Control of Expression of One-carbon Metabolism Genes of Saccharomyces cerevisiae Is Mediated by a Tetrahydrofolate-responsive Protein Binding to a Glycine Regulatory Region Including a Core 5’-CTTCTT-3’ Motif*

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Expression of yeast genes involved in one-carbon metabolism is controlled by glycine, by l-methionine, and by nitrogen sources. Here we report a novel control element containing a core CTTCTT motif mediating the glycine response, demonstrating that a protein binds this element, that binding is modulated by tetrahydrofolate, and that folate is required for the in vivo glycine response. In an heterologous CYC1 promoter the region needed for the glycine response of GCV2 (encoding the P-subunit of glycine decarboxylase) mediated repression that was relieved by glycine. It was also responsible for l-methionine control but not nitrogen repression. GCV1 and GCV2 have an homologous region in their promoters. The GCV1 region conferred a glycine response on an heterologous promoter acting as a repressor or activator depending on promoter context. A protein was identified that bound to the glycine regulatory regions of GCV1 and GCV2 only if the CTTCTT motif was intact. This protein protected a 17-base pair region in their promoters. The regulatory regions of A protein was identified that bound to the glycine regulatory region or activator depending on promoter context. fol1 drofolate, and use of a folate in the biogenesis of methyl groups (1). It is located in the mitochondrial inner membrane of animals (2) and plants (3) and in the cytosol of bacteria (4), where it catalyzes the reversible conversion of glycine and NAD⁺ to CO₂, NH₄⁺, 5,10-CH₂-Hfolate, and NADH (5).

The GDC is composed of four subunits designated P- (pyridoxal phosphate containing), H- (lipoic acid containing carrier), L- (lipoamide dehydrogenase), and T- (tetrahydrofolate-requiring) proteins. The roles of these proteins in the reaction mechanism have been described by Hiraga and Kikuchi (6). Genes encoding the subunits of the GDC have been identified from many organisms; in Saccharomyces cerevisiae GCV1 encodes the T-protein (7), GCV2 the P-protein (8), GCV3 the H-protein (9) and LPD1 the L-protein (10). Although the first three genes are unique to the GDC complex, the LPD1 gene product acts in several other complexes including pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and a branched chain o xo-acid dehydrogenase (11, 12).

In most organisms, a major source of one-carbon units is the C-3 of serine, which is transferred to Hfolate in a reversible reaction catalyzed by serine hydroxymethyltransferase generating 5,10-CH₂-Hfolate and glycine. In S. cerevisiae there are two other significant pathways for generation of one-carbon units: from glycine via the GDC (13) or from formiate via the cytoplasmic C₅-tetrahydrofolate synthase (a trifunctional polypeptide encoded by ADE3) (14, 15). The GDC serves an important role in balancing cellular requirements for glycine and one-carbon units. In S. cerevisiae for example, glycine can substitute for the serine requirement in a ser1 strain in which synthesis of serine from glycolytic intermediates is blocked (16). In this mutant the GDC cleaves some of the glycine to CO₂ and 5,10-CH₂-Hfolate, providing the one-carbon units required for other reactions in the cell. Glycine can also serve as a source of nitrogen in the absence of other more readily metabolized nitrogen sources (17).

In yeast the expression of GCV1, GCV2, and GCV3 is regulated by glycine (7–9) In Escherichia coli expression of the gcv operon encoding the glycine cleavage system is induced by glycine and repressed by purines (18), whereas a mixture of one-carbon metabolites has been reported to repress the yeast GCV3 gene (9). GCV2 expression is also greatly reduced in cells grown in rich medium or in good nitrogen sources compared with defined minimal medium. Because glycine can act as a poor nitrogen source, expression of the GCV genes may be subject to some form of nitrogen catabolite repression (19, 20) mediated by readily assimilated nitrogen sources such as l-glutamine and l-asparagine.

Here we report a detailed characterization of the molecular mechanism of GCV2 regulation by glycine and show that the gene is also subject to control by l-methionine and nitrogen.
source. The elements in the promoter of GCV2 responsible for glycine and l-methionine regulation have been identified by deletion analysis and footprinting, and the gene was shown to be negatively regulated by repression, which is relieved in the presence of glycine. These elements act negatively in a heterologous promoter to bring it under glycine control. Similar elements have been identified in the GCV1 and GCV3 genes.

