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Distinct upstream activation regions for glucose-repressed and derepressed expression of the yeast citrate synthase gene *CIT1*

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Abstract. The yeast *CIT1* (mitochondrial citrate synthase) gene is subject to glucose repression and is further repressed by glucose plus glutamate. Based on deletion analysis of a *CIT1-lacZ* gene fusion, DNA sequences between −548 and −273 are required for full expression of *CIT1*. The region of transcription initiation and the putative TATA element are located at −150 to −100 and −195 respectively. A restriction fragment containing DNA sequences between −457 and −211 conferred activation and glucose-glutamate regulation when placed in either orientation upstream of a UAS-less heterologous yeast gene. Deletion of DNA sequences between −291 and −273 specifically eliminated derepression of *CIT1*, and destroyed one of two closely-spaced, potential binding sites for the HAP2,3,4 transcriptional activator protein. Ten-base-pair block substitutions in the region −367 to −348 reduced glucose-repressed expression. Thus, it appears that distinct DNA sequences upstream of *CIT1* activate expression in glucose-repressed and derepressed cells. Possible mechanisms of regulation by glutamate plus glucose, are discussed.

Key words: Yeast – Citrate synthase – Transcriptional regulation – HAP2,3,4

Introduction

In eukaryotic cells, utilization of nonfermentable carbon sources requires the tricarboxylic acid (TCA) cycle and the electron transport chain, both located in the mitochondria. Almost all of the proteins required for these functions are encoded by nuclear genes (Tzagoloff and Myers 1986; Pon and Schatz 1991). During growth in glucose medium, the levels of many of these proteins are reduced (Fraenkel 1982). Catabolite repression of nuclear genes encoding components of the electron transport chain has been explored by several groups, and molecular mechanisms have been described (Forsburg and Guarente 1990; Guarente 1993; Pinkham and Keng 1993). Transcription of several of these nuclear genes has been shown to be under global control mechanisms involving activation by heme and the HAP1 activator protein or derepression in the absence of glucose involving the HAP2,3,4 activator protein (Forsburg and Guarente 1990; Mattoon et al. 1990; Pinkham and Keng 1993). Regulation by glucose and HAP2,3,4 appears to be specific for nuclear genes encoding mitochondrial components, but is under control of general glucose repression mediated by *SNF1* and *SSN6/CYC8* (Carlson 1987; Forsburg and Guarente 1990; Wright and Poyton 1990; Trumbly 1992).

While there has been considerable study of the regulation of nuclear genes encoding components of the electron transport chain in *Saccharomyces cerevisiae*, less is known about the nuclear genes encoding tricarboxylic acid (TCA) cycle enzymes. We have been studying the synthesis of citrate synthase, which catalyzes the first and rate-limiting step of the TCA cycle, the condensation of acetyl-CoA and oxaloacetate to form citrate (Krebs and Lowenstein 1960; Walsh and Koshland 1985). We previously reported that *S. cerevisiae* has two genes encoding functional citrate synthase isozymes. *CIT1* encodes the major, mitochondrial form while *CIT2* encodes a nonmitochondrial form which appears to be peroxisomal (Kim et al. 1986; Rickey and Lewin 1986; Rosenkrantz et al. 1986; Kispal et al. 1988; Lewin et al. 1990; Singh et al. 1992). There is evidence for and against a role of the *CIT2*-encoded form in the glyoxylate cycle, allowing utilization of acetate or fatty acids (Kim et al. 1986; Kispal et al. 1988, 1989; Lewin et al. 1990). The glyoxylate cycle is found in glyoxysomes in some plant cells (Beevers 1979) and may be localized in the peroxisomal fraction from yeast (McCammon et al. 1990).

The level of mRNA from both *CIT1* and *CIT2* is reduced synergistically in response to supplementation with glucose and glutamate (Kim et al. 1986). This regulation reflects the roles of the TCA cycle in catabolism and biosynthesis (e.g., α-ketoglutarate is a precursor for

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the biosynthesis of glutamate). Transcription of the *CIT1* gene, but not of *CIT2*, is also partially repressed by glucose alone (Kim et al. 1986). Similar regulation has been found in bacteria (Gray et al. 1966; Flechtner and Hanson 1969; Hanson and MacKechnie 1969; Nimmo 1987), and occurs at the transcriptional level (Rosenkrantz et al. 1985; Wilde and Guest 1986).

We show here that expression and regulation of *CIT1* requires activation sequences located upstream of the TATA box and the transcription initiation region. These activation sequences resemble other yeast upstream activation sequences (UASs) in being located far upstream of the transcription initiation sites, in being capable of conferring activation and regulation upon a heterologous transcription initiation region, and in functioning at variable distance and in either orientation (Guarente 1987, 1988). The results of promoter-deletion analysis and block-nucleotide substitutions, are that glucose-repressed expression and derepression of *CIT1* are dependent upon separate upstream sequences. Two potential binding sites for the HAP2,3,4 activator protein lie in or near the upstream DNA region required for derepression. The region required for glucose-repressed expression appears to define a novel element. Possible mechanisms for regulation by glutamate plus glucose are discussed.

Materials and methods

Strains and growth media. All experiments were performed with *S. cerevisiae* strain BWG1-7a *MATa ade1-100 his4-519 leu2-2 leu2-112 ura3-52* (Guarente and Mason 1983). Recombinant DNA constructions were performed with *E. coli* strain YMC9 (Δ*lacU169 hsdR*$^-$ *hsdM*$^+$) or DH10B [F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*-ΔM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara,leu*)7697 *galU galK λ-rpsL nupG* (supplied by GIBCO BRL]. Yeast-rich medium (YEP) and yeast-minimal medium (YMM) were prepared with the carbon source (glucose, galactose, or lactate) at 2% and supplemented with growth requirements (Sherman et al. 1986). When indicated, sodium glutamate was added at 0.2% (Kim et al. 1986; Rosenkrantz et al. 1986).

Transformation. Transformations of yeast and *E. coli* were performed as described previously (Kim et al. 1986; Rosenkrantz et al. 1986) except that, after ligation to the vector of fragments generated by the polymerase chain reaction, DNA was introduced into DH10B cells by electroporation, using a GIBCO BRL *E. coli* Pulser (as recommended by the manufacturer).

