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Saccharomyces cerevisiae Mutants Provide Evidence of Hexokinase PII as a Bifunctional Enzyme with Catalytic and Regulatory Domains for Triggering Carbon Catabolite Repression†

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A selection system has been devised for isolating hexokinase PII structural gene mutants that cause defects in carbon catabolite repression, but retain normal catalytic activity. We used diploid parental strains with homozygotic defects in the hexokinase PI structural gene and with only one functional hexokinase PII allele. Of 3,000 colonies tested, 35 mutants (*hex1*^{r}) did not repress the synthesis of invertase, maltase, malate dehydrogenase, and respiratory enzymes. These mutants had additional hexokinase PII activity. In contrast to *hex1* mutants (Entian et al., Mol. Gen. Genet. **156**:99–105, 1977; F. K. Zimmermann and I. Scheel, Mol. Gen. Genet. **154**:75–82, 1977), which were allelic to structural gene mutants of hexokinase PII and had no catalytic activity (K.-D. Entian, Mol. Gen. Gent. **178**:633–637, 1980), the *hex1*^{r} mutants sporulated hardly at all or formed aberrant cells. Those ascospores obtained were mostly inviable. As the few viable *hex1*^{r} segregants were sterile, triploid cells were constructed to demonstrate allelism between *hex1*^{r} mutants and hexokinase PII structural gene mutants. Metabolite concentrations, growth rate, and ethanol production were the same in *hex1*^{r} mutants and their corresponding wild-type strains. Recombination of hexokinase and glucokinase alleles gave strains with different specific activities. The defect in carbon catabolite repression was strongly associated with the defect in hexokinase PII and was independent of the glucose phosphorylating capacity. Hence, a secondary effect caused by reduced hexose phosphorylation was not responsible for the repression defect in *hex1* mutants. These results, and those with the *hex1*^{r} mutants isolated, strongly supported our earlier hypothesis that hexokinase PII is a bifunctional enzyme with (i) catalytic activity and (ii) a regulatory component triggering carbon catabolite repression (Entian, Mol. Gen. Genet. 178:633–637, 1980; K.-D. Entian and D. Mecke, J. Biol. Chem. **257**:870–874, 1982).

The yeast *Saccharomyces cerevisiae* is valuable for elucidating the complex systems regulating gene activity in eucaryotic organisms. Cells can be easily cultivated and are accessible to genetic manipulation. Carbon catabolite repression (glucose repression) describes a phenomenon that adapts the carbon metabolic machinery of *S. cerevisiae* to the utilization of the most convenient carbon source. That is, great differences in the activities of certain enzymes occur between cells growing on glucose and those on nonfermentable carbon sources such as ethanol or acetate. Such striking differences have been reported for the enzymes of the glyoxylate shunt (3), the tricarboxylic acid cycle (27), respiratory enzymes (28), gluconeogenic enzymes (20, 21, 31), alpha-glucosidases (30), and invertase (beta-fructofuranosidase) (22).

Genetic analysis of glucose repression has progressed well since Zimmermann and Scheel (36) developed a powerful selection system for isolating mutants resistant to carbon catabolite repression. Three classes of mutants, *hex1*, *hex2*, and *cat80* (wild-type designations *HEX1*, *HEX2*, and *CAT80*, respectively) with pleiotropic effects on glucose repression have been identified (16, 36).

In the *hex1* mutants, glucose does not repress invertase, maltase, malate dehydrogenase, or respiratory enzymes. Hexokinase activity is reduced to about two-thirds that of the wild type (18). These mutants are allelic to structural gene mutants of the hexokinase isoenzyme PII; another designation is hexokinase B (12, 15). There are two hexokinase isoenzymes in *S. cerevisiae* which phosphorylate glucose and fructose, called PI and PII, and a so-called glucokinase which phosphorylates glucose specifically (for review of hexokinases, see reference 10). Structural gene mutants of these isoenzymes were isolated, and genes were designated *HXK1*, *HXK2*, and *GLK1*, respectively (24). *HXK2* is allelic to *HEX1*, and gene designations are interchangeable (12, 15). Transformation of *S. cerevisiae* cells lacking hexokinase isoenzymes PI and PII with an isolated hexokinase PII gene also restores carbon catabolite repression (18a).

The second class of mutants have been assigned to gene *HEX2*, a centromere-linked locus close to the *TRP1* gene on chromosome IV (Zimmermann, unpublished data). These *hex2* mutants did not show repression of the synthesis of maltase, invertase, malate dehydrogenase, and respiratory enzymes. They had elevated hexokinase activity and were extremely sensitive to maltose (13). Increased hexokinase activity could be attributed to increased hexokinase PII synthesis in the presence of glucose in the medium (14). The *cat80* mutants had normal hexokinase activity and were not repressible for invertase, maltase, and malate dehydrogenase (16). However, a functional *CAT80* gene product is necessary for elevated hexokinase PII synthesis in *hex2* mutants (17).

Since the mutants so far described were unaffected in the repression of certain repressible enzymes, e.g., fructose-1,6-bisphosphatase, malate synthase, and isocitrate lyase, a branched regulatory system could be inferred. This dual regulatory system was also consistent with the finding of entirely different types of mutants, mainly affected in the repression of respiratory and mitochondrial enzymes (6, 9, 26).

