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Research Article

Influence of low glycolytic activities in *gcr1* and *gcr2* mutants on the expression of other metabolic pathway genes in *Saccharomyces cerevisiae*

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Abstract

A complex of the transcription factors Gcr1p and Gcr2p coordinately regulates the expression of glycolytic genes in *Saccharomyces cerevisiae*. To understand the effects of *gcr* mutations on other metabolic pathways, genome-wide gene expression profiles in *gcr1 and gcr2* mutants were examined. The biggest effects of *gcr1* and *gcr2* mutations were observed on the glycolytic genes and the expressions of most of the glycolytic genes were substantially decreased compared to those in the wild-type strain in both glucose and glycerol + lactate growth conditions. On the other hand, the expressions of genes encoding the TCA cycle and respiration were increased in *gcr* mutants when the cells were grown in glucose. RT-PCR analyses revealed that the expression of *SIP4* and *HAP5*, which are known to affect the expression of some of the gluconeogenic, TCA cycle and respiratory genes, were also increased under this condition. The growth of *gcr* mutants on glucose was impaired by adding respiration inhibitor antimycin A, whereas the growth of the wild-type strain was not. The conversion of glucose to biomass was higher and, to the contrary, ethanol yield was lower in the *gcr2* mutant compared to those in the wild-type strain. These results suggest the possibility that the *gcr* mutants, in which glycolytic activities are low, changed their metabolic patterns under glucose growth condition to enhance the expression of TCA cycle and respiratory genes to produce more energy.

Keywords: ***Saccharomyces cerevisiae*; glycolysis; *GCR* genes; transcription**

Introduction

Glycolysis is the major route of carbohydrate metabolism in *S. cerevisiae* and the glycolytic enzyme genes are highly expressed (Fraenkel, 1982). Whether their expression is regulated may be strain-dependent, with reports ranging from virtual constitutivity (Clifton and Fraenkel, 1981; Baker, 1986; Uemura and Fraenkel, 1990) to apparent induction by glucose of at least some of the enzymes (Maitra and Lobo, 1971; Cohen *et al*., 1987; Chambers *et al*., 1989; Nishizawa *et al*., 1989). Since many of the steps of glycolysis are shared by gluconeogenesis, most glycolytic genes, including those with glucose induction, are also highly expressed in media containing non-fermentable carbon sources.

The core elements of the upstream activation sequence (UAS) of glycolytic enzyme genes consist of the binding sites for Rap1p and Gcr1p (Huie *et al*., 1992). The observations that the UAS elements of most of the glycolytic genes contain binding sites for Rap1p and Gcr1p in close vicinity, and that these binding sites display a strong synergism for each other (Chambers *et al*., 1995), suggest that Rap1p and Gcr1p are intimately involved in glycolytic gene expression. Since the binding sites for Rap1p is not restricted to glycolytic enzyme genes, and Rap1p is capable of carrying out many diverse cellular functions, such as activation and

repression of transcription and control of telomere length depending on the sequence context of its binding site (Shore, 1994), it has been suggested that its function may be determined by the interaction with other regulatory proteins. Such transcription factors involved in specific regulatory mechanisms for glycolytic gene expression are *GCR1* (Clifton *et al*., 1978) and *GCR2* (Uemura and Fraenkel, 1990). In contrast to Rap1p, the binding sites for Gcr1p, the CTTCC motifs (Baker, 1991), are more or less restricted to genes encoding glycolytic enzymes and Ty elements (Lopez and Baker, 2000), and *gcr1* mutants are severely reduced in expression of most glycolytic enzymes at the transcriptional level (Baker, 1986; Clifton and Fraenkel, 1981; Kawasaki and Fraenkel, 1982; Uemura and Fraenkel, 1990).

It was thought that the conformational change of DNA by the binding of Rap1p helps the binding of other regulatory factors to DNA, because the binding of Rap1p induces local bending and distortion of the DNA helix (Vignais and Sentenac, 1989; Gilson *et al*., 1993), and it has been proposed that Rap1p plays a role in maintaining the accessibility of transcription factor binding sites within chromatin (Devlin *et al*., 1991). Alternatively, protein–protein interactions between Gcr1p and Rap1p (Tornow *et al*., 1993) may be important for the regulation of the glycolytic gene expression. *In vivo* binding studies demonstrated that Gcr1p is unable to bind at its binding site in the absence of an appropriately spaced and bound Rap1p-binding site (Drazinic *et al*., 1996), and we showed that oligonucleotides composed of tandem Gcr1p-binding sites in the absence of Rap1p-binding sites can function as strong UAS elements (Uemura *et al*., 1997). These lines of evidence suggest that the most likely role of Rap1p at glycolytic promoters is to facilitate the binding of Gcr1p (Drazinic *et al*., 1996; Uemura *et al*., 1997; Lopez *et al*., 1998).

In addition to Rap1p, Gcr1p also interacts with Gcr2p to mediate high-level expression of glycolytic genes. Genetic studies suggest that *GCR2* also regulates the expression of most glycolytic genes, and the pattern of reduction of glycolytic enzymes in *gcr2* mutants is similar to that in *gcr1* mutants (Uemura and Fraenkel, 1990). Uemura and Jigami (1992) presented genetic evidence for a physical interaction of Gcr2p and Gcr1p and postulated that Gcr2p likely functions as a co-activator in the Gcr1p–Gcr2p complex (Uemura and Jigami, 1995), presumably by changing the conformation of Gcr1p (Deminoff and Santangelo, 2001).

In this report we examined the effect of *gcr* mutations, which decrease the entire glycolytic gene expression, on the other metabolic pathways and showed that the decrease of glycolytic flux affects the expression of the TCA cycle and the respiratory genes.

Materials and Methods

Yeast strains and media

Yeast strain 2845 (*MATα leu2-3 leu2-112 ura3-52 his6*) (Uemura and Fraenkel, 1990), its isogenic *gcr1* disruptant strain C179-15C-F16 (*MATα*Δ *gcr1::ura3 leu2-3 leu2-112 ura3-52 his6*) (Uemura and Jigami, 1995), and *gcr2* disruptant strain YHU3002-8C-F16 (*MATα*Δ*gcr2-3F leu2-3 leu2-112 ura3-52 his6*) (Uemura *et al*., 1997) were used for this study. Yeast cells were grown in rich medium (Uemura and Wickner, 1988) and synthetic complete medium (SC) (Sherman *et al*., 1986) or SC dropout medium, depending on the selective pressure required to maintain the plasmids. As carbon sources, glucose (Glc) or glycerol + lactate (GL) were added as indicated. Yeast cells were transformed by the method of Ito *et al*. (1983).

Growth conditions

Since the growth of *gcr* mutants, especially the *gcr1* mutant, is slow on glucose, cells were routinely grown in rich medium supplemented with 2% each of glycerol and lactate (YPAGL). For the analysis of cells grown in glucose, yeast strains were grown at 30 °C in 100 ml rich medium supplemented with 2% glucose (YPAD) in 500 ml Erlenmeyer flasks, starting from OD_{600} = 0.001 [for 2845 (WT)], 0.1 [for C179-15C-F16 (Δ*gcr1::ura3*)] and 0.01 [for YHU3002-8C-F16 (Δ*gcr2::ura3*)] after subculture in YPAGL. For the analysis of cells grown in glycerol + lactate, all the strains were grown at 30 °C in 100 ml YPAGL starting from $OD_{600} = 0.01$ after subculture in YPAGL. At $OD_{600} = 0.5$, cells were rapidly chilled on ice and recovered by centrifugation at 4 °C after washing once with 40 ml ice-cold water. Cell pellets were immediately frozen in liquid

nitrogen and kept stored at −80 °C until processed for RNA purification.

RNA isolation and preparation of Cy3 and Cy5 labelled cDNA

Total RNA was extracted by the hot phenol method (Schmitt *et al*., 1990). Briefly, the frozen cells were resuspended in 10 ml of sodium acetate buffer (pH 5.2) with 0.5% SDS; 12 mL 65 °C phenol saturated with sodium acetate buffer were added quickly to the cell suspension, and total RNA was extracted by short vortexing (every 30 s at room temperature) of samples while incubating them at 65 °C for 10 min. After standing tubes on ice for 5 min, the aqueous phase was separated by centrifugation and was further extracted with chloropane (50% phenol, 50% chloroform, 0.5% 8-hydroxychinoline equilibrated with ANE buffer), and then with chloroform:isoamyl alcohol (24 : 1). Total RNA was precipitated by adding 1/5 volume of 3 M sodium acetate (pH 5.2) and one volume of isopropanol. PolyA RNA was subsequently purified using the Oligotex-dT30 mRNA purification kit (Takara Bio, Japan), according to the protocol recommended by the manufacturer.

