Structure and Regulation of KGD1, the Structural Gene for Yeast α-Ketoglutarate Dehydrogenase

BARBARA REPETTO AND ALEXANDER TZAGOLOFF*

Department of Biological Sciences, Columbia University, New York, New York 10027

Received 12 January 1989/Accepted 23 March 1989

Nuclear respiratory-defective mutants of Saccharomyces cerevisiae have been screened for lesions in the mitochondrial α-ketoglutarate dehydrogenase complex. Strains assigned to complementation group G70 were ascertained to be deficient in enzyme activity due to mutations in the KGD1 gene coding for the α-ketoglutarate dehydrogenase component of the complex. The KGD1 gene has been cloned by transformation of a representative kgd1 mutant, C225/1, with a recombinant plasmid library of wild-type yeast nuclear DNA. Transformants containing the gene on a multicyclone plasmid had three to four-times-higher α-ketoglutarate dehydrogenase activity than did wild-type S. cerevisiae. Substitution of the chromosomal copy of KGD1 with a disrupted allele (kgd1::URA3) induced a deficiency in α-ketoglutarate dehydrogenase. The sequence of the cloned region of DNA which complements kgd1 mutants was found to have an open reading frame of 3,042 nucleotides capable of coding for a protein of Mw 114,470. The encoded protein had 38% identical residues with the reported sequence of α-ketoglutarate dehydrogenase from Escherichia coli. Two lines of evidence indicated that transcription of KGD1 is catabolite repressed. Higher steady-state levels of KGD1 mRNA were detected in wild-type yeast grown on the nonrepressible sugar galactose than in yeast grown on high glucose. Regulation of KGD1 was also studied by using different 5′-flanking regions of KGD1 to the lacZ gene of E. coli and measuring the β-galactosidase in yeast. Transformants harboring a fusion of 693 nucleotides of the 5′-flanking sequence expressed 10 times more β-galactosidase activity when grown under derepressed conditions. The response to the carbon source was reduced dramatically when the same lacZ fusion was present in a hap2 or hap3 mutant. The promoter element(s) responsible for the regulated expression of KGD1 has been mapped to the −354 to −143 region. This region contained several putative activation sites with sequences matching the core element proposed to be essential for binding of the HAP2 and HAP3 regulatory proteins.

The α-ketoglutarate dehydrogenase complex (KGD1) of mitochondria catalyzes the oxidative decarboxylation of α-ketoglutarate to succinyl-coenzyme A and carbon dioxide, a key reaction of the tricarboxylic acid (TCA) cycle (31). The three enzymatic components of KGD1 are α-ketoglutarate dehydrogenase (KE1), dihydrolipoyl transsuccinylase (KE2), and dihydrolipoyl dehydrogenase (KE3). Each component is present in multiple copies in a macromolecular complex with an estimated molecular weight of 2.5 × 106 to 2.8 × 106 (16, 31).

Even though a good deal of information is available about the structural and catalytic properties of the bacterial (31) and mammalian (16) complexes, very few studies have addressed the question of how the synthesis of the three different subunits is regulated and how the extravesicular structure of the holoenzyme is assembled in vivo. In Escherichia coli, the genes coding for the KE1 and KE2 components are part of the suc operon located near some other genes for TCA cycle enzymes (6, 7). The KE3 component is encoded by lpd, which lies immediately downstream of the ace operon coding for the pyruvate dehydrogenase and dihydrolipoyltransacylase components of the pyruvate dehydrogenase complex (41). The lpd gene is transcribed from both the ace promoter and from its own separate promoter (39). In the eucaryote Saccharomyces cerevisiae, the structural genes of KGD1 are probably linked and separately regulated. At present, only the LPD1 gene for dihydrolipoyl dehydrogenase has been characterized and shown to be transcriptionally regulated by glucose (36). The concentrations of the LPD1 transcript and the KE3 polypeptide have been reported to be reduced in yeast grown on glucose as the carbon source (36). In addition, the 5′-flanking region of LPD1 has a sequence homologous to the upstream activation sites (UAS) of CYC1 involved in catabolite regulation of iso-1-cytochrome c (34).