The greater complexity of the genetic control of regulation

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† Dedicated to D. Mecke on the occasion of his 50th birthday.

TABLE 1. *S. cerevisiae* strains[a]

Strain	Genotype						
H.38-4B	**a** *leu1 his4*	*MAL2-8*^{c}	*MAL3*	*SUC3*	*HXK1*	*HEX1*	*GLK1*
308.14A-11C	**a** *trp1 his2 his4*				*HXK1*	*HEX1*	*GLK1*
hxk0.P1-2C	**a** *trp1 his2*		*MAL3*	*SUC3*	*HXK1*	*hex1*	*GLK1*
hxk.0-7C	α *trp1 his2 his4*	*MAL2-8*^{c}			*hxk1*	*hex1*	*GLK1*
hxk.0-3B	**a** *trp1 his2 his4*	*MAL2-8*^{c}	*MAL3*	*SUC3*	*hxk1*	*hex1*	*GLK1*
P1.2-3A	α *trp1 his4*	*MAL2-8*^{c}	*MAL3*	*SUC3*	*HXK1*	*hex1*	*GLK1*
P1.1-2B	**a** *trp1 leu1*	*MAL2-8*^{c}	*MAL3*	*SUC3*	*hxk1*	*HEX1*	*glk1*
D.308-2A	α *trp1*	*MAL2-8*^{c}	*MAL3*	*SUC3*	*hxk1*	*hex1*	*GLK1*
P1.6-2C	**a** *his4*	*MAL2-8*^{c}	*MAL3*	*SUC3*	*HXK1*	*hex1*	*GLK1*
D18.6	α/γ *ade1/ade1 trp1/TRP1 his2/HIS2 met14/MET14 MAL2-8*^{c}*/MAL2-8*^{c} *MAL3/MAL3 SUC3/SUC3 hxk1/hxk1 HEX1/hex1 GLK1/GLK1*						
D19.6	α/**a** *ade1/ade1 met14/met14 his2/HIS2 his4/HIS4 trp1/TRP1 mal2/MAL2-8*^{c} *MAL3/mal3 SUC3/mal3 hxk1/hxk1 HEX1/hex1 GLK1/GLK1*						

[a] **a** and α refer to mating type, and *his2*, *his4*, *leu*1, *trp1*, and *ade1* cause nutritional requirements for amino acids and adenine, respectively. *MAL2-8*^{c} causes largely constitutive, but still glucose-repressible, synthesis of maltase (34) and is closely linked to *SUC3*, the structural gene for invertase (23). *HXK1*, mutant allele *hxk1*, is the structural gene of hexokinase PI (another designation is hexokinase A) (24), and *HEX1*, mutant allele *hex1*, causes pleiotropic defects in carbon catabolite repression and is the structural gene of hexokinase PII (another designation is hexokinase B) (15). *HEX1* is allelic to gene *HXK2*, which was also identified as the structural gene for hexokinase PII (24).

became apparent when three different gene loci, *CAT1*, *CAT2*, and *CAT3*, were identified whose function was specifically involved in derepression after a shift to derepression conditions (17, 35). The *cat1* and *cat3* mutant alleles prevented derepression of enzymes of the glyoxylate shunt, gluconeogenesis, the maltose uptake system, and retarded derepression of maltase synthesis. The *cat2* mutant allele acted as a suppressor allele for the *cat1* mutant allele. Similar mutant alleles were described previously (8).

So far, carbon catabolite repression appears as a highly ramified system for regulating gene activity. Overlapping repression defects in different mutants indicated that the branches of the repression system are interconnected. Surprisingly, analysis of extragenic suppressor mutants of the *hex2* mutant allele demonstrated that mutant alleles *cat1* and *cat3* acted as epistatic suppressor alleles on all *hex2* mutant effects, such as defects in carbon catabolite repression, maltase inhibition, and increased hexokinase PII synthesis (17). Clearly, the repressing and derepressing genes are also interdependent.

All of our results have shown that hexokinase PII is either directly connected with the gene systems regulating carbon catabolite repression and derepression as indicated by *hex1* mutants, or indirectly; as shown by *hex2*, *cat80*, *cat1*, and *cat3* mutants. Since hexose phosphorylation is the first enzymatic step in the utilization of the fermentable substrates glucose, fructose, and mannose acting as repressing carbon sources, we have suggested that hexokinase PII is the recognition site of carbon catabolite repression giving the triggering signal (12, 15). This hypothesis requires that hexokinase PII is a bifunctional enzyme having (i) a catalytic domain for hexose phosphorylation and (ii) a regulatory domain giving the triggering signal for carbon catabolite repression. In the present investigation we report the isolation of mutants that were altered in the regulatory part of the hexokinase PII molecule, but retained catalytic activity. The behavior of these mutants gave strong support to our hypothesis.

MATERIALS AND METHODS

Strains. Strains were derived from strains originating from the Department of Genetics, University of Washington, Seattle, Wash., and from the Yeast Genetic Stock Center, Donner Laboratory, University of California, Berkeley, Calif. They are shown in Table 1.

