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GCR3 Encodes an Acidic Protein That Is Required for Expression of Glycolytic Genes in *Saccharomyces cerevisiae*

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Screening of a mutagenized strain carrying a multicopy *ENO1-'lacZ* fusion plasmid revealed a new mutation affecting several glycolytic enzyme activities. The recessive single nuclear gene mutation, named *gcr3*, caused an extremely defective growth phenotype on fermentable carbon sources such as glucose, while growth on respiratory media was almost normal. The *GCR3* gene was obtained by growth complementation from a genomic DNA library, and the complemented strains had normal enzyme levels. *GCR3* gene was sequenced, and a 99,537-Da protein was predicted. The predicted *GCR3* protein was fairly acidic (net charge, −34). The C-terminal region was highly charged, and an acidic stretch was found in it.

The glycolytic pathway is a major metabolic route in *Saccharomyces cerevisiae*, and most of the genes are highly expressed (10, 12). Analyses of the regulatory regions of many glycolytic genes have provided evidence for a common *cis*-acting regulatory element(s) and for binding of the *trans*-acting factor RAP1 (also called GRF1 or TUF1) (5, 6, 22, 26, 29, 30). The isolation of *gcr1* (7, 8) and *gcr2* (32) mutants, in which amounts of many glycolytic enzymes are severely reduced at the transcriptional level (7, 8, 20), also suggests that there is a coordinated mechanism governing expression of glycolytic genes. Both *GCR1* and *GCR2* have been cloned (15, 20, 32) and sequenced (1, 15, 33), and they are thought to contain transcriptional activators.

This paper describes the isolation of another gene, named *GCR3*, which is also involved in the expression of several glycolytic genes. *GCR3* was cloned and sequenced, and the sequence predicted a 99,537-Da protein rich in acidic amino acid residues.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* strains used in this study are listed in Table 1. *Escherichia coli* DH5α [F⁻ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacU169* φ80 *dlacA*Δ *M15*)] was used to propagate all plasmids (11).

Media. *E. coli* cells were grown in Luria broth (9). Yeast cells were grown in rich medium (35) and in synthetic complete (SC) medium (31) or SC dropout medium, depending on the selective pressure required to maintain the plasmids. Two percent glucose or 2% glycerol plus 2% lactate was added as indicated.

Mutagenesis and isolation of mutants. Mutagenesis and isolation of mutants were performed by using *ENO1*-derived β-galactosidase activity as a screening marker as described previously (32).

Matings, diploid selection, sporulation, and dissection were carried out by the usual methods (23). Yeast cells were transformed by the method of Ito et al. (17). Growth on different carbon sources was scored by measuring the average colony size under the microscope.

DNA manipulation. Standard techniques of DNA manipulation used in this study are described in Sambrook et al. (28a). *S. cerevisiae* chromosomal DNA was prepared by the method of Sherman et al. (31). Isolation of plasmid DNA from yeast cells was done by the method of Hoffman and Winston (14).

Plasmids. Plasmids pHU15002 (34) and pSH530 (25) are multicopy plasmids containing *ENO1-'lacZ* and *HIS5-'lacZ* fusions, respectively. An *ENO2-'lacZ* fusion plasmid was constructed by fusing the *Hin*dIII site of *ENO2* (at codon 44 of *ENO2*) to the *Hin*dIII site of YEp366 (24). Likewise, a *PYK-'lacZ* fusion plasmid was constructed by fusing the *Hpa*I site of *PYK* (at codon 247 of *PYK*) to the *Sma*I site of YEp368 (24). YEp351 (13), a multicopy plasmid with a *LEU2* selection marker, was used to construct a yeast genomic DNA library, employing *Sau*3A partially digested chromosomal DNA (average length of 10 to 20 kb) and the *Bam*HI site of YEp351. PD87, PD94, and PD95 were obtained from this DNA library. pML86-2, pML89-1, pML90-2, and pML92-1 are "dropout" derivatives of PD87. pL27-1, pL28-2, pL29-2, and pL30-2 are YEp351 derivatives containing recloned fragments of PD87. The structures of these plasmids are shown in Fig. 1. YIp351 (13), an integrative plasmid with a *LEU2* selection marker, was used to examine the linkage between the cloned gene and the *gcr3* mutations.