PolyA RNAs (2 μg aliquots) were fluorescently labelled with Cy3-dUTP or Cy5-dUTP and unincorporated substrates were removed by gel filtration, using the RNA Fluorescence Labeling Core Kit (Takara Bio, Japan), according to the manufacturer's protocol. PolyA RNA from the wild-type strain was labelled with Cy3 and the RNA from the mutant strains was labelled with Cy5.

cDNA microarray hybridization

DNA microarrays spotting 5818 PCR-amplified yeast open reading frames onto glass microscope slide glasses were obtained from DNA chip research laboratory (Yokohama, Japan). Mixture of Cy3- and Cy5-labelled cDNAs derived from 2 μg each of polyA RNAs was suspended in 50 μl hybridization solution (5× SSC, 0.1% SDS, 0.9 μg/μl polyA DNA), and hybridization was performed at 65 °C for 15 h in a hybridization chamber. After hybridization, cover glasses were removed in 2× SSC-0.1% SDS solution, and arrays were washed (2× SSC-0.1% SDS for 10 min at room temperature (twice), 0.2× SSC-0.1% SDS for 10 min at room temperature (twice), 0.2× SSC-0.1% SDS for 20 min at 60 °C (twice), 0.2× SSC for 20 min for room temperature (twice), rinse with 0.2× SSC for 5 min at room temperature, rinsed with 0.05× SSC for 5 min at room temperature), and immediately dried by centrifugation.

Signal detection and analysis

Arrays were scanned by ScanArray 4000 (GSI Lumonics) and image analysis was performed by QuantArray. Cy3 and Cy5 fluorescent intensities were normalized by using global normalization methods. The overall fluorescent intensities (the total hybridization signals) were scaled to be equal and then the ratio between adjusted signals of each spot was calculated. Three replicate hybridizations were performed in each condition, using independently prepared RNA samples.

Gene functions were classified according to the functional categories of SGD (**http://yeastgeneme.org/**), MIPS (**http://mips.gsf.de/genre/proj/yeast/index.jsp**) and YPD (**https://www.incyte.com/control/tools/proteome**).

RT-PCR

RT-PCR analysis was performed by using the SuperScript One-Step RT-PCR System with platinum Taq DNA polymerase (Invitrogen). RT-PCR reactions were performed in a 15 μl volume containing 0.2 mM each of deoxynucleotide triphosphates, 0.2 μM each of sense and anti-sense primers, 0.3 μl RT/Taq mixture, and an appropriate amount of polyA RNA. Fragments were amplified by incubation at 50 °C for 30 min (reverse-transcription), 94 °C for 2 min (pre-treatment), *n* cycles of 94 °C for 30 s/55 °C for 30 s/72 °C for 90 s, and additionally 72 °C for 5 min (extension). Amplified fragments were run on 1% agarose gels and the intensity of each band was examined. Primer sets used for amplification of genes are listed in Table 1.

Glucose and ethanol assays

For the analysis of glucose and ethanol concentration, cells were grown in SC containing 0.5% glucose after subculture in SC(GL). Glucose and ethanol concentrations were determined spectrophotometrically by using the Glucose CII test

Table 1. List of primers used for RT-PCR

Gene name	Sequence of the forward primer (5′ to 3′)	Position	Sequence of the reverse primer (5′ to 3′)	Position	Amplified size (bp)
GPM1	ACGCTTCTTCTCCATTCTCTCAAAA	374–398	CCAAGTGCTTAACCAAACCTCTCAA	580–556	207
*ENO1/2**	TAACCCAACCGTCGAAGTCGAATTAACC	48–75	GATTCTCAACAATTGGTTCAATTTAGCC	1248–1221	1201
TDH1	CTGCTCCATCTTCTTCTGCTCCAA	359–382	CAGCACCGGTAGAGGATGGGATAA	634–611	276
HXK2	TTCCCACAAGGTATCTCTGAGCCAA	427–451	TTGATTGGGTAGTCGTCTAGTGAGG	1355–1331	929
ACO1	CATCCATTGTCAAGGATGCTGCTGC	1181–1205	AGCGAAAGACTTTGTGATGATAGCG	2067–2043	887
SDH1	TCAGCGCTCTCCAAGTTGACTTTGC	19–43	CTGCCGTAGATCTTGAACCATTAGC	1507–1483	1489
SDH4	CCATGAAATTTATGACTGGAAGGAG	17–41	AAACCAACAACACCATCATTCTCGG	482–458	466
ATP1	CAAGAGCTCAAGTCAAAGCACCAGG	491–515	GGCAACTTCATAAATGGTGGAGACC	1358–1334	868
HAP1	TGGTTGTCTATCATGAAAGGTGACC	715–739	GTCAAGGTAGTCAAATCATTAGAGG	1991–1967	1277
HAP2	ACGAAACGGATGCGAAATTTCATCC	11–35	TCATGACTATCGTCAGGATCATCGC	716–692	706
HAP3	TACCAACGAGTCCGAACATGTTAGC	6–30	ATACATTAGTTGATTCTTCAGCGCC	408–384	403
HAP4	CATAACAATAGTTCGCTGGAATTCG	121–145	TGAACCTAACTCGTCATGGTGATGC	1341–1317	1221
HAP5	ATTTCTCACCACAACAAGGACAAGG	14–38	ACATCTTCATCCGTCTTCATGACC	524–501	511
SIP4	GAATTTCAACCGAAATCAATCTCGG	1177–1201	ATTGGAGTTAGAACTCGAGTGTTGC	2085–2061	909
SNF1	ACGATTGGTTCAAAGTTGACCTGCC	908–932	ATCTCCATAACTACTTTCCCAGCCG	1785–1761	878
SNF3	GACAGGCAATCTAATATGACATCCG	184–208	GATCGTATTGGAATTTAAGTGCGCG	1747–1723	1564
RGT2	TTATCAGTGGGCTATCACTATTGGG	711–735	TATCGATGATGTTTACTAACGAGCC	1942–1918	1232
ACT1	AGGTTGCTGCTTTGGTTATTGATAACGG	11–38	AGCCAAGATAGAACCACCAATCCAGACG	1031–1004	1021

* These primers hybridize with both *ENO1* and *ENO2* transcripts.

WAKO kit (Wako, Japan) and the ethanol assay F-kit (Roche diagnostics), according to the protocols of the manufacturers.

Results and Discussion

The biggest effects of *gcr* mutations are observed on glycolytic enzyme gene levels

Genetical studies showed that expressions of most glycolytic enzymes are severely reduced in *gcr1* and *gcr2* mutants and the levels of reduction of most glycolytic enzymes are similar in these mutants (Baker, 1986; Clifton and Fraenkel, 1981; Kawasaki and Fraenkel, 1982; Uemura and Fraenkel, 1990). However, there is a lesser growth defect in *gcr2* mutants compared to the severe growth defect in *gcr1* mutants (Uemura and Fraenkel, 1990). Thus, we are quite interested in the genome-wide gene expression patterns in *gcr1* and *gcr2* mutants, and compared the gene expression levels in the mutants to those in the isogenic wild-type strain under fermentable (2% glucose) and non-fermentable (2% glycerol plus 2% lactate) carbon source growth conditions.

PolyA RNAs were prepared from wild-type and *gcr* mutant cells grown in YPAD and YPAGL, as described in Materials and methods. 2 μg each of polyA RNAs were fluorescently labelled with Cy3 (for the wild-type strain) and Cy5 (for the mutant strains) and competitively hybridized to DNA microarrays. The expression ratio to the wild-type was calculated based on the average of triplicated experiments by using the mean of the log2 ratio. A total of 212 genes showed the expression ratio to the wild-type more than three-fold (log2 ratio $> +1.58$) or smaller than 1/3-fold (log2 ratio < -1.58) in *gcr1* mutant strain grown in glucose (*gcr1* Glc down = 91, *gcr1* Glc up = 121), 50 genes in *gcr1* mutant grown in glycerol + lactate (*gcr1* GL down = 24, *gcr1* GL up = 26), 146 genes in *gcr2* mutant grown in glucose (*gcr2* Glc down = 69, *gcr2* Glc up = 77), and 34 genes in *gcr2* mutant grown in glycerol + lactate (*gcr2* GL down = 19, *gcr2* GL up = 15). By using the annotation resources of SGD, MIPS and YPD, the genes affected by *gcr* mutations were categorized. A group of genes, which were most consistently and substantially altered in the *gcr* mutant strains, has functions in glycolysis. Their f values are 9.98×10^{-11} (*gcr1* mutant grown in glucose), 1.64×10^{-20} (*gcr1* mutant grown in glycerol + lactate), 2.63×10^{-8} (*gcr2* mutant grown in glucose), and 1.58×10^{-24} (*gcr2* mutant grown in glycerol + lactate).