The control region of the GCV2 promoter has been found to bind a protein that is present in yeast nuclear extracts or in heparin-Sepharose purified cell extracts, and a similar complex is formed with the putative GCV1 control region. The formation of the complex seen between a protein and the promoter has been shown to be responsive to H4folate in vitro. Use of a fol1 deletion mutant, which is inhibited in H4folate biosynthesis, indicated the need of a folate for the glycine response in vivo, indicating that glycine-specific control may be mediated by monitoring the balance of one-carbon intermediates present in the cell at the folate level.

**EXPERIMENTAL PROCEDURES**

**Materials**—Amino acids, 2-mercaptoethanol, poly(dI-dC), folic acid, sodium tetrahydrofolate (H4folate), and folinic acid (5-formyltetrahydrofolate) were obtained from Sigma. Sodium tetrahydrofolate stock solution (70 mM) was prepared by adding H4folate to 1 M 2-mercaptoethanol. The solution was adjusted to pH 7.0 with 2 N NaOH. The solution was bubbled with argon, and the tubes remained sealed until used. [32P]dATP/dCTP (3000 Ci/mmol) was obtained from DuPont. Restriction and modifying enzymes were obtained from New England BioLabs or Boehringer Mannheim. Taq polymerase was from Perkin-Elmer. All other materials were of high quality and obtained from various commercial vendors.

**Strains and Media—** *E. coli* strain JM101 was used as host for plasmids. Yeast strain BWG1–7A (MATa ade1–100 his4–S19 leu2–3, 112 ura3–52) was from Dr. Leonard Guarente, and YUG1 (MATa ara3–52 leu2–3 trp1–389 hisA-delta1, fol1::KanMX4) was from Dr. Johannes Hegemann. The yeast media used for the strain have been described previously (8). Auxotrophic requirements were added at a concentration of 40 mg/liter. Minimal media with an amino acid as the sole nitrogen source (GLNmin, GLNmin, ASNmin, or PRomin) consisted of 2% (w/v) of 40 mg/liter. Minimal media with an amino acid as the sole nitrogen source. The elements in the promoter of GCV2 responsible for glycine and l-methionine regulation have been identified by deletion analysis and footprinting, and the gene was shown to be negatively regulated by repression, which is relieved in the presence of glycine. These elements act negatively in a heterologous promoter to bring it under glycine control. Similar elements have been identified in the GCV1 and GCV3 genes.

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**Construction of the GCV1-, GCV2-, and GCV3-lacZ Gene Fusion Plasmids—** Plasmid pGSD2.5 was used to prepare plasmid pH1 (EcoRI/XbaI fragment: −351 to −378 bp of GCV2) and pH2 (1.3-kilobase pair (kb) fragment of plasmid YRH1). The sequence between two potential Gcn4p binding sites (GCV2) and a set of synthetic oligonucleotides for ligation into pH3 upstream of the lacZ gene in YIp358 (23).

A PCR-based technique was employed to make further deletion constructs with pGSD2.5 as a template. For each construct, an oligonucleotide 5′-acgtagattacacctc-3′ (+427 to +444 of GCV2) and a set of oligonucleotides harboring EcoRI recognition sequences was used to introduce a restriction site by site-directed mutagenesis. YIp358 as well as the PCR products were cut with EcoRI/XbaI and ligated to yield the constructs. The resulting pH4, pH5, and pH6 contained 649, 611, and 576-bp fragments of GCV2, respectively.

Using pH3 as a template, potential Gcn4p-binding sites (GCN4) at −312 and −291 and the CTTCTT motif of GCV2 were mutated to KpnI restriction sites as described (25), and the PCR products were cloned into YIp358. Constructs with the GCN4 mutated at −312 and −291 were designated pH7 and pH8, respectively, and the CTTCTT mutated construct was pH9. Further deletions were constructed from pH7 and pH8 by cloning the 690- and 669-bp KpnI/XbaI fragments into YIp358 to produce pH10 and pH11, respectively. To localize the glycine regulatory element, five sets of double-stranded oligonucleotides with EcoRI overhangs were prepared from pairs of annealed synthetic oligonucleotides for ligation into pH8 upstream of the 5′ end of the truncated GCV2 gene. The resulting constructs were sequenced to determine the orientation of each insertion.

