GENETICS OF YEAST GLUCOKINASE

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ABSTRACT

Mutants of *Saccharomyces cerevisiae* lacking glucokinase (EC 2.7.1.2) have no discernible phenotypic difference from the wild-type strain; in a hexokinaseless background, however, they are unable to grow on any sugar except galactose. Reversion studies with glucokinase mutants indicate that the yeast *S. cerevisiae* has no other enzyme for phosphorylating glucose except the two hexokinases, P1 and P2, and glucokinase. Spontaneous revertants of *hxk1 hxk2 glk1* strains collected on glucose regain any one of these three enzymes. The majority of glucokinase revertants synthesize species of enzyme activity that are kinetically or otherwise indistinguishable from the wild-type enzyme. In a few cases the reverted enzyme is very perceptibly altered in properties with a K_m for glucose two orders of magnitude higher than that of the enzyme from the wild-type parent. These recessive, noncomplementing mutants, thus, define a single structural gene *GLK1* of glucokinase. Yeast diploids lacking all of the three enzymes for glucose phosphorylation fail to sporulate. Heterozygosity of either of the hexokinase genes *HXK1* or *HXK2*, but not *GLK1*, restores sporulation. The location of *GLK1* on chromosome *III* was indicated by loss of this chromosome when hexokinaseless diploids heterozygous for *glk1* were selected for resistance to 2-deoxyglucose; the homologue of chromosome *III* carrying *GLK1*, the mating-type allele and other nutritional markers on this chromosome was lost. Meiotic mapping of glucokinase executed with heterozygosity of one of the hexokinases indicated that the gene *GLK1* defining the structure of glucokinase protein is located on the left arm of chromosome *III* 24 cM to the left of *his4* in the order: *leu2—his4—glk1*.——Only two of 206 independent glucokinase mutants are nonsense ochre, both of which map at one end of the gene. In *hxk1* only one of 130 isolates is a nonsense mutation, whereas in *hxk2* none has been found among 220 independent mutants. These results raise the possibility that the protein products of these genes have some other essential function.——An earlier mapping result for *hxk2* has been corrected. The new location is on the left arm of chromosome *VII*, 17 cM distal to *ade5* in the order: *lys5—ade5—hxk2*.

INTEREST in the problem of glucose phosphorylation is matched by its complexity. Both in animal systems and in microbial, the process is mediated by multiple protein catalysts, some of which control the entry and certain others control the chemical conversion of the sugar to the phosphate ester. Tissues of higher organisms bring about this conversion by ATP-linked kinases, often including isozymic forms (COLOWICK 1973). Among the prokaryotes a large number of bacteria employ instead an enzyme complex called the phosphotransferase system that uses *P*-enol pyruvate as the phosphate donor

and a number of enzymes (KUNDIG, GHOSH and ROSEMAN 1964). Genetic studies with mutants have revealed the intimate relationship of the bacterial sugar phosphorylation system with a wide spectrum of cellular control processes (see SAIER, SIMONI and ROSEMAN 1976). The common denominator of this linkage is perhaps the bacterial membrane that somehow controls intracellular 3′,5′-cyclic AMP levels consequent to the interaction of glucose with the cell surface.

The yeast *Saccharomyces cerevisiae* is known to have three enzymes for the phosphorylation of glucose. Two of these are hexokinase P1 and hexokinase P2 (LOBO and MAITRA 1977a; GANCEDO, CLIFTON and FRAENKEL 1977), and the third is glucokinase (MAITRA 1970). However, it is not clearly established whether yeast has any other enzyme such as the bacterial phosphotransferase system for sugar phosphorylation. We report here a genetic study of yeast glucokinase and show that the two hexokinases and the glucokinase constitute the entire complement of enzymes involved in glucose phosphorylation in the yeast *S. cerevisiae*. A preliminary report has been published (MAITRA and LOBO 1981).

MATERIALS AND METHODS

Strains: The strains of *S. cerevisiae* used in this work are given in Table 1.

Growth of yeast: The yeast extract-peptone (YEP) medium and the minimal medium have been described (LOBO and MAITRA 1977a). Since the fermentable carbon sources, glucose, fructose and mannose, produced ethanol, scoring of growth on plates containing these sugars was not unequivocal because glucose-negative spores were growing by cross-feeding on ethanol produced from the neighboring glucose-positive colonies (LOBO and MAITRA 1977a). The inclusion of 0.3 mM sodium azide as an inhibitor of oxidative metabolism eliminated this ambiguity. This concentration of azide completely arrests the growth of yeast on alcohol without affecting the fermentative growth on sugars (LOBO and MAITRA 1977a). All of the segregants scored as negatives on fructose-azide plates were tested by growing them individually in test tubes containing YEP glucose medium. The tubes were incubated at 30° without shaking, and growth was scored every day for 4 days in order to be able to detect a sudden surge of growth whenever revertants appeared. Doubtful cases were always resolved by assay of glucose/fructose phosphorylation in cells rendered permeable by toluene. Mutants lacking all of the glucose-phosphorylating enzymes were maintained on YEP medium containing 150 mM ethanol as a carbon source.

