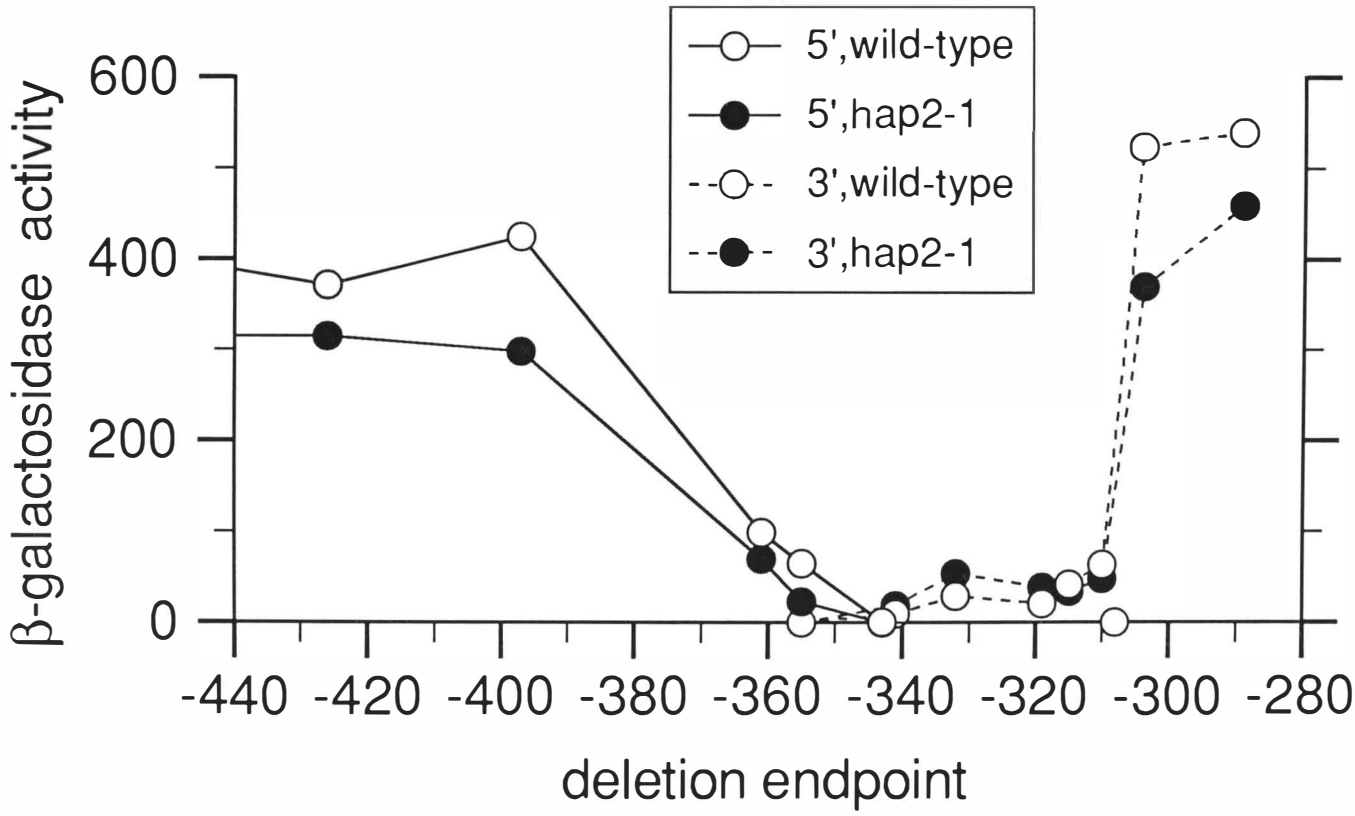
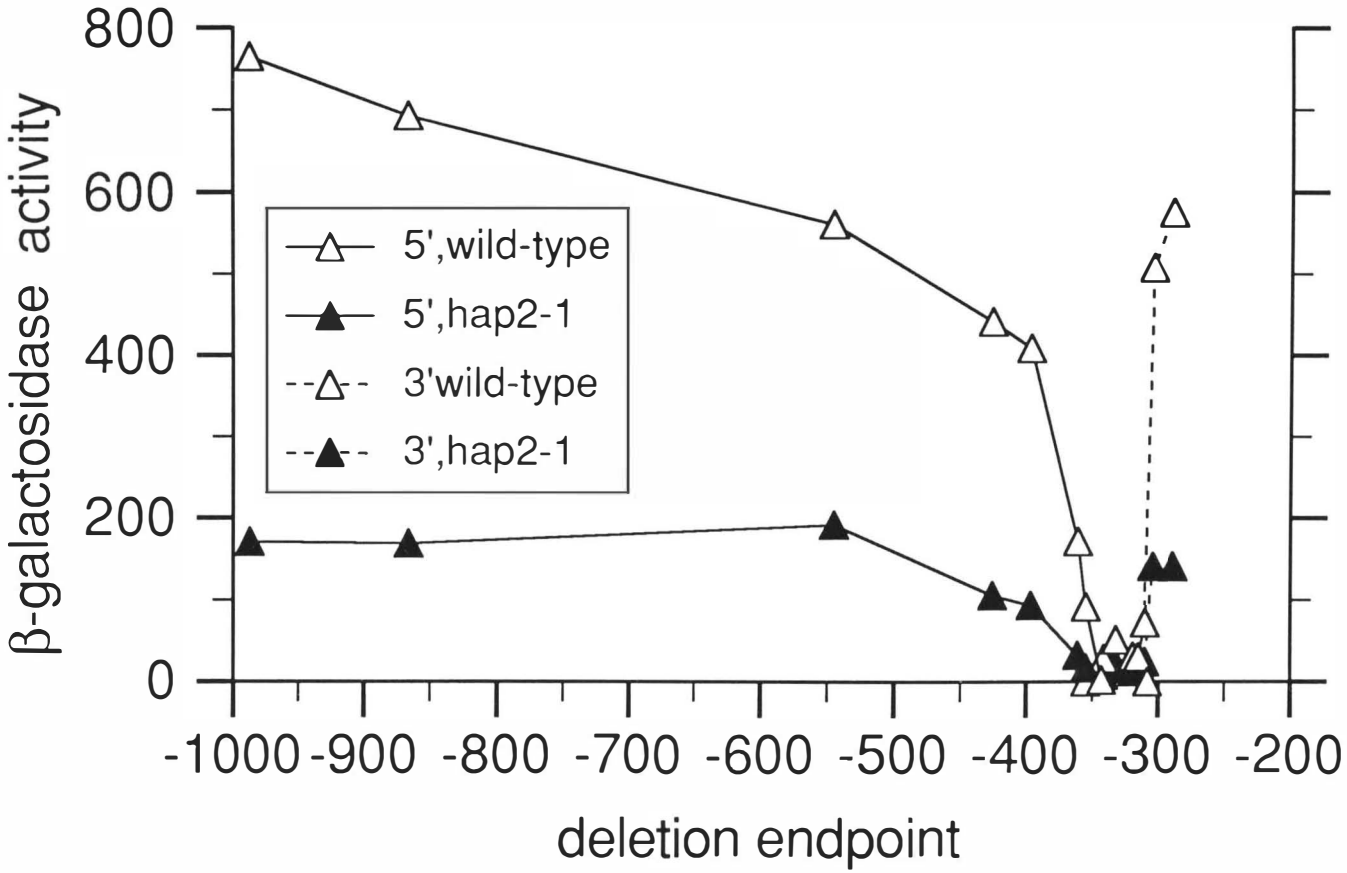
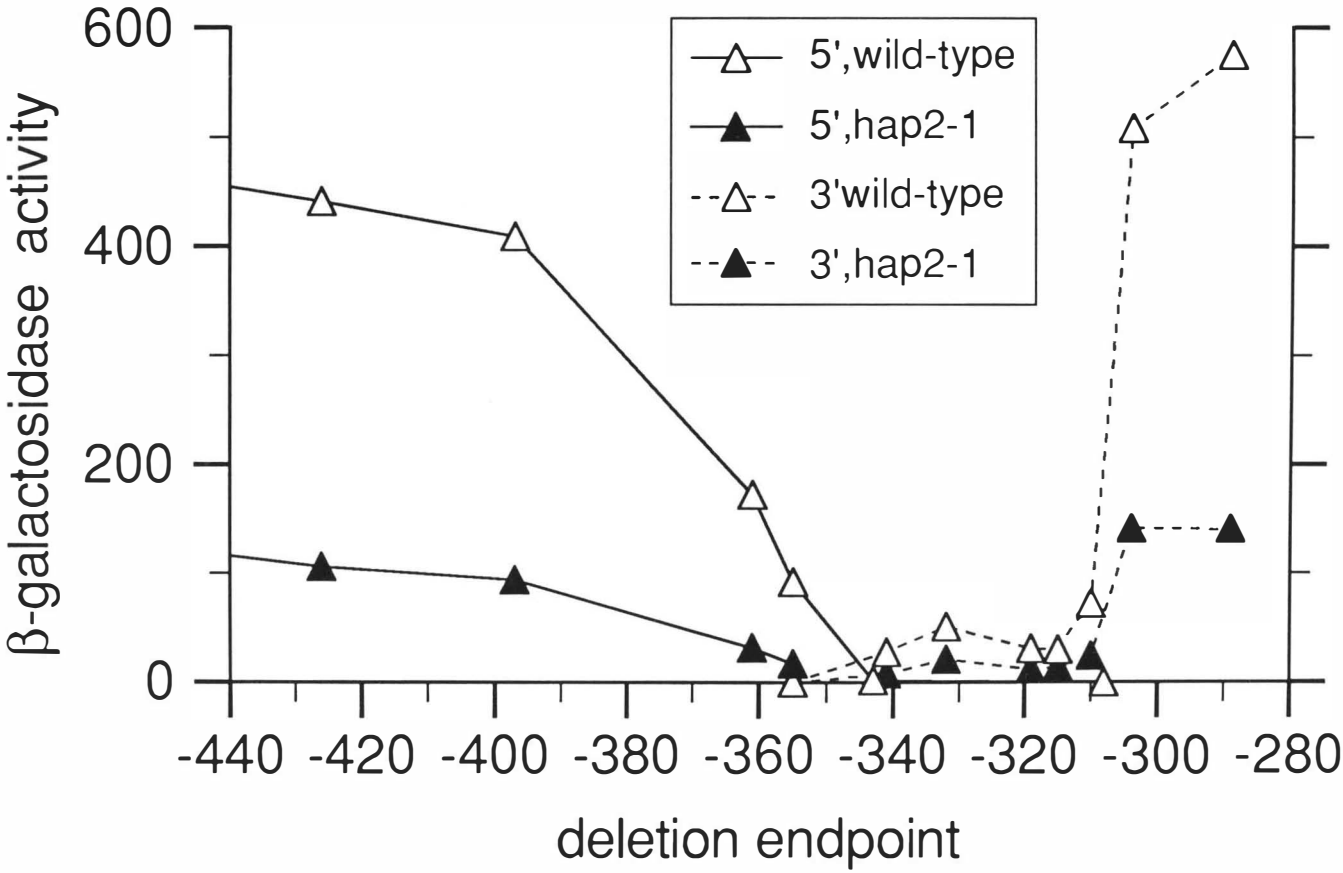

