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

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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/U1, with a recombinant plasmid library of wild-type yeast nuclear DNA. Transformants containing the gene on a multicopy 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 M_w 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 fusing different 5′-flanking regions of *KGD1* to the *lacZ* gene of *E. coli* and measuring the expression of 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 (KGDC) 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 KGDC 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 2.5×10^6 to 2.8×10^6 (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 extravagant 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 dihydrolipoyltransacetylase 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 KGDC are probably unlinked 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 KGDC 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), YEPG (2% glycerol, 2% ethanol, 1% yeast extract, 2% peptone), WO (2% glucose, 0.67% Difco yeast nitrogen base without

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TABLE 1. Genotypes and sources of *S. cerevisiae* strains

Strain	Genotype	Source or reference
D273-10B/A1	α *met6*	43
W303-1A	**a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100*	R. Rothstein[a]
W303-1B	α *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100*	R. Rothstein[a]
C225	α *met6 kgd1-1*	This study
C225/U1 or U2	α *ura3-1 kgd1-1*	C225 × W303-1A
W303Δ*KGD1*	**a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 kgd1::URA3*	This study
BWG1-7a	**a** *ade1-100 his4-519 leu2-3 ura3-52*	17
LGW1	**a** *ade1-100 his4-519 leu2-3,112 ura3-52 hap2-1*	17
JP40-1	**a** *ade1-100 his4-519 leu2-3,112 ura3-52 hap3-1*	17
WCZ	**a** *leu2 his3 ura3 ade2 trp1 CYC1-lacZ(URA3)*	23

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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 kgd1-1*) and C225/U2 (α *ura3-1 kgd1-1*) with a yeast genomic library prepared by ligation of partial *Sau*3A 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, New York, kindly provided this plasmid bank. Cells were grown in 2% galactose liquid media (YPGal) 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 KGDC 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 Berk 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 S1 buffer and treated with different concentrations of S1 nuclease for 30 min at 37 or 44°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 *Pvu*II site at −143 to the *Hin*dIII site at +173. This construct, pG70/Z3, was used to introduce longer deletions in the 5′-flanking region. pG70/Z3 was linearized at the *Eco*RI site immediately adjacent to the *Pvu*II site. This *Eco*RI 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 *Xba*I linker. The extent of the deletions created by the BAL 31 treatment was determined by sequence analysis of the *Xba*I-*Hin*dIII 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 *kgd1* mutants. C225 is one of five respiratory-deficient mutant isolates assigned to complementation group G70. Mutants in this group were selected for their inability to utilize the nonfermentable substrate glycerol as their carbon source. The G70 mutants were complemented by a [rho^0] tester, indicating that the Gly^- phenotype is the result of

TABLE 2. α-Ketoglutarate dehydrogenase (KE1) activity in wild-type and *kgd1* mutants of *S. cerevisiae*

Strain	Genotype	KE1 activity[a]
W303-1A	*KGD-1*	0.439 ± 0.038
C225	*kgd1-1*	0
C225/U2	*kgd1-1*	0
C225/U2/T1[b]	*KGD1*	1.58 ± 0.26
W303ΔKGD1	*kgd1::URA3*	0

[a] 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.

[b] C225/U2/T1 is a transformant harboring the autonomously replicating plasmid pG70/T1 containing the *KGD1* gene.

recessive mutations in a nuclear gene. C225 has a functional respiratory chain and wild-type levels of oligomycin-sensitive ATPase. Mitochondrial protein synthesis was unaffected in the mutant as judged by the in vivo cycloheximide-resistant incorporation of [^{35}S]methionine into all the known products of this translational system (data not shown).

Mitochondria of C225 were assayed for the TCA cycle enzymes aconitase, isocitrate, α-ketoglutarate, succinate, and malate dehydrogenase. With the exception of α-ketoglutarate dehydrogenase, the specific activities of all the other enzymes tested were either similar or slightly lower than in the wild type. Assays of KGDC performed by measuring the reduction of NAD by α-ketoglutarate revealed that C225 mitochondria had no detectable activity. The mutant mitochondria were also unable to reduce ferricyanide by α-ketoglutarate (Table 2), a reaction catalyzed by the KE1 component of the complex (32). In contrast, dihydrolipoyl dehydrogenase activities were comparable in the mutant and wild-type mitochondria. The dihydrolipoyl transsuccinylase was also assayed, but the results were not conclusive due to the slow rate of the reaction even in wild-type mitochondria. KGDC and KE1 activities were not detected in the uracil auxotrophic derivative of C225/U1 obtained from a cross of C225 to W303-1A. These results suggested that the genetic lesion of C225 is in the *KGD1* gene for the KE1 component of the KGDC. This conclusion was confirmed by the absence of KE1 activity in mitochondria of W303ΔKGD1 (a mutant construct with a disrupted chromosomal copy of *KGD1*) and elevated KE1 activity in C225/U2/T1 (a *kgd1* mutant transformed with the *KGD1* gene on a multicopy plasmid) (see below).

Mutations in *KGD1* resulted in the expression of different growth phenotypes depending on the nuclear genetic background of the strains. The growth properties of C225, C225/U1, C225/U2, and W303ΔKGD1 on glucose, rich glycerol, minimal ethanol, minimal glycerol, and minimal media supplemented with either glutamate or succinate are summarized in Table 3. Growth of the four strains on the rich-glycerol medium was only noticeable after 2 to 3 days of incubation. C225 and C225/U1 did not exhibit any growth on the minimal media with or without the supplements even after incubation at 30°C for up to 7 days. C225/U2 and W303ΔKGD1, however, grew slowly on minimal ethanol; the growth of these strains on minimal ethanol was not enhanced by the addition of glutamate or succinate. The difference in growth phenotypes of the *kgd1* mutants probably reflects the different genetic backgrounds of the strains. Slow growth on ethanol but not on glycerol has also been reported by Subik et al. (42) for a mutant with a lesion in an unidentified component of the KGDC and by Dickinson et al. for an *lpd1* mutant (8).