Previous studies demonstrated that the expressions of most glycolytic enzymes are severely reduced in both glucose and non-fermentable carbon sources, such as glycerol and lactate, in *gcr1* and *gcr2* mutants (Baker, 1986; Clifton and Fraenkel, 1981; Kawasaki and Fraenkel, 1982; Uemura and Fraenkel, 1990). The fact that their growth defect was observed only on glucose but not on non-fermentable carbon sources suggested that the targets of Gcr1p and Gcr2p were specific to glycolytic genes (Clifton *et al.*, 1978; Clifton and Fraenkel, 1981; Uemura and Fraenkel, 1990). In the *gcr1* mutant, Lopez and Baker (2000) demonstrated that the effect of *gcr1* mutation was highly specific to glycolytic genes and Ty elements by using macroarrays.

Consistent with the previous observations, expression of the genes encoding glycolytic enzymes was most drastically decreased in both Δ*gcr1* and Δ*gcr2* mutants relative to those in the wild-type strain, in both glucose and glycerol + lactate media. They are similar, but the effect was more pronounced in the *gcr1* mutant than in the *gcr2* mutant in general. The behaviour of *TDH1* is an exception. In the *gcr1* mutant its expression was decreased when the cells were grown in glycerol + lactate, but it increased when the cells were grown in glucose. Tdh1p is the least abundant glyceraldehyde-3-phosphate dehydrogenase in the cell (McAlister and Holland, 1985) and it has been reported that *TDH1* may be involved in the stress response because the expression of *TDH1* is induced by heat, stationary phase and glucose starvation (Boucherie *et al.*, 1995). Effect of *gcr* mutations on Ty expression was dominant in glycerol + lactate condition. Our expression profiles in the *gcr1* mutant agreed well with the macroarray result by Lopez and Baker (2000), including the exceptional behaviour of *TDHl* and the glycerol + lactate dominant behaviour of Ty expression.

To further confirm the reduction of glycolytic gene expression in *gcr1* and *gcr2* mutants, amount of *GPM1* and *ENO1/2* mRNAs was measured by RT-PCR. Expression level of *ACT1* was also examined as a control. Based on the pilot PCR reactions, numbers of cycles were fixed to 15, 18 and 21 for

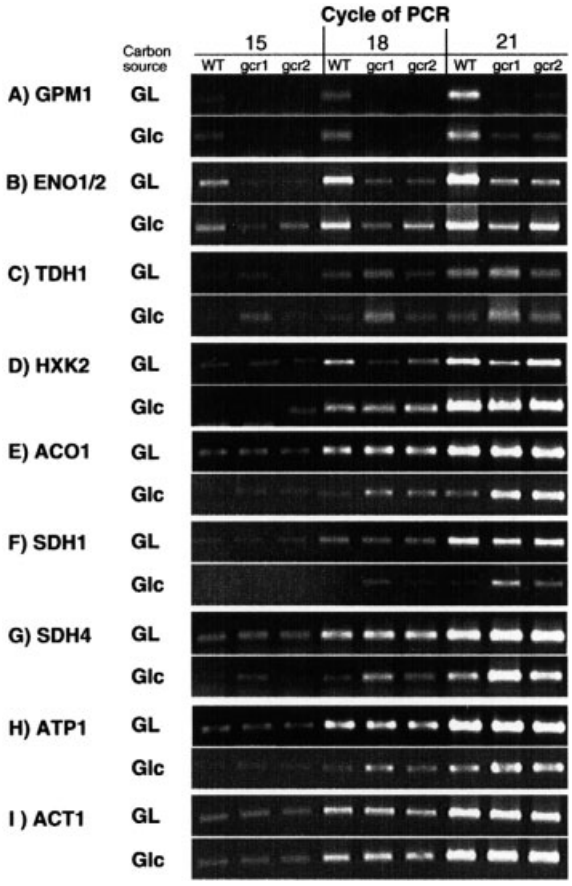

Figure 1. RT-PCR analysis of the expression levels of glycolytic, TCA cycle, and respiratory genes in Δ*gcr1* and Δ*gcr2* mutants. (A–I) Levels of *GPM1, ENO1/2, TDH1, HXK2, ACO1, SDH1, SDH4, ATP1*, and *ACT1* mRNAs in the wild-type, Δ*gcr1*, and Δ*gcr2* strains were examined by RT-PCR. mRNA was prepared from the cells grown in YPAD or YPAGL at $OD_{600} = 0.5$. Genes were amplified by RT-PCR and analysed by 1% agarose gels, as described in Materials and Methods. Cycles of PCR were fixed to 15, 18 and 21 for amplification of linear range. For PCR amplification, 2 ng polyA RNAs were used for *ACO1, SDH4, ATP1* and *ACT1* and 1 ng polyA RNAs was used for the rest of the genes

their amplification with 1 ng polyA RNA as a linear range of amplification. Likewise, the numbers of cycles were fixed to 15, 18 and 21 for *ACT1* amplification with 2 ng polyA RNA. Compared to the wild-type strain, expression levels of *GPM1* and *ENO1/2* were drastically reduced in *gcr1* and *gcr2* mutant strains in both glucose and glycerol + lactate growth conditions (Figure 1A,B), whereas almost the same levels of *ACT1* fragments were observed in both strains (Figure 1I). Absence of genomic DNA in RNA preparations was verified by omitting the reverse transcriptase in the reaction (data not shown). Consistent with the array analysis described above, these results clearly showed that the expression of glycolytic genes was affected in both *gcr1* and *gcr2* mutants. In contrast to the drastic reduction of *TDH2* and *TDH3* expression in *gcr* mutants (data not shown), increased expression of *TDH1* in *gcr1* mutant grown in glucose was also confirmed by RT-PCR (Figure 1C).

The activation mechanism by a complex of transcription factors Gcr1p and Gcr2p is due to the binding of Gcr1p to the *cis*-element containing the CTTCC motif (Baker, 1991), and Gcr2p mediates high level expression of glycolytic genes by making complex with Gcr1p (Uemura and Jigami, 1992, 1995; Uemura *et al*., 1997). Expression of Ty elements is also regulated by binding of Gcr1p with CTTCC motif (Turkel *et al*., 1997). Thus, the effects of *gcr* mutations on glycolytic genes and Ty elements are related to the CT-box. However, the effects on other genes are thought to be secondary. Therefore, in this paper we have focused on the behaviour of those other genes. From this point of view, most of the genes of known functions that were consistently and substantially altered in the mutant strains have functions in ribosomal proteins, TCA cycle, ATP synthesis and oxidative phosphorylation.

Effect of *gcr* mutations on ribosomal protein genes

The most distinct difference between *gcr1* and *gcr2* mutants was observed in the expression of ribosomal protein genes. Expressions of 12 ribosomal protein genes were exclusively decreased more than three-fold in the *gcr1* mutant grown in glucose. If the genes affected more than two-fold were included 57 of 120 ribosomal protein genes were decreased under this condition ($p = 2.50 \times 10^{-12}$). In contrast, only three genes were affected between two- and three-fold when the *gcr1* mutant was grown in glycerol + lactate. Similar results were obtained in the macroarray analysis of the *gcr1* mutant (Lopez and Baker, 2000). Since the expressions of ribosomal protein genes are known to be regulated by the growth rate (Herruer *et al*., 1987; Kief and Warner, 1981; Kraakman *et al*., 1993; Mager and Planta, 1991) and the growth of *gcr1* mutant was severely reduced in glucose, Lopez and

Baker concluded that this is the indirect role of Gcr1p due to the slow growth of the *gcr1* mutant in glucose.