5',wild-type
5',hap2-1
3',wild-type
3',hap2-1
β-galactosidase activity
600
400
200
0
-440 -420 -400 -380 -360 -340 -320 -300 -280
deletion endpoint

Figure 13A: 5' and 3' deletion mapping of CIT2 upstream regulatory regions in wild-type and hap2-1 strains resuspended in lactate medium. ß-galactosidase is measured in Miller Units (Miller, 1972). Deletion endpoints are in distance in bp from the translational start site. Deletion constructs were made in the -426 background.

13B: Enlargement on graph 12A to show the region from -426 to -200.


β-galactosidase activity
800
600
400
200
0
-1000
-900
-800
-700
-600
-500
-400
-300
-200
deletion endpoint
5',wild-type
5',hap2-1
3'wild-type
3',hap2-1


600
400
200
0
β-galactosidase activity
5',wild-type
5',hap2-1
3'wild-type
3',hap2-1
-440 -420 -400 -380 -360 -340 -320 -300 -280
deletion endpoint

HAP2,3,4 may be indirect.

Effect of glutamate on CIT1

Glutamate is known to reduce transcription of CIT1 during growth on glucose (Kim, et al., 1986) The wild-type strain was transformed with the 5' and 3' deletion constructs, and grown on glucose minimal medium with and without glutamate to see if a region involved in glutamate regulation could be detected. (Figure 14) None of the deletions reduced regulation by glucose plus glutamate.

Possibly, glutamate negative regulatory elements overlap or lie in the region required for activation of expression in glucose-grown cells (i.e., -459 to -291). Alternatively, glutamate may act in a negative fashion indirectly (e.g., by inhibiting binding of an activator to a UAS). Regulation by glutamate occurs in glucose-grown cells (Fig. 14), but not in lactate-grown cells (Fig. 15) indicating that either the intracellular regulatory signal is missing in lactate-grown cells (i.e., it is derived from both glucose and glutamate) or that only the mechanism of activation of CIT1 in glucose-grown cells is subject to regulation by glutamate (i.e., the HAP2,3,4-independent mechanism). Figure 16 shows that the regulation by glutamate does not require HAP2,3,4.

Generation of trans-acting mutations affecting CIT1 expression

In order to identify novel trans-acting factors involved in the control of transcription of CIT1 or CIT2 in glucose-grown cells, the wild-type strain was mutagenized, and mutants were isolated that were altered in expression of either of these genes. Strain BWG1-7a was transformed with either a high-copy

plasmid of CIT1-lacZ or an integrating plasmid of CIT2-lacZ and mutagenized using ethylmethylsulfonate as described in the Materials and Methods. Cells were plated on X-gal minimal medium containing glucose, and only those colonies with a lighter or darker blue color than the wild-type strain were studied further. The protocol shown in Figure 17 was used to characterize the mutants isolated. Mutants with decreased expression (lighter blue) were isolated, but no mutants were isolated that reproducibly had increased expression (i.e. darker blue) of CIT1 or CIT2.