TABLE 3. Growth properties of wild-type and *kgd1* mutants of *S. cerevisiae*

Strain	Genotype	Growth on rich media[a]		Growth on minimal media[a,b]			
		YPD	YEPG[c]	Et	Gly	Gly + Glut	Gly + Succ
W303-1A	*KGD1*	+++	++	++	++	++	++
C225	*kgd1-1*	++	+/−	−	−	−	−
C225/U1	*kgd1-1*	++	+/−	−	−	−	−
C225/U2	*kgd1-1*	+++	+/−	+/−	−	−	−
W303ΔKGD1	*kgd1::URA3*	+++	+/−	+/−	−	−	−

[a] Growth was scored after incubation at 30°C. +++, Heavy growth after 1 day; ++, moderate growth after 1 day; +/−, moderate growth after 3 days; −, no growth after 7 days.

[b] Minimal media contained 2% glycerol (Gly) or ethanol (Et), 0.67% yeast nitrogen base without amino acids (Difco), auxotrophic requirements, and 2% agar. Glutamate (Glut) and succinate (Succ) were added to final concentrations of 2 and 20 mg/ml, respectively.

[c] YEPG, 2% glycerol–2% ethanol–1% yeast extract–2% peptone.

Cloning of the *KGD1* gene. The *KGD1* gene was cloned by transformation of two *kgd1* 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 Gly$^+$ and Ura$^+$ 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 Gly$^+$ and Ura$^+$ 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 *kgd1* mutant. The inability of pG70/ST1 and pG70/ST2 to complement indicated that the gene must span the unique *Sph*I 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 *Xba*I sites and the proximal *Eco*RI site (Fig. 1).

Sequence of the *KGD1* gene. A region of approximately 3.7 kb of the pG70/T1 insert starting from the leftmost *Bam*HI site and ending at the distal *Eco*RI 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

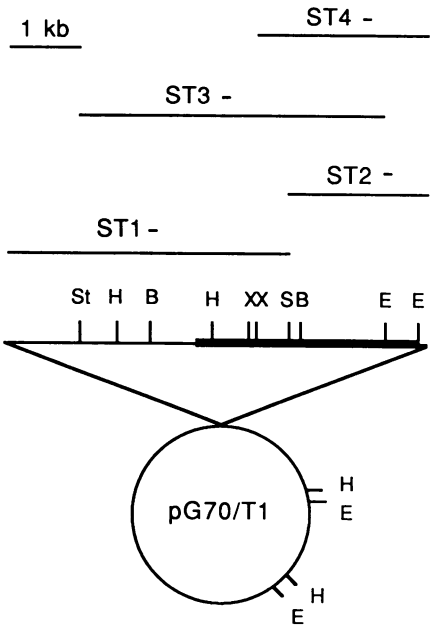


FIG. 1. Localization of *KGD1* within the genomic insert of pG70/T1. The lower part of the figure shows a partial restriction map of the nuclear DNA insert of pG70/T1. The physical limits of the reading frame coding for α-ketoglutarate dehydrogenase are depicted by the heavy line in the insert of pG70/T1. Transcription of the gene is from left to right. The restriction sites shown are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Sph*I (S), *Sst*I (St), and *Xba*I (X). The various restriction fragments subcloned in the shuttle vector YEp352 are shown by the lines in the upper part of the figure. These constructs were tested for their ability to complement the respiratory deficiency of C225/U1. Lack of complementation is indicated by a minus sign.

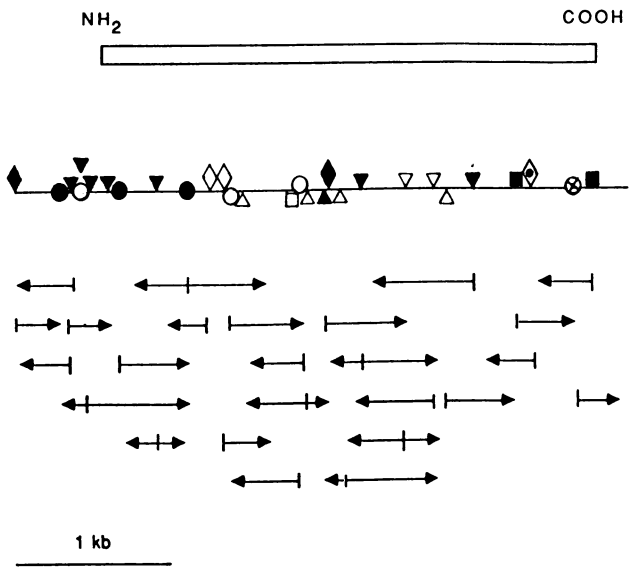


FIG. 2. Sequencing strategy. Arrows depict directions and lengths of sequences obtained from the various 5′-end-labeled fragments. The restriction sites shown are *Bam*HI (◆), *Bst*NI (⊗), *Dde*I (▽), *Eco*RI (■), *Hae*III (○), *Hin*fI (▼), *Hpa*II (●), *Sph*I (□), *Taq*I (◇), and *Xba*I (◇). The open reading frame encoding α-ketoglutarate dehydrogenase is shown at the top of the figure.