As part of a study aimed at understanding the genetic determinants governing the biogenesis and maintenance of oxidatively competent mitochondria, we have screened by biochemical assays a collection of respiratory-deficient strains of S. cerevisiae for TCA cycle enzyme mutants. Mutants in three different complementation groups have been ascertained to lack α-ketoglutarate dehydrogenase activity. We report the properties of one group of mutants in which the absence of functional KGD1 has been correlated with a defective KE1 component. The mutants have enabled us to clone the KGD1 gene coding for the yeast KE1 component and to study its regulation. In this paper, we present evidence showing that transcription of KGD1 is regulated by glucose and activated by the products of HAP2 and HAP3 (14, 27).

MATERIALS AND METHODS

Yeast strains and growth media. The genotypes and sources of yeast strains used in this study are presented in Table 1. The nuclear pet (respiratory-defective) mutants were obtained by mutagenesis of the wild-type strain D273-10B/A1 with either ethylmethane sulfonate or nitrosoguanidine (44). The media used for routine growth of yeast were as follows:YPD (2% glucose, 1% yeast extract, 2% peptone),YPGal (2% galactose, 1% yeast extract, 2% peptone),YPEG (2% glycerol, 2% ethanol, 1% yeast extract, 2% peptone), WO (2% glucose, 0.67% Difco yeast nitrogen base without...
TABLE 1. Genotypes and sources of *S. cerevisiae* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D273-10B/A1</td>
<td><em>a met6</em></td>
<td>43 R. Rothstein*</td>
</tr>
<tr>
<td>W303-1A</td>
<td><em>ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100</em></td>
<td>R. Rothstein*</td>
</tr>
<tr>
<td>W303-1B</td>
<td><em>ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100</em></td>
<td>R. Rothstein*</td>
</tr>
<tr>
<td>C225</td>
<td><em>met6 kgdl-1</em></td>
<td>This study</td>
</tr>
<tr>
<td>C225/U1 or U2</td>
<td><em>ura3-1 kgdl-1</em></td>
<td>C225 × W303-1A This study</td>
</tr>
<tr>
<td>W303ΔKGD1</td>
<td><em>ade2-1 his3-11,15 leu2-3,112 trp1-1 kgdl::URA3</em></td>
<td>This study</td>
</tr>
<tr>
<td>BWG1-7a</td>
<td><em>ade1-100 his4-519 leu2-3 ura3-S2</em></td>
<td>17</td>
</tr>
<tr>
<td>LGW1</td>
<td><em>ade1-100 his4-519 leu2-3,112 ura3-S2 hap2-1 ura3-1</em></td>
<td>17</td>
</tr>
<tr>
<td>JF40-1</td>
<td><em>ade1-100 his4-519 leu2-3,112 ura3-S2 hap2-1</em></td>
<td>17</td>
</tr>
<tr>
<td>W303-2</td>
<td><em>ade6-1 leu2 his3 ura3 ade2 trp1 CYC1-lacZ(URA3)</em></td>
<td>23</td>
</tr>
</tbody>
</table>

* College of Physicians and Surgeons, Columbia University, New York, N.Y.

amino acids), and WOGal (2% galactose, 0.67% Difco yeast nitrogen base without amino acids). Solid media contained 2% agar. Amino acid supplements were added at a final concentration of 20 μg per ml.

**Cloning of KGD1.** The KGD1 gene was cloned by transformation of C225/U1 (α *ura3-1 kgdl-1*) and C225/U2 (α *ura3-1 kgdl-1*) with a yeast genomic library prepared by ligation of partial *Sac*III fragments (7 to 10 kilobase pairs [kb]) of nuclear DNA to the *Bam*HI site of the shuttle vector YEp24 (4). Marian Carlson of the Department of Human Genetics, Columbia University, New York, N.Y., kindly provided this plasmid bank. Cells were grown in 2% galactose liquid media (YEPGal) to early log phase and transformed with plasmid DNA by the method of Beggs (2). Transformants complemented for the uracil auxotrophy and respiratory deficiency were selected on minimal glycerol medium. Two *Ura*+ *Gly*+ transformants were found to have recombinant plasmids with the same nuclear DNA insert of approximately 6 kb. This plasmid, pG70/T1, was used to characterize the complementing gene.

**Enzyme assays of wild-type and mutant mitochondria.** Mitochondria were isolated from yeast cells grown to stationary phase in liquid YPGal. Cells were collected and converted to spheroplasts by treatment with Zymolyase 20,000 (Miles Corp.), and mitochondria were prepared by the procedure of Faye et al. (10). The overall activity of KGD was assayed spectrophotometrically by measuring NAD reduction at 340 nm in the presence of α-ketoglutarate as the reducing substrate (37). The activity of the α-ketoglutarate dehydrogenase component was assayed by the α-ketoglutarate-dependent reduction of ferricyanide measured at 410 nm (32). Aconitase activity was determined by the conversion of citrate to cis-aconitate (30). Published procedures were used to measure malate (9), isocitrate (5), and succinate (38) dehydrogenases.