Construction of CIT1-lacZ gene fusions. The *CIT1* gene was obtained as a *Sau*3a fragment cloned into the *Bam*H1 site of shuttle vector pFL1 (Suissa et al. 1984). This plasmid, pFCS2, was partially restricted with *Sma*1 and a *Bgl*2 linker was placed at the *Sma*1 site located 806 bp upstream of the *CIT1* coding sequence. To create the *Bam*H1 end to be fused to *lacZ* (β-galactosidase), *Hin*d3 or *Cla*1 were used to cut within the *CIT1* coding sequence, and *Bal*31 was used to chew toward the initiation codon of *CIT1*. A *Bam*H1 linker was ligated to the chewed ends. Plasmid pLG669Z is an *E. coli* and *S. cerevisiae* shuttle vector with the yeast 2μ origin of replication and the *URA3* gene, as well as a *CYC1-lacZ* gene fusion (Guarente and Ptashne 1981). A *Bgl*2 linker was inserted at the *Sma*1 site just upstream of the *CYC1* DNA. Then the *Bgl*2–*Bam*H1 fragment carrying all the *CYC1* DNA was replaced with the *Bgl*2–*Bam*H1 fragment carrying *CIT1* DNA (see above). Yeast colonies expressing *lacZ* were identified by screening for blue colonies on plates containing Xgal (Guarente 1983). One expressed *CIT1-lacZ* fusion contains the first 164 bp of the coding sequence of *CIT1* ("+164" fusion). This fusion is regulated by glucose and glutamate, but does not derepress in lactate-grown cells (it inhibits growth in lactate medium; data not shown). This fusion contains the amino-terminal mitochondrial signal sequence of *CIT1* (Rosenkrantz et al. 1986; Singh et al. 1992), and targeting of high levels of β-galactosidase to the mitochondria has been shown to interfere with growth (Douglas et al. 1986). Integration of this +164 *CIT1-lacZ* fusion at the *CIT1* locus, lowered expression of β-galactosidase in glucose-grown cells by 6-fold, and resulted in normal growth and derepression in lactate medium (data not shown). A second plasmid-borne *CIT1-lacZ* fusion produced approximately 15-fold lower levels of β-galactosidase than the +164 plasmid in glucose-grown cells, and resulted in normal growth and derepression in lactate medium. Surprisingly, it lacks the *CIT1* translational start codon and starts at the tenth base pair upstream of the coding sequence ("−10" fusion). Presumably translation begins at the first downstream ATG which is located at position 347 of the *lacI* gene, a portion of which is fused in-frame to *lacZ* in pLG669Z (Rogers Yocum et al. 1984). The −10 *CIT1-lacZ* fusion, which is regulated correctly, was used for all regulatory site mapping studies (deletion and base substitution analysis). The +164 *CIT1-lacZ* fusion was used for transcript mapping and for mutant screening by colony color on Xgal glucose medium (where its higher-level expression was advantageous).

β-galactosidase assays. Yeast transformants were grown for 2–3 days in YEP medium supplemented with 2% galactose, and then diluted into yeast-minimal medium containing 2% glucose or 2% lactate (and 0.2% sodium glutamate where indicated), and grown overnight. Cells were assayed for β-galactosidase activity at an OD_{600} of about 0.5. β-galactosidase was assayed in SDS-chloroform-permeabilized yeast cells (Guarente 1983), and activity is expressed as the change in $OD_{420} \times 1000$ divided by (min × ml of culture used × OD_{600} of the culture). Assays were performed in duplicate and were averaged over at least 4–6 experiments. Standard deviations were typically 20–40%.

Generation of promoter deletions using Bal31 exonuclease. 5′ deletions of the −10 *CIT1-lacZ* fusion were constructed using *Bal*31 nuclease in a unidirectional manner, by employing a temporary upstream "buffer fragment" to protect the yeast *URA3* gene (and its transcription terminator) from digestion. A 3-kbp *Bgl*2–*Bgl*2 fragment containing the yeast *LEU2* gene (Hsu and Kohlhaw 1982) was cloned into the unique *Bgl*2 restriction site located just upstream of the *CIT1* DNA. Then, this plasmid was opened at a unique *Xho*1 site located close to the end of the *LEU2* buffer fragment nearest to the *CIT1* DNA. DNA was deleted in both directions using *Bal*31 nuclease. A *Bgl*2 linker was added to the deleted ends and the chewed buffer fragment was removed using *Bgl*2. Thus, all fusions contain identical *URA3* DNA upstream of the deletion endpoint.

Mapping of 5′ ends of CIT1 RNAs. Cells containing the +164 *CIT1-lacZ* multicopy fusion plasmid were grown in glucose or glucose plus glutamate media (the +164 fusion does not derepress in lactate-grown cells, see above). Total RNA was isolated as described previously (Guarente and Mason 1983). An 18-bp primer homologous to the portion of *lacI* present at the beginning of *lacZ* (Rogers Yocum et al. 1984) (starting 16 bp downstream from the *Bam*H1 site) was labeled at its 5′ end using ^{32}P-ATP and T4 polynucleotide kinase. The labeled primer was hybridized to RNA and was extended using reverse transcriptase (Hahn et al. 1985). The products were denatured and electrophoresed in a polyacrylamide-urea gel with denatured pBR322/*Hpa*2 size markers.

Fusion of CIT1 upstream-DNA to a UAS-less CYC1-lacZ fusion. The −457 deletion derivative of *CIT1-lacZ* was partially digested with *Eco*R5 and a *Xho*1 linker was placed at the *Eco*R5 site located at −210 in *CIT1*. Then, the *Xho*1–*Sac*1 fragment containing the *CIT1* TATA element (−195), the transcription initiation region (−150 to −100), and the beginning of *lacZ* was replaced with a

*Xho*1–*Sac*1 fragment from plasmid pLG669Z (Guarente and Ptashne 1981), which contains the analogous region (−178 and downstream) of a *CYC1-lacZ* fusion, and lacks both UAS_1 and UAS_2 (Guarente et al. 1984). Inversion of *CIT1* upstream-DNA was accomplished by adding a *Bgl*2 linker at the *Xho*1 site and removing and recloning the resulting *Bgl*2–*Bgl*2 fragment in either orientation. In these heterologous constructs, the nearest *CYC1* TATA element is about 60 bp further downstream from the *CIT1* upstream fragment being tested than is the putative TATA element in the native *CIT1* gene.

Mutagenesis by the polymerase chain reaction (PCR). *Amplitaq* polymerase (Perkin-Elmer) or *Pfu* polymerase (Stratagene) were used as suggested by the manufacturer. Amplification was carried out in 3 mM $MgSO_4$, using 300 ng of each primer, 100 ng of plasmid template, and 50 μm dNTPs (to reduce misincorporation). After incubation for 5 min at 95 °C, samples were cycled 25 times for 30 s at 95 °C, 30 s at 37 °C, and 30 s at 72 °C, followed by incubation at 72 °C for 7 min (for complete extension). Deletions within the *Bgl*2 to *Xho*1 region upstream of *CIT1* were created as follows. A primer homologous to a portion of this region and carrying a *Bgl*2 or *Xho*1 restriction site (for 5′ or 3′ deletion, respectively) plus an opposing primer external to this region were used for amplification. Subsequently, the PCR product was cut and cloned in place of the wild-type *Bgl*2 to *Xho*1 fragment, upstream of a −10 *CIT1-lacZ* fusion. Multiple base substitutions were made by a similar strategy, using a primer with 10 bp of wild-type sequence on either side of the substituted nucleotides. In cases, where the region to be mutagenized was far from either restriction site, a two-step, three-primer PCR strategy was employed (Sarkar and Sommer 1990). An initial amplification was used to make a portion of the *Bgl*2 to *Xho*1 region. One primer (within the *Bgl*2 to *Xho*1 region) included the desired base substitutions, while the second, opposing, primer was entirely homologous to sequences outside of this region. Subsequently, the PCR product (5% of it) was extended in the presence of a third, opposing, primer and wild-type plasmid template, to make a full length *Bgl*2 to *Xho*1 fragment. This mutagenized fragment was used to replace the wild-type fragment upstream of a −10 *CIT1-lacZ* fusion. For these two-step PCR constructions, *Pfu* proofreading polymerase was substituted for *Taq* polymerase since the latter exhibited a high frequency of misincorporation in the region synthesized during the second round of PCR. All PCR-generated constructs were checked for the desired mutation and lack of misincorporations by determining the DNA sequence of the entire *Bgl*2 to *Xho*1 fragment. In some cases a mobility-shift assay was used to detect single-strand conformation polymorphisms (SSCP) (Orita et al. 1989) as a rapid screen for mutant clones. The *Bgl*2 to *Xho*1 fragment was amplified by PCR from bacterial colonies (Joshi et al. 1991). One-tenth of the PCR product was denatured in 50 mM NaOH, 1 mM EDTA at 42 °C for 5 min, and loaded in 10% formamide, 0.5% xylene cyanol, 5% bromophenol blue onto a 10% nondenaturing polyacrylamide gel. After electrophoresis, DNA was detected by staining with 0.5 μg/ml of EtBr (Yap and McGee 1993).