In the *gcr2* mutant, on the contrary, only eight genes were affected two- to three-fold in glucose and no gene was affected in glycerol + lactate growth conditions. *gcr1* and *gcr2* mutants show similar growth rate to the wild-type strain in YPAGL (doubling times of wild-type, *gcr1* and *gcr2* strains in YPAGL were 4.5 ± 0.1, 5.4 ± 0.1, and 4.7 ± 0.1 h, respectively (Figure 2B), but their growth rates differ greatly in glucose. In contrast to the severe growth defect of the *gcr1* mutant in glucose, growth of the *gcr2* mutant was not so much affected (doubling times of WT, *gcr1* and *gcr2* strains in YPAD are 1.6 ± 0.1, 8.4 ± 0.2 and 3.0 ± 0.2 h, respectively; Figure 2A). Thus, the difference of the ribosomal protein gene expression between *gcr1* and *gcr2* mutants can be explained based on the difference of their growth rate.

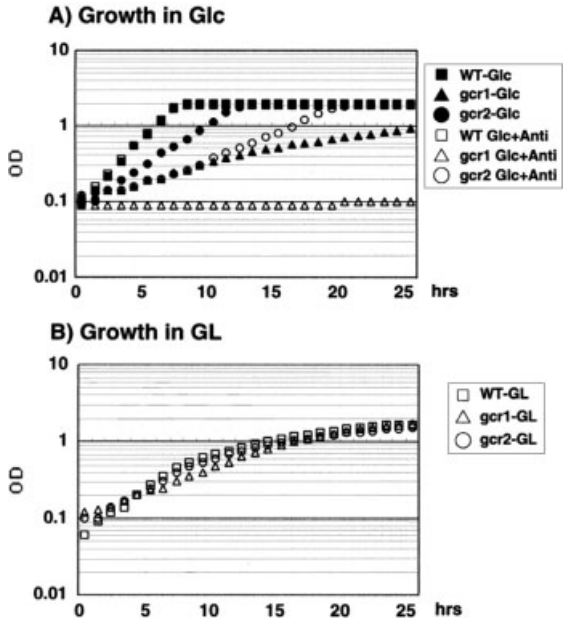

Figure 2. Growth of *gcr* mutant strains in rich media. (A) Cells were grown in rich medium with 2% glucose at 30 °C. Turbidity of the cells was monitored at 630 nm by an automatic detector (Bio-Plotter, Toyo-Sokki, Japan). Closed squares, closed triangles, and closed circles indicate the growth of 2845(wild-type), C179-15C-F16 (Δ*gcr1*), and YHU3002-8C-F16 (Δ*gcr2*) strains in the absence of antimycin A, respectively. Their growth in the presence of antimycin A is indicated by open symbols. (B) Cells were grown in rich medium with 2% each of glycerol and lactate at 30 °C. Open squares, open triangles, and open circles indicate the growth of 2845 (wild-type), C179-15C-F16 (Δ*gcr1*) and YHU3002-8C-F16 (Δ*gcr2*) strains, respectively

Effect of *gcr* mutations on TCA cycle, ATP synthesis and oxidative phosphorylation

In contrast to the decrease of glycolytic gene expression, expression of genes specifying the enzymes of TCA cycle, ATP synthesis and oxidative phosphorylation was increased in *gcr1* and *gcr2* mutants. Interestingly, the increase of these genes was observed only when the cells were grown in glucose. *p* Values for oxidative phosphorylation were 5.74×10^{-11} (*gcr1* mutant grown in glucose) and 4.51×10^{-10} (*gcr1* mutant grown in glucose); for ATP synthesis-coupled electron transport they were 6.53×10^{-7} (*gcr1* mutant grown in glucose), 8.22×10^{-7} (*gcr1* mutant grown in glucose); for the TCA cycle they were 1.20×10^{-6} (*gcr1* mutant grown in glucose) and 1.12×10^{-3} (*gcr2* mutant grown in glucose). Since the binding sites for Gcr1p (CT-box) are not found in the upstream region of these genes, these results suggest that the effects of *gcr* mutations on these genes are not direct, but a secondary effect of the change of glycolytic flux to the closely related metabolic pathways.

To clarify the impact of *gcr* mutations on these pathways, we selected genes involved in glycolysis, TCA cycle and respiration, and compared their expression levels in Δ*gcr1* and Δ*gcr2* mutants (Table 2). Values (in log2 ratio) which were increased or decreased by more than two-fold compared to the wild-type cells grown in the same conditions are indicated. Table 2 clearly shows that the majority of genes involved in the TCA cycle, ATP synthesis and oxidative phosphorylation are affected in both *gcr1* and *gcr2* mutants, but it is limited to the cells grown in glucose. As a typical example, well-characterized genes of glucose metabolism and energy production pathways that fulfil our selection criteria in the *gcr1* mutant grown in glucose are displayed on the metabolic map (Figure 3). Almost all the increased (red) genes are not affected when the cells were grown in glycerol + lactate. Decreased (green) genes are approximately the same in both growth conditions (Table 2).

Expression levels of some of these genes (*ACO1, SDH1, SDH4* and *ATP1*) were also examined by

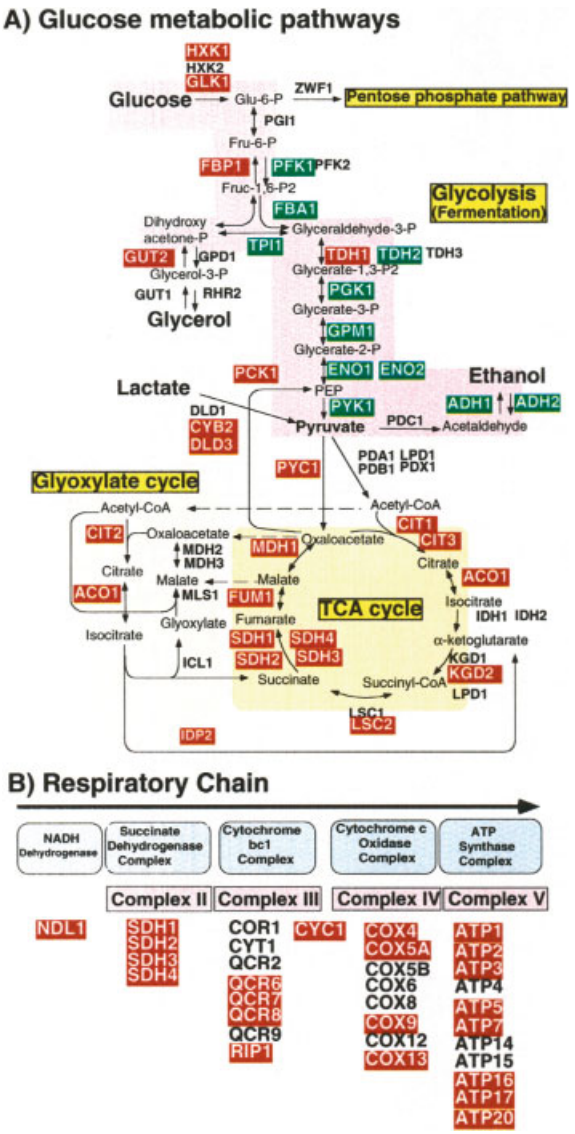

Figure 3. Gene expression profile in the *gcr1* mutant grown in glucose. Well-characterized components of glucose metabolism pathway (A) and respiratory chain (B) that fulfil our selection criteria are displayed on the metabolic map. Green genes and red genes indicate their expression levels were decreased or increased more than two-fold compared to the wild-type strain under glucose growth condition

RT-PCR analysis. Since the expression of these genes is known to be repressed by glucose, their expression was very low in the wild-type strain grown in glucose (Figure 1E–H). In *gcr* mutants, expression levels of *ACO1, SDH1, SDH4* and *ATP1* were increased compared to those in the wild-type strain under the glucose growth condition, but their expression levels were not as high as their expression in the wild-type strain grown in glycerol + lactate (Figure 4). In contrast, when the cells were grown in glycerol + lactate their expression levels were not affected (Figure 4). Lopez and Baker (2000) performed a comparison between before and after the addition of glucose in the *gcr1* mutant, and concluded that glucose repression was normal in the *gcr1* mutant. In our experiments, we performed a comparison between *gcr* mutants and the wild-type strain, and we observed that the TCA cycle and respiratory genes are more expressed in the *gcr* mutants compared to those in the wild-type strain. Since our RT-PCR analysis demonstrated that the increased levels of most of the genes in glucose-grown *gcr* mutants were lower than those in the glycerol + lactate-grown cells, our results confirmed that the glucose repression was still functioning in *gcr* mutants, although there was a difference in the degree of repression. Hence, our results do not contradict the results of Lopez and Baker (2000). We interpret that the overall changes in the gene expression patterns suggest a change of the metabolism for efficient utilization of glucose as an energy source under a low flux of glycolysis, because the yield of ATP per mole of glucose is much higher when glucose is oxidized than when it is fermented.