Media. YEP medium consisting of 1% yeast extract, 2% Bacto-Peptone (both from Difco Laboratories) was used as the basic medium supplemented with 2% glucose, 2% fructose, 4% fructose, or 2% raffinose. If necessary, antimycin A was added to a final concentration of 2 ppm. For solid media 1.5% agar (Difco) was used. Selection media for the isolation of *hex1*^{r} mutants consisted of YEP–2% raffinose and 200 ppm (200 μg/ml) 2-deoxyglucose with or without antimycin A. Minimal medium contained 0.67% yeast nitrogen base (Difco), 2% glucose, and those substances needed, for the auxotrophic strains used, at concentrations given elsewhere (17a). Sporulation medium consisted of 1% potassium acetate and 1.5% agar.

Repression of enzymes was estimated in cells grown on YEP–4% glucose medium for more than 16 h. Only logarithmically growing cell suspensions were harvested. Crude extracts were prepared by shaking cells with glass beads (7). We added 2 ml of potassium phosphate buffer (pH 6.5) to the suspension before centrifuging it at 1,000 × *g* for 10 min if respiratory enzymes were to be tested and at 5,000 × *g* for all other enzymes. The supernatant was used as a crude extract.

Enzyme assays. Total alpha-glucosidases were tested with *p*-nitrophenyl-alpha-D-glucopyranoside (maltase [E.C. 3.2.1.20] and isomaltase [E.C. 3.2.1.10]), invertase (E.C. 3.2.1.26) was tested by incubation with 0.1 M sucrose as the substrate in 0.1 M potassium acetate (pH 4.5) for different periods at 30°C in a water bath. Reactions were stopped in a boiling water bath for 30 s. Free glucose was estimated with 0.6 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid (Sigma Chemical Co.; A-1888), 2 U of horseradish peroxidase (Boehringer Mannheim; 108 090), and 4 U of glucose oxidase (Boehringer Mannheim; 108 081). Reaction mixtures were incubated for 30 min at 30°C. Hexokinase activity was tested with glucose as substrate (4) or with 2.2% fructose. Malate dehydrogenase (E.C. 1.1.1.37) was assayed in glycine-hydrazine buffer, pH 9.5 (32)–fructose-1,6-bisphosphatase (E.C. 3.1.3.11) (19), malate synthase (E.C. 4.1.3.2) and isocitrate lyase (E.C. 4.1.3.1) (G. H. Dixon and H. L. Kornberg, Biochem. J. 72:3p, 1959), succinate dehydrogenase with cytochrome c as an electron acceptor (1), and NADH

dehydrogenase (11). Protein was estimated with bovine serum albumine as a standard by the microbiuret method (33). Absorption was measured at 290 nm. Specific activities of enzymes are expressed as nanomoles of substrate converted per minute per milligram of protein.

Sporulation was induced by transferring cells from a YEP–2% glucose medium in the early stationary phase to sporulation plates. Cells were centrifuged, and the pellet was transferred without intermediate washings. After 2 to 4 days at 30°C at least 70% of cells showed asci when diploid wild-type strains were used. Tetrad analysis was done as described previously (35).

Isolation of *hex1*^{r} mutants. Strains D18.6 and D19.6 were grown on YEP–2% fructose with or without 2 ppm of antimycin A giving a cell titer of 1×10^8 to 3×10^8/ml. Cells were washed twice with sterile distilled water, suspended in 1% ethylmethansulfonate (Merck-Schuchardt, Darmstadt; 820774) dissolved in 1.1 M potassium phosphate buffer (pH 6.5) and incubated for 1 h at 30°C. These conditions yielded about 50% viable cells when haploid strains were used and gave 99% survivals in the diploid strains D18.6 and D19.6. Mutagenization was stopped by washing twice with potassium phosphate buffer (pH 6.5). For fixation of the mutation and additional derepression of invertase in the required mutants, cells were incubated for 8 h in YEP–8% fructose medium at a cell titer of 5×10^8/ml, which is about two generation times before stationary phase. No derepression of invertase was observed in the wild type with these conditions. After this treatment cells were washed once with potassium phosphate buffer (pH 6.5) and plated on selection medium at a density of 10^7 per plate. Colonies appeared after 3 to 8 days at 30°C and were transferred to YEP–2% glucose agar. Each colony was tested for repression of invertase and maltase and for hexokinase activity with glucose and fructose as substrates.

Triploid cells were obtained after mating diploid strain 19.6 with two homozygotic auxotrophic mutations and a haploid strain lacking at least two complementary auxotrophies. Cells were mated on YEP–2% glucose agar and replica plated on synthetic minimal medium agar. Triploids occurred as a result of mating type homozygotic mutations in the diploid strain.

Metabolite concentrations (17a) and harvesting of cells and NADH determination (16) were as described previously.

For determining ethanol excretion into the medium, logarithmically growing cells were harvested by centrifugation (1 min at 5,000 × *g*; Christ Labofuge 6000), washed twice, and inoculated into new medium giving an optical density of 0.75 at 550 nm (about 10^6 cells per ml). After 4 h of growth cells were again centrifuged, and ethanol in the supernatant was estimated as described previously (5).