Window deletion constructs pH12 and pH14 were made from pH7 and pH8, respectively. After cutting pH7 and pH8 with KpnI, the termini were blunt-ended by the 3′ to 5′ exonuclease activity of T4-polymerase, pH4 was first cut with EcoRI and also blunt-ended by treatment with Klenow fragment. Then a 650-bp GCV2 fragment from pH4 and fragments of pH7 containing −351 to −313 of GCV2 and pH8 containing −351 to −259 of GCV2 were isolated after cutting with XhoI. Subsequent blunt-end ligation created pH12 and pH14. pH9 was used to make another window deletion construct (pRH13) by cutting with KpnI and then religating the larger fragment. This deleted the sequence between two potential Gcn4p binding sites (−310 and −289) of GCV2.

pRH16 was constructed by inserting annealed oligonucleotides into the EcoRI site of pH4. The sequence −194 to −157 harbored the GCV1
glycine regulatory region as determined by broad deletion and computer analysis.

The GCV3 gene sequence from -410 to +1328 (relative to the start codon) was amplified from genomic DNA by PCR and the BamHI fragment of GCV3 (-371 to +1291) was cloned into pTZ19. Subsequently, the GCV3 BamHI to SphI (-371 to +53) fragment was subcloned into Ylp356 (23) and fused in frame to the lacZ reporter gene.

The GCV1 gene cloned into YEp13 was a gift from Dr. Andrew Bogner. The -1026 to +96 (SphI-KpnI) fragment of GCV1 was cloned into Ylp357R (23) and fused to lacZ in this study. Deletion constructs were also made from HindIII-KpnI (-310 to +96 of GCV1) and Xhol-KpnI (-130 to +96) fragments by cloning into the same vector. The LPD1-lacZ fusion reporter construct (pDS1) has been described previously (8).

The GCV2 fragment -322 to -248 (relative to start codon) was amplified by PCR using primers harboring Xhol restriction sites and the internal 42-bp Xhol fragment was cloned into the Xhol site within the CYC1 gene from YEp13. The pLG1-312S (26). The GCV1 fragment -193 to -162 with Xhol cohesive ends was produced by annealing appropriate oligonucleotides and after phosphorylation was cloned into pLG1-312S or pLG1-312SS, which is identical to pLG1-312S except that the UAS sites in the promoter region of the CYC1 gene were removed by cutting with SmaI and SfiI, and religating after filling in the SfiI site.

β-Galactosidase Assay—β-Galactosidase assays were performed as described previously (8). Yeast strain BWG1–7A was either grown to an A<sub>600</sub> of 0.5 (for nitrogen source regulation) or to an A<sub>600</sub> of 1.0 and then transferred to fresh medium and incubated a further 2 h before harvest (for glycine and L-methionine response analysis). Cells of strain YUG1 were grown to an A<sub>600</sub> of 1.3 and then transferred to fresh medium after centrifugation and washing and incubated for appropriate time before harvest.

Gel Mobility Shift Assay—Gel mobility shift assays were performed as described (27). Nuclear protein extracts (28) and protein extracts using heparin-Sepharose (29) were prepared from strains BWG1–7A grown in minimal medium (Dmin) to an A<sub>600</sub> of 1.0 at 30 °C. A 74-bp fragment (-322 to -248) of GCV2 was amplified by PCR and was cut with appropriate restriction enzymes. Termini were filled using the Klenow fragment of DNA polymerase in the presence of [α-<sup>32</sup>P]dATP/ dCTP, and the fragment was isolated by polyacrylamide gel electrophoresis. 8 fmol of the labeled DNA was added if not specified otherwise, and 2 μg of poly (dI-dC) was added to prevent nonspecific binding of proteins to the DNA. Reaction mixtures containing footprint buffer (27) were incubated at room temperature for 20 min before loading on a 5% (w/v) polyacrylamide gel and separated by electrophoresis at 7.5 V cm<sup>-1</sup> for 2 h. Gels were dried, and radioactivity was scanned by a Phosphoimager (Bio-Rad) to quantify the amount of the retarded species as a DNA-protein complex in comparison with the free DNA.
and the DNA digestion was proceeded for 1 min at room temperature. 

At room temperature, freshly diluted DNaseI (4.5 units) was added, isolated by polyacrylamide gel electrophoresis. After a 20-min incubation at room temperature, the same fragment (30) and the terminal was end-labeled with \( ^{32} \text{P} \) dATP, and the fragment was added to give the same amount of protein in each reaction. The DNA fragment used was prepared by isolating the EcoRI-XhoI GCV2 fragment (−351 to −148) from pRH4 and then cutting this fragment with AluI generating an EcoRI-AluI fragment (−351 to −218). The 5′ EcoRI terminus was end-labeled with [\( ^{32} \text{P} \)]dATP, and the fragment was isolated by polyacrylamide gel electrophoresis. After a 20-min incubation at room temperature, freshly diluted DNAseI (4.5 units) was added, and the DNA digestion was proceeded for 1 min at room temperature. The reaction was stopped by adding 140 μl of stop solution containing 192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, and 64 mM/μl yeast RNA. DNA fragments were extracted once with phenol/chloroform (1:1) and precipitated with ethanol and 9.3 μl of protein extract. The DNA fragments were rinsed with 95% ethanol and resuspended in 4 μl of loading dye (95% v/v formamide, 10 mM EDTA, 1% bromphenol blue, 1% xylene cyanol). The DNA fragments were denatured for 1 min at 90 °C prior to electrophoresis on an 8% sequencing gel. The G + A sequencing was prepared from the same fragment (30).