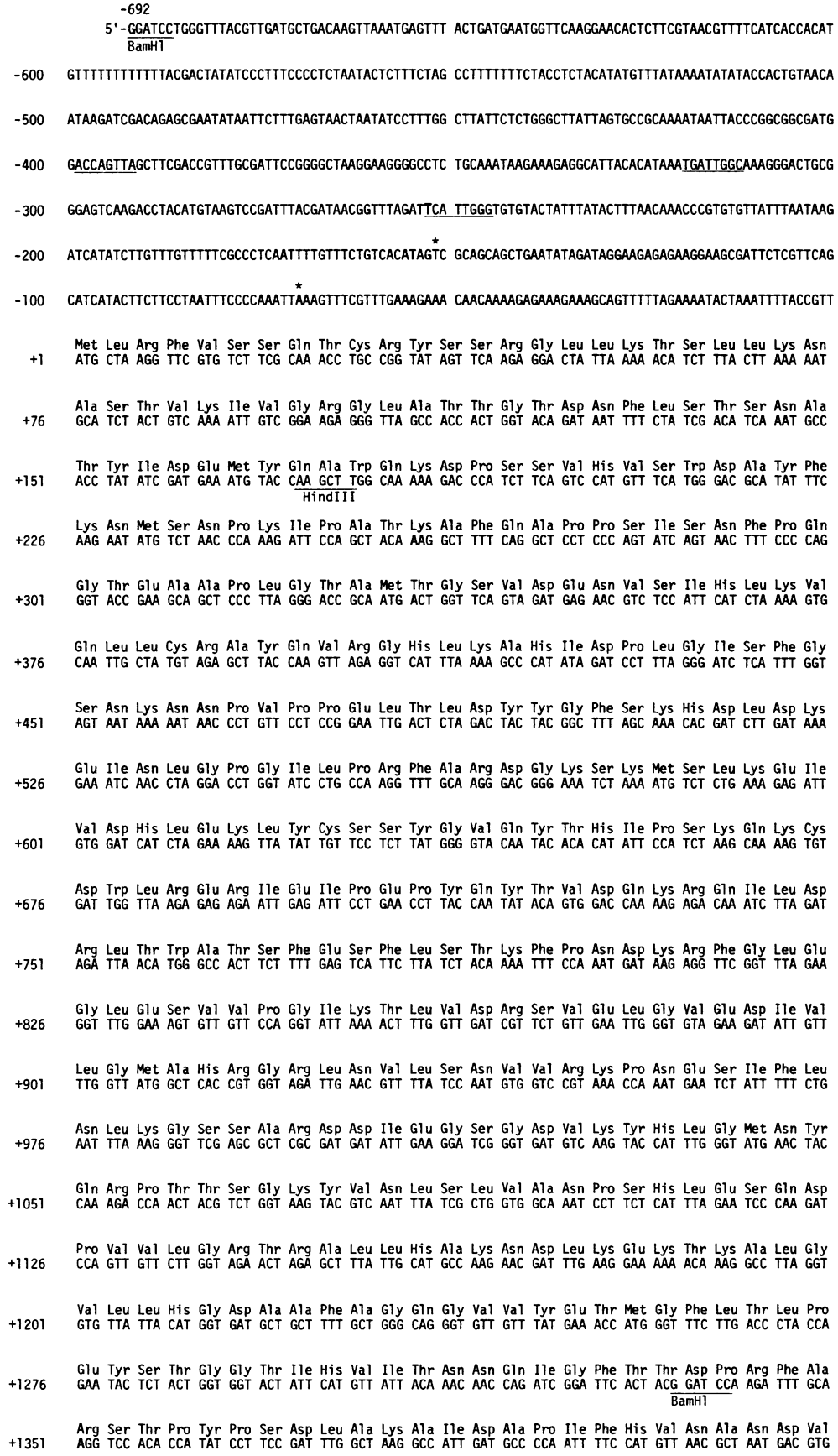
few deletions, the most substantial of which was 18 residues long and occurred in the *E. coli* protein. The primary sequence homology combined with the absence of α-ketoglutarate dehydrogenase activity in C225 constitutes strong evidence for the identity of the reading frame as the structural gene for the KE1 component of yeast KGDC. This gene has previously been given the designation *KGD1* (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 *KGD1* gene. The one-step gene replacement procedure (35) was used to disrupt the wild-type *KGD1* gene. To construct the disrupted allele *kgd1*::*URA3* illustrated in Fig. 5, the 2-kb *Bam*HI 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 *Bam*HI site). The new plasmid was digested with *Hin*dIII and was ligated to a 1.2-kb *Hin*dIII fragment containing the yeast *URA3* gene. This ligation yielded a plasmid in which the coding sequence of *KGD1* was disrupted at nucleotide +173 (residue 58). The linear 3.2-kb *Bam*HI 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*^{0}] and *kgd1* testers to have acquired an integrated copy of the *kgd1*::*URA3* allele.

The presence of the mutant allele in two of the respiratory-deficient transformants (W303ΔKGD1) 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 *Bam*HI and *Pst*I and probed with the 2-kb *Bam*HI fragment containing part of the 5′-flanking and coding region of *KGD1*. The probe detected the 2-kb *Bam*HI fragment in wild-type and the expected 1.1- and 2.1-kb fragments in W303ΔKGD1. 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 *KGD1* 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 *KGD1* 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-

FIG. 3. Nucleotide sequence of *KGD1* and flanking regions. The sequence extends over 3.78 kb of the genomic insert of pG70/T1. The reading frame identified as *KGD1* starts with a methionine initiation codon at nucleotide +1 and terminates with an ochre codon at nucleotide +3043. Only the sequence of the sense strand is shown. The amino acid sequence of α-ketoglutarate dehydrogenase is shown above the nucleotide sequence. The restriction sites for *Bam*HI and *Hin*dIII are underlined. The initiation sites at −70 and −152 determined by S1 analysis are marked by asterisks. The three putative UAS sites in the 5′-flanking sequence of *KGD1* are underlined.