To insure that mutants isolated carried trans-acting mutations, and not plasmid-borne or cis-acting mutations, the mutants were cured of the original plasmid and transformed with a non-mutagenized plasmid. Activity was measured by ß-galactosidase assays on liquid cultures. Those isolates having reduced levels of activity were tested further.

Characterization of mutants isolated

In order to further characterize the mutants, each was transformed with CIT1-lacZ, CIT2-lacZ, LEU2-lacZ, and HIS4-lacZ fusion plasmids and expression of each measured in glucose-grown cells (Fig. 18). The LEU2 and HIS4 genes encode enzymes involved in biosynthesis of leucine and histidine respectively. They are regulated by general amino acid control (and leucine control for LEU2)(Hinnebusch et al., 1985; Guarente et al., 1984). Mutants with reduced expression of both of these plasmids were generally eliminated from this study.

Of 130,000 colonies screened by X-gal colony color for CIT1 expression, 44 were confirmed as having reduced expression of the CIT1-lacZ fusion

Figure 14: 5' and 3' deletion mapping of CIT1 upstream regulatory region in the wild-type strain grown in glucose plus glutamate medium. ß-galactosidase activity is measured in Miller Units (Miller, 1972). Deletion endpoints are the distance in bp from the translational start site.

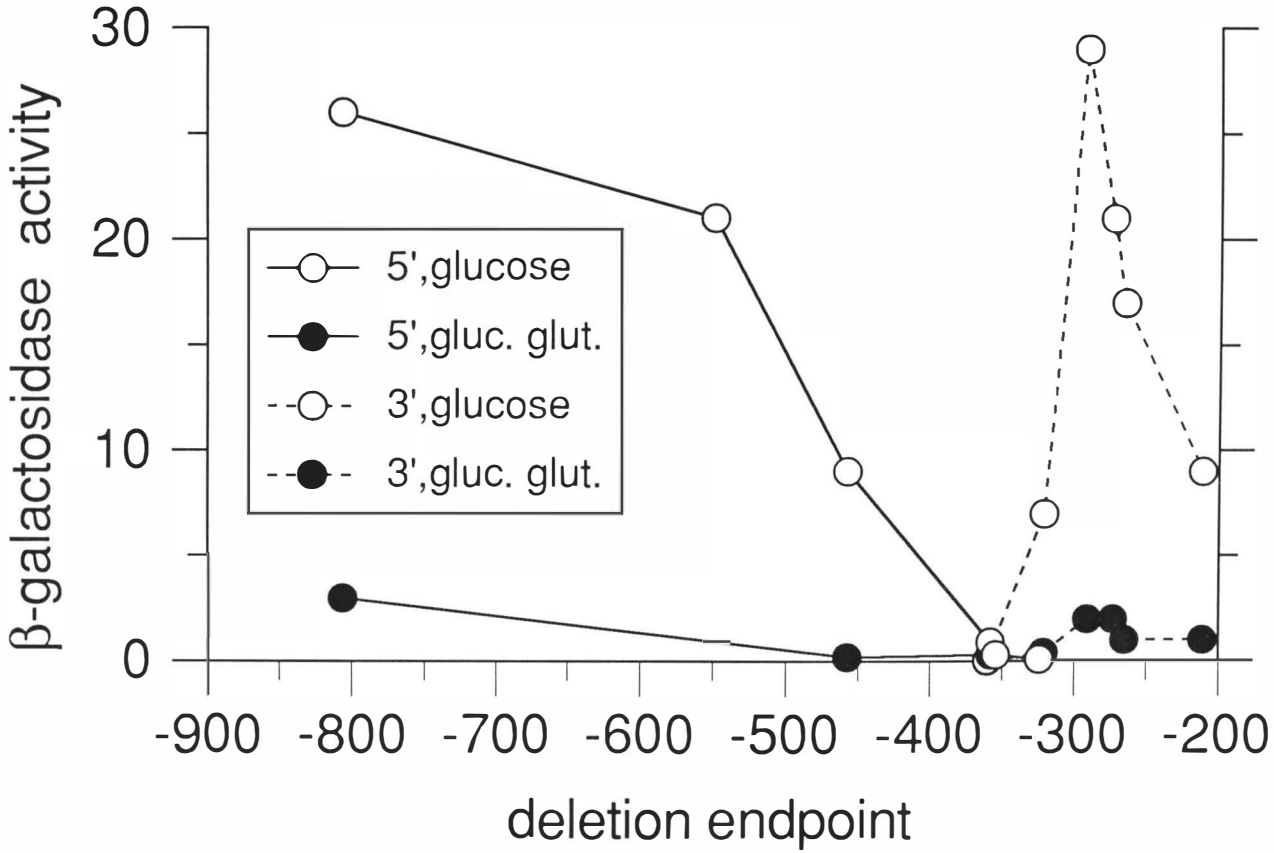

β-galactosidase activity
30
20
10
0
-900
-800
-700
-600
-500
-400
-300
-200
deletion endpoint
5',glucose
5',gluc. glut.
3',glucose
3',gluc. glut.

Figure 15: 5' and 3' deletion mapping of CIT1 upstream regulatory region in the wild-type strain grown in lactose plus glutamate medium. ß-galactosidase activity is measured in Miller Units (Miller, 1972). Deletion endpoints are the distance in bp from the translational start site.

