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The 15 N-Terminal Amino Acids of Hexokinase II Are Not Required for In Vivo Function: Analysis of a Truncated Form of Hexokinase II in *Saccharomyces cerevisiae*

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ABSTRACT **The function of the N-terminal amino acids of *Saccharomyces cerevisiae* hexokinase II was studied in vivo using strains producing a form of hexokinase II lacking its first 15 amino acids (short form). This short form of hexokinase II was produced from a fusion between the promoter region of the *PGK1* gene and the *HXK2* coding sequence except the first 15 codons. As expected, the in vitro analysis of the short form protein by gel filtration chromatography indicates that the short protein does not form dimers under conditions where the wild-type protein dimerizes. Kinetic studies show that the enzymatic activities are very similar to wild-type behavior. The physiological experiments performed on the strains containing the fusion allele demonstrate that the short form of the enzyme is similar to the wild-type both in terms of phosphorylation of hexoses and glucose repression. We conclude that the N-terminal amino acids of hexokinase II are not required in vivo either for phosphorylation of hexoses or for glucose repression.**

Key words: yeast hexokinase II, dimerization, in vivo functions, glucose repression

INTRODUCTION

In the budding yeast *Saccharomyces cerevisiae*, hexokinase II is required for glucose repression[1,2] and, in the absence of hexokinase I, for growth on fructose.[3,4] In vitro results indicate that hexokinase II exists in a monomer dimer equilibrium that can vary with the assay conditions.[5,6] One hypothesis to explain glucose repression for several genes is that it involves alterations in the enzyme's monomer dimer equilibrium. Hexokinase II monomers are the catalytically active species in in vitro assays[7]; the active form in vivo has not been identified. However, the native intact hexokinase II dimerizes over a broad pH and concentration range.[8,9] A shortened enzyme missing its N-terminal 11 amino acids, resulting from mild in vitro proteolysis, exists in the monomer form unless the pH is below 5.5.[8,10,11] The proteolyzed form is enzymatically active and in vitro it has specific activities[12] similar to native isoenzymes. Since the physiological significance of the N-terminal amino acids is not clear, we have now analyzed glucose repression and in vivo phosphorylation of hexoses when the N-terminal amino acids have been removed by genetic deletion.

We constructed a fusion of the yeast *PGK1* promoter to the *HXK2* gene coding sequence beginning at the sixteenth codon, ATG, for the purpose of examining the in vivo role of the N-terminal amino acids directly. The first 15 amino acids were removed because that is the most straightforward limited deletion construction to make. We anticipated that the effects of removing the N-terminal amino acids would be apparent if either 11 or 15 amino acids were removed. Here we report that we can find no phenotypic differences between wild-type and strains expressing only the shortened hexokinase II enzyme.

MATERIALS AND METHODS

Strains and Plasmids

The yeast strains used in this study are isogenic to S288C (*MAT*a *SUC2 gal2*). The null alleles of the hexokinase genes, *hxk1::LEU2* and *hxk2-202*, have been described before.[2] The plasmid pRB312 is a derivative of YCp50 (C. Mann, personal communication; see Fig. 1 in ref. 13 for a restriction map) with a 3.7 kb *Eco*RI *HXK2* fragment subcloned into the *Eco*RI site (Fig. 1). The plasmid pCGS521 was kindly provided by Dr. Don Moir at Collaborative Research, Inc. It is a derivative of YEp420 (previously called β72; see Fig. 1 in ref. 13 for a restriction map) having a *Hin*dIII–*Nco*I fragment of the yeast

