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Effects of Null Mutations in the Hexokinase Genes of *Saccharomyces cerevisiae* on Catabolite Repression

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***Saccharomyces cerevisiae* has two homologous hexokinases, I and II; they are 78% identical at the amino acid level. Either enzyme allows yeast cells to ferment fructose. Mutant strains without any hexokinase can still grow on glucose by using a third enzyme, glucokinase. Hexokinase II has been implicated in the control of catabolite repression in yeasts. We constructed null mutations in both hexokinase genes, *HXK1* and *HXK2*, and studied their effect on the fermentation of fructose and on catabolite repression of three different genes in yeasts: *SUC2*, *CYC1*, and *GAL10*. The results indicate that *hxk1* or *hxk2* single null mutants can ferment fructose but that *hxk1 hxk2* double mutants cannot. The *hxk2* single mutant, as well as the double mutant, failed to show catabolite repression in all three systems, while the *hxk1* null mutation had little or no effect on catabolite repression.**

In the budding yeast *Saccharomyces cerevisiae*, there are two homologous but nonidentical isoenzymes of the glycolytic enzyme hexokinase: hexokinase I and II, encoded respectively by the genes *HXK1* and *HXK2*. These are the only enzymes that phosphorylate fructose in *S. cerevisiae*. Maitra and colleagues (31) found that strains with point mutations in both the *HXK1* and *HXK2* genes failed to use fructose as a carbon source. These mutants can still grow on glucose presumably by using the glucose-specific enzyme glucokinase, specified by the *GLK1* gene (35).

Catabolite repression, or glucose repression, is a global regulation of the genes controlling the metabolism of many carbon sources. Cells grown on glucose generally express lower levels of the enzymes for metabolizing other carbon sources than when they are grown on poorer, "nonrepressing" carbon sources such as glycerol or lactate. This phenomenon has been observed in many microorganisms, including *Escherichia coli* (See reviews in references 15, 33, and 34) and *S. cerevisiae* (e.g., see references 8, 9, 13, 14, 18, 25, 38, 39, and references therein). Hexokinase II, in addition to its glycolytic activity, has been implicated in the control of catabolite repression in *S. cerevisiae*. Work in other laboratories has found that point mutations in the *HXK2* gene are able to relieve catabolite repression (17–19, 21).

Both of the *HXK1* and *HXK2* genes have been cloned independently by other groups (20, 22, 60) and in this laboratory (55), and the sequences of the two genes are now available (23, 29, 55). The availability of the cloned genes allows the use of powerful molecular and genetic manipulations (4) including in vitro mutagenesis (6, 52, 53), which we have used to generate a large number of mutants. Here we report the phenotypic characterizations of the in vitro-generated null mutations of the *HXK1* and *HXK2* genes, after they are introduced back into the yeast genome. We found that the *hxk1 hxk2* double null strain, as expected, failed to ferment fructose. Our results also indicate that the null phenotype of the *HXK1* gene is not much different from that of wild type but that the null mutants of the *HXK2* gene fail to show catabolite repression in all three systems that were examined: the *SUC2* gene (7, 8, 10) encoding invertase, the *CYC1* gene (25) of iso-1-cytochrome *c*, and *GAL10* (16), the gene specifying epimerase, one of the galactose-metabolizing enzymes.

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MATERIALS AND METHODS

Strains and plasmids. The yeast strains used in this study, their genotypes, and their sources are listed in Table 1. Subclones of the *HXK1* and *HXK2* loci were made for various purposes by standard procedures (36). A 6.2-kilobase (kb) *Cla*I-*Bam*HI fragment of the *HXK1* locus was inserted into the *Cla*I-*Bam*HI sites of pBR322 (3) to give pJJ101 (Fig. 1A). A 3.7-kb *Eco*RI fragment of the *HXK2* locus (23, 55) was subcloned into the *Eco*RI site of pRB290 (YIp5 [5] without its *Hin*dIII site [50]) to give pRB309 (Fig. 1B). The same *Eco*RI fragment was also inserted into the *Eco*RI site of YCp50 (C. Mann, personal communication; see reference 30 for map) to give a yeast centromere plasmid carrying the *HXK2* gene (pRB313).