DNA sequence determination. All plasmid constructions were checked and deletion endpoints determined by performing DNA sequencing (Sanger et al. 1977) on denatured plasmid DNA, using Sequenase (as suggested by United States Biochemical Corporation). Primers homologous to the end of *URA3* or the TATA region of *CIT1* were used to determine the DNA sequence on both strands, between the *Bgl*2 and *Xho*1 sites. Our *CIT1* sequence numbering differs slightly from that in the original sequence report (Suissa et al. 1984), due to differences at four positions. In addition, we have extended the DNA sequence beyond −375. Additional *CIT1* upstream-DNA sequence data is available as Genbank/EMBL accession number X63746 (Lalo et al. 1993). 5′ and 3′ deletion derivatives made by *Bal*31 exonuclease treatment or PCR are numbered by the last contiguous wild-type base pair remaining (even when contributed by a restriction-site linker).

Screen for mutants with trans-acting mutations affecting expression of CIT1. Cells containing the +164 *CIT1-lacZ* fusion plasmid were treated with ethylmethanesulfonate to about 95% lethality (Sherman et al. 1986) and were spread on minimal-medium plates (pH 7) containing salts, vitamins, strain-dependent requirements, 2% glucose, and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) (Guarente 1983). The Xgal phenotype was checked by restreaking and β-galactosidase activity was assayed in liquid cultures. Mutations were characterized as cis- or trans-acting by curing the plasmid from the strain, retransforming with the *CIT1-lacZ* fusion plasmid, and assaying for β-galactosidase activity. Plasmid-cured mutants were also transformed with plasmids bearing other yeast gene fusions, *CIT2-lacZ* (unpublished), *CYC1-lacZ* (Δ312) (Guarente et al. 1984), *LEU2-lacZ* (pLG1) (Guarente et al. 1984), and *HIS4-lacZ* (HYC3) (Hinnebusch et al. 1985), to determine the specificity of the mutation. Mutations were characterized as dominant or recessive by mating with an α strain (lacking plasmid) and assaying β-galactosidase activity. Out of 130000 colonies screened, expression of *CIT1-lacZ* was reduced in 44 mutants (increased expression was not observed). All but six of these reduced the expression of both the *LEU2-lacZ* and *HIS4-lacZ* fusions. An additional mutant, J40-86, was isolated in a similar screen for reduced expression of a *CIT2-lacZ* fusion in glucose-grown cells (unpublished).

Results

A CIT1-lacZ gene fusion is regulated by glucose and glutamate

We previously demonstrated that *CIT1* (mitochondrial citrate synthase) mRNA is reduced approximately 6-fold in glucose-grown cells compared with cells grown on a nonfermentable carbon source such as lactate (Kim et al. 1986). Expression is reduced approximately 10-fold further in cells supplied with both glucose and 0.2% glutamate (Kim et al. 1986). This regulation reflects the catabolic and biosynthetic roles of the first three enzymes of the TCA cycle (the TCA cycle intermediate α-ketoglutarate is a precursor for the biosynthesis of glutamate). To simplify identification of the DNA sequences and transcription factors required for regulation of the *CIT1* gene, we desired a more convenient assay for expression. Although assays for citrate synthase activity exist (Parvin 1969; Srere 1969), *S. cerevisiae* contains a second gene, *CIT2*, which also encodes citrate synthase (Kim et al. 1986; Rosenkrantz et al. 1986). We fused the *CIT1* gene to the *E. coli lacZ* gene which encodes β-galactosidase. This fusion is carried on a multicopy yeast-*E. coli* shuttle vector derived from pLG669Z (Fig. 1) (Guarente and Ptashne 1981). The initial fusion construct carried DNA sequences from −806 to −10 (relative to the *CIT1* coding sequence) (see Materials and methods).

Expression of β-galactosidase from the −806 *CIT1-lacZ* gene fusion was regulated similarly to mRNA from the native *CIT1* gene (Kim et al. 1986). β-galactosidase activity was reduced 4–6-fold in glucose-grown cells compared with lactate or lactate plus glutamate-grown cells, and was reduced an additional 13-fold in cells supplied with both glucose and 0.2% glutamate (Fig. 2, first data point, −806). There was a slight deviation from the expected regulation, in that expression of the *CIT1-lacZ* fusion was approximately 1.4-fold higher in lactate-grown cells when glutamate was also supplied. This phe-

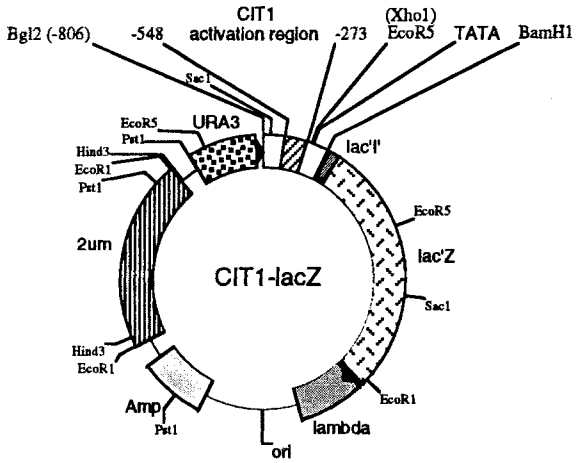

Fig. 1. *CIT1-lacZ* fusion plasmid. The *CIT1* gene of *S. cerevisiae* (mitochondrial citrate synthase) was fused to the *lacZ* gene of *E. coli* (β-galactosidase) on a multicopy plasmid. Initially 806 bp of *CIT1* upstream-DNA were included. Deletion and nucleotide substitution derivatives are similar in structure to the map shown, except that the *Bgl*2 and *Xho*1 sites mark the new ends of 5′ and 3′ deletion derivatives, respectively