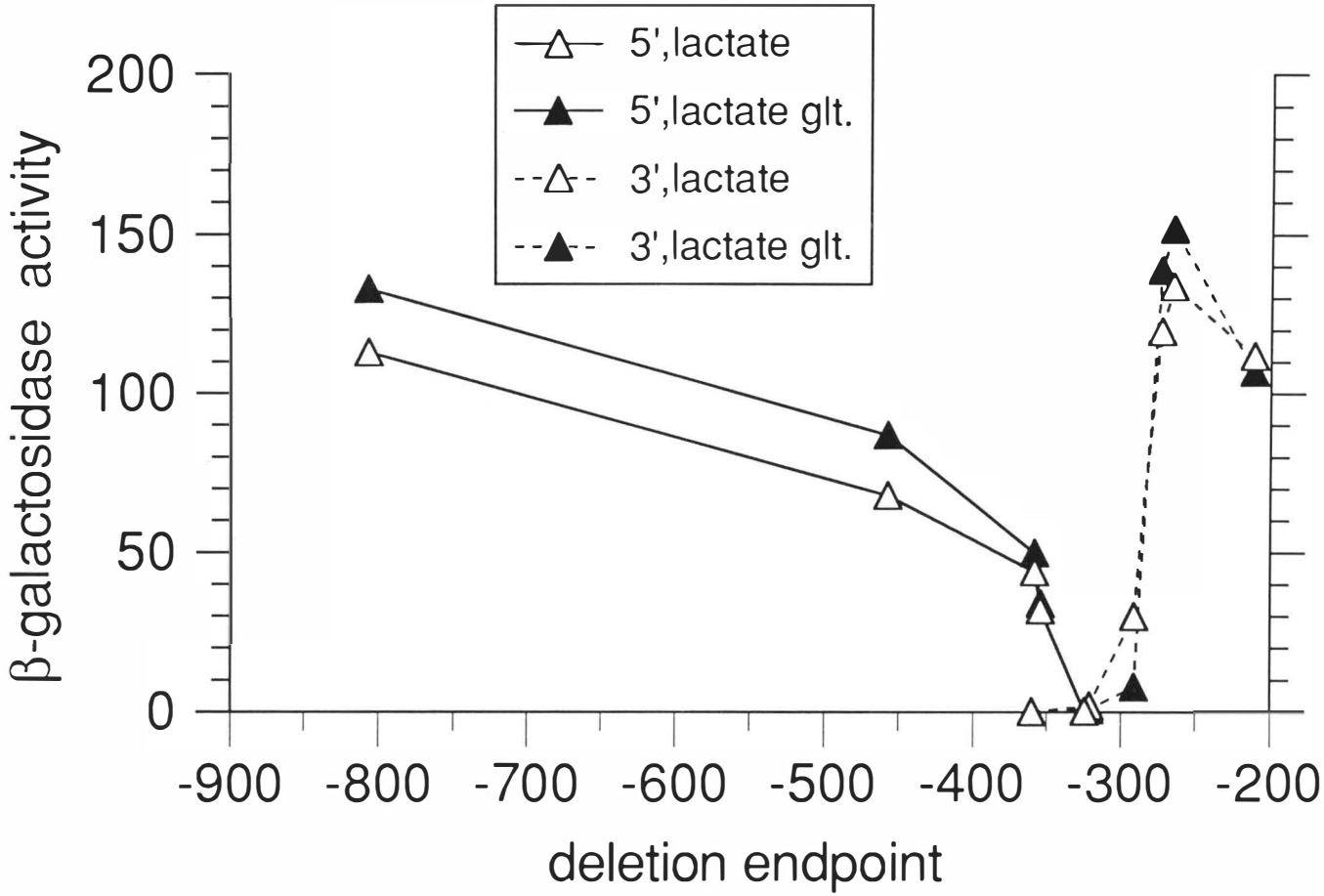
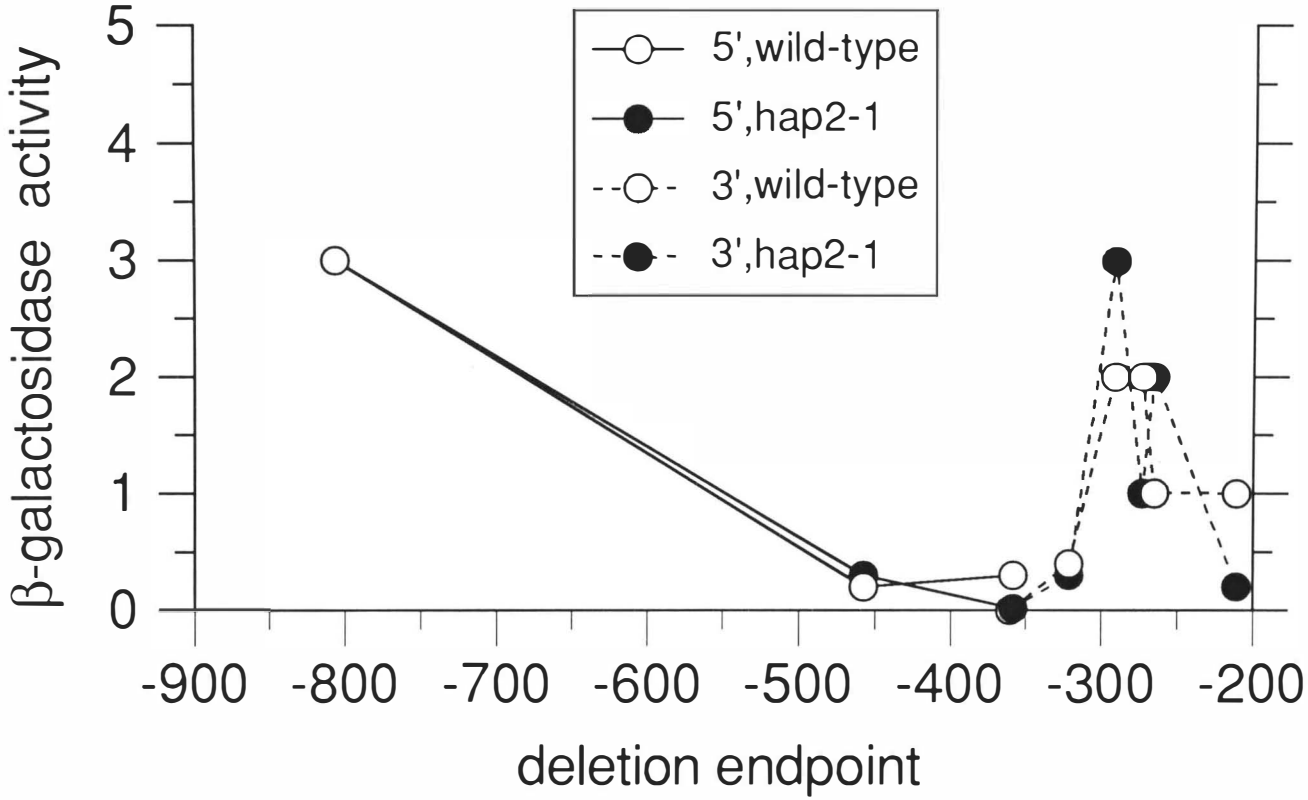

5',lactate
5',lactate glt.
3',lactate
3',lactate glt.
β-galactosidase activity
200
150
100
50
0
-900
-800
-700
-600
-500
-400
-300
-200
deletion endpoint

Figure 16: 5' and 3' deletion mapping of CIT1 upstream regulatory region in the wild-type and hap2-1 strains grown in glucose plus glutamate medium. ß-galactosidase activity is measured in Miller Units (Miller, 1972). Deletion endpoints are the distance in bp from the translational start site.

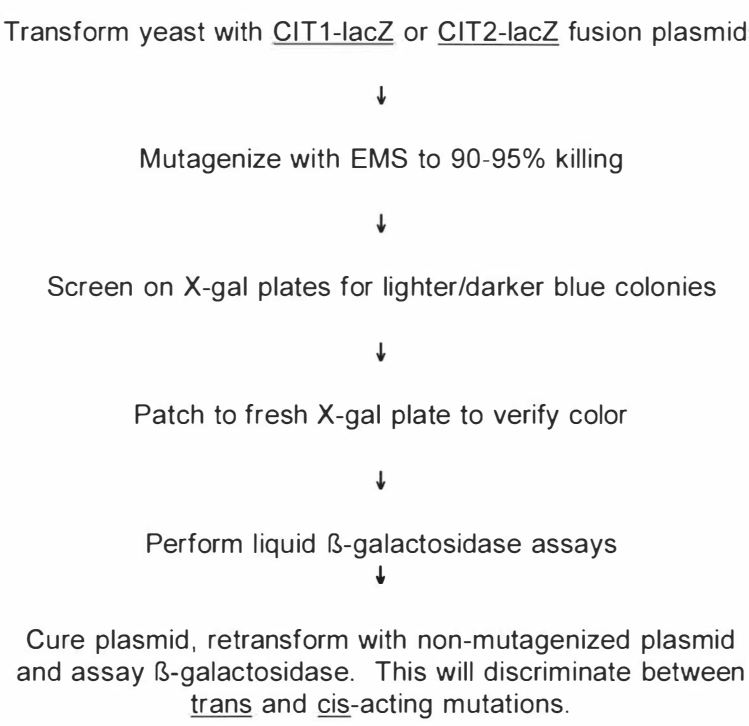

β-galactosidase activity
5
4
3
2
1
0
-900
-800
-700
-600
-500
-400
-300
-200
deletion endpoint
5',wild-type
5',hap2-1
3',wild-type
3',hap2-1

plasmid. 20,000 colonies were screened for CIT2, and one isolate, J40-86, had reduced expression of a CIT2-lacZ fusion plasmid. Of the 45 mutants, only 7 had wild-type levels with either HIS4 or LEU2 CYC1-lacZ fusions (Fig. 18).