RESULTS

Selection of *hex1*^{r} mutants. The selection system was based on that of Zimmermann and Scheel (36). This system used the toxic agent 2-deoxy-D-glucose, which is taken up by the glucose carrier and phosphorylated by hexokinase and leads, even at low concentrations such as 150 ppm, to severe defects especially in the yeast cell wall (reviewed in reference 2). Glucose or fructose, if also available in the medium at concentrations of at least 5,000 ppm, abolish this toxic effect, mainly by competition at the glucose uptake carrier (K.-D. Entian, doctoral thesis, TH Darmstadt, Federal Republic of Germany, 1978). If cells have high invertase activity and hydrolyze raffinose effectively, yielding high concentrations of fructose, the effects of 2-deoxy-D-glucose can also be eliminated by raffinose. Since invertase is located in the cell wall, outside the plasmalemma, the effects of adding raffinose are like those of adding fructose. Additionally, invertase is a strongly repressible enzyme. After growth with glucose as carbon source, wild-type cells have no measurable invertase activity. When cells grown on glucose medium are plated on medium containing 2-deoxy-D-glucose and raffinose, they are unable to hydrolyze raffinose and consequently are inhibited by 2-deoxy-D-glucose. In contrast, mutants having high invertase activity can hydrolyze raffinose and thus surround the cell with free fructose, which counteracts the action of 2-deoxy-D-glucose. The mutants obtained are not 2-deoxy-D-glucose resistant; they are defective only in carbon catabolite repression. For the isolation of *hex1*^{r} mutants a special diploid strain was constructed. The diploid strains D18.6 and D19.6 carried homozygotic defects in the hexokinase PI structural gene (*hxk1*/*hxk1*) and were heterozygotic with respect to hexokinase isoenzyme PII alleles, having only one hexokinase PII allele in functional condition (*HEX1*/*hex1* or *HXK2*/*hxk2*). Further, the genes for glucokinase, which phosphorylates glucose specifically, were present in a homozygotic state (*GLK1*/*GLK1*). Since these strains had one *HEX1* allele, they could grow with fructose as carbon source. For selecting *hex1*^{r} mutants, these strains were used in the 2-deoxy-D-glucose–raffinose selection system. Since the cells were diploid, no recessive mutants should be obtained; that is to say, *hex2* and *cat80* mutants were unlikely to occur. Only the functional allele for hexokinase isoenzyme PII was haploid in the figurative sense. Since no dominant mutant alleles had been obtained so far after mutant selection with diploid cells (Entian, doctoral thesis), only a mutation in the functional *HEX1* allele could give rise to carbon catabolite repression mutants. However, those mutants were viable only if they could utilize the fructose liberated after raffinose hydrolysis. Hence, there was strong selection pressure for the isolation of mutants that were defective in carbon catabolite repression and had additional fructose phosphorylating activity. Such mutants, if they existed, would have been very rare, since only a small region on the hexokinase PII gene was accessible for mutation. In the first series of experiments nearly all colonies defective in carbon catabolite repression showed no hexokinase PII activity. Comparable experiments, after transformation of *S. cerevisiae* cells with an plasmid containing the hexokinase PII structural gene, showed that cells could derepress invertase even without any carbon source (18a). Degradation of reserve carbohydrates provided the energy necessary for derepression (unpublished observations), and mutants with defective hexokinase PII were protected from 2-deoxy-D-glucose uptake by raffinose hydrolysis. However cells could grow by oxidative degradation of substrates contained in the complete medium, so antimycin A, a respiratory inhibitor, was added to the selection media to minimize this growth. In a second series of experiments, about 100 colonies were isolated which had defects in carbon catabolite repression and had additional hexokinase activity; 35 were stable and could be used for further analysis. Of the enzymes tested in these mutants, invertase, maltase, malate dehydrogenase, and the respiratory enzymes succinate dehydrogenase and NADH dehydrogenase were not repressed (Table 2). Repression of malate synthase and fructose-1,6-bisphosphatase was as in the wild type. Thus, repression in these mutants was similar to that in *hex1* mutants. However, these new mutants retained catalytic hexokinase PII activity as shown by the *Q*

TABLE 2. Repression of maltase, invertase, malate dehydrogenase, and hexokinase activities after growth on YEP–2% glucose in original strain (D19.6), an *hex1* homozygote strain (D19.6 880), and *hex1*^{r} strains

Strain	Genotype	Sp act[a]					*Q F/G*[b]
		Maltase	Invertase	Malate dehydrogenase	Hexokinase		
					Glucose	Fructose	
D19.6	*hxk1/hxk1 HEX1/hex1*	2	25	121	261	402	1.54
D19.6 880	*hxk1/hxk1 hex1/hex1*	239	700	432	68	bd[c]	bd[c]
D18.6 59	*hxk1/hxk1 hex1*^{r}*/hex1*	388	1,044	321	353	455	1.29
D19.6 1663	*hxk1/hxk1 hex1*^{r}*/hex1*	72	542	195	272	422	1.55
D19.6 1568	*hxk1/hxk1 hex1*^{r}*/hex1*	468	698	166	241	362	1.5
D19.6 1709	*hxk1/hxk1 hex1*^{r}*/hex1*	66	1,009	370	189	280	1.48
D19.6 877	*hxk1/hxk1 hex1*^{r}*/hex1*	22	540	378	142	173	1.22
D19.6 777	*hxk1/hxk1 hex1*^{r}*/hex1*	25	329	369	130	155	1.19
D19.6 1804	*hxk1/hxk1 hex1*^{r}*/hex1*	262	649	480	115	120	1.04
D19.6 936	*hxk1/hxk1 hex1*^{r}*/hex1*	128	494	496	98	100	1.02

[a] Specific activity is expressed in nanomoles of substrate converted per minute per milligram of protein.
[b] *Q F/G* is the ratio of fructose to glucose phosphorylating activity.
[c] bd, Below detection.