Although the expressions of some of the hexose transporters were decreased in *gcr* mutants (Table 2), we do not think that it caused the increase of TCA cycle, ATP synthesis and oxidative phosphorylation gene expression. Several lines of evidence suggest that glucose signalling is functional in *gcr* mutants. First, relative glucose flux in *gcr1* and *gcr2* mutants to the wild-type cells are 33% and 89%, respectively, when cells grown in lactate and exposed to glucose for a limited period prior to harvest were assayed under the resting cell condition (Uemura and Fraenkel, 1999). Second, the doubling time of the *gcr2* mutant in glucose is only the half of that of the wild-type (1.6 vs. 3.0 h). Furthermore, it has been reported that glucose repression in *S. cerevisiae* is triggered by the extracellular glucose concentration and not related to glucose flux (Meijer *et al.*, 1998). In our experiments, cells were grown in media with a 2% initial concentration of glucose, and the glucose concentrations when the cells were harvested (OD_{600} = 0.5) were 1.80%, 1.90% and 1.88% in wild-type,

Table 2. Expression levels of glycolysis, TCA cycle, ATP synthesis and oxidative phosphorylation genes in Δ*gcr1* and Δ*gcr2* mutants

Function	ORF name	Gene name	Enzyme/function	Log 2 ratio (mutant/wild-type)			
				gcr1GL	gcr1Glc	gcr2GL	gcr2Glc
Hexose transporters	YHR094C	HXT1	Hexose transporter (low-affinity)	NC	−1.50	NC	NC
	YMR011W	HXT2	Hexose transporter (high/intermediate affinity)	NC	−2.33	NC	2.38
	YDR345C	HXT3	Hexose transporter (low-affinity)	NC	−1.10	NC	−1.32
	YHR092C	HXT4	Hexose transporter (intermediate-affinity)	1.08	NC	NC	NC
	YHR096C	HXT5	Hexose transporter (not well known, low expression level)	NC	NC	NC	NC
	YDR343C	HXT6	Hexose transporter (high affinity)	NC	NC	NC	−1.56
	YDR342C	HXT7	Hexose transporter (high affinity)	NC	NC	NC	−1.91
Glycolysis	YCL040W	GLK1	Glucokinase	1.01	3.64	NC	3.38
	YFR053C	HXK1	Hexokinase	NC	1.36	NC	NC
	YGL253W	HXK2	Hexokinase	NC	NC	NC	−1.55
	YBR196C	PGI1	Phosphoglucoisomerase	−2.06	NC	−3.47	−1.36
	YGR240C	PFK1	6-Phosphofructokinase, α-subunit	−1.53	−1.93	NC	−1.98
	YMR205C	PFK2	6-Phosphofructokinase, β-subunit	NC	NC	−1.76	NC
	YKL060C	FBA1	Fructose 1,6-bisphosphate aldolase	−3.07	−1.56	−2.98	−1.25
	YDR050C	TPI1	Triosephosphate isomerase	−2.98	−2.15	−4.43	NC
	YJL052W	TDH1	Glyceraldehyde-3-phosphate dehydrogenase	−1.01	2.79	−2.62	NC
	YJR009C	TDH2	Glyceraldehyde-3-phosphate dehydrogenase	−2.66	−2.36	−1.47	NC
	YGR192C	TDH3	Glyceraldehyde-3-phosphate dehydrogenase	−1.64	NC	−2.08	NC
	YCR012W	PGK1	Phosphoglycerale kinase	−3.02	−1.92	−3.25	−2.37
	YKL152C	GPM1	Phosphoglycerate mutase	−2.49	−3.62	−2.75	−3.94
	YGR254W	ENO1	Enolase	−3.14	−3.43	−3.55	−1.94
	YHR174W	ENO2	Enolase	−3.72	−4.64	−4.03	−2.92
	YAL038W	PYK1	Pyruvate kinase	−2.36	−2.39	−3.54	−2.47
Fermentation	YLR044C	PDC1	Pyruvate decarboxylase	NC	NC	NC	NC
	YOL086C	ADH1	Alcohol dehydrogenase I	−1.11	−2.54	NC	−2.05
	YMR303C	ADH2	Alcohol dehydrogenase II	NC	−2.00	NC	−1.34
Lactate utilization	YML054C	CYB2	Cytochrome b2 (L-lactate cytochrome c oxidoreductase)	NC	1.26	NC	1.42
	YDL174C	DLD1	D-Lactate dehydrogenase	NC	NC	NC	NC
	YEL071W	DLD3	D-Lactate dehydrogenase	NC	2.03	NC	1.69
Glycerol metabolism	YDL022W	GPD1	Glycerol-3-phosphate dehydrogenase	NC	NC	NC	1.37
	YHL032C	GUT1	Glycerol kinase	NC	NC	NC	NC
	YIL155C	GUT2	Glycerol-3-phosphate dehydrogenase	NC	1.10	−1.07	NC
	YIL053W	RHR2	DL-glycerol phosphate phosphatase	−1.17	−1.12	−1.20	NC
Gluconeogenesis	YLR377C	FBP1	Fructose-1,6-bisphosphatase	NC	1.34	NC	1.17
	YGL062W	PYC1	Pyruvate carboxylase	NC	1.52	NC	NC
	YKR097W	PCK1	Phosphoenolpyruvate carboxylkinase	NC	1.09	NC	NC

Table 2. Continued

Function	ORF name	Gene name	Enzyme/function	Log 2 ratio (mutant/wild-type) gcr1GL	gcr1Glc	gcr2GL	gcr2Glc
Pyruvate metabolism	YER178W	PDA1	Pyruvate dehydrogenase α subunit (E1 α)	NC	NC	NC	NC
	YBR221C	PDB1	Pyruvate dehydrogenase, β subsunit (E1 β)	NC	NC	NC	NC
Acetyl-CoA biosynthesis	YGR193C	PDX1	Pyruvate dehydrogenase, complex protein X component	NC	NC	NC	NC
	YFL018C	LPD1	Pyruvate dehydrogenase, E3 component	NC	1.10	NC	NC
TCA cycle	YNR001C	CIT1	Citrate synthase	NC	3.13	NC	1.57
	YPR001W	CIT3	Citrate synthase	NC	1.85	NC	NC
	YLR304C	ACO1	Aconitase	NC	2.21	NC	1.64
	YNL037C	IDH1	Isocitrate dehydrogenase (NAD+) subunit 1	−1.52	NC	NC	NC
	YOR136W	IDH2	Isocitrate dehydrogenase (NAD+) subunit 2	−1.77	NC	NC	NC
	YIL125W	KGD1	α-Ketoglutarate dehydrogenase complex E1 component	NC	NC	NC	NC
	YDR148C	KGD2	α-Ketoglutarate dehydrogenase complex KE2 component	NC	NC	NC	NC
	YOR142W	LSC1	Succinyl-CoA ligase, α-subunit	NC	NC	NC	NC
	YGR244C	LSC2	Succinyl-CoA ligase, β-subunit	NC	1.19	NC	NC
	YKL148C	SDH1	Succinate dehydrogenase flavoprotein subunit	NC	1.48	NC	NC
	YLL041C	SDH2	Succinate dehydrogenase (ubiquinone) iron–sulphur protein subunit	NC	2.29	NC	1.16
	YKL141W	SDH3	Succinate dehydrogenase cytochrome *b*	NC	1.68	NC	1.43
	YDR178W	SDH4	Succinate dehydrogenase membrane anchor subunit	NC	2.16	NC	1.34
	YPL262W	FUM1	Fumarase	NC	1.41	NC	NC
	YKL085W	MDH1	Malate dehydrogenase	NC	1.00	NC	NC
Glyoxylate cycle	YCR005C	CIT2	Citrate synthase	NC	3.37	NC	1.87
	YER065C	ICL1	Isocitrate lyase	NC	NC	NC	NC
	YNL117W	MLS1	Malate synthase	NC	NC	NC	NC
	YOL126C	MDH2	Malate dehydrogenase	NC	NC	NC	NC
	YDL078C	MDH3	Malate dehydrogenase	NC	NC	NC	NC
Isocitrate metabolism	YLR174W	IDP2	NADP-dependent isocitrate dehydrogenase	NC	1.67	NC	NC
NADH dehydrogenase	YML120C	NDI1	NADH dehydrogenase (ubiquinone)	NC	2.30	NC	1.03
Respiratory chain complex III	YBL045C	COR1	Ubiquinol cytochrome *c* reductase	NC	NC	NC	NC
	YOR065W	CYT1	Cytochrome *c1*, member of cytochrome *bc1* complex	NC	NC	NC	NC
	YPR191W	QCR2	Ubiquinol cytochrome *c* reductase core protein 2	NC	1.34	NC	NC
	YFR033C	QCR6	Ubiquinol cytochrome *c* oxidoreductase subunit 6	NC	1.02	NC	1.83
	YDR529C	QCR7	Ubiquinol cytochrome *c* oxidoreductase subunit 7	NC	1.43	NC	1.48
	YJL166W	QCR8	Ubiquinol cytochrome *c* reductase subunit 8	NC	1.45	NC	1.17

