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# A Hexose Transporter Homologue Controls Glucose Repression in the Methylotrophic Yeast *Hansenula polymorpha**

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**Peroxisome biogenesis and synthesis of peroxisomal enzymes in the methylotrophic yeast *Hansenula polymorpha* are under the strict control of glucose repression. We identified an *H. polymorpha* glucose catabolite repression gene (*HpGCR*1) that encodes a hexose transporter homologue. Deficiency in *GCR1* leads to a pleiotropic phenotype that includes the constitutive presence of peroxisomes and peroxisomal enzymes in glucose-grown cells. Glucose transport and repression defects in a UV-induced *gcr1-2* mutant were found to result from a missense point mutation that substitutes a serine residue ($Ser^{85}$) with a phenylalanine in the second predicted transmembrane segment of the Gcr1 protein. In addition to glucose, mannose and trehalose fail to repress the peroxisomal enzyme, alcohol oxidase in *gcr1-2* cells. A mutant deleted for the *GCR1* gene was additionally deficient in fructose repression. Ethanol, sucrose, and maltose continue to repress peroxisomes and peroxisomal enzymes normally and therefore, appear to have *GCR1*-independent repression mechanisms in *H. polymorpha*. Among proteins of the hexose transporter family of baker's yeast, *Saccharomyces cerevisiae*, the amino acid sequence of the *H. polymorpha* Gcr1 protein shares the highest similarity with a core region of Snf3p, a putative high affinity glucose sensor. Certain features of the phenotype exhibited by *gcr1* mutants suggest a regulatory role for Gcr1p in a repression pathway, along with involvement in hexose transport.**

If provided with a mixture of carbon substrates, yeast preferentially utilizes the one that supports the fastest growth rate. This is achieved by several coordinated regulatory mechanisms of metabolic adaptation. They include: (i) the induction of enzymes involved in the metabolism of a preferred substrate and (ii) repression and/or inactivation of enzymes involved in the metabolism of less preferred carbon sources. Carbon source-triggered repression (or catabolite repression) generally affects expression of the corresponding target genes at the transcriptional level. Among co-repressor substrates in *Saccharomyces cerevisiae*, glucose is best known (for review see Refs. 1 and 2). The main targets of glucose repression in *S. cerevisiae* are enzymes of gluconeogenesis and the glyoxylate cycle, mitochondrial enzymes involved in oxidative phosphorylation, and enzymes involved in transport and metabolism of alternative carbon substrates, such as galactose, sucrose, and maltose. Despite extensive studies and a growing number of genes known to be involved in glucose repression in this and other species, its actual mechanism, especially in the early stages of sensing and signal transduction, is not fully understood.

In methylotrophic yeasts, unique peroxisomal and cytosolic enzymes of methanol metabolism are under strict control of repression by various carbon substrates, *e.g.* glucose and ethanol (3, 4). Glucose and ethanol also trigger inactivation of peroxisomal enzymes by a process that involves the autophagic degradation of whole peroxisomes by vacuolar proteases (5–7). Previously, in the methylotrophic yeasts *Pichia methanolica* (formerly *P. pinus*) and *Candida boidinii*, we and others showed that glucose and ethanol repression and degradative inactivation of peroxisomal enzymes are controlled by separate carbon source-specific sets of regulatory genes (4, 8, 9). One complementation group of *P. methanolica* mutants defective in glucose repression was found to be deficient in phosphofructokinase activity. We proposed that this enzyme has a second signaling function in repression that is distinct from its catalytic activity (8).

The methylotrophic yeast *Hansenula polymorpha* (syn. *Pichia angusta*) is an important organism for biotechnological use, *e.g.* heterologous gene expression, and for basic research on peroxisome biogenesis and degradation (7, 10). There are several reports describing *H. polymorpha* mutants defective in glucose repression (11–13). Kramarenko *et al.* (11) demonstrated that glucose has to be phosphorylated in order to cause repression in *H. polymorpha*, and mutants impaired in activities for hexo- or glucokinases are insensitive to repression. However, the molecular nature of other mutations that impair glucose repression in *H. polymorpha* has not been determined (12, 13).

In a previous report, we described the isolation and characterization of *H. polymorpha* mutants in a gene named *GCR1*[1] that are defective in glucose repression (14).[2] Glucose-grown

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY465112.*

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[1] The abbreviations used are: GCR, glucose catabolite repression; AO, alcohol oxidase; TM, transmembrane domain; $P_{MOX}$, promoter of the *AO* gene; EGFP, enhanced green fluorescent protein; PTS, peroxisomal targeting signal; ORF, open reading frame.

[2] The authors chose to use the name of the gene as it appeared in their original reports (14, 15), despite the fact that it coincides with the name of a different *S. cerevisiae GCR1* gene, involved in the translational activation of glycolytic proteins.

TABLE I
*H. polymorpha strains used in this study*

All strains were derived from *H. polymorpha* NCYC 495. The genotype of a strain is not shown when it coincides with the strain's name. In biochemical experiments, *gcr1-2 leu1-1* and the isogenic wild-type *leu1-1* strains were utilized throughout this study, except that strains *gcr1-2 leu10* and isogenic AS8 (*leu10*) were used in glucose uptake experiments, and mixed substrate utilization experiments (referred to in text as *gcr1-2* and a wild type, respectively).

| Strain | Genotype | Ref. |
|---|---|---|
| AS8 | *leu10* | 14 |
| *leu1-1* | -//- | 16 |
| *met6* | -//- | 16 |
| *leu1-1 met6* | -//- | This study |
| *ade11* | -//- | 16 |
| *leu1-1 ade11* | -//- | This study |
| *gcr1-2* | *gcr1-2 leu10* | 14 |
| *gcr1-2* | *gcr1-2 leu1-1* | This study |
| *gcr1*Δ | *gcr1*Δ::*ScLEU2 leu1-1 met6* | This study |
| *ade11* (GFP-PTS1) | *ade11*:$P_{MOX}$-*GFP-PTS1*:*ScLEU2 leu1-1* | This study |
| wt (GFP-PTS1) | $P_{MOX}$-*GFP-PTS1*:*ScLEU2 leu1-1* | This study |
| *gcr1-2* (GFP-PTS1) | *gcr1-2*:$P_{MOX}$-*GFP-PTS1*:*ScLEU2 leu1-1* | This study |
| *gcr1*Δ (GFP-PTS1) | *gcr1*Δ::*ScLEU2*:$P_{MOX}$-*GFP-PTS1*:*ScLEU2* | This study |

cells of *gcr1* mutants exhibit pleiotropic alterations in cellular metabolism, namely: (i) the constitutive synthesis of the peroxisomal enzymes, alcohol oxidase (AO) and catalase, and the constitutive presence of peroxisomes; (ii) a decrease in growth rate; and (iii) a decrease in levels of glycolytic intermediates but wild-type levels of activity for each of the primary glycolytic enzymes. In addition, and unlike in wild-type cells, cytosolic enzymes required for methanol metabolism (formaldehyde and formate dehydrogenases) and α-glucosidase are not repressed in *gcr1* mutants grown in medium containing glucose along with either methanol or maltose, respectively. When shifted from methanol to glucose medium, AO is not inactivated in *gcr1* mutants. We suggested that a glucose transport defect might be responsible for the phenotypes displayed by our *gcr1* mutants (14, 15). Here, we further characterize the phenotype of *gcr1* mutants, describe the cloning and sequence analysis of the *GCR1* gene and its product, Gcr1p, the construction of a *GCR1* deletion strain, and the identification of a missense mutation in a UV-induced mutant, *gcr1-2*. Finally, we discuss the possible involvement of Gcr1p in glucose sensing of repression in *H. polymorpha*.