F/G (ratio of fructose to glucose phosphorylating activity), which was between 1.0 and 1.55. The specific hexokinase activity, with fructose as substrate, varied between 100 and 455 mU/mg in the different mutant strains (Table 2). Mutants with low specific activities indicated that the mutation was localized inside the hexokinase PII structural gene. In addition to the postulated regulatory compartment, the catalytic domain of the enzyme was also defective. On the other hand, those mutants with catalytic activity comparable to that of the wild type, if allelic to the hexokinase PII structural gene, would clearly indicate that the proposed regulatory domain acts separately from the catalytic domain. Remarkably, the repression defects in these so-called *hex1*^{r} mutants were not expressed uniformly in all mutants. For example, *hex1*^{r}*-777* mutant had only about 10 times higher maltase activity, whereas other mutants, such as *hex1*^{r}*-59* or *hex1*^{r}*-1568*, had at least 100 times higher maltase activity than did the wild type after growth on glucose. However, invertase activities were comparable in each of these mutants, whereas malate dehydrogenase activity was only increased slightly in *hex1*^{r}*-1568* mutants, but much more in *hex1*^{r}*-59* and *hex1*^{r}*-777* mutants. Hence, the repressible enzymes may show different sensitivities to the triggering signal given by the proposed regulatory domain.

Allelism tests of *hex1*^{r} mutants. The *hex1*^{r} mutants obtained were diploid and should have been tested for allelism to *hxk2*, the structural gene of hexokinase PII, by sporulation of zygotes, tetrad analysis, and mating with a hexokinase PII mutant strain. However, unlike diploid cells, which were homozygotic for the *hex1* mutant allele, most of the *hex1*^{r} mutants obtained did not sporulate. About 15 of these mutants did not show any response to sporulation conditions with potassium acetate (see above); 14 mutants formed large aberrant cells after 3 days on potassium acetate. Five mutants sporulated, but gave no viable spores in about 100 tetrads segregated. Only one mutant, *hex1*^{r}*-877*, yielded viable spores which could be analyzed. Segregants of 20 tetrads were tested for carbon catabolite repression and hexokinase activity. As expected, all segregants were defective in catabolite repression. About one-half showed no hexokinase activity, as a consequence of the segregating *hxk2* allele, and the other segregants had hexokinase PII

TABLE 3. Allelism tests of original strain (D19.6), and *hex1* homozygote strain (D19.6 880), and *hex1*^{r} strains with different genotype of hexokinase isoenzymes[a]

Strain	Genotype	Haploid mating strains	Genotype	Sp act (range)				*Q F/G*
				Maltase	Invertase	Hexokinase		
						Glucose	Fructose	
D19.6	*HEX1/hex1*	308.14A-11C and H.38-4B	*HXK1 HEX1*	2–3	17–25	618–653	765–744	1.24–1.31
D19.6 880	*hex1/hex1*			1–2	23–30	516–542	725–753	1.40–1.47
D19.6 777, 877, 936, 1568, 1804	*hex1*^{r}*/hex1*			1–5	21–33	499–610	699–841	1.23–1.35
D19.6	*HEX1/hex1*	hxk.0-3B and hxk.0-7C	*hxk1 hex1*	2–4	18–24	310–337	327–382	1.05–1.12
D19.6 880	*hex1/hex1*			99–186	839–1,210	158–172	bd	bd
D19.6 777, 877, 936, 1568, 1804	*hex1*^{r}*/hex1*			38–85	578–1,411	311–431	214–356	0.74–1.32
D19.6	*HEX1/hex1*	P1.2-2C and P1.2-3A	*HXK1 hex1*	1–3	16–26	531–566	825–873	1.55–1.65
D19.6 880	*hex1/hex1*			175–332	1,004–1,423	198–223	547–570	2.48–2.89
D19.6 777, 877, 936, 1568, 1804	*hex1*^{r}*/hex1*			40–111	1,006–1,715	243–529	440–843	1.72–2.14

[a] Units and abbreviations are as in Table 2.

TABLE 4. Generation times, metabolite concentrations, and amount of ethanol excreted into the medium in various mutant strains