Table 2. Continued

Function	ORF name	Gene name	Enzyme/function	Log 2 ratio (mutant/wild-type)			
				gcr1GL	gcr1Glc	gcr2GL	gcr2Glc
	YGR183C	QCR9	Ubiquinol cytochrome *c* oxidoreductase complex subunit 9	NC	NC	NC	1.75
	YEL024W	RIP1	Ubiquinol cytochrome *c* reductase iron–sulphur protein	NC	1.31	NC	NC
	YJR048W	CYC1	Cytochrome-*c* isoform 1	NC	2.52	NC	2.22
Respiratory chain complex IV	YGL187C	COX4	Cytochrome *c* oxidase subunit IV	NC	1.31	NC	NC
	YNL052W	COX5A	Cytochrome *c* oxidase chain Va	NC	1.29	NC	1.06
	YIL111W	COX5B	Cytochrome *c* oxidase chain Vb	NC	NC	NC	NC
	YHR051W	COX6	Cytochrome *c* oxidase subunit VI	NC	NC	NC	NC
	YLR395C	COX8	Cytochrome *c* oxidase chain VIII	NC	1.89	NC	NC
	YDL067C	COX9	Cytochrome *c* oxidase subunit VIIa	NC	1.02	NC	NC
	YLR038C	COX12	Cytochrome *c* oxidase subunit VIb	NC	1.08	NC	NC
	YGL191W	COX13	Cytochrome *c* oxidase subunit VIa	NC	2.26	NC	1.24
Respiratory chain complex V	YBL099W	ATP1	α-subunit of the F1 subunit of ATP synthase–mitochondrial respiratory complex V	NC	2.42	NC	1.30
	YJR121W	ATP2	β-subunit of the F1 subunit of ATP synthase–mitochondrial respiratory complex V	NC	1.39	NC	NC
	YBR039W	ATP3	Gamma subunit of the F1 subunit of ATP synthase–mitochondrial respiratory complex V	NC	1.14	NC	NC
	YPL078C	ATP4	Subunit 4 of the F0 subunit of ATP synthase–mitochondrial respiratory complex V	NC	NC	NC	1.09
	YDR298C	ATP5	Subunit 5 of the F0 subunit of ATP synthase–mitochondrial respiratory complex V	NC	1.52	NC	1.08
	YKL016C	ATP7	Subunit 7 of the F0 subunit of ATP synthase–mitochondrial respiratory complex V	NC	1.41	NC	NC
	YLR295C	ATP14	Subunit h of the F0 subunit of ATP synthase–mitochondrial respiratory complex V	NC	NC	NC	NC
	YPL271W	ATP15	ϵ-subunit of the F1 subunit of ATP synthase–mitochondrial respiratory complex V	NC	NC	NC	NC
	YDL004W	ATP16	δ-subunit of the F1 subunit of ATP synthase–mitochondrial respiratory complex V	NC	1.10	NC	NC
	YDR377W	ATP17	Subunit f of the F0 subunit of ATP synthase–mitochondrial respiratory complex V	NC	1.04	NC	1.19
	YPR020W	ATP20	Subunit g of the F0 subunit of ATP synthase–mitochondrial respiratory complex V	NC	1.03	NC	1.12

Genes are ordered by means of metabolic pathways. The log2 ratios are the average of three experiments performed under the same condition. Values of the genes which were increased or decreased by more than two-fold compared to the wild-type strain grown in the same conditions are indicated, and others are indicated as NC (not changed).

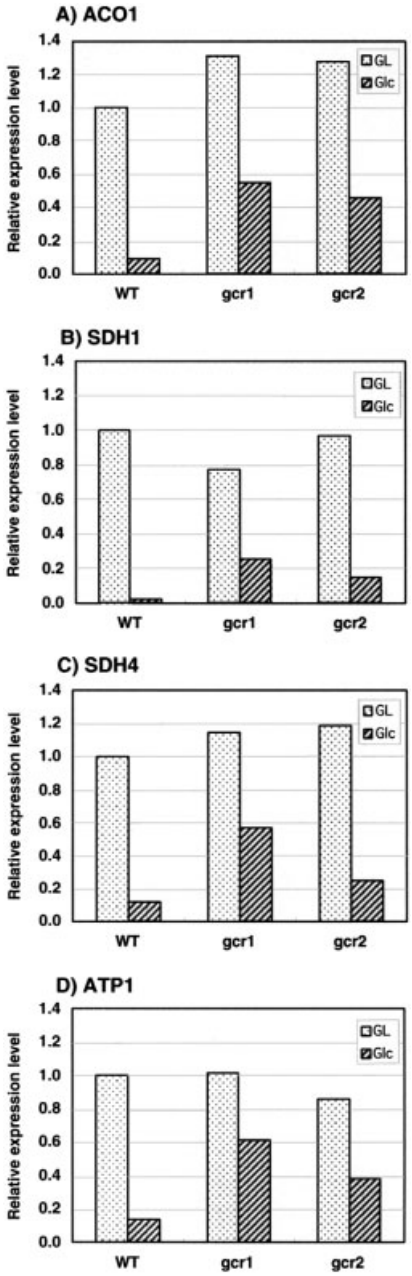

Figure 4. Expression levels of *ACO1, SDH1, SDH4* and *ATP1* in Δ*gcr1* and Δ*gcr2* mutants. The mRNA levels of *ACO1, SDH1, SDH4* and *ATP1* were examined by RT-PCR, and mRNA levels were quantified based on the intensities of bands at cycle 18 of Figure 1E–H

gcr1 and *gcr2* strains, respectively. Thus, we interpreted that the increase of TCA cycle and respiratory genes were not simply due to the release of glucose repression.

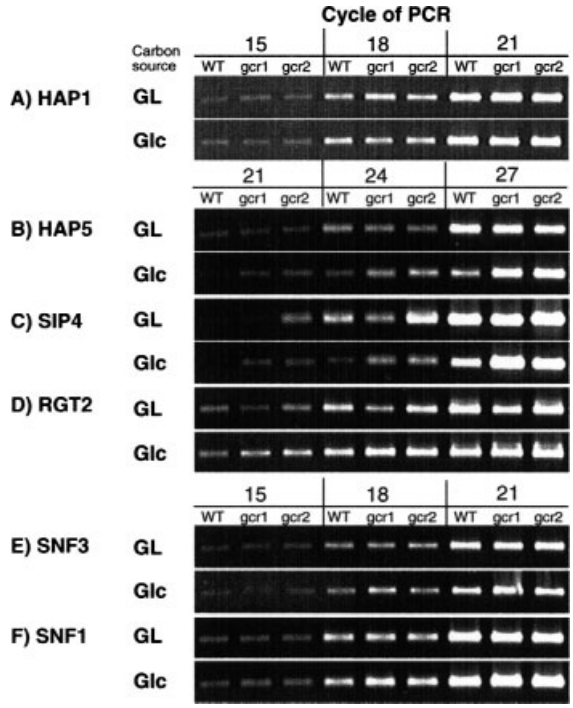

Figure 5. RT-PCR analysis of the expression levels of regulatory genes in Δ*gcr1* and Δ*gcr2* mutants. (A–F) Levels of *HAP1, HAP5, SIP4, RGT2, SNF3* and *SNF1* mRNAs in the wild-type, Δ*gcr1* and Δ*gcr2* strains were examined by RT-PCR. mRNA was prepared from the cells grown in YPAD or YPAGL at $OD_{600} = 0.5$. Cycles of PCR were fixed to 15, 18 and 21 (for *HAP1, SNF3* and *SNF1*) and 21, 24 and 27 (for *HAP5, SIP4* and *RGT2*) for amplification of linear range by using 10 ng polyA RNAs. Amplified fragments were analysed by 1% agarose gels and quantified