Nucleotide sequence analysis. Restriction fragments were cloned into M13mp18 and M13mp19 (27), and the sequences were determined by the dideoxy chain termination method using ABI 373A DNA sequencer (Applied Biosystems, Foster City, Calif.) with *Taq* DNA polymerase (AmpliTaq; Cetus Corp., Norwalk, Conn.) and the 18-nucleotide sequencing primer −21M13 (ABI). Sequences of both strands for the region −732 to 2813 were determined.

Enzyme assays. Glycolytic enzyme assays (32) and β-galactosidase assays (34) have been described elsewhere.

Preparation of yeast RNA and Northern (RNA) blot analysis. Yeast cells were grown to mid-log phase (A_{580}, 2.0) in SC medium plus glucose and SC medium plus glycerol and lactate. Cells were disrupted by vortexing them with glass beads, and RNA was extracted by using an RNA isolation kit (Stratagene) according to the protocol recommended by the manufacturer. RNAs were electrophoresed through a 1.2% agarose–1 M formaldehyde gel, and Northern blot analysis was performed as described by Sambrook et al. (28a). The DIG (digoxygenin) luminescence detection kit

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Comments or source
2845	α *leu2-3 leu2-112 ura3-52 his6*	32
YHU2012	a *leu2-3 leu2-112 ura3-52*	Isogenic with 2845; 32
C110-1	a/α *leu2-3/leu2-3 leu2-112/leu2-112 ura3-52/ura3-52 his6/HIS6*	2845 × YHU2012
MWGL29	α *gcr1-6 leu2-3 leu2-112 ura3-52 his6*	*gcr1* mutant of 2845; 32
NW9-19-1	α *gcr2-1 leu2-3 leu2-112 ura3-52 his6*	*gcr2* mutant of 2845; 32
NW4-23-1	α *gcr3-2 leu2-3 leu2-112 ura3-52 his6*	*gcr3* mutant of 2845; see text
NW10-7-1	α *gcr3-1 leu2-3 leu2-112 ura3-52 his6*	*gcr3* mutant of 2845; see text
C122-5A	a *gcr3-2 leu2-3 leu2-112 ura3-52* HIS^+	Segregant of NW4-23-1 × YHU2012
YHU3003-2B	α Δ*gcr3::URA3 leu2-3 leu2-112 ura3-52 his6*	Δ*gcr3::URA3* mutant of 2845; see text

(Boehringer Mannheim) was used for labeling of DNA probes with DIG-dUTP, hybridization, and signal detection.

Nucleotide sequence accession number. The sequence shown in Fig. 3 has been given GenBank-EMBL-DDBJ accession number D10224.

RESULTS AND DISCUSSION

Original mutants. To study the regulation of *ENO1* expression, yeast strain 2845 carrying *ENO1-'lacZ* fusion plasmid pHU15002 was mutagenized and screened for putative mutations decreasing *ENO1* expression by measuring *ENO1*-derived β-galactosidase activity as a screening marker. Among putative mutants which showed lower β-galactosidase activity derived from the *ENO1* promoter but not from the *HIS5* promoter (pSH530), we have already isolated general regulatory mutants of glycolysis, *gcr1* and *gcr2* (32). NW4-23-1 and NW10-7-1 were isolated by the same screening. These mutants also showed lower β-galactosidase activity and poor growth on glucose. Both mutations were recessive, with normal growth and β-galactosidase activity in diploids formed with the isogenic wild-type strain YHU2012. For NW4-23-1 and NW10-7-1, in nine and eight tetrads examined, respectively, the slow-growth-on-glucose phenotype and low β-galactosidase activity cosegregated 2:2 in segregants, indicating single nuclear gene mutations.