Isolation of mutants: The first mutant 611 representing allele *glk1-1* was isolated from a *hxk1 hxk2* strain as a glucose-negative progeny (LOBO and MAITRA 1977a). Others were obtained as clones resistant to 4 mM 2-deoxyglucose on YEP alcohol plates (LOBO and MAITRA 1977b). 2-Deoxyglucose, a substrate of glucose-phosphorylating enzymes of yeast, prevents growth at 0.1 mM concentration. Presumably, it causes abortive expenditure of ATP with the accumulation of 2-deoxyglucose 6-phosphate that cannot be productively metabolized. The rationale for choosing this concentration of 2-deoxyglucose will be summarized here briefly. The resistance of yeast to 4 mM 2-deoxyglucose comes about not only by lesions in each of the three genes, *hxk1*, *hxk2* and *glk1*, determining the three glucose-phosphorylating enzymes, but also by mutation at two other unlinked loci conferring deoxyglucose resistance, *DGR1* and *DGR4*. The mutant allele *dgr1* confers resistance to only 1 mM 2-deoxyglucose; resistance to a concentration of 4 mM requires both the mutant alleles, *dgr1* and *dgr4* (LOBO and MAITRA 1977a). When, therefore, a cell of the genotype *DGR1 DGR4 hxk1 hxk2 GLK1* is challenged with 4 mM 2-deoxyglucose, the great majority of the survivors are, in fact, single-gene mutants at *glk1* rather than double mutants *dgr1 dgr4*. More than 200 independent mutants were isolated by this method using ethyl methane sulfonate as the mutagen. The method also yielded several spontaneous mutants.

TABLE 1

List of strains of Saccharomyces cerevisiae

Strain	Genotype	Source
HSC	**a** prototroph	LOBO and MAITRA (1977a)
711	**a** *hxk1-1 hxk2-1*	LOBO and MAITRA (1977a)
611	**a** *hxk1-1 hxk2-1 glk1-1*	LOBO and MAITRA (1977a)
D308	α *ade1 his2 trp1 met14 hxk1-2 hxk2-2*	LOBO and MAITRA (1977a)
D3010	**a** *ura3 met14 hxk1-2 hxk2-2*	This laboratory
X2182-3D (C2)	**a** *can1-100 trp5-48 his5-2 ade2-1 lys1-1 met1-1 leu1-12 SUP7*	Yeast Genetic Stock Center
X2182-3D (C8)	**a** *can1-100 trp5-48 his5-2 ade2-1 lys1-1 met1-1 leu1-12 SUP8*	Yeast Genetic Stock Center
X2182-3D (C59)	**a** *can1-100 trp5-48 his5-2 ade2-1 lys1-1 met1-1 leu1-12 SUP4*	Yeast Genetic Stock Center
XS144-S19	**a** *ade5 lys5 aro2 met13 cyh2 trp5 leu1*	Yeast Genetic Stock Center
N248-1C	α *ade1 gal1 trp1 ura3 his2 leu1 met14*	G. R. FINK
FM11	α *met8-1 his4-280 lys1-1 ade2-1 SUP4-3a*	G. R. FINK
5B IV	$\frac{\alpha \text{ his4-}\Delta 29 \text{ leu2}}{\alpha \text{ his4-290 } +}$ *ade2*	G. R. FINK
D603-1A	α *his7 ade2*	F. SHERMAN
D649	$\frac{\text{a MAL2 trp1 pet6 ade2 lys2}}{\alpha \text{ mal his4 leu2 ade1 thr4}}$	F. SHERMAN

Genetic analysis: Standard procedures as outlined by SHERMAN, FINK and LAWRENCE (1974) were followed.

Enzyme Assays: These have been described (LOBO and MAITRA 1977a).

RESULTS

Properties of glk1 *mutants*: A mutant strain lacking glucokinase grows normally on glucose because yeast has two other hexokinases. The triple mutant, *hxk1 hxk2 glk1*, lacking both the hexokinases as well as glucokinase grows on galactose but fails to grow on glucose (Figure 1). The parent strain D308 containing the wild-type allele *GLK1* grows on glucose and galactose with equal facility. Mutants lacking glucokinase and the hexokinases fail to grow also on maltose, sucrose and other sugars that generate glucose or fructose on hydrolysis. Loss of glucokinase did not affect growth on alcohol. The weak growth of the first mutant allele *glk1-1* on alcohol reported earlier (LOBO and MAITRA 1977a) was due to a gene unlinked to *glk1*.

Enzyme levels: The majority of the mutants reported here had less than 1% of the glucokinase activity synthesized by the *GLK1* parent strains, whereas the other glycolytic enzyme activities were normal. This was expected because the selection for resistance to 2-deoxyglucose was stringent (LOBO and MAITRA 1977b). The first glucokinase mutant 611 (*glk1-1*), however, retained approximately 2% of the wild-type activity. The levels of glucokinase and another glycolytic enzyme, phosphoglucose isomerase, in various haploid and diploid