A variety of phenotypes were exhibited by the mutants. Mutants were reduced in expression of either CIT1 (CKH-10, CKH-16) or both CIT1 and CIT2 (CK2-7, CK144, CK2-2, CKh-7, J40-86). None of the mutations reduced expression of only CIT2, but only J40-86 was isolated as having altered expression of a CIT2-lacZ fusion. Strains CK2-2, CKH-7, and J40-86, in which expression of one or both CIT genes was reduced below 25%, may be the best candidates for identifying and studying novel factors which directly regulate CIT1 or CIT2. The weaker effects in the other mutant strains might be due to indirect (e.g., physiological) effects on expression of CIT1 and CIT2, or might represent loss of only one of several activation mechanisms which may be present in glucose-grown cells. CYC1-lacZ fusions driven by the HIS4 UAS or LEU2 UAS (which are not glucose-regulated) were used to determine specificity of the mutant phenotypes. Unfortunately, the mutants most severely reduced in expression of CIT1 or CIT2 (Fig.18) also exhibited reduced expression of one or both of these control fusions. Also, many other mutants exhibited reduced activation by both the HIS4 UAS and the LEU2 UAS (data not shown). Expression of a CYC1-lacz fusion (UAS1 and UAS2 present) was also significantly reduced in CK144 and CKH-7 (and perhaps in CKH-10 and J40-86). Mutation of the HAP1 gene could explain reduced expression of CYC1, but not of CIT1 in glucose-grown cells.

Figure 17

IDENTIFICATION OF trans-ACTING REGULATORY MUTATIONS



Expression of CIT1, CIT2, and CYC1-lacZ fusions in lactate-resuspended cells were all reduced sharply in all seven mutants studied. Mutants deficient in HAP2,3,4 would exhibit a reduction in derepressed expression of all three genes (Fig. 10, Guarente, 1992). However, the strong reductions in glucose-repressed expression would have to be due to an additional mutation in at least one other gene. The mutants were found to be rho^- (by genetic cross with rho^+ and rho^0 strains), except J40-86. Expression of CIT1 in lactate-resuspended cells is reduced sharply by a rho^- genotype in the BWG1-7a strain background (Table 7). However, the reduction in derepressed expression of CIT2 is probably not due to the rho^- genotype, since expression of a CIT2-lacZ fusion is increased by rho^-, at least in raffinose (derepression) medium (Table 6). The effect of the rho status on expression of CYC1 is not known.

Analysis of diploids

To determine if the phenotypes were dominant or recessive, the mutants (MAT**a**, **a** mating type) were mated to PSY142***α*** (MAT***α***,***α*** mating type) lacking plasmid. The diploids were transformed with a CIT1-lacZ fusion plasmid. In all cases the expression was wild-type, indicating that this phenotype was recessive (data not shown).

Determination of respiratory sufficiency of mutants

When the haploids were plated on rich medium with lactate as the carbon source, only J40-86 grew. ß-galactosidase assays of haploid cells transformed with CIT1-lacZ, CIT2-lacZ, and CYC1-lacZ and resuspended in lactate medium indicated that no derepression was occurring (see Fig. 19).

Figure 18: All strains were transformed with the plasmids indicated, grown in glucose medium overnight and then assayed for beta-galactosidase activity. For the graph, activity is presented as % of wild-type.

