Pleiotropic Mutations Regulating Resistance to Glucose Repression in *Saccharomyces carlsbergensis* Are Allelic to the Structural Gene for Hexokinase B

CORINNE A. MICHELS,* KAREN M. HAHNENBERGER, AND YOLANDE SYLVESTRE

Department of Biology, Queens College, Flushing, New York 11367

Received 1 September 1982/Accepted 15 October 1982

Previously, we described a mutation *glr1-1* in *Saccharomyces carlsbergensis* which pleiotropically relieves the synthesis of the following enzymes from glucose repression: maltase, galactokinase, α -galactosidase, NADH:cytochrome *c* reductase, and cytochrome *c* oxidase (C. A. Michels and A. Romanowski, J. Bacteriol. 143:674-679, 1980.) In this report, we demonstrate that *glr1-1* and two other alleles, *glr1-3* and *glr1-16*, are also insensitive to the glucose repression of invertase synthesis. Determinations of the levels of hexokinase activity and the rate of glucose transport in these mutants show that both are reduced as compared with the parent strain. Complementation tests and genetic analysis indicate that the *glr1* mutations are allelic to *HXX2*, the structural gene for hexokinase B. The significance of this result is discussed with regard to the mechanism of glucose repression in *S. carlsbergensis*.

The addition of glucose to a growing yeast culture causes two types of metabolic changes. The first effect, called glucose inactivation (12), involves the proteolytic inactivation of a specific set of enzymes, including maltose permease (23), fructose 1,6-diphosphatase (22), isocitrate lyase (3), and cytoplasmic malate dehydrogenase (23). The second effect, called glucose repression, produces a reduction in the rate of synthesis of a wide variety of enzymes. These include the sugar fermentation enzymes maltase (24), galactokinase (1), invertase (2), and α -galactosidase (18); mitochondrial functions such as the Krebs cycle enzymes, electron transport, and mitochondrial ATPase (20, 21); and some of the enzymes of gluconeogenesis and the glyoxalate cycle, including those glucose-inactivated enzymes listed above (21). The "glucose effect" clearly represents a global control mechanism in yeasts.

In a previous report (18), we described the isolation of a mutant with the mutation *glr1-1*, which pleiotropically relieves the repressing effects of glucose on the following enzymes: maltase, galactokinase, α -galactosidase, NADH:cytochrome *c* reductase, and cytochrome *c* oxidase. Two new mutant alleles at this locus have been isolated: *glr1-3*, a nitrous acid-induced mutation, and *glr1-16*, a spontaneous mutation. Both were isolated from the *Saccharomyces carlsbergensis* strain E56-2C based on their ability to grow on maltose in the presence of D-glucosamine. Both are as resistant as *glr1-1*

to the glucose repression of the same enzymes listed above. In addition, the synthesis of invertase in all three *GLR1* mutants is resistant to glucose repression (see Table 1).

Glucose transport and phosphorylation in *glr1* mutants. The mutations in *GLR1* described here are phenotypically similar to three classes of mutations described by Zimmerman and Scheel (25) and Entian and co-workers (5-9). The *hex1* mutants they describe do not have detectable levels of hexokinase B activity (7) and do not complement mutations in the *HXX2* locus which codes for hexokinase B (14). The *hex2* mutations they describe have been shown to exhibit elevated levels of hexokinase B (6), but mutations in *CAT80* show no altered hexokinase activity (8). Based on these results, we investigated glucose transport and phosphorylation in the *glr1* mutant strains. Table 2 shows both of these activities to be reduced in the *glr1* mutants. The reduction in glucose-phosphorylating activity is equivalent to that seen in the *hex1* mutants.

Genetic analysis of allelism between *glr1* mutations and the genes coding for glucose-phosphorylating enzymes. Three glucose-phosphorylating enzymes are present in *Saccharomyces* spp.: two hexokinase isoenzymes called A and B (or PI and PII, respectively) and glucokinase (10, 14). The structural genes encoding these proteins are *HXX1* (hexokinase A), *HXX2* (hexokinase B), and *GLK1* (glucokinase). To further investigate the reduced rate of glucose phosphorylation found in *glr1* mutants, complemen-

TABLE 1. Pleiotropic glucose repression insensitivity of invertase synthesis in *glr1* mutant strains

Strain	Genotype	Invertase ^a (U/mg of protein)	
		Repressed ^b	Derepressed ^c
E56-2C	<i>GLR1</i>	0.06	1.25
E6-2	<i>GLR1</i>	0.04	0.99
M1	<i>glr1-1</i>	1.21	1.73
M3	<i>glr1-3</i>	0.83	1.80
M16	<i>glr1-16</i>	1.38	1.78

^a Invertase was assayed according to the method described by Goldstein and Lampen (11), using crude cell extract prepared as described by Michels and Romanowski (18), except that the final centrifugation was for 15 min at 15,000 rpm.

