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Structure and Regulation of *KGD2*, the Structural Gene for Yeast Dihydrolipoyl Transsuccinylase

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Yeast mutants assigned to the *pet* complementation group G104 were found to lack α-ketoglutarate dehydrogenase activity as a result of mutations in the dihydrolipoyl transsuccinylase (KE2) component of the complex. The nuclear gene *KGD2*, coding for yeast KE2, was cloned by transformation of E250/U6, a G104 mutant, with a yeast genomic library. Analysis of the *KGD2* sequence revealed an open reading frame encoding a protein with a molecular weight of 52,375 and 42% identities to the KE2 component of *Escherichia coli* α-ketoglutarate dehydrogenase complex. Disruption of the chromosomal copy of *KGD2* in a respiratory-competent haploid yeast strain elicited a growth phenotype similar to that of G104 mutants and abolished the ability to mitochondria to catalyze the reduction of NAD^+ by α-ketoglutarate. The expression of *KGD2* was transcriptionally regulated by glucose. Northern (RNA) analysis of poly(A)$^+$ RNA indicated the existence of two *KGD2* transcripts differing in length by 150 nucleotides. The concentrations of both RNAs were at least 10 times lower in glucose (repressed)- than in galactose (derepressed)-grown cells. Different 5′-flanking regions of *KGD2* were fused to the *lacZ* gene of *E. coli* in episomal plasmids, and the resultant constructs were tested for expression of β-galactosidase in wild-type yeast cells and in *hap2* and *hap3* mutants. Results of the *lacZ* fusion assays indicated that transcription of *KGD2* is activated by the HAP2 and HAP3 proteins. The regulated expression of *KGD2* was found to depend on sequences that map to a region 244 to 484 nucleotides upstream of the structural gene. This region contains two short sequence elements that differ by one nucleotide from the consensus core (5′-TN[A/G]TTGGT-3′) that has been proposed to be essential for binding of the HAP activation complex. These data together with earlier reports on the regulation of the *KGD1* and *LPD1* genes for the α-ketoglutarate and dihydrolipoyl dehydrogenases indicate that all three enzyme components of the complex are catabolite repressed and subject to positive regulation by the HAP2 and HAP3 proteins.

The oxidative decarboxylation of α-ketoglutarate to succinyl coenzyme A is catalyzed by the α-ketoglutarate dehydrogenase complex (KGDC) (35). The core structure of this macromolecular enzyme consists of multiple copies of dihydrolipoyl transsuccinylase (KE2) to which dimers of α-ketoglutarate dehydrogenase (KE1) and dihydrolipoyl dehydrogenase (E3) are noncovalently bound (35). These three catalytic subunits form a complex whose molecular weight has been estimated to be 2.5×10^6 to 2.8×10^6 (17, 35).

The genes for the KE1 and KE2 components of the *Escherichia coli* KGDC are located in a single operon (*suc*), thereby ensuring a coordinate output of the respective mRNAs (6, 7, 43). The E3 subunit of KGDC is encoded by *lpd*, which also codes for the identical subunit of the pyruvate dehydrogenase complex (45). Even though it is adjacent to the pyruvate dehydrogenase operon (*ace*), *lpd* is transcribed from its own promoter (43).

The KGDC of mitochondria is composed of the same three catalytic subunits as the bacterial complex. In *Saccharomyces cerevisiae*, the genes coding for KE1, KE2, and E3 are unlinked and therefore separately transcribed and regulated (38, 41). This is also true of the genes coding for the analogous components of yeast pyruvate dehydrogenase complex (5, 29, 39). The *KGD1* and *LPD1* genes, coding for the KE1 and E3 subunits, respectively, of the yeast complex have recently been cloned, and their sequences have been determined (5, 38, 39). Initial efforts to understand how the synthesis of the complex is phased to the metabolic requirements of yeast cells have revealed that transcription of both genes is severely repressed by glucose and is under the control of the HAP2 and HAP3 proteins, which also regulate the expression of numerous other mitochondrial constituents of yeast cells (34). Thus, only very low levels of the mRNAs for KE1 and E3 are detected under conditions in which yeast cells derive their ATP fermentatively and the complex is used exclusively for biosynthetic purposes (38, 41).

To complete the genetic characterization of the yeast KGDC, we have extended our screen of respiratory-deficient *pet* mutants with the aim of identifying strains defective in the KE2 component. In this communication, we show that the respiratory defect of mutants previously placed in complementation group G104 is due to lesions in this subunit of the complex. The mutants have enabled us to clone and characterize the *KGD2* gene, coding for the KE2 subunit of the yeast KGDC. We also present evidence that transcription of *KGD2*, like that of *KGD1* and *LPD1*, is subject to regulation by HAP2 and HAP3.

MATERIALS AND METHODS

Yeast strains and growth media. Table 1 lists the genotypes and sources of the yeast strains used. Nuclear respiratory-deficient (*pet*) mutants were obtained by mutagenesis of the respiratory-competent haploid strain *S. cerevisiae* D273-10B/A1 with either ethylmethane sulfonate or nitrosoguanidine (49). The media used for routine cultivation of yeast cells had the following compositions: 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 amino acids), and WOGal (2% galactose, 0.67% Difco yeast nitrogen base without amino

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

Strain	Genotype	Source or reference
D273-10B/A1	α *met6*	47
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]
E250	α *met6 kgd2-1*	This study
E250/U6	α *ura3-1 kgd2-1*	E250 × W303-1A
W303ΔKGD2	**a** *ade2-1 leu2-3,112 trp1-1 ura3-1 can1-100 kgd2::HIS3*	This study
BWG1-7a	**a** *ade1-100 his4-519 leu2-3,2-112 ura3-52*	18
LGW1	**a** *ade1-100 his4-519 leu2-3,2-112 ura3-52 hap2-1*	18
JP40-1	**a** *ade1-100 his4-519 leu2-3,2-112 ura3-52 hap3-1*	18
WCZ	**a** *leu2 his3 ura3 ade2 trp1, CYC1-lacZ(URA3)*	24

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acids). Solid media contained 2% agar. Amino acid supplements were added to a final concentration of 20 μg/ml.

Cloning of *KGD2*. A yeast genomic library prepared by the ligation of partial *Sau*3A fragments (7 to 10 kilobase pairs [kb]) of nuclear DNA to the *Bam*HI site of YEp24 (4), kindly supplied by Marian Carlson (Department of Human Genetics, Columbia University), was used to clone the *KGD2* gene, coding for yeast mitochondrial KE2. A plasmid containing the *KGD2* gene was isolated by transformation of E250/U6 (α *ura3-1 kgd2-1*) with the yeast genomic library. Approximately 5×10^8 cells grown to early log phase in YPGal medium were transformed with 5 μg of DNA by the procedure of Beggs (2). Two independent transformants complemented for the respiratory deficiency and uracil dependence were obtained. Both transformants were found to have plasmids with nuclear inserts differing by only 0.5 kb. The shorter plasmid, pG104/T1, with an insert of approximately 6 kb, and was used to subclone *KGD2*.

Enzyme assays of wild-type and mutant mitochondria. Yeast cells grown to stationary phase in liquid YPGal were used to prepare mitochondria by the procedure of Faye et al. (9) except for the use of Zymolyase 20000 (Miles Corp.) instead of glusulase for the preparation of spheroplasts. The overall activity of KGDC was assayed spectrophotometrically by measuring NAD^+ reduction at 340 nm in the presence of α-ketoglutarate (42). Dihydrolipoate dehydrogenase was assayed by monitoring the oxidation of NADH in the presence of lipoic acid (37).

Hybridization analyses and S1 nuclease mapping. Southern blot analysis was done on yeast genomic DNA isolated by the procedure of Myers et al. (25). The DNA was digested with restriction endonucleases and separated electrophoretically on a 1% agarose gel. After transfer to nitrocellulose, the blot was hybridized with a nick-translated probe containing most of the *KGD2* gene as described previously (26).