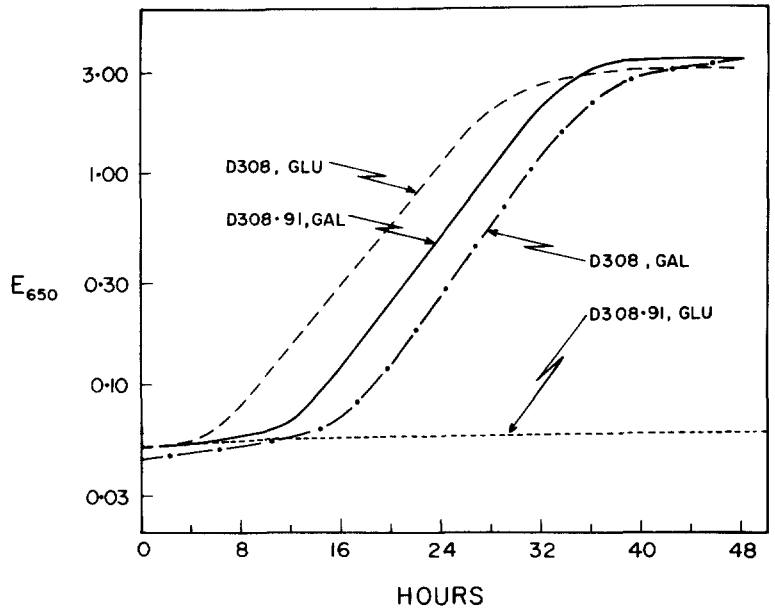

FIGURE 1.—Growth curve on galactose and glucose of a mutant of *S. cerevisiae* lacking all of the glucose-phosphorylating enzymes. A minimal medium complete in respect of auxotrophic markers containing either 50 mM glucose (GLU) or galactose (GAL) was inoculated, respectively, with strain D308 (*hxk1 hxk2 GLK1*) or a nonsense mutant D308.91 (*hxk1 hxk2 glk1-91*). Growth was followed by measuring the absorbance at 650 nm (E_{650}) during aerobic incubation on a gyrotory shaker at 30°.

strains are presented in Table 2. The observation that a $\frac{glk1}{GLK1}$ diploid had about half the glucokinase activity of the wild-type strains points to the recessive nature of the *glk1* mutation. The phosphoglucose isomerase activity, in contrast, was approximately the same in all of the strains. The activities of glucokinase do not depend appreciably on whether the alcohol-grown cultures are in stationary or exponential phase of growth.

Segregation of glk1: The gene *GLK1* was found to segregate $2^+:2^-$ in crosses of the type *hxk1 hxk2 GLK1* × *hxk1 hxk2 glk1*. The ability to grow on glucose and glucokinase activity were associated with *GLK1*. Although this suggested that *GLK1* was a single nuclear gene, we have examined this question closely. This was necessary because glucokinase displays a diffuse profile during migration in a centrifugal or electric field (MAITRA 1970; MAITRA and LOBO 1977). Furthermore, the specific activity of this enzyme was found to vary over a large range (120 to 795 milliunits (mu)/mg of protein) between meiotic segregants. Could this be due to multiple molecular forms of the enzyme? If the variation was due to expression of multiple unlinked genes each specifying glucokinase activity, intercrosses between segregants bearing glucokinase should have given rise to enzyme-negative progeny. The majority of hybrids of the genotype $\frac{hxk1\ hxk2\ GLK}{hxk1\ hxk2\ GLK}$, however, turned out to be meiotically incom-

TABLE 2

Glucokinase activity in haploids and diploids

Strain	mu/mg protein	
	GK	PGI
hxk1 hxk2 glk1	8	1230
hxk1 hxk2 GLK1	330	1530
hxk1 hxk2 GLK1 / *hxk1 hxk2 GLK1*	375	1120
hxk1 hxk2 glk1 / *hxk1 hxk2 GLK1*	110, 170	1180
hxk1 hxk2 glk1 / *hxk1 hxk2 glk1*	3	1200

Stationary cultures of strains grown on YEP alcohol were disrupted by a French press and the centrifuged crude extracts assayed for enzyme activities. Results are expressed in nmoles substrate per minute (milliunits) per milligram of protein. GK, glucokinase; PGI, phosphoglucose isomerase; *glk1* refers to the mutant allele *glk1-1*.

petent (see *Sporulation*). We could analyze only six different diploids arising out of some paired combinations of six haploids of the genotype *hxk1 hxk2 GLK* which sporulated with a frequency ranging from 10^{-3} to 10^{-2}. No glucokinase-negative clone was found among 177 such asci. Although this was consistent with a single determinant for glucokinase, it was still possible that only a particular activity of unique glucokinase present in these segregants permitted meiotic analysis. Fortunately, heterozygosity of either *HXK1* or *HXK2* in these crosses allowed sporulation. A total of ten strains of the genotype *HXK1 hxk2 glk1* or *hxk1 HXK2 glk1* were crossed to glucokinase-positive haploids of the genotype *hxk1 hxk2 GLK*. Sporulation of the resultant diploids gave in each case a single-gene segregation for growth on fructose and two-gene segregation for growth on glucose. These results are, thus, consistent with the presence of a single gene specifying glucokinase. The observation that all glucokinase activity in *hxk1 hxk2 GLK* strains could be lost by spontaneous mutation leaves little doubt that *S. cerevisiae* has a unique Mendelian locus for glucokinase, viz., GLK1. Whether the observed variation in specific activity of the enzyme arose from mitotic segregation of any cytoplasmic determinant or simply reflected experimental variations remains undetermined.