Expression levels of the regulatory factors involved in glucose repression

To examine whether the activation of TCA cycle and respiratory genes are related to the increase of the expression of known transcription factors involved in these pathways, expression levels of *HAP* genes and *SIP4* were examined by RT-PCR. Since the expression levels of transcription factors are low in microarray analyses, we compared their expression levels by RT-PCR, and band intensities of PCR products were measured to quantitate their expressions. Expression levels of *HAP1* (Figures 5A, 6A), *HAP2, HAP3* and *HAP4* (Figure 6B–D) were not affected by *gcr* mutations, but the expression levels of *HAP5* and *SIP4* in *gcr* mutants were higher than those in the wild-type strain when the cells were grown in glucose (Figure 5B, C). Expression levels of *HAP5* in the

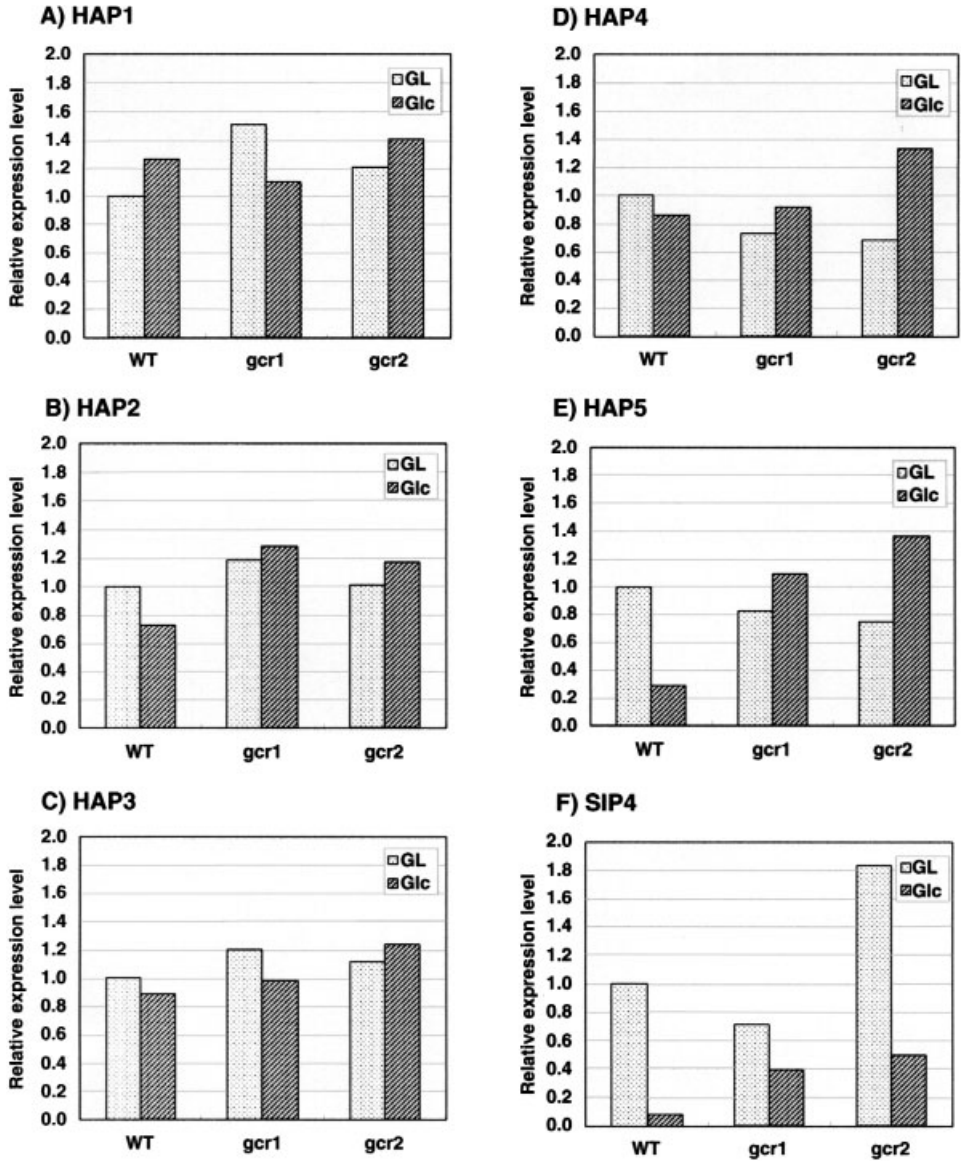

Figure 6. Expression levels of *HAP* genes and *SIP4*. The mRNA levels of *HAP* genes and *SIP4* were examined by RT-PCR, and mRNA levels were quantified based on the intensities of bands at cycle 24 for *HAP1, HAP5* and *SIP4* (Figure 5A–C), and at cycle 18 for *HAP2, HAP3* and *HAP4*

mutants were completely derepressed to the level of *HAP5* in the wild-type strain grown in glycerol + lactate (Figure 6E), but the expression of *SIP4* was partially derepressed (Figure 6F).

We also examined the expression levels of *HXK2*, because its product, hexokinase isozyme PII, not only phosphorylates glucose but also plays a regulatory role in the glucose repression, and the null mutant of *HXK2* is deficient in glucose repression (Gancedo, 1998). However, at least from the result of RT-PCR (Figure 1D), expression of *HXK2* was not so much affected in *gcr* mutants. Expression levels of *SNF1* (a protein kinase that has a central role in the signal transduction pathway for glucose repression), *SNF3* (high-affinity glucose sensor) and *RGT2* (low-affinity glucose sensor) were also examined, but we could not detect any differences in their expression level

(Figure 5D–F). However, since the activity of Hxk2p and Snf1p are known to be regulated by phosphorylation (Randez-Gil *et al*., 1998; Ludin *et al*., 1998), the possibility of their contribution still remains.

Efficiency of glucose utilization

The increase of TCA cycle, ATP synthesis and oxidative phosphorylation genes suggested that the growth of *gcr1* and *gcr2* mutants under glucose was more dependent on respiration than the wild-type strain. It has been known that the growth of *gcr2* mutants on glucose was affected by the respiratory inhibitor antimycin A (Uemura and Fraenkel, 1990). Figure 2 shows the growth of *gcr* mutants in glucose in the presence of antimycin A (1 μg/ml). Growth of the wild-type strain was almost the same with or without antimycin A (doubling times were 1.6 ± 0.1 and 1.7 ± 0.1 h, respectively), but the growth of *gcr* mutants was slowed down by adding antimycin A (Figure 2A). Doubling times of *gcr2* mutant were elongated from 3.0 ± 0.2 to 4.4 ± 0.1 h, and the *gcr1* mutant (8.4 ± 0.2 h in glucose) hardly grew in the presence of antimycin A, strongly indicating that the respiratory pathway is functioning more actively in *gcr* mutants than in the wild-type strain. These phenotypes agreed well with the observation in DNA microarray experiments that the expression of TCA cycle, ATP synthesis and oxidative phosphorylation genes increased in *gcr* mutants.

If the growth of *gcr* mutants in glucose is more dependent on respiration, it is expected that the increase of OD (cell growth) and the production of ethanol would be affected in the *gcr* mutants. To address these points, the relationship among growth, glucose utilization and ethanol production was measured. In the wild-type strain, almost the same amount of ethanol was produced in the presence or absence of antimycin A when the cells were growing in log phase (1.74 and 1.77 mM EtOH/mM consumed glucose; Table 3, line 1). After consumption of glucose, a clear diauxic shift was observed and ethanol was used as the second carbon source for the cells to grow in the absence of antimycin A. However, the amount of ethanol stayed the same level when the antimycin A was present (Figure 7A).