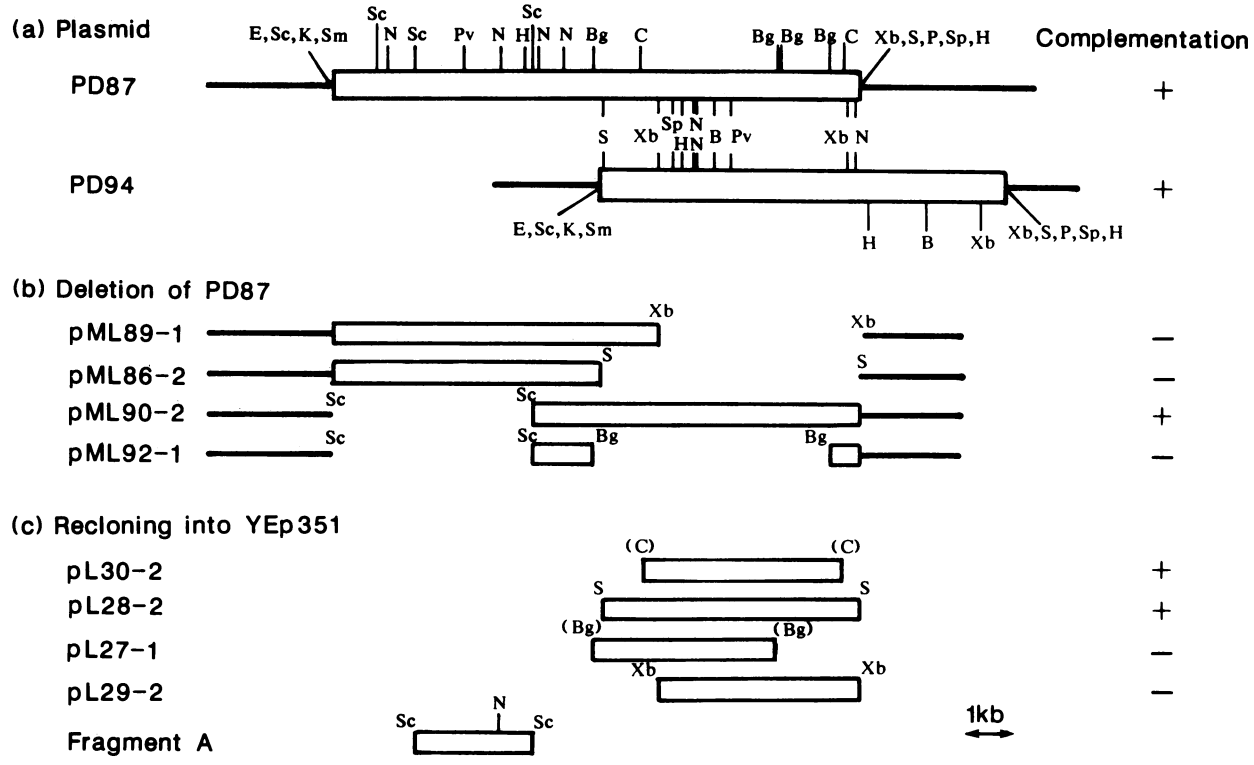

FIG. 1. Restriction maps of PD87 and PD94 and results of complementation analysis of PD87. Plasmids PD87 and PD94 were isolated from a genomic DNA library in YEp351. Plasmids pML89-1, pML86-2, pML90-2, and pML92-1 are dropout subclones of PD87. Plasmids pL30-2, pL28-2, pL27-1, and pL29-2 were constructed by subcloning *Cla*I, *Sal*I, *Bgl*II, and *Xba*I fragments into the *Sma*I, *Sal*I, *Bam*HI, and *Xba*I sites of YEp351, respectively. Results of growth complementation are summarized on the right: +, fully complemented; −, not complemented. Thick bars and thin lines indicate genomic DNA fragments and YEp351 DNA, respectively. Fragment A was used for the linkage test (for details, see the text). Restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; P, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Xb, *Xba*I. *Bgl*II, *Cla*I, and *Pst*I sites in PD94 insert are not indicated. Restriction enzyme sites in parentheses are not conserved.