-692
5'-GGATCCTGGGTTTACGTTGATGCTGACAAGTTAAATGAGTTT ACTGATGAATGGTTCAAGGAACACTCTTCGTAACGTTTTCATCACCACAT
BamH1
-600 GTTTTTTTTTTTTACGACTATATCCCTTTCCCCTCTAATACTCTTTCTAG CCTTTTTTTCTACCTCTACATATGTTTATAAAATATATACCACTGTAACA
-500 ATAAGATCGACAGAGCGAATATAATTCTTTGAGTAACTAATATCCTTTGG CTTATTCTCTGGGCTTATTAGTGCCGCAAAATAATTACCCGGCGGCGATG
-400 GACCAGTTAGCTTCGACCGTTTGCGATTCCGGGGCTAAGGAAGGGGCCTC TGCAAATAAGAAAGAGGCATTACACATAAATGATTGGCAAAGGGACTGCG
-300 GGAGTCAAGACCTACATGTAAGTCCGATTTACGATAACGGTTTAGATTCA TTGGGTGTGTACTATTTATACTTTAACAAACCCGTGTGTTATTTAATAAG
-200 ATCATATCTTGTTTGTTTTTCGCCCTCAATTTTGTTTCTGTCACATAGTC GCAGCAGCTGAATATAGATAGGAAGAGAGAAGGAAGCGATTCTCGTTCAG
-100 CATCATACTTCTTCCTAATTTCCCCAAATTAAAGTTTCGTTTGAAAGAAA CAACAAAAGAGAAAGAAAGCAGTTTTTAGAAAATACTAAATTTTACCGTT
Met Leu Arg Phe Val Ser Ser Gln Thr Cys Arg Tyr Ser Ser Arg Gly Leu Leu Lys Thr Ser Leu Leu Lys Asn
+1 ATG CTA AGG TTC GTG TCT TCG CAA ACC TGC CGG TAT AGT TCA AGA GGA CTA TTA AAA ACA TCT TTA CTT AAA AAT
Ala Ser Thr Val Lys Ile Val Gly Arg Gly Leu Ala Thr Thr Gly Thr Asp Asn Phe Leu Ser Thr Ser Asn Ala
+76 GCA TCT ACT GTC AAA ATT GTC GGA AGA GGG TTA GCC ACC ACT GGT ACA GAT AAT TTT CTA TCG ACA TCA AAT GCC
Thr Tyr Ile Asp Glu Met Tyr Gln Ala Trp Gln Lys Asp Pro Ser Ser Val His Val Ser Trp Asp Ala Tyr Phe
+151 ACC TAT ATC GAT GAA ATG TAC CAA GCT TGG CAA AAA GAC CCA TCT TCA GTC CAT GTT TCA TGG GAC GCA TAT TTC
HindIII
Lys Asn Met Ser Asn Pro Lys Ile Pro Ala Thr Lys Ala Phe Gln Ala Pro Pro Ser Ile Ser Asn Phe Pro Gln
+226 AAG AAT ATG TCT AAC CCA AAG ATT CCA GCT ACA AAG GCT TTT CAG GCT CCT CCC AGT ATC AGT AAC TTT CCC CAG
Gly Thr Glu Ala Ala Pro Leu Gly Thr Ala Met Thr Gly Ser Val Asp Glu Asn Val Ser Ile His Leu Lys Val
+301 GGT ACC GAA GCA GCT CCC TTA GGG ACC GCA ATG ACT GGT TCA GTA GAT GAG AAC GTC TCC ATT CAT CTA AAA GTG
Gln Leu Leu Cys Arg Ala Tyr Gln Val Arg Gly His Leu Lys Ala His Ile Asp Pro Leu Gly Ile Ser Phe Gly
+376 CAA TTG CTA TGT AGA GCT TAC CAA GTT AGA GGT CAT TTA AAA GCC CAT ATA GAT CCT TTA GGG ATC TCA TTT GGT
Ser Asn Lys Asn Asn Pro Val Pro Pro Glu Leu Thr Leu Asp Tyr Tyr Gly Phe Ser Lys His Asp Leu Asp Lys
+451 AGT AAT AAA AAT AAC CCT GTT CCT CCG GAA TTG ACT CTA GAC TAC TAC GGC TTT AGC AAA CAC GAT CTT GAT AAA
Glu Ile Asn Leu Gly Pro Gly Ile Leu Pro Arg Phe Ala Arg Asp Gly Lys Ser Lys Met Ser Leu Lys Glu Ile
+526 GAA ATC AAC CTA GGA CCT GGT ATC CTG CCA AGG TTT GCA AGG GAC GGG AAA TCT AAA ATG TCT CTG AAA GAG ATT
Val Asp His Leu Glu Lys Leu Tyr Cys Ser Ser Tyr Gly Val Gln Tyr Thr His Ile Pro Ser Lys Gln Lys Cys
+601 GTG GAT CAT CTA GAA AAG TTA TAT TGT TCC TCT TAT GGG GTA CAA TAC ACA CAT ATT CCA TCT AAG CAA AAG TGT
Asp Trp Leu Arg Glu Arg Ile Glu Ile Pro Glu Pro Tyr Gln Tyr Thr Val Asp Gln Lys Arg Gln Ile Leu Asp
+676 GAT TGG TTA AGA GAG AGA ATT GAG ATT CCT GAA CCT TAC CAA TAT ACA GTG GAC CAA AAG AGA CAA ATC TTA GAT
Arg Leu Thr Trp Ala Thr Ser Phe Glu Ser Phe Leu Ser Thr Lys Phe Pro Asn Asp Lys Arg Phe Gly Leu Glu
+751 AGA TTA ACA TGG GCC ACT TCT TTT GAG TCA TTC TTA TCT ACA AAA TTT CCA AAT GAT AAG AGG TTC GGT TTA GAA
Gly Leu Glu Ser Val Val Pro Gly Ile Lys Thr Leu Val Asp Arg Ser Val Glu Leu Gly Val Glu Asp Ile Val
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Gln Arg Pro Thr Thr Ser Gly Lys Tyr Val Asn Leu Ser Leu Val Ala Asn Pro Ser His Leu Glu Ser Gln Asp
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Pro Val Val Leu Gly Arg Thr Arg Ala Leu Leu His Ala Lys Asn Asp Leu Lys Glu Lys Thr Lys Ala Leu Gly
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Glu Tyr Ser Thr Gly Gly Thr Ile His Val Ile Thr Asn Asn Gln Ile Gly Phe Thr Thr Asp Pro Arg Phe Ala
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BamH1
Arg Ser Thr Pro Tyr Pro Ser Asp Leu Ala Lys Ala Ile Asp Ala Pro Ile Phe His Val Asn Ala Asn Asp Val
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        Glu Ala Val Thr Phe Ile Phe Asn Leu Ala Ala Glu Trp Arg His Lys Phe His Thr Asp Ala Ile Ile Asp Val
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        Val Gly Trp Arg Lys His Gly His Asn Glu Thr Asp Arg Pro Ser Phe Thr Gln Pro Leu Met Tyr Lys Lys Ile
+1501   GTT GGT TGG AGA AAA CAT GGA CAT AAT GAA ACC GAT CGA CCA TCG TTT ACT CAA CCA TTA ATG TAC AAA AAA ATT

        Ala Lys Gln Lys Ser Val Ile Asp Val Tyr Thr Glu Lys Leu Ile Ser Glu Gly Thr Phe Ser Lys Lys Asp Ile
+1576   GCA AAA CAA AAA TCT GTC ATT GAC GTC TAT ACG GAA AAA TTG ATA AGT GAA GGC ACA TTT TCT AAA AAA GAT ATT

        Asp Glu His Lys Lys Trp Val Trp Asn Leu Phe Glu Asp Ala Phe Glu Lys Thr Lys Asp Tyr Val Pro Ser Gln
+1651   GAT GAG CAC AAG AAA TGG GTA TGG AAC TTA TTT GAA GAT GCT TTC GAA AAG ACA AAG GAT TAC GTC CCA TCT CAA