For Northern (RNA) blot and S1 nuclease analysis, total RNA was isolated from the wild-type strain D273-10B/A1 harvested either from YPGal in early stationary phase or from YPD containing 10% glucose in log phase. The RNAs were enriched for poly(A)$^+$ RNA by fractionation on poly(U) Sepharose 4B (Pharmacia, Inc.) (20). The poly(A)$^+$ RNAs were separated electrophoretically on a 1% agarose gel under nondenaturing conditions and transferred to diazobenzyloxymethyl-paper (1) for hybridization with nick-translated probes.

The method of Berk and Sharp (3) was used to map the 5′ termini of *KGD2* transcripts. Two different 5′-end-labeled single-stranded DNA probes were hybridized to poly(A)$^+$ RNA at 44°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 hybrids were digested with several concentrations of S1 nuclease at 37°C for 30 min. The protected fragments were separated on a 7% sequencing gel next to a sequencing ladder consisting of untreated probe derivatized by the A+G-specific reaction of Maxam and Gilbert (22).

Construction of *lacZ* fusions and assays of β-galactosidase activity in permeabilized yeast cells. Four different fragments of the 5′-flanking region of *KGD2* ranging from 1 kb (pG104/Z1) to 244 base pairs (bp) (pG104/Z2) were fused in frame to the seventh codon of the *lacZ* gene of *E. coli* in the episomal plasmid YEp366 (27). A fifth fusion (pG104/Z5) was made by insertion of a 1-kb *Bam*HI fragment of DNA from the *MSL1* gene (48) into the *Bam*HI site at pG104/Z1. The same fragment was also inserted into the *Sma*I site upstream of the *CYC1* promoter in the episomal plasmid YEpCZ312 (24). Each plasmid construct was introduced into wild-type and mutant yeast strains by transformation, and β-galactosidase activity was measured by the procedure of Guarente (13) in cells grown in either liquid YPGal or YPD containing 10% glucose. Under these conditions of growth, plasmid retention ranged from 75 to 85%.

Miscellaneous methods. Standard procedures were used for restriction endonuclease analysis of DNA, preparation and ligation of DNA fragments, transformation of *E. coli*, and isolation of plasmid DNA from *E. coli* transformants (21). DNA was sequenced by chemical derivatization of 5′-end-labeled single-stranded restriction fragments (22). Antibodies against KE1 and KE2 were prepared by immunizing rabbits with hybrid proteins expressed from *trpE* fusion genes (T. J. Koerner, J. E. Hill, A. M. Myers, and A. Tzagoloff, Methods Enzymol., in press). A 675-bp *Bcl*I fragment from *KGD2* and an 1,162-bp *Hin*dII-*Bam*HI fragment from *KGD1* (38) were ligated to the *trpE* gene, coding for the amino-terminal half of the protein (Koerner et al., in press). The hybrid proteins expressed in *E. coli* were purified on a sizing column and used as antigens (Koerner et al., in press).

RESULTS

Phenotype of *kgd2* mutants. Mutants representative of approximately 30 different *pet* complementation groups were screened for lesions in KE2. Mitochondria were isolated from cells grown in YPGal and assayed for α-ketoglutarate-dependent reduction of NAD^+ (42). Representative isolates from several complementation groups had recessive mutations in nuclear genes and displayed either a complete absence of or greatly reduced KGDC activity. Although attempts were made to assay KE2 directly by published procedures (36), we were unable to measure this partial activity even in mitochondria of wild-type yeast cells.

In the absence of a suitable enzymatic assay for KE2, KE2 mutants were identified by transformation of different KGDC-deficient strains with a genomic library and characterization of the complementing gene. As shown below, the respiratory and KGDC deficiencies of E250/U6, a mutant from complementation group G104, were restored by a gene that codes for a protein with high primary sequence similar-

ity to the KE2 component of the bacterial KGDC. On the basis of this homology and the biochemical and genetic properties of a mutant construct with a disrupted copy of the cloned gene (see below), we conclude that mutants in complementation group G104 have lesions in the gene coding for the KE2 component of the KGDC. This gene has been named *KGD2*, in keeping with the previous convention (12, 38).

Complementation group G104 consists of three independent mutant isolates, of which E250 has been the most extensively studied. E250 and other *kgd2* mutants display leaky growth on rich glycerol medium but fail to grow on minimal glycerol. This growth phenotype is similar to that previously reported for mutants in the KE1 component of the complex (38).

Cloning and sequence analysis of *KGD2*. E250/U6 was transformed with a yeast genomic library, and clones complemented for the respiratory defect and uracil auxotrophy were selected on minimal glycerol medium. The Gly^+ Ura^+ phenotype of two transformants (E250/U6/T1 and E250/U6/T2) was verified by segregation tests to be a function of autonomously replicating plasmids. The complementing plasmid of each transformant was isolated and amplified in *E. coli*, and its restriction map was determined. The two plasmids analyzed were almost identical except that pG104/T2 obtained from E250/U6/T2 had a nuclear DNA insert 0.5 kb longer at one end. The slightly shorter plasmid pG104/T1 was used to subclone the gene.

To localize the complementing gene, different segments of the insert in pG104/T1 were transferred to the shuttle vector YEp352 (16), and the resultant constructs were tested for complementation of the Gly^- phenotype of E250/U6. Of the plasmids made, the smallest (pG104/ST4) capable of complementing the E250/U6 mutant has an insert with the region from the unique *Bam*HI to the *Hin*dIII site of the original insert in pG104/T1 (Fig. 1). The inability of pG104/ST5 to complement the mutant indicates that the gene must cross the *Bal*I site proximal to the downstream *Bgl*II site of the insert.

The region of the pG104/ST4 insert suspected to contain the gene was sequenced in both strands by the method of Maxam and Gilbert (22). The sequence reported in Fig. 2 starts approximately 300 bp to the left of the *Bam*HI site and extends for an additional 1.8 kb to the right of the site (Fig. 1). This region of DNA has only one open reading frame of sufficient length to qualify as a protein-coding gene. The reading frame starts with an ATG at nucleotide +1 and ends with an opal termination codon at nucleotide +1426 of the sequence reported in Fig. 2. The fact that the reading frame is initiated downstream of the *Bam*HI site and crosses the two *Bal*I sites is consistent with the result of the subcloning experiments (Fig. 1).

The product encoded by the reading frame is 475 amino acids long and has a calculated molecular weight of 52,375. A comparison of the deduced amino acid sequence with the previously reported sequence of the *E. coli* KE2 (7) revealed that the two proteins have very similar primary structures. The alignment obtained with the MFALGO program (50) indicates that the two proteins share 42% identical and 11% conserved residues at equivalent positions of the polypeptide chains. The primary sequence similarity with bacterial KE2 and the ability of the cloned gene to complement a mutant lacking KGDC activity provides strong evidence that the encoded product is the KE2 component of the KGDC.

The yeast KE2 is some 70 residues longer than the *E. coli* protein at the amino-terminal end. Part or all of this sequence may be a mitochondrial targeting signal. This amino-terminal domain has a high proportion of basic and hydroxylated residues, a common feature of mitochondrial signal sequences (15).