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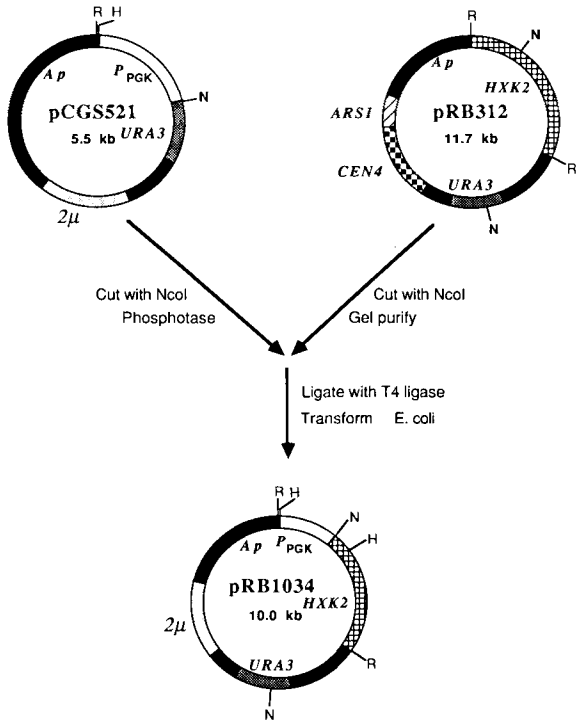

Fig. 1. Construction of P_{PGK}–*HXK2* fusion. The *Nco*I fragment containing most of *HXK2* gene, pBR322 sequence and the N-terminal portion of the *URA3* gene was released from pRB312, and ligated to pCGS521 cut with *Nco*I and treated with calf intestinal phosphatase. The product pRB1034 carries a *PGK1–HXK2* fusion and a functional *URA3* gene. The solid bars are pBR322 sequences, the other sequences are individually indicated. Restriction sites: H, *Hin*dIII; N, *Nco*I; R, *Eco*RI.

PGK1 promoter region with an engineered *Nco*I site, replacing the vector fragment from the *Hin*dIII site of pBR322 sequence to the *Nco*I site of the *URA3* gene (Fig. 1); the *PGK1* promoter is fused to a large C-terminal portion of the *URA3*.[14] Because the *URA3* part of the fusion lacks the N-terminal 208 base pairs of the coding region, the plasmid does not confer uracil prototrophy.

Construction of a Fusion Between *PGK1* Promoter and *HXK2*

In vitro DNA manipulations were performed as previously described.[15] In order to make the construction for in vivo production of a shortened form of hexokinase II, we used a convenient *Nco*I site at codons for amino acid residues 15–17 of *HXK2* to make a fusion between the promoter of *PGK1* and the coding region of *HXK2*. As shown in Figure 1, the plasmid pRB312 was cut with *Nco*I endonuclease to release a 4.5 kb *Nco*I fragment containing most of the *HXK2* coding and 3′ noncoding regions, as well as the 5′ noncoding region and N-terminal part of the coding region of *URA3* sequence and the intervening pRB312 sequence. This fragment was separated from the other fragment by electrophoresis in low-melting-point agarose, and ligated to *Nco*I cleaved pCGS521 in melted agarose according to Struhl.[16] The *PGK1–HXK2* junction was verified by sequencing. The *URA3* was restored as indicated by the fact that the plasmid confers URA^+ phenotype to cells of chromosomal *ura3-52* background.

Construction of Other Plasmids by Recombination in Yeast

In order to examine the phenotypes of strains with different amounts of either wild-type or the short form of hexokinase II, we constructed plasmids of different copy number carrying the wild-type *HXK2* gene or the *PGK1–HXK2* fusion. Various YCp50, YEp420 derivatives for this purpose were constructed by an in vivo method based on homologous recombination in yeast, as previously described.[13] Figure 2 illustrates how such a construction is carried out. The preparation of these plasmids is summarized in Table I. The plasmids were recovered from yeast transformants and their structures confirmed by restriction analysis.

Yeast Transformation and Plasmid Recovery From Yeast

Yeast transformation was done essentially according to the modified procedure[17] of the alkali cation method.[18] Plasmids were recovered from yeast strains essentially as described by Hoffman and Winston.[19] The aqueous supernatant from the yeast prep was used to transform *E. coli* cells.