Strain	Genotype	Generation time (min)	Metabolite[a] (μmol/g dry wt)												EtOH excreted (mmol/g dry wt)
			G-6-P	F-6-P	F-1, 6-P_2	GAP/DHAP	3-PG	2-PG	PEP	PYR	ATP	ADP	NAD	NADH	
D19.6	*HEX1/hex1*	110	2.57	0.9	4.62	1.85	0.8	0.2	1.35	2.6	3.83	3.87	2.0	0.3	28,670
D19.6 880	*hex1/hex1*	130	1.90	0.67	5.7	1.57	1.38	0.34	1.34	2.9	2.77	3.0	1.2	0.2	20,930
D18.6 59	*hex1^r/hex1*	115	2.33	0.78	3.91	1.09	0.7	0.2	1.33	2.3	4.6	3.6	2.11	0.2	25,060
D19.6 1663	*hex1^r/hex1*	120	1.88	0.82	5.69	1.48	0.53	0.45	1.23	2.21	3.27	3.24	1.4	0.3	27,280
D19.6 1568	*hex1^r/hex1*	110	2.41	0.9	4.8	1.80	0.6	0.3	1.25	2.33	3.8	4.0	1.8	0.3	28,700
D19.6 1709	*hex1^r/hex1*	120	2.70	1.1	4.42	1.52	0.68	0.46	1.1	2.02	3.19	3.6	2.0	0.1	25,230
D19.6 1804	*hex1^r/hex1*	125	1.54	0.73	3.52	1.0	0.69	0.45	1.14	3.26	3.66	2.36	1.9	0.2	21,470
D19.6 936	*hex1^r/hex1*	140	1.10	0.76	2.87	0.93	0.93	0.2	1.02	2.54	2.6	2.72	1.7	0.1	19,480

[a] Abbreviations: G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; F-1,6-P_2, fructose 1,6-bisphosphate; GAP/DHAP glyceraldehyde 3-phosphate/dihydroxyacetone phosphate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate, PYR, pyruvate.

activity coded by the *hex1^r* allele. Since *MAL2-8^c* and *SUC3* were heterozygous in the original diploid strain (see above), only one-half of the segregants had invertase and maltase activity. Clearly the mutation obtained was at a locus identical with or closely linked to the hexokinase PII gene locus. The *hex1^r* segregants were sterile, and they could not be used for allelism tests with *hxk2* alleles. To circumvent these problems, triploid strains were constructed. For that reason the diploid cells that had two homozygote auxotrophies were mated with haploid strains having two different auxotrophies. Triploids occured as a result of mating type homozygotic mutation in the diploid strains (see above). When the *HXK2* gene was present in the triploids carbon catabolite repression was completely restored, whereas triploids having *hex1^r/hxk2/hxk2* alleles were not repressible (Table 3). This clearly indicated allelism between the *hex1^r* allele and the *hxk2* allele.

Physiological characterization of *hex1^r* mutants. The *hex1^r* mutants had different growth rates. Those mutants with reduced hexokinase PII activity also had longer generation times than did the wild type, whereas those mutants with hexokinase PII activities comparable to that of the wild type had unchanged generation times. Glycolytic metabolite concentrations and ATP, ADP, NAD, and NADH concentrations were similar in all mutants and the wild type. Only the rate of ethanol production, as an index of fermentative activity, increased with hexose phosphorylating capacity in *hex1^r* mutants. In mutants with normal hexokinase activity ethanol production was as in wild-type cells (Table 4). Hence, the defect in carbon catabolite repression in *hex1^r* mutants was independent of glycolytic flux. This was confirmed in strains with different hexokinase activities, which were obtained by combining different mutant alleles of the hexokinase isoenzymes. Recombinants of genotype *hxk1 HEX1 glk1*, having only hexokinase PII in functional condition, had the same specific activity with glucose as the substrate as recombinants of genotype *HXK1 hex1 GLK1* having isoenzymes PI and glucokinase in functional condition. The latter strain had even a higher phosphorylation rate with fructose as the substrate. Although *hxk1 HEX1 glk1* strains were normally repressible after growth with glucose and fructose as carbon source, *HXK1 hex1 GLK1* recombinants were defective in carbon catabolite repression. Metabolite concentrations, growth rate, and ethanol production were similar in both classes of recombinants (Table 5). These results are clearly consistent with the defect in carbon catabolite repression depending only on the availability of the hexokinase PII wild-type allele.

DISCUSSION

In previous investigations we reported on a large number of genes whose wild-type function is necessary for carbon catabolite repression or derepression of certain enzymes (or both) (12, 13, 15–18, 35, 36). Additionally, all of these mutants had direct or indirect influences on hexokinase isoenzyme PII (hexokinase B). Whereas in *hex1* mutants the structural gene of hexokinase PII was mutated, in the other mutants *hex2*, *cat80*, *cat1*, and *cat3* were involved in the regulation of hexokinase PII synthesis. Hence, hexokinase PII appeared to be of major importance in the process of carbon catabolite repression. No significant changes in glycolytic flux and metabolite concentrations were detected when repression mutants were compared with their respective wild types. Hence, an increase in glucose metabolite concentrations was unlikely to trigger carbon catabolite repression in *S. cerevisiae*, as described by Magasanik (25) for *Escherichia coli*. The finding of the special role of hexokinase isoenzyme PII, the first glycolytic enzyme, enabled us to state an alternative hypothesis, namely, that the hexokinase PII molecule might give the repression signal itself. Clearly, this requires changes in the enzyme during hexose phosphorylation which produce the triggering signal. To elucidate the molecular basis of this postulated reaction, it was necessary to find out whether the proposed regulatory and catalytic sites act independently. Proof of the hypothesis depended on finding that the regulatory site could be mutated separately, with no or only few effects on the catalytic active site of the enzyme. The findings with the *hex1^r* mutants indicated that hexokinase PII is a bifunctional enzyme having (i) a catalytic domain for hexose phosphorylation and (ii) a regulatory domain triggering carbon catabolite repression. As shown by mutants with reduced phosphorylating activity these two domains are closely associated. In those *hex1^r* mutants with uneffected phosphorylating activity, glycolytic flux and energy content were the same as those in the corresponding wild-type cells. In addition, strains were constructed with different phosphorylating activity. These experiments also clearly showed that carbon catabolite repression depended solely on the presence or absence of the hexokinase isoenzyme PII and was independent of the hexose phosphorylating capacity of the cell. Altogether, the hexokinase isoenzyme PII is highly complex. Interestingly, hexokinase isoenzyme PI has only catalytic function. After peptide mapping hexokinase PI and PII shared 27 common bands (29). Clearly, these isoenzymes were derived by gene duplication. Perhaps, the hexokinase PII isoenzyme was derived from PI and, thereafter, evolved