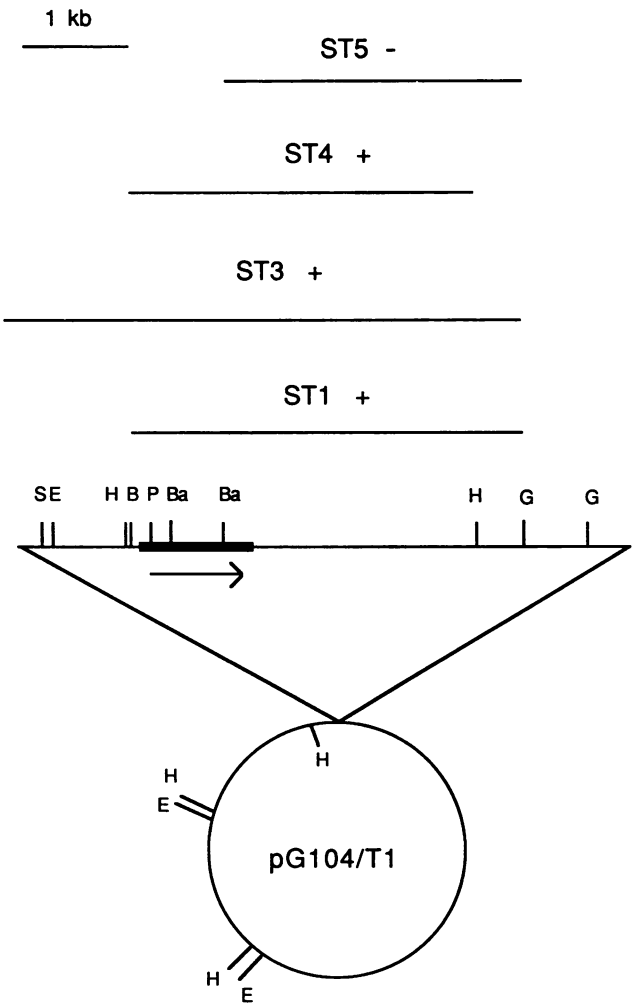

FIG. 1. Localization of *KGD2* within the genomic insert of pG104/T1. A partial restriction map of the DNA insert in pG104/T1 is shown at the bottom. The restriction sites shown are *Bal*I (Ba), *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), and *Sst*I (S). The heavy bar indicates the physical limits, and the arrow shows the direction of transcription of the open reading frame corresponding to *KGD2*. The restriction fragments cloned into YEp352 (16) are depicted by the bars in the upper part of the figure. Cloned regions that complement the respiratory deficiency of E250/U6 are marked by the plus signs, and the region that fails to complement the mutant is marked by the minus sign.

According to the model proposed for the domain structure of *E. coli* KE2 and the dihydrolipoyl acetyltransferase (PE2) of the pyruvate dehydrogenase complex, both are composed of at least three distinct functional regions: an N-terminal domain with covalently attached lipoic acid, followed by a short domain responsible for binding the lipoyl dehydrogenase component (E3), and a third long domain constituting the catalytic core structure of each protein (31, 32). The dot matrix alignment of the bacterial and yeast mitochondrial KE2 components shown in Fig. 3 shows the most highly conserved region to be in their carboxyl-terminal halves corresponding the catalytic core domain. The primary se-

FIG. 2. Nucleotide sequence of *KGD2* (GenBank accession number M34531) and of flanking regions. The sequence of 2.1 kb of the genomic insert of pG104/T1 is reported. Only the sequence of the sense strand is shown. The reading frame identified as the *KGD2* gene starts with the methionine codon at nucleotide +1 and terminates with the opal termination codon at nucleotide +1426. The amino acid sequence of the KE2 component of the yeast KGDC is shown above the gene sequence. The two CCAAT boxes (TN[A/G]TTGGT consensus sequence in the complementary strand) are underlined. The major transcriptional start sites at −109 and −254 are indicated by the asterisks. On the basis of the homology with *E. coli* KE2, the lipoyl moiety is probably covalently attached to the lysine residue marked by the arrow. The region of yeast KE2 with the internally duplicated KKLLQ sequence is marked by the dashed underline.

quence similarity in the E3 binding region is nonexistent and in the N-terminal region is only marginal except for a short stretch of amino acids near the conserved lysine (Lys-114 in yeast KE2) that binds lipoic acid (28).

In situ disruption of *KGD2*. Even though *KGD2* complements G104 mutants deficient in KGDC activity, this result by itself does not establish that such strains have mutations in the KE2 component. Overproduction of KE2 in transformants harboring *KGD2* on a multicopy plasmid could compensate for or suppress a mutation in some other gene. To confirm that the absence of KGDC activity in G104 strains is due to mutations in *KGD2*, we constructed a genetic tester in which the chromosomal copy of *KGD2* was disrupted with the yeast *HIS3* gene by the one-step gene replacement procedure (40). A 675-bp fragment internal to the coding sequence of *KGD2* was removed by digestion of pG104/ST4 with *Bal*I. The gapped plasmid was ligated with a blunt-ended 1.8-kb *Sma*I-*Hin*cII fragment containing the yeast *HIS3* gene. The resultant deletion-disruption allele *kgd2*::*HIS3* was isolated as an *Eco*RI-*Nar*I fragment (Fig. 4) and used to transform the respiratory-competent haploid strain of yeast W303-1A.

Transformation of W303-1A with the linear fragment yielded the mutant W303ΔKGD2, with a His^+ Gly^- phenotype. This strain was verified by Southern analysis to have acquired the *kgd2*::*HIS3* allele. Genomic DNA from W303ΔKGD2 and the parental strain W303-1A was digested with a combination of *Sst*I and *Bgl*II. A 3-kb *Pst*I-*Hin*dIII probe detected the wild-type 4.3-kb fragment. The appearance in the mutant of novel fragments of approximately 2.1 and 2.2 kb is explained by cleavage of the disrupted allele at the *Bgl*II sites within the *HIS3* insert (Fig. 4). In addition to the expected fragments, the probe hybridized to a larger fragment (>9.3 kb) whose size was not affected in the mutant. We presume that this fragment probably originates from some other region of the genome but contains sequences homologous to *KGD2*. The weaker signal seen in the smallest band of the mutant may be due to the fact that

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        Glu Ala Pro Ala Glu Gly Ser Gly Glu Ser Lys Pro Glu Pro Thr Glu Gln Ala Glu His Arg Lys Val Ser Pro
+451    GAA GCA CCT GCT GAG GGT TCT GGA GAA TCT AAG CCA GAG CCT ACC GAA CAA GCG GAG CAT CGC AAG GTG TCG CCG

        Gln Gly Lys Thr Gln Val Arg Lys Arg Leu Gln Arg Lys Lys Leu Leu Gln Arg Lys Lys Pro Leu Gln Arg Lys
+526    CAA GGG AAA ACT CAA GTG AGG AAA CGG CTT CAA AGA AAG AAG CTG CTC CAA AGA AAG AAG CCG CTC CAA AGA AAG
                               ----------------------------------------------------------------------

        Lys Leu Gln Asn Gln Lys Arg Thr Asp Gln Pro Lys Lys Thr Val Ser Lys Ala Gln Glu Pro Pro Val Ala Ser
+601    AAG TTA CAG AAC CAA AAA AGG ACT GAT CAA CCA AAG AAG ACC GTC TCT AAG GCG CAG GAA CCC CCA GTA GCC TCT
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        Asn Ser Phe Thr Pro Phe Pro Arg Thr Glu Thr Arg Val Lys Met Asn Arg Met Arg Leu Arg Ile Ala Glu Arg
+676    AAC TCT TTC ACA CCA TTT CCA CGT ACA GAA ACC AGG GTC AAA ATG AAC CGT ATG AGA TTG AGG ATT GCC GAA AGA

        Leu Lys Glu Ser Gln Asn Thr Ala Ala Ser Leu Thr Thr Phe Asn Glu Val Asp Met Ser Ala Leu Met Glu Met
+751    TTA AAA GAG TCT CAA AAC ACT GCT GCT TCC TTA ACC ACA TTC AAC GAA GTT GAC ATG TCA GCT TTG ATG GAA ATG

        Arg Lys Leu Tyr Lys Asp Glu Ile Ile Lys Lys Thr Gly Thr Lys Phe Gly Phe Met Gly Leu Phe Ser Lys Ala
+826    AGG AAA CTG TAT AAA GAT GAG ATT ATT AAG AAG ACC GGT ACT AAA TTC GGA TTC ATG GGT CTT TTC TCC AAA GCA

        Cys Thr Leu Ala Ala Lys Asp Ile Pro Ala Val Asn Gly Ala Ile Glu Gly Asp Gln Ile Val Tyr Arg Asp Tyr
+901    TGT ACC TTG GCC GCC AAG GAT ATT CCA GCC GTC AAT GGT GCC ATT GAA GGT GAC CAG ATT GTT TAT CGT GAT TAC
                                                                      BstEII