RESULTS

GCV2 Is Induced by Glycine and Methionine and Repressed in Rich Medium by Nitrogen Sources—Previous work has shown that the GCV2 gene encoding the P component of the GDC is regulated by excess glycine in the external medium, that this effect is restricted almost solely to glycine (8), and that similar responses have been demonstrated for expression of GCV1 and GCV3 (7, 9). We have determined whether the LPD1 gene encoding the fourth component of the glycine dehydrogenase complex is regulated by glycine. LPD1 showed a fairly high level of expression in cells grown in minimal medium, and no significant effect on expression with the addition of glycine (Fig. 1A). This is not surprising because in yeast LPD1 encodes lipamide dehydrogenase, which is a subunit for at least three other multienzyme complexes including pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and the branched chain 2-oxoacid dehydrogenase (11, 12). Fig. 1A illustrates the extent of the glycine control over GCV1, GCV2, GCV3, and LPD1 expression in the same strain.

This paper is primarily concerned with the glycine control system; however, given the role of the GCV genes in one-carbon metabolism and because glycine can act as a nitrogen source, we also investigated whether any other products of one-carbon metabolism or nitrogen sources affected control of GCV2. Fig. 1B illustrates the levels of expression seen when cells are grown in Dmin alone, Dmin supplemented with one of the one-carbon metabolites (L-methionine), rich glucose medium (YPD), and Dmin medium in which glycine, L-proline, L-glutamine, or L-asparagine replaced NH₄⁺ as the nitrogen source (GLYmin, PROmin, GLNmin, and ASNmin, respectively) or a mixture of good nitrogen sources (GLN + ASN + NH₄⁺)min. These results show that the GCV2 gene can be regulated over an 150-fold range, of which about 20-fold is due to repression in rich medium and 7-fold due to glycine induction. No significant change was observed for the addition of separate components of the one-carbon metabolites to Dmin except for L-methionine, which caused an approximate 2-fold induction of GCV2. Repression in rich medium is probably due to a form of nitrogen-catabolite repression because growth of cells on L-glutamine as nitrogen source led to a 3-fold decrease in GCV2 expression and on L-proline a 50% increase relative to that seen on NH₄⁺ as nitrogen source. A mixture of L-glutamine, L-asparagine, and NH₄⁺ repressed GCV2 to the level seen in YPD (Fig. 1B). Therefore, GCV2 expression is induced by glycine and l-methionine and is repressed by rich nitrogen substrates.

Localization of Control Elements in the GCV2 Gene—Important control regions were located by deletion analysis of the upstream region of the GCV2 gene. Plasmid pRH2 contains 1.37 kilobase pairs of GCV2 sequence fused to the E. coli lacZ reporter, including 1 kilobase pair of GCV2 upstream sequence. This construct when integrated as a single copy at the URA3 locus retained all of the known control elements. Unidirectional

FIG. 3. Sequences of GCV2 (between −291 and −266 relative to the start codon) that can restore the glycine response to the truncated GCV2 promoter in pRH4. A, the fragments from 1 to 5 indicated in the diagram were cloned in both forward and reverse orientations at the 5′ boundary of the GCV2 sequence in pRH4. The resulting constructs were integrated as single copies in strain BWG1–7A and assayed for β-galactosidase in triplicate as described in the legend to Fig. 1A; all errors were less than 26%. B, comparison of the promoter regions of the glycine-responsive genes (GCV1, −296 to −132; GCV2, −315 to −240; GCV3, −314 to −241). The asterisks indicate identity to the central GCV2 sequence of either GCV1 or GCV3. 

Densitometric scanning was performed for the H₄folate effect analysis by Imaging Densitometer (Bio-Rad).