## EXPERIMENTAL PROCEDURES

*Strains, Media, and Microbial Techniques—H. polymorpha* strains used are listed in Table I. Auxotrophic strains AS8 (*leu10*), kindly supplied by Dr. P. Sudbery (University of Sheffield), and *leu1−1* (both derived from NCYC495), were used in this study as the wild-type strains as indicated. The cells were grown at 37 °C in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or a minimal medium (0.17% w/v) yeast nitrogen base without amino acids (Difco, Detroit, MI) with 0.5% w/v ammonium sulfate as a nitrogen source. Concentration of carbon sources was 1% if not indicated otherwise. Amino acids were added to a final concentration of 50 μg/ml as required. For solid media, agar was added to 2% (w/v). Sporulation/mating media and techniques were essentially as described (16). Absorbance was determined at 600 nm and yeast cells density calculated as mg dry weight per ml using a calibration curve. Cultivation of *Escherichia coli DH5α* and standard recombinant DNA techniques were performed essentially as described (17).

*Cloning and Sequence Analysis of the GCR1 Gene and Gcr1-2 Mutant Allele*—For the *GCR1* gene cloning, a recombinant mutant strain *gcr1-2 leu1-1* was isolated from spore progeny after crossing the original *gcr1-2 leu10* mutant (14) with *leu1-1* wild-type strain. To isolate the *GCR1* gene, the mutant *gcr1-2 leu1-1* was transformed with a *H. polymorpha* genomic pYT3-based DNA library carrying *ScLEU2* gene as a selectable marker (18) by the electroporation method (19). Following selection for leucine prototrophy on YND plates with low, 5 mM glucose (l-Glc), growing colonies were transferred to liquid minimal glucose medium. Genomic DNA from mid-logarithmic cells was extracted and transformed into *E. coli DH5α* by electroporation. Individual plasmids were recovered from ampicillin-resistant colonies and retransformed into the *gcr1-2 leu1-1* strain. The prototrophic transformants were analyzed for Gcr$^-$ phenotype by AO qualitative assay in colonies as described (20, 21). The complementing plasmids were analyzed with selected restriction enzymes (BamHI, EcoRI, SalI, PstI, KpnI). The region present in genomic DNA fragments on all plasmids and predicted to complement *gcr1-2* mutation, was sequenced starting with primer SO61 (5′-TTCCTTCTTTGCTATCAC-3′), specific for adjusted *HpARS* region on pYT3, and plasmid pOS22 as a template (see main text). Double stranded DNA sequencing was performed with gene-specific 18–21 bp primers. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). DNA sequencing was performed at the Oregon Regional Primate Research Center, Molecular Biology Core Facility (Beaverton, OR). For analysis of DNA and amino acid sequences, MacVector software (IBI, New Haven, CT) was used. Sequence alignments were performed using the ClustalW version 1.6 algorithm (22). The BLAST Network Service of the National Center for Biotechnology Information (Bethesda, MD) was used to search for amino acid sequence similarities.

To localize a putative mutation in the *gcr1-2* mutant allele, a number of pOS22 fragments comprised of different portions of *GCR1* ORF were analyzed for ability to functionally complement the corresponding *gcr1-2 leu1-1* mutant. The fragments were isolated after digestion of pOS22 with selected restriction enzymes, and as PCR products, with pOS22 as a template and primers: SO53 (5′-AATCGAAGCTCCCTTGAC-3′), SO56 (5′-TTCTTCGTCACCATCGGATT-3′), SO72 (5′-AACACCATGCAAATGTCGAG-3′). The fragments were co-transformed along with plasmid pYT3 (molar concentration ratio of fragment *versus* plasmid was ~1:10 in each case) by electroporation into *gcr1-2 leu1-1* strain. Transformants were selected for leucine prototrophy on minimal YNB plates with sucrose. Colonies were further replicated on l-Glc plates, and high, 55 mM glucose plates (h-Glc), for AO colony assay. To isolate the mutated *gcr1-2* gene and identify the mutation, the total genomic DNAs of *gcr1-2 leu1-1*, and original *gcr1-2 leu10* mutant were extracted and used as templates in PCR reactions with TaqDNA polymerase (Invitrogen, Life Technologies, Inc.), primers SO58 (5′-CCCAAGCTTAAACGGAGTAAATCCT-3′) and SO72. Resulting 2.75-kb fragments were sequenced with the same *GCR1*-specific primers as used for sequencing of the wild-type gene. To exclude potential PCR amplification mistakes, two independently amplified fragments were sequenced in both directions. In addition, the wild-type fragment isolated by analogous PCR from *leu10* parental strain was sequenced and served as a control. Nucleotide sequences were aligned using MacVector software to identify the site of a putative mutation.

*Construction of a GCR1 Deletion Strain*—A vector capable of deleting most of the *HpGCR1* ORF was constructed in two steps. In the first step, a 0.5-kb fragment containing sequences just 5′ of the methionine initiator ATG of *GCR1* was amplified by PCR using plasmid pOS22 as a template with Vent DNA polymerase (NEB, Beverly, MA). The primers for this PCR, SO58 and SO57 (5′-ATCCTGCAGGTTTGTAATAGTTGATTG-3′) included restriction sites for HindIII and PstI, respectively. The 5′-flanking fragment was inserted into HindIII/PstI-digested plasmid pYT1 (18), carrying *ScLEU2* gene as a selectable marker, to create pOS28. For the second step, a 1.2-kb SalI-SalI fragment of pOS22 composed of sequences from the 3′ terminus of *GCR1*, beginning at nucleotide 1480 of the ORF and adjusted 3′-flanking region of ~1.1-kb was inserted in the correct orientation into SalI-digested pOS28 to create pOS29. The latter plasmid was digested with

