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Regulation of the *GCV3* gene in *Saccharomyces cerevisiae*

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ABSTRACT

Regulation of the *GCV3* gene in *Saccharomyces cerevisiae*

Yun Zheng

The glycine cleavage system (GCS) is a multienzyme complex containing four proteins. The GCS, which is important for the growth and viability of organisms ranging from bacteria to humans, catalyses the oxidative cleavage of glycine into CO_2 and NH_3. Concomitantly it generates the C1-donor 5,10-methylenetetrahydrofolate and the electron donor NADH. NH_3 is an important precursor for cellular nitrogen metabolism. The C1-donor 5,10-methylenetetrahydrofolate is a precursor for the biosynthesis of C1-end products such as adenine, thymidylate, serine and methionine. The goal of my research was to study the regulatory mechanisms controlling GCS activity.

The expression of *GCV3*, a yeast gene that codes for one of the four GCS subunits, was analyzed in detail. This revealed that *GCV3* expression is regulated by the availability of glycine, and cellular demand for the metabolic products of glycine cleavage. 10 mM glycine in minimal medium (SD) induced *GCV3* expression about 4-fold. Supplementing with the C1-metabolic end products repressed *GCV3* expression about 3-fold. Both glycine induction and repression by the C1-end products were found to be Bas1p-dependent. The upstream promoter elements required for regulation by Bas1p were localized. Expression of *GCV3* is also subject to the general control system in a Gcn4p-dependent fashion. The elements utilized by Gcn4p have been characterized. A GATAA sequence located at –167bp upstream of the start codon is used by the

nitrogen regulation system. Gcr1p, a transcription activator for glycolytic genes, is involved in regulating *GCV3* in the presence of glucose. Evidences are also presented for an as yet unidentified regulator that represses expression in SD. Additional results presented in this thesis suggest that Rap1p, Nil1p, Ure2p and Deh1p also regulate *GCV3*.

For My Love

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Introduction

Regulation of transcription plays a critical role in the control of growth and differentiation in eukaryotic cells. In eukaryotic cells, the transcription machinery consists of RNA polymerase and a number of other general transcription factors needed for initiation of transcription. Specific regulators activate or repress the transcription apparatus by specific binding to DNA elements in promoters and/or the core transcription apparatus. Since individual genes can have several elements that bind gene-specific regulators, eukaryotic promoters can be complex, with many different regulators influencing transcription.

One of the major themes to emerge from studies of transcriptional regulation in eukaryotes in recent years is the remarkable conservation of this process from yeast to mammals. This conservation includes the components of the transcriptional machinery, specific activator or repressor proteins and their cognate DNA-binding sites, and the very mechanisms of transcriptional activation and repression. *Saccharomyces cerevisiae* (Yeast) is a single celled organism, that is easy to genetically modify. Recent completion of the genome-sequencing project of *S. cerevisiae* further enhances yeast's utility as a model organism for genetic analysis. Yeast therefore affords one of the best experimental systems in which to unravel fundamental mechanisms of transcriptional control that could be applied to all eukaryotic cells.

Yeast promoters contain the TATA box, transcription initiation sites, upstream activation sites (UAS), and upstream repression sites (URS). Most yeast promoters rely on the TATA box for RNA polymerase binding to initiate transcription. One

distinguishing feature of the action of yeast TATA boxes is that they are not always located 20 nucleotides upstream of the initiation site as in higher eukaryotes. Rather, initiation occurs within a window of 40-120 nucleotides downstream from the TATA element. The precise position where transcription begins is determined by the initiation site itself. Consensus sequences of 5'-TCGA-3' and PuPuPyPuPu (Pu: purine; Py: pyrimidine), which account for the majority of initiation sites, will function as initiators if inserted downstream of a TATA box (for a review, see Guarente, 1992).

So far, very little is known about how the transcription apparatus actually initiates transcription. The level of expression of structural genes is governed by signals that are received from the environment and integrated by cellular machinery to yield a response. These signals are transduced through specific pathways to the cytoplasm or the nucleus to regulate the production, location or activity of transcriptional regulators that in turn control gene expression through interaction with regulatory sequences located upstream of the target gene. Promoters may contain several UASs or URSs that respond to different signals.

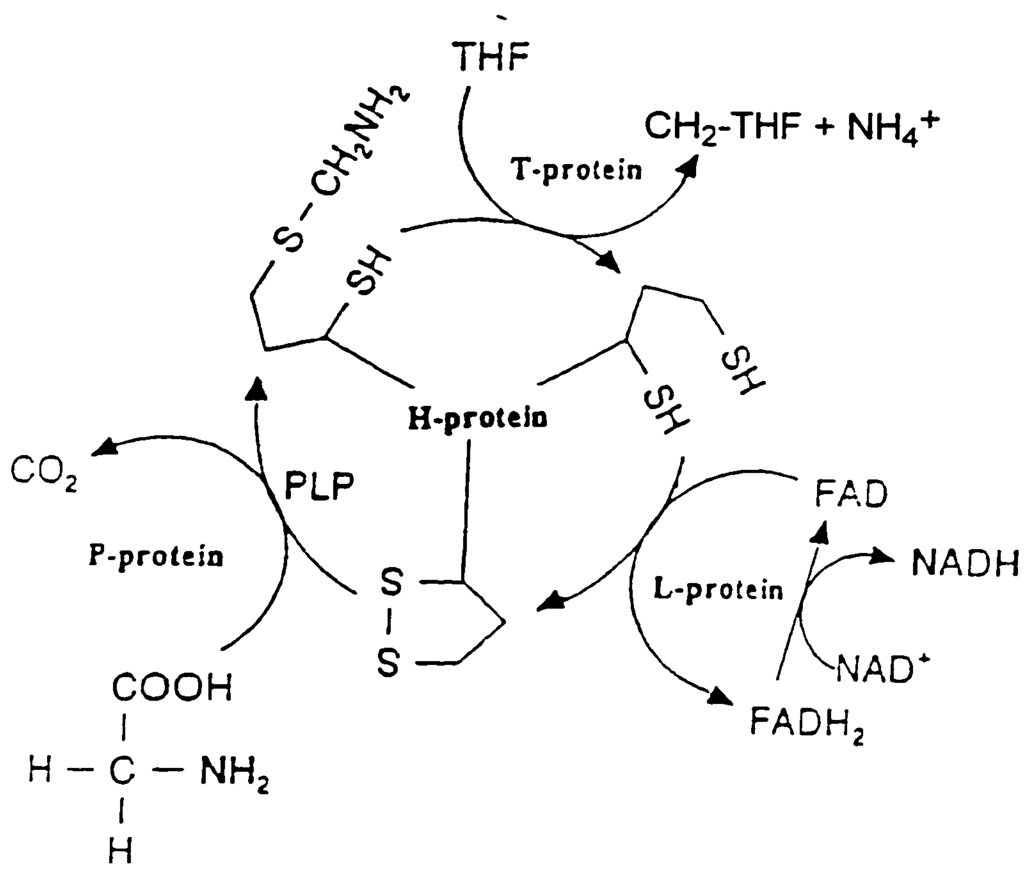
In this thesis, I present a molecular characterization of a yeast gene promoter and outline possible regulatory mechanisms that control its expression.