Footprinting Analysis—The footprint assay mixture (final volume, 50 μl) contained 2–3 ng (~20,000–30,000 cpm) of [\( ^{32} \text{P} \)]dATP, and the fragment was added to give the same amount of protein in each reaction. The DNA fragment used was prepared by isolating the EcoRI-XhoI GCV2 fragment (−351 to −148) from pRH4 and then cutting this fragment with AluI generating an EcoRI-AluI fragment (−351 to −218). The 5′ EcoRI terminus was end-labeled with [\( ^{32} \text{P} \)]dATP, and the fragment was isolated by polyacrylamide gel electrophoresis. After a 20-min incubation at room temperature, freshly diluted DNAseI (4.5 units) was added, and the DNA digestion was proceeded for 1 min at room temperature. The reaction was stopped by adding 140 μl of stop solution containing 192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, and 64 mM/μl yeast RNA. DNA fragments were extracted once with phenol/chloroform (1:1) and precipitated with ethanol and 9.3 μl of protein extract. The DNA fragments were rinsed with 95% ethanol and resuspended in 4 μl of loading dye (95% v/v formamide, 10 mM EDTA, 1% bromphenol blue, 1% xylene cyanol). The DNA fragments were denatured for 1 min at 90 °C prior to electrophoresis on an 8% sequencing gel. The G + A sequencing was prepared from the same fragment (30).
deletion analysis (Fig. 2A) indicated that the glycine response was mediated by sequences between −313 and −267, with complete loss of the glycine effect on deletion to −267. Glycine induction is actually due to a loss of repression of GCV2 because deletion led to a constitutively higher level of gene expression as is clearly seen in Fig. 2B, which shows the response of the deleted constructs to different concentrations of external glycine.

Repression in the absence of glycine was partially relieved (2-fold) on deletion of sequences to −289. This partial derepression was also observed in the window deletion construct pRH13 (Fig. 2A) with deletion of sequences between −310 to −289. Window deletions from −313 to −267 and from −289 to −267 showed complete loss of repression. Therefore, sequences between −289 and −267 are absolutely required for glycine-specific repression of GCV2 expression and an additional important sequence between −310 and −289 affects the level of this control because its deletion led to a consistent 2-fold increase in basal expression on Dmin without loss of glycine control.

To further dissect the glycine-specific control region, overlapping fragments between −291 and −266 were inserted in both forward and reverse orientation at the 5′ end of the truncated GCV2-lacZ in pRH4 (the largest promoter lacking glycine-specific control). Among these constructs, those with the 11-bp fragment (5′-TGACTCTTCTT-3′) inserted in either forward or reverse orientation restored the glycine response of GCV2 (Fig. 3A). However, this fragment did not completely repress GCV2 expression, which is consistent with the window deletion data above, because it only showed a 2-fold effect compared with the wild-type (pRH1), which showed about 4-fold repression. This fragment contains the motif 5′-TGACTCTTCTT-3′, which is a potential binding site for Gcn4p, Yap1p, and Bas1p transcription factors, as well as an additional 5′-CTTCTT-3′ motif. This 11-bp sequence was sought in the other known glycine responsive genes, GCV1 and GCV3 (7, 9). The promoter of GCV1 shares remarkably strong homology with GCV2 over about 50 bp including the 5′-CTTCTT-3′ motif (at −177; Fig. 3B). For GCV3 there is less homology with either GCV1 or GCV2 in the promoter region, although there is a region around a CTTCTT motif (at −262) that shows a fair degree of sequence conservation across all three genes.

The constructs shown in Figs. 2 and 3 were also used to locate the l-methionine response region of the GCV2 gene (Fig. 1B). The 11-bp sequence that was important for the glycine response was also involved in the l-methionine response, and the same sequences are responsible for the regulation of GCV2 when cells are grown in glycine- or l-methionine-supplemented medium. A GCV1-lacZ fusion construct was also found to respond to l-methionine, whereas a GCV3-lacZ construct did not (data not shown).

To further delimit the important bases in the 11-bp sequence that partly restored the glycine response, the region was subject to site-directed mutagenesis. The mutations made included one that altered 4 bp in the GCN4B core 5′-TGACTCTTCTT-3′ consensus to 5′-GGTACC-3′ and another that altered the 5′-CTTCTT-3′ hexanucleotide sequence to 5′-GGTACC-3′ (Fig. 2A; RH8 and RH9). Mutation of the GCN4B site made a small difference to the level of basal expression; however, the change to the 5′-CTTCTT-3′ sequence led to over 2-fold increase in the basal level expression, reflecting a partial loss of glycine repression. Because there were also effects on basal GCV2 expression from sequences between −313 to −289 (just upstream of the 11-bp sequence) and this contains another potential GCN4 site, this (GCN4A; at −312) was also mutated to 5′-GGTACC-3′. There was no effect of this change on the basal level or glycine responsiveness of GCV2 expression (Fig. 2A; RH7).