**Hybridization analyses and S1 nuclease mapping.** Yeast genomic DNA was isolated according to the procedure of Myers et al. (24) for the Southern blot analysis. The DNA was digested with restriction endonucleases, electrophoretically separated on a 1% agarose gel, blotted to nitrocellulose, and hybridized with a nick-translated DNA probe containing part of the *KGD1* coding sequence as described previously (25).

For Northern (RNA) blot analysis, RNA was isolated from the wild-type strain D273-10B/A1 and fractionated on poly(U) Sepharose 4B (Pharmacia, Inc.) (19). Poly(A)+ RNA was electrophoretically separated under nondenaturing conditions on a 1% agarose gel. The RNA was blotted to diazobenzyloxymethyl-paper (1) and hybridized to a nick-translated DNA probe with part of the *KGD1* gene. The 5' termini of the *KGD1* transcripts were mapped by the method of Bard and Sharp (3). Poly(A)+-enriched RNA was hybridized to a single-stranded 5'-end-labeled restriction fragment complementary to the RNA. The fragment included the sequence from −354 to +173. The mixture was incubated at 45°C for 3 h in a solution containing 80% formamide, 0.4 M NaCl, 0.04 M PIPES ([piperazine-N,N'-bis(2-ethanesulfonic acid)] pH 6.5), and 1 mM EDTA. The mixture was diluted with 1× buffer and treated with different concentrations of S1 nuclease for 30 min at 37°C or 45°C. The protected fragments were separated on a 7% polyacrylamide sequencing gel. A sample of the untreated probe derivatized by the A+G-specific reaction of Maxam and Gilbert (21) was used as a sequencing ladder.

**Construction of lacZ fusions and assays of β-galactosidase activity in permeabilized yeast cells.** The episomal lacZ fusion vectors YEp353 and YEp366 (26) were used to construct in-frame fusions of different 5'-flanking regions of *KGD1* to the seventh codon of the *E. coli* lacZ gene. The shortest fragment cloned encompassed the sequence from the *PvuII* site at −143 to the *HindIII* site at +173. This construct, pG70/Z3, was used to introduce longer deletions in the 5'-flanking region. pG70/Z3 was linearized at the *EcoRI* site immediately adjacent to the *PvuII* site. This *EcoRI* site is part of the multiple cloning sequence of YEp353 (26). The linear plasmid was treated with BAL 31 nuclease and religated in the presence of an *XbaI* linker. The extent of the deletions created by the BAL 31 treatment was determined by sequence analysis of the *XbaI-HindIII* fragments recovered from the different plasmids.

Plasmids with the lacZ fusions were introduced into wild-type and mutant yeast strains by transformation and selection of either *Leu*+ or *Ura*+ clones. Segregation tests indicated 75% or higher plasmid retention after growth of the transformants in liquid glucose or galactose medium.

β-Galactosidase activity expressed from the lacZ fusions was measured in cells grown to early log phase in YPD medium containing 10% glucose or in YPGal. Cells were permeabilized, and β-galactosidase was assayed as described by Guarente (13).

**DNA manipulations and sequence analysis.** Standard procedures were used for the isolation of plasmid DNA, digestion of DNA with restriction nucleases, electrophoresis of DNA fragments, ligation of DNA, and transformation of *E. coli* (20). The *KGD1* gene was sequenced by the method of Maxam and Gilbert (21). Restriction fragments labeled at the 5' ends were either cleaved at internal sites or separated into the single strands before being chemically derivatized.