TABLE 2. Relative enzyme activities of wild-type and mutant strains

Medium and strain[a]	Relevant genotype	Relative activity[b] of:													
		Hxk (Glu)	Hxk (Fru)	Zwf	Pgi	Pfk	Fbp	Fba	Tpi	Gld	Pgk	Gpm	Eno	Pyk	Icd
Rich (Gly Lac)															
2845	Wild type	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
		0.75	1.12	0.20	2.14	0.39	0.023	1.26	9.84	6.23	2.81	0.58	0.68	2.24	
NW4-23-1	*gcr3-2*	0.88	1.36	0.87	0.58	0.63	1.47	0.85	1.06	0.92	0.82	1.18	0.44	0.80	
NW10-7-1	*gcr3-1*	2.58	1.67	0.65	0.73	0.73	0.96	1.05	1.48	1.25	1.07	1.26	0.88	1.15	
Rich (Glu)															
2845	Wild type	1.0	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		1.04	1.75	0.12	1.83	0.44	ND	0.97	10.57	4.42	2.78	0.77	0.50	3.17	0.031
NW4-23-1	*gcr3-2*	0.76	0.89	1.95	0.63	0.59	ND	1.39	0.64	0.64	0.62	0.49	0.29	0.82	1.32
NW10-7-1	*gcr3-1*	2.07	1.29	2.16	0.31	0.65	ND	0.87	0.48	1.32	0.89	0.22	0.24	0.82	1.77
SC (Glu)															
2845	Wild type	1.0	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		0.70	0.78	0.15	1.97	0.47	ND	1.22	16.66	3.81	3.07	0.61	0.84	3.77	0.052
NW4-23-1(YEp351)	*gcr3-2*	1.98	1.91	1.75	0.70	0.64	ND	0.77	1.17	0.65	0.72	0.48	0.01	0.88	0.81
NW4-23-1(PD87)	*gcr3-2 GCR3*	1.0	1.05	0.94	0.87	0.89	ND	1.12	1.05	1.11	1.12	1.23	1.02	0.93	0.79
NW10-7-1(YEp351)	*gcr3-1*	2.33	2.44	1.56	0.25	0.61	ND	0.48	0.32	1.27	0.71	0.12	0.09	0.33	1.15
NM10-7-1(PD94)	*gcr3-1 GCR3*	1.14	1.13	1.11	1.08	0.90	ND	0.71	0.97	1.39	1.03	1.25	1.14	1.08	0.81

[a] Cultures were harvested in rich medium with 2% glycerol plus 2% lactate at an A_{580} of 17 to 24, in rich medium with 2% glucose at an A_{580} of about 2, or in SC medium with 2% glucose at an A_{580} of 5 to 7.

[b] Values are normalized to those of the wild-type strain. Specific activities (in micromoles per minute per milligram of protein) for the wild-type strains are given in the second line of each group. Abbreviations: Hxk, hexokinase (with glucose or fructose as indicated); Zwf, glucose-6-phosphate dehydrogenase; Pgi, phosphoglucose isomerase; Pfk, phosphofructokinase; Fbp, fructose-1,6-bisphosphate phosphatase; Fba, fructose 1,6-bisphosphate aldolase; Tpi, triose phosphate isomerase; Gld, glyceraldehyde-3-phosphate dehydrogenase; Pgk, phosphoglycerate kinase; Gpm, phosphoglycerate mutase; Eno, enolase; Pyk, pyruvate kinase; Icd, isocitrate dehydrogenase; ND, not detected.

Like *gcr1* and *gcr2* mutants, the expression of both the *ENO2-'lacZ* fusion and the *PYK-'lacZ* fusion was affected. However, the mutations in these strains were different from *gcr1* or *gcr2*, because the impaired growth of NW4-23-1 and NW10-7-1 was not complemented by multicopy plasmids carrying *GCR1* or *GCR2*. Mutations in NW4-23-1 and NW10-7-1 were allelic, and a diploid between NW10-7-1 and C122-5A, a segregant from NW4-23-1 × YHU2012 with a slow-growth phenotype, grew poorly, as did all segregants from 11 tetrads.

Table 2 shows enzyme activities of the two strains grown in rich medium with 2% glycerol plus 2% lactate or 2% glucose and in SC medium with 2% glucose compared with their isogenic wild-type parental strain 2845. In both mutants, enzyme levels were almost normal in rich medium with glycerol plus lactate (Table 2), but the levels of some enzymes were reduced when cells were grown in glucose. The effect was greater when cells were grown in SC medium. In strain NW10-7-1, expression of enolase and phosphoglycerate mutase was drastically affected, while levels of phosphoglucose isomerase, triose phosphate isomerase, and pyruvate kinase were ca. 30% of normal, and a similar pattern was observed for a null mutant (see below); the effect was narrower in the allelic mutant strain NW4-23-1. Since the pattern in some ways resembles those of *gcr1* and *gcr2* mutant strains, we named the locus *GCR3*. Both mutant strains (as well as the null mutant) were strongly impaired in their growth on glucose, fructose, and mannose but almost normal on respiratory media (Table 3). In this regard, they resemble *gcr1* mutants more than *gcr2* mutants.