TABLE 5. Specific activities, metabolite concentrations, ethanol excreted into the medium, and generation times in recombinants with different hexokinase and glucokinase mutant alleles

Strain	Genotype	Generation time (min)	Sp act[a]				Metabolite[b] (μmol/g dry weight)										EtOH excreted (mmol/g dry wt)
			Hexokinase		Maltase	Invertase	G-6-P	F-6-P	F-1,6-P_2	GAP/DHAP	3-PG	2-PG	PEP	PYR	ATP	ADP	NAD	NADH	
			Glucose	Fructose															
H.38-4B	*HXK1 HEX1 GLK1*	150	1,239	1,857	3.9	28	2.5	0.5	3.6	1.2	0.8	0.1	1.1	2.7	3.8	2.4	2.0	0.3	38,500
P1.2.3A	*HXK1 hex1 glk1*	205	380	1,193	98	1,101	2.1	0.38	3.5	0.9	0.7	0.3	1.0	2.9	3.6	2.7	2.2	0.2	26,300
P2.1-2B	*hxk1 HEX1 GLK1*	220	520	738	6.2	19	2.3	0.45	3.0	0.8	0.9	0.2	1.2	2.3	3.0	3.3	1.9	0.1	25,800
D.308-2A	*hxk1 hex1 GLK1*	255	100	bd	112	1,290	1.5	0.3	1.9	0.5	0.3	0.2	1.4	2.6	2.0	2.9	2.1	0.1	17,400
P1.6-2C	*HXK1 hex1 GLK1*	195	505	1,251	126	997	2.3	0.5	3.2	1.3	0.6	0.3	1.1	2.3	3.2	2.5	1.9	0.2	28,900

[a] Units and abbreviations are as in Table 2.
[b] Abbreviations are as in Table 4.

a second function for regulating carbon catabolite repression. The *hex1*^{r} mutants with their catalytic activity make purification and characterization of these enzymes practicable. Comparison with wild-type hexokinase PII could provide a basis for elucidating the molecular mechanism of the triggering reaction for carbon catabolite repression.

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LITERATURE CITED

1. **Arrigoni, O., and T. P. Singer.** 1962. Limitation of the phenazine methosulfate assay for succinic and related dehydrogenases. Nature (London) **193**:1256–1258.
2. **Barnett, J. A.** 1976. The utilization of sugars by yeasts. Adv. Carbohydr. Chem. Biochem. **32**:126–234.
3. **Barnett, J. A., and H. L. Kornberg.** 1960. The utilization by yeasts of acids of the tricarboxylic cycle. J. Gen. Microbiol. **23**:65–82.
4. **Bergmeyer, H. U.** 1970. Methoden der enzymatischen Analyse, 2nd ed., p. 432. Verlag Chemie, Weinheim, Germany.
5. **Bergmeyer, H. U.** 1970. Methoden der enzymatischen Analyse, 2nd ed., p. 1457. Verlag Chemie, Weinheim, Germany.
6. **Böker-Schmitt, E., S. Francisci, and R. J. Schweyen.** 1982. Mutations releasing mitochondrial biogenesis from glucose repression in *Saccharomyces cerevisiae*. J. Bacteriol. **151**:303–310.
7. **Ciriacy, M.** 1975. Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae*. I. Isolation and genetic analysis of mutants. Mutat. Res. **29**:315–326.
8. **Ciriacy, M.** 1977. Isolation and characterization of yeast mutants defective in intermediary carbon metabolism and in carbon catabolite derepression. Mol. Gen. Genet. **154**:213–220
9. **Ciriacy, M.** 1978. A yeast mutant with glucose-resistant formation of mitochondrial enzymes. Mol. Gen. Genet. **129**:329–335.
10. **Colowick, S. P.** 1973. The hexokinases, p. 1–48. *In* P. D. Boyer (ed.), The enzymes. vol. 9, 3rd ed. Academic Press, Inc., New York.
11. **Duncan, H. M., and B. J. Mackler.** 1966. Electron transport systems of yeast. J. Biol. Chem. **241**:1694–1697.
12. **Entian, K.-D.** 1980 Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in carbon catabolite repression. Mol. Gen. Genet. **178**:633–637.
13. **Entian, K.-D.** 1980. A defect in carbon catabolite repression associated with uncontrollable and excessive maltose uptake. Mol. Gen. Genet. **179**:169–175.
14. **Entian, K.-D.** 1981. A carbon catabolite repression mutant of *Saccharomyces cerevisiae* with elevated hexokinase activity: evidence for a regulatory control of hexokinase PII synthesis. Mol. Gen. Genet. **184**:278–282.
14a. **Entian, K.-D., L. Droll, and D. Mecke.** 1983. Studies on rapid reversible and non-reversible inactivation of fructose-1,6-bisphosphatase and malate dehydrogenase in wild-type and glycolytic block mutants of *Saccharomyces cerevisiae*. Arch. Microbiol. **134**:187–192.
15. **Entian, K.-D., and D. Mecke.** 1982. Genetic evidence for a role of hexokinase isozyme PII in carbon catabolite repression in *Saccharomyces cerevisiae*. J. Biol. Chem. **257**:870–874.
16. **Entian, K.-D., and F. K. Zimmermann.** 1980. Glycolytic enzymes and intermediates in carbon catabolite repression mutants of *Saccharomyces cerevisiae*. Mol. Gen. Genet. **177**:345–350.
17. **Entian, K.-D., and F. K. Zimmermann.** 1982. New genes involved in carbon catabolite repression and derepression in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **151**:1123–1128.
18. **Entian, K.-D., F. K. Zimmermann, and I. Scheel.** 1977. A partial