In the growth of the *gcr2* mutant, smaller amount of ethanol was produced in the absence of antimycin A and no clear diauxic shift was observed. However, almost the same amount of ethanol as the wild-type strain was produced in the presence of antimycin A, concomitantly with the decrease of the growth rate. In agreement with the microarray results, this result suggested that the metabolic pathway was changed to the TCA cycle rather than fermentation in the *gcr2* mutant. As mentioned in the previous section, no growth was observed in the *gcr1* mutant when the antimycin A was present in the medium.

To confirm the difference of glucose utilization in the wild-type and *gcr2* mutant strains, yields of OD and ethanol against consumed glucose were calculated by using the range where cells were growing logarithmically. Compared to the wild-type strain, production of ethanol per consumed glucose was lower and the increase of OD per consumed glucose was higher in the *gcr2* mutant, indicating that the efficiency of biomass production was higher in the *gcr2* mutant. The efficiency

Table 3. Efficiency of glucose utilization

Line	Strain (relevant genotype)	OD increased/M glucose consumed (ratio*)		mM EtOH produced/mM glucose consumed (ratio*)	
		Glc	Glc + Anti	Glc	Glc + Anti
1	2845 (WT)	98.39 ± 0.72 (1)	72.82 ± 1.57 (0.74)	1.74 ± 0.18 (1)	1.77 ± 0.12 (1.02)
2	YHU3002-8C-F16 (Δ*gcr2*)	153.61 ± 2.17 (1.56)	55.53 ± 0.85 (0.56)	1.34 ± 0.03 (0.77)	1.96 ± 0.20 (1.13)
3	C179-15C-F16 (Δ*gcr1*)	112.99 ± 2.54 (1.15)	NA NA	0.84 ± 0.01 (0.48)	NA NA

Cells were grown in synthetic 0.5% glucose medium with or without antimycin A, and OD_{600}, glucose concentration, and ethanol concentration were measured as described.

* The value of 2845 grown in glucose was used as a standard to calculate the relative ratio.

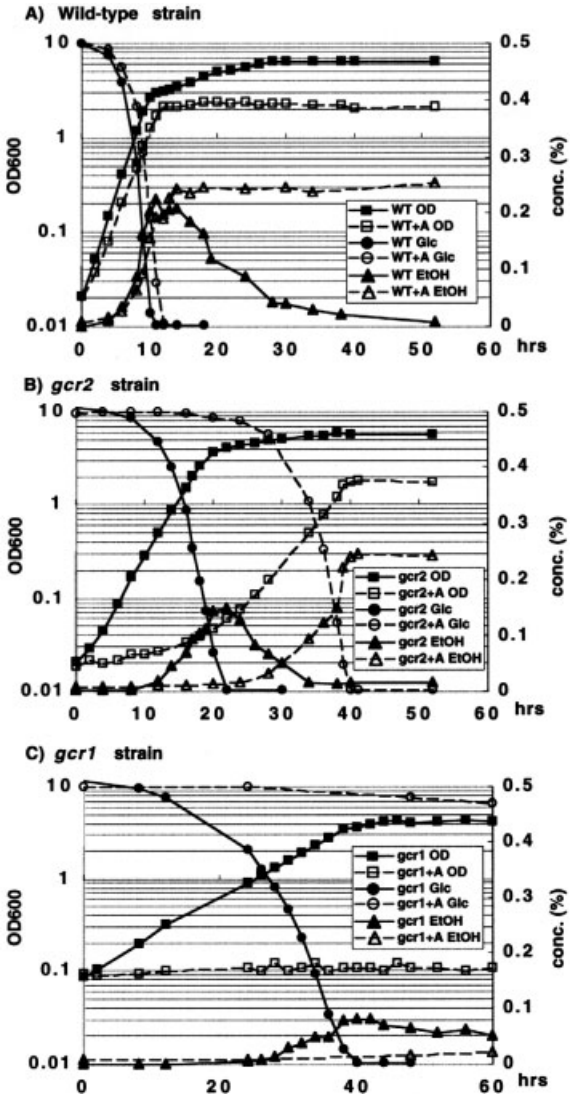

Figure 7. Growth, glucose consumption, and ethanol production in Δ*gcr1* and Δ*gcr2* mutants. Cells were grown in 100 ml of SC medium with 0.5% glucose in the presence or absence of antimycin A (1 μg/ml) at 30 °C. Turbidity of the cells was monitored at 600 nm. Glucose and ethanol concentrations were assayed as described in Materials and Methods. (A) Growth of the 2845 (wild-type) strain. Squares, circles, and triangles indicate OD, glucose concentration, and ethanol concentration, respectively. Open and closed symbols indicate growth with or without antimycin A. (B) Growth of the YHU3002-8C-F16 (Δ*gcr2*) strain. (C) Growth of the C179-15C-F16 (Δ*gcr1*) strain

of biomass production increased 1.6-fold and the ethanol production decreased 0.8-fold in the *gcr2* mutant (Table 3, line 2). This phenomenon was not observed when the cells were grown in the presence of antimycin A (Table 3, line 2, columns with antimycin A). Since observation of cells under the microscope did not show any differences in size and morphology between the mutants and the wild-type cells (data not shown), these data strongly suggest that the consumed glucose was more efficiently converted to biomass instead of producing ethanol in the *gcr2* mutant. After the diauxic shift in the absence of antimycin A, the biomass of the wild-type strain was larger than that of the *gcr2* mutant. OD at $t = 52$ h was 6.43 and 5.66 in the wild-type and *gcr2* mutant strains, respectively. Their cell numbers and wet weights of 50 ml cultures were 1.6×10^8 and 1.2×10^8cells/ml, 0.55 and 0.51 g, in the wild-type and *gcr2* mutant cells, respectively.

Conclusions

DeRisi *et al*. (1997) examined the change of gene expression during the diauxic shift, in which glucose in the batch culture was exhausted and the cells begin to oxidize the produced ethanol during fermentation. Ferea *et al*. (1999) examined the changes in gene expression after a long incubation in a glucose-limited chemostat. Many of the same genes (glycolysis, TCA cycle, ATP synthesis, oxidative phosphorylation) showed parallel changes in expression levels in these two experiments. However, the meanings of the change of gene expression patterns are different in two cases. In the diauxic shift experiments, cells physiologically adapted to the gradual reduction of glucose in the media. In the experiments of Ferea *et al*., in contrast, the cells were grown in a glucose limitation condition in the chemostat for more than 500 generations, and the cells evolutionarily adapted to the condition. Very recently, Dunham *et al*. (2002) showed that the increase of gene expression was due to genome rearrangements in the experimentally evolved *S. cerevisiae*. It has been shown that aneuploidy affects gene expression levels (Hughes *et al*., 2000).

In our experiments, cells were grown in the batch culture with 2% initial concentration of glucose, and the cells were harvested at the early stage of log phase (OD = 0.5). Thus, the amount of glucose was still enough, but it was suggested that glucose flux was decreased, especially in the *gcr1* mutant, because of the reduced expression of glycolytic

genes (Uemura and Fraenkel, 1999). Comparison of our data with the microarray analysis during the diauxic shift by DeRisi *et al*. (1997) and the adaptive evolution under low glucose by Ferea *et al*. (1999) showed similarities to some extent. However we can point out some clear differences, too.

For example, *HAP4* expression was increased in the experiments of Ferea *et al*. (1999) and DeRisi *et al*. (1997), but in our experiments expression levels of *HAP1–HAP4* were not changed in either the *gcr1* or *gcr2* mutants. On the other hand, increase of *HAP5* expression was observed in our experiments, but its expression was rather decreased in the experiments of Ferea *et al*. (1999) and DeRisi *et al*. (1997). Another noteworthy point in the diauxic shift experiment was the general induction of genes encoding mitochondrial ribosomal proteins, in contrast to the repression of ribosomal protein genes. However, in our experiments, expression of mitochondrial ribosomal protein genes was not induced in all conditions. Too much detailed comparison should be avoided, because the growth conditions were so very different: Ferea *et al*. (1999) used defined medium and the cells were growing in a steady state, whereas DeRisi *et al*. (1997) examined gene expression in batch cultures under changing conditions of glucose concentration, pH, other nutrition, etc. Although we could not identify specific genes that might cause the difference of growth phenotype between *gcr1* and *gcr2* mutants, our findings suggested that the degree of glycolysis affected the switching of metabolic pathways, and these observations will give hints to the manipulation of metabolic pathways in the future.

Acknowledgements

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