1.1. *GCV3* codes for the H-protein subunit of the glycine cleavage system in *S. cerevisiae*.

1.1.1. Glycine cleavage system.

The glycine cleavage system (GCS; EC 2.1.2.10), also known as the glycine decarboxylase complex or serine synthase, is a multienzyme complex that is located in the mitochondrial inner membrane (Okamura-Ikedia et al., 1987). It catalyzes the reversible oxidation of glycine resulting in the production of CO_2, NH_3, NADH and N^5, N^{10}-methylene tetrahydrofolate (Oliver et al., 1990). The system is composed of four component proteins termed P-protein, T-protein, L-protein, and H-protein. P-protein is a pyridoxal phosphate-dependent decarboxylase. It catalyzes the first reaction, forming a Schiff base with the amino group of glycine, releasing carbon dioxide from glycine and transferring the methylamine moiety to the lipoyl prosthetic group of the H-protein (a lipoamide-containing carrier protein). The lipoic acid group is reduced during the transfer. T-protein (a tetrahydrofolate transferase) catalyzes the release of ammonia and transfer of the one-carbon group from the lipoyl residue of the H-protein to tetrahydrofolate. Finally, L-protein, a lipoamide dehydrogenase, catalyzes oxidation of the H-protein dihydrolipoyl residue and the reduction of NAD^+. The H-protein provides the attachment site for the lipoic acid cofactor that interacts with the active sites of the other three enzymes (Okamura-Ikedia et al., 1982; Walker and Oliver, 1986). The mechanism of this reaction has been summarized in Figure 1. In addition to its role in the GCS, L-protein serves a similar biochemical function in both the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes (Dickinson et al., 1986; Dickinson and Dawes, 1992).

Figure 1. Schematic representation of the glycine cleavage system (GCS) multienzyme complex (taken from Oliver, 1994). Abbreviations in the figure designate peptides, co-factors, metabolic intermediates and side chain as follows: P-protein (glycine decarboxylase); H-protein (hydrogen carrier protein); T-protein (aminomethyltransferase); L-protein (dihydrolipoamide dehydrogenase); THF (tetrahydrofolate); PLP (pyridoxal phosphate); SH (sulfhydryl group); NAD (nicotinamide adenine dinucleotide); NADH (nicotinamide adenine dinucleotide reduced form); FAD (flavin adenine dinucleotide); FADH (flavin adenine dinucleotide reduced form). H-protein plays a central role through its lipoamide co-factor, which carries the reaction intermediates between the reactive sites of the P-protein, T-protein and L-protein.


THF
CH_2-THF + NH_4^+
T-protein
S – CH_2NH_2
SH
SH
SH
H-protein
CO_2
PLP
P-protein
S
S
COOH
H – C – NH_2
H
FAD
NADH
L-protein
NAD^+
$FADH_2$

The biochemistry of the glycine cleavage system has been studied in organisms ranging from *Escherichia coli* (Okamura-Ikedia et al., 1993) to plants such as peas (Macherel et al., 1990) and *Arabidopsis thaliana* (Srinivasan and Oliver, 1992) and to animals such as chickens (Yamamoto et al., 1991), cows (Fujiwara et al., 1990) and humans (Fujiwara et al., 1991). The biochemistry of the reaction is known in detail, but very little is known about the physical structure of the GCS or the subunit interactions required for complex assembly in mitochondria. In plants, the weakly bound complex appears to have a stable subunit ratio of P-protein dimers: H-protein monomers: T-protein monomers: L-protein dimer of 2:27:9:1 (Oliver et al., 1990).

1.1.2. Physiological importance of the glycine cleavage system.

The four products of the glycine cleavage system are precursors for many important cellular metabolic processes. Ammonia is a nitrogen source for cells. The role of ammonia in nitrogen source catabolism is unique - it is the only product that is absolutely required. The nitrogen atoms of amino acids, purines, pyrimidines, and other biomolecules come from ammonia. Ammonia is assimilated into amino acids by way of glutamate and glutamine. Ammonia can be directly incorporated into both glutamine and glutamate by glutamine synthetase (GS, Figure 2 reaction 1) and $NADP^+$-glutamate dehydrogenase ($NADP^+$-GDH1 or $NADP^+$-GDH3, Figure 2 reaction 2), respectively (Tempest et al., 1970; Avendano et al., 1997). Then, by transamination, the α-amino group of glutamate provides the α-amino group of most amino acids. Glutamine, the other major nitrogen donor, contributes its side-chain nitrogen to the biosynthesis of a

Figure 2. Ammonia assimilation reactions (Tempest et al., 1970; Avendano et al., 1997). Abbreviations: NH_3 (ammonia); ATP (adenosine triphosphate); GS (glutamine synthetase); ADP (adenosine diphosphate); P_i (inorganic phosphate); NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate); H^+ (proton); $NADP^+$-GDH1 ($NADP^+$-glutamate dehydrogenase); $NADP^+$-GDH3 (an isoenzyme of $NADP^+$-GDH1).

$$NH_3 + \text{glutamate} + \text{ATP} \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + P_i \quad (1)$$