Hexokinase Purification

Both the wild-type and short form hexokinase II were purified using the following procedure. Cells containing a single form of hexokinase were grown to early stationary phase and then harvested. The cells were washed with water twice and with 50 mM Tricine pH 8.0 buffer once. The pellet was then resuspended in freshly prepared 50 mM Tricine pH 8.0, 8 mM PMSF (1:20 dilution of PMSF stock in ethanol), and transferred to a glass-bead mill (The Bead-Beater™, Biospec Products, P.O. Box 722, Bartlesville, Ok, 74003, 918-333-2166) 80 ml chamber with an ice-water jacket. About 45 ml of acid-washed glass beads was added to the chamber. The cells were then subjected to five to seven 1 minute pulses of bead-mill homogenization, with 3–5 minutes in between pulses to avoid overheating the homogenate. The cells were checked for cell lysis under a light microscope, and more than 90% of the cells were lysed. The homogenate was recovered; the glass beads were washed with additional buffer and the wash was combined with the homogenate. The total lysate from 1 liter culture was about 40–50 ml. The lysate was centrifuged at 15,000 rpm and 4°C for 30 minutes. The supernatant of 30–40 ml was saved as extract; it can be frozen at −78°C until the column is run. The extract is treated with 1 ml of 80 mg/ml protamine sulfate for every 40 ml of extract and in-

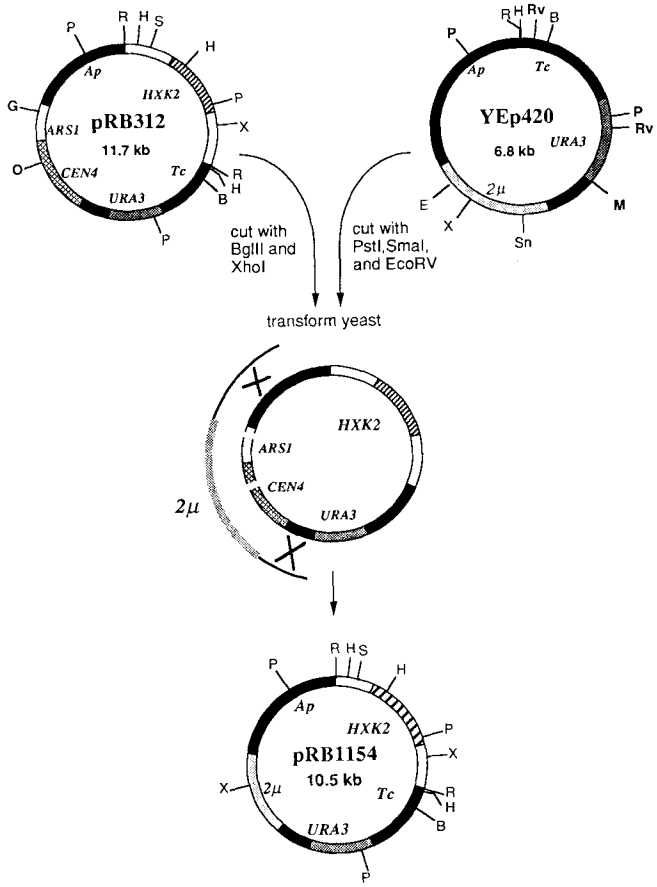

Fig. 2. Scheme for plasmid construction in yeast. A *ura3 hxkl hxk2* yeast strain was transformed with pRB312 linearized by cutting at *Bgl*II and *Xho*I sites, and with a fragment containing 2μm sequence from YEp420 generated by cutting at *Eco*RV, *Pst*I, and *Sma*I. Ura$^+$ transformants were selected and plasmids were recovered from these transformants. Restriction analysis indicated that they have the expected structure. The solid bars are pBR322 sequences, where homologous recombination occurs; the open bars are 5′ and 3′ regions at the *HXK2* locus; the other sequences are indicated specifically. Restriction site keys: B, *Bam*HI; G, *Bgl*II; H, *Hind*III; M, *Sma*I; O, *Xho*I; P, *Pst*I; R, *Eco*RI; Rv, *Eco*RV; S, *Sac*I; X, *Xba*I.