^b For repressed conditions, the cells were grown to mid-log phase in YEP medium (1% yeast extract and 2% peptone) plus 2% glucose.

^c To derepress invertase synthesis, the culture was grown to early log phase in YEP plus 2% glucose, harvested, resuspended in YEP plus 0.05% glucose, and allowed to grow for two doublings.

tation tests were done between the *glr1* mutant alleles and mutations in the *HXK1*, *HXK2*, and *GLK1* loci. Table 3 summarizes the genotypes of each of the strains used for this analysis. The strains PIT8C, P2T22D, D308, and D308.3 carry various combinations of the *hxx1*, *hxx2*, and *glk1* mutant alleles isolated by Lobo and Maitra (14). DFY-64 was isolated by Gancedo et al. (10) and is mutant at both the *hxx1* and *hxx2* loci and wild type at the *GLK1* locus. Diploids of the genotype *glr1/hxx2* are insensitive to the glucose repression of α -galactosidase synthesis (Table 4). All of the remaining diploids are glucose repression sensitive. Thus, mutations at the *GLR1* locus are not complemented by mutations at the *HXK2* locus.

Genetic analysis also supports the conclusion that *glr1* mutations are allelic to mutations in the *HXK2* locus. The fermentation of fructose by *Saccharomyces* spp. is initiated by using either hexokinase A or hexokinase B for phosphorylation. An *hxx1 hxx2* double mutant strain is a

TABLE 2. Glucose transport and phosphorylation in *glr1* mutant strains

Strain	Genotype	Glucose phosphorylation ^a (U/mg of protein)	Sugar transport ^b (μ g of xylose transported per ml of cell water per s)
E56-C2	<i>GLR1</i>	2.62	6.9
M1	<i>glr-1</i>	0.71	4.9
M3	<i>glr1-3</i>	1.19	4.7
M16	<i>glr1-16</i>	0.83	3.3

^a The cells were grown as described for Table 1 in YEP plus 2% glucose. Hexokinase was assayed according to Maitra and Lobo (15), using crude cell extract (18).

^b Glucose transport was measured by following the intracellular accumulation of [¹⁴C]xylose based on the procedure described by Cirillo (4).

fructose nonfermenter. The following series of crosses uses this to demonstrate that *glr1* is allelic to *hxx2*.

Cross 1. In the first cross, a strain of the genotype *hxx1 hxx2 MEL* (fructose nonfermenter) was crossed with a strain of the genotype *glr1-16 MEL* (fructose fermenter). The analysis of the resulting tetrads is shown in Table 5. The 2:2 segregation of fructose-fermenting to fructose-nonfermenting segregants indicates that the *glr1-16* mutation is allelic to either *hxx1* or *hxx2*. All four segregants of the tetrad are glucose insensitive, indicating that the *glr1-16* allele and either its *hxx1* or *hxx2* allele are responsible for this phenotype.

Crosses 2 and 3. In crosses 2 and 3, a *glr1* mutant strain is crossed with *hxx1* and *hxx2* tester strains constructed from the Lobo and Maitra (14) mutants. If *glr1* is allelic to *hxx2*, a cross between *hxx2* and *glr1* mutant strains should yield only fructose-fermenting segregants upon sporulation. Likewise, if *glr1* is allelic to *hxx1*, it is mutant in the hexokinase B structural gene, and a cross between strains carrying mutations at *glr1* and *hxx1* should yield some fructose-nonfermenting segregants upon sporulation. Eight four-spored and two three-spored

TABLE 3. Strain list

Strain	Genetic marker	Source
E56-2C	α <i>adel ura MAL6^c GAL MEL SUC</i>	A. M. A. ten Berge
E6-2	α <i>lys MAL6^c GAL MEL SUC</i>	Laboratory strain
M1	α <i>adel ura glr1-1 MAL6^c GAL MEL</i>	C. A. Michels and A. Romanowski
PIT8C	α <i>adel his2 HXK1 hxx2 glk1</i>	Z. Lobo and P. K. Maitra
P2T22D	α <i>adel met14 hxx1 HXK2 glk1</i>	Z. Lobo and P. K. Maitra
D308	α <i>adel his2 trp1 met14 hxx1 hxx2</i>	Z. Lobo and P. K. Maitra
D308.3	α <i>adel his2 trp1 met14 hxx1 hxx2 glk1</i>	Z. Lobo and P. K. Maitra
DFY-64	α <i>leu2 aro7 hxx1 hxx2</i>	D. Fraenkel