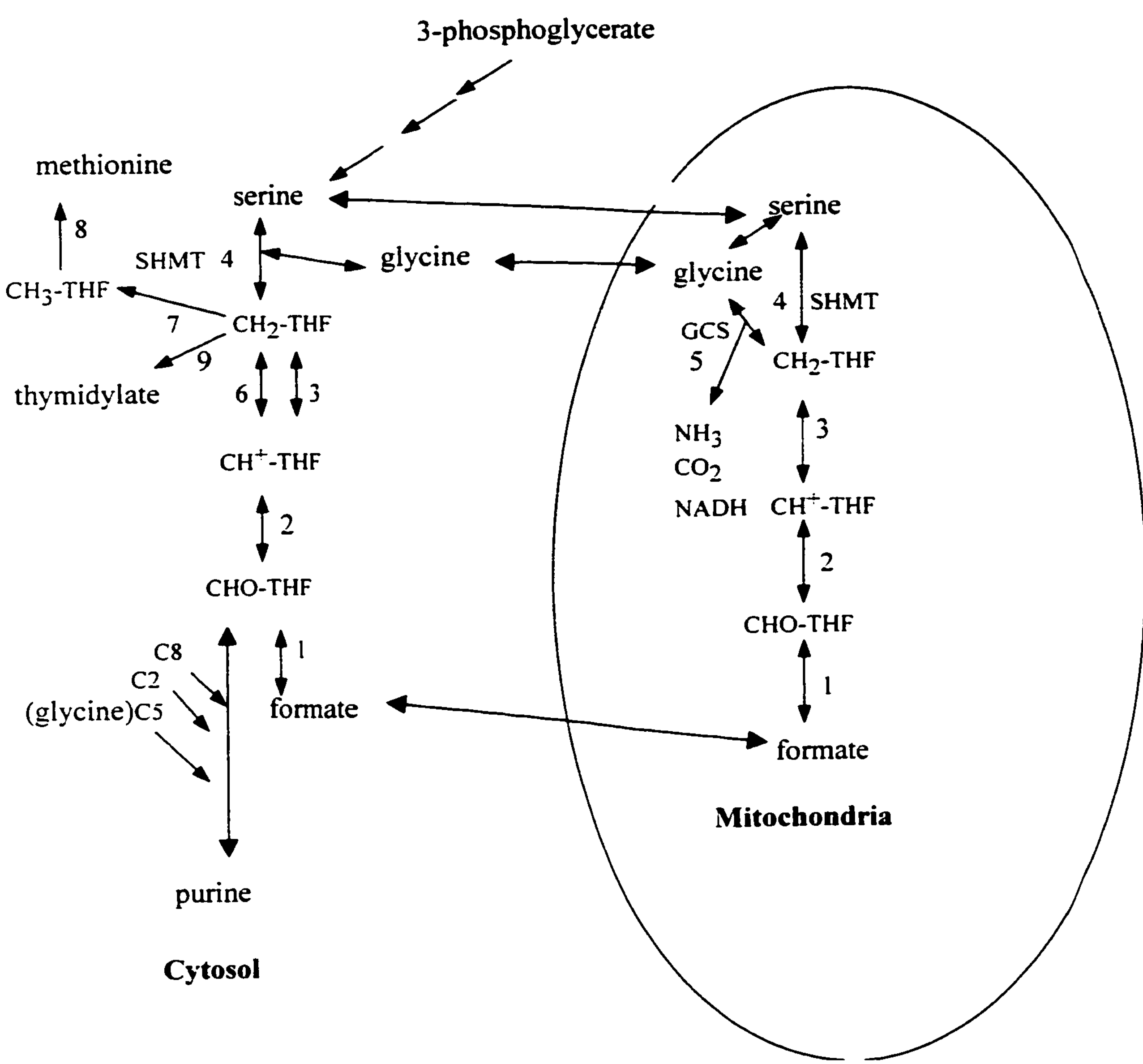
$$NH_3 + \alpha\text{-ketoglutarate} + \text{NADPH} + H^+ \xrightarrow{\text{NADP}^+\text{-GDH1/ NADP}^+\text{-GDH3}} \text{glutamate} + \text{NADP}^+ \quad (2)$$

wide range of important compounds, such as tryptophan, histidine, carbamoyl phosphate, glucosamine 6-phosphate, CTP and ATP.

NADH is an electron donor predominantly used for oxidative phosphorylation (Hatefi, 1985). The glycine cleavage system is thus also involved in energy synthesis since NADH is a product of glycine oxidation.

Another important product from glycine oxidation is N^5, N^{10}-methylenetetrahydrofolate, a carrier of activated C1-units. One-carbon units play essential roles in several major cellular processes including nucleic acid biosynthesis, organelle protein biosynthesis, methyl group biogenesis, amino acid metabolism, and vitamin metabolism (Appling, 1991). Serine, methionine, adenine, histidine and formate have been called C1-end products. Serine, glycine and formate are the only products of mitochondrial one-carbon metabolism that may have access to the cytoplasm (Shannon and Rabinowitz, 1988; McNeil et al., 1996). Although the cytoplasm is the major cellular compartment for synthesis of C1-units, 25% of the C1-units utilized for cytoplasmic purine synthesis is derived from mitochondrial formate (Pasternack et al., 1994; McNeil et al., 1996). The glycine cleavage system, like the mitochondrial glycine hydroxymethyltransferase (SHMTm), contributes to the synthesis of mitochondrial formate (McNeil et al., 1996; Nagarajan and Storms, 1997). Furthermore, the observation that glycine supplementation can satisfy serine requirements in *ser1* yeast (McNeil et al., 1996; Nagarajan and Storms, 1997) indicates that serine is another important indirect product of the glycine cleavage system. The role of the GCS in C1-unit metabolism is illustrated in Figure 3.