        Arg Glu Trp Leu Thr Ala Ala Trp Glu Gly Phe Lys Ser Pro Lys Glu Leu Ala Thr Glu Ile Leu Pro His Glu
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        Pro Thr Asn Val Pro Glu Ser Thr Leu Lys Glu Leu Gly Lys Val Leu Ser Ser Trp Pro Glu Gly Phe Glu Val
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        His Lys Asn Leu Lys Arg Ile Leu Lys Asn Arg Gly Lys Ser Ile Glu Thr Gly Glu Gly Ile Asp Trp Ala Thr
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        Gly Glu Ala Leu Ala Phe Gly Thr Leu Val Leu Asp Gly Gln Asn Val Arg Val Ser Gly Glu Asp Val Glu Arg
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        Asn Asn Glu Lys Ala Asp Phe Thr Ile Ala Asn Ser Ser Leu Ser Glu Tyr Gly Val Met Gly Phe Glu Tyr Gly
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        Tyr Ser Leu Thr Ser Pro Asp Tyr Leu Val Met Trp Glu Ala Gln Phe Gly Asp Phe Ala Asn Thr Ala Gln Val
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        Ile Ile Asp Gln Phe Ile Ala Gly Gly Glu Gln Lys Trp Lys Gln Arg Ser Gly Leu Val Leu Ser Leu Pro His
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        Arg Tyr Phe Pro Ser Glu Glu Lys Leu Gln Arg Gln His Gln Asp Cys Asn Phe Gln Val Val Tyr Pro Thr Thr
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        Pro Ala Asn Leu Phe His Ile Leu Arg Arg Gln Gln His Arg Gln Phe Arg Lys Pro Leu Ala Leu Phe Phe Ser
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        Lys Gln Leu Leu Arg His Pro Leu Ala Arg Ser Ser Leu Ser Glu Phe Thr Glu Gly Gly Phe Gln Trp Ile Ile
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        Glu Asp Ile Glu His Gly Lys Ser Ile Gly Thr Lys Glu Glu Thr Lys Arg Leu Val Leu Leu Ser Gly Gln Val
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        Tyr Thr Ala Leu His Lys Arg Arg Glu Ser Leu Gly Asp Lys Thr Thr Ala Phe Leu Lys Ile Glu Gln Leu His
+2701   TAC ACT GCC CTA CAT AAA AGA CGT GAA AGT TTG GGT GAT AAG ACC ACT GCT TTC TTA AAG ATT GAA CAG CTG CAC

        Pro Phe Pro Phe Ala Gln Leu Arg Asp Ser Leu Asn Ser Tyr Pro Asn Leu Glu Glu Ile Val Trp Cys Gln Glu
+2776   CCA TTC CCA TTT GCT CAG CTA CGT GAT TCA TTA AAT TCT TAT CCA AAC TTG GAA GAA ATT GTT TGG TGC CAG GAA

        Glu Pro Leu Asn Met Gly Ser Trp Ala Tyr Thr Glu Pro Arg Leu His Thr Thr Leu Lys Glu Thr Asp Lys Tyr
+2851   GAG CCA TTG AAC ATG GGT TCG TGG GCA TAC ACA GAA CCA CGC TTA CAC ACA ACA TTA AAA GAA ACG GAT AAA TAT

        Lys Asp Phe Lys Val Arg Tyr Cys Gly Arg Asn Pro Ser Gly Ala Val Ala Ala Gly Ser Lys Ser Leu His Leu
+2926   AAG GAT TTC AAG GTC AGA TAC TGT GGT AGA AAC CCA AGT GGT GCT GTT GCT GCC GGT AGC AAA TCA CTA CAT TTG

        Ala Glu Glu Asp Ala Phe Leu Lys Asp Val Phe Gln Gln Ser Och
+3001   GCC GAA GAA GAT GCC TTT TTG AAA GAT GTT TTC CAA CAA TCC TAA AGATGAATTC
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FIG. 3—*Continued*

translated 2-kb *Bam*HI fragment containing part of the *KGD1* gene and a 600-bp *Eco*RI-*Hin*dIII 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

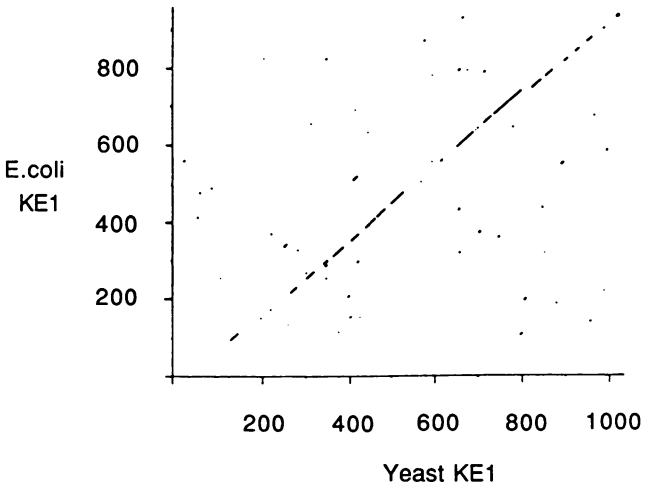

FIG. 4. Dot matrix of the *S. cerevisiae* and *E. coli* α-ketoglutarate dehydrogenases. The program used to generate this graph scored a dot for every five identities out of 10 residues scanned.