TABLE 4. Complementation tests between *glr1* and *hxx1*, *hxx2* and *glk1* mutant strains

Diploid	Cross ^a	Relevant genotype	α -Galactosidase activity ^b	
			Repressed ^c	Derepressed ^d
KH1	PIT8C $\times$ E56-2C	<i>HXX1 hxx2 glk1/GLR1</i>	0.06	2.3
KH2	PIT8C $\times$ M1	<i>HXX1 hxx2 glk1/glr1-1</i>	0.58	2.3
KH3	D308 $\times$ E6-2	<i>hxx1 hxx2 GLK1/GLR1</i>	0.03	2.1
KH4	D308 $\times$ D18-6B	<i>hxx1 hxx2 GLK1/glr1-16</i>	0.34	2.6
KH5	D308 $\times$ D18-10D	<i>hxx1 hxx2 GLK1/glr1-16</i>	0.29	1.7
KH6	D308.3 $\times$ E6-2	<i>hxx1 hxx2 glk1/GLR1</i>	0.06	2.0
KH7	D308.3 $\times$ D18-6B	<i>hxx1 hxx2 glk1/glr1-16</i>	0.35	2.2
KH8	D308.3 $\times$ D18-10D	<i>hxx1 hxx2 glk1/glr1-16</i>	0.43	2.3
KH9	P2T22D $\times$ E6-2	<i>hxx1 HXX2 glk1/GLR1</i>	0.07	3.1
KH10	P2T22D $\times$ D18-6B	<i>hxx1 HXX2 glk1/glr1-16</i>	0.01	1.9
KH11	P2T22D $\times$ D18-10D	<i>hxx1 HXX2 glk1/glr1-16</i>	0.02	1.9
KH12	DFY-64 $\times$ E6-2	<i>hxx1 hxx2 GLK1/GLR1</i>	0.05	3.3
KH13	DFY-64 $\times$ D18-6B	<i>hxx1 hxx2 GLK1/glr1-16</i>	1.00	2.5
KH14	DFY-64 $\times$ D18-10D	<i>hxx1 hxx2 GLK1/glr1-16</i>	0.99	3.9

^a Standard genetic techniques were used (19).

^b α -Galactosidase is an external enzyme making it possible to assay for this enzyme by using whole cells. The assay procedure used is described by Kew and Douglas (13). Activity is expressed as the ratio of the change in optical density at 400 nm per minute to the optical density at 650 nm.

^c Glucose (5%) plus 2% galactose.

^d Galactose (2%).

tetrads were obtained from the cross KH35-29 (*HXX1 hxx2*) $\times$ D18-10D (*glr1-16*). All of the segregants from this cross were fructose fermenters, as expected if *glr1* and *hxx2* are allelic. The results of the cross D18-10D (*glr1-16*) $\times$ KH27-9 (*hxx1*) could only be analyzed by random spore analysis because of poor spore viability. Nonetheless, 14% (17 of 120 segregants) of the random haploid segregants were fructose nonfermenters. Since *HXX1* and *HXX2* are unlinked, 25% of the segregants would be expected to be fructose nonfermenters if *hxx2* and *glr1* were alleles. The results of crosses 2 and 3 are consistent with the allelism of *glr1* and *hxx2*.

Crosses 4 and 5. Crosses 4 and 5 were carried out to further demonstrate that mutations at the *HXX1* locus do not relieve glucose repression. In cross 4, strain P2T22D (*hxx1 HXX2 mel*) was crossed to strain PIT8C (*HXX1 hxx2 MEL*). The results from five four-spored and seven three-spored tetrads show the expected dihybrid segregation pattern for fructose fermentation. If only mutations at the *HXX2* locus affect glucose repression, then one-third of the fructose-fermenting segregants should be glucose insensitive, as opposed to two-thirds of the fructose-fermenting segregants if mutations at either *HXX1* or *HXX2* could affect glucose repression. Our results show that 4 of the 23 fructose- and melibiose-fermenting segregants exhibited glucose-insensitive α -galactosidase synthesis.