Construction of null mutations. The in vitro manipulations were done by standard procedures as described by Maniatis et al. (36). A 2.3-kb *Sal*I-*Xho*I *LEU2* fragment (1) was inserted into a *Sal*I site in the coding region of the *HXK1* gene (29, 55). The transplacement to introduce the *hxk1*::*LEU2*$^+$ allele onto the chromosome was performed essentially as previously described (49) by selecting for leucine prototrophy in a *leu2-3,2-112* background (DBY1315). Three null mutations of the *HXK2* gene were constructed in pRB309, a derivative of the integrating vector YIp5 (5) with the *URA3* gene (47, 48), by deleting various extents of the *HXK2* gene (Fig. 1B). Plasmids carrying these null mutations of the *HXK2* gene were then integrated at the *HXK2* locus as previously described (44) selecting for uracil prototrophy in a *ura3-52* and *hxk1*::*LEU2*$^+$ background. Subsequently, the Ura$^-$ segregants were selected on medium containing 5-fluoroorotic acid, and the *hxk1 hxk2* double mutants were screened for the inability to ferment fructose. The single mutants with different *hxk2* alleles were then obtained by transformation with a wild-type *HXK1* fragment under selection for fructose fermentation.

The *gal80*::*HIS3*$^+$ disruption was constructed by replacing a 0.56-kb internal *Bgl*II fragment of the *GAL80* gene in clone pRY176 (62) with the 1.8-kb *Bam*HI fragment containing the *HIS3* gene (58). The transplacement (49) of *gal80*::*HIS3*$^+$

TABLE 1. Strains used in this study

Strain	Genotype[a]	Source
DBY1315	α *ura3-52 leu2-3,2-112 lys2-801 gal2*	This laboratory
DBY2052	α *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 leu2-3,2-112 lys2-801 gal2*	This work
DBY2053	α *hxk1*::*LEU2*$^+$ *ura3-52 leu2-3,2-112 lys2-801 gal2*	This work
DBY2184	α *hxk2-202 ura3-52 leu2-3,2-112 lys2-801 gal2*	This work
DBY2219	**a** *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 lys2-801 gal10-120 LEU2*$^+$::(pJP100, *CYC1-lacZ*$^+$)::*leu2-3,2-112*[b]	This work
DBY2221	**a** *hxk1*::*LEU2*$^+$ *ura3-52 lys2-801 gal10-120 LEU2*$^+$::(pJP100, *CYC1-lacZ*$^+$)::*leu2-3,2-112*[b]	This work
DBY2226	α *hxk2-202 ura3-52 lys2-801 gal10-120 LEU2*$^+$::(pJP100, *CYC1-lacZ*$^+$)::*leu2-3,2-112*[b]	This work
DBY2228	α *ura3-52 lys2-801 gal10-120 LEU2*$^+$::(pJP100, *CYC1-lacZ*$^+$)::*leu2-3,2-112*[b]	This work
DBY2311	**a** *ura3-52 lys2-801 his3-200 ade2-101 tyr1-501 gal80*::*HIS3*$^+$ *GAL*$^+$ *LEU2*$^+$::(pRY183, *GAL10-lacZ*$^+$)::*leu2-3,2-112*[c]	This work
DBY2312	α *hxk1*::*LEU2*$^+$ *ura3-52 lys2-801 his3-200 gal80*::*HIS3*$^+$ *GAL*$^+$ *LEU2*$^+$::(pRY183, *GAL10-lacZ*$^+$)::*leu2-3,2-112*[c]	This work
DBY2313	α *hxk2-202 ura3-52 lys2-801 his3-200 tyr1-501 gal80*::*HIS3*$^+$ *GAL*$^+$ *LEU2*$^+$::(pRY183, *GAL10-lacZ*$^+$)::*leu2-3,2-112*[c]	This work
DBY2314	α *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 lys2-801 his3-200 GAL*$^+$ *LEU2*$^+$::(pRY183, *GAL10-lacZ*$^+$)::*leu2-3,2-112*[c]	This work
DBY2315	**a** *ura3-52 lys2-801 his3-200 ade2-101 GAL*$^+$ *Leu2*$^+$::(pRY183, *GAL10-lacZ*$^+$)::*leu2-3,2-112*[c]	This work
DBY2316	**a** *hxk2-202 ura3-52 lys2-801 his3-200 tyr1-501 GAL*$^+$ *LEU2*$^+$::(pRY183, *GAL10-lacZ*$^+$)::*leu2-3,2-112*[c]	This work
DBY2317	α *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 lys2-801 his3-200 tyr1-501 GAL*$^+$ *gal80*::*HIS3*$^+$ *LEU2*$^+$::(pRY183, *GAL10-lacZ*$^+$)::*leu2-3,2-112*[c]	This work
DBY2318	**a** *hxk1*::*LEU2*$^+$ *ura3-52 lys2-801 his3-200 GAL*$^+$ *LEU2*$^+$::(pRY183, *GAL10-lacZ*$^+$)::*leu2-3,2-112*[c]	This work
DBY2319	α *ura3-52 lys2-801 leu2-3,2-112*; pRB194::(2μm, *URA3*$^+$ *HXK2-lacZ*$^+$)	This work
DBY2320	α *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 lys2-801 gal10-120*; pRB522::(CEN4, *URA3*$^+$ *hxk2-522*[d])	This work
DBY2321	α *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 lys2-801 gal10-120*; pRB313::(CEN4, *URA3*$^+$ *HXK2*$^+$)	This work
DBY2322	α *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 lys2-801 gal10-120 his3-200 leu2-3,2-112 gal80*::*HIS3*$^+$; pRB138::(2μm, *URA3*$^+$ *HXK2*$^+$)[e]	This work
DBY2323	α *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 lys2-801 gal10-120 his3-200 leu2-3,2-112 gal80*::*HIS3*$^+$; pRB141::(2μm, *URA3*$^+$ *HXK1*$^+$)[e]	This work
DBY2324	α *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 lys2-801 gal10-120 his3-200 leu2-3,2-112 gal80*::*HIS3*$^+$; pRB142::(2μm, *URA3*$^+$ *HXK1*$^+$)[e]	This work
DBY2325	α *hxk1*::*LEU2*$^+$ *hxk2-202 ura3-52 lys2-801 gal10-120 his3-200 leu2-3,2-112 gal80*::*HIS3*$^+$; pRB143::(2μm, *URA3*$^+$ *HXK1*$^+$)[e]	This work