**RESULTS**

**Phenotype of *kgdI* mutants.** C225 is one of five respiratory-deficient mutant isolates assigned to complementation group G by the Author in this paper. Two clones were selected for their inability to utilize the nonfermentable substrate glycerol as their carbon source. The G70 mutants were complemented by a [rho0'] tester, indicating that the Gly phenotype is the result of
TABLE 2. α-Ketoglutarate dehydrogenase (KE1) activity in wild-type and kgdl mutants of S. cerevisiae

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>KE1 activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1A</td>
<td>KGD1</td>
<td>0.439 ± 0.038</td>
</tr>
<tr>
<td>C225</td>
<td>kgdl-1</td>
<td>0</td>
</tr>
<tr>
<td>C225/U2</td>
<td>kgdl-1</td>
<td>0</td>
</tr>
<tr>
<td>C225/U2/T1</td>
<td>KGDI</td>
<td>1.58 ± 0.26</td>
</tr>
<tr>
<td>W303AKGD1</td>
<td>kgdl::URA3</td>
<td>0</td>
</tr>
</tbody>
</table>

*KE1 activity refers to micromoles of ferricyanide reduced per hour per milligram of mitochondrial protein. The values shown are averages of two independent assays plus or minus the standard error.

Cloning of the KGDI gene. The KGDI gene was cloned by transformation of two kgdl mutants, C225/U1 and C225/U2, with a yeast genomic library. Transformants were selected for the acquisition of respiratory competence and uracil prototrophy. Two independent Glys+ and Ura+c clones (C225/U1/T1 and C225/U2/T2) were obtained. Vegetative progeny of both transformants grown in nonselective medium (rich glucose) showed cosegregation of the Glys+ and Ura+c phenotypes, indicating that complementation of the two markers is a function of a single autonomously replicating plasmid. Plasmid DNA isolated from each transformant was amplified in _E. coli_ and analyzed by restriction mapping. Both plasmids were found to have the same nuclear DNA insert of approximately 6 kb. This recombinant plasmid was designated pG70/T1 (Fig. 1).

The complementing region of pG70/T1 was localized by transferring different regions of the nuclear DNA insert to the shuttle vector YEp352 (15) and testing the new constructs for complementation of the respiratory deficiency of C225/U1. None of the four plasmid constructs tested were able to confer a respiratory-competent phenotype on the kgdl mutant. The inability of pG70/ST1 and pG70/ST2 to complement indicated that the gene must span the unique _SpfI_ site in the insert. Lack of complementation by pG70/ST3 and pG70/ST4 further localized the gene to a region of at least 2 kb defined by one of the _XbaI_ sites and the proximal _EcoRI_ site (Fig. 1).

Sequence of the KGDI gene. A region of approximately 3.7 kb of the pG70/T1 insert starting from the leftmost _BamHI_ site and ending at the distal _EcoRI_ site was sequenced by the method of Maxam and Gilbert (21). All the restriction sites used for 5'-end labeling were crossed from neighboring sites, and most of the sequence was confirmed from the complementary strands by the strategy shown in Fig. 2.

Analysis of the sequence revealed an open reading frame 3,042 nucleotides long (Fig. 3). This reading frame starts at nucleotide +1 and ends with an ochre termination codon at nucleotide +3,043. The translated protein had a molecular weight of 114,470. This size is consistent with the reported sizes of the KE1 subunits of the _E. coli_ (31) and mammalian α-ketoglutarate dehydrogenase complexes (40). The primary sequence of the yeast protein is homologous to the _E. coli_ KE1 subunit encoded by the _sucA_ gene (Fig. 4). When aligned by the MFALGO program (45), the two proteins shared 381 (38%) identities and 348 (34%) conservative substitutions. The best alignment was obtained with only a
few deletions, the most substantial of which was 18 residues long and occurred in the *E. coli* protein. The primary sequence homology combined with the absence of \( \alpha \)-ketoglutarate dehydrogenase activity in C225 constitutes strong evidence for the identity of the reading frame as the structural gene for the KE1 component of yeast KGDC. This gene has previously been given the designation KGDI (12). The amino-terminal sequence (30 to 40 residues) is basic and has no counterpart in the *E. coli* protein, suggesting that it might be a mitochondrial targeting signal.

**In situ disruption of the KGDI gene.** The one-step gene replacement procedure (35) was used to disrupt the wild-type KGDI gene. To construct the disrupted allele *kgdi::URA3* illustrated in Fig. 5, the 2-kb BamHI fragment of the pG70/T1 insert was transferred to the shuttle vector YEp352B (this vector is identical to YEp352 except that the multiple cloning region was replaced by a single BamHI site). The new plasmid was digested with HindIII and was ligated to a 1.2-kb HindIII fragment containing the yeast URA3 gene. This ligation yielded a plasmid in which the coding sequence of KGDI was disrupted at nucleotide +173 (residue 58). The linear 3.2-kb BamHI fragment containing the disrupted gene was recovered from the plasmid and used to transform W303-1A and W303-1B. Approximately 10 uracil-independent clones were obtained from each transformation. Gly+ Ura- clones were further verified by crosses to \( \rho^+ \) and *kgdl* testers to have acquired an integrated copy of the *kgdl::URA3* allele.