cubated on ice for 20–40 minutes; the extract is clarified by centrifugation at 11,000 rpm for 15 minutes. Then 1 M $MgCl_2$ was added to bring the extract to a final concentration of 50 mM in $MgCl_2$, before loading the extract from 1–2 liter culture onto a 50 ml Amicon Red dye column preequilibrated with 50 mM Tricine, 50 mM $MgCl_2$ pH 8.0 buffer at a flow rate of 2 ml/minutes. After loading, wash column with at least 2 bed volumes of 50 mM Tricine, 50 mM $MgCl_2$ pH 8.0. Elute the hexokinase II with 50 mM Tricine pH 8.0 (no Mg) and collect fractions. Hexokinase II elutes as soon as the $MgCl_2$ is washed out of the column. Elution with a shallow salt gradient produces a sharper peak, but several contaminating proteins also come off in higher concentrations. The hexokinase II can be further purified by passing it through the column again under the same conditions. Hexokinase II containing fractions were determined by activity assays and frozen at −78°C for storage.

Gel Filtration

Purified hexokinase II wild-type and purified short form were analyzed by gel filtration chromatography. Protein samples (200 μl) in 50 mM Tricine pH 8.0 were run through a Sepharose 12 HR 10/30 column (Pharmacia, Inc.) equilibrated in 50 mM Tricine pH 8.0 at a flow rate of 0.75 ml/minute. The column was standardized with individual samples of blue dextran, ovalbumn, bovine serum albumin, and catalase (Pharmacia Gel Filtration Standards) prepared in 50 mM Tricine. The concentrations of the samples applied to the column were as follows: wild-type = 73 μg/ml, short form = 17 μg/ml, and purchased proteolyzed enyzme = 109 μg/ml (Sigma, C302). The apparent molecular weights of the samples were calculated based on the standard curve generated using the molecular weights of the standards and their retention times according to the formula $[K_{av} = (V_e - V_0)/(V_t - V_0)]$ (Pharmacia Gel Filtration Calibration Kit–Instruction Manual).

Enzyme Assays

In order to determine the levels of hexokinase II and invertase, cells were grown in media containing 5% glucose, fructose, or mannose and harvested in the mid-exponential phase (OD_{600} of 1.0–2.0). Invertase activity was measured on whole cells according to Goldstein and Lampen.[20] Hexokinase activity was assayed on crude extracts and purified enzymes essentially as previously described,[21] with the slight modifications of volume and concentrations of reagents. For each hexokinase sample, the following were included in the assay reaction mix: 500 μl of 50 mM triethanolamine, pH 7.6; 500 μl of 100 mg/ml sugar in 50 mM triethanolamine; 40 μl of 10 mg/ml ATP, Na salt; 40 μl of 10 mg/ml NADP, Na salt; 10 μl of 1 M $MgCl_2$; and 1 μl of 1 mg/ml glucose-6-phosphate dehydrogenase. For fructose as substrate, 1.5 μl of 2 mg/ml phosphoglucose isomerase was also added. The protein content was determined by the Lowry method,[22] the Bio-Rad Bradford protein assay,[23] or by measuring the absorption of the purified enzyme at 278 nm. Kinetic experiments were done at 30°C.