        Thr Asp Ile Ser Val Ala Val Ala Thr Pro Lys Gly Leu Val Thr Pro Val Val Arg Asn Ala Glu Ser Leu Ser
+976    ACA GAT ATT TCT GTT GCT GTG GCC ACT CCA AAG GGT TTG GTT ACC CCC GTC GTT CGT AAT GCA GAG TCA TTG AGT
                                                    BstEII

        Val Leu Asp Ile Glu Asn Glu Ile Val Arg Leu Ser His Lys Ala Arg Asp Gly Lys Leu Thr Leu Glu Asp Met
+1051   GTT TTA GAT ATT GAG AAC GAA ATT GTT CGC TTG AGT CAT AAA GCG CGT GAT GGC AAA TTA ACC CTA GAA GAT ATG

        Thr Gly Gly Thr Phe Thr Ile Ser Asn Gly Gly Val Phe Gly Ser Leu Tyr Gly Thr Pro Ile Ile Asn Ser Pro
+1126   ACG GGT GGT ACT TTC ACC ATA TCT AAT GGT GGT GTT TTT GGT TCA TTA TAC GGT ACT CCT ATC ATC AAT TCA CCA

        Gln Thr Ala Val Leu Gly Leu His Gly Val Lys Glu Arg Pro Val Thr Val Asn Gly Gln Ile Val Ser Arg Pro
+1201   CAA ACA GCC GTC CTA GGC TTG CAT GGT GTC AAA GAG AGA CCT GTC ACT GTT AAT GGA CAA ATT GTC TCA AGA CCA

        Met Met Tyr Leu Ala Leu Thr Tyr Asp His Arg Leu Leu Asp Gly Glu Lys Leu Leu Ser Phe Leu Lys Thr Val
+1276   ATG ATG TAC TTG GCT TTG ACT TAT GAT CAT AGA TTG CTA GAT GGT GAG AAG CTG TTA TCC TTC TTG AAG ACT GTT

        Lys Glu Leu Ile Glu Asp Pro Arg Lys Cys Cys Tyr Gly Asp Leu Lys Phe Ala Ala His Thr Asn Leu Ile Ser
+1351   AAA GAG TTG ATT GAA GAC CCT AGA AAA TGT TGT TAT GGT GAT TTG AAA TTT GCA GCC CAT ACC AAT CTG ATT TCA

        Opa
+1426   TGA TTATTCTTGTCCGCTATTACTGTGTGAATCTCTTCTATTTAAGTA TGATATTTAAAGATATGCTATGATGATAAATGGAACTATGAATTAATGTT

+1524   TTCTTTTTATAATGAGATATGGAAAAGCGC
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the probe contained only some 300 bp of complementary sequences. Other digests confirmed the presence of the disrupted *kgd2* allele in W303ΔKGD2 and of another region of DNA that cross-hybridizes with the probe but is not affected in the mutant. Whether this second gene codes for the dihydrolipoyl transacetylase component of the pyruvate dehydrogenase complex or for some other enzyme involved in the metabolism of α-ketoglutarate has not been examined.

In addition to the results of the Southern analysis, the presence of the *kgd2*::*HIS3* allele was also evidenced by our failure to detect the 52 kilodalton *KGD2* product in mitochondria of W303ΔKGD2 (Fig. 5). The respiratory defect of W303ΔKGD2 was complemented by a [*rho*^{0}] tester strain, indicating that the mutation is recessive. The recessive nature of the *kgd2*::*HIS3* allele was also confirmed by the respiratory-competent phenotype of diploid cells obtained from crosses of the mutant to a wild-type haploid strain.

Partial restoration of KGDC activity in mutants transformed with *KGD2* on a high-copy-number plasmid. The KGDC activity of mitochondria was measured in a wild-type strain, in *kgd2* mutants, and in the same strains transformed with the *KGD2* gene on a high-copy-number plasmid. Nei-

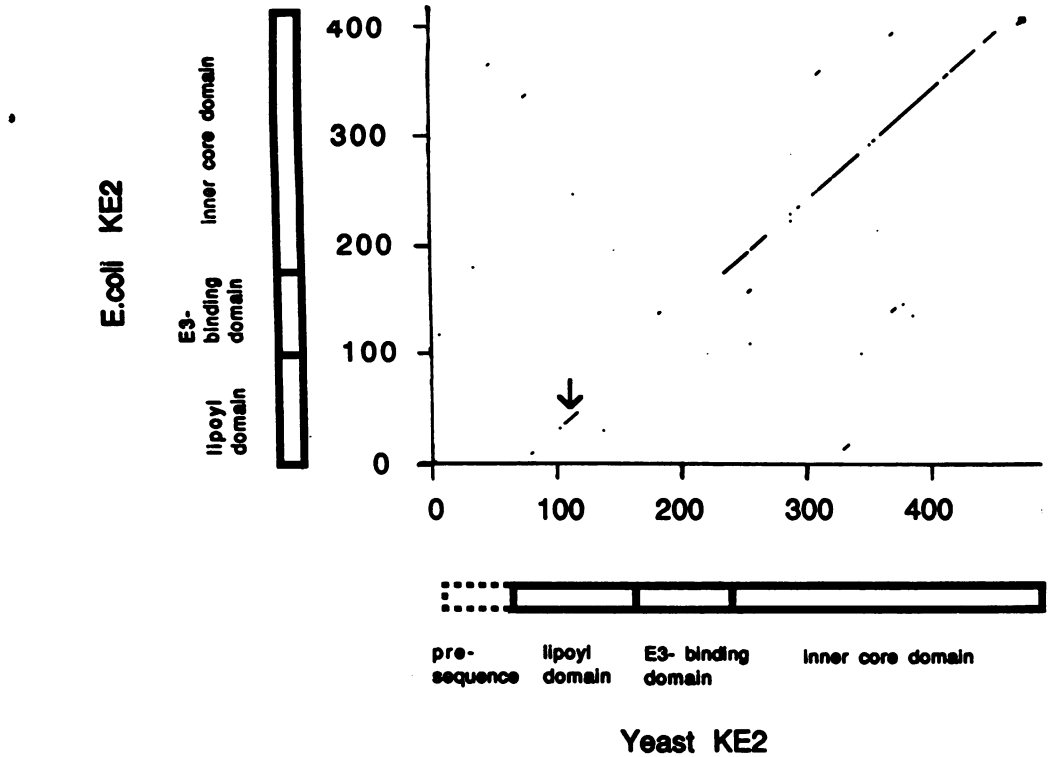

FIG. 3. Homology of yeast and *E. coli* KE2. The program used to align the two proteins scores a dot for every five identities out of 10 residues scanned. The three functional domains of the bacterial KE2 are indicated next to the *y* axis, and the corresponding regions of the yeast protein are shown on the *x* axis. The amino-terminal sequence absent in *E. coli* KE2 is depicted by the dashed box. The arrow indicates the lysine residue with covalently bound lipoic acid.

ther the mutants with the *kgd2-1* or those with the *kgd2*::*HIS3* allele had any detectable enzyme activity (Table 2). Transformation of W303ΔKGD2 with either pG104/T1 or pG104/ST4 restored less than wild-type KGDC activity even though both clones had at least 10 times more immunologically detectable KE2 in their mitochondria than did the wild-type strain (Fig. 5). The lower KGDC activity in the transformants cannot be explained by loss of plasmid, since the yeast cultures used for isolation of mitochondria were determined to have 75 to 80% cells with plasmid DNA.