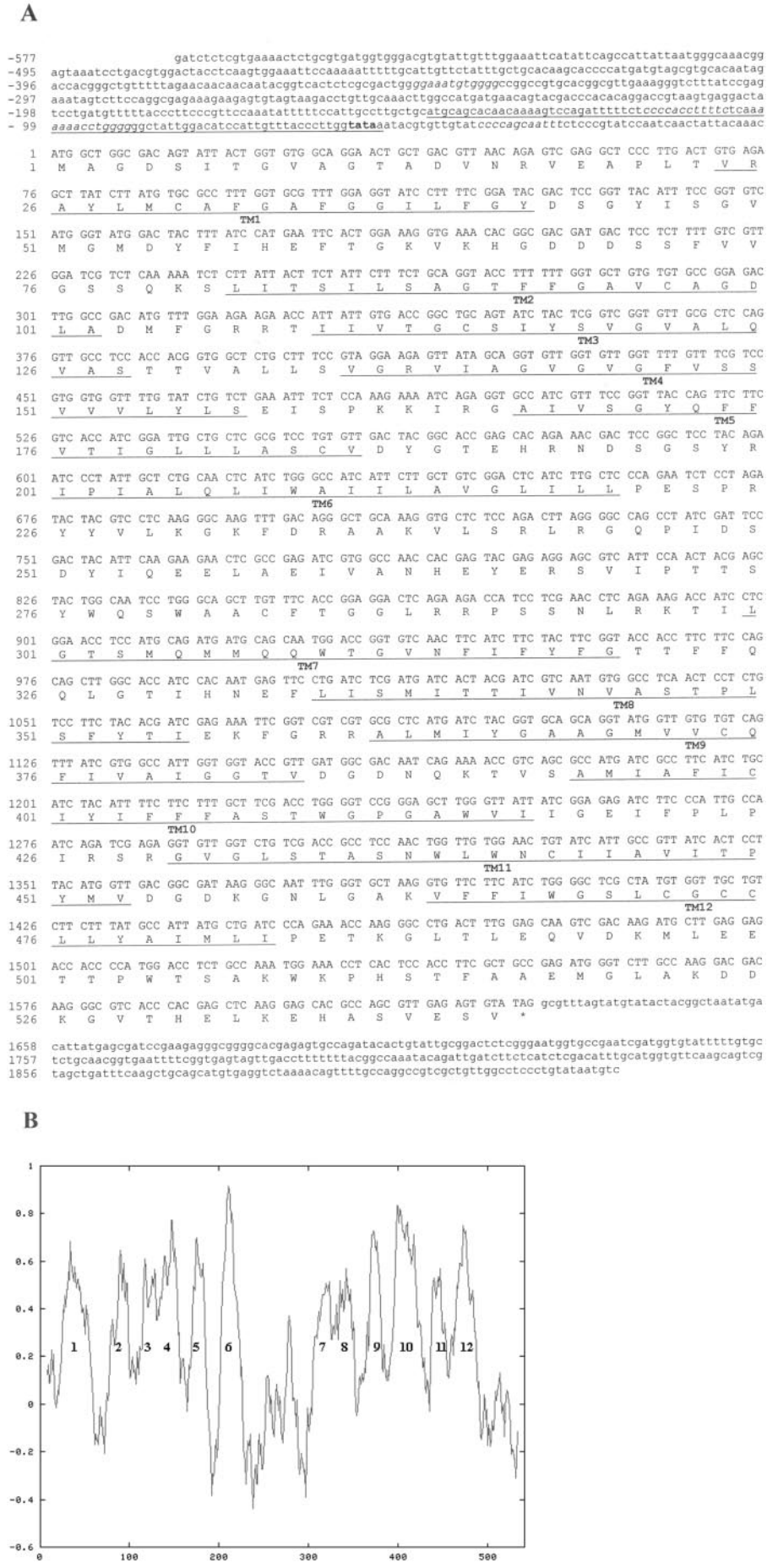


FIG. 1. **Nucleotide and amino acid sequences of the *H. polymorpha GCR1* gene and its product.** *A*, twelve predicted membrane-spanning segments (*TM 1–12*) are *numbered* and *underlined*. A putative uORF is *underlined*, a potential TATA box is shown in *bold*, and potential binding sites for a Mig1p-like repressor protein are shown in *italics* in the GCR1 5′-region. *B*, hydrophobicity profile derived from the predicted amino acid sequence of Gcr1p according to the method of Eisenberg *et al.* (37) with a window size of 17 amino acids. Hydropathy values are on the *y*-axis, and the residue numbers are on the *x*-axis.

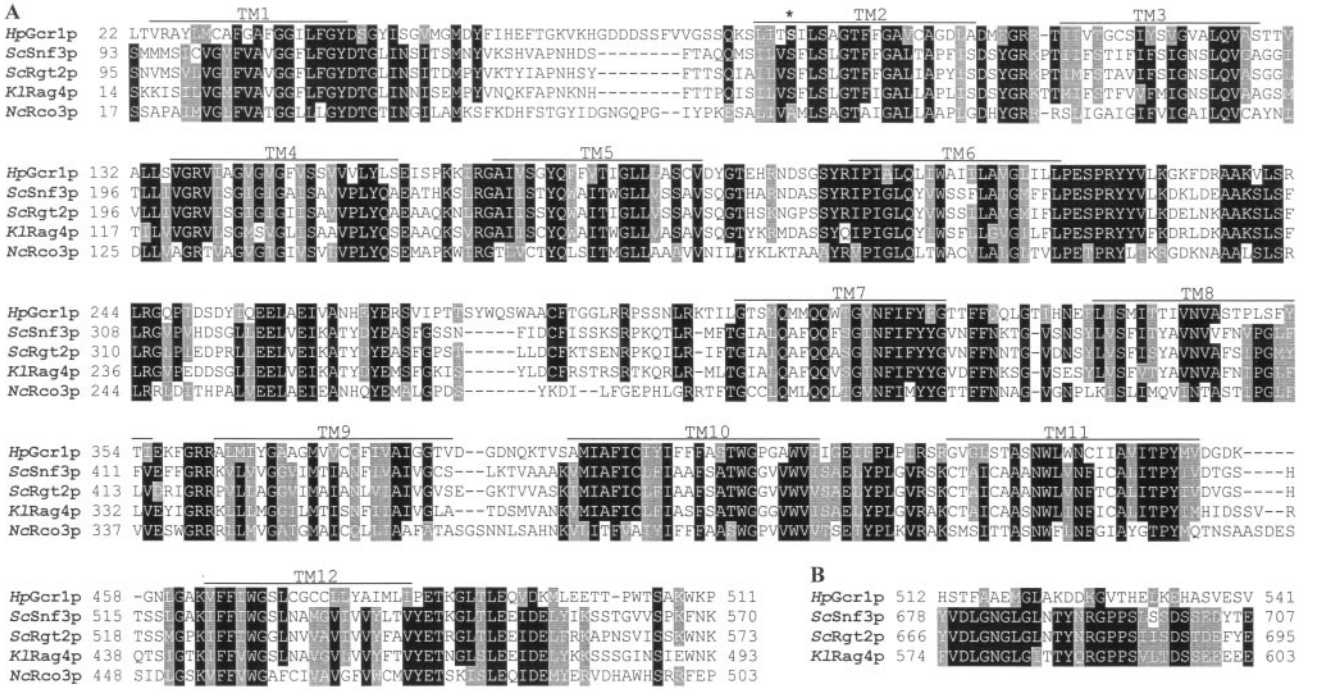


FIG. 2. **Alignment of selected amino acid sequence regions of *H. polymorpha* Gcr1p, *S. cerevisiae* Snf3p and Rgt2p, *K. lactis* Rag4p, and *N. crassa* Rco3p.** *A*, transmembrane domain regions. The serine residue (*S85*) altered in the *gcr1-2* mutant to phenylalanine by a missense mutation is shown in *bold* and indicated by an *asterisk*. Predicted membrane spanning segments designated as TM 1–12 are shown with an *overline*. *B*, C-terminal regions. Conserved amino acid residues are shown with *black shaded areas* indicating identical residues, and *light gray areas* indicating similar residues.

HindIII and XbaI, releasing a 3.9-kb fragment comprised of *ScLEU2* flanked by *GCR1* 5′ and 3′ sequences and transformed into the *leu1-1 met6* strain by electroporation. This double auxotrophic hybrid was isolated from the spore progeny of a diploid strain resulting from crossing of *leu1-1* and *met6*. Transformants were selected and analyzed for Gcr$^-$ phenotype as described in the main text. To confirm deletion of the *GCR1* gene, genomic DNAs were isolated from several Gcr$^-$ transformants and used as templates in PCR reactions with two sets of oligonucleotide primers. One set was composed of primer SO56, complementary to sequences in the 3′-flanking region of the *GCR1* ORF, present in both, the wild type and a deletion allele. Second primer, SO72, hybridized to sequences in the 5′ region of the wild-type *GCR1* ORF that were absent in the *gcr1*Δ allele. The other set of primers contained the same 3′-flanking sequence primer SO56 and a second primer complementary to a sequence in *ScLEU2*, SO90 (5′-TAAGAAGATCGTCGTTTTGCC-3′). The first set of primers produced a 1.3-kb long fragment with only the wild-type genomic DNA as template, while the second set generated a 1.9-kb long fragment with genomic DNAs of each of the putative candidate *gcr1*-deletion transformants as template, but not with wild-type DNA. One transformant, *gcr1*Δ::*ScLEU2 leu1-1 met6*, was utilized throughout this study as a *gcr1* deletion strain.