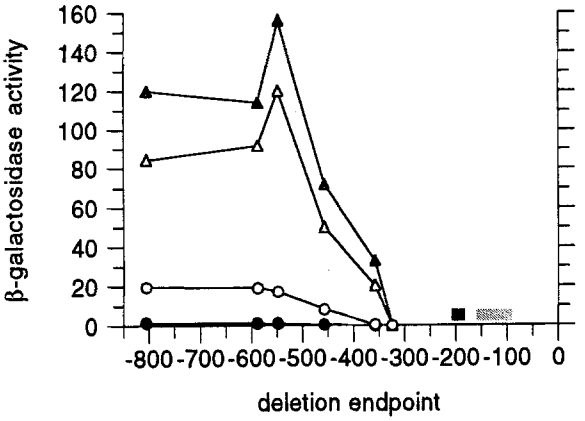

Fig. 2. 5′ deletions of DNA upstream of a *CIT1-lacZ* fusion. Upstream DNA sequences were deleted from a *CIT1-lacZ* fusion plasmid, starting at the *Bgl*2 site at −806 (Fig. 1). β-galactosidase activity was assayed in cells grown in minimal medium containing glucose (○), glucose plus glutamate (●), lactate (△), or lactate plus glutamate (▲). Deletion endpoints are numbered by the last remaining wild-type base pair, as measured from the translation initiation codon of *CIT1*. The locations of the putative active TATA element (*black box*) and transcription initiation region (*gray box*) are also shown

nomenon was consistent, though less pronounced in deletion derivatives (Fig. 2 and below), and may be due to the presence of multiple copies of the fusion plasmid or to slightly different growth rates in these media. The effect of varying the concentration of glutamate in glucose-minimal medium on the expression of the *CIT1-lacZ* fusion was determined. Glutamate concentrations of 0.01% or 0.05% reduced *CIT1-lacZ* expression by about 55% or 80%, respectively, while concentrations of 0.1% to 0.5% reduced expression by 85–90%, and noticeably increased the growth rate. Glutamate auxotrophs are typically supplemented with glutamate in the range of 0.01–0.1% (Sherman et al. 1986; Folch et al. 1989).

Mapping the transcription initiation region

Reverse transcriptase mapping was used to determine the locations of the 5′-ends of transcripts from a plasmid-borne *CIT1-lacZ* fusion (Fig. 3). Several major transcripts initiate between 150 bp and 100 bp upstream of the *CIT1* translation initiation codon. At this level of resolution, it appears that all *CIT1* transcripts are coordinately regulated by the combination of glucose and glutamate. A candidate TATA element (TATAAA at −195) has the appropriate sequence (Chen and Struhl 1988) and the positioning (Hahn et al. 1985; Nagawa and Fink 1985) required for these transcripts. There was no appreciable level of products below 200 bases, which would have indicated transcripts encoding a truncated product lacking the amino-terminal mitochondrial targeting signal (Rosenkrantz et al. 1986; Singh et al. 1992). Thus, although some *CIT1*-encoded citrate synthase activity is detected in the post-mitochondrial supernatant (Rosenkrantz et al. 1986), it is not the result of a truncated protein encoded by a distinct transcript. The locations of the region of transcription initiation and the predicted functional TATA element (Hahn et al. 1985) are indicated in Fig. 2.

5′ deletion analysis of CIT1 upstream-DNA

To identify regulatory elements involved in regulation of *CIT1*, deletion analysis was performed on the −806 to −10 *CIT1-lacZ* plasmid-borne fusion, which is regulated correctly (see above). 5′ deletions were made starting at the *Bgl*2 site at −806, unidirectionally downstream toward *lacZ* (Fig. 1). Removal of DNA sequences from −806 to −548 did not reduce expression or alter regulation (expression was increased; Fig. 2). Further deletion gradually reduced all expression, until −324, where expression was eliminated. Thus, expression of *CIT1* is dependent on activation sequences between −548 and −324.

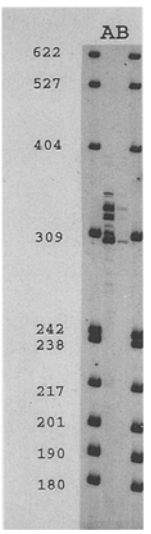

Fig. 3. Mapping of 5′ ends of *CIT1* mRNA. RNA was isolated from cells containing a *CIT1-lacZ* fusion plasmid, after growth in glucose-minimal medium without (*A*) or with (*B*) glutamate. A ^{32}P end-labeled primer was hybridized to the RNA and was extended using reverse transcriptase. The products were denatured and electrophoresed in a polyacrylamide-urea gel with denatured pBR322/*Hpa*2 fragments as size markers (bases)

Like other yeast upstream activation sequences (UASs), this region lies far upstream of the transcription initiation region and the TATA box. Since these deletions were all made from the 5′ side, we could not decide whether sequences between −324 and the TATA box (−195) are also required, but not sufficient, for expression (see below).

Some deletions ending between −548 and −324 had different effects on glucose-repressed and derepressed expression. Deletion from −548 to −457 reduced both glucose-repressed and derepressed expression of *CIT1* (2–2.5-fold) (Fig. 2). Further deletion from −457 to −358 reduced expression much more sharply in glucose-grown cells (16-fold) than in lactate-grown cells (2.5-fold). Another way of stating this is that derepression was 4–7-fold for deletion constructs with endpoints from −806 to −457, but was 41-fold for the −358 deletion derivative. Further deletion to −324 reduced expression in lactate-grown cells (140-fold) more than in glucose-grown cells (4.5-fold), and essentially eliminated all expression. These data are consistent with a model in which distinct DNA elements activate glucose-repressed expression and derepression, with elements critical for the former located upstream of elements critical for the latter. The region −548 to −457 appears to contribute to both kinds of expression, and might contain activation elements of both types or else constitutive activation sites.

While *CIT1* is negatively regulated by glucose and glucose plus glutamate, there was little evidence from the deletion analysis for negative regulatory elements. Deletion from −588 to −548 increased expression in lactate-grown cells, but did not affect regulation by glucose or glucose plus glutamate (Fig. 2). The −358 and −324 deletions reduced the difference between expression levels in glucose-grown and glucose plus glutamate-grown cells, but this is probably not significant since it was due to decreased expression in the former rather than increased expression in the latter. Negative elements for regulation by glucose or glucose plus glutamate could have gone undetected if located within DNA sequences also critical for expression (e.g., −457 to −324). Alternatively, negative regulation might be accomplished by reduced levels, DNA-binding, or the function of an activator protein which interacts with an upstream activation element.

CIT1 upstream-sequences confer regulated expression upon a heterologous gene

In general, activation of transcription by yeast UASs is largely independent of precise distance, orientation, and the source of the transcription initiation region and TATA element (Guarente 1988; Forsburg and Guarente 1990). We tested the ability of *CIT1* upstream-DNA to activate transcription of a heterologous gene lacking its own UAS. *CIT1* DNA between −457 and −211 (*Xho*1 site just upstream of the TATA element, Fig. 1), was placed in either orientation upstream of the TATA elements of the yeast *CYC1* gene fused to *lacZ* (Guarente and Ptashne 1981). This portion of the *CYC1* gene lacks both *CYC1* UASs and is not expressed on its own (Guarente et al. 1984).