[a] These strains are isogenic to S288C (*MAT*α *SUC2*$^+$ *gal2*). See references for the origins of the following alleles: *GAL2*$^+$ and *lys2-801* (8), *ura3-52* and *ade2-101* (9). The sources of the other alleles are: *leu2-3,2-112* from G. Fink; *his3-200*, a deletion made by M. Fasullo and P. Hieter (56; see map in reference 57); and *gal10-120* (28) and *tyr1-501* from M. Johnston. The *gal80*::*HIS3*$^+$ disruption was constructed for this study (see text for details) with a *GAL80* DNA clone (43, 62) generously provided by R. Yocum.

[b] These have the plasmid pJP100 (45) carrying a *CYC1-lacZ*$^+$ fusion (from J. Pinkham and L. Guarente) integrated at the *LEU2* locus. The order of the two alleles of the *LEU2* gene is not known.

[c] These have the plasmid pRY183 (61) carrying the *GAL10-lacZ*$^+$ fusion (kindly provided by M. Lamphier and M. Ptashne) integrated at the *LEU2* locus. The order of the two alleles of the *LEU2* gene is not known.

[d] This is a mutation isolated on pRB313; it behaves like *hxk2* null mutations in catabolite repression.

[e] The *HXK1* DNA clones pRB141 and pRB142 (55) and pRB143 and an *HXK2* DNA clone, pRB138 (coisolate of pRB62; see reference 55), are YEp24 (5) derivatives isolated by J. Swan in this laboratory from a yeast library (7) containing inserts of a partial *Sau*3A digest of DNA from the yeast strain S288C into the *Bam*HI site of YEp24.

was done by selecting for histidine prototrophy in a *his3-200* background.

DNA transformations of yeast cells were performed by the lithium acetate method described by Ito et al. (27), with the modifications of Kuo and Campbell (30).

Enzyme assays. For various enzyme assays, cells were harvested in the mid-exponential phase (Klett 50 to 70). Hexokinase assays were performed as previously described (2) with crude extract from cells grown in YEP–5% glucose medium (51), and the protein content was determined by the Lowry method (32). Invertase assays were carried out as before (24) on cells grown on either repressing media containing 5% glucose or derepressing media containing 2% lactate, as were β-galactosidase assays (40) for strains with the *CYC1-lacZ*$^+$ fusion. For β-galactosidase assays of strains with the *GAL10-lacZ*$^+$ fusion, cells were grown in repressing media containing 5% glucose or 5% glucose plus 2% galactose or in derepressing medium containing 2% galactose.