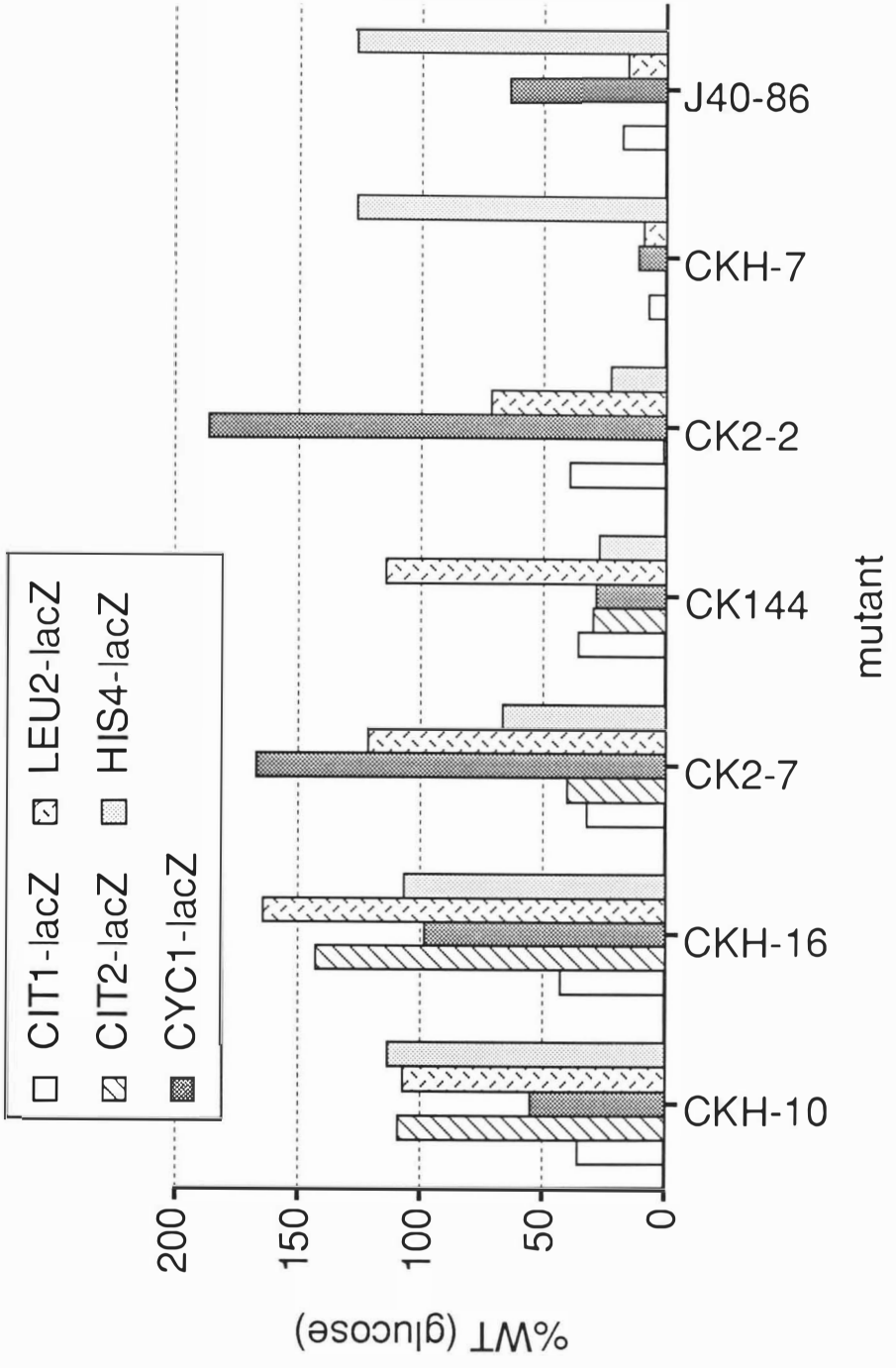
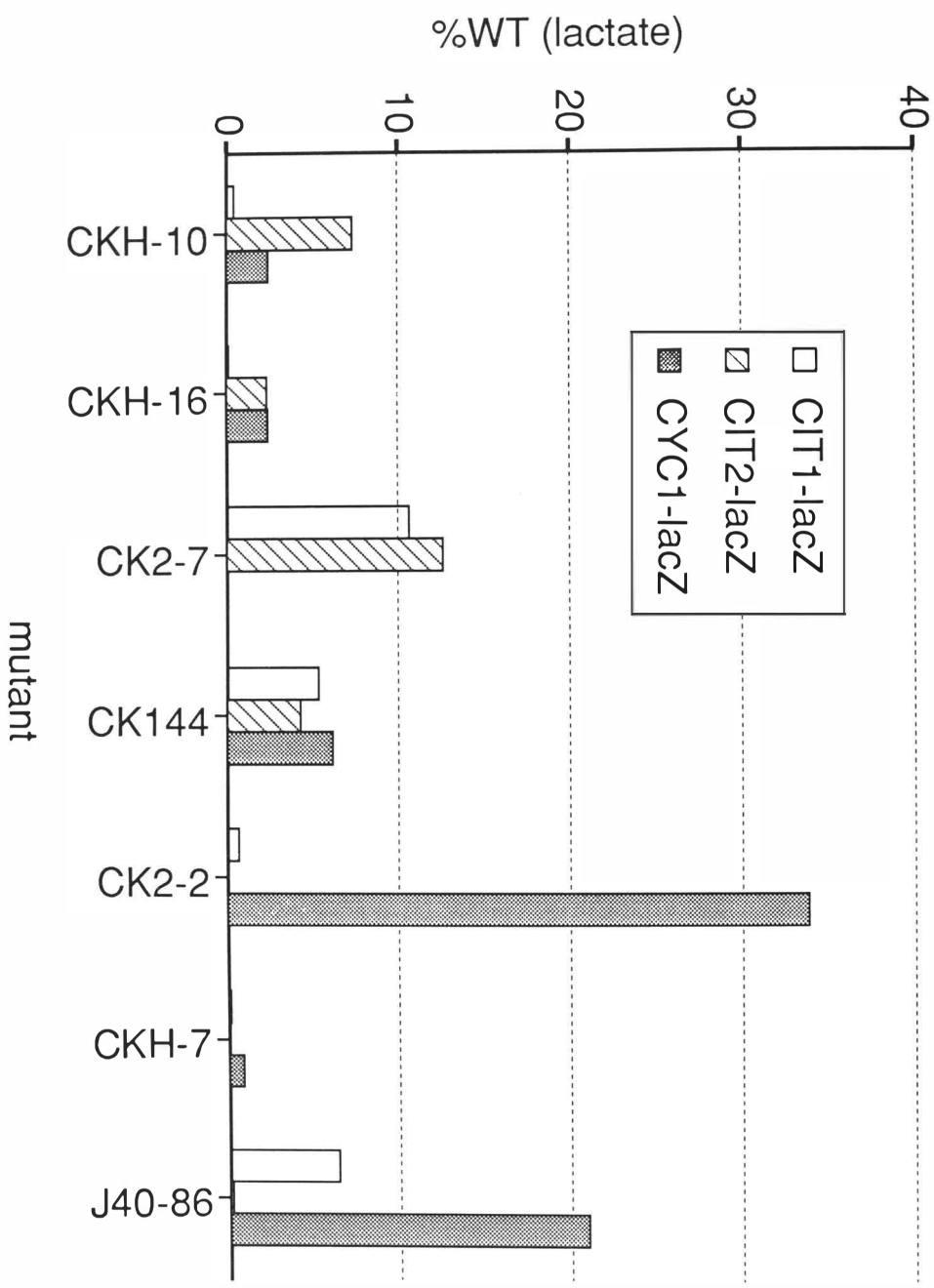

CIT1-lacZ
LEU2-lacZ
CIT2-lacZ
HIS4-lacZ
CYC1-lacZ
200
150
100
50
0
%WT (glucose)
J40-86
CKH-7
CK2-2
CK144
CK2-7
CKH-16
CKH-10
mutant

Figure 19: All strains were transformed with the plasmids indicated, grown in glucose medium overnight, resuspended in lactate medium for 5 hours, and assayed for beta-galactosidase activity. For the graph, activity is presented as % of wild-type.


%WT (lactate)
0
10
20
30
40
mutant
CKH-10
CKH-16
CK2-7
CK144
CK2-2
CKH-7
J40-86
CIT1-lacZ
CIT2-lacZ
CYC1-lacZ

The inability of the mutants to grow on lactate could be attributed to nuclear mutations in lactate uptake or utilization or mitochondrial mutations rendering the cells rho$^-$ or rho^0 instead of a mutation specific for trans-acting factors involved in regulation of CIT1. As mentioned previously rho mutants are a frequent result of using classical mutagens (Dujon 1981). One method used to test for mitochondrial deficiency was to mate the mutants to a rho$^+$ and a rho$^-$ strain. All diploid strains from the rho$^+$ cross were able to grow in lactate medium; however, none of the diploids from the rho^0 cross were able to grow in lactate medium. If the mutants had been rho$^+$ then all the diploids should have grown. Therefore the mutants are rho-. This genotype would result in reduced levels of derepression with CIT1-lacZ and CYC1-lacZ; but should result in increased expression of CIT2.

Gel-binding results for mutants

The final characterization of the seven mutants was a comparison of gel-binding patterns to upstream DNA of CIT1 to see if a complex was absent or altered in mobility. The mutants were grown on YEPD for 24 hours and these cultures were used for protein preparations as described in Materials and Methods. Results of the gel-binding assay on glucose-grown cells are shown in Figure 20. The bands for CKH-16, CKH-7, and 2-7 were similar to wild-type. Bands are missing or altered in mobility for CK144. No bands were detected for J40-86, CK2-2, or H-10. Further work need to be done to confirm these results, before any conclusions can be made.

Regulation of CIT2 in CIT1 disrupted strain and rho^0 strains

Although the primary focus of this research has been regulation of CIT1, the effect of disruption of CIT1 and its effect on CIT2 regulation was also examined.