Data in Table 3 summarize the results of segregation in a cross between the triply negative *hxk1 hxk2 glk1* mutant 611 with a wild-type strain D-603-1A. Of the 70 four-spore tetrads analyzed, growth on fructose-azide plates indicated the segregation of two unlinked genes, *hxk1* and *hxk2*. The fructose-azide-negative class should contain segregants of *hxk1 hxk2 glk1* as well as *hxk1 hxk2 GLK1* genotypes. To distinguish between these two kinds of segregants, all of the fructose-azide negatives were individually tested for growth in test tubes containing YEP glucose medium. Of the 74 fructose-azide negatives about half (42) grew and were also found to be glucokinase positive by enzyme assay. The remaining (32) did not grow on glucose but grew on galactose as well as

on alcohol. These did not have any glucokinase activity. Thus, 32 of 280 segregants from a cross of *hxk1 hxk2 glk1* × wild type were negative for all glucose-phosphorylating enzyme activity. These data are consistent with three-gene control of growth on glucose: *HXK1*, *HXK2* and *GLK1*.

An additional observation made on these segregants was that resistance to 4 mM 2-deoxyglucose and inability to grow on glucose were fully correlated. All of the spores resistant to 2-deoxyglucose were glucose negative, and all of the glucose-positive ones were 2-deoxyglucose sensitive.

Complementation: A large number of independent *glk1* mutants were tested for intragenic complementation of glucokinase activity in diploids whose general configuration was $\frac{hxk1\text{-}1\ hxk2\text{-}1\ glk1\text{-}1 \text{ to } glk1\text{-}5}{hxk1\text{-}1\ hxk2\text{-}1\ glk1\text{-}6 \text{ to } glk1\text{-}130}$. That is, the first five mutant alleles of *glk1* were crossed individually to 125 independent mutants representing *glk1-6*, *glk1-7* ... and *glk1-130*, respectively. Diploids were obtained on alcohol plates either by complementation of auxotrophic markers or by zygote isolation. The purified diploids were tested for growth on glucose and sensitivity to 2-deoxyglucose; by both of these criteria the diploids were found to be glucokinase negative. Enzyme assays of some of these hybrids confirmed this result, because the enzyme activity in the heteroallelic diploids never exceeded that of the respective haploid or homoallelic diploid parents. In view of the multimeric nature of glucokinase protein (MAITRA and LOBO 1977) this result was surprising, although very reminiscent of the absence of interallelic complementation in *hxk1* and *hxk2* mutants (LOBO and MAITRA 1977b). We consider this result to indicate that the monomeric form of the glucokinase protein must participate at least in one of the elementary steps in the glucokinase-mediated catalysis.

Nonsense mutation: Studies on *glk1* revertants, to be described in a subsequent section, indicate the gene *GLK1* to specify the structure of the glucokinase enzyme. Since the large majority of the mutants had no detectable enzyme activity and they failed to show interallelic complementation, it was expected that some of these mutants might carry translation-terminating codons. Each of 206 *glk1* mutants in our collection had been examined for suppression by the ochre suppressor *SUP7* and the temperature-sensitive amber suppressor *SUP4-3a* (RASSE-MESSENGUY and FINK 1973); a few had been tested also by two other ochre suppressors *SUP4* and *SUP8*. Strains *hxk1-1 hxk2-1 glk1-1* bearing the suppressors were constructed and confirmed to be 2-deoxyglucose resistant. Additional *glk1* alleles were isolated in the nonsuppressible *hxk1-2 hxk2-2* background. The latter group of strains was hybridized to the former, and the diploids were tested for growth on glucose. Only two mutations, *glk1-91* and *glk1-121*, were found to be suppressed by all of the three ochre suppressors, whereas none was suppressed by the amber suppressor. This was confirmed by the coincident suppression of the particular *glk1* allele and the known ochre mutation *lys1-1*. Since the hexokinaseless diploids carrying suppressed glucokinase activity did not sporulate, the following diploids were constructed using a leaky mutant allele *hxk1-20* that allowed their sporulation: $\frac{hxk1\text{-}20\ hxk2\text{-}2\ glk1\text{-}91 \text{ or } glk1\text{-}121 \quad + \quad lys1\text{-}1}{hxk1\text{-}71\ hxk2\text{-}2\ glk1\text{-}1 \qquad\qquad SUP7\ lys1\text{-}1}$. All meiotic segregants which

TABLE 3

Tetrad analysis of a cross between a wild-type strain and a mutant lacking all three glucose-phosphorylating enzymes

Growth on:	Ascus type		
	$4^+:0^-$	$3^+:1^-$	$2^+:2^-$
Alcohol	70	0	0
Fructose-azide	12	42	16

were glucose positive were also lysine independent. Those that required lysine for growth were unable to grow on glucose.

In another experiment a 2-deoxyglucose-sensitive strain of the genotype *hxk1 hxk2 glk1-91 SUP7* was crossed to another sensitive strain of genotype *hxk1 hxk2 GLK1*. Sporulation of this diploid yielded progeny that was resistant to 2-deoxyglucose as follows: three asci of $3^s:1^r$ and two asci of $2^s:2^r$. This showed that the suppression of *glkl1-91* was extragenic.