Cloning of *GCR3*. Plasmids carrying the *GCR3* gene were isolated from a genomic DNA library by complementation from an *S. cerevisiae* genomic DNA library in the multicopy Leu$^+$ vector YEp351. Leu$^+$ transformants of NW4-23-1 and NW10-7-1 with normal growth were selected on glucose, and complementation was confirmed by retransformation. Three plasmids carrying overlapping segments of cloned yeast DNA were recovered: PD87 from NW4-23-1, and PD94 and PD95 (identical to PD87) from NW10-7-1. PD94 also complemented NW4-23-1. The plasmids restored normal enzyme levels to the *gcr3* mutants (Table 2).

The restriction maps are shown in Fig. 1a. To delimit the

TABLE 3. Growth of various *gcr* mutants on plates

Strain	Relevant genotype	Growth (colony size [mm]) on plates[a] containing:						
		Glucose	Fructose	Mannose	Pyruvate	Glycerol + lactate	Ethanol	No addition
2845	Wild type	2.1	2.0	2.1	0.8	0.8	0.7	0.5
NW4-23-1	*gcr3-2*	0.3	0.4	0.3	0.8	0.8	0.7	0.5
NW10-7-1	*gcr3-1*	0.3	0.4	0.3	0.7	0.8	0.7	0.4
MWGL29	*gcr1-6*	0.3	0.4	0.3	0.7	0.8	0.7	0.5
NW9-19-1	*gcr2-1*	1.3	1.3	1.5	0.8	0.8	0.7	0.5

[a] Strains were grown on rich medium plates supplemented with the indicated carbon source (2% each), and average colony sizes were measured after incubation at 30°C for 3 days.

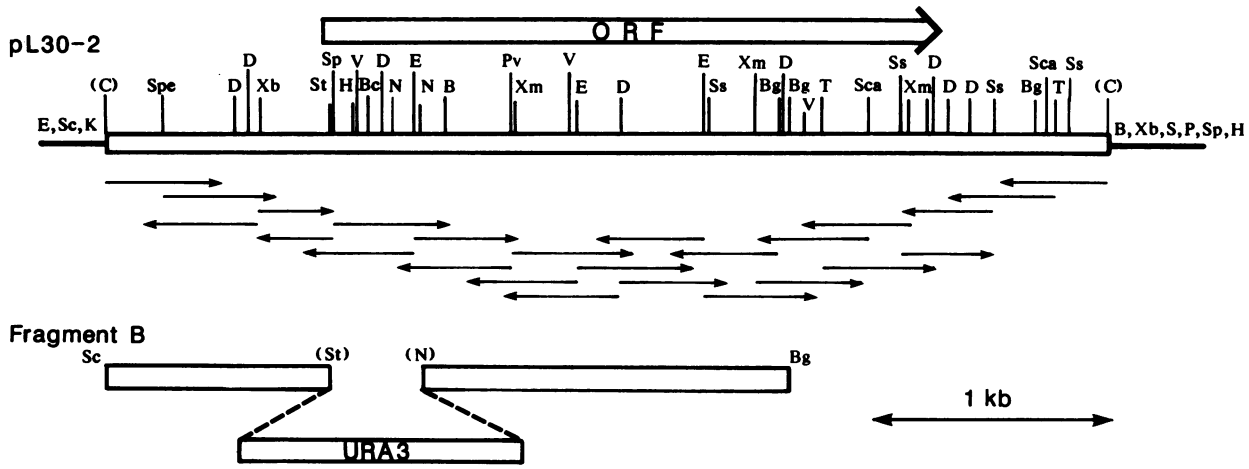

FIG. 2. DNA-sequencing strategy. Plasmid pL30-2 was used for DNA sequencing. Thick and thin lines indicate cloned yeast DNA and YEp351 DNA, respectively. The open arrow above the map denotes the long open reading frame (ORF) proposed to encode the *GCR3* gene product. The arrows below the map represent the directions and extents of sequences obtained from a given M13 clone. Fragment B was used to construct a *URA3* substitution mutant of *GCR3* (see the text). Restriction enzyme sites: Bc, *Bcl*I; D, *Dra*I; Sca, *Sca*I; Spe, *Spe*I; Ss, *Ssp*I; St, *Stu*I; T, *Eco*T22I; V, *Eco*RV; Xm, *Xmn*I. Abbreviations for other restriction enzyme sites are the same as in Fig. 1.