Liao, et al. (1991) found increased activity of CIT2 in raffinose medium when CIT1 was disrupted or when the mitochondrial phenotype was rho°. To localize these effects, rho$^+$ and rho° strains were transformed with 5' deletion constructs of a CIT2-lacZ fusion plasmid, a CIT1::LEU2 disruption strain (1-7a-L), and a rho° strain and grown in raffinose minimal medium. Results are shown in Table 7. The disrupted strain 1-7a-L was unable to grow on raffinose minimal medium. Therefore, we were unable to map a regulatory site between -993 and -308 responsible for regulation of CIT2 by CIT1 status. In agreement with the work of Butow (Liao and Butow, 1993), we found no affects of CIT1$^-$ or rho$^-$ on CIT2 expression in glucose-grown cells (data not shown). In raffinose-grown cells, the effects of CIT1$^-$ and rho$^-$ were small, but reproducible. Deletion to -369 appeared to remove rho regulation of CIT2. This needs to be examined further.

Figure 20: Gel-binding assay using protein extracts prepared from mutants grown in glucose medium. Protein concentration used was 50ug/lane. A 180bp fragment of CIT1 upstream DNA was labelled internally by PCR as described in the Materials and Methods and used for these assays.

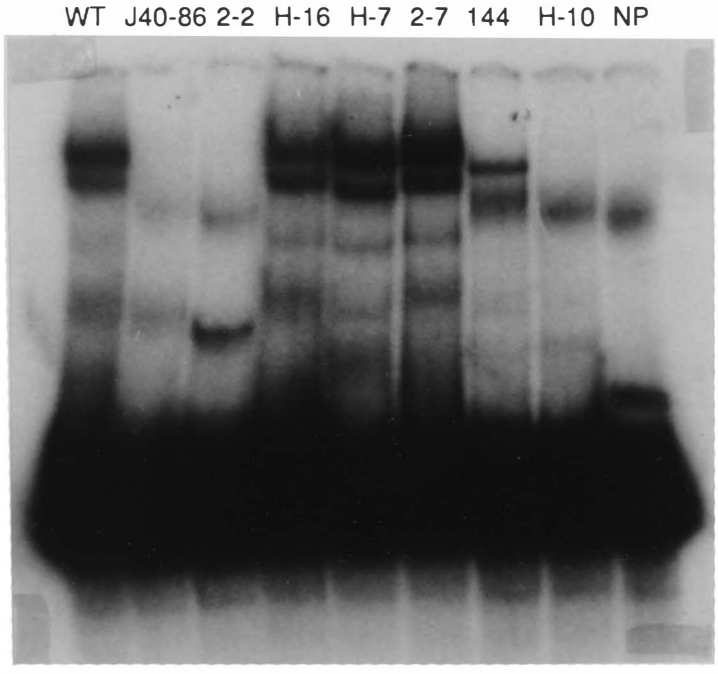

WT
J40-86
2-2
H-16
H-7
2-7
144
H-10
NP

TABLE 6

Effect of rho status on expression* of a CIT1-lacZ fusion

	wild-type	rho$^-$
glucose	28	44
lactate-resuspension	81	0.1

* - measured in Miller Units (Miller, 1972) of beta-galactosidase activity

TABLE 7

Effect of rho status and CIT1 disruption on expression of CIT2

strain	-993*	-866*	-369*	-361*	-308*
1-7a	83	87	118	14	0.2
1-7a-L**	228	252	310	71	0.1
1-7a rho^o	144	179	113	NG	NG
1-7a-L** rho^o	NG	NG	NG	NG	NG

** 1-7a-L has CIT1 disrupted by insertion of LEU2 into the gene (Rosenkrantz, et al., 1986).
NG = no growth
* = 5' deletion constructs of CIT2 used to transform wild-type strain

DISCUSSION

The major aim of this research was to investigate the molecular mechanisms of regulation of transcription of CIT1 and CIT2, the genes encoding citrate synthase in yeast. Specifically, we addressed the questions of (1) localization of cis-acting sites required for expression or regulation, (2) the roles of HAP1 and HAP2,3,4 in expression of both genes, (3) identification of other trans-acting factors involved in expression of either gene, particularly in glucose-grown cells, (4) localization of cis-acting sites involved in up regulation of CIT2 in response to disruption of CIT1 or rho° status (Liao, et al., 1991, Liao and Butow, 1993).

Regulation of CIT1 by Hap2,3,4

Deletion studies (Fig. 6 and Fig. 7) suggested that derepression of CIT1 might depend on binding of the HAP2,3,4 transcriptional activator to one or both near consensus sites at -290 and -310. Deletion to -291 specifically prevents derepression, deletion to -321 (which eliminates the -310 site as well) decreased activity in both glucose-grown and lactate-grown cells.

By assaying expression of CIT1-lacZ fusions in hap2-1, hap3-1, and HAP4::LEU2 strains, it was possible to determine that HAP2,3,4 is specifically required for derepression of CIT1, above the glucose-repressed level.

Furthermore, deletion to -291 (removing 1/2 of the -290 site) also prevented derepression, with the remaining derepressed expression equivalent in the wild-type and HAP2,3,4-deficient strains. The role of the -310 candidate site remained unclear.

These candidate HAP2,3,4 sites were also investigated by base substitutions (Figure 10). The effects of mutations toward or away from consensus, on derepressed expression of CIT1-lacZ fusions are generally consistent with binding of HAP2,3,4 to both sites, and contribution by both sites to derepressed expression. Based on a limited number of mutations, the -290 site, which has the CCAAT core, may be the more important of the two sites.

Surprisingly, some multiple-base substitutions in either site also dramatically reduced glucose-repressed expression of CIT1. Glucose-repressed expression is not reduced by deletion to -291, and is only slightly reduced by mutations in HAP2, HAP3, and HAP4. This effect does not appear to involve binding of HAP2,3, since it also occurs in a hap2-1 strain (Fig. 10). Perhaps another activator protein binds to sequences overlapping the -310 and -290 sites in glucose-grown cells.

This arrangement of two closely spaced HAP2,3,4 recognition sites is novel and raises the possibility of interaction of proteins at the two sites. The center to center distance is about 18 bp which would place proteins at both sites roughly on the same side of the helix. One way to address this question would be to insert bases between the two sites, so the sites would be on opposite sides of the helix. Unfortunately, attempts to construct a 3 bp insertion between

the sites, by PCR, were not successful. An alternative approach is to use a 2-step (3 primer) PCR (Sarkov and Sommer, 1990). The product of the first amplification would then be used as one of the primers for the second PCR amplification. This technique is currently being used in this laboratory with success.

Other work in our laboratory involves saturation mutagenesis of the two sites and neighboring sequences, introducing single basepair mutations, to determine which bases are essential. This should provide further evidence, for or against, recognition by HAP2,3,4. In addition, it may be possible to dissect apart bases required for derepression or glucose-repressed expression.