*Construction of Strains with Fluorescently Labeled Peroxisomes*—pOS18, an *E. coli-H. polymorpha* shuttle vector capable of expressing a peroxisome-targeted red shifted form of the green fluorescent protein (EGFP-PTS1) under control of the *H. polymorpha AO* promoter ($P_{MOX}$) was constructed. As a first step, plasmid pOGP-2 was constructed by introducing an adapter fragment encoding the last nine amino acids and stop codon of *Pichia pastoris* Pex8p (including the PTS1 sequence, C-terminal AKL) into a pEGFP-C3 vector (Clontech Laboratories, Inc., Palo Alto, CA) as described (23). It resulted in a chimeric gene encoding EGFP with the last nine amino acids of Pex8p fused in-frame to its C terminus (EGFP-PTS1). As the next step, the EGFP-PTS1 0.8-kb fragment was amplified from pOGP2 by PCR with primers SO40 (5′-GTGAAGCTTATGGTGAGCAAGGGCGAG-3′) and SO2 (5′-AGCTTACCGGTTTATAACTTTGCGGTTGATTGGGCG-3′) that introduced HindIII flanking sites immediately 5′ and 3′ of the EGFP fusion gene. The fragment was inserted into the unique HindIII site of pET1 (18) downstream of $P_{MOX}$ in required orientation to produce pOS18. The latter was linearized in the unique StuI site in the $P_{MOX}$ sequence and transformed into *leu1-1* wild type and *gcr1-2 leu1-1* strains. Isolated prototrophic transformants were grown on methanol plates to induce $P_{MOX}$, and fluorescence was examined directly in yeast colonies with fluorescent microscope. To identify stable integrants, individual fluorescent clones were examined for mitotic stability by repeated shifting from selective methanol minimal to non-selective YPD medium. Two strains, WT (GFP-PTS1), a wild type, and *gcr1-2* (GFP-PTS1) (Table I.) were further utilized in this study. To isolate a *gcr1*Δ GFP-PTS1-expressing strain, *ade11* (GFP-PTS1) wild-type strain was isolated first in the same way as described above, by transforming *leu1-1 ade11* with pOS18. The resulting strain was crossed with a *gcr1*Δ *met6*-null mutant, and recombinant prototrophic *gcr1*Δ (GFP-PTS1) was isolated from spore progeny as a fluorescent clone on glucose medium also unable to grow on l-Glc plates.

TABLE II
*Effect of extracellular glucose concentration on AO activity in gcr1 mutants*

AO activity was measured in cell-free extracts of *H. polymorpha* strains pregrown in YNB medium with sucrose and incubated with selected concentrations of glucose for 18 h. Data are mean values from three independent experiments. AO activity is expressed in units/mg of protein.

| Glucose | AO activity | | |
|---|---|---|---|
| | 5 mM | 25 mM | 55 mM |
| Wild type | 0 | 0 | 0 |
| *gcr1-2* | 2.0 | 2.4 | 2.5 |
| *gcr1*Δ | 1.0 | 1.0 | 1.5 |

*Biochemical Methods*—Preparation of crude cell free extracts was performed as described previously (23). AO activity was measured in cell-free extracts as described (24), and expressed as micromoles of product/min/mg of protein, or in permeabilized whole cells (8), and expressed in micromoles of product/min/g dry weight. Protein concentration was determined by the method of Lowry (25). Extracellular glucose concentration was measured with a glucose oxidase-based enzymatic kit Diagluc (UBT Ltd., Lviv, Ukraine), and methanol concentration in culture media with the alcohol oxidase-based enzymatic kit Alcotest as described (26).

*Glucose Uptake Assays*—For glucose transport assays, cells were grown on 1% glucose YNB medium until mid-logarithmic phase and harvested at a cell density of 1–1.5 mg dry weight/ml. Cells were washed twice by centrifugation in distilled water at 3,000 × *g*. Sugar transport was measured at 20 °C, starting with the addition of 0.1 ml of

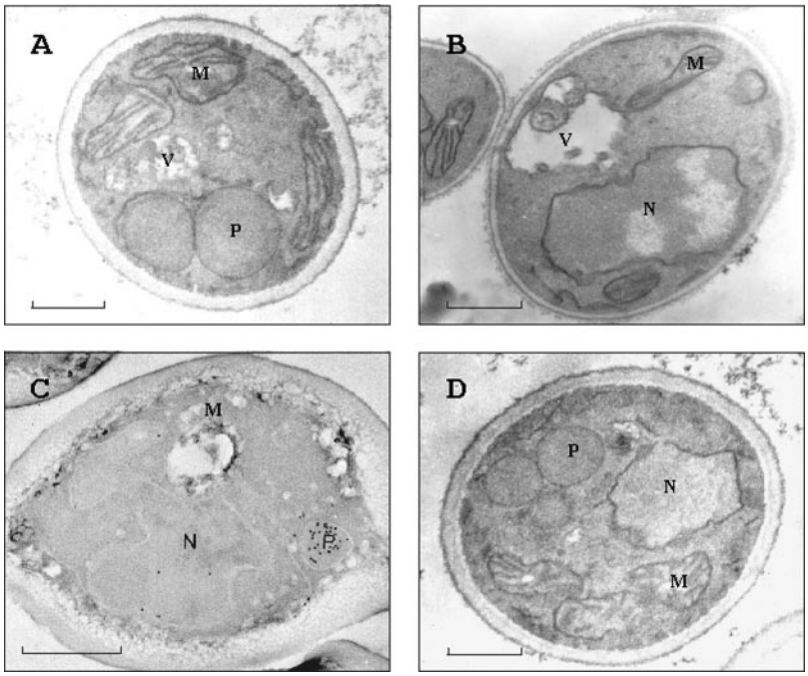


FIG. 3. **Peroxisome biogenesis in a *gcr1*Δ mutant.** Electron microscopic images of cells of wild-type strain grown in batch culture with (*A*) methanol and (*B*) glucose; *C*, immunogold detection of AO in glucose-grown *gcr1*Δ mutant cell section; *D*, *gcr1*Δ mutant grown in glucose medium. *P*, peroxisome; *V*, vacuole; *N*, nucleus; *M*, mitochondrion. *Bar*, 1 micron.

TABLE III

*Effect of carbon source on AO activity and growth rate in wild-type and gcr1 strains*

AO activity (AO, in units/g dry weight of cells) and cell doubling time (d.t., in hours) were determined for mid-exponential cells grown in shake-flask cultures on the carbon substrates shown.

| Carbon source[a] | Fructose | | Mannose | | Xylose | | Maltose | | Trehalose | | Sucrose | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | d.t. | AO | d.t. | AO | d.t. | AO | d.t. | AO | d.t. | AO | d.t. | AO |
| Wild type | 2.6 | 0 | 2.6 | 0 | 15 | 15 | 4.6 | 0 | 6.6 | 0 | 4.3 | 0 |
| *gcr1-2* | 2.6 | 0 | 5.5 | 140 | 28 | 120 | 4.6 | 0 | 11.3 | 95 | 4.3 | 0 |
| *gcr1*Δ | 6.2 | 55 | 5.8 | 90 | 30 | 98 | 4.6 | 0 | 15.0 | 66 | 4.5 | 0 |

[a] Carbon source, 1% w/v each.

the uniformly labeled [$^{14}$C]glucose or [$^{14}$C]fructose in a final volume of 0.2 ml, in 0.1 M potassium phosphate buffer (pH 6.0). Cell concentration was 50 mg/ml. Ten seconds later, transport was stopped by adding 8 ml of ice-cold 0.8 M glucose in the same buffer essentially as described (27). Samples were immediately filtered under vacuum and washed twice with 10 ml of ice-cold glucose solution. The treatment of control samples differed in that the cold glucose was added first to the cells and labeled sugar. Thereafter, the reaction was kept at 0 °C. Samples were transferred to scintillation vials with 2 ml of scintillation liquid. A portion of the reaction mixture served as a reference to determine the total radioactivity. The radioactivity was measured with a liquid scintillation counter (Rac-Beta 1219, LKB). The final glucose concentration ranged from 0.5 to 50 mM. The glucose consumption rate ($V_{Glc}$) was expressed as grams per hour per gram of dry weight.