Gel Electrophoresis and Western Blots

Western blotting was performed according to Burnette.[24] Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed following the Laemmlli[25] protocol and DNA gel electrophoresis was performed using TBE buffer according to Maniatis et al.[15]

Chemicals and Enzymes

Most chemicals are purchased from Sigma Chemical Co. NADP, glucose-6-phosphate dehydrogenase, and phosphoglucose isomerase are from Boehringer

TABLE I. Construction of Derivatives of YCp50 and YEp420 Containing Different Alleles of *HXK2*

New plasmid	Linearized Plasmid		Homologous fragment		Selection‡
	Plasmid*	Cut with	Plasmid†	Cut with	
pRB1154	pRB312	*Bgl*II, *Xho*I	YEp420	*Eco*RV, *Pst*I, *Sma*I	Ura$^+$
pRB1168	pRB1034	*Spe*I	YCp403	*Eco*RI, *Bam*HI	Ura$^+$
pRB1169	pRB1034	*Sna*BI	YCp403	*Eco*RI, *Bam*HI	Ura$^+$

*These are the plasmids from which the selective marker and *HXK2* in the products are derived.
†These are the plasmids from which the *CEN4 ARS1* or 2μm sequences in the products are derived.
‡The transformants were selected on media lacking uracil.

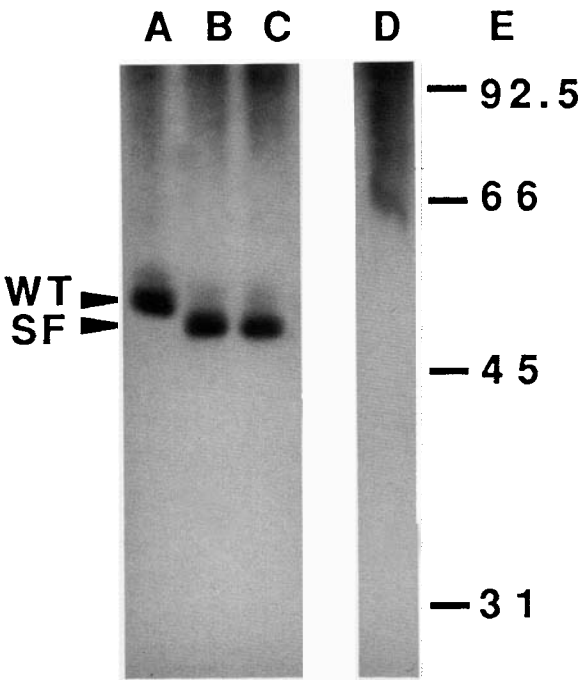

Fig. 3. Autoradiogram of an immunoblot of extracts containing either wild-type or short form hexokinase II. Extracts from (A) wild type, (B) + (C) the short form, and (D) a null mutant were loaded on a polyacrylamide gel. A Western blot experiment was performed as described in Materials and Methods using a polyclonal and antihexokinase II antibody. WT indicates the position of wild-type hexokinase II and SF is the short form. The molecular weight standards are in lane E.

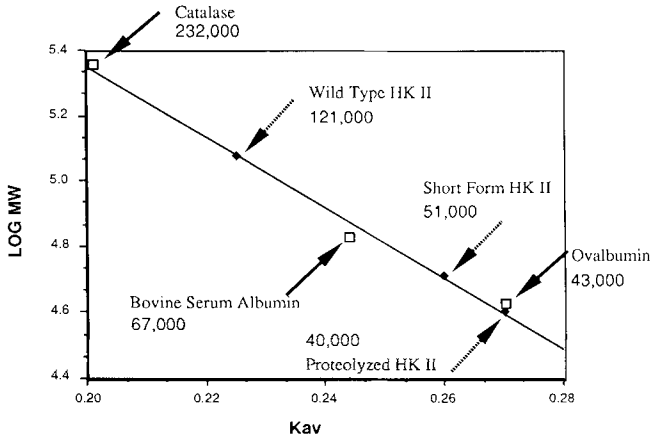

Fig. 4. Standard curve for gel filtration based on the mobility of catalase, bovine serum albumin, and ovalbumin. The calculated molecular weights for wild-type hexokinase II, short form hexokinase II, and proteolyzed hexokinase II are indicated.

Mannheim Biochemicals. The ^{125}I-labeled protein A is from Amersham.