The above results indicate that sequences between −313 and −267 affect the overall response to glycine (and l-methionine) and that an 11-bp fragment 5′-TGACTCTTCTT-3′ can confer some glycine response with the 5′-CTTCTT-3′ at −286 playing an important part in this glycine effect. There are, however, additional sequences within the −313 to −289 region that are involved in setting the level of basal expression to accomplish the full response.

The main deletion analysis (Fig. 2A) also revealed control elements regulating GCV2 expression under other conditions. In YPD medium, the gene is repressed over 20-fold relative to expression on minimal medium (Fig. 1B). This repression occurred in deletions down to −227 but was lost on further deletion to −205 (data not shown). This response to growth on rich medium is therefore separate from that to one-carbon metabolites such as glycine and l-methionine. The nitrogen source repression seen in l-glutamine-containing minimal medium was localized to the same region as that for the rich medium response using the deletion constructs (Fig. 2A).

The above results have localized sequences essential for repression of GCV2 in rich medium including nitrogen catabolite regulation (up to 20-fold). For the glycine response, an essential “core” 11-bp region has been identified, although flanking upstream sequence(s) also appear to play a role. Because the glycine response is common to GCV1, GCV2, and GCV3 genes and very little is known about the molecular mechanisms regulating expression of genes for one-carbon metabolism in S. cerevisiae, we have concentrated on this aspect of the regulation.

The Glycine Regulatory Region from GCV2 Mediates Repression When Inserted in a Heterologous CYC1 Promoter, and This Repression Is Relieved by Glycine and l-Methionine—To test
whether the 11-bp essential region and adjacent sequences involved in glycine induction have activity in a different sequence context, the 42-bp fragment (−309 to −267) spanning the important core and flanking sequences was inserted into the XhoI site located between the UASC and TATA box elements of a complete CYC1 promoter fused to a lacZ reporter (Fig. 4). Two constructs were tested; one contained one 42-bp fragment, the other carried two inserts in a tandem duplication. In this relatively strong promoter, insertion of one element led to a 2-fold repression of expression, and much of this repression was relieved on adding glycine to the medium (Fig. 4). When two 42-bp fragments were inserted, repression was greater than 4-fold, and the addition of glycine caused a 3-fold “induction” to 75% of the level seen in the intact CYC1 promoter. Similar results were obtained on addition of l-methionine instead of glycine as an inducer (data not shown). These results demonstrate clearly that the elements confer a glycine response, and this is true even if changes in promoter spacing were affecting expression.

These results confirmed that the 42-bp glycine regulatory region (GRR) of GCV2 from −309 to −267 is capable of causing repression of gene expression in an heterologous system with a more highly expressed promoter than that of GCV2 and that it carries element(s) needed for glycine and l-methionine to relieve this repression as is seen in the native GCV2 gene.

The GCV1 Gene Is Regulated Positively by a Very Similar Element to the GRR That Regulates the GCV2 Gene Negatively—Deletion analysis of the GCV1 upstream sequence gave the interesting result that unlike the negative control seen for GCV2, deletion of sequences (between −206 to −130) led to a loss of glycine induction (data not shown). Because the GCV1 sequence can act as an activator, −193 to −162 of GCV1 was inserted into the blind (UAS-less) CYC1-lacZ reporter plasmid pLGΔ-312SS at the XhoI site with results that were virtually the opposite of those seen for the homologous GCV2 element (Fig. 5A). One copy caused strong activation even in the absence of glycine in the medium, and addition of glycine caused a modest induction. When four copies of the construct were present in tandem array, there was a very substantial activation of the reporter in the absence of glycine and a greater than 10-fold further induction on the addition of glycine. The homologous elements from GCV1 and GCV2 therefore both introduce glycine responsiveness but differ in whether they act to repress or promote transcription, reflecting the situation in the native genes. Because this region contained the sequence homologous to the functional 42-bp element in GCV2 (Fig. 3B), the region of strong homology (−193 to −162) of GCV1 was also inserted into the XhoI site of the CYC1-lacZ reporter plasmid pLGΔ-312SS. In this context (Fig. 5B) the sequence now more closely resembled the situation seen with GCV2, because it acted as a repressor with a greater effect of more copies, still retaining glycine responsiveness. The effect of context was further highlighted by inserting the GCV1 glycine regulatory region (−193 to −162) in front of the truncated GCV2 promoter in pRH4. This restored a glycine response to the GCV2 construct, and in this context the GCV1 element was acting in a similar way to the GCV2 element (Fig. 2A; RH15).