Reversion: When the glucose-negative triple mutant strains *hxk1 hxk2 glk1* were plated heavily on YEP glucose plates, revertant colonies appeared. The majority were revertants for either of the two hexokinase activities, as tested by examining their growth on YEP fructose plates containing azide. Approximately one-fifth of such revertants failed to grow on fructose but grew on glucose; these were tested for the presence of ATP:sugar-6-phosphotransferase activity using glucose as well as fructose as substrates in toluene lyzates. Those which phosphorylated glucose but not fructose were provisionally considered to be revertants for glucokinase. However, a small fraction of hexokinase revertants at the *hxk1* or *hxk2* locus synthesized an activity for glucose phosphorylation that phosphorylated fructose weakly. When a total of three such revertants were crossed to each of *HXK1 hxk2 glk1* and *hxk1 HXK2 glk1* strains, they turned out to be harboring the reversion mutations at *hxk1* (two revertants) and *hxk2* (one revertant) loci. The alteration in the relative velocity of fructose and glucose phosphorylation reflected a structural defect in hexokinase (LOBO and MAITRA 1977a). In other words, the lack of fructose phosphorylation could not be taken as a criterion for restoration of glucokinase activity in revertants. Therefore, we resorted to genetic analysis. Results in Table 4 illustrate this in respect of glucokinase revertants from three mutant alleles of *glk1*, *viz.*, missense mutations *glk1-1* and *glk1-2* and the nonsense mutation *glk1-91*. The revertant GK2 R48, for example, showed two-gene segregation in crosses with single mutants lacking the hexokinases, whereas no segregation was seen when crossed with the *hxk1 hxk2 GLK1* strain. This showed that GK2 R48 was very likely an intragenic revertant of *glk1-2*. The three revertants from the strain bearing the nonsense mutation *glk1-91* represented three prototype classes: R18 was an intragenic revertant, whereas R22 and R23 were extragenic. The suppressor in R22 appeared to be linked to the centromere, unlike in R23. It should be noted that meiotic analysis of glucokinase revertants was rendered difficult whenever the diploid was hexokinase negative (lines 1, 4, 7, 8, 9 and 10). A large number of tester strains had to be used

TABLE 4

Genetic analysis of revertants of glk1

GK revertant	Crossed to:	Segregation of tetrads		
		$4^+:0^-$	$2^+:2^-$	$3^+:1^-$
GK1 R29	*hxk1 hxk2 GLK1*	29	0	0
GK2 R48	*HXK1 hxk2 glk1*	3	1	11
	hxk1 HXK2 glk1	4	2	15
	hxk1 hxk2 GLK1	9	0	0
GK2 R49	*HXK1 hxk2 glk1*	3	4	9
	hxk1 HXK2 glk1	3	2	8
	hxk1 hxk2 GLK1	20	0	0
GK91 R18	*hxk1 hxk2 GLK1*	7	0	0
GK91 R22	*hxk1 hxk2 GLK1*	2	5	3
GK91 R23	*hxk1 hxk2 GLK1*	1	2	12

GK1 R29 designates revertant no. 29 from mutant *glk1-1*, GK2 R48 revertant no. 48 from mutant allele *glk1-2*, etc. Their genotypes were: *hxk1 hxk2 glk1* (*sup*). Tetrads were germinated on YEP alcohol and scored for growth on glucose.

in these experiments because most, but not all, such diploids failed to sporulate.

Do the glucokinase revertants resemble the wild-type strain in regard to the glucokinase they synthesize? Crude cell-free extracts from revertants were examined for a number of distinguishing characteristics of glucokinase: high affinity for glucose, a pronounced protection against thermolability conferred by glucose, and the relative velocity of phosphorylation of several sugar substrates of this enzyme (MAITRA 1970). We have examined a large number of spontaneous and mutagen (ethyl methane sulfonate; *N*-methyl *N'*-nitro *N*-nitrosoguanidine; ultraviolet radiation)-induced revertants from at least 30 independently derived *glk1* mutants for several of these properties. The specific activities and catalytic properties of glucokinase synthesized by most revertants were similar to those of the wild-type strain. However, two revertants, R48 and R49 from *glk1-2*, were found to synthesize altered enzymes. The glucokinase from these revertants as well as from a *hxk1 hxk2 GLK1* strain was partially purified by ammonium sulfate fractionation followed by a gel filtration step using Sephadex G200 to ensure absence of any hexokinase activity (MAITRA 1975). The enzyme preparations were examined for their sedimentation properties (MAITRA 1970) and several kinetic characteristics. Results shown in Table 5 indicate that, unlike the revertants GK1 R15 and GK2 R50, revertants R48 and R49 from *glk1-2* synthesized perceptibly altered enzyme species whose affinity for glucose was reduced by one or two orders of magnitude. The observation that their sedimentation characteristics were similar to that of glucokinase ensured that no artifact of contamination of either hexokinase P1 or P2 was involved. The fact that altered glucokinase was obtained by intragenic suppression (Table 4) is a strong argument for the idea that GLK1 specifies the glucokinase structural gene and renders unlikely the possibility that it codes for a modifying enzyme required for glucokinase activity.

Sporulation: An observation made in the course of these experiments was

that loss of hexokinase activity in diploids led to a pronounced reduction in their ability to sporulate. Results in Table 6 show that either of the hexokinases P1 and P2 is necessary for sporulation. In the absence of all of three enzymes for the phosphorylation of glucose, sporulation was completely lost (line 6). Presence of *GLK1* either in one copy or in two copies led to a marginal increase in sporulation.