This upstream-DNA region of *CIT1* activated the transcription of *CYC1-lacZ* and conferred regulation by glucose and glutamate (Fig. 4). The level of β-galactosidase activity produced (Fig. 4 A) was much higher than that from the −806 *CIT1-lacZ* fusion (Fig. 2). However, this is probably due in large part to the lack of a native yeast translation initiation site in this *CIT1-lacZ* fusion (see Materials and methods). Activation and regulation were independent of the precise distance from the TATA element and orientation. In construct A (native orientation) the distance between the *CIT1* upstream-DNA region and the nearest *CYC1* TATA element was approximately 60 bp longer than in the native *CIT1* gene. In construct C, inverted *CIT1* DNA also activated and regulated expression. Expression was reduced in construct C compared with construct A, but this appears to be due to the addition of a *Bgl*2 linker (construct B) between the *CIT1* upstream-DNA and the *CYC1* TATA elements (to allow inversion of the *CIT1* DNA in construct C). We do not have data on the mechanism of this linker effect. Thus, inversion of the *CIT1* DNA actually increased expression, especially in glucose-grown cells (Fig. 4 B, C). Perhaps inversion of the *CIT1* DNA has placed activation elements, functional in glucose-grown cells, closer to the *CYC1* TATA elements, where they might function more efficiently (Ruden et al. 1988).

Fine 5′ deletion analysis of the CIT1 activation region

To further localize upstream regulatory sequences, additional 5′ deletions were constructed from −548, with endpoints every 10 to 20 bp. These deletions produced a slow, steady decline in glucose-repressed expression, until −354 where expression was lost (Fig. 5). Derepressed

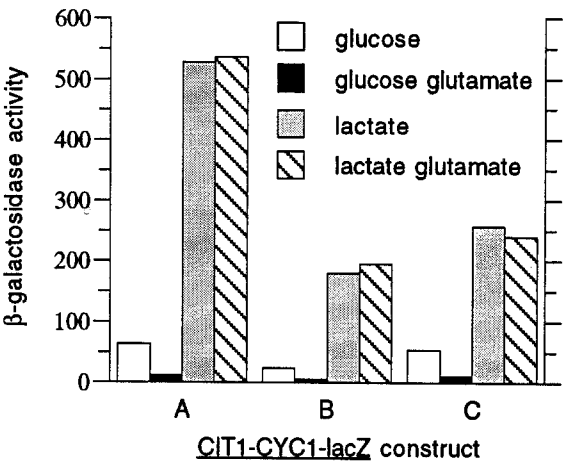

Fig. 4. Effect of *CIT1* upstream-DNA sequences on a UAS-less *CYC1-lacZ* fusion. β-galactosidase was assayed in cells grown in minimal medium containing glucose or lactate, with or without glutamate. **A** *CIT1* sequences from −457 to −211 were placed, in their natural orientation, at the *Xho*1 site upstream of the TATA elements and the transcription initiation region of *CYC1* (cytochrome c) fused to the *E. coli lacZ* gene. **B** As an intermediate construction step, the *Xho*1 site was filled-in and a *Bgl*2 linker was inserted. **C** The *Bgl*2-*Bgl*2 fragment containing *CIT1* upstream-DNA was cloned in inverted orientation

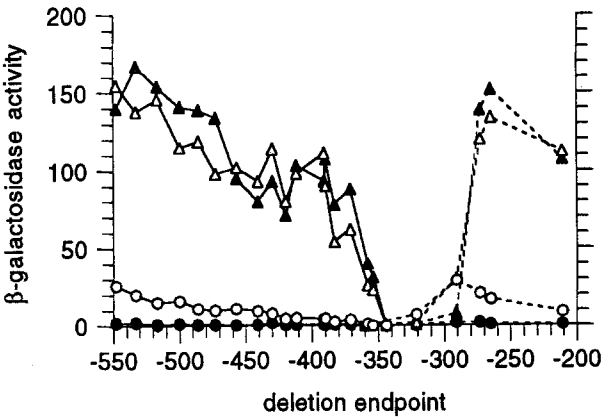

Fig. 5. Fine 5′ and 3′ deletion mapping of the *CIT1* upstream activation region. 5′ deletions (*solid lines*) heading downstream into the activation region of *CIT1* were generated starting from a *Bgl*2 site at −548 (Fig. 1). 3′ deletions (*dashed lines*) heading upstream into this region were made from the −457 5′ deletion, starting at a *Xho*1 linker placed at −211 (Figs. 1, 6). β-galactosidase activity was assayed in cells grown in minimal medium containing glucose (○), glucose plus glutamate (●), lactate (△), or lactate plus glutamate (▲). Deletion endpoints are numbered by the last remaining wild-type base pair, as measured from the translation initiation codon of *CIT1*

expression declined slowly from −548 to approximately −473, remained steady from −473 to −390, and declined sharply from −390 to −344, where expression was lost. Thus, expression appeared to depend on multiple activation elements.

Again, differential effects on glucose-repressed and derepressed expression were observed. Deletion from −548 to −390 reduced expression in glucose-grown cells by 5.5-fold and expression in lactate-grown cells by 1.7-fold. Deletion from −390 to −358 reduced expression in both glucose-grown and lactate-grown cells, by 3.5–4-fold. Further deletion from −358 to −354 essentially eliminated expression in glucose-grown cells (reduced 6-fold), but had no effect on expression in lactate-grown cells. Finally, deletion from −354 to −344 essentially eliminated expression in lactate-grown cells (reduced 230-fold). Once again, the data is suggestive that there are distinct sequences for activation of expression in glucose-grown and lactate-grown cells.

None of the deletions appeared to remove a negative element critical for repression by glucose or glucose plus glutamate. Instead, as DNA was deleted, the derepression ratio increased, from six to about 20, due to a faster decline in glucose-repressed expression than in derepressed expression. The effect of glutamate on glucose-grown cells declined gradually, from a 13-fold reduction to no reduction, but this was due to reduced expression in glucose-grown cells rather than increased expression in glucose plus glutamate-grown cells.

Identification of an activation region required specifically for derepression

We also undertook a 3′ deletion analysis of the activation region. Deletions were made unidirectionally by PCR, starting at the *Xho*1 site at −211 and heading upstream, away from the putative TATA element at −195 (Fig. 1). To simplify DNA sequence analysis of deletion derivatives, these 3′ deletions were made from the 5′ deletion construct −457. Therefore, their levels of expression should be compared to those of the −457 construct. (The −211 construct is identical to the −457 construct except that a *Xho*1 linker has been inserted at the *Eco*R5 site at −211).