GCR3 gene on the cloned DNA, subclones were constructed from PD87 and tested by transformation for their abilities to complement the defects in growth on glucose of NW4-23-1 and NW10-7-1 (Fig. 1b and c). The same complementation was obtained with both strains. These results suggest that the gene lies in the 4.2-kb *Cla*I fragment of pL30-2, which spans the right *Bgl*II site of pL27-1 and the left *Xba*I site of pL29-1 (Fig. 1c).

The cloned gene was shown by genetic analysis to be *GCR3* and not a suppressor. Since the complementation by pL30-2 and pL28-2 showed that the neighboring *Sac*I fragment (fragment A in Fig. 1c) was not in the *GCR3* gene, fragment A was cloned into YIp351. After linearization of the resulting plasmid with the unique *Nco*I site in fragment A, the plasmid was used to transform the original mutants NW4-23-1 and NW10-7-1 to Leu$^+$. Transformants were crossed with YHU2012 (*GCR3 leu2*), and segregants were analyzed. In six tetrads from each of four transformants, Leu$^+$ cosegregated with Gcr3$^-$.

Sequence of the *GCR3* gene. The nucleotide sequence of the 4.2-kb *Cla*I fragment of pL30-2 was determined. The sequencing strategy is shown in Fig. 2. A single open reading frame containing 858 codons was identified. The size and the location of this open reading frame accorded well with the results of the complementation test. Since the left-hand *Xba*I site of pL29-2 (Fig. 1c) is located 266 bp upstream of the open reading frame, this *Xba*I site is probably in the regulatory region of *GCR3*. Figure 3 shows the nucleotide sequence and the amino acid sequence of the predicted 99,537-Da protein. The nucleotide sequence was compared with the sequences in GenBank, but no significant homology to other genes was found. Comparison of the deduced amino acid sequence of *GCR3* with other sequences in the EMBL protein data base showed no significant homologies, either. The codon usage of *GCR3* is not biased, the codon bias index (3) being 0.152.

The predicted Gcr3 protein seems fairly acidic. It contains 128 acidic residues (14.9%) and 94 basic residues (11.0%), with a net charge of −34. The C-terminal region (residues 771 to 858) is highly charged (40%); of the 88 residues, 21 are acidic and 14 are basic. This region includes an acidic stretch (net charge, −12 over 28 amino acids from 771 to 798) and a following basic region (net charge, +6 over 24 amino acids from 799 to 822). Acidic regions have been shown to function in activation of transcription (16, 21), and charged clusters are common among nuclear transcription factors (4). If *GCR3* affects transcription by a direct mechanism, the protein might be expected to reside in the nucleus. A possible nuclear localization signal comprises residues 19 to 27. Figure 4 shows the comparison of this sequence with other known signal sequences.

Disruption of *GCR3* is not lethal. To determine the phenotype of a null mutation at the *GCR3* locus, we used the cloned DNA to construct a *URA3* substitution mutation. Nucleotide sequence analysis allowed us to construct a deletion by removing the first part of *GCR3* gene. As shown in Fig. 2, pL30-2 was digested with *Stu*I plus *Nco*I, and the *Stu*I-*Nco*I region was replaced with the *URA3* gene. The resulting plasmid was digested with *Bgl*II and *Sac*I, and the fragment (fragment B in Fig. 2) was introduced into the genome of wild-type diploid strain C110-1. Diploids were sporulated and subjected to tetrad analysis. Analysis of 18 tetrads derived from two independently transformed diploids showed 2+:2− segregation for growth on glucose, and poor growth on glucose cosegregated with the Ura$^+$ phenotype. All segregants grew normally on glycerol plus lactate. Assay data on a couple of enzymes show the null mutant to resemble one of the mutants, NW10-7-1. The enzyme levels were almost normal when cells were grown in glycerol plus lactate, but the levels of some enzymes were reduced when cells were grown in glucose, and the effect was greater in SC medium, the enzyme activities relative to that of wild type in the latter medium being 0.12, 0.40, and 0.58 for enolase, phosphoglycerate mutase, and pyruvate kinase, respectively. The *URA3*-substituted mutants failed to complement *gcr3-1* and *gcr3-2* for growth on glucose, indicating that the fragment was integrated in the *GCR3* locus. These analyses showed that *GCR3* is an important but not an essential gene.