The presence of the mutant allele in two of the respiratory-deficient transformants (W303\( \Delta \)KGDI) was also confirmed by analysis of their genomic DNAs. Nuclear DNA obtained from the transformants and from the wild-type parent strain W303-1A was digested with a combination of BamHI and *Pst*I and probed with the 2-kb BamHI fragment containing part of the 5'-flanking and coding region of KGDI. The probe detected the 2-kb BamHI fragment in wild-type and the expected 1.1- and 2.1-kb fragments in W303\( \Delta \)KGDI. The two novel fragments present in the transformant resulted from cleavage at the *Pst*I site within the URA3 gene (Fig. 5).

The Southern blot analysis also showed the presence of a larger hybridizing band in the wild-type and mutant DNAs. The presence of a sequence capable of cross-hybridizing with the *kgdl* probe was also indicated in other digests. At present we have no information about the identity of this other gene.

**Northern analysis and S1 nuclease mapping of the KGDI transcript.** Total RNA was isolated from the wild-type strain D273-10B/A1 grown in galactose and high-glucose media. The two RNA preparations were enriched for poly(A)+ RNA and separated on a 1% agarose gel under nondenaturing conditions. After transfer to diazobenzyloxymethyl paper, the blot was hybridized with a mixture of the nick-
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATG</strong></td>
<td>Met (Methionine)</td>
</tr>
<tr>
<td><strong>CTG</strong></td>
<td>Leu (Leucine)</td>
</tr>
<tr>
<td><strong>GAA</strong></td>
<td>Glu (Glutamic Acid)</td>
</tr>
<tr>
<td><strong>GAT</strong></td>
<td>Asp (Aspartic Acid)</td>
</tr>
<tr>
<td><strong>GCT</strong></td>
<td>Ala (Alanine)</td>
</tr>
<tr>
<td><strong>GTC</strong></td>
<td>Val (Valine)</td>
</tr>
<tr>
<td><strong>TCT</strong></td>
<td>Ser (Serine)</td>
</tr>
<tr>
<td><strong>TTA</strong></td>
<td>Leu (Leucine)</td>
</tr>
</tbody>
</table>
translated 2-kb BamHI fragment containing part of the KGD1 gene and a 600-bp EcoRI-HindIII fragment of the yeast actin gene. Two prominent transcripts were detected by the probes; one, of 1.4 kb, corresponded to the processed actin mRNA, and a second larger transcript of 4.3 kb corresponded to the KGD1 mRNA (Fig. 6). The identities of the two transcripts were verified by hybridizations with the single probes. Although Southern analysis indicated the presence of a second cross-hybridizing gene, no other transcript was detected by the KGD1 probe. The sizes of the
transcripts were estimated on the basis of the migration of known double-stranded DNA standards and are therefore probably not accurate. The Northern blot also showed that the concentration of the KGD1 transcript was higher in the galactose-grown cells when normalized to the actin mRNA. The higher abundance of KGD1 transcript in the galactose-grown cells suggests that transcription of KGD1, like that of LPDI, is catabolite repressed.

To characterize the KGD1 transcript, wild-type poly(A)⁺ RNA from either repressed (10% glucose) or derepressed (2% galactose) cells was hybridized to a 5'-end-labeled single-stranded fragment of DNA covering the region from nucleotides −354 to +173. The hybrids were digested with S1 nuclease at either 37 or 44°C and separated on a 7% sequencing gel. The S1 mapping analysis indicated two discrete size families of transcripts; the longer transcripts had 5' termini located at approximately −152, and the shorter transcripts had 5' termini centered at −70 (Fig. 5). Identical results were obtained at the two different temperatures. Both transcripts were present in poly(A)⁺ RNA of derepressed cells. However, only transcripts initiating near −70 were detected in cells grown under repressed conditions. That the two protected ends detected by the S1 analysis represent different-size transcripts was also supported by measurements of β-galactosidase expressed from different lacZ fusions (see next section).