*Electron and Fluorescence Microscopy*—Cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously (28). Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells using specific antibodies against *H. polymorpha* AO and goat anti-rabbit antibodies conjugated to 15 nm gold particles (Amersham Biosciences) according to the instructions of the manufacturer. Fluorescence microscopy was performed essentially as described (23).

## RESULTS

*Cloning and Sequence Analysis of the GCR1 Gene*—The *GCR1* gene was isolated by functional complementation of a *gcr1-2 leu1-1* mutant (see "Experimental Procedures") with an *H. polymorpha* genomic DNA library (18). To clone the gene, we made use of the severe growth defect of the mutant at low extracellular glucose concentrations (14, 15). Library transformants were selected simultaneously for leucine prototrophy (Leu$^+$) and for restored ability to grow on low glucose (5 mM) agar medium (l-Glc$^+$). Four transformants displaying a Leu$^+$ l-Glc$^+$ phenotype were further examined for AO activity in colonies grown on high (55 mM) glucose plates. All displayed a wild-type phenotype, *i.e.* full repression of AO synthesis by glucose (Aog$^-$). To isolate the *GCR1* gene, total DNA was extracted from the transformants, and, after transformation of the total genomic DNA preparations into *E. coli* and amplification, four plasmids were recovered. All four were able to transform the *gcr1-2 leu1-1* strain to Leu$^+$, l-Glc$^+$, and Aog$^-$ phenotypes at high efficiency, suggesting that the plasmids most likely each harbored the complementing *GCR1* gene. Restriction mapping of these four plasmids revealed identical 2.0-kb PstI and 1.2-kb SalI fragments in the genomic DNA inserts in each. Both restriction fragments were found to originate from within an ~3.3-kb long region of genomic DNA present in one of the plasmids, named pOS22. Subsequent sequence analysis of this fragment revealed a single open reading frame (ORF) of 1,623 bp, the putative *GCR1* gene, predicted to encode a polypeptide of 541 amino acids (Fig. 1*A*). This ORF was subsequently shown to be *GCR1* (see below).

A search of the protein databases revealed significant sequence similarity between the deduced amino acid sequence of Gcr1p and a number of proteins belonging to the large family of hexose transporters from different organisms (30, 31). The protein with the strongest similarity was AmMst-1p from the fungus *Amanita muscaria* (48% identity, 65% similarity) (GenPept accession no. CAB06078). Gcr1p was also found to share 44% identity and 62% similarity with a core region of 478 amino acids from the *S. cerevisiae* high affinity glucose sensor, Snf3p (32, 33). Other proteins with strong similarity included:

*S. cerevisiae* Rgt2p (34), *Kluyveromyces lactis* Rag4p (35), and *Neurospora crassa* Rco3p (36). Gcr1p exhibited less than 36% identity to other proteins of the hexose transporter family. Gcr1p is predicted to contain twelve membrane-spanning domains (TM) (Fig. 1*B*). These TMs are characteristic of the hexose transporters and related carriers (30, 31). Alignment of the putative Gcr1p homologues showed that the TMs were also the most conserved regions in the primary sequences (Fig. 2*A*). Gcr1p and its putative homologues, *Sc*Snf3p, *Sc*Rgt2p, *Kl*Rag4p, and *Nc*Rco3p, had in common several unique conserved amino acid residues not found in other hexose transporters. They included Gcr1p tyrosine residues $Tyr^{199}$ and $Tyr^{266}$, with $Tyr^{266}$ predicted to be the target of a tyrosine protein kinase (38). An interesting feature of Gcr1p is that its hydrophilic region between TM6 and TM7, is larger relative to the other homologues and consists of 80 amino acid residues, whereas this region in the other proteins ranges from 71 to 74 amino acids. In addition, Gcr1p lacks a C-terminal extension present in its putative yeast homologues. However, a short sequence of amino acids exhibiting similarity to the so-called "glucose-sensing" domains of *Sc*Snf3p, *Sc*Rgt2p, and *Kl*Rag4p is present in the Gcr1p C terminus (Fig. 2*B*) (33). The consensus sequence from this region is (M/L)G(L/I)$X_4$(K/R)G, with two glycine residues ($Gly^{520}$ and $Gly^{527}$ in Gcr1p) that are conserved in each protein. Such a conserved amino acid sequence is not found at the C termini of other hexose transporters. In the promoter region of the *GCR1* gene, a small ORF of 93 bp was identified at positions −146 to −84 bp upstream of the *GCR1* translational start site (Fig. 1*A*). Small uORFs with distinctive sequences are also found in the *ScSNF3* and *NcRCO3* promoter regions and have been proposed to play regulatory functions in the expression of the associated ORFs (36). A potential TATA box for the *GCR1* gene is located at position −58 bp. Also, four hypothetical binding sites for a putative Mig1-like repressor protein exist at −24, −89, −105, and −335 bp upstream of the *GCR1* translational start codon (Fig. 1*A*). The consensus sequence for the four sites is (A/G)(A/G)AAA$N_1$(C/G)TGGGG, which corresponds well to that suggested for *Sc*Mig1p and found also in the *ScSNF3* promoter region (39).

*Construction of a GCR1 Deletion Strain*—To confirm that the identified ORF was the *GCR1* gene, a deletion mutant was constructed by the gene replacement method. For this, plasmid pOS29 was constructed in which 1479 bp of the *GCR1* coding sequence (encoding amino acid residues 1–493) were replaced by a fragment containing the *S. cerevisiae LEU2* gene as described under "Experimental Procedures." This *gcr1*Δ::*ScLEU2* allele was released with two restriction enzymes on a 3.9-kb DNA fragment and transformed into *H. polymorpha leu1-1 met6*. $Leu^+$ transformants were selected on a sucrose-containing medium without leucine and subsequently analyzed for typical $Gcr^-$ (l-$Glc^-$, $Aog^+$) phenotypes. Total genomic DNA was isolated from several transformants unable to grow on l-Glc medium (l-$Glc^-$) and displaying high AO activity on h-Glc plates ($Aog^+$). With this DNA used as a template, PCR analysis indicated a correctly targeted chromosomal integration of the *gcr1*Δ::*ScLEU2* fragment (not shown). Subsequently, the *gcr1*Δ::*ScLEU2 leu1-1 met6* strain was crossed with a *gcr1-2 leu1-1* strain and prototrophic diploid cells were examined for the $Gcr^-$ phenotype. All were l-$Glc^-$ and $Aog^+$. Additionally, after sporulation, ~1,000 spore products were grown on sucrose plates, then replica plated onto l-Glc plates. No colonies were observed. Together, these results demonstrated that the *gcr1-2* and *gcr1*Δ alleles were tightly linked and most probably mutant alleles of the same gene.