Effect on mRNA. Figure 5 shows a Northern blot analysis with an enolase 1 probe (*ENO1*) for wild-type and Δ*gcr3*::*URA3* strains grown with glucose or with glycerol plus lactate. In the hybridization with the *ENO1* probe,

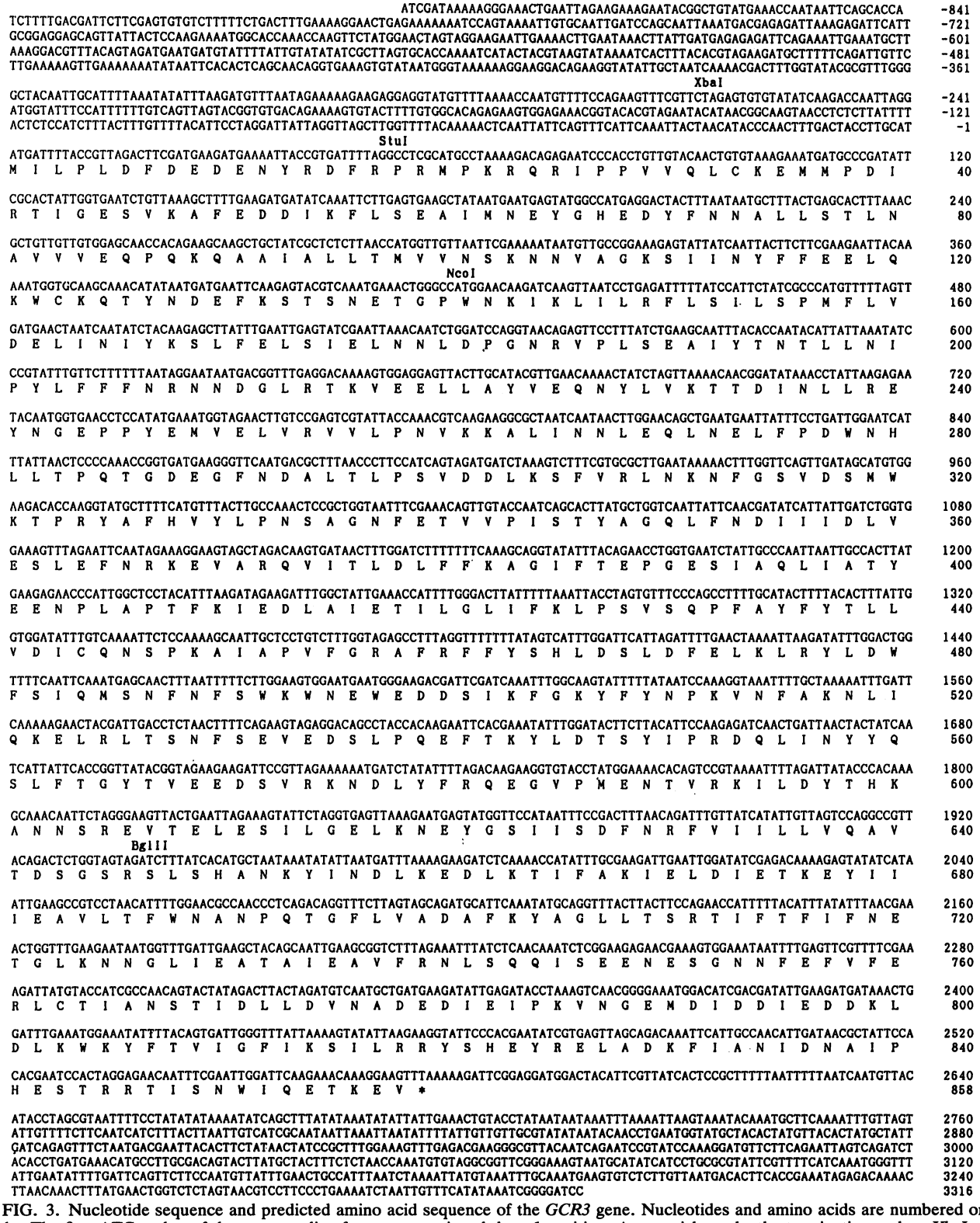

FIG. 3. Nucleotide sequence and predicted amino acid sequence of the *GCR3* gene. Nucleotides and amino acids are numbered on the right. The first ATG codon of the open reading frame was assigned the +1 position. An asterisk marks the termination codon. *Xba*I, *Stu*I, *Nco*I, and *Bgl*II sites relevant to the construction of plasmids are marked.

SV40 large T	124	Thr Pro Pro Lys Lys Lys Arg Lys Val
SV40 VP1	1	Ala Pro Thr Lys Arg Lys Gly Ser
Polyoma large T	188	Pro Val Ser Arg Lys Arg Pro Arg Pro
Polyoma large T	278	Thr Pro Pro Lys Lys Ala Arg Glu Asp
GCR3	19	Arg Met Pro Lys Arg Gln Arg Ile Pro

FIG. 4. Homology of Gcr3 sequence to nuclear localization signals. The Gcr3 sequence from amino acids 19 to 27 is compared with sequences that have been identified as nuclear localization signals in simian virus 40 (SV40) large T antigen (18, 19), SV40 VP1 protein (36), and polyomavirus large T antigen (28). Numbers at the left indicate the position of the first residue in each sequence.

transcripts of enolase of about 1.6 kb were observed, but the level was reduced in the Δ*gcr3*::*URA3* mutant when cells were grown in glucose; the degree of reduction was marginal for cultures grown in glycerol plus lactate, as was also reflected in enzyme levels. However, the degree of reduction was less for the mRNA than for the enzyme. The densities of the bands were measured by densitometer (Shimadzu CS-930 Dual-Wavelength Chromato Scanner) and normalized with the amount of actin mRNA (ca. 1.4 kb), which was relatively unaffected (data not shown). The relative mRNA levels in the Δ*gcr3*::*URA3* strain were 0.87 and 0.49 for glycerol-lactate- and glucose-grown cells, respectively, whereas the relative enolase activities were 0.66 and 0.12.