Figure 3. Proposed organization of the enzymes of one-carbon metabolism in *Saccharomyces cerevisiae* (Kastanos et al., 1997; Nagarajan and Storms, 1997). Reactions 1, 2 and 3, are catalyzed by cytoplasmic or mitochondrial CHO-THF synthetase (EC 6.3.4.3), CH^+-THF cyclohydrolase (EC 3.5.4.9) and NADP-dependent CH_2-THF dehydrogenase (EC 1.5.1.5), respectively. Reaction 4 is catalyzed by SHMT (EC 2.1.2.1) present in both compartments. Reaction 5 is catalyzed by the GCS (EC 2.1.2.10). Reaction 6 is catalyzed by the monofunctional NAD-dependent CH_2-THF dehydrogenase. Reaction 7 is cataltzed by CH_2-THF reductase (EC 1.5.1.45). Reaction 8 is catalyzed by methionine synthase (EC 2.1.1.14). Reaction 9 is catalyzed by thymidylate synthase (EC 2.1.1.45). The reactions from 3-phosphoglycerate to serine are catalyzed by 3-phosphoglycerate dehydrogenase (EC 1.1.195), phosphoserine aminotransferase (EC 2.6.1.52) and phosphoserine phosphatase (EC 3.1.3.3), respectively. Carbon 2 (C2) and 8 (C5) of the purine backbone are derived from CHO-THF, and carbon 5 (C5) is derived from glycine. Abbreviations: THF (tetrahydrofolate), SHMT (glycine hydroxymethyltransferase), GCS (glycine cleavage system), CH_2-THF (N^5, N^{10}-methylenetetrahydrofolate), CH^+-THF (N^5, N^{10}-methyltetrahydrofolate), CHO-THF (N^{10}-formyltetrahydrofolate), CH_3-THF (N^5-methyltetrahydrofolate).


3-phosphoglycerate
methionine
serine
8
SHMT 4
glycine
CH3-THF
7
CH2-THF
9
thymidylate
6
3
CH+-THF
2
CHO-THF
C8
C2
(glycine)C5
1
formate
purine
Cytosol
serine
glycine
4
SHMT
GCS
5
CH2-THF
NH3
CO2
NADH
3
CH+-THF
2
CHO-THF
1
formate
Mitochondria

Finally, CO_2 produced by the GCS may not be very important for cellular metabolism in yeast, but it plays an important role in C_3 plants. CO_2 generated from oxidation of glycine through the GCS is required for carbon fixation in illuminated leaves during photosynthesis (Canvin et al.,1976; Somerville and Ogren, 1982; Huisic et al., 1987).

It has been suggested that the glycine cleavage system provides a second pathway for synthesis of one carbon (C1) units (Kikuchi, 1973). In *E. coli*, a strain defective in the glycine cleavage enzyme system excretes glycine (Plamann et al., 1983; Ghrist and Stauffer, 1995). This implies that the amount of glycine produced from serine exceeds the amount used for protein synthesis and purines and heme biosynthesis. Stauffer and Stauffer (1999) suggested that the role for the GCS enzyme complex is to balance the cell's requirements for glycine and the active C1 units used for various methylation reactions (Stauffer and Stauffer, 1999).

In yeast, deficiency in the GCS is not lethal, provided that the cells do not have glycine as the sole nitrogen source (Sinclair and Dawes, 1995). Furthermore, in *S. cerevisiae* (as well as *E. coli*) strains blocked in the synthesis of serine from glycolytic intermediates (*ser* mutants), a defect in the GCS renders them unable to grow on glycine minimal medium unless supplied with an alternate source of C1-units (Ogur et al., 1977; Plamann et al., 1983; Sinclair and Dawes, 1995; Nagarajan and Storms, 1997).

In humans, a deficiency of the GCS causes severe neurological damage in the neonatal period. The metabolic disorder resulting from a defective GCS is called non-ketotic hyperglycinaemia (NKH). It shows autosomal recessive inheritance, and is often fatal without intervention. Patients suffering from this disease accumulate 10- to 30-fold

elevated levels of glycine in plasma, urine, and particularly cerebrospinal fluids (Hamosh et al., 1995; Kure et al., 1997).

1.1.3. Genes that code for the glycine cleavage system in yeast.

In yeast, the genes encoding the four GCS components have been identified. *GCV1, GCV2, GCV3* and *LPD1* encode the T-protein, P-protein, H-protein and L-protein, respectively (McNeil et al., 1997; Sinclair et al., 1996; Nagarajan and Storms, 1997; Sinclair and Dawes, 1995). Table 1 lists the GCS subunits, their genes, the predicted molecular masses, their chromosomal locations, and their GenBank accession numbers.

1.2. Transcriptional regulators implicated in the control of the GCS expression.

Because the products of the GCS are involved in nitrogen metabolism, energy synthesis, C1-unit metabolism and possibly carbon metabolism, studies of the regulation of the GCS genes should provide a better understanding about how the cellular requirement for GCS activity is satisfied.

The regulation of the glycine cleavage system in various species has been shown to respond to a variety of stimuli (Kim et al., 1991; Wilson et al., 1993a and b). In *E. coli*, transcription of three GCS subunits, encoded by the *GCV* operon (H, P, and T) (Wilson et al., 1993a and b), is positively regulated in response to glycine, by four DNA-binding proteins: Lrp, Purp, CRP, and GcvA (Lin et al., 1992; Wilson and Stauffer, 1994; Wonderling and Stauffer, 1999). GcvA and GcvR negatively regulate the *GCV* operon in the presence of purines (Ghrist and Stauffer, 1995; Stauffer and Stauffer, 1999). H-

Table 1. The glycine cleavage system in *Saccharomyces cerevisiae*.

Component	Gene name	Protein molecular weight[a]	Chromosome location	Intergenic region (nt)[b]	GenBank accession numbers	Protein names
T-protein[c]	*GCV1*	44469	IV	389	X95966	tetrahydrofolate transferase
P-protein[d]	*GCV2*	114451	XIII	637	Z49808	glycine decarboxylase
H-protein[e]	*GCV3*	18793	I	232	U12980	hydrogen carrier protein
L-protein[f]	*LPD1*	54010	VI	863	D50617	dihydrolipoamide dehydrogenase

[a]Molecular weight (Da). [b]Size of the promoter (nt). [c]Reference: McNeil et al., 1997. [d]Reference: Sinclair and Dawes, 1995. [e]References: Nagarajan and Storms, 1997; this study. [f]Reference: Sinclair et al., 1996.

protein transcripts from pea and chicken show developmental and tissue-specific regulation, respectively (Macherel et al., 1992; Kure et al., 1991). However, information on the regulation of the glycine cleavage system in *S. cerevisiae* was very limited. The genes coding for the four subunits of the GCS were only recently identified and cloned. The *GCV1*, *GCV2* and *GCV3* genes are all induced by glycine (McNeil et al., 1997; Sinclair et al., 1996; Nagarajan and Storms, 1997). A core 5'-CTTCTT-3' motif in the *GCV1* and *GCV2* promoters was reported to mediate the response to glycine, and a protein was shown to bind to this region (Hong et al., 1999). This same region was also required for mediating the repression that could be relieved by glycine and methionine, but not the nitrogen repression (Hong et al., 1999). Recently, a study showed that C1-unit metabolism was involved in the expression of *GCV1*, *GCV2* and *GCV3* and the C1-end products have been shown to repress *GCV3* expression *in vivo* (Nagarajan and Storms, 1997). Gel mobility shift experiments demonstrated that the affinity of a putative glycine responsive protein to the *GCV1* and *GCV2* promoters is increased by the presence of tetrahydrofolate, and that glycine-induced transcription of *GCV2* is dependent on folate synthesis (Hong et al., 1999).