Chromosome loss: The frequency of second division segregation of *glk1* measured with respect to *trp1* on chromosome *IV* was 71% (45 of 63). Mitotic experiments were, therefore, undertaken to determine its chromosomal location. A diploid homozygous for *hxk1* and *hxk2* but heterozygous for *glk1* was sensitive to 2-deoxyglucose. Induction of mitotic crossovers would lead to homozygosity of this marker coincident with other known markers located on the same chromosome arm (MORTIMER and HAWTHORNE 1973). A large number of diploids were constructed by mating the hexokinase mutant strain D3010 with 2-deoxyglucose-resistant haploid strains (*hxk1 hxk2 glk1*) carrying nutritional markers on various chromosomes. The diploids of the constitution $\frac{\textit{hxk1-2 hxk2-2 glk1-1 trp1 thr4 his4 leu2 ura3}}{\textit{hxk1-2 hxk2-2} \quad + \quad + \quad + \quad + \quad + \quad +}$, etc., were exposed to ultraviolet light for approximately 70% survival and plated on YEP alcohol medium containing 4 mM 2-deoxyglucose, and the mitotic progeny was analyzed to determine which of the recessive nutritional markers such as *trp1*, *thr4*, etc., were expressed. Such experiments located *glk1* on the left arm of chromosome *III*, because a significant fraction of the 2-deoxyglucose-resistant mitotic progeny became auxotrophic for both histidine (*his4*) and leucine (*leu2*) markers, whereas some resistant clones were auxotrophic for histidine alone. The yield of deoxyglucose-resistant clones ranged from a frequency of 5×10^{-6} to 7.7×10^{-5} of the input sensitive cells depending on the diploid used. A typical diploid had the following genotype:

$$\frac{\mathbf{a} \quad + \quad + \quad + \quad + \quad \textit{hxk1-2 hxk2-2 ura3 met14} \quad +}{\alpha \quad \textit{glk1} \quad \textit{his4} \quad \textit{leu2 thr4} \quad \textit{hxk1-2 hxk2-2} \quad + \quad + \quad \textit{lys2}}.$$

The unexpected observation was that about 20 to 50% of the 2-deoxyglucose-resistant cells obtained from such diploids were not only auxotrophic for both histidine (*his4*) and leucine (*leu2*) markers on the left arm of chromosome *III* but also expressed the recessive marker *thr4* on the right arm of this chromosome (Figure 2). However, they remained prototrophic for markers on other chromosomes. When examined for their mating proficiency these clones behaved as haploids, mating as α in the above example, or as **a** haploids when the glucokinase-positive parent was of the α mating type. This indicated that the entire homologue of chromosome *III* carrying *GLK1* was lost in these mitotic progenies, generating strains monosomic for this chromosome. That these strains were diploids for other chromosomes was shown by the recovery of recessive markers such as *ura3*, *lys2* in crosses with haploid strains prototrophic for these markers. In a total of 56 tetrads dissected from chromosome *III* monosome *X* haploid crosses as few as six diads and ten single spores could

TABLE 5

Properties of glucokinase from revertants and the wild-type strain

Strain	$S_{20,w}$	K_m, mM		$t_{1/2}$, min at 44°	
		Glucose	ATP	−Glucose	+Glucose 10 mM
Wild type	8.0	0.02	0.05	3	>50
GK1 R15		0.03	0.05	2.5	>50
GK2 R50		0.02		1.5	>50
GK2 R48	8.1	3.30	0.55	0.3	0.3
GK2 R49	7.6	0.25	0.67	1	5

TABLE 6

Effect of glucose-phosphorylating enzymes on sporulation

Genotype of diploid			% Sporulation
hxk1	*hxk2*	*glk1*	
+ / +	+ / +	+ / +	30–95
+ / −	+ / −	+ / −	45
− / −	+ / −	− / −	37
− / −	− / −	+ / +	1–3
− / −	− / −	+ / −	0–1
− / −	− / −	− / −	0

Sporulated cultures from potassium acetate-yeast extract plates were examined microscopically for asci until the 7th day of incubation at 30°. At least 1000 cells were counted.

be recovered. The poor viability of spores from such crosses was also consistent with this conclusion.

From 287 2-deoxyglucose-resistant clones obtained from four different diploids such as that shown (having identical chromosome *III* markers but varying with respect to auxotrophic markers on the other chromosomes), 114 were found to be chromosome *III* monosomes. The remaining 173 clones behaved as $\frac{\mathbf{a}}{\alpha}$ diploids in which mitotic crossover had taken place on the left arm of chromosome *III*. The distribution of crossovers was as follows: between *his4* and *glk1*, 148; between *leu2* and *his4*, 11; between the centromere and *leu2*, 13; and double crossover in the regions *leu2-his4* and *his4-glk1*, 1. These data accord with the map order: *leu2—his4—glk1*.

Meiotic mapping of glk1: Since hexokinaseless diploids sporulate poorly, the meiotic mapping of *glk1* was done by incorporating one of the hexokinase genes

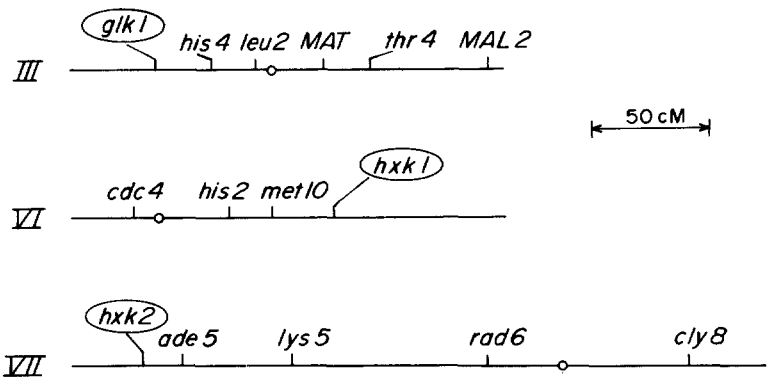

FIGURE 2.—Chromosomal locations of the structural genes for hexokinases P1 (*hxk1*) and P2 (*hxk2*) and glucokinase (*glk1*) in *S. cerevisiae*. The map position of *hxk1* is from LOBO and MAITRA (1977a).