Genetic methods. Standard genetic procedures of crossing, sporulation, and tetrad analysis were followed (41, 51). For selecting the Ura$^+$ transformants, the yeast synthetic medium SD (51) was supplemented with 0.2% Casamino Acids (Difco Laboratories, Detroit, Mich.) or 0.2% of a mixture of equal amounts of all amino acids plus adenine. Scoring for fructose fermentation was carried out under anaerobic conditions in a GasPak disposable anaerobic system (BBL Microbiology Systems, Cockeysville, Md.).

DNA manipulations. DNA gel transfer (36, 54) and hybridization (46, 59) experiments were performed as previously described. Yeast DNA was isolated by the small-scale yeast DNA preparation precedure described by Holm et al. (26).

RESULTS

Construction of null mutations. Null mutations in the *HXK1* and *HXK2* genes were constructed in vitro as described above. Figure 1A shows the plasmid pJJ101, which contains the *HXK1* gene on a 6.2-kb *Cla*I-*Bam*HI fragment, as well as the insertion mutation (the 2.3-kb *LEU2* fragment

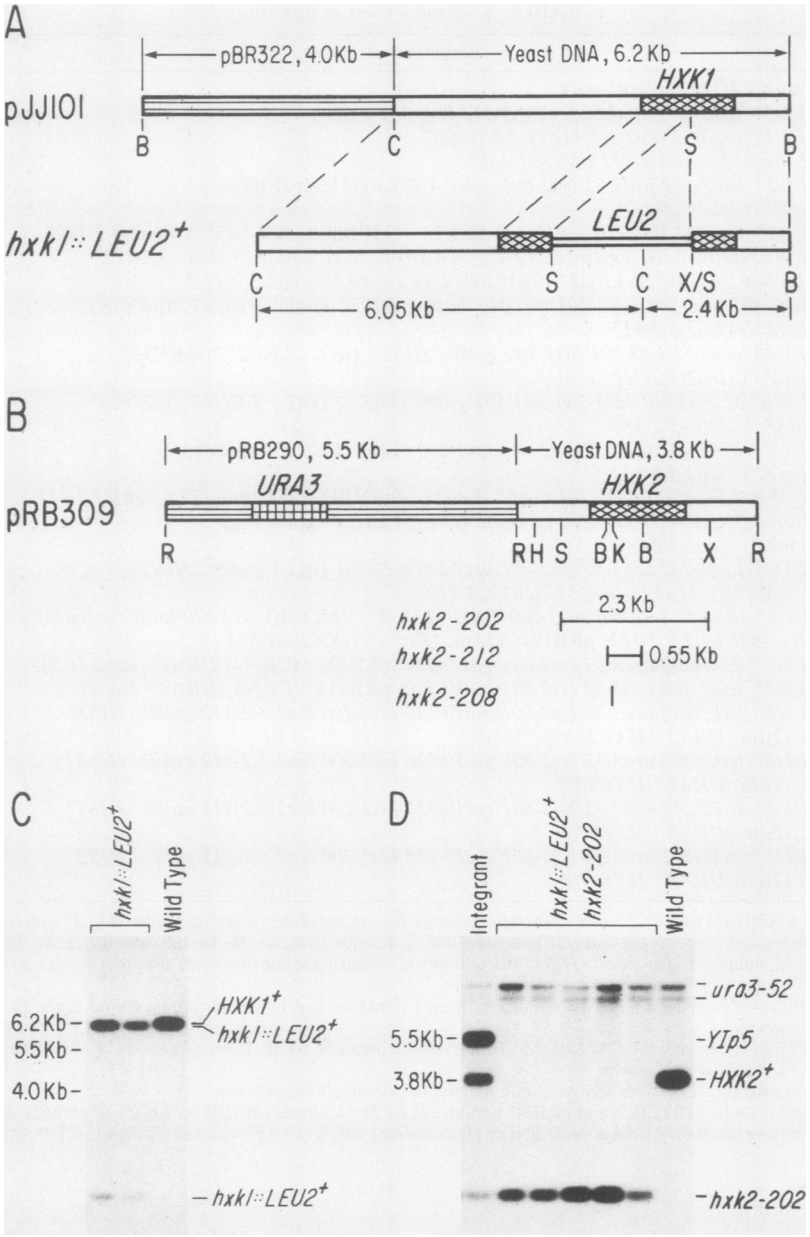

FIG. 1. (A) *HXK1* clone and null mutation *hxk1*::*LEU2*$^+$. Restriction enzymes: B, *Bam*HI; C, *Cla*I; S, *Sal*I; X, *Xho*I. (B) *HXK2* clone and deletions. Restriction enzymes: B, *Bal*I; H, *Hin*dIII; K, *Kpn*I; R, *Eco*RI; S, *Sac*I; X, *Xba*I. The large deletion *hxk2-202* is from the *Sac*I site to the *Xba*I site, and the small internal deletion *hxk2-212* is between the *Bal*I sites. The third mutation *hxk2-208* is a frameshift mutation from a 4-base-pair deletion at the *Kpn*I site. (C) Autoradiogram of DNA hybridization with radioactive pJJ101. Yeast DNA was isolated from strains containing either the *hxk1*::*LEU2*$^+$ allele or the wild-type allele of the *HXK1* gene and was digested with *Cla*I and *Bam*HI. (D) Autoradiogram of DNA hybridization with radioactive pRB309. Yeast DNA was isolated from the integrant containing the *hxk1*::*LEU2*$^+$ allele and a duplication of the *HXK2*$^+$ and *hxk2-202* alleles with YIp5 sequence in between, or from strains containing mutant alleles *hxk1*::*LEU2*$^+$ and *hxk2-202*, or from the wild-type strain. Restriction digests were done with *Eco*RI.

into the *Sal*I site). The insertion mutation in the *HXK1* gene was then introduced onto the chromosome as described in the Materials and Methods. Several null mutations of the *HXK2* gene were constructed by different means in the YIp5 derivative pRB309 containing a 3.7-kb *Eco*RI fragment carrying the *HXK2* gene (23, 55). The *HXK2*-bearing plasmid pRB309, the deletions *hxk2-202* and *hxk2-212*, and the frameshift mutation *hxk2-208* are shown in Fig. 1B. These null mutations were introduced back into the yeast genome by integration directed at the *HXK2* locus followed by excision of the plasmid and loss of the wild-type allele. DNA gel transfer and hybridization experiments were performed, and the results showed the expected hybridization patterns for the null mutations (Fig. 1C and D).