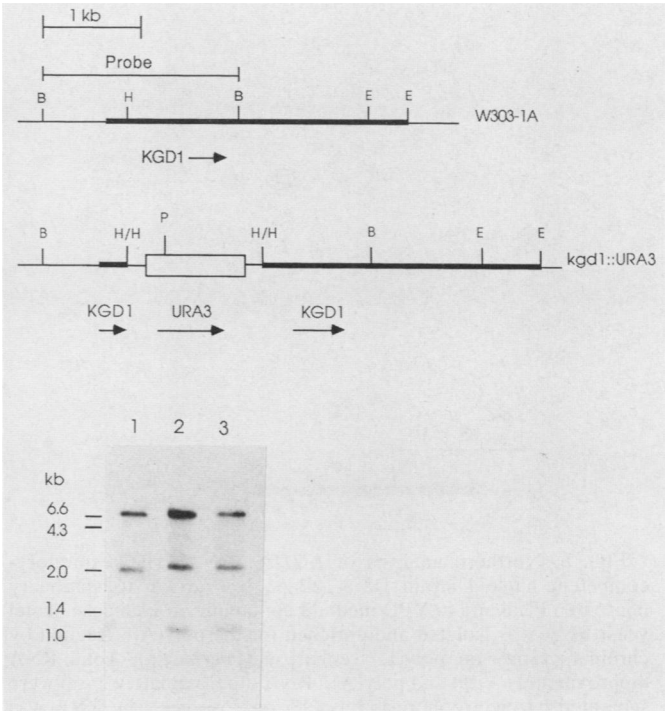

FIG. 5. Southern analysis of chromosomal DNA in the wild-type strain and in strains with the *kgd1::URA3* allele. The *kgd1::URA3* allele was constructed and introduced into the chromosomal DNA of the respiratory-competent strains W303-1A and W303-1B as described in the text. Genomic DNA was prepared from W303-1A and from several independent Ura$^+$ Gly$^-$ transformants and was digested with a combination of *Bam*HI and *Pst*I. The digests were separated electrophoretically on a 1% agarose gel, transferred to nitrocellulose paper, and hybridized to the nick-translated 2-kb *Bam*HI fragment (Probe). In the upper part of the figure, the locations of the *KGD1* (▬) and *URA3* genes (▭) are indicated. The *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), and *Pst*I (P) sites are marked on the restriction maps of wild-type and disrupted *KGD1* genes. The direction of transcription is indicated by the arrows. The results of the Southern analysis are shown in the lower part of the figure. Lanes: 1, W303-1A; 2, aW303ΔKGD1; 3, αW303ΔKGD1. The migration of DNA size standards is shown on the left.

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 *LPD1* (36), 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 centered at approximately −152, and the shorter transcripts had 5′ termini centered at −70 (Fig. 7). 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 in 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

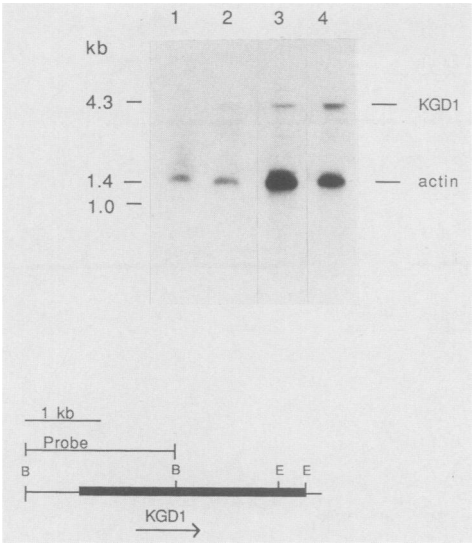

FIG. 6. Northern analysis of *KGD1* mRNA. The respiratory-competent haploid strain D273-10B/A1 was grown to stationary phase in YPGal and in YPD medium containing 10% glucose. Total yeast RNA was isolated and enriched for the poly(A)$^+$ fraction by chromatography on poly(U) sepharose (Pharmacia). Total RNA (approximately 3 μg) and poly(A)$^+$ RNA (approximately 2 μg) were separated by electrophoresis on a 1% agarose gel. The RNA was blotted to diazobenzyloxymethyl-paper and hybridized with a mixture of nick-translated 2-kb *Bam*HI fragment with the *KGD1* gene (see legend to Fig. 5) and a 600-bp fragment of the yeast actin gene. Lanes: 1, total RNA from glucose-grown cells; 2, total RNA from galactose-grown cells; 3, poly(A)$^+$ RNA from glucose-grown cells; 4, poly(A)$^+$ RNA from galactose-grown cells. The migration of DNA size standards is 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, *Bam*HI site; E, *Eco*RI 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/GTTGGT (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).

TABLE 4. β-Galactosidase activities of strain W303-1A transformed with various *lacZ* fusions[a]

Construct	Vector	5′-flanking region of *KGD1*	β-galactosidase activity[b] in:	
			YPGal	YPD
pG70/Z1	YEp366	−693 to +173	233 ± 10.5	22 ± 1.0
pG70/Z2	YEp353	−354 to +173	304 ± 40.5	70 ± 11.3
pG70/Z3	YEp353	−143 to +173	86 ± 2.5	51 ± 8.5
pG70/Z4	YEp353	−101 to +173	22 ± 2.0	16 ± 8.4
pG70/Z5	YEp353	−40 to +173	0	0

[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).
[b] Values are averages of two independent experiments plus or minus standard error. β-galactosidase activities are expressed in Miller units (22).

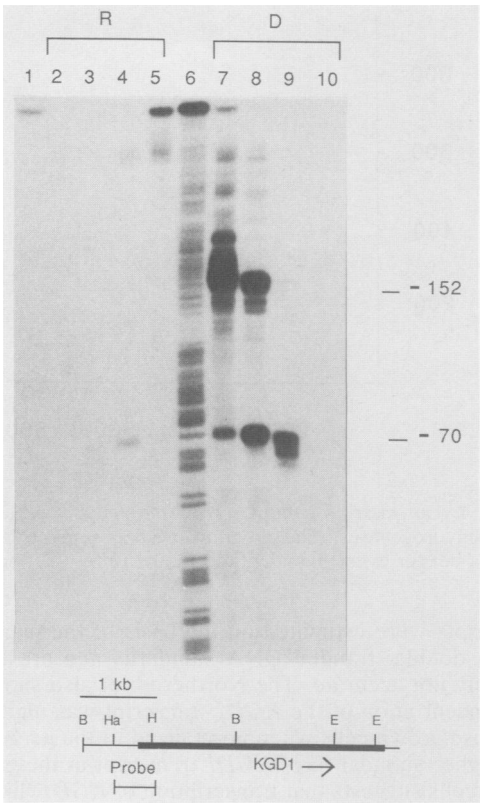

FIG. 7. S1 nuclease mapping of *KGD1* transcripts. Poly(A)$^+$-enriched RNA from *S. cerevisiae* D273-10B/A1 grown under repressed (R) (lanes 2 to 5) or derepressed (D) (lanes 7 to 10) conditions was hybridized to the single-stranded 5′-end-labeled *Hae*III-*Hin*dIII fragment from −354 to +173 (probe) under the conditions described in Materials and Methods. The hybrids were digested at 44°C with 1 U (lanes 5 and 7), 10 U (lanes 4 and 8), 100 U (lanes 3 and 9) or 300 U (lanes 2 and 10) of S1 nuclease per ml and separated on a 7% polyacrylamide gel. The single-stranded fragment derivatized for the A+G-specific reactions (21) was used as a sequencing ladder (lane 6). Lane 1 contains single-stranded DNA probe digested with 100 U of S1 nuclease per ml. The locations of the *Bam*HI (B), *Hin*dIII (H), *Eco*RI (E), and *Hae*III (Ha) sites are indicated on the restriction map in the lower part of the figure. The *KGD1* gene is also depicted (▬).