defect in carbon catabolite repression in mutants of *Saccharomyces cerevisiae* with reduced hexose phosphorylation. Mol. Gen. Genet. **156**:99–105.

18a. **Frohlich, K.-U., K.-D. Entjan, and D. Mecke.** 1984. Cloning and restriction analysis of the heterokinase gene of the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **194**:144–148.

19. **Gancedo, J. M., and C. Gancedo.** 1971. Fructose-1,6-diphosphatase, phosphofructokinase and glucose-6-phosphate dehydrogenase from fermenting and non fermenting yeast. Arch. Microbiol. **76**:132–138.

20. **Gancedo, C., M. L. Salas, A. Giner, and A. Sols.** 1965. Reciprocal effects of carbon sources on the level of an AMP-sensitive fructose-1,6-diphosphatase and phosphofructokinase in yeast. Biochem. Biophys. Res. Commun. **20**:15–20.

21. **Gancedo, C., and N. Schwerzmann.** 1976. Inactivation by glucose of phosphoenolpyruvate carboxykinase from *Saccharomyces cerevisiae*. Arch. Microbiol. **109**:221–225.

22. **Gascón, S., N. P. Neumann, and J. O. Lampen.** 1968. Comparative study of the purified internal and external invertases from yeast. J. Biol. Chem. **243**:1573–1577.

23. **Grossmann, M. K., and F. K. Zimmermann.** 1979. The structural genes of internal invertases in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **175**:223–229.

24. **Lobo, Z., and P. K. Maitra.** 1977. Genetics of yeast hexokinase. Genetics **86**:727–744.

25. **Magasanik, B.** 1961. Catabolite repression. Cold Spring Harbor Symp. Quant. Biol. **26**:249–256.

26. **Mishra, S. D., and C. A. Michels.** 1982. Glucosamine-resistant mutations in yeast affecting the glucose repression sensitivity of electron transport enzymes. Curr. Genet. **6**:209–218.

27. **Polakis, E. S., and W. Bartley.** 1965. Changes in enzyme activities in *Saccharomyces cerevisiae* during aerobic growth on different carbon sources. Biochem. J. **97**:284–297.

28. **Polakis, E. S., W. Bartley, and G. A. Meek.** 1965. Changes in the activities of respiratory enzymes during the aerobic growth of yeast on different carbon sources. Biochem. J. **97**:298–302.

29. **Schmidt, J. J., and S. P. Colowick.** 1973. Chemistry and subunit structure of yeast hexokinase isoenzymes. Arch. Biochem. Biophys. **158**:458–470.

30. **Wijk, R. van, J. Ouwehand, T. van den Bos, and V. V. Konigsberger.** 1969. Induction and catabolite repression of alpha-glucosidase synthesis in protoplasts of *Saccharomyces carlsbergensis*. Biochim. Biophys. Acta **186**:178–191.

31. **Witt, I., R. Kronau, and H. Holzer.** 1966. Repression von Alkohol-dehydrogenase, Malatdehydrogenase, Isocitratlyase und Malatsynthase in Hefe durch Glucose. Biochim. Biophys. Acta **118**:522–537.

32. **Wolfe, R. G., and J. B. Neilands.** 1956. Some molecular and kinetic properties of heart malic dehydrogenase. J. Biol. Chem. **221**:61–69.

33. **Zamenhoff, S.** 1957. Preparation and assay of desoxyribonucleic acids from animal tissue. Methods Enzymol. **3**:696–704.

34. **Zimmermann, F. K., and N. R. Eaton.** 1974. Genetics of induction and catabolite repression of maltase synthesis in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **134**:261–272.

35. **Zimmermann, F. K., I. Kaufmann, H. Rasenberger, and P. Haußmann.** 1977. Genetics of carbon catabolite repression in *Saccharomyces cerevisiae*: genes involved in the derepression process. Mol. Gen. Genet. **151**:95–103.

36. **Zimmermann, F. K., and I. Scheel.** 1977. Mutants of *Saccharomyces cerevisiae* resistant to carbon catabolite repression. Mol. Gen. Genet. **154**:75–82.