Growth on fructose and hexokinase activities. In yeasts, the hexokinases are the only two enzymes that phosphorylate fructose, thus allowing fructose to enter glycolysis and to be used as a carbon and energy source. The null mutants showed the expected phenotypes for growth on media containing fructose as the carbon and energy source: the

TABLE 2. Expression of *SUC2* and *CYC1*

Strain	Genotype[a]		Hexokinase activity[b]		*SUC2* invertase activity[c]		*CYC1-lacZ*$^+$ β-galactosidase activity[d]	
	HXK1	*HXK2*	Glu[e]	Fruct[e]	Repressed	Derepressed	Repressed	Derepressed
DBY2228	+	+	80	86	0.3	110	0.2	55
DBY2221	−	+	85	85	0.8	130	0.6	45
DBY2226	+	−	24	60	75	50	16	65
DBY2219	−	−	8	2	135	55	30	50

[a] *hxk1* = *hxk1::LEU2*$^+$; *hxk2* = *hxk2-202*.
[b] 1 unit = 1 μmol of glucose or fructose phosphorylated/min per g of protein.
[c] 1 unit = 1 μg of glucose produced/min per 1.0 ml of cells of 1.0 optical density unit at 600 nm (OD_{600}).
[d] 1 unit = 1,000 × OD_{420}/min per 1.0 ml of cells of 1.0 OD_{600}.
[e] These indicate substrates of hexokinase; Glu, glucose; Fruct, fructose.

single *hxk1* or *hxk2* null mutants grew, as did the wild type, so either one of the hexokinases is enough to provide the phosphorylating activity for growth on fructose; on the other hand, the *hxk1* and *hxk2* double mutant did not grow on fructose.

Hexokinase assays were performed with the wild type, the single mutants, and the double mutant, and the activities were consistent with the genotypes (Table 2).

Effect of *HXK* null mutations on catabolite repression of *SUC2*. To examine the possible effects of the various null mutations of the *HXK1* and *HXK2* genes on the expression of *SUC2*, a catabolite repression-sensitive gene (7–9), we assayed the specific activity of invertase, the product of the *SUC2* gene, in the wild type and the various *hxk* mutant strains under repressing and derepressing conditions. We found that under the repressing condition of high glucose the *hxk2* single mutant (strain DBY2226) and the *hxk1 hxk2* double mutant (DBY2219) had high levels of invertase activity, while the *hxk1* single mutant and the wild type had very low levels of invertase activity (Table 2). Thus, the *hxk2* null phenotype is apparently complete loss of catabolite repression.