**Mapping of the 5'-flanking regions necessary for the regulated transcription of KGD1.** The higher concentration of KGD1 mRNA in cells grown on the nonrepressible sugar galactose compared with cells grown on glucose suggested that the gene is transcriptionally regulated by the carbon source. This was verified by quantitating β-galactosidase expression in cells transformed with plasmids containing different 5'-flanking sequences of KGD1 fused to the E. coli lacZ gene. The longest construct (pG70/Z1) had 693 base pairs of the upstream sequence and 173 base pairs of the KGD1 coding sequence fused in frame to the seventh codon of lacZ in the shuttle vector YEp366 (26). The respiratory-competent strain W303-1A transformed with this plasmid was grown to early log phase in liquid YPGal and in YPD medium containing 10% glucose. Assays of β-galactosidase indicated 10 times more activity in the cells grown on galactose than in the cells grown on glucose (Fig. 8). Under the same conditions there was an 11-fold increase in expression of lacZ fused to the control region of CYC1.

To define the region of DNA necessary for regulated expression of KGD1, several other lacZ fusions were constructed with shorter 5'-flanking sequences (Table 4). W303-1A harboring the different lacZ fusions was grown under repressed (glucose) and derepressed (galactose) conditions and assayed for β-galactosidase activity. A fusion containing 354 base pairs of the 5'-noncoding sequence (pG70/Z2) allowed maximal expression of the lacZ gene in galactose (Table 4). The basal activity of glucose-grown cells transformed with this shorter fusion, however, was three times higher than that of transformants containing the longer fusion of pG70/Z1. A significant decrease in the derepressed levels of β-galactosidase was seen in cells transformed with pG70/Z3, which had the lacZ gene fused to a fragment of DNA containing only 143 nucleotides of the 5'-flanking sequence of KGD1. This fusion also expressed 30% lower basal levels of β-galactosidase in glucose-grown cells. The lower basal activity is consistent with the absence of pG70/Z3 of the transcriptional initiation site mapped at −152. The nominal effect of carbon source on the expression of the
haploid phase chromatography (approximately 3 μg) and poly(A)+ cells; galactose-grown transcripts
These separated by lacZ fusions pG70/Z3 fusion indicated size standards is indicated DNA
The were times nucleotides of fusion lacZ
indicated on the left. The KGD1 and actin transcripts are identified on the right. The probe used is indicated in the diagram below the lanes. B. BamHI site: E. EcoRI site.

pG70/Z3 fusion indicated the presence of a regulatory element in the region between −354 and −143. Two additional lacZ fusions were made by BAL 31 treatment of pG70/Z3. These constructs, pG70/Z4 and pG70/Z5, had 101 and 40 nucleotides of 5′-flanking sequence, respectively. The β-galactosidase activity expressed from pG70/Z4 was four times less than that expressed from pG70/Z2 when the transformants were grown on glucose. Furthermore, the lacZ fusion in pG70/Z4 was not induced by galactose. No β-galactosidase activity was detected in either glucose- or galactose-grown cells harboring pG70/Z5, a construct lacking the transcriptional start site at −70.

Effect of hap2 and hap3 mutations on regulation of KGD1. The Northern analysis and lacZ fusion experiments suggested that transcription of KGD1 is controlled by the global regulatory pathway responsible for glucose repression of a large number of genes involved in mitochondrial oxidative metabolism (29). Transcription of such genes is activated by at least two regulatory proteins encoded by HAP2 and HAP3 (14, 27). The HAP2 and HAP3 proteins bind to UASs, one of whose features is the core consensus sequence 5′-TNA/GTTGTT (11). The regulated expression of KGD1 inferred from the lacZ fusion assays was found to depend on the 5′-flanking sequence located between −354 and −143. A scan of this region revealed three putative binding sites for the HAP2 and HAP3 proteins (Fig. 9).

The involvement of the HAP2 and HAP3 regulatory proteins in transcription of the KGD1 gene was examined by comparing the expression of a KGD1-lacZ fusion in the wild type and in hap2 and hap3 mutants. The mutant and parental wild-type strains were transformed with pG70/Z1 and grown under derepressed conditions in YPGal, and their β-galactosidase activities were assayed. The level of β-galactosidase measured in either the hap2 or hap3 genetic background was at least five times lower than in the wild type.