LPD1 is also induced by glycine, but not as dramatically as the other GCS genes (Nagarajan and Storms, 1997; Nagarajan and Storms, unpublished data). General amino acid control, the nitrogen regulation system and Gcr1p were also shown to regulate the GCS (Nagarajan and Storms, 1997; this study).

1.2.1. General amino acid control system..

Previous studies in our lab have found that *GCV3* was regulated by the general amino acid control system. Under conditions of amino acid starvation the general amino acid control system activates the transcription of several genes coding for amino acid biosynthetic enzymes. So far, the expression of more than 30 genes involved in the biosynthesis of 11 different amino acids and of several genes for purine biosynthesis, such as *ADE1, ADE4, ADE5, ADE7* and *ADE8*, have been found to be coregulated by the general amino acid system (Rolfes and Hinnebusch, 1993). Transcription of these genes in response to starvation of any one of several amino acids, including histidine, arginine, lysine, isoleucine, valine, leucine, serine, phenylalanine, tryptophan, methionine and proline, is stimulated from 2- to 10-fold. Gcn4p is the proximal positive regulator of gene expression in this system. It acts directly to stimulate expression of genes subject to general control. The production of Gcn4p is regulated in response to amino acid availability. In the absence of amino acid starvation, the translation of *GCN4* is almost abolished, but Gcn4p levels increase in response to amino acid starvation. Thus, an effective way to test if a gene is subject to general amino acid control is to grow wild-type cells in the presence of an inhibitor of an amino acid biosynthetic enzyme. For example, treatment with 3-amino 1,2,4-triazole (3-AT), a competitive inhibitor of the histidine biosynthetic enzyme encoded by *HIS3*, causes histidine starvation and thereby stimulates Gcn4p translation. If a gene is subject to the general control system, its expression will be induced by 3-AT (Hinnebusch, 1992).

Gcn4p is a leucine zipper protein (Agre et al., 1989) that shares homology to the jun oncoprotein and the human activation factor AP-1 (Mösch et al., 1990; Arndt et al.,

1987). Gcn4p activates transcription of responsive genes by binding to an upstream consensus sequence called the GCRE (General Control Regulation Element). The core sequence of this element is 5'-TGACTC-3'. (Hinnebusch, 1988; Hope and Struhl, 1985; Hope et al., 1988; Hill et al., 1986; Arndt and Fink, 1986; Arndt et al., 1987; Tice-Baldwin et al., 1989).

1.2.2. Basal level of transcription activation system.

The regulation of amino acid biosynthesis genes in yeast and bacteria is very different. When amino acids are present in the growth medium, bacteria transcribes amino acid biosynthetic genes at very low rates. Under similar growth conditions, yeast cells still maintain a significant level of transcription from amino acid biosynthetic genes, often referred to as basal level expression. This basal level expression is controlled by two transcription factors, Bas1p and Bas2p.

Bas1p has a myb-like DNA-binding motif near its N-terminal end (Tice-Baldwin et al., 1989). It has been reported to be involved in regulating both basal and induced expression of histidine and adenine biosynthesis genes (Tice-Baldwin et al., 1989; Devlin et al., 1991; Daignan-Fornier and Fink, 1992; Stotz et al., 1993; Rolfes et al., 1997; Denis et al., 1998). *In vitro* DNase I footprinting analysis revealed that the myb-motif of Bas1p binds to a conserved sequence 5'-TGACTC-3', which is required *in vivo* for Bas1p-dependent activation (Daignan-Fornier and Fink 1992; Zhang et al., 1997). Interference footprinting experiments have further demonstrated the specific interaction of Bas1p with the 5'-TGACTC-3' sequence and with a 3-bp extension on the 3' side which can affect the affinity of Bas1p binding (Hovring et al., 1994). Interestingly, this hexanucleotide

sequence is also the core of the binding site for Gcn4p, although flanking nucleotides differentially affect the binding affinity of these two proteins (Hovring et al., 1994; Hinnebusch, 1992). Whereas Gcn4p has a preference for the sequence 5'-RRTGACTCATTT-3' (R represents A or G; Hinnebusch, 1992), none of the known Bas1p binding sites or any predicted sites in other *ADE* gene promoters have an A nucleotide at the 3' base following the conserved hexanucleotide core.

Bas2p, also known as Pho2p and Gfr10p, is a homeodomain type protein which is closely related to the engrailed protein of *Drosophila* (Daignan-Fornier and Fink, 1992). Bas2p binds to DNA via its N-terminal homeodomain. The binding site for Bas2p is less well defined than are the Gcn4p and Bas1p binding sites. Bas2p binding to the *PHO5, HIS4, TRP4, HO* and *ADE5,7* promoters has been studied by DNase I footprinting (Vogel et al., 1989; Tice-Baldwin et al., 1989; Braus et al., 1989; Barbaric et al., 1996; McBride et al., 1997) and *in vitro* scanning mutagenesis (Rolfes et al., 1997). From these studies it appears that Bas2p binds to AT-rich sequences, but no clear consensus can be derived.

Bas2p cooperatively activates basal and induced transcription of histidine and adenine biosynthesis genes by interacting with Bas1p. Recent studies have shown that the interaction between a LexA-Bas1p fusion protein and Bas2p is independent of the DNA-binding activity of Bas1p, since the LexA-Bas1p fusion protein activates transcription of a lexA operator-controlled reporter gene in a Bas2p-dependent and adenine-repressible manner. That overexpression of Bas2p can overcome the adenine repression indicates that Bas1p-Bas2p complex formation is inhibited by excess adenine (Zhang et al., 1997). Site-specific and deletion mutations in the carboxy terminus of Bas1p make Bas1p activity independent of Bas2p and unrepressible by adenine. This

implies that the repression by adenine probably operates by modifying interactions between the Bas1p and Bas2p transcription factors (Zhang et al., 1997).