The small variations in the constitutive invertase level of strains DBY2226 and DBY2219 are not related to catabolite repression but instead are the consequence of high osmolarity of the 5% glucose medium. In control experiments (data not shown), addition of sorbitol to 5% eliminated these differences.

The strains DBY2226 and DBY2219 carry a large deletion of the *HXK2* gene that extends well beyond the ends of the coding sequence. This raised the possibility that neighboring gene(s) might be affected. To test whether the failure to repress invertase expression in high-glucose medium is due solely to the absence of the *HXK2* function, we tested the invertase activity of *hxk2* mutants carrying internal null alleles *hxk2-208* and *hxk2-212* (Fig. 1B). The results again indicate that removal of the *HXK2* gene alone is sufficient to allow constitutive expression of the *SUC2* gene (data not shown).

Effect of multicopy *HXK1* in the absence of *HXK2* on catabolite repression. Because the specific activity of hexokinase I on glucose is lower than that of hexokinase II (Table 2), the difference in their effects on catabolite repression could be due to the different levels of specific activity. To test this hypothesis, we examined the effect of the *HXK1* gene on a multicopy plasmid. An *hxk1 hxk2 ura3-52* strain was transformed with YEp24 (5) derivatives containing the *HXK1* gene (pRB141, pRB142, or pRB143), a YEp24 derivative containing the *HXK2* gene (pRB138), or a YCp50 derivative containing the *HXK2* gene (pRB313), selecting for uracil prototrophy. All these transformants were able to grow on fructose, as expected. The transformants with the *HXK2* gene on either YEp24 or YCp50 had normal catabolite repression. The YEp24 derivatives carrying the *HXK1* gene, however, gave only partial catabolite repression (Table 3), even though the specific activity of hexokinase I in the cells on glucose now is comparable to that of single-copy hexokinase II.

Effect of *HXK* null mutations on catabolite repression of *CYC1*. We examined the effects of the null mutations on the expression of another catabolite repression-sensitive gene, *CYC1* (25). A *CYC1-lacZ*$^+$ fusion (containing UAS2 only [45]) was integrated at the *LEU2* locus and subsequently put into different *HXK1* and *HXK2* backgrounds by crosses. The UAS2 in this fusion contains a mutation (UP1 [25]) which increases both the repressed and derepressed expression by 5- to 10-fold but does not affect catabolite repression or other aspects of the regulation of the *CYC1* gene expression (25). The expression of the *CYC1* gene was monitored by assaying for β-galactosidase activity in different strains. The results were similar to those for the *SUC2* gene (Table 2).

Expression of *GAL10* in different *HXK* backgrounds. The *SUC2* and *CYC1* genes are similar in that they do not require the function of an external inducer for expression. To test whether hexokinase II is involved in the catabolite repression of a gene requiring an external inducer for expression, we examined the expression of the *GAL10* gene (16) using a *GAL10-lacZ*$^+$ fusion (61). If the cells were *gal80*, the pattern of the *GAL10-lacZ*$^+$ fusion expression paralleled those of the *SUC2* and *CYC1* genes (Table 4). The elimination of the *HXK2* function alone resulted in enzyme levels being con-

TABLE 3. Effect of *HXK1* on multicopy plasmid

Strain	Genotype		Hexokinase activity[a]		Invertase activity[b] (repressed)
	Chromosome	Plasmid	Glu[c]	Fruct[c]	
DBY2228	*HXK1*$^+$ *HXK2*$^+$	None	80	86	0.3
DBY2319	*HXK1*$^+$ *HXK2*$^+$	2μm(*hxk2*)	60	80	0.4
DBY2321	*hxk1 hxk2*	CEN(*HXK2*$^+$)	NT[d]	NT[d]	2.5
DBY2322	*hxk1 hxk2*	2μm(*HXK2*$^+$)	920	1,000	2.3
DBY2323	*hxk1 hxk2*	2μm(*HXK1*$^+$)	94	260	21
DBY2324	*hxk1 hxk2*	2μm(*HXK1*$^+$)	77	210	27
DBY2325	*hxk1 hxk2*	2μm(*HXK1*$^+$)	86	277	26
DBY2320	*hxk1 hxk2*	CEN(*hxk2*)	NT	NT	220

[a] 1 unit = 1 μmol of glucose or fructose phosphorylated/min per g of protein.
[b] 1 unit = 1 μg of glucose produced/min per 1.0 ml of cells of 1.0 OD_{600}.
[c] These indicate the substrates of hexokinase; Glu, glucose; Fruct, fructose.
[d] NT, Not tested.