Cross 5 was a cross between P2T22D (*hxx1*

HXX2 mel) and KH3C (*hxx1 hxx2 MEL*). Germination was poor, and only two four-spored tetrads were obtained, but both showed the expected segregation pattern of two fructose fermenters: two fructose nonfermenters. Determinations of the level of α -galactosidase activity under glucose-repressed conditions were done on three fructose- and melibiose-fermenting segregants, and all were glucose sensitive. The one fructose-nonfermenting, melibiose-fermenting segregant assayed was found to be insensitive to glucose repression.

The results of crosses 4 and 5 indicate that mutations in the *HXX1* locus do not relieve glucose repression as do mutations in the *HXX2* locus. Based on these results, it appears that the mechanism of glucose repression in the *Saccharomyces* yeasts is different from that seen in *Escherichia coli*. In *E. coli*, the level of glucose in the growth medium controls intracellular cyclic AMP levels, which in turn appear to control enzyme synthesis. Recent reports indicate that cyclic AMP does not play an equivalent role in the *Saccharomyces* yeasts. The glucose repression sensitivity of mutants which transport and utilize cyclic AMP as an adenine source is unaffected by the presence or absence of cyclic AMP in the growth medium (17). More significantly, mutants defective in the synthesis of cyclic AMP remain sensitive to glucose repression (16). Consistent with these results, we find that in preliminary measurements of the levels of

TABLE 5. Tetrad analysis of the diploid KH41

No. of asci analyzed	KH25-9D					D18-10D					×					(glr1-16 MEL)														
	(hsk1 hsk2 MEL)																													
	Segregation of fructose fermenter:fructose nonfermenter					Segregation of glucose resistant:glucose sensitive																								
	Four-spored asci					Three-spored asci					Four-spored asci					Three-spored asci					Four-spored asci					Three-spored asci				
	4:0	3:1	2:2	1:3	0:4	3:0	2:1	1:2	0:3	4:0	3:1	2:2	1:3	0:4	3:0	2:1	1:2	0:3	4:0	3:1	2:2	1:3	0:4	3:0	2:1	1:2	0:3			
13	0	0	7	0	0	0	0	2	4	0	7	0	0	0	0	0	0	0	7	0	0	0	0	6	0	0	0			

adenyl cyclase and cyclic AMP phosphodiesterase, the *glr1* mutant strains show no significant differences from the E56-2C parent.

It appears that the independently isolated *hxx2*, *glr1*, and *hex1* mutations described in the literature (14, 18, 25) are all, in fact, mutations at the same locus, the structural gene coding for hexokinase B. All were isolated by using different selection methods and are pleiotropically insensitive to glucose repression. Clearly, hexokinase B plays a central role in this global control mechanism. The primary question to be answered is, what is this role?

We would like to present two possible explanations for the function of hexokinase B in the regulation of glucose repression. The first possibility is that hexokinase B mutants reduce the rate of glucose metabolism by lowering the rate of glucose phosphorylation and transport, and that this results in a loss of glucose repression. Our results showed the rate of glucose transport in the hexokinase B mutants (*glr1*) to be approximately 60% of the parental rate with no measurable effect on the growth rate on glucose of these mutant strains. Entian and Zimmerman (8) report only a very slight reduction in intracellular levels of glucose-6-phosphate and other glycolysis intermediates in *hex1* mutants. These findings argue against the possibility that hexokinase B exerts its control on glucose repression simply by controlling the rate of glucose metabolism.

The second possibility explaining the effect of hexokinase B mutations on glucose repression is that hexokinase B is a regulatory protein acting as a repressor of all glucose-sensitive functions or is responsible for the synthesis of a corepressor of these functions. This hypothesis is consistent with the recessive character of the *glr1* mutations and with the pleiotropic loss of glucose sensitivity seen in these mutants but is confused by the characterization of the *hex2* mutants described by Entian (6). These mutants elevate the level of hexokinase B, but rather than exhibiting a super-repressed state, they are pleiotropically insensitive to glucose repression. This conflict requires more attention.

Our current research is aimed at deciding between these two explanations of the role of hexokinase B in glucose repression in *S. carlsbergensis*.

This work was supported by Public Health Service grant GM-28216 from the National Institute of General Medical Sciences and in part by grant RR-07064.

We acknowledge the technical assistance of Susanne Haupt. The determinations of adeny cyclase and cyclic AMP phosphodiesterase activity were kindly done by Hans Liao.