In addition to participating with Bas1p in transcriptional activation of the *HIS4* and *ADE* genes, Bas2p stimulates transcription of the *PHO5* (Johnston and Carlson, 1992) and *HO* (Brazas and Stillman, 1993a and b) genes. Bas2p interacts with Pho4p to induce *PHO5* transcription under phosphate starvation condition (Hirst at al., 1994) and can cooperatively bind with Swi5p to the *HO* promoter *in vitro* (Brazas and Stillman, 1993a and b; Brazas et al., 1995).

1.2.3. Nitrogen regulation system.

S. cerevisiae is able to use a wide variety of nitrogen sources for growth. Growth on medium containing ammonia, glutamine or asparagine results in relatively fast growth compared to growth on medium containing proline or urea. Consequently, ammonia, glutamine and asparagine are classified as preferred nitrogen sources or "good nitrogen sources", in contrast to proline and urea. The later are called the non-preferred nitrogen sources or "poor nitrogen sources". Glycine can serve as a poor nitrogen source (Sinclair and Dawes, 1995).

S. cerevisiae, like many other eukaryotic and prokaryotic microorganisms, is able to adjust its enzymatic composition according to the quality of its nitrogen source. During growth on good nitrogen sources, the activities of enzymes involved in the utilization of poor nitrogen sources are decreased. This phenomenon is regulated by the nitrogen catabolite repression or nitrogen regulation system (NCR), and the genes regulated in response to the quality of the nitrogen source are called the NCR-sensitive genes

(Cooper, 1982). One type of regulation of these enzyme activities occurs at the level of gene expression. It is now well established that the expression of many genes of *S. cerevisiae* whose products are responsible for the utilization of various compounds as sources of nitrogen is regulated by the GATA family of proteins. The GATA proteins recognize elements with the core sequence 5'-GATA-3' and are present in organisms from *S. cerevisiae* to humans. These proteins contain one or more characteristic C4 zinc finger motifs, which are capable of binding to DNA (Arceci et al., 1993; Coffman et al., 1994; Cunningham and Cooper, 1991; Detrich et al., 1995; Evans and Felsenfeld, 1989; Ko et al., 1991; Lee et al., 1991). So far, four proteins of the GATA family have been found in *S. cerevisiae*. They are Gln3p, Gat1p/Nil1p, Dal80p/Uga43p, and Deh1p/Nil2p (Blinder and Magasanik, 1995; Coffman et al., 1995; Courchesne and Magasanik, 1988; Cunningham et al., 1994, 1996; Rowen et al., 1997).

The nitrogen regulation system is quite complex. In the presence of good nitrogen sources, the nitrogen regulation system represses NCR-sensitive genes, while in the presence of poor nitrogen sources it activates NCR-sensitive genes. Gln3p and Gat1p/Nil1p both contain one zinc finger motif and a highly acidic amino-terminal domain. The latter is characteristic of the activation domain of many transcription factors (Coffman et al., 1995, 1996; Stanbrough et al. 1995). Gln3p and Gat1p/Nil1p positively regulate the NCR-sensitive genes, but in different manners. Gln3p is required for the transcription of a wide variety of genes involved in nitrogen metabolism, including *GDH1* (NADPH-dependent glutamate dehydrogenase), *GDH2* (NAD-dependent glutamate dehydrogenase), *GLN1* (glutamine synthetase) and *GAP1* (general amino acid permease) (Daugherty et al., 1993). When growing with glutamate, urea, or proline as

the nitrogen source, Gln3p activates NCR-sensitive gene expression. It is believed that high intracellular glutamate concentrations may stimulate Gln3p and consequently induce the transcription of Gln3p-regulated genes (Courchesne and Magasanik, 1988). In the presence of glutamine or ammonia, Gln3p is in an inactive form due to the high level of intracellular glutamine. It has been suggested that Gln3p is inactivated by Ure2p through modification of the thiol groups in Gln3p (Coschigano and Magasanik, 1991; Magasanik, 1992). Experimental evidence has shown that Ure2p acts directly on Gln3p in blocking its ability to activate transcription in the presence of high levels of intracellular glutamine (Stanbrough and Magasanik, 1995; Blinder et al., 1996).

Nil1p/Gat1p is capable of activating some of the same promoters as Gln3p (for example, *GDH1*, *GDH2*, *CAP1*, *DAL80* and *PUT4*) (Coffman et al., 1995; Stanbrough and Magasanik, 1996), but it also activates *GAP1* and *GLN1* in the absence of Gln3p with urea as the nitrogen source (Stanbrough and Magasanik, 1995; Stanbrough et al., 1995). Nil1p/Gat1p likely plays a supplementary role to Gln3p in activating the NCR-sensitive genes needed to use non-preferred nitrogen sources, but functions as a weak transcription factor (Coffman et al., 1996; Rowen et al., 1997). It is inactivated by elevated intracellular glutamate (Stanbrough and Magasanik, 1995). This inactivation is caused by Deh1p/Nil2p/Gzf3p which acts as an antagonist (Rowen et al., 1997).

Dal80p/Uga43p and Deh1p/Nil2p/Gzf3p also contain zinc fingers with high homology to those of Gln3p and Nil1p, but they lack the acidic amino-terminal portion (Cunningham and Cooper, 1991; Rasmussen, 1995; Soussi-Boudekou et al., 1997). Dal80p acts as an antagonist of the Gln3p transcriptional activator on a subset of Gln3p-activated genes (Andre et al., 1993; Cunningham and Cooper, 1993; Rowen et al., 1997;

Soussi-Boudekou et al., 1997). It can form a homodimer or heterodimer with Deh1p and requires two GATA sequences oriented tail-to-tail or head-to-tail 15 to 35 bp apart from each other in order to exert an effect (Cunningham and Cooper, 1993; Svetlov and Cooper, 1998). It is believed that the leucine zipper motif at the C-terminus of Dal80p is responsible for homomeric and heteromeric interaction (Svetlov and Cooper, 1998).