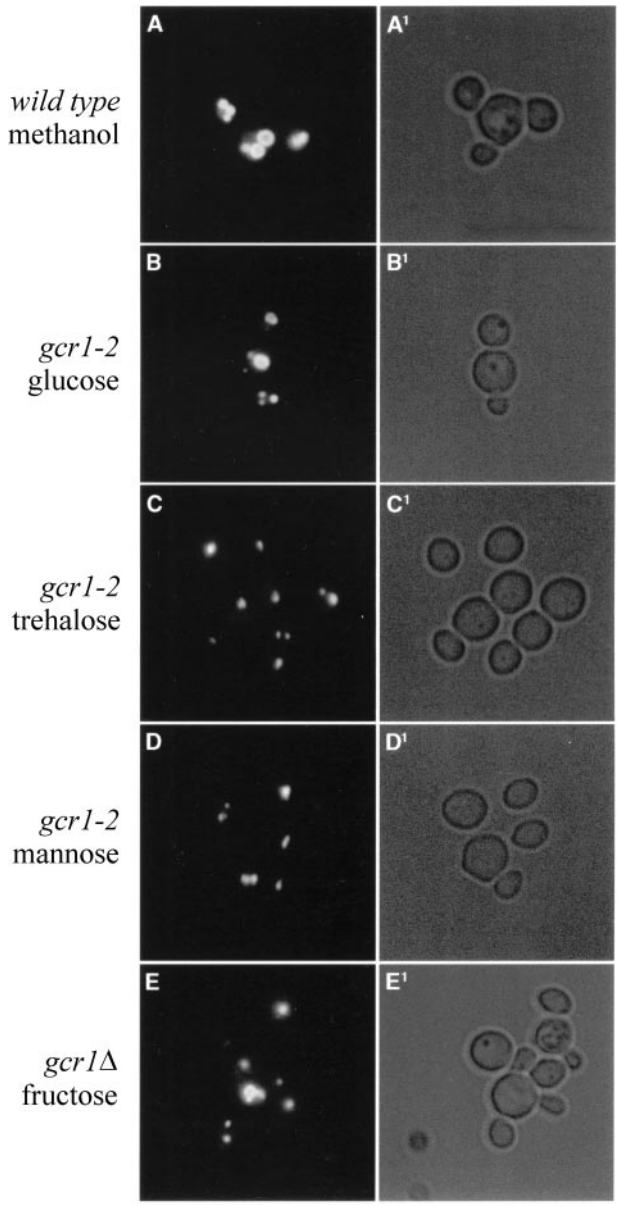


FIG. 4. **Fluorescence microscopy images of wild-type, *gcr1-2*, and *gcr1*Δ strains expressing peroxisome-targeted EGFP-PTS1 fusion protein on different carbon substrates.** *Left panels* (*A–E*), mid-exponential cells were analyzed for the presence of fluorescent peroxisomes with an excitation light wavelength of 490 nm. *Right panels* (*$A^1$–$E^1$*), the same field of cells in visible light. The same fluorescence intensity setting was used for all images.

*Cloning of the gcr1-2 Mutant Allele*—The phenotype of the *gcr1-2* mutant was similar but not identical to that of a *gcr1*Δ strain. We determined the molecular nature of the *gcr1-2* mutation by isolating the mutated gene from genomic DNA of *gcr1-2* by PCR, and sequencing (see "Experimental Procedures" for details). A point mutation was identified that caused a transition from C to T at position 254 of the *GCR1* ORF. In addition, only those fragments of pOS22 that contained the N-terminal part of the wild-type *GCR1* gene including nucleotide 254, were able to rescue our *gcr1-2* mutation when integrated into the mutant genome, thus confirming the 5′ location of the site of the mutation in the gene (not shown). The mutation resulted in the substitution of a semi-conserved serine residue ($Ser^{85}$) with a phenylalanine (Fig. 2*A*). At this position, only one of three amino acid residues is found in hexose transporters: alanine, glycine, or, in a majority of proteins, serine.

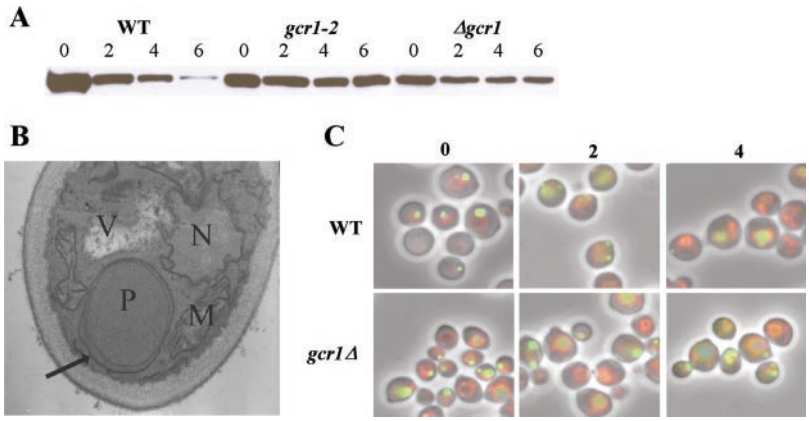


FIG. 5. **Peroxisome degradation in *gcr1* mutants.** *A*, Western blot analysis of AO degradation. Cells pregrown in YPS medium were induced in methanol medium for 19 h and shifted to fresh glucose medium. Aliquots were taken at time points 0, 2, 4, and 6 h to measure AO activity and for Western blotting for AO. Equal amounts of protein were loaded per lane. The initial AO activity (time point 0) for the wild-type strain was 2.77 units/mg, *gcr1-2*; 1.5 units/mg and Δ*gcr1*, 0.78 units/mg. The residual AO specific activity in time point 6 was for the wild-type strain, 1.5%; *gcr1-2*, 46%; and Δ*gcr1*, 35% of the corresponding initial activity. *B*, electron microscopic image of Δ*gcr1* cell 2 h after transfer from methanol medium to fresh glucose medium. Additional membrane enwrapping autophagic peroxisome (specific for macropexophagy) is indicated by *arrow*. *P*, peroxisome; *V*, vacuole; *N*, nucleus; *M*, mitochondrion. *C*, combined fluorescent microscopic images of the wild type and Δ*gcr1* cells with GFP-PTS1 labeled peroxisomes and FM64-labeled vacuoles. Time points indicated on the *top* correspond to methanol-induced cells (0), and those after 2 and 4 h of glucose adaptation.

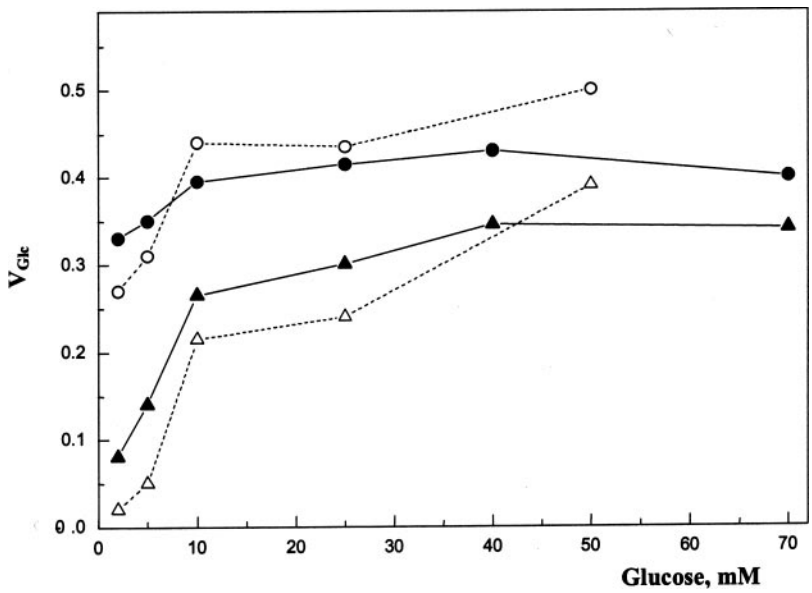


FIG. 6. **Glucose uptake and utilization as a function of extracellular glucose concentration.** Glucose uptake (*open symbols*, *dashed lines*) and consumption from the medium (*solid symbols* and *lines*) were measured in wild-type (*circles*) and *gcr1-2* (*triangles*) strains as described under "Experimental Procedures." The glucose consumption rate ($V_{Glc}$) is given in grams per hour per gram of cells (dry weight).

*Effect of Carbon Substrates on AO Repression and Peroxisome Biogenesis in gcr1 Mutants*—Defects in the *GCR1* gene lead to synthesis of the peroxisomal enzyme AO in glucose-grown cells (Table II and Ref. 14). The level of AO induction in glucose-grown mutant cells was comparable or higher relative to wild-type cells induced by methanol under analogous conditions (1.7 units/mg). Remarkably, the AO-repression defect was more pronounced in the missense *gcr1-2* mutant relative to the *gcr1*Δ mutant (Table II). The defects in repression were associated with the presence of AO-containing peroxisomes in mid-exponential glucose-grown cells of *gcr1* mutants (Fig. 3 and Ref. 14).