Table 4. β-Galactosidase activities of strain W303-1A transformed with various lacZ fusions

<table>
<thead>
<tr>
<th>Construct</th>
<th>Vector</th>
<th>5′-flanking region of KGD1</th>
<th>β-galactosidase activitya in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>YPGal</td>
</tr>
<tr>
<td>pG70/Z1</td>
<td>YEp366</td>
<td>−693 to +173</td>
<td>233 ± 10.5</td>
</tr>
<tr>
<td>pG70/Z2</td>
<td>YEp353</td>
<td>−354 to +173</td>
<td>304 ± 40.5</td>
</tr>
<tr>
<td>pG70/Z3</td>
<td>YEp353</td>
<td>−143 to +173</td>
<td>86 ± 2.5</td>
</tr>
<tr>
<td>pG70/Z4</td>
<td>YEp353</td>
<td>−101 to +173</td>
<td>22 ± 2.0</td>
</tr>
<tr>
<td>pG70/Z5</td>
<td>YEp353</td>
<td>−40 to +173</td>
<td>0</td>
</tr>
</tbody>
</table>

a Yeast cells were grown at 30°C in 10 ml of YPD (containing 10% glucose) or YPGal (containing 2% galactose) to an optical density at 600 nm of 1.0 to 1.5, and β-galactosidase activity was assayed as described by Guarente (13), with standard error. β-galactosidase activities are expressed in Miller units (22).
The KGDC-integrated in the URA3 gene regions 5'-flanking HindIII CYCJ averages and plasmid in YPGal transcription in yeast grown reported (17). Maps locations of three hydrogenase subunit coenzyme regulation of pyruvate carboxylase C225, KE1 component has two isoforms of citrate synthase, has been characterized (36). This component is derived from LPD1, which also codes for the dihydrolipoamide dehydrogenase subunit of the pyruvate dehydrogenase complex (36). In order to study the regulation of the yeast KGDC genes, we have screened a collection of respiratory-deficient pet mutants by assaying their mitochondria for α-ketoglutarate dehydrogenase activity. Mutants of three complementation groups were found to lack active KGDC.

In this communication, we demonstrate that the absence of KGDC activity in strains assigned to complementation group G70 is due to mutations in the KGDI gene coding for the α-ketoglutarate dehydrogenase subunit of the complex. This conclusion was supported by the following evidence. (i) C225, a representative G70 mutant, lacked KE1 activity. (ii) The respiratory defect of C225 was complemented by a yeast gene (KGDI) whose encoded product is homologous to the KE1 component of E. coli KGDC. (iii) Transformation of C225 with KGDI on a multicopy plasmid resulted in mitochondrial KE1 activity three to four times higher than that in wild-type yeast. (iv) Disruption of KGDI induced a respiratory-deficient phenotype; this mutant construct had no KE1 activity and was not complemented by C225.

The wild-type genes conferring respiratory competence to mutants of two other KGDC-deficient complementation groups have also been cloned, and their sequences have been determined. The primary structures of the proteins derived from their respective gene sequences bear no similarity to the known subunits of the E. coli KGDC. The functions of these novel proteins in the synthesis of a functional complex are currently being studied. Several TCA cycle enzymes of yeast appear to have a dual distribution in mitochondria and in the soluble cytosolic phase. The FUMI gene has been shown to code for mitochondrial and cytoplasmic fumarase (46). Similarly, S. cerevisiae has two isoforms of citrate synthase, only one of which is a mitochondrial enzyme (33). The two isoenzymes are encoded by separate genes (18). We have examined the possibility that KGDC may also have a dual localization but have not been able to obtain any evidence of significant extramitochondrial KE1 activity in the postmitochondrial fraction of wild-type yeast or of yeast transformed with the

Table 5: Effect of hap2 and hap3 on the derepression of KGDI

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>YPGal</th>
<th>YPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BWG1-7a</td>
<td>HAP2 HAP3</td>
<td>231</td>
<td>43</td>
</tr>
<tr>
<td>LGW-1</td>
<td>hap2-1</td>
<td>27</td>
<td>30</td>
</tr>
<tr>
<td>JF40-1</td>
<td>hap3-1</td>
<td>47</td>
<td>48</td>
</tr>
</tbody>
</table>

* The indicated strains were transformed with the construct pG70/Z1 and grown in either YPD (containing 10% glucose) or YPGal.

β-Galactosidase activity was assayed as described by Guarente (13). Activities are reported in Miller units (22).