In the presence of repressive nitrogen sources (glutamine, glutamate, ammonia), Deh1p negatively regulates the expression of some genes involved in utilization of nitrogen, such as *GAP1* (Coffman et al., 1997) and possibly functions as an antagonist that prevents transcription activation by Nil1p (Rowen et al., 1997). It has a predicted leucine zipper motif at its C-terminus, and has been shown to interact with Dal80p in a two-hybrid assay (Svetlov and Cooper, 1998). Unlike Dal80p, however, it requires a single GATA site for its function (Rowen et al., 1997).

One of the most intriguing characteristics of the family of GATA binding transcription regulators (except Gln3p) is their regulation by other GATA family members. The *NIL1*, *DEH1* and *DAL80* genes contain GATA elements in their promoters (Coffman et al., 1996, 1997; Cunningham and Cooper, 1991). The expression of *DAL80* exhibits the most complex pattern of regulation. Furthermore, its regulation is the most similar to that observed for genes encoding nitrogen catabolic pathway enzymes and permeases. It is Gln3p and Nil1p dependent, and Dal80p and Deh1p regulated (Coffman et al., 1997; Soussi-Boudekou et al., 1997). Transcriptional regulation of the remaining GATA family genes is more stringent. *NIL1* expression is regulated by Gln3p and Dal80p, but not by Nil1p or Deh1p (Coffman et al., 1996). *DEH1* expression is largely Gln3p independent, modestly Nil1p dependent, and highly regulated by Dal80p

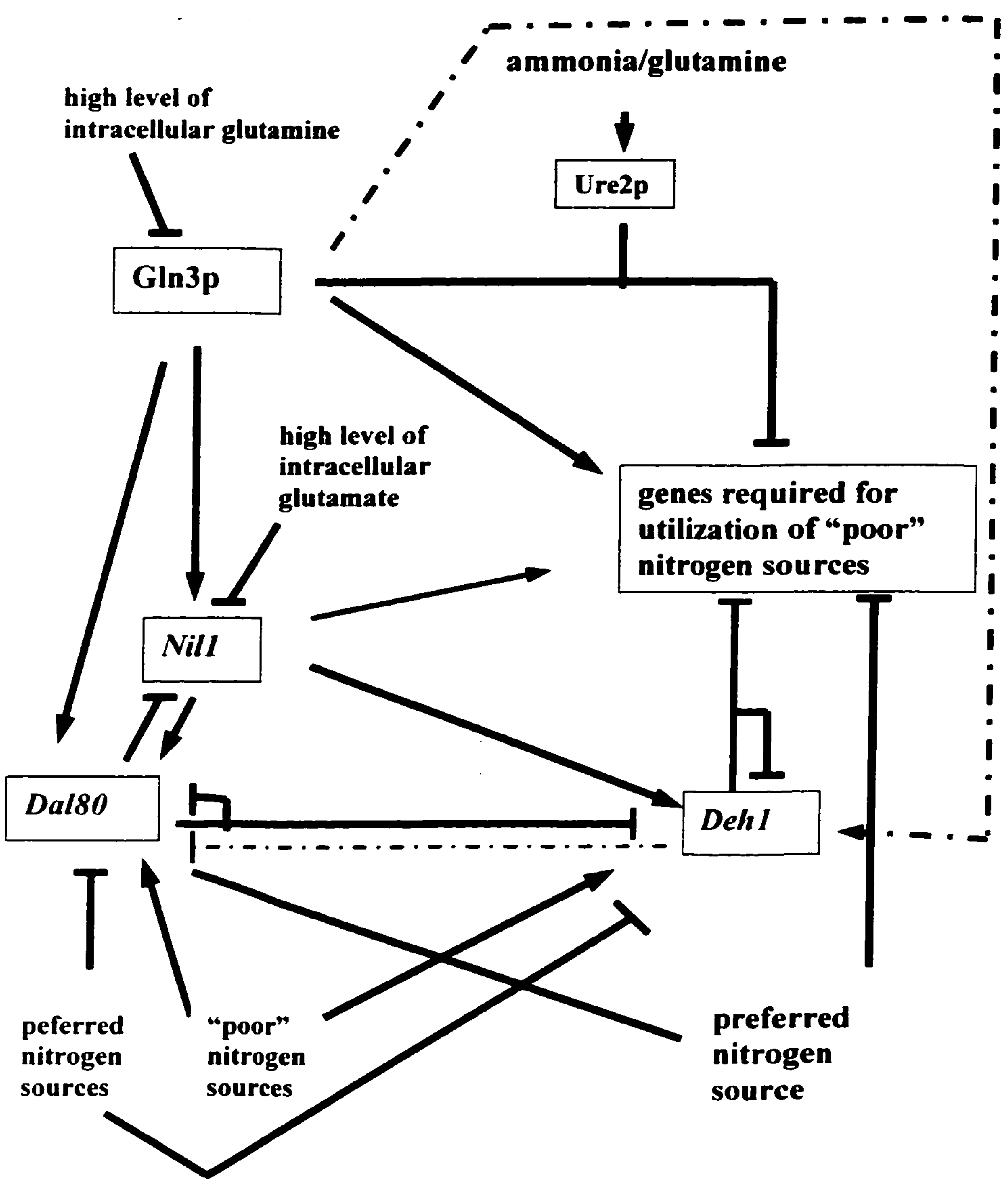
(Coffman et al., 1997). *GLN3* expression is marginally, if at all, regulated in response to changing the nitrogen source (Minehart and Magasanik, 1991, 1992). A working model of the nitrogen regulation system is illustrated in Figure 4 (Coffman et al., 1997; Soussi-Boudekouet et al., 1997; Rowen et al., 1997).

1.2.4. Carbon source regulation system.

Yeast utilizes various carbon sources. The central route of carbon catabolism is the glycolytic pathway. In this pathway, glucose is converted via glycolysis to pyruvate, which is then converted either to ethanol and CO_2 (fermentation) or to acetyl-CoA and thence to CO_2 and H_2O (respiration, through the TCA cycle and oxidative phosphorylation). Carbon sources, such as glucose and fructose, which enter carbon catabolism via the glycolytic pathway, are called fermentable carbon sources. Ethanol, acetate and lactate are called nonfermentable carbon sources because they are directly converted to acetyl-CoA or pyruvate and therefore bypass glycolysis. To obtain energy from nonfermentable carbon sources, yeast must be able to respire to obtain ATP by oxidative phosphorylation. Glycerol enters glycolysis after its conversion to glyceraldehyde-3-phosphate and also requires respiration in order to serve as an energy source.