Both *gcr1-2* and *gcr1*Δ cells were impaired in the repression of AO synthesis in response to a number of other carbon substrates that are strong AO repressors in the wild-type strain (Table III). This defect was accompanied by retarded growth of both *gcr1* mutants on these substrates relative to the wild-type strain. In addition to glucose, other sugars that no longer repressed AO in *gcr1* mutants included mannose, xylose, and trehalose. Substrates that continued to normally repressed AO synthesis were sucrose, maltose, and ethanol. Remarkably, we observed that repression in response to fructose was defective in the deletion strain but was normal in the *gcr1-2* mutant (Table III).

To confirm that the AO repression defect in *gcr1* mutants on different carbon sources corresponded to altered transcriptional regulation of the *AO* promoter ($P_{MOX}$), we constructed strains with fluorescently labeled peroxisomes. These strains expressed peroxisome-targeted enhanced green fluorescent protein (EGFP-PTS1) under control of $P_{MOX}$. As expected, microscopic examination revealed fluorescent spots in methanol grown wild-type cells, as well as in *gcr1* mutant cells grown on sugars that failed to repress AO synthesis (Fig. 4). Cells of the *gcr1-2* mutant grown on fructose, of both *gcr1* missense and deletion mutants grown on either sucrose, maltose, or ethanol, as well as cells of the wild-type strain grown on the above sugars or ethanol, did not exhibit fluorescence (not shown). It appears that Gcr1p is selectively involved in repression of $P_{MOX}$, triggered only by a subset of carbon substrates.

*Ultrastructural Studies of Peroxisome Degradation in gcr1 Mutants*—When shifted to fresh glucose medium, methanol-grown cells of the wild-type strain exhibit a fast decrease in AO

activity due to the selective autophagic degradation of peroxisomes (termed pexophagy) (5). We addressed the question as to whether deficiency in the *GCR1* gene also affects the pexophagic process. We observed a decrease in AO specific activity and AO protein levels in *gcr1* cells upon glucose adaptation, but residual AO levels were higher in the *gcr1* mutants relative to wild type (Fig. 5*A*). However, these data do not demonstrate a direct involvement of Gcr1p in pexophagy since in our *gcr1* strains, *de novo* peroxisome synthesis occurs due to the defect in glucose repression.

A time course examination of cell morphology revealed clear signs that pexophagy in *gcr1* mutants proceeds. Some peroxisomes were observed sequestered by additional membrane layers typical for initial stages of macroautophagic peroxisome degradation in *H. polymorpha* (Fig. 5*B*) (7). Also, in *gcr1* cells with fluorescently labeled peroxisomes, the pexophagic process was evident upon glucose adaptation. Shortly after the shift, GFP fluorescence was observed in vacuoles, while in methanol-growing cells it is confined to peroxisomes (Fig. 5*C*). These data led to the conclusion that Gcr1p is not directly involved in pexophagy. Both *gcr1* mutants continued to exhibit normal wild-type peroxisome degradation in response to ethanol (not shown).

*Glucose Uptake and Consumption in H. polymorpha gcr1-2*—The glucose repression defect in the *gcr1-2* mutant was accompanied by retarded growth on glucose (14). Since glucose phosphorylation activity was normal in *gcr1-2* cells, we suggested that a defect in glucose transport might be the primary cause of mutant catabolite repression deficiency (14, 15). In further investigations of this phenotype, we have determined that the rate of glucose consumption by the *gcr1-2* mutant relative to the wild-type strain was decreased at all extracellular sugar concentrations (Fig. 6). The relative difference between the two strains was most pronounced at low extracellular glucose concentrations (*e.g.* at 5 mM the rate was 3.5-fold slower in the mutant relative to wild type), but this effect diminished with increasing glucose concentrations (*e.g.* at 55 mM the rate was only 1.3-fold slower than wild type) (Fig. 6). Using [$^{14}$C]glucose, we observed that the kinetics of glucose uptake in the *gcr1-2* mutant closely matched that of glucose consumption (Fig. 6). In contrast, fructose was transported and consumed by the mutant at wild-type rates (not shown).

Consistent with a relative decrease in the rate of glucose transport at low extracellular glucose concentrations (Fig. 6), the *gcr1-2* mutant was able to grow on methanol or maltose media containing 1 mM, but not 10 mM 2-deoxyglucose (not shown). Either concentration of 2-deoxyglucose, a toxic glucose analogue capable of exerting a repression effect, completely blocked growth of wild-type cells in these two media.

*Effect of Extracellular Glucose Concentration on AO Repression in gcr1 Mutants*—The deficiency of the *gcr1-2* mutant in AO repression is not a function of extracellular glucose concentration and, consequently, glucose uptake. In cells incubated in high-glucose (55 mM) medium, where the transport defect in the *gcr1-2* mutant is less pronounced, AO activity was the highest (Table II). In low-glucose (5 mM) medium, where glucose uptake in the *gcr1-2* mutant is severely impaired, AO activity was lower. A similar pattern of AO activity levels relative to glucose concentration was also displayed by our *gcr1*Δ strain. In the wild-type strain, any of these glucose concentrations was sufficient to completely repress AO synthesis (Table II).

*Growth of the gcr1-2 Mutant in Glucose/Methanol Mixtures*—Consistent with the classical catabolite repression paradigm (29), a wild-type strain of *H. polymorpha* utilizes glucose first when incubated in a glucose/methanol mixture, while enzymes of methanol utilization remain repressed. In the *gcr1-2* mutant, we observed that the utilization of these carbon sources was reversed, *i.e.* glucose consumption initiates following exhaustion of methanol from the medium (Fig. 7). Furthermore, methanol, when administrated as a pulse to mid-exponential glucose-grown *gcr1-2* cells, transiently blocked growth and glucose utilization of the strain. After a lag-period of several hours, *gcr1-2* cells resumed growth, utilizing methanol as carbon substrate (not shown). Addition of methanol to glucose-grown wild-type cells did not affect the growth and substrate utilization pattern. Consistent with our previous data, fructose remained the preferred substrate in the *gcr1-2* mutant grown in fructose/methanol mixtures (not shown).

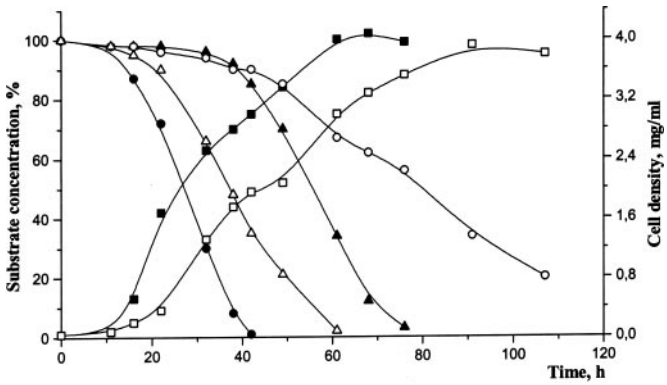


FIG. 7. **Growth and carbon substrate consumption in glucose and methanol medium mixture.** Kinetics of growth (*squares*), glucose (*circles*), and methanol (*triangles*) consumption in wild-type (*solid symbols*) and *gcr1-2* (*open symbols*) strains grown in batch culture. Initial glucose and methanol concentrations were 55 mM and 1% v/v respectively.

## DISCUSSION

The selection procedure utilized to isolate the *gcr1* mutants presumed a pleiotropic phenotype. One mutant phenotype was the ability to grow on methanol in the presence of 2-deoxyglucose. Growth on methanol in the presence of this compound requires deregulation of genes encoding enzymes and other proteins essential for methanol metabolism (5). Another phenotype is insensitivity to the accumulation of toxic phosphorylated derivates of 2-deoxyglucose, *e.g.* by reducing the rate of glucose uptake or early steps in glucose catabolism. In this report, we demonstrate that the *gcr1-2* mutant is deficient both in glucose repression and glucose uptake, and that the *GCR1* gene encodes a protein homologous to other hexose transporters. The most remarkable feature of *gcr1* mutants is the constitutive presence of AO-containing peroxisomes in glucose-grown cells. Such a phenotype confirms that the *H. polymorpha AO* gene is controlled primarily at the transcriptional level by represion/derepression mechanisms and that full expression of the gene is not dependent on a methanol induction mechanism (see also Ref. 4).