RESULTS

Construction of a Fusion Between the *PGK1* Promoter and *HXK2*

It is known that mild proteolysis during or after isolation of the yeast hexokinase II protein can lead to cleavage of its first 11 amino acids. In order to test the function of the hexokinase II N-terminal amino acids in vivo, we constructed a fusion between the *PGK1* promoter and the *HXK2* coding sequence beginning with amino acid sixteen (Fig. 1), as described in the Materials and Methods section. The construction of plasmid pRB1034 also restored a functional *URA3* gene. The predicted nucleotide sequence at the fusion junction was confirmed by sequence analysis (data not shown).

The fusion construction was used to transform a *ura3* yeast strain with *hxk1 hxk2* double null mutations, selecting for Ura$^+$. Crude protein extracts from these cells were compared to the wild-type protein by Western blot analysis, and the results indicate that the fusion produces a shorter form of hexokinase II as expected (Fig. 3).

The Short Form of Hexokinase II Is a Monomer at pH 8.0 in 50 mM Tricine

Purified hexokinase II enzymes from both the wild-type and fusion containing strains were analyzed by gel filtration. Under these conditions, the wild-type enzyme behaved as a dimer; the short form enzyme from the fusion was a monomer, as was the purchased proteolyzed form (Fig. 4).

The In Vitro Activity of the Short Form

Both wild-type and the short form hexokinase II were studied to determine the K_m values for glucose, fructose, and mannose. The specific activity of each form was also obtained. The kinetic properties for wild type and short form hexokinase II are shown in Table II. The results indicate that the short form is very much like the wild-type in every respect.

TABLE II. Enzymatic Properties of Wild-Type and Short Form Hexokinase II

Substrate	K_m (mM)*		Specific activity† (unit/mg)	
	Wild-type	Short form	Wild-type	Short form
Glucose	0.31 ± 0.03	0.23 ± 0.01	730	590
Fructose	2.2 ± 0.2	2.4 ± 0.1	1030	708
Mannose	0.08 ± 0.01	0.067 ± 0.003	133	211

*The K_m values were calculated using linear regression with the Macintosh statistic program, StatWorks™. The errors reflect the degree of deviation of data from the fitted straight line; the actual errors may be larger.
†The specific activity is calculated from V_{max} and enzyme concentration.

TABLE III. Growth Rates of Cells Contaning Wild-Type or Shortened Forms of Hexokinase II

Plasmid*	Hexokinase II	Doubling time† (hours)		
		On glucose‡	On fructose‡	On mannose‡
pRB312 Cen	Wild-type	2.00 ± 0.03	2.06 ± 0.02	2.91 ± 0.04
pRB1154 2μm	Wild-type	1.94 ± 0.01	2.01 ± 0.06	2.76 ± 0.06
pRB1168 CEN	Short	2.04 ± 0.05	2.44 ± 0.08	3.17 ± 0.10
pRB1169 CEN	Short	2.06 ± 0.04	2.24 ± 0.05	3.11 ± 0.07
pRB1034 2μm	Short	1.97 ± 0.05	1.93 ± 0.04	2.73 ± 0.09
YEp420 2μm	Null	5.3 ± 0.2	No growth	No growth

*The cells have null mutations in the chromosomal copies of *HXK1* and *HXK2*. The CEN plasmids are derivatives of YCp50; the 2μm plasmids are derivatives of YEp420.
†The values of doubling time are from growth curves; cell density was followed by Klett meter turbidity measurements. The errors reflect the degree of deviation from the fitted straight line; the actual errors may be greater.
‡These are the carbon source in the media. The media contained 0.2% cas amino acid and lacked uracil.