Despite considerable effort, binding of the Hap2,3,4 protein complex to CIT1 upstream DNA could not be demonstrated. L. Guarente's laboratory has been able to demonstrate binding to UAS2up1 (Olesen et al., 1987) X. Ling and L. Guarente (Massachusetts Institute of Technology) provided us with purified HAP2 and HAP3 (expressed in E. coli) and crude yeast protein extract which must also be added (for unknown reasons). HAP4 is not required for binding (Forsburg and Guarente, 1989). Using a variety of combinations of their purified proteins and crude protein extracts, and our protein preparations made similarly, we were unable to detect an alteration in the gel shift pattern which would indicate binding of HAP2,3,4 to CIT1 upstream DNA or to the positive control, CYC1 UAS2up1. It is CIT1 is not regulated by heme (T. Keng, personal communication). Deletion-mapping studies (Fig. 14 and Fig. 15) were performed to identify a region required for activation by HAP1. 3' deletion to -291 reduced

expression in the wild-type strain to below the level in hap1-1 strains. Repetition of this result and further investigation of the region is required.

Glutamate regulation

CIT1 is regulated in a synergistic manner by glucose and glutamate, reflecting the role of the TCA cycle in biosynthesis of amino acids (Kim et al., 1986). Negative regulatory elements for glutamate regulation were not identified by deletion analysis (Fig. 14). It is possible that a negative element exists between -371 and -321, the region required for glucose-repressed expression. Alternatively, glutamate may act indirectly by inhibiting binding of an activator in this region. Using the hap2-1 strain, it was determined that HAP2,3,4 is not required for regulation by glutamate (Fig. 15). Finally, it should be noted that the actual intracellular signal has not been identified, and may be some compound or ratio of compounds which is influenced by both glucose and glutamate.

Trans-acting regulatory mutations generated by EMS mutagenesis

One of the goals of this research was to isolate mutants that had mutations in novel trans-acting factors (e.g. DNA-binding proteins) affecting transcription of CIT1 or CIT2 or both. 130,000 colonies were screened for altered expression of a CIT1-lacZ fusion plasmid, and 20,000 were screened for altered expression of an integrated CIT2-lacZ fusion plasmid. Of these, 44 mutants had reduced expression of the CIT1 vector and one (J40-86) had reduced expression of the CIT2 vector. These mutants were then screened for expression of a CYC1-lacZ fusion plasmid containing the UAS of either LEU2 or HIS4..

A variety of phenotypes were exhibited by the mutants. Mutants were reduced in expression of either CIT1 (CKH-10, CkH-16) or both CIT1 and CIT2 (CK2-7, CK144, CK2-2,, CKH-7,, J40-86). None of the mutants reduced expression of only CIT2, but only J40-86 was isolated as altering expression of a CIT2-lacZ fusion. Strains CK2-2, CKH-7, and J40-86 in which expression of one or both CIT genes was reduced below 25%, may be the best candidates for identifying and studying novel factors which directly regulate CIT1 or CIT2. The weaker effects in the other mutant strains might be due to indirect (e.g. physiological) effects on expression of CIT1 and CIT2, or might represent loss of only one of several activation mechanisms which may be present in glucose-grown cells.

Surprisingly, expression of CIT1, CIT2, and CYC1-lacZ fusions in lactate-resuspended cells were all sharply reduced in all seven mutants studied. The mutants were found to be rho^- (by genetic crosses with rho^+ and rho^0 strains). This probably is not responsible for the reduction in expression of CIT2, since expression of a CIT2-lacZ fusion is increased by rho^- (Table 7).

In conclusion it is necessary to determine whether these mutant phenotypes are due to one mutation (2:2 meiotic segregation) and whether the effects on expression of the different gene fusions cosegregate. Also, additional gel-mobility shift assays are required to determine conclusively whether any of these mutants are altered in proteins binding at the -310 or -290 candidate HAP2,3,4 binding sites or at the activation region for glucose-repressed expression (roughly -367 to -348). The reductions in expression of CIT1 in

glucose-grown or lactate-grown cells were found to be recessive (by crossing with PSY142). This should allow direct cloning of the wild-type alleles by complementation with existing yeast genomic libraries.

Regulation of CIT2 by HAP2,3,4

Regulation of CIT2 was also investigated. HAP2,3,4 does appear to be required to activate transcription of CIT2, especially in lactate-grown cells (Figures). This is surprising since expression of CIT2 is approximately the same in both glucose and lactate medium. Furthermore, expression of CIT2 in the hap2-1 strain was lower in lactate than glucose. Further analysis is required to understand this. A HAP2,3,4 site was not mapped by deletion analysis, and may lie in the region critical for all expression, -370 to -300.

Effect of disruption of CIT2 and rho control on CIT2

Liao and Butow have identified the region from -370 to -300 in the upstream DNA of CIT2 as responsible for retrograde regulation and for CIT1 regulation of CIT2 and have designated this element UASr (Liao and Butow, 1993). They also have cloned and sequenced two genes, RTG1 and RTG2, involved in communication between mitochondria, peroxisomes, and the nucleus. Liao et al. (1991) had previously found that disruption of CIT1 resulted in an increase in expression of CIT2, and partially compensates for the mitochondrial citrate synthase deficiency. The proteins encoded by RTG1 and RTG2 are required for both increases in CIT2 expression. Our results were similar to those of Liao et al. (1991), that disruption of CIT1 in a rho^+ strain results in an increase of transcription of CIT2 when cells were grown in raffinose medium.

However, the effect in our strain was not as strong as that seen by Liao. Surprisingly, the CIT1$^-$, rho$^-$ disrupted strain was unable to grow on glucose or raffinose. Deletion studies mapped the region responsible for activation by RTG1 and RTG2 of the rho^0 strain to be between -370 and -300 (Liao and Butow, 1993). The increase was not as pronounced in the nondisrupted rho^0 strain, but a similar pattern of increase in transcription was seen and declined when the DNA was deleted to -369 to the wild-type levels. Additionally, we found that deletion from the 5' end to -361 rendered the rho$^-$ cells unable to grow in both glucose and lactate medium. Deletion of the element identified by Liao may result in an inability of BWG1-7a cells to grow. Liao et al. saw a decrease in retrograde regulation, but not in growth, when they deleted the site in their strain, PSY142α (Liao and Butow, 1993).

In summary, we have shown that derepression of CIT1 requires HAP2,3,4 and identified the -290 and -310 regions as being required for full derepression. Further work is being conducted to mutate both regions with single basepair changes to see which bases are involved in regulation. Also, further work is needed to see if binding of the HAP2,3,4 complex can be detected using the gel-binding assay.

Additional experiments are also being conducted by others on regulation of transcription of CIT2. We verified the finding by Liao et al. (1991) that disruption of CIT1 increased transcription of CIT2, however our results showed less of an effect. We were unable to grow the disrupted rho$^-$, CIT1$^-$disrupted strain on raffinose, so could not confirm their work on this configuration (Liao

and Butow, 1993). We did see a decline in transcriptional activity of CIT2 when deleted to -361.

Many of the genes involved in the electron transport system and the TCA cycle contain potential HAP2,3,4 binding sites, and several of these genes are known to be regulated by HAP2,3,4 (Table 1). Many of these genes are subject to glucose repression. SNF1 is a protein kinase involved in regulation of glucose repression in SUC2, and thought to regulate HAP2,3,4.

A pathway of coordinated regulation of glycolysis, the TCA cycle, and the electron transport system allows the yeast to use the most energy efficient method of catabolism under varying oxygen concentrations through changes at the level of transcription of genes involved in these pathways.

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