The following observations suggest that the high molar excess of KE2 over KE1 in the transformants, due to the presence of *KGD2* on a multicopy plasmid, results in a smaller fraction of fully assembled or fully active KGDC. The introduction of *KGD2* on a high-copy-number plasmid into a wild-type or mutant strain elicited a lower KGDC activity in mitochondria (Table 2). The observation that the final specific activity of KGDC was higher in the wild type transformed with pG104/T1 than in the mutant W303ΔKGD2 transformed with the same plasmid can be explained by the fact that in the case of the wild-type strain, the segregants (15 to 20%) that lost plasmid were still able to synthesize KGDC as a result of the presence of the chromosomal copy of *KGD2*. Second, sedimentation analyses of mitochondrial extracts from the wild type and from W303ΔKGD2 transformed with either pG104/T1 or pG104/ST4 indicate the latter strains to have substantially less KE1 sedimenting as part of the KGDC. Sedimentation of wild-type mitochondrial extracts through sucrose gradients showed that KE1 and KE2 cosedimented as a high-molecular-weight complex of approximately 2×10^6 (Fig. 6A). Under the same conditions, the KE1 and KE2 components in the extract of the transformant sedimented as heterodispersed proteins with wider size distribution (Fig. 6B). Furthermore, KE1 and KE2 in the transformant had apparent molecular weights lower than that of the complex, and their peak fractions did not coincide.

Northern and S1 nuclease mapping of *KGD2* transcripts. Transcription of *LPD1* (41) and *KGD1* (38) has been shown to be catabolite repressed. To determine whether the KE2 component of the complex is similarly regulated, the abundance of *KGD2* transcripts was assessed by Northern hybridization analysis in cells grown under repressed and under derepressed conditions. Total RNA was isolated from the wild-type strain D273-10B/A1 grown in YPGal (derepressed) and in YPD containing 10% glucose (repressed). The two preparations were enriched for poly(A)$^+$ RNA, separated on a 1% nondenaturing gel, and transferred to diazobenzyloxymethyl-paper. The blotted RNAs were hybridized with a mixture of nick-translated DNA probes for *KGD2* and for the yeast actin gene. The latter served as an internal standard, since its transcription is not affected by glucose. The *KGD2* probe detected two transcripts of approximately equal abundance in the poly(A)$^+$ fraction of derepressed cells (Fig. 7). The identical transcripts were detected when the *Bam*HI-*Pst*I fragment from the upstream region of *KGD2* was used as the probe. The concentration of the *KGD2* transcripts was much lower in glucose- than in galactose-grown cells when normalized to the actin mRNA level, indicating that transcription of *KGD2* is severely catabolite repressed.

The two transcripts observed by Northern hybridization analysis suggested that *KGD2*, like *KGD1*, might have two close but distinct transcriptional start sites (38). The 5′ termini of the *KGD2* transcripts were determined by S1 nuclease mapping with the two 5′-labeled protection probes

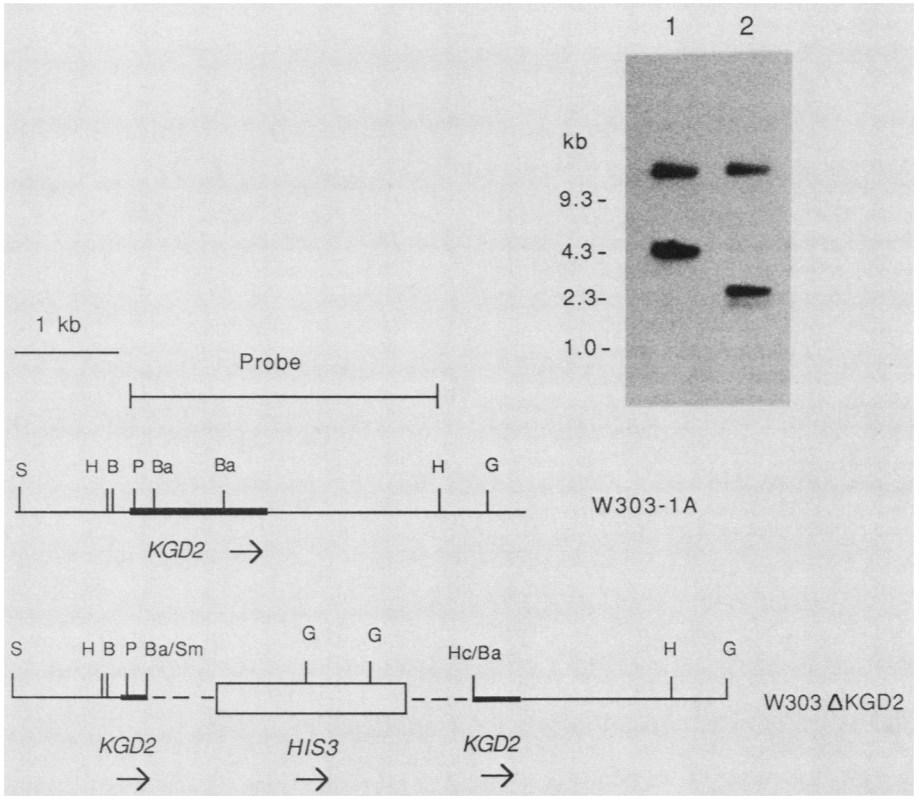

FIG. 4. Southern analysis of chromosomal DNA. Shown are restriction maps of the wild-type *KGD2* gene and of the mutant allele *kgd2*::*HIS3* constructed by insertion of a 1.8-kb fragment of DNA with the yeast *HIS3* gene between the two *Bal*I sites of *KGD2*. The *KGD2* and *HIS3* gene are depicted by the solid and open bars, respectively. The direction of transcription of genes is indicated by the arrows. Locations of the restriction sites for *Bal*I (Ba), *Bam*HI (B), *Bgl*II (G), *Hin*dIII (H), *Pst*I (P), and *Sst*I (S) and the junctions of *Bal*I and *Sma*I (Ba/Sm) and *Hin*cII and *Bal*I (Hc/Ba) are indicated. The autoradiogram shows the results of the Southern analysis of DNA isolated from the wild-type W303-1A parental strain (lane 1) and from the Gly^- His^+ mutant (lane 2) obtained by transformation of W303-1A with a linear fragment containing the disrupted *KGD2* gene. The two DNA preparations were digested with a combination of *Sst*I and *Bgl*II and separated by electrophoresis on a 1% agarose gel. After transfer to nitrocellulose, the blot was hybridized with the nick-translated *Pst*I-*Hin*dIII fragment (probe) with the *KGD2* gene. The migration of known size standards is marked in the margin.

shown in Fig. 8. The first probe (A), covering the region from nucleotides −244 to +130, protected a family of transcripts with 5′ termini centering at nucleotide −109. These transcripts were detected only in the poly(A)$^+$ RNA of cells grown under derepressed conditions (Fig. 8). The second probe (B), spanning the more distal upstream region from −560 to −195, revealed a family of longer transcripts, with transcriptional initiating sites clustering near −254. The results of the S1 analyses confirm the much higher abundance of *KGD2* transcripts in derepressed yeast cells. The two size classes of *KGD2* RNAs differ by 140 to 150 nucleotides, consistent with the difference in the electrophoretic migration of the two transcripts (Fig. 7).

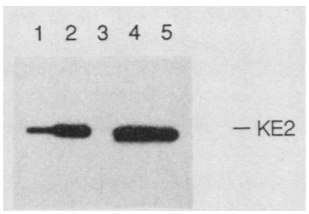

FIG. 5. Western immunoblot analysis of KE2. Mitochondria were isolated from the wild type, mutant, and transformants. Total mitochondrial proteins (10 μg) were separated on a 10% polyacrylamide gel (19). After transfer to nitrocellulose, the blotted proteins were reacted sequentially first with antiserum to a *trpE-KGD2* fusion protein, followed by ^{125}I-protein A. Lanes: 1, mitochondria from the wild-type parental strain W303-1A; 2, W303-1A transformed with pG104T1; 3, W303ΔKGD2; 4, W303ΔKGD2 transformed with pG104T1; 5, E250/U6 transformed with pG104T1.