Conclusions. Mutants with mutations in *GCR3* are drastically affected in their growth on glucose and, depending on allelic and cultural conditions, show a pattern of levels of reduction of several glycolytic enzyme activities which resembles in some ways those in *gcr1* and *gcr2* mutants. *GCR3* was obtained by complementation, and sequencing showed an open reading frame of 858 amino acids for a protein which, as mentioned above, would have certain characteristics of a transcriptional activator.

It is not known how *GCR3* acts, and a relatively small transcriptional effect was observed for *ENO1* mRNA. However, preliminary gel shift experiments have shown *gcr3* to alter the patterns of gel shift obtained with crude extracts and UAS of *ENO1* (data not shown), which fits with the possibility of the *GCR3* product being a DNA-binding protein or affecting the interaction of other proteins. It is interesting that neither Gcr1 nor Gcr2 was revealed as influencing DNA binding in this same assay with crude extracts or in the previous similar assays (22, 30). However, specific DNA binding by Gcr1 has since been reported (2).

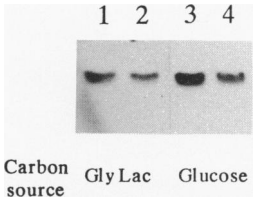

FIG. 5. Comparison of enolase mRNA levels in wild-type and Δ*gcr3*::*URA3* strains. Total RNAs (25 μg) from strains 2845 (wild type) (lanes 1 and 3) and YHU3003-2B (Δ*gcr3*::*URA3*) (lanes 2 and 4) were electrophoresed through a 1.2% agarose–1 M formaldehyde gel. RNAs from lanes 1 and 2 were isolated from glycerol-lactate-grown cells, and RNAs from lanes 3 and 4 were isolated from glucose-grown cells. After transfer of RNA to a nylon membrane, the RNA was hybridized with an *ENO1* probe complementary to the 5′ half of the *ENO1* transcript.

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