TABLE 4. Expression of *GAL10-lacZ*$^+$ fusion

Strain	Genotype[a]			β-Galactosidase activity[b]			
	HXK1	*HXK2*	*GAL80*	Glu → Glu + Gal		Gal → Glu + Gal	
DBY2311	+	+	−	2	2.1	127	2.1
DBY2312	−	+	−	4.5	4.5	186	4.5
DBY2313	+	−	−	100	105	165	105
DBY2317	−	−	−	160	165	182	165
DBY2315	+	+	+	<0.01	0.1	80	0.2
DBY2318	−	+	+	<0.01	0.02	129	0.1
DBY2316	+	−	+	<0.01	6	127	9
DBY2314	−	−	+	<0.01	18	144	30

[a] *hxk1* = *hxk1*::*LEU2*$^+$; *hxk2* = *hxk2-202*; and *gal80* = *gal80*::*HIS3*$^+$.
[b] 1 unit = 1,000 × OD_{420}/min per 1.0 ml of cells of 1.0 OD_{600}. The arrows indicate shifts of carbon sources in the medium. Glu, Glucose; Gal, galactose.

stitutively high, comparable to the wild-type fully derepressed level (on galactose). This is true whether the cells were induced on galactose first or not. Furthermore, the presence of galactose in the repressing medium makes no difference.

However, if the cells were *GAL80*$^+$, the levels of expression were below detection in the absence of the inducer galactose. Even in the presence of galactose, cells had much lower levels in high glucose than the corresponding *gal80* strains of the *HXK* genotype; even the *hxk1 hxk2* double mutant had a level much lower than the wild-type derepressed level (Table 4), in contrast to the *SUC2* and *CYC1* genes (Table 2). These observations are consistent with an effect of glucose on galactose uptake, as described below.

DISCUSSION

Previous results on the function of the hexokinases in catabolite repression have come from studies with point mutations (17–19, 21). Entian and colleagues (17) have isolated mutations in the *HXK2* gene causing low hexokinase activity and found them to lead to failure of catabolite repression. They have also isolated mutations both in the *HXK2* gene and in a second gene *HEX2* which lead to high hexokinase activity and lack of catabolite repression (18, 19). Mutations of the first kind could be loss of function mutations but need not be so; those of the second kind do not lead to loss of the hexokinase activity. It is therefore not clear whether the lack of catabolite repression is due to loss of function or altered function. This makes the interpretation of the results difficult, especially in terms of determining whether hexokinase II acts as a regulator in a positive or negative fashion. Here, we found that one of the null phenotypes of the *HXK2* gene is the failure of catabolite repression; loss of hexokinase II alone caused a 100-fold increase of the expression of both the *SUC2* and *CYC1* genes under repressing condition. That the null phenotype of the *HXK2* gene is constitutive expression of the *SUC2* and *CYC1* gene argues that hexokinase II acts in a negative fashion in catabolite repression. In contrast, the effect of loss of the *HXK1* function on catabolite repression was insignificant.

Nevertheless, the results with the *HXK1* gene on multicopy plasmids indicate that hexokinase I has the potential of conferring partial catabolite repression. The observation that the restorative effect of the multicopy *HXK1* gene is smaller than that of the single-copy *HXK2* gene suggests that there is some qualitative difference between the two hexokinases. Models have been proposed (19) hypothesizing that hexokinase I and II are sufficiently different from each other so that hexokinase II is the only one of the two that is involved in catabolite repression. Our results are consistent with those models because the normal single copy of the *HXK1* gene has little or no effect on catabolite repression. On the basis of the presence of mutations in the *HXK2* gene that cause a defect in catabolite repression but retain high hexokinase activity, Entian and Frohlich (19) proposed that hexokinase II is a bifunctional protein with separate domains for catalytic and regulatory activities. Our results here that null mutations in the *HXK2* gene cause loss of both catabolite repression and hexokinase activity of hexokinase II do not contradict such a model; however, they do not rule out other explanations either. Our preliminary results with point mutations of the *HXK2* gene show that catabolite repression-defective mutations with high hexokinase activity do not cluster to one part of the gene (data not shown). These results do not support the two-domain model.