These results indicate that the GCV1 GRR element may
function as either an activator or a repressor depending on its context, but the response to glycine is retained. This has interesting implications for the way that this cis-acting element functions.

**Protein Specifically Binds to the GCV2 Sequence (−309 to −267)**

To determine whether any protein bound to the GRR of GCV2, gel mobility shift analysis was performed initially using a DNA fragment spanning the region of GCV2 from −322 to −248. Nuclear extracts were obtained from cells grown in minimal medium with or without glycine. The DNA sequence from −322 to −248 was amplified by PCR and in a mobility shift assay was found to form one major complex with the nuclear protein extract. Similar complex formation was observed using the internal 42-bp XhoI fragment (−309 to −267; Fig. 6A). This complex was not formed in assays using cell extracts treated with proteinase K (data not shown).

Two fragments were generated by cutting the −322 to −248 fragment at an MboII site (cuts at −295). No protein bound to the smaller fragment (Fig. 6A), and although binding occurred to the larger one, it was not as extensive as that to the longer XhoI fragment (−309 to −267). This indicated that although the protein could bind between −267 and −295, for strong binding there was also a requirement for bases up to 14 bp further upstream. This correlated with the in vivo results from the genetic studies, which showed that although the major control element of GCV2 was between −289 and −267, flanking sequence up to −310 were needed for the full response.

This complex could be effectively competed by the unlabeled DNA sequence from −309 to −267 but not by the same sequence with a mutation of the CTTCTT motif (Fig. 6B). DNaseI footprinting of the protein-DNA complex (Fig. 7) showed that the region from the 5'-CATCN-CTTCTT-3' motif was protected and that binding of the protein led to an increase in DNaseI susceptibility of the bases immediately 3' of the footprint (asterisk in Fig. 7), indicating an effect of protein binding on the topology of the DNA in this region. From Fig. 3B it can be seen that the protected region corresponds to the sequence of greatest homology between GCV1, GCV2, and GCV3, with the flanking CATC and
CTTCTT motifs most conserved. These results are fully in accord with the genetic data presented above because the binding of the protein depends on the region containing the CTTCTT motif but is augmented by the bases further upstream to include the CATC. The protein is therefore an excellent candidate for a transcription factor that mediates the glycine control.

There was no difference in the DNA-protein complexes formed using extracts prepared from cells grown on inducing concentrations of glycine and those from cells grown in the absence of glycine (data not shown). This may indicate that the putative transcription factor is present constitutively and is activated by modification or ligand binding in the presence of excess glycine or methionine in the medium. The availability of an in vitro binding assay enabled screening for possible interactions between the GRR-binding protein and low molecular mass metabolites related to one-carbon metabolism.

Tetrahydrofolate Affects the Binding of the Putative Glycine Response Protein to the Control Region of Both the GCV2 and GCV1 Genes—Complicated signal transduction pathways often mediate activation or repression of a transcription factor in response to a stimulus. For a few systems, however, low molecular mass metabolites can act as ligands to activate a transcription factor (e.g. the Hap1p, heme-activated protein; Ref. 31). We therefore tested products or intermediates of one-carbon metabolism, including glycine, l-methionine, folic acid, and H$_4$folate. Of the compounds tested, H$_4$folate had a marked effect on the binding of the protein in the gel mobility shift assays.

Complex formation responded at concentrations of H$_4$folate between 10 and 50 mM and was responsive 3-fold in the range up to 1 mM (Fig. 8B). Control experiments adding the same concentrations of buffer and 2-mercaptoethanol needed to stabilize H$_4$folate did not affect formation of the complex. Other compounds tested, including folic acid, folic acid, glycine, and l-methionine had no effect at concentrations up to 10 mM, which were beyond physiological levels. Because the GCV1 gene is also regulated by glycine, we repeated the above experiments with a 31-bp fragment (−193 to −162) of GCV1 encompassing the GRR region that contains the core CTTCTT motif. Similar results were obtained (Fig. 8C), which indicated that the putative GCV1 promoter region can bind the same proteins as GCV2 and that there is a similar effect of H$_4$folate on the binding.