**DISCUSSION**

The α-ketoglutarate dehydrogenase complex is composed of three functionally distinct subunits, each catalyzing a different step in the conversion of α-ketoglutarate to succinyl coenzyme A (31). At present, only the dihydrolipoyl dehydrogenase subunit of the yeast complex has been characterized (36). This component is derived from LPD1, which also codes for the dihydrolipoyl dehydrogenase subunit of the pyruvate dehydrogenase complex (36). In order to study the regulation of the yeast KGDC genes, we have screened a collection of respiratory-deficient pet mutants by assaying their mitochondria for α-ketoglutarate dehydrogenase activity.

![Diagram](http://mcb.asm.org)
**KGDI** gene on a multicopy plasmid. It is interesting, however, that a probe from the **KGDI** coding region hybridized to a second gene in wild-type genomic DNA. This unknown gene must have significant sequence similarity to **KGDI**, since it was detected by the **KGDI** probe under fairly stringent hybridization conditions.

The TCA cycle serves two important functions in obligate aerobes and facultative anaerobes such as *S. cerevisiae*. First, it supplies intermediates for a large number of different biosynthetic pathways. Second, it is the major route for the oxidative degradation of carbohydrates, fats, and amino acids. In the latter capacity, the TCA cycle plays a crucial role in the aerobic energy metabolism of a cell. In yeast, the synthesis of a large number of mitochondrial proteins, particularly those necessary for aerobic metabolism, is known to be repressed when glucose is metabolized fermentatively. This global regulatory system allows yeast to economize on an unwise outlay of energy for the biosynthesis of proteins with questionable metabolic utility. The prediction that yeast grown on glucose would have lower levels of TCA cycle enzymes compatible with its anabolic needs is not unreasonable. Earlier studies indicated that many TCA cycle enzymes are glucose repressed (28). More recently, several TCA cycle enzymes, such as citrate synthase (18) and the dihydrolipoyl dehydrogenase component of KGDC (36), have been shown to be transcriptionally regulated by glucose.

Northern blot analysis of **KGDI** transcripts in cells grown on glucose or on the nonrepressible sugar galactose indicate that **KGDI**, like **LPD1**, is subject to catabolite repression. S1 nuclease mapping has further revealed two different-sized transcripts. The longer transcripts have 5′ termini at approximately −152 and are detected only in cells grown under derepressed conditions. The second transcriptional initiation site has been mapped at −70. Although transcription from this site is also regulated by the carbon source, repression by glucose is not as severe. Presumably, the low levels of **KGDI** transcripts with 5′ termini at −70 seen in glucose-repressed cells are representative of KGDC needed for anabolic purposes.

The extent to which transcription of **KGDI** is regulated by glucose was also estimated by measurements of β-galactosidase activity expressed from fusions of the upstream region of **KGDI** to the lacZ gene of *E. coli*. Cells harboring a lacZ construct with 693 nucleotides of the 5′-flanking sequence of **KGDI** had 10 times more β-galactosidase activity when grown on galactose than when grown on glucose. The regulated expression of the gene is dependent on the HAP2 and HAP3 proteins previously shown to be positive activators of **CYC1** (14, 27) and of several other genes whose transcription is repressed by glucose (11). It is of interest that the shorter fusion of 354 nucleotides of the 5′-flanking region caused a threefold increase in basal (glucose) β-galactosidase activity even though the magnitude of derepression was only marginally affected. This suggests the presence of a regulatory element in the −693 to −354 region responsible for modulating the basal expression of the gene. The β-galactosidase activity of transformants harboring still shorter lacZ fusions, with only 143 nucleotides of the 5′-flanking sequence of **KGDI**, was only marginally increased under derepressed conditions compared with that in transformants harboring longer constructs extending to −354. These data suggest the presence of one or more UASs in the region between −354 and −143. Analysis of the DNA in this span disclosed several short sequences which deviated by only 1 nucleotide from the core consensus of identified UASs regulated by the HAP2 and HAP3 proteins. A finer deletion analysis of the region, however, is needed to further localize the HAP-binding element(s) in **KGDI**.

The presence of two separate promoters implicit from the S1 nuclease mapping data was also supported by the results of lacZ fusions. The basal expression of β-galactosidase was decreased in cells transformed with a construct containing 101 nucleotides of the upstream region and was completely abolished when the deletion was extended to −40, which removed the transcriptional start site of the shorter transcripts at −70. These results suggest that part of the second promoter responsible for initiation at −70 may lie in the region between −143 and −70.

**ACKNOWLEDGMENTS**

This research was supported by Public Health Service research grant HL22174 from the National Institutes of Health. We thank Ronald Butow for providing the yeast actin probe.

**LITERATURE CITED**