The effect of the hexokinases on the expression of the *GAL10* gene seems to be somewhat more complicated at first glance. The results for the *GAL10-lacZ*$^+$ fusion in the absence of the *GAL80* function are very similar to those found with the *SUC2* and *CYC1* genes. However, when the *GAL80* function was present, the results were quite different. The *GAL80* gene product acts as an inhibitor of the *GAL4* protein in the absence of the inducer galactose (16). Matern and Holzer (37) found that glucose has a strong inhibitory effect on the function of the galactose permease encoded by the *GAL2* gene. This could explain why the *hxk1 hxk2 GAL80*$^+$ strain has activity in medium containing glucose and galactose that is 20-fold lower than the fully derepressed level. The high glucose inhibits the permease, preventing enough galactose from entering the cells to counter the inhibitory effect of the functional *GAL80* protein that is present. Matsumuto et al. (38, 39) have identified three genes that affect the catabolite repression of the galactose genes, *GAL82*, *GAL83*, and *REG1*. They found the *GAL82* and *GAL83* functions to be in the same pathway and the *REG1* function to be in a separate pathway also affecting the *SUC2* gene. In the absence of the *GAL80* function,

TABLE 5. Comparison of effect of *HXK2* and *GAL82*, *GAL83* and *REG1*

Gene examined	Genotype	Expression of galactose genes (% of wild-type derepressed level) on[a]:		
		Glu	Glu + Gal	Gal
GAL1	*GAL80*$^+$ *GAL82*$^+$ *REG1*$^+$	<0.1	<0.1	100
	GAL80$^+$ *gal82 REG1*$^+$	0.1	0.1	89
	GAL80$^+$ *GAL82*$^+$ *reg1*	<0.1	<0.1	88
	GAL80$^+$ *gal82 reg1*	<0.1	11	88
	gal80 GAL82$^+$ *REG1*$^+$	4	6	96
	gal80 gal82 REG1$^+$	38	28	81
	gal80 GAL82$^+$ *reg1*	40	46	77
	gal80 gal82 reg1	80	81	78
GAL10	*GAL80*$^+$ *HXK2*$^+$	<0.1	0.2	100
	GAL80$^+$ *hxk2*	<0.1	11	160
	gal80 HXK2$^+$	2.5	2.5	160
	gal80 hxk2	130	130	200

[a] The results for the *GAL1* gene are calculated from the data in Table 3 of Matsumoto et al. (39) by normalizing to the wild-type derepressed level; the results for the *GAL10* gene are calculated from data in Table 4 of this study in the same way. Glu, Glucose; gal, galactose.

recessive mutations in either of the two pathways resulted in partial derepression (Table 5), and combination of mutations in both pathways gave full derepression. In the presence of the *GAL80* function, however, mutations in either or both of the pathways had much smaller effects (Table 5). The combination of mutations in both the *GAL82*, *GAL83* pathway and the *REG1* pathway showed results remarkably similar to those of the *hxk2* mutant (Table 5).

In this study, we examined the effects of null mutations of the *HXK1* and *HXK2* genes on three different genes: *SUC2*, *CYC1*, and *GAL10*. Invertase is the enzyme that hydrolyzes sucrose, while cytochrome *c*, the product of the *CYC1* gene, is involved in the electron transport chain of respiration. The *GAL10* gene product is the third enzyme of galactose metabolism, epimerase. Furthermore, the expression of the *SUC2* and *CYC1* genes does not require an external inducer, but the expression of the third gene, *GAL10*, does need the function of the inducer galactose. Our results indicate that hexokinase II is required for the catabolite repression of all three genes. Moreover, mutation in *SNF1* (11, 12), a gene affecting the catabolite repression of several systems including the *SUC2* gene, the galactose genes, and the maltose genes (8), was found to be epistatic to a recessive *hxk2* mutation (42). This argues that hexokinase II functions earlier than the *SNF1* gene product in the regulatory pathway(s) of catabolite repression. The striking similarity of the effects on the galactose genes between our *hxk2* null mutation and the combination of *gal82* (*gal83*) and *reg1* mutations again supports the notion that hexokinase II is common to all these pathways and that it functions at a stage before pathways branch for different systems. Because hexokinase is intimately involved in the early metabolism of glucose, it is most plausible that hexokinase II is involved in the earliest steps of catabolite repression, the assessment of available metabolizable carbon source.

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