hxk1 or *hxk2* in the heterozygous state. This permitted sporulation of diploids heterozygous for *glk1*. Tetrads segregating *glk1* and *hxk1* in PD and NPD configurations were selected and examined for the two nutritional markers *his4* and *leu2* on the left arm of chromosome *III*. The tetratype class was omitted since the two genotypes, *HXK1 glk1* and *HXK1 GLK1*, could not be distinguished on the basis of growth. The tetrads were also tested on YEP alcohol plates containing 1 mM 2-deoxyglucose; spores that did not grow on this plate were invariably able to grow on either glucose or fructose plates depending on the parental or nonparental configuration of *hxk1* and *glk1* genotype. The diploid had the genotype $\frac{glk1 \quad hxk1 \quad hxk2 \quad lys10 \quad + \quad + \quad +}{+ \quad + \quad hxk2 \quad + \quad his4 \quad leu2 \quad thr4}$. Results in Table 7 confirm the linkage of *glk1* to *his4*. The NPD tetrads between *glk1-his4* and *glk1-leu2* helped to order these three markers unambiguously. The one that was NPD for *glk1-his4* was also NPD for the marker pair *glk1* and *leu2* but PD for *his4-leu2*. The other tetrad that was NPD for *glk1-leu2* showed a tetratype combination between both *glk1-his4* and *his4-leu2*. Inspection of the individual T tetrads between *glk1-his4* and between *his4-leu2* was also consistent with the order: *glk1—his4—leu2*. The marker *glk1* has not been mapped with respect to *HML*, but a comparison of reported distance from *his4* of both this marker and of *glk1* (MORTIMER and SCHILD 1980) suggests these two genes to be closely linked.

Figure 2 summarizes the chromosomal locations of the structural genes responsible for glucose phosphorylation.

Interallelic crosses between glucokinase mutants: Diploids were constructed by micromanipulation of a mating mixture of a number of *glk1* allele pairs of the genotype *hxk1-2 hxk2-2 glk1*. These were grown up in YEP alcohol plates containing 4 mM 2-deoxyglucose, washed and suspended in water before plating on YEP glucose. Mitotic crossing over was induced with ultraviolet irradiation for 20 sec which gave 85% survival on YEP alcohol plates. The fraction of

TABLE 7

Meiotic linkage of glk1 *to* his4 *and* leu2

Gene pair	Tetrad class			Map distance (cM)
	PD	NPD	T	
glk1–his4	43	1	29	24
glk1–leu2	20	2	48	43
his4–leu2	44	0	30	20

Only asci with PD and NPD association of *glk1* and *hxk1* were used.

survivors able to form colonies on YEP glucose plates was determined on the fifth day. The individual haploids were also plated the same way after ultraviolet treatment to test reversion frequency. Revertants arose at frequencies no higher than 1% of that of mitotic recombination for the alleles chosen. The reversion frequency was ignored in the calculation of results (Figure 3).

The frequency of mitotic crossing over between sites of mutation in the *glk1* gene provides a qualitatively consistent map of this gene despite quantitative ambiguities. Of interest is the observation on the location of the two nonsense mutations defined by alleles *glk1-91* and *glk1-121*. They map closer to each other than does any other pair of alleles and constitute the two terminal loci among the set of six mutant sites tested here.

DISCUSSION

The experiments described here provide fairly firm evidence for the conclusion that the three enzymes, hexokinase P1, hexokinase P2 and glucokinase, are the only instruments for glucose phosphorylation in *S. cerevisiae*. Reversion of triply negative mutants for growth on glucose led invariably to the restitution of one of these three enzymes. Thus, it is unlikely that yeast has any other enzyme for glucose phosphorylation such as the bacterial phosphotransferase system (KUNDIG, GHOSH and ROSEMAN 1964). Why this organism should have three enzymes for phosphorylating glucose where one of the hexokinases could cover the spectrum of sugar substrates is not clear. Since many of the glycolytic steps are catalyzed by a single enzyme, the idea that multiplicity ensures against accidental loss is not convincing. On the other hand, it is possible that each of them has some special function in cell physiology; our early attempts (LOBO and MAITRA 1977c), however, failed to uncover any such feature.