We demonstrated that deficiency in *GCR1* does not block autophagic peroxisome degradation upon adaptation of methanol-grown cells to glucose. Nevertheless, *gcr1* mutants are capable of preferentially utilizing methanol in the presence of glucose. This fact suggests that glucose metabolism is required for pexophagy to proceed. The *gcr1* mutants may serve as a unique model to study molecular mechanisms of peroxisome homeostasis. An intriguing question is how the fate of pre-existing and newly formed peroxisomes is regulated in the mutants under pexophagy-triggering conditions.

A *gcr1*Δ mutant retains the capability to grow well on elevated concentrations of glucose, suggesting that other sugar transporter(s) facilitate the uptake of glucose in the absence of

Gcr1p. Two kinetically distinct glucose transport systems have been described in *H. polymorpha*: a high and a low-affinity system (40). Our data suggest that Gcr1p could be primarily involved in high-affinity glucose transport, as growth of *gcr1* mutants at glucose concentrations of less than 5 mM is severely hampered. However, to determine whether Gcr1p is, in fact, a functional glucose transporter, further studies are required.

For *S. cerevisiae* hexose transport mutants, glucose uptake capacity determines the strength of the repression signal (41, 42). However, in our *H. polymorpha gcr1-2* mutant, the defect in AO repression did not correlate with a concentration-dependent glucose transport capacity, as AO levels were higher in cells fed with higher concentrations of glucose. Thus, it is possible that Gcr1p somehow functions directly in AO repression, rather than just as a sugar carrier. The phenomenon of methanol inhibition of glucose utilization in the *gcr1-2* mutant, also suggests a regulatory function for Gcr1p. We hypothesize that the glucose transport deficiency in the *gcr1-2* mutant alone is not sufficient to cause this regulatory effect. Alternatively, this phenomenon may be a common physiological feature of *H. polymorpha* mutants deficient in glucose repression (13), and may result from altered carbon fluxes through glycolysis and gluconeogenesis pathways.

All closely related potential orthologues of Gcr1p are integral proteins of the plasma membrane and are involved in glucose sensing and/or glucose repression. This sensing/repression function and sequence similarity separates these transporters into their own subgroup within the hexose transporter family (30). The closest *GCR1* homologues in *S. cerevisiae*, *Sc*SNF3, and *Sc*RGT2, are thought to encode high and low affinity glucose sensors, respectively, that are non-functional as sugar carriers. Both gene products are involved primarily in the regulation of glucose transport via differential induction of other hexose transporters in response to changes in extracellular glucose concentrations, but are not thought to act directly in the repression pathway (33, 34). Two other close Gcr1p homologues, *K. lactis* Rag4p and *N. crassa* Rco3p, seem to be directly involved in a signaling mechanism for repression, in addition to sugar transport (35, 36). For instance, *Nc*Rco3p regulates catabolite repression of conidiation genes, the synthesis of several repressible enzymes, as well as glucose transport in this fungus (36). Like *ScSNF3* and *ScRGT2*, *NcRCO3* is expressed at a low level (36). However, there are no conclusive data, as to whether it is a functional transporter or a sensor protein.

*Hp*Gcr1p, in contrast with the other putative sensor homologues, *Sc*Snf3p, *Sc*Rgt2p, *Nc*Rco3p and *Kl*Rag4p, lacks an elongated protein-specific C-terminal region (43). For *Sc*Snf3p, this region is functionally essential for glucose signaling (33). A unique short amino acid sequence at the Gcr1p C terminus exhibits similarity to the putative C-terminal "glucose-sensing" domains of *Sc*Snf3p, *Sc*Rgt2p, and *Kl*Rag4p (Fig. 2*B*). Although, this similarity is too low to conclude that Gcr1p plays a role in glucose sensing from sequence comparison alone, the concept that Gcr1p is a sensor protein that affects glucose transport via a signaling mechanism similar to *Sc*Snf3p and *Sc*Rgt2p is a reasonable working hypothesis.

The mutation in *gcr1-2* is predicted to result in the substitution of a serine residue for a phenylalanine at position 85 in the amino acid sequence of Gcr1p. This amino acid change resides in the second predicted transmembrane segment (see Fig. 2*A*), which overlaps with a leucine zipper motif in many hexose transporters (30). The leucine zipper motif has been proposed to be essential for hetero- or homo-oligomerization of hexose transporters (30). It was also shown that the glucose-sensing function of *Sc*Snf3p requires the presence of at least one of the *HXT* gene products, suggesting their possible interaction (44). Although this putative leucine zipper motif appears to be rather degenerate in Gcr1p and its closest homologues, $Gcr1^{S85F}p$ may be unable to form oligomeric structures or to correctly interact with other downstream components involved in signaling for repression. Alternatively, a mutation in the second TM may lead to cytoplasmic mislocalization of Gcr1p, as demonstrated for one of the *Sc*Snf3p mutants, namely in *snf3-72* (45). This question will be addressed in further studies. The second membrane-spanning segment of hexose transporters is not thought to be involved in formation of a channel pore (31).

Remarkably, the glucose repression defect in the *gcr1-2* mutant is more profound than in the *gcr1*Δ strain. In addition, the *gcr1-2* strain is not defective in fructose-mediated repression, while the *gcr1*Δ strain is. Similarly, certain mutants in *ScSNF3* and *NcRCO3* also exhibit phenotypes different from their respective null mutant strains. For example, an $rco3^{1}$ mutant was able to conidiate within the agar of solid medium, while the deletion mutant, $rco3^{3}$, like the wild-type strain, could not. Other glucose-repressible phenotypes were the same between *N. crassa rco3* mutants (36). These results can be explained if one assumes that the peculiar and distinct phenotypes of the missense mutants are the result of an abnormal function conferred on the protein, whereas null phenotypes result from the abolition of all functions of that protein.

Results with the *gcr1* mutants suggest the existence of specific *GCR1*-independent repression pathway(s) in *H. polymorpha* for sucrose, maltose and ethanol. In both the missense *gcr1-2* and *gcr1*Δ mutants, these substrates continue to repress AO synthesis and to support a wild-type growth rate on these substrates. In contrast, trehalose, mannose, fructose, and xylose seem to have *GCR1*-dependent repression and transport mechanisms. This observation suggests that, in *H. polymorpha*, hydrolysis of a disaccharide to hexose residues precedes the transport step for trehalose, but not maltose and sucrose, a conclusion that is consistent with other reports (40).

The sugar-specific phenotype of *gcr1* mutants provides an opportunity for their exploitation as hosts for $P_{MOX}$-directed expression of heterologous proteins (7), as demonstrated in this report with EGFP-PTS1. Such an expression system would combine the advantages of the strong regulatable $P_{MOX}$ with the utilization of convenient sugar substrates (*i.e.* sucrose and glucose, respectively) for growth and induction of *gcr1*-based production strains, while avoiding the use of toxic and flammable methanol. We have also successfully utilized the glucose repression-deficient mutants derived from *gcr1*, for AO production in glucose medium (46, 47).

In conclusion, we have identified and characterized a gene essential for glucose repression in *H. polymorpha*. This gene, *GCR1*, encodes a hexose transporter homologue. We have also demonstrated that deletion of this gene, or a point mutation that causes a single amino acid substitution relieves repression triggered by several but not all sugar substrates. Our data implicate Gcr1p in an early stage of the repression mechanism, functioning either in glucose transport or glucose signaling.

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