Limitation of Folate Synthesis Affects the Glycine Response of GCV2 in Vivo—To test whether the above in vitro results with H$_4$folate binding were relevant to the control of one-carbon metabolism in vivo, we tested the effect of inhibiting H$_4$folate biosynthesis on glycine induction of GCV2. For this we used the recently generated fol1 mutant (YUG1) provided by Dr. Johannes H. Hegemann (Heinrich-Heine University, Düsseldorf, Germany). This strain requires folic acid for growth. Complex formation responded at concentrations of H$_4$folate between 10 and 50 mM and was responsive 3-fold in the range up to 1 mM (Fig. 8B). Control experiments adding the same concentrations of buffer and 2-mercaptoethanol needed to stabilize H$_4$folate did not affect formation of the complex. Other compounds tested, including folic acid, folic acid, glycine, and l-methionine had no effect at concentrations up to 10 mM, which were beyond physiological levels. Because the GCV1 gene is also regulated by glycine, we repeated the above experiments with a 31-bp fragment (−193 to −162) of GCV1 encompassing the GRR region that contains the core CTTCTT motif. Similar results were obtained (Fig. 8C), which indicated that the putative GCV1 promoter region can bind the same proteins as GCV2 and that there is a similar effect of H$_4$folate on the binding.

Cells of YUG1 transformed with the GCV2::lacZ construct were grown to late exponential phase (A$_{600}$ of 1.3) in Dmin containing 50 µg/ml of folic acid and then transferred to fresh medium in the presence and absence of glycine (10 mM) and folic acid (100 µg/ml), and the level of induction of GCV2 was determined by measuring β-galactosidase activity at intervals.

From Table I, it can be seen that under conditions in which folate was limiting or depleted the GCV2 gene was not inducible by glycine but that a response was obtained in the presence of folic acid. The results support the hypothesis that a folate species plays a role in the cell in the glycine response of GCV2 and therefore in regulation of one-carbon metabolism.

DISCUSSION

Previous studies of the expression of GCV1, GCV2, and GCV3 have shown them to be regulated in response to the presence of glycine in the external medium (7–9). We have here shown that GCV2 can be regulated sensitively over a broad (about 150-fold) range from maximal induction in cells growing in minimal medium with glycine as the sole nitrogen source to maximum repression in rich medium. The glycine decarboxylase complex thus appears to be controlled to balance the requirement for one-carbon metabolites versus the need for nitrogen.
In addition to the glycine response we have identified a control capable of modulating expression up to 20-fold in response to growth on different nitrogen sources or on rich medium. Most of this effect is localized to a 23-bp region (227 to 240) that includes a 9-bp palindromic sequence adjacent to one copy of a GATA element typically associated with genes regulated by nitrogen catabolite repression (19). There may be some involvement of a protein binding to the GATA site, although the situation would be different from that seen for most other genes for which multiple copies of these sites are required (20, 32). Adding L-methionine to the medium also led to an elevation of GCV2 and GCV1 expression to a lesser extent than glycine. This control is mediated through the same motifs in the promoters as the glycine response. Although the advantages to the cell of up-regulating the glycine metabolic genes in response to excess glycine is obvious, those resulting from elevated expression in response to L-methionine are less so. L-Methionine seems metabolically rather remote from the sensing system involved in regulating transcription because in another strain of S. cerevisiae we have seen L-methionine work in the opposite direction to repress the GCV2 gene. This and the nitrogen control require more extensive investigation because here we have concentrated in much more detail on the glycine response.

Deletion analysis has indicated that glycine control is a response to the binding of a protein at a GRR. Site-directed mutagenesis localized the main effects to a CTTCTT element within GRR. However, it is also clear that there is a requirement for additional upstream sequences for full wild-type response to glycine of GCV2. These sequences cannot on their own confer any response on the gene and are not absolutely essential but serve to modulate the degree of expression from the adjacent CTTCTT element. It appears therefore that a glycine response protein transcription factor binds to the 6-bp motif, and this binding is stabilized by flanking sequences. The footprinting data are consistent with the genetic analysis because they indicate the existence of a protein that protects the 22-bp sequence from 227 to 249, which includes the CT-
The GRR of GCV genes, 5'-CATCN7CTTCTT-3' is also found in the promoter of the DFR1 gene encoding dihydrofolate reductase (at –377), which catalyzes de novo synthesis of H$_2$folate. The spacing between the CATC and CTTCCT motifs seen in the GRR of GCV1 and GCV2 was also conserved in the DFR1 promoter. The importance of H$_2$folate in the regulation of GCV genes, and the existence of the GRR in the DFR1 may therefore give an insight into the regulation of one-carbon metabolism in S. cerevisiae.

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