Localization of the *KGD2* promoter. The location of the promoter(s) responsible for the regulated transcription of the *KGD2* mRNAs was studied by measuring the β-galactosidase activity of yeast cells harboring plasmids with fusions of different 5′-flanking regions of *KGD2* to the *E. coli lacZ* gene. The expression of β-galactosidase from the *lacZ* fusions was examined in wild-type and mutant yeast cells grown under repressed and derepressed conditions.

The regions of *KGD2* fused to the *lacZ* gene are shown in Fig. 9. The longest construct (pG104/Z1) contained approximately 1 kb of 5′-flanking sequence from the *Sst*I to the *Pst*I site just inside the reading frame. In the shortest construct (pG104/Z2), *lacZ* was fused to the region from the *Bam*HI site at nucleotide −244 to the same *Pst*I site. This region has only one of the two transcriptional start sites mapped with S1 nuclease. Two other fusions had 5′-flanking regions intermediate between those of pG104/Z1 and pG104/Z2, and a third had the 5′-flanking region of *KGD2* disjoined by insertion of a 1-kb fragment of DNA into the *Bam*HI site of pG104/Z1. The plasmid constructs were introduced into the

TABLE 2. KGDC activity in wild-type and *kgd2* mutants of *S. cerevisiae*

Strain	Genotype	KGDC activity[a]	
		Expt 1	Expt 2
W303-1A	*KGD1 KGD2*	0.303	0.150
W303-1A/T1[b]	*KGD1 KGD2* +(*KGD2*)	ND	0.087
E250	*KGD1 kgd2-1*	0	0
E250/U6	*KGD1 kgd2-1*	0	0
E250/U6/T1[c]	*KGD1 kgd2-1* +(*KGD2*)	ND	0.029
W303ΔKGD2	*KGD1 kgd2::HIS3*	0	0
W303ΔKGD2/T1[d]	*KGD1 kgd2::HIS3* +(*KGD2*)	0.071	0.059
W303ΔKGD2/ST4[e]	*KGD1 kgd2::HIS3* +(*KGD2*)	0.037	ND
C225/U2/T1[f]	*kgd1-1 KGD2* +(*KGD1*)	0.210	ND

[a] Micromoles of NAD reduced per minute per milligram of mitochondrial protein. Values are averages of duplicate assays ±5%. ND, Not determined.
[b] W303-1A transformed with pG104/T1 containing *KGD2*.
[c] E250/U6 transformed with pG104/T1.
[d] W303ΔKGD2 transformed with pG104/T1.
[e] W303ΔKGD2 transformed with pG104/ST4 containing *KGD2*.
[f] C225/U2 transformed with pG70/T1 (38) containing *KGD1*.

two respiratory-competent strains W303-1A and BWG1-7A and into the *hap2* mutant LGW1 and the *hap3* mutant JP40-1. Transformants harboring the *lacZ* fusions were grown to early logarithmic phase in 10% glucose medium (repressed) and to stationary phase in 2% galactose medium

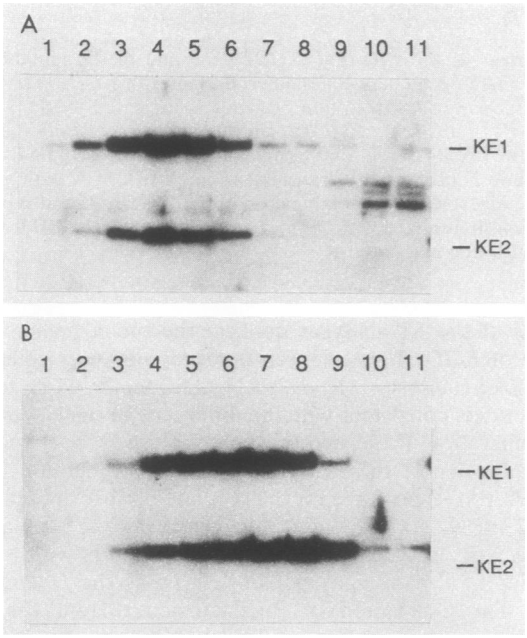

FIG. 6. Sedimentation of KE1 from wild type and in a transformant overexpressing KE2. Mitochondria were prepared from the wild-type strain W303-1A (A) and from W303ΔKGD2 transformed with pG104/T1 (B). The two preparations were suspended at a protein concentration of 10 mg/ml in 10 mM Tris hydrochloride (pH 7.5) and were disrupted by sonic irradiation. The clear supernatants (200 μl) obtained after centrifugation at 105,000 × g_{av} for 10 min were applied on 5-ml columns of a linear 6 to 20% sucrose gradient prepared in the presence of 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, and 0.02% Triton X-100. The gradients were centrifuged at 4°C for 45 min at 65,000 rpm in a Beckman Sw65Ti rotor. Eleven equal (0.5-ml) fractions were collected and analyzed for the distribution of KE1 and KE2 by the Western technique as described in the legend to Fig. 5. KE1 and KE2 are identified in the margin. Sedimentation was from right to left.

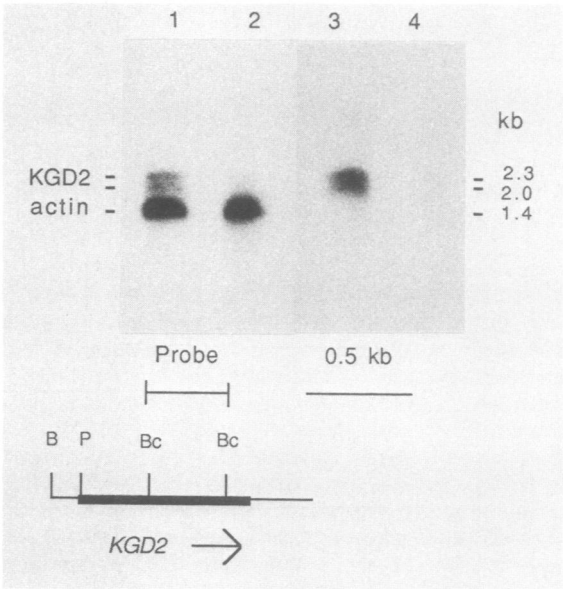

FIG. 7. Northern analysis of *KGD2* mRNA. The respiratory-competent strain D273-10/A1 was grown under repressed (YPD containing 10% glucose) or derepressed (YPGal) conditions. Total RNA was prepared and enriched for poly(A)$^+$ RNA by affinity chromatography on poly(U) Sepharose (Pharmacia). Approximately 2 to 3 μg of poly(A)$^+$ RNA was separated on a 1% agarose gel. The RNAs were transferred to diazobenzyloxymethyl-paper by blotting and hybridized to nick-translated probes specific for *KGD2* and the yeast actin gene. A 675-bp *Bcl*I fragment internal to *KGD2* was used as the hybridization probe for *KGD2*. The restriction sites for *Bam*HI (B), *Bcl*I (Bc), and *Pst*I (P) are indicated. The actin probe is an internal 600-bp *Eco*RI-*Hin*dIII fragment. Lanes: 1, RNA from derepressed yeast cells hybridized with a mixture of the *KGD2* and actin probes; 2, RNA from repressed yeast cells hybridized with a mixture of the *KGD2* probe and the actin probe; 3, RNA from derepressed cells hybridized with the *KGD2* probe; 4, RNA from repressed cells hybridized with the *KGD2* probe. The migration of DNA size standards is shown in the margin.

(derepressed), and β-galactosidase activity was assayed in permeabilized cells.

The activities expressed from the different constructs in the wild-type strain W303-1A are reported in Fig. 9. Eight times more β-galactosidase activity was measured when cells transformed with pG104/Z1 were grown on galactose as compared with glucose. This magnitude of repression by glucose is comparable to that observed in a control strain with *lacZ* fused to the *CYC1* promoter. Cells containing the shortest fusion (pG104/Z2) with the upstream region of *KGD2* starting from the *Bam*HI site at −244 exhibited only a threefold difference in the two carbon sources because of the higher expression of β-galactosidase under glucose-repressed conditions. No β-galactosidase was produced when the 5′-flanking region was disrupted with a 1-kb fragment of foreign DNA at the *Bam*HI site (pG104/Z5). The same fragment inserted into the *Sma*I site 5′ to *CYC* had no effect on transcription of this gene (data not shown).