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

Strains	Units of β-galactosidase	
	YPGal	YPD
WCZ	309 ± 25.9	28 ± 2.8
W303-1A + pG70/Z1	248 ± 15.3	24 ± 2.6

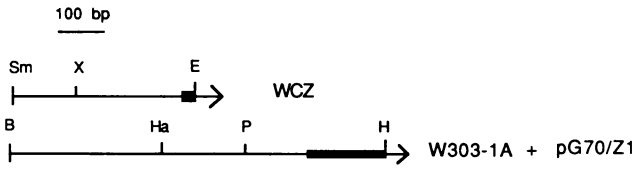

FIG. 8. Expression of *CYC1-lacZ* and *KGD1-lacZ* fusions in yeast grown under repressed and derepressed conditions. The 5'-flanking regions of *CYC1* and *KGD1* fused to the *lacZ* gene are shown in the lower part of the figure. Strain WCZ has a *CYC1-lacZ* fusion integrated in the *URA3* gene of *S. cerevisiae* W303-1A (23). The *KGD1-lacZ* fusion was introduced into W303-1A on the episomal plasmid YEp366 (26) by transformation. Cells were grown either in YPGal or YPD containing 10% glucose as described in Materials and Methods and were assayed for β-galactosidase. The activities reported are in Miller units (22). The values reported are the averages of two independent experiments plus or minus standard errors. *CYC1* and *KGD1* coding regions are depicted (▬). The locations of the *Sma*I (Sm), *Xba*I (X), *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Pvu*II (P), and *Hae*III (Ha) sites are shown on the restriction maps of the two constructs.

(Table 5). Similar effects of *hap2* and *hap3* mutations on the transcription of other catabolite-repressed genes have been reported (17).

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. Mutants of three complementation groups were found to lack active KGDC.

TABLE 5. Effect of *hap2* and *hap3* on the derepression of *KGD1*[a]

Strain	Genotype	β-Galactosidase activity[b] in:	
		YPGal	YPD
BWG1-7a	*HAP2 HAP3*	231	43
LGW-1	*hap2-1*	27	30
JP40-1	*hap3-1*	47	48

[a] The indicated strains were transformed with the construct pG70/Z1 and grown in either YPD (containing 10% glucose) or YPGal.
[b] β-Galactosidase activity was assayed as described by Guarente (13). Activities are reported in Miller units (22).

In this communication, we demonstrate that the absence of KGDC activity in strains assigned to complementation group G70 is due to mutations in the *KGD1* 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 (*KGD1*) whose encoded product is homologous to the KE1 component of *E. coli* KGDC. (iii) Transformation of C225 with *KGD1* on a multicopy plasmid resulted in mitochondrial KE1 activity three to four times higher than that in wild-type yeast. (iv) Disruption of *KGD1* 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 *FUM1* 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

CYC1 UAS2UP1	-225	T C A T T T G G C G A G C G T T G A T T G G T G G A T C A A G C C C A C G C		-188
COX4	-584	T G G A A T C C G C T G A T C T T A T T G G T G G A C A G T C A G C T G A C		-617
HEM1	-390	G G C C G C C T T C G T C G C T C A T T G G T C T G C G G C C G C G G G C G		-357
KGD1 site 1	-383	G C A A A C G G T C G A A G C T A A C T G G T C C A T C G C C G C C G G G T		-413
KGD1 site 2	-335	G G C A T T A C A C A T A A A T G A T T G G C A A A G G G A C T G C G G G A		-298
KGD1 site 3	-268	G A T A A C G G T T T A G A T T C A T T G G G T G T G T A C T A T T T A T A		-231

FIG. 9. Putative HAP2-HAP3-binding sites in the 5'-flanking region of *KGD1*. The sequences from the 5'-flanking region of *KGD1* have been aligned with the UAS2UP1 of *CYC1* and the UAS of *COX4* and *HEM1* (11). The consensus nucleotides proposed to be critical for the binding of the HAP2-HAP3 complex (27) are boxed.

KGD1 gene on a multicopy plasmid. It is interesting, however, that a probe from the *KGD1* coding region hybridized to a second gene in wild-type genomic DNA. This unknown gene must have significant sequence similarity to *KGD1*, since it was detected by the *KGD1* 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 *KGD1* transcripts in cells grown on glucose or on the nonrepressible sugar galactose indicate that *KGD1*, 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 *KGD1* 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 *KGD1* is regulated by glucose was also estimated by measurements of β-galactosidase activity expressed from fusions of the upstream region of *KGD1* to the *lacZ* gene of *E. coli*. Cells harboring a *lacZ* construct with 693 nucleotides of the 5′-flanking sequence of *KGD1* 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 *KGD1*, 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 *KGD1*.

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.

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LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. USA **74**:5350–5354.
2. **Beggs, J. D.** 1978. Transformation of yeast by a replicating hybrid plasmid. Nature (London) **275**:104–109.
3. **Berk, A. J., and P. A. Sharp.** 1978. Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. USA **75**:1274–1278.
4. **Botstein, D., and R. W. Davis.** 1982. Principles and practice of recombinant DNA research with yeast, p. 607–636. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Chen, R. F., and G. W. E. Plaut.** 1963. Activation and inhibition of DPN-linked isocitrate dehydrogenase of heart by certain nucleotides. Biochemistry **2**:1023–1032.
6. **Darlison, M. G., M. E. Spencer, and J. R. Guest.** 1984. Nucleotide sequence of the *sucA* gene encoding the 2-oxoglutarate dehydrogenase of *E. coli* K12. Eur. J. Biochem. **141**:352–359.
7. **Darlison, M. G., M. E. Spencer, and J. R. Guest.** 1984. Nucleotide sequence of the *sucB* gene encoding the dihydrolipoamide succinyltransferase of *E. coli* K12 and homology with the corresponding acetyltransferase. Eur. J. Biochem. **141**:361–374.
8. **Dickinson, J. R., D. J. Roy, and I. W. Dawes.** 1986. A mutation affecting lipoamide dehydrogenase, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **204**:103–107.
9. **Englard, S., and L. Lewis.** 1969. Mitochondrial malate dehydrogenase of beef heart. Methods Enzymol. **13**:99–106.
10. **Faye, G., C. Kujawa, and H. Fukuhara.** 1974. Physical and genetic organization of *petite* and *grande* yeast mitochondrial DNA. J. Mol. Biol. **88**:185–203.
11. **Forsburg, S. L., and L. Guarente.** 1988. Mutational analysis of upstream activation sequence 2 of the *CYC1* gene of *S. cerevisiae*: a *HAP2-HAP3* responsive site. Mol. Cell. Biol. **8**:647–654.
12. **Fraenkel, D. G.** 1982. Carbohydrate metabolism, p. 1–38. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Guarente, L.** 1983. Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. Methods Enzymol. **101**:181–191.
14. **Hahn, S., and L. Guarente.** 1988. Yeast HAP2 and HAP3: transcriptional activators in a heteromeric complex. Science **240**:317–321.
15. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff.** 1986. Yeast *E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2**:163–167.
16. **Hirashima, M., T. Hayakawa, and M. Koike.** 1967. Mammalian