Perhaps the most striking result seen in this study was the great paucity of nonsense mutations in these genes. Examination of a large number of mutations in *hxk1* and *hxk2* genes described earlier (LOBO and MAITRA 1977b) revealed only one of 130 *hxk1* mutants, *hxk1-61*, to be suppressible by ochre suppressors. No suppression, either ochre or amber, was seen among 220 independent isolates of *hxk2* mutation. The observation that a number of ochre nonsense mutants were indeed obtained demonstrates that our failure to find larger frequencies of nonsense mutations was not due to methodological or trivial reasons such as the presence of a resident suppressor in the strains. Among a collection of 50 *pyk1*

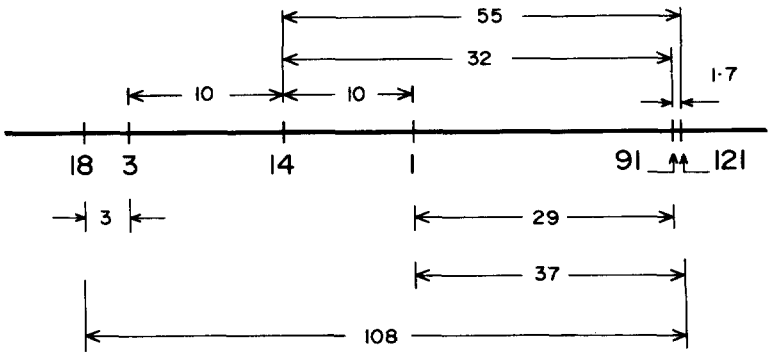

FIGURE 3.—A fine structure map of the gene *GLK1* for glucokinase of *S. cerevisiae*. The bold-face numerals 18, 3, 14, 1, 91 and 121 represent mutant alleles and the smaller sized numerals indicate the number of glucose-positive recombinants from 1000 cells of the heteroallelic diploids.

mutants lacking the glycolytic enzyme pyruvate kinase, none could be suppressed by nonsense suppressors (Z. LOBO, unpublished observation). Yeast genes are known to vary a great deal in their yield of nonsense mutations. This has been discussed by SNOW (1978), CHATTOO *et al.* (1979) and DONAHUE and HENRY (1981). Certain genes such as *his1* and *ino1* are characterized by having no nonsense mutations at all, whereas for certain others such as *ura2* the frequency can be as high as 100%. The genes discussed here, *viz.*, those responsible for specifying the structure of the glucose-phosphorylating enzymes, seem to belong to the former category, the difference being that we have examined a larger collection of mutants. Could this be due to a second essential function encoded in these genes? Our experiments have not uncovered any such function as being controlled by these genes. However, recent work by ENTIAN (1981) suggests that hexokinase P2, in association with the product from an unlinked gene, *HEX2*, is involved in catabolite repression in yeast and may in fact be a bifunctional protein. This is consistent with the observation that no nonsense mutation of *HXK2* has been seen among 220 isolates. In *GLK1* the only two nonsense mutations, alleles *91* and *121*, happen to constitute the terminal markers among a set of six mutational sites examined. In the absence of any evidence for the presence of cross-reacting proteins in these mutants, the question of whether the nonsense mutations mark the start or the terminus of the gene cannot be settled. If the nonsense alleles define the end of the gene, and the cross-reacting material test is positive, this would argue in favor of the idea that the glucokinase polypeptide serves a second, indispensable function. Our attempts to isolate nonsense mutations by looking for 2-deoxyglucose-resistant derivatives of singly heterozygous diploids, such as, $\frac{hxk1 \quad hxk2 \quad glk1}{hxk1 \quad hxk2 \quad +}$ were not successful; most such clones turned out to be mitotic crossover products involving the mutant gene *glk1*, whereas the others had lost chromosome *III* of the *GLK1* parent.

A number of spontaneous mutants lacking the hexokinases and glucokinase were obtained during this work. Some of these were unstable mutations, reverting to glucose positivity at frequencies of the order of 10^{-2}. It remains to be seen

whether these are mediated by insertion sequences of the kind described by CHALEFF and FINK (1980).

The lack of sporulation in diploids lacking the hexokinases remains unexplained. Diploid strains of *S. cerevisiae* were induced to sporulate in liquid sporulation media during aerobic incubation and the cell-free sporulation media examined for glucose and fructose after 3 days. When the diploid $\frac{hxk1\ \ hxk2\ \ glk1}{hxk1\ \ +\ \ glk1}$ sporulated the medium contained approximately 0.25 mM glucose and 0.02 mM fructose. In a similar experiment with a nonsporulating diploid lacking the hexokinases and heterozygous for glucokinase the medium contained 1.5 mM glucose and 0.1 mM each of fructose and mannose. The content of glucose and fructose in acid extracts of the incubated diploid cells was also in nearly the same ratio. That is, the requirement of hexokinase for sporulation was not reflected in the pool sizes of fructose either in the cells or in the sporulation medium.

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APPENDIX

Mapping of hxk2, *a correction*

An earlier report by us (LOBO and MAITRA 1977a) had placed the gene *hxk2* on the chromosome *III* right arm distal to *MAL2*. We have since discovered this result to be erroneous due to several unrelated reasons: (1) the disomic strain 5B IV was possibly also a chromosome *VII* disome; (2) the putative mitotic crossover at the mating-type locus was more likely due to loss of one of the homologues of chromosome *III*; (3) the increased α-glucosidase activity in *hxk2* mutants (ENTIAN 1981) led to a spurious meiotic linkage between this gene and *MAL2*. The following table shows the experiments on the meiotic mapping of *hxk2* and places this gene instead on the chromosome *VII* left arm in the order: centromere—*lys5*—*ade5*—*hxk2*.

Meiotic mapping of hxk2

Gene pair	Tetrad classes			Distance (cM)
	PD	NPD	T	
hxk2-ade5	41	0	21	17
hxk2-lys5	11	4	55	56
ade5-lys5	17	3	44	48

The parental association in the diploid was

hxk1	*glk1*	*hxk2*	*lys5*	*ade5*
hxk1	*glk1*	+	+	+