TABLE IV. The Effect of the Removal of First 15 Amino Acid Residues on Glucose Repression

Plasmid*	Hexokinase II form†	Hexokinase activity‡			Invertase Activity§		
		Gluc**	Fruct	Mann	Gluc	Fruct	Mann
pRB312 CEN	Wild-type	2.4	2.8	3.3	2.5	7.0	21
pRB1135 CEN	Short	1.9	2.8	1.4	2.3	2.8	18
pRB1034 2μm	Short	1.9	2.9	3.0	1.1	1.3	30
YCp50 CEN	Null	0.0	0.0	0.0	90	50††	80††

*The cells containing these plasmids have null mutations in the chromosomal copies of the *HXK1* and *HXK2* genes. The CEN plasmids are YCp50 derivatives; the 2μm plasmid is a YEp420 derivative.
†These are the only forms of hexokinase in the cells.
‡Hexokinase activity was assayed using fructose as substrate. One unit = μmol of glucose phosphorylated/min/mg of total protein.
§Invertase activity was assayed using whole cells. One unit = 1 μg of glucose produced/min/1 ml cells of 1 OD_{600}.
**These refer to the carbon sources used in media: gluc = glucose, fruct = fructose, mann = mannose.
††These results were obtained by assaying cells for invertase activity after they were incubated in either 5% fructose or mannose containing medium for several hours.

Growth and Glucose Repression

In order to study the in vivo functions of the short form of hexokinase II from the fusion of the *PGK1* promoter to *HXK2*, we examined the growth of cells containing either the wild-type or the short form of hexokinase II using three different substrates of hexokinase: glucose, fructose, or mannose, as the carbon source. Table III shows the results from this experiment. The short form can provide the catalytic activity for growth on these sugars. Strains without hexokinase failed to grow on either fructose or mannose, but the strains with the short form can grow quite well, as rates similar to those of wild-type.

For the purpose of analyzing the effect of removing the first 15 amino acid residues on glucose repression, we have also determined the levels of invertase activity in cells containing various amounts of the two forms of hexokinase II. Table IV shows that the short form can provide glucose repression, with 1 to 2 units of invertase when cells were grown on glucose as does the wild-type. When cells were grown on fructose, both wild-type and the short form hexokinase II have higher invertase levels because fructose is less repressive; mannose is even less repressive then fructose.

DISCUSSION

The results reported here show that a short form of hexokinase II, with the N-terminal 15 residues deleted, produced from a fusion between *PGK1* pro-

moter and *HXK2*, is able to provide cells with sufficient catalytic activity to grow on glucose, fructose, or mannose at rates that are not much different from wild-type yeast cells. Cells can grow on glucose without hexokinase because of glucokinase; nevertheless, in the absence of hexokinase, cells grow much more slowly than in its presence (last line in Table III). Growth on fructose absolutely requires hexokinase function, as indicated by the failure to grow of the strain without hexokinase. Mannose is a substrate of glucokinase,[4] however, strains of S288C background fail to grow on mannose in the absence of hexokinase; the reason for this is not known, although it is possible that the cells are defective in the import of mannose in the absence of hexokinase.

Our results indicate that, in addition to being catalytically active, the N-15 short form can confer glucose repression to cells lacking wild-type hexokinase II. Levels of invertase, an index enzyme for catabolite repression/derepression, are virtually indistinguishable for cells grown in the presence of the wild type hexokinase II and the short form of hexokinase II. Clearly, the N-terminal amino acids of hexokinase II are not required for either catalysis or glucose-mediated cataboliate repression. Since the gel filtration result of this short form of hexokinase II shows that its apparent molecular weight is slightly less than half of that of the wild-type enzyme, and its structure is extremely similar to the proteolyzed enzyme that dimerizes only under extremes of pH, it is highly unlikely that the dimer is necessary for glucose repression or catalysis in vivo. These results thus render very unlikely a role for hexokinase II monomer–dimer equilibrium as a switch for glucose-mediated catabolite gene repression. The apparent molecular weight of the in vitro proteolyzed form is smaller than the short form; this could be due to the fact that the purchased proteolyzed form has lower specific activity, and possibly has higher fraction of denatured protein. Alternatively, the difference in the apparent molecular weights can simply reflect the properties of gel filtration assays.

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