The region −211 to −273 is not essential for activation. Deletion from −211 to −265 or −273 actually increased expression of *CIT1* under all growth conditions (Fig. 5). The increase was greater for glucose-grown cells (2-fold) than for lactate-grown cells (1.1–1.2-fold). This increase might be due to the deletion of negatively-acting sequences or might simply reflect the closer spacing of the TATA box to upstream activation sequences (Ruden et al. 1988).

Deletion beyond −273, to −291, specifically eliminated *CIT1* derepression, by slightly increasing expression in glucose-grown cells and reducing expression in lactate-grown cells by 4-fold (Fig. 5). It therefore appears that DNA sequences at least partially between −273 and −291 are specifically required for derepression of *CIT1* expression in lactate-grown cells. This deletion replaces half of a potential binding sequence (TCCAATAA) for the HAP2,3,4 transcriptional activator protein (Forsburg and Guarente 1990; Guarente 1992, 1993; Pinkham and Keng 1993) (Fig. 6). The new junction sequence created is CGAGATAA. A second candidate binding site for HAP2,3,4 (ACCAAAAA) lies just upstream, with only

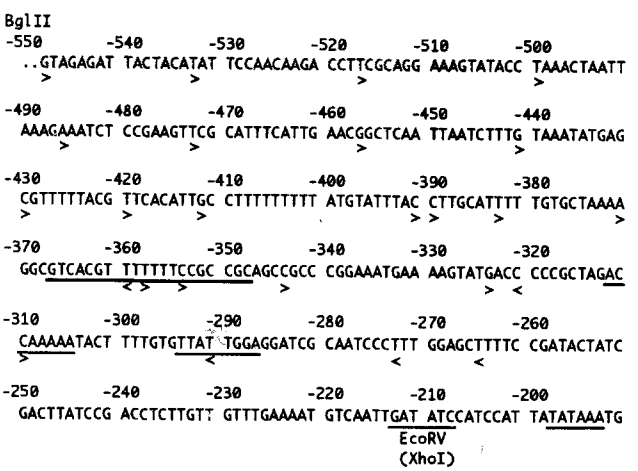

Fig. 6. The DNA sequence upstream of *CIT1*. The region of *CIT1* upstream-DNA under study is shown. The positions of deletion endpoints (Figs. 2, 5) are shown as follows. 5′ deletions were made from the *Bgl*2 site (−548) and relocated this site to the positions indicated (>). 3′ deletions were made from a *Xho*1 linker placed at the *Eco*R5 site in the −457 5′ deletion construct, and relocated this *Xho*1 site to the positions indicated (<). Deletions are numbered by the last remaining wild-type base pair, relative to the translation initiation codon of *CIT1*. DNA downstream from the *Xho*1 site, including the putative functional TATA element at −195 (*TATAAA*), is maintained in all deletion constructs. The location of two 10-bp substitutions which reduce glucose-repressed expression of *CIT1* (−367 to −348) and the locations of two candidate HAP2,3,4-binding sites (−310 and −290) are *underlined*. The DNA sequence reported here differs at several positions from the published *CIT1* sequence (Suissa et al. 1984)

10 bp between the two sites. Both candidate HAP2,3,4-binding sites differ from consensus (ACCAATNA or reverse complement) by 1 bp. The upstream candidate site differs in the same position as the original *CYC1* UAS_2 sequence (ACCAACCA) (Guarente et al. 1984) (which was a weak HAP2,3,4 activation site) and as the proposed HAP2,3,4 site of the *CYT1* gene (ACCAACCA) (Schneider and Guarente 1991). Further deletion, to −321, removed both candidate HAP2,3,4 sites and 8 bp in addition, and severely reduced expression under all growth conditions (Fig. 5). Finally, further deletion to −360 essentially eliminated all expression.

None of the 3′ deletions strongly affected regulation by glutamate plus glucose, which remained 9–18-fold in all cases. However, the −291 and −321 deletions not only reduced expression in lactate-grown cells, they also caused this remaining expression to be repressed by glutamate (2.5–3.8-fold). Thus, it appears that the signal for repression by glutamate is present (to some extent) in lactate-grown cells, and that other explanations must be sought for the lack of an effect of glutamate on the derepressed expression of *CIT1* (see Discussion).

Identification of an activation region required for expression in glucose-grown cells

5′ deletions ending in the region −390 to −344, reduced and ultimately eliminated all expression of *CIT1*. To further investigate this region, we chose a less intrusive method of mutagenesis involving substitution of blocks of nucleotides. This was done by PCR, in a manner which preserves the entire *Bgl*2 (−548) to *Xho*1 (−211) region with the exception of substitution of the complementary DNA sequence in a 10-bp block (see Materials and methods).

Replacement of the 10 bp from −367 to −358 or from −357 to −348 reduced *CIT1* expression 4–5-fold in glucose-grown cells (Table 1). Expression in lactate-grown cells appeared to be reduced 20–25%, but this change was within the standard deviation of the data. Thus, these substitutions alter an element or elements critical for activation of expression in glucose-repressed cells but making, at most, only a small contribution to expression in derepressed cells. The basal expression in glucose plus glutamate-grown cells was unaffected by either substitution (Table 1).

The results of the substitution experiments conflict with those from the 5′ deletion analysis. Deletion from −371 to −358 (or to −354) reduced glucose-repressed expression 3-fold (or 18-fold), but also reduced derepressed expression 7-fold (or 33-fold). Also, deletion from −371 to −344 eliminated all expression, rather than just reducing it. We do not believe that these discrepancies are due to the slightly different endpoints in the two techniques (−371 versus −367 and −348 versus −344), as our preliminary results are that substitution of the 5 bp on either side of the −367 to −348 region reduces glucose-repressed expression only by 20–40% (data not shown). We believe the results of this base-substitution analysis to be more reliable than those from the deletion analysis (see Discussion).

Trans-acting mutations affecting expression in glucose-grown cells

A genetic approach was undertaken to identify factors required for expression or regulation of *CIT1*. For this study, we focused on expression in glucose-grown cells. Cells transformed with a −806 to +164 *CIT1-lacZ* fusion were mutagenized, and screened for increased or reduced expression by colony color, on glucose-minimal medium containing Xgal. Effects were checked and quantified by β-galactosidase assays on liquid cultures. Only mutants with reduced expression were successfully isolated. Expression of *CIT1-lacZ* was reduced 2–14-fold in several mutants with trans-acting mutations (Table 2, CK series). For all of these mutants, reduced expression of the *CIT1-lacZ* fusion is recessive (see Materials and methods).