LITERATURE CITED

1. Adams, B. G. 1972. Induction of galactokinase in *Saccharomyces cerevisiae*: kinetics of induction and glucose effects. *J. Bacteriol.* 111:308-315.
2. Carlson, M., and D. Botstein. 1982. Two differentially

- regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. *Cell* **28**:145-154.
3. Chapman, C., and W. Bartley. 1968. The kinetics of enzyme changes in yeast under conditions that cause loss of mitochondria. *Biochem. J.* **107**:455-465.
 4. Cirillo, V. P. 1962. Mechanism of glucose transport across the yeast cell membrane. *J. Bacteriol.* **84**:485-491.
 5. Entian, K. D. 1980. Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in carbon catabolite repression in yeast. *Mol. Gen. Genet.* **178**:633-637.
 6. Entian, K. D. 1981. A carbon catabolite repression mutant of *Saccharomyces cerevisiae* with elevated hexokinase activity: evidence for regulatory control of hexokinase PII synthesis. *Mol. Gen. Genet.* **184**:278-282.
 7. Entian, K. D., and D. Mecke. 1982. Genetic evidence for a role of hexokinase isoenzyme PII in carbon catabolite repression in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **257**:870-874.
 8. Entian, K. D., and F. K. Zimmerman. 1980. Glycolytic enzymes and intermediates in carbon catabolite repression mutants of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **177**:345-350.
 9. Entian, K. D., F. K. Zimmerman, 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.
 10. Gancedo, J., D. Clifton, and D. G. Fraenkel. 1977. Yeast hexokinase mutants. *J. Biol. Chem.* **252**:4443-4444.
 11. Goldstein, A., and J. O. Lampen. 1979. β -Fructofuranoside fructohydrolase from yeast. *Methods Enzymol.* **43**:504-511.
 12. Holzer, H. 1976. Catabolite inactivation in yeast. *Trends Biochem. Sci.* **1**:178-180.
 13. Kew, O. M., and H. C. Douglas. 1976. Genetic co-regulation of galactose and melibiose utilization in *Saccharomyces*. *J. Bacteriol.* **125**:33-41.
 14. Lobo, Z., and P. K. Maitra. 1977. Genetics of yeast hexokinase. *Genetics* **86**:726-744.
 15. Maitra, P. K., and Z. Lobo. 1971. A kinetic study of glucolytic enzyme synthesis in yeast. *J. Biol. Chem.* **246**:475-489.
 16. Matsumoto, K., I. Uno, Y. Oshima, and T. Ishikawa. 1982. Isolation and characterization of yeast mutants deficient in adenyl cyclase and cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **79**:2355-2359.
 17. Matsumoto, K., I. Uno, A. Toh-e, T. Ishikawa, and Y. Oshima. 1982. Cyclic AMP may not be involved in catabolite repression in *Saccharomyces cerevisiae*: evidence from mutants capable of utilizing it as an adenine source. *J. Bacteriol.* **150**:277-285.
 18. Michels, C. A., and A. Romanowski. 1980. Pleiotropic glucose repression-resistant mutation in *Saccharomyces carlsbergensis*. *J. Bacteriol.* **143**:674-697.
 19. Mortimer, R. K., and D. C. Hawthorne. 1969. Yeast genetics, p. 385-460. In A. H. Rose and J. S. Harrison (ed.), *The yeasts*, vol. 1. Academic Press, Inc., New York.
 20. Perlman, P. S., and H. R. Mahler. 1974. Derepression of mitochondria and their enzymes in yeast: regulatory aspects. *Arch. Biochem. Biophys.* **162**:248-271.
 21. Polakis, E. S., and W. Bartley. 1965. Changes in the enzyme activities of *Saccharomyces cerevisiae* during aerobic growth on different carbon sources. *Biochem. J.* **97**:284-297.
 22. van de Poll, K. W., A. Kerkenaar, and D. H. J. Schamhart. 1974. Isolation of a regulatory mutant of fructose-1,6-diphosphatase in *Saccharomyces carlsbergensis*. *J. Bacteriol.* **117**:965-970.
 23. van Rijn, J., and R. van Wijk. 1972. Differential sensitivities of the two malate dehydrogenases and the maltose permease to the effect of glucose in *Saccharomyces carlsbergensis*. *J. Bacteriol.* **110**:477-484.
 24. van Wijk, R., J. Ouwehand, T., van de Bos, and V. V. Koningsberger. 1969. Induction and catabolite repression of α -glucosidase synthesis in protoplasts of *Saccharomyces carlsbergensis*. *Biochim. Biophys. Acta* **186**:178-191.
 25. Zimmerman, F. K., and I. Scheel. 1977. Mutants of *Saccharomyces cerevisiae* resistant to carbon catabolite repression. *Mol. Gen. Genet.* **154**:75-82.