α-keto acid dehydrogenase complexes. II. An improved procedure for the preparation of 2-oxoglutarate dehydrogenase complex from pig heart muscle. J. Biol. Chem. **242**:902–907.
17. **Keng, T., and L. Guarente.** 1987. Constitutive expression of the yeast *HEM1* gene is actually a composite of activation and repression. Proc. Natl. Acad. Sci. USA **84**:9113–9117.
18. **Kim, K. S., M. S. Rosenkrantz, and L. Guarente.** 1986. *Saccharomyces cerevisiae* contains two functional citrate synthase genes. Mol. Cell. Biol. **6**:1936–1942.
19. **Laughon, A., and R. F. Gesteland.** 1982. Isolation and preliminary characterization of the *GAL4* gene, a positive regulator of transcription in yeast. Proc. Natl. Acad. Sci. USA **79**:6827–6831.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Myers, A. M., M. D. Crivellone, T. J. Koerner, and A. Tzagoloff.** 1987. Characterization of the yeast *HEM2* gene and regulation of *COX4* and *COR1* by heme. J. Biol. Chem. **262**:16822–16829.
24. **Myers, A. M., L. K. Pape, and A. Tzagoloff.** 1985. Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae*. EMBO J. **4**:2087–2092.
25. **Myers, A. M., and A. Tzagoloff.** 1985. MSW, a yeast gene coding for mitochondrial tryptophanyl-tRNA synthetase. J. Biol. Chem. **260**:15371–15377.
26. **Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty.** 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. Gene **45**:299–310.
27. **Olesen, J., S. Hahn, and L. Guarente.** 1987. Yeast *HAP2* and *HAP3* activators both bind to the *CYC1* upstream activation site UAS2, in an interdependent manner. Cell **51**:953–961.
28. **Perlman, P. S., and H. R. Mahler.** 1974. Derepression of mitochondria and their enzymes in yeast: regulatory aspects. Arch. Biochem. Biophys. **162**:248–271.
29. **Pinkham, J. L., and L. Guarente.** 1985. Cloning and molecular analysis of the *HAP2* locus: a global regulator of respiratory genes in *S. cerevisiae*. Mol. Cell. Biol. **5**:3410–3416.
30. **Racker, E.** 1950. Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim. Biophys. Acta **4**:211–214.
31. **Reed, L. J.** 1974. Multienzyme complexes. Acc. Chem. Res. **7**:40–46.
32. **Reed, L. J., and B. B. Mukherjee.** 1969. α-Ketoglutarate dehydrogenase complex from *Escherichia coli*. Methods Enzymol. **13**:55–61.
33. **Rickey, T. M., and A. S. Lewin.** 1986. Extramitochondrial citrate synthase activity in bakers yeast. Mol. Cell. Biol. **6**:488–493.
34. **Ross, J., R. A. Graeme, and I. W. Dawes.** 1988. The nucleotide sequence of the *LPD1* gene encoding the lipoamide dehydrogenase in *S. cerevisiae*: comparison of eukaryotic and prokaryotic sequences for related enzymes and identification of potential upstream control sites. J. Gen. Microbiol. **134**:1131–1139.
35. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–211.
36. **Roy, D. J., and I. W. Dawes.** 1987. Cloning and characterization of the gene encoding lipoamide dehydrogenase in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **133**:925–933.
37. **Sanadi, D. R.** 1969. α-Ketoglutarate dehydrogenase from pig heart. Methods Enzymol. **13**:52–55.
38. **Slater, E. C., and W. D. Bonner.** 1952. The effect of fluoride on the succinic oxidase system. Biochem. J. **52**:185–196.
39. **Spencer, M. E., and J. R. Guest.** 1985. Transcription analysis of the *suc*AB, *ace*EF and *lpd* genes of *Escherichia coli*. Mol. Gen. Genet. **200**:145–154.
40. **Stanley, C. J., and R. N. Perham.** 1980. Purification of 2-oxo acid dehydrogenase multienzyme complexes from ox heart by a new method. Biochem. J. **191**:147–157.
41. **Stephens, P. E., H. M. Lewis, M. G. Darlison, and J. R. Guest.** 1983. The pyruvate dehydrogenase complex of *Escherichia coli* K12. The nucleotide sequence of the lipoamide dehydrogenase component. Eur. J. Biochem. **133**:519–527.
42. **Subik, J., J. Kolarov, and T. M. Lachowicz.** 1972. A mutant of *Saccharomyces cerevisiae* lacking α-ketoglutarate dehydrogenase activity. FEBS Lett. **27**:81–84.
43. **Tzagoloff, A., A. Akai, and F. Foury.** 1976. Assembly of the mitochondrial membrane system. XVI. Modified form of the ATPase proteolipid in oligomycin-resistant mutants of *S. cerevisiae*. FEBS Lett. **65**:391–396.
44. **Tzagoloff, A., A. Akai, and R. B. Needleman.** 1975. Assembly of the mitochondrial membrane system. Isolation of nuclear and cytoplasmic mutants of *Saccharomyces cerevisiae* with specific defects in mitochondrial functions. J. Bacteriol. **122**:826–831.
45. **Woodbury, N. W., and R. F. Doolittle.** 1980. Sequence homology among *E. coli* ribosomal proteins. Evidence for evolutionarily related groupings and internal duplications. J. Mol. Evol. **15**:129–148.
46. **Wu, M., and A. Tzagoloff.** 1987. Mitochondrial and cytoplasmic fumarases in *Saccharomyces cerevisiae* are encoded by a single nuclear gene *FUM1*. J. Biol. Chem. **262**:12275–12282.