Table 1. The effects of 10-bp substitutions on expression of a *CIT1-lacZ* fusion

Region substituted[a]	β-galactosidase activity[b]		
	Glucose	Glucose glutamate	Lactate
Wild-type	24±6	2±1	112±24
−357 to −348 sub.	5±2	2±1	86±23
−367 to −358 sub.	6±2	2±1	87±20

[a] 10-bp substitutions of the complementary sequence were introduced by PCR into a *CIT1-lacZ* fusion retaining 548 bp of upstream DNA

[b] β-galactosidase activity is expressed as the change in $OD_{420} \times 1000$ divided by (min × ml of culture × OD_{600} of the culture). Standard deviations are indicated (±)

Table 2. Effects of trans-acting regulatory mutations on expression of *CIT1-lacZ* and other yeast gene fusions

Strain[a]	β-galactosidase activity[b]				
	CIT1-lacZ	*CIT2-lacZ*	*CYC1-lacZ*	*LEU2-lacZ*	*HIS4-lacZ*
WT	28	770	480	140	150
CKH10	10	730	270	150	170
CKH16	12	1020	470	230	160
CK2-7	9	310	800	170	100
CK144	10	42	140	160	41
CK2-2	11	13	900	100	34
CKH7	2	36	55	13	190
J40-86	5	1	300	22	190

[a] Mutants were isolated by screening for decreased expression of a *CIT1-lacZ* fusion, except for J40-86, which was isolated using a *CIT2-lacZ* fusion

[b] Cells were grown in glucose-minimal medium. β-galactosidase activity is expressed as the change in $OD_{420} \times 1000$ divided by (min × ml of culture × OD_{600} of the culture). The standard deviation was usually 20–40% of the value

To check the specificity of these mutations, mutants were cured of plasmid and transformed with other yeast genes fused to *lacZ*. Expression of *CIT2-lacZ* (nonmitochondrial citrate synthase) and *CYC1-lacZ* (iso-1-cytochrome c) fusions were also reduced in some of the mutants. *CIT2* is regulated by glucose plus glutamate but not by glucose alone (Kim et al. 1986), while *CYC1* is regulated by glucose (Guarente 1992, 1993) but not by glutamate (unpublished results). Transcription of *CYC1* in glucose-grown cells depends on UAS_1 and the HAP1 transcriptional activator protein (and perhaps other proteins). The mutants were also tested for expression of *LEU2-lacZ* (pLG1) (Guarente et al. 1984) and *HIS4-lacZ* (HYC3) (Hinnebusch et al. 1985) fusions. These genes do not encode mitochondrial proteins and are not repressed by glucose. A variety of specificity patterns were detected among the mutants, and there was no correlation between the effects on different genes. Unfortunately, the mutants most severely reduced in expression of *CIT1* or *CIT2* also exhibited reduced expression of the *LEU2-lacZ* or *HIS4-lacZ* control fusions, indicating a lack of specificity. J40-86, which was isolated in a similar screen for reduced expression of a *CIT2-lacZ* fusion (unpublished), also exhibited reduced expression of the *CIT1-lacZ* and *LEU2-lacZ* fusions (Table 2). Biochemical experiments are in progress to determine whether any of these mutations define genes encoding proteins which bind to *CIT1* or *CIT2* upstream-DNA.

Discussion

A fundamental problem in eukaryotic cells is the regulation of synthesis of proteins required for mitochondrial function. Most of these proteins are encoded by nuclear genes, and expression of many of these genes is repressed by glucose (Forsburg and Guarente 1990; Pon and Schatz 1991; Pinkham and Keng 1993). The *CIT1* gene of *S. cerevisiae* encodes mitochondrial citrate synthase, which catalyzes the first and rate-limiting step of the TCA cycle (Krebs and Lowenstein 1960; Walsh and Koshland 1985). The level of *CIT1* mRNA is reduced 5–7 fold in glucose-grown cells and is reduced an additional 10-fold by supplying glutamate in addition to glucose (Kim et al. 1986). This complex, synergistic regulation reflects the roles of the TCA cycle in the catabolism of nonfermentable carbon sources and in providing precursors for biosynthetic pathways (e.g., α-ketoglutarate is a precursor for the synthesis of glutamate).

We have shown here that upstream DNA sequences activate and regulate expression of *CIT1*. Based on mapping the 5′ ends of *CIT1* transcripts, transcription initiates at multiple sites between −150 and −100 (relative to the *CIT1* coding sequence), and all transcripts appear to be regulated by glucose plus glutamate (Fig. 3). The putative functional TATA element is located at −195 (Fig. 6). Based on 5′ and 3′ deletion analysis of upstream DNA, sequences critical for expression of a *CIT1-lacZ* fusion were mapped to the region −548 to −273 (Figs. 2, 5). The DNA fragment −457 to −211 (located just upstream of the putative TATA element and retaining half the expression of the −548 construct) was shown to confer activation and regulation by glucose and glutamate, when placed in either orientation upstream of a UAS-less version of the yeast *CYC1* gene (iso-1-cytochrome c) (Fig. 4).

Since closely-spaced 5′ deletions resulted in gradual declines in glucose-repressed and derepressed expression, we were unable to localize critical activation elements by this method. An alternative method, the introduction of 10-bp substitutions by PCR, proved more useful. The approach used here offers several advantages over the original linker-scanning method (McKnight and Kingsbury 1982): every nucleotide within the block is mutated, a large set of opposing deletions is not required, and the number of nucleotides substituted per block can be chosen, depending on the size of the region to be analyzed. The two-step, three-primer PCR method employed (Sarkar and Sommer 1990) allows mutagenesis of sequences far away from convenient restriction sites, without the use of very-large oligonucleotide primers.

We initially focused our substitution analysis on the region −390 to −344, where closely-spaced 5′ deletion endpoints caused a rapid decline in, and ultimately eliminated, expression (Fig. 5). Substitution of the complementary 10-bp DNA sequence from −367 to −358 or from −357 to −348 strongly reduced expression in glucose-grown cells, and appeared to decrease expression in lactate-grown cells by 20–25% (Table 1). Preliminary results indicate that adjacent sequences on either side of this 20-bp region, −367 to −348, are less critical.

Deletion and substitution analyses gave conflicting conclusions as to the importance of the −367 to −348 activation region for derepressed expression (see Results). We believe deletion data to be less reliable than base substitution data. Substitutions leave intact the DNA upstream of the altered region, while 5′ deletions replace them, in this case with the *URA3* sequences present upstream (Fig. 1). These foreign sequences, when moved closer, might reduce expression of the downstream *CIT1-lacZ* fusion. Such inhibition could be due to binding of a protein or to transcription from *URA3*.

The region for activation of *CIT1* in glucose-repressed cells, −367 to −348, does not contain any obvious binding sites for known yeast regulatory proteins (Fig. 6) (Verdier 1990). As one approach to the identification of proteins which interact with this regulatory region, we have generated trans-acting mutations affecting expression of *CIT1* in glucose-grown cells (Table 2). Experiments are underway to determine whether any of these mutants are defective in a protein required for activation by the −367 to −348 region, and if so, whether the protein specifically binds this DNA sequence. Some of the mutants also exhibited reduced expression of *CIT2* (nonmitochondrial citrate synthase) or *CYC1* (iso-1-cytochrome c), indicating possible links between the regulation of these genes.