Transcription of *KGD2* in cells harboring pG104/Z2 but not pG104/Z5 suggests that the omission of sequences upstream of the *Bam*HI site leads to the creation of a moderately glucose regulated promoter as a result of either fusion to vector sequences or activation of some latent promoter sequences downstream of the *Bam*HI site. Whatever the nature of this promoter, its physiological significance is

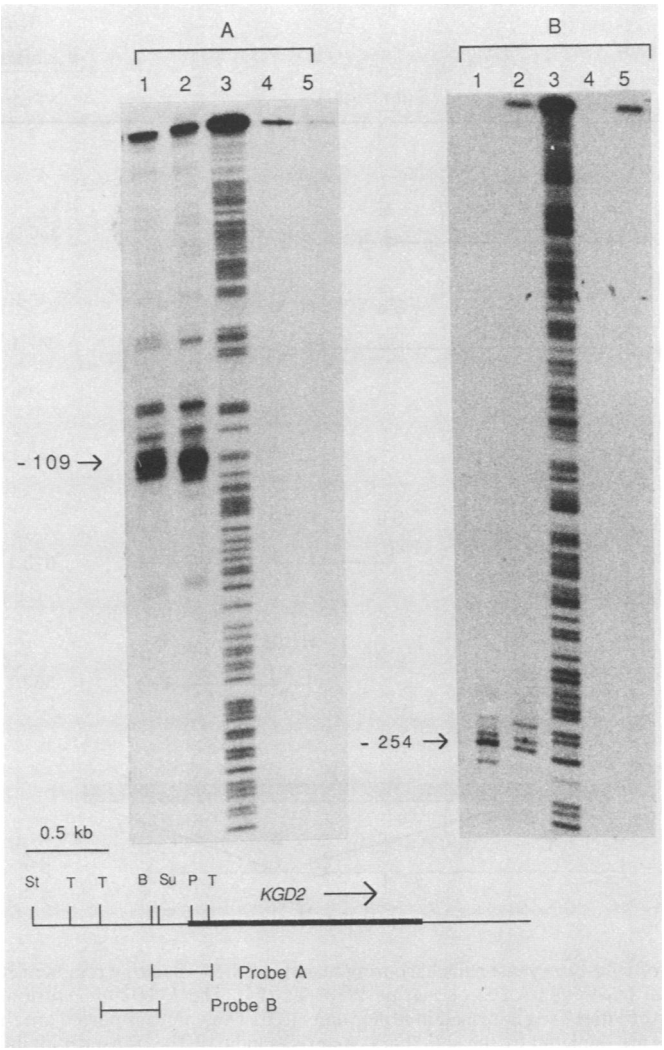

FIG. 8. S1 nuclease mapping of *KGD2* transcripts. Poly(A)$^+$-enriched RNAs from D273-10B/A1 grown under derepressed (lanes 1 and 2) and repressed (lanes 4 and 5) conditions were hybridized to two different 5′-end-labeled probes (A and B in the diagram at the bottom) under the conditions described in Materials and Methods. Probe A is a *Bam*HI-*Taq*I fragment with the sequence from −244 to +130, and probe B is a *Taq*I-*Stu*I fragment spanning the sequence from −560 to −195. In panel A, the hybrids protected by probe A were digested at 37°C with 10 U (lanes 1 and 4) or 100 U (lanes 2 and 5) of S1 nuclease per ml. In panel B, the hybrids were protected with probe B and digested at 37°C with 10 U (lanes 2 and 5) or 100 U (lanes 1 and 4). Each probe was derivatized for the A+G-specific reactions (22) and used as a sequencing ladder (lane 3). The locations of the restriction sites for *Bam*HI (B), *Sst*I (St), *Stu*I (Su), *Pst*I (P), and *Taq*I (T) are shown.

difficult to assess because of the marginal repression by glucose and lack of responsiveness to activation by HAP2 and HAP3 (see below).

Further definition of the glucose-regulated promoter region was achieved with two other *lacZ* fusions, one with an additional 80 bp (pG104/Z4) and the other with 250 bp (pG104/Z3) upstream of the *Bam*HI site. Both constructs supported maximal expression of β-galactosidase that was fully repressed by glucose (Fig. 9; Table 3). These results indicate that the 5′-flanking region starting from the *Hae*III site at −322 has the regulatory elements necessary for glucose-repressible transcription of the gene.

Transcription of *KGD2* is activated by HAP2 and HAP3 proteins. Transcription of many yeast nuclear genes coding for proteins involved in respiration is positively regulated by the products of the *HAP2*, *HAP3*, and *HAP4* genes (11, 14, 30, 34). These proteins form a complex that binds to the upstream activation sites and enhances transcription of the genes in derepressed but not glucose-repressed yeast cells (11, 14, 30).

To assess whether transcription of *KGD2* is subject to activation by the HAP complex, the *lacZ* fusions were assayed in *hap2* and *hap3* mutants and in the parental wild-type strain. Fusions containing at least 484 nucleotides of the *KGD2* 5′-flanking sequence expressed 10 to 20 times less β-galactosidase in the mutants, indicating a HAP-responsive promoter (Table 3). The shorter fusion (pG104/Z4) with the sequence starting from −322 also expressed maximal β-galactosidase in wild-type background. In this case, however, the *hap2* and *hap3* mutations had a much less pronounced effect on the production of β-galactosidase. These results suggest that HAP-regulated transcription of *KGD2* requires sequences located between the *Sau*3A site at −484 and the *Hae*III site at −322. This region has two potential upstream activation sites (Fig. 2) which differ by