3′ deletion beyond −273, to −291, specifically eliminated derepression above the glucose-repressed level of expression (Fig. 5). The −291 deletion replaces half of a candidate binding site for the HAP2,3,4 transcriptional activator protein, which has previously been shown to derepress transcription of genes encoding components of

the mitochondrial electron transport chain, in the absence of glucose (Forsburg and Guarente 1990; Mattoon et al. 1990; Guarente 1993; Pinkham and Keng 1993). Transcription of several other nuclear genes encoding TCA cycle enzymes has been found to be repressed by glucose: *ACO1* (aconitase) (Gangloff et al. 1990), *KGD1* (α-ketoglutarate dehydrogenase) (Repetto and Tzagoloff 1989), *KGD2* (dihydrolipoyl transsuccinylase) (Repetto and Tzagoloff 1990), *LPD1* (lipoamide dehydrogenase) (Roy and Dawes 1987), and *MDH1* (malate dehydrogenase) (McAlister-Henn and Thompson 1987). HAP2,3,4 has been shown to be required for the derepression of *KGD1*, *KGD2*, and *LPD1* (Repetto and Tzagoloff 1989, 1990), but the mechanism of expression of these genes in glucose-repressed cells is unknown.

The remaining expression of the −291 deletion construct in lactate-grown cells may indicate that we have only partially incapacitated the element(s) responsible for derepressed expression. For instance, the −291 deletion retains a second candidate HAP2,3,4-binding site 10 bp upstream, at −312 to −305 (Fig. 6). Both candidate sites differ by only one nucleotide from the consensus binding site (TNATTGGT or ACCAATNA) (Forsburg and Guarente 1990; Guarente 1992, 1993; Pinkham and Keng 1993). However, if the remaining HAP2,3,4 consensus site were active in the −291 deletion construct, it would be expected to give derepressible expression rather than the observed constitutive expression (Fig. 5). The −312 to −305 candidate HAP2,3,4 element may normally activate *CIT1* expression, but not in the −291 deletion construct, where the other candidate HAP2,3,4 element at −290 has been removed and the TATA element has been moved very close by.

A second possibility is that the remaining expression of the −291 deletion construct in lactate-grown cells is due to a different activation region, located further upstream. Since expression of the −291 deletion construct is about the same in both glucose-repressed and derepressed cells (about 30 units of β-galactosidase activity; Fig. 5), it is possible that a single upstream region activates constitutively. The region −367 to −348 (see above) may be this constitutive activation region. Substitutions in this region did appear to reduce both glucose-repressed and derepressed expression by 20 to 25 units (Table 1). However, the apparent decrease in derepressed expression is similar to the standard deviation of the data, and is thus suspect. Construction of a *CIT1-lacZ* fusion with both the −291 deletion and either substitution in the −367 to −348 region should answer whether both regions contribute to derepressed expression of *CIT1*.

It is possible to construct a simple model explaining the data on regulation of *CIT1* by glucose and glutamate. The region −367 to −348 activates at low level in glucose-repressed cells, and perhaps equally in derepressed cells. DNA sequences located at least partially between −291 and −273 mediate derepression, perhaps by binding HAP2,3,4. What mediates repression of *CIT1* by glutamate and how is it limited to glucose-repressed cells? The fact that the remaining expression of the −291 and −321 deletion constructs is repressed by glutamate not only in glucose-grown cells, but also in lactate-grown cells (Fig. 5), indicates that the intracellular signal for regulation by glutamate is not lacking in lactate-grown cells. Perhaps a negative element located in or near the −367 to −348 activation region mediates repression by glutamate. The activation region mediating derepression might escape repression by glutamate by virtue of its distance or location downstream from the glutamate repression element. Alternatively, perhaps the synthesis, binding, or function of the cognate transcriptional activator protein for the −367 to −348 activation region is reduced by glutamate, while the transcriptional activator protein for derepression mediated by the −291 and −273 region (perhaps HAP2,3,4) is not affected by glutamate.

This dual activation model for the regulation of *CIT1* resembles that for the yeast *CYC1* gene (iso-1-cytochrome c), where glucose-repressed expression requires the HAP1 protein bound to UAS_1 (in the presence of heme) and derepression requires HAP2,3,4 bound to UAS_2 (Forsburg and Guarente 1990; Pinkham and Keng 1993). *ACO1* (aconitase), like *CIT1*, is regulated by glucose and by glucose plus glutamate, and has a candidate HAP2,3,4-binding site (ACCAATCA) at −288 (Gangloff et al. 1990). The molecular mechanism of regulation of *CIT2* (nonmitochondrial citrate synthase) by glucose plus glutamate (but not by glucose alone) is also under investigation in this laboratory (Kim et al. 1986).

According to the model proposed above, if the region −367 to −348 activates constitutively with respect to glucose, addition of glutamate to lactate cultures should slightly reduce expression of a *CIT1-lacZ* fusion, rather than have no effect or increase it, as observed (Figs. 2, 4, 5). The predicted decrease is about 20 to 25 units of β-galactosidase activity (the level of activation by the −367 to −348 region; Table 1). This amount is similar to, or less than, the standard deviation of the data, and therefore difficult to confirm or deny. Also, the slightly-faster growth rate of lactate cultures supplied with glutamate may increase expression of the *CIT1-lacZ* fusion sufficiently to obscure the predicted small decrease in expression.

What is the role of DNA sequences upstream of the activation sequences identified (−291 to −273 and −367 to −348)? Closely-spaced deletions from −548 to −371 (Fig. 5) produced a slow decline in glucose-repressed expression (7-fold) and derepressed expression (2.5-fold), suggesting that this region contains multiple activation elements. Also, 5′ deletion to −344 eliminates derepressed expression (as well as glucose-repressed), even though the −291 to −273 region and the candidate HAP2,3,4 elements at −310 and −290 remain intact. Thus, the identified activation regions and DNA further upstream both appear to be critical for expression (i.e., they function synergistically). The construction of 10-bp substitutions from −548 to −457 has failed to identify additional critical upstream activation regions (data not shown), despite the fact that deletion of this region reduces all expression 2-fold (Figs. 2, 5). Perhaps, this region contains multiple weak activation sequences. An alternative explanation for the gradual decline in expression observed with deletions from −548 to −371 is that it is an artifact of the 5′ deletion analysis. Indeed, we observed differences be-

tween the results from 5′ deletion analysis and 10-bp substitutions (see above and Results), and believe substitution data to be more reliable.

Finally, although the *CIT1* and *CIT2* coding sequences share homology and appear to be related through a multigenic duplication (Rosenkrantz et al. 1986; Lalo et al. 1993), the upstream sequences and regulation of these genes have diverged substantially. Expression of *CIT2* is reduced by glucose plus glutamate, but, unlike *CIT1*, not by glucose alone (Kim et al. 1986). Furthermore, expression of *CIT1* and *CIT2* responds to the state of the mitochondrial DNA, but the response is opposite. *CIT1* expression is reduced in *rho*^{0} strains (deficient in mitochondrial DNA), while *CIT2* expression is increased in *rho*^{0} strains (and in strains defective in the *CIT1* gene) (Liao et al. 1991; Liao and Butow 1993). Clarification of the relationship between the regulatory elements and regulatory mutants described here and those involved in regulation of *CIT2* by glucose plus glutamate or by *rho*° status, requires further work.

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