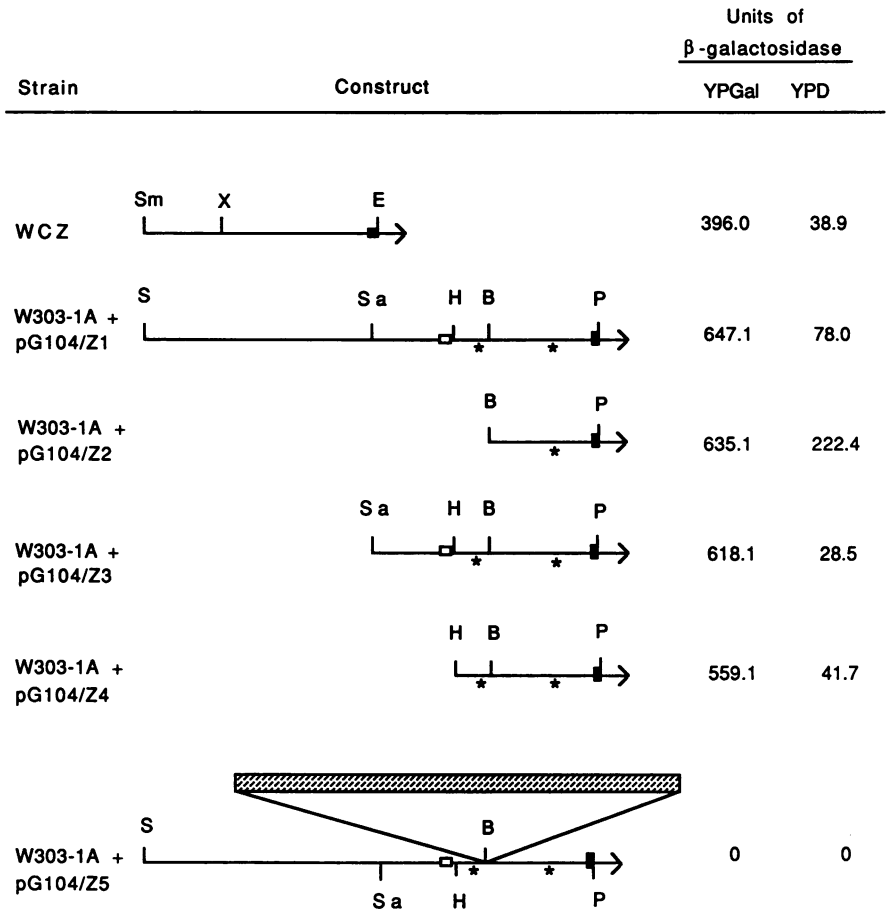

FIG. 9. β-Galactosidase activity of wild-type yeast cells harboring fusions of the 5'-flanking region of *KGD2*. Strain WCZ has a *CYC1-lacZ* fusion integrated in the chromosomal *URA3* gene of *S. cerevisiae* W303-1A (24). The *KGD2-lacZ* fusions were introduced into W303-1A on the episomal plasmid YEp366 (27). Activities are expressed in Miller units (23). The values reported are the average of duplicate assays ±5%. The *CYC1* and *KGD2* coding regions are depicted by the solid bars. Asterisks indicate the transcription initiation sites at −109 and −254. The open box represents the region which contains the two putative HAP2 and HAP3 binding sites. The dashed bar in pG104/Z5 represents the 1-kb piece of DNA from the yeast *MSL1* gene (48) inserted at the *Bam*HI site. The locations of *Bam*HI (B), *Eco*RI (E), *Hae*III (H), *Pst*I (P), *Sau*3AI (Sa), *Sma*I (Sm), *Sst*I (S), and *Xho*I (X) sites are shown.

one nucleotide from the consensus core sequence that has been proposed to bind the HAP activation complex (10).

DISCUSSION

Because mutants of *S. cerevisiae* defective in the KE1 or E3 component of the mitochondrial KGDC are unable to grow on nonfermentable carbon sources (8, 38), we reasoned that mutations in KE2, the third component of the complex, would express a similar phenotype. To identify such mutants, some 30 different complementation groups from a larger collection of *pet* strains were screened for KGDC activity.

The following evidence has convinced us that the respiratory defect of *pet* mutants previously assigned to complementation group G104 is a consequence of mutations in the structural gene for KE2. (i) G104 mutants have no KGDC activity. (ii) A gene capable of complementing the respiratory deficiency of G104 mutants and of restoring their KGDC activity has been cloned by transformation with a yeast genomic library. This gene, designated *KGD2*, codes for a protein homologous to the KE2 component of the *E. coli* KGDC. (iii) A yeast strain with the disrupted allele

TABLE 3. Effects of *hap2* and *hap3* on the derepression of *KGD2*[a]

Strain	Plasmid	Genotype	β-Galactosidase activity			
			Expt 1		Expt 2	
			YPGal	YPD	YPGal	YPD
BWG1-7a	pG104/Z1	*HAP2 HAP3*	783.0	45.2	243.2	16.0
LGW-1	pG104/Z1	*hap2-1 HAP3*	30.8	19.5	41.4	36.2
JP40-1	pG104/Z1	*HAP2 hap3-1*	67.8	18.7	44.1	17.5
BWG1-7a	pG104/Z2	*HAP2 HAP3*	797.3	268.3	405.4	100.6
LGW-1	pG104/Z2	*hap2-1 HAP3*	418.4	99.0	375.0	223.3
JP40-1	pG104/Z2	*HAP2 hap3-1*	431.2	97.6	578.1	107.4
BWG1-7a	pG104/Z3	*HAP2 HAP3*	629.3	78.2	901.0	89.9
LGW-1	pG104/Z3	*hap2-1 HAP3*	89.9	35.6	84.0	30.0
JP40-1	pG104/Z3	*HAP2 hap3-1*	63.9	10.4	93.0	23.4
BWG1-7a	pG104/Z4	*HAP2 HAP3*	710.8	87.0	702.2	50.9
LGW-1	pG104/Z4	*hap2-1 HAP3*	437.4	156.8	288.0	109.4
JP40-1	pG104/Z4	*HAP2 hap3-1*	237.6	42.3	187.0	97.2

[a] The transformed strains were grown in either YPD (containing 10% glucose) or YPGal. In two independent experiments, β-galactosidase activity was assayed as described by Guarente (13). Activities are reported in Miller units (23). Values are averages of duplicate assays ± 5%.

kgd2::*HIS3* has been constructed. This mutant construct lacks KGDC activity and fails to be complemented by the *kgd2* mutant E250.

The primary structure of the yeast KE2 component deduced from the sequence *KGD2* suggests a domain structure similar to that described for the homologous protein of the *E. coli* KGDC and the PE2 component of the pyruvate dehydrogenase complex (31, 32). The most highly conserved domain is in the carboxyl half of the yeast protein, where 54% of the residues are identical with those of the KE2 of *E. coli*. This region has been proposed to determine the catalytic core structures of bacterial KE2 and PE2 (31, 32). Also conserved is a shorter amino-terminal domain with the lysine residue that anchors the lipoyl group (28). As judged from its amino acid sequence, the KE2 component of *S. cerevisiae*, like its *E. coli* homolog, has only one lipoyl domain, whereas the acyltransferase (PE2) of the *E. coli* pyruvate dehydrogenase complex (44) has multiple lipoyl moieties. The least well conserved region of yeast KE2 occurs near the central part of the protein. The corresponding regions of the bacterial KE2 and PE2 have been implicated to bind E3 (31, 32). It is interesting that even though completely divergent in sequence, this region of both the yeast and bacterial KE2 has evolved by internal duplications of a short sequence. The repeated sequence is K/R-K-L/P/R-L-Q in yeast KE2 and a proline followed by an alanine-rich stretch in *E. coli* KE2.

The synthesis of some tricarboxylic acid cycle enzymes, including KGDC, is known from earlier studies to be repressed when yeast cells metabolize glucose fermentatively (33). More recently, transcription of *LPD1* (41) and *KGD1* (38), coding, respectively, for the E3 and KE1 components of the yeast KGDC, was found to be regulated by carbon source and to be activated by the HAP2 and HAP3 proteins (30). In this study, we show that the KE2 component of the complex is similarly regulated. Northern blot analyses of poly(A)$^+$ RNA isolated from yeast cells grown under derepressed conditions indicate the existence of two different-size *KGD2* transcripts. The synthesis of both RNAs is strongly catabolite repressed, as evidenced by their virtually complete absence in poly(A)$^+$ RNA prepared from cells grown in high concentrations of glucose. The effect of carbon source on *KGD2* transcription was confirmed by *lacZ* fusion assays. Fusions containing 322 bp to 1 kb of sequences from the upstream of region of *KGD2* expressed 8 to 15 times more β-galactosidase activity in derepressed than in glucose-repressed yeast cells. Comparable magnitudes of catabolite repression have been reported for *KGD1* (38) and *LPD1* (41). The lower KGDC activity in glucose-grown cells (33) therefore reflects inhibition of transcription of the genes for all three components of the complex.

The regulated transcription of *KGD2* depends on sequences located between the *Bam*HI site at −244 and the *Sau*3A site at −484. On the basis of the still somewhat crude dissection of the 5′-flanking region, two functionally distinct elements of the promoter are discerned. The region between −322 and −244, which also includes one of the transcription initiation sites, is sufficient for optimal regulation by glucose in wild-type yeast cells. The fusion lacking sequences upstream of −322 (pG104/Z4), however, allows efficient transcription even in a *hap2* or *hap3* mutant background. Thus, removal of the more upstream element leads to a partial loss of HAP2- and HAP3-dependent transcription. A similar loss of HAP2- but not glucose-regulated transcription has been noted in deletions of sequences upstream of the *COX6* gene (46).

The shortest construct exhibiting both full repression of transcription by glucose and HAP2 and HAP3 dependence has an additional 160 nucleotides upstream of the *Hae*III site at −322. This region contains two eight-nucleotide-long sequences, with one deviation from the consensus sequence reported to be involved in the binding of the HAP activation complex (10, 11). Both of these putative binding sites lie upstream of the two transcriptional initiation sites. Whether both or only one of the sequences is required for transcription of *KGD2* will require a finer molecular dissection of the region.

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