Glucose is a preferred carbon source because it can support rapid growth of yeast. However, growth on glucose reduces the activities of a large number of proteins that are dispensable in yeast cells. This can occur by either or both of two mechanisms: i) glucose inactivation, which rapidly inhibits the function of some proteins by their

Figure 4. Model of the regulatory circuit for activation and repression of nitrogen catabolic genes in *Saccharomyces cerevisiae* (adapted from Coffman et al., 1997, Soussi-Boudekou et al., 1997 and Rowen et al., 1997). Arrows indicate positive regulation; closed bars indicate negative regulation. Thick lines (━━━━) indicate strong effects. intermediate thickness lines (────) indicate moderate effects and dashed lines (▬ • ▬ •) indicate weak effects.


ammonia/glutamine
high level of
intracellular glutamine
Ure2p
Gln3p
high level of
intracellular
glutamate
genes required for
utilization of "poor"
nitrogen sources
Nil1
Dal80
Deh1
peferred
nitrogen
sources
"poor"
nitrogen
sources
preferred
nitrogen
source

modification and/or degradation, and ii) glucose repression, which reduces expression of many genes at the transcriptional and post-transcriptional level. The genes repressed in the presence of glucose include i) those required to metabolize carbon sources that are utilized less efficiently than glucose, such as glycerol (*GUT*, Sprague and Cronan, 1977) and ethanol (*ADH2*, Denis et al., 1981), ii) genes encoding proteins required for mitochondrial respiration by oxidative phosphorylation (that is, glucose represses energy biosynthesis in mitochondria) (Polakis and Bartley 1965; Beck and von Meyenberg 1968; Haarasilta and Oura 1975; Wales et al., 1980; McAlister-Henn and Thompson 1987; Roy and Dawes, 1987; Lombardo et al., 1990; Johnston and Carlson, 1992; Scheffler et al, 1998) and iii) genes coding for enzymes responsible for gluconeogenesis (Haarasilta and Oura, 1975; Sedivy and Fraenkel 1985; de la Guerra et al., 1988; Valdés-Hevia et al., 1989). Glucose as carbon source also induces the transcription of a limited set of genes, such as genes for the glycolytic pathway enzymes (McAlister and Holland 1982; Denis et al., 1983; Schmitt et al., 1983; Cohen et al., 1986; Kellermann et al., 1986; Butler and McConnell 1988; Chambers et al., 1989; Nishizawa et al., 1989). In the presence of glucose, the glycolytic pathway is the major source for biosynthesis of serine and glycine (Ulane and Ogur, 1972).

Gcr1p (Glycolysis Regulatory Protein 1) activates the glycolytic genes in the presence of glucose (Santangelo and Tornow, 1990; Baker, 1991; Huie et al., 1992). It has also been found that Gcr1p is required to activate *GCV1*, *GCV2* and *LPD1* expression (Nagarajan and Storms, unpublished data). Gcr1p activates the transcription of genes by binding to the consensus sequence 5'-CTTCC-3' (CT-box) (Baker, 1991; Huie et al., 1992; Huie and Baker, 1996). However, Gcr1p doesn't bind efficiently to a single CT-

box, and efficient Gcr1p binding requires a second nearby protein (Scott and Baker, 1993; Chambers et al., 1995; Turkel et al., 1997). A single CT-box does not show any UAS activity (Buchman et al., 1988; Bitter et al., 1991), but strong UAS activity was associated with a single 5'-CTTCC-3' site when another transcription factor, Rap1p (Repressor Activator Protein 1), binds to an adjacent RPG-box (5'-A/G A/C ACCCANNCAC/T C/T-3', where N is any nucleotide) and increases the binding specificity of Gcr1p to the CT-box (Uemura et al., 1997). Gcr2p is a third protein required for Gcr1p/Rap1p function. It interacts with Gcr1p and enhances Gcr1p binding to the CT-box (Zeng et al., 1997). Some argue that there may not be an absolute requirement for Rap1p for the activation of Gcr1p-dependent genes. The major contribution of Rap1p is to facilitate the binding of Gcr1p. Uemura and colleages found that five CT-boxes could exert UAS activity and overcome the requirement for Rap1p (Uemura et al., 1997). Apparently, Gcr1p itself binds cooperatively to CT-boxes. Although Gcr1p acts as the primary activator, proteins other than Rap1p may serve as Gcr1p binding partners to activate Gcr1p-dependent gene expression. Since there are two potential CT-boxes located at −191 bp and −123 bp from the start codon in the *GCV3* promoter region, Gcr1p may regulate *GCV3* expression.

1.3. Experimental approach.

The objectives of this investigation were to delineate the general features of *GCV3* regulation, and to understand the physiological importance of the glycine cleavage system. I have determined the *GCV3* transcription start sites by primer extension, and found that the *GCV3* coding region is 513 base pairs and encodes a 170 amino acid

residue protein. The regulation of *GCV3* in the different growth conditions was analyzed by placing its promoter upstream of a *lacZ* reporter gene. To localize the cis-acting elements that regulated transcription, various *GCV3* promoter deletion mutants and point mutants were constructed. Expression studies with these various promoter mutants demonstrated that *GCV3* expression is regulated by its substrate and the products of the glycine cleavage system. The 132 bp region immediately upstream of the start codon is very important for *GCV3* expression.

Materials and methods

2.1. *S. cerevisiae* strains.

The strains used in this study are described in Table 2.

2.2. Media and culture conditions:

Saccharomyces cerevisiae rich medium (YEPD) consisted of 2% Bacto-peptone (Difco), 1% yeast extract (Difco) and 2% dextrose (BDH). Three types of minimal media were routinely used. i) Standard SD (synthetic dextrose), consisting of 0.175% yeast nitrogen base without amino acids and ammonium sulphate (Difco), 2% dextrose and 0.5% ammonium sulphate as the nitrogen source (Sigma). ii) SD supplemented with 10 mM glycine (SD + 10 mM glycine). iii) SD with ammonium sulphate replaced by various separate amino acids as the sole nitrogen source. The most commonly used alternative nitrogen source was 250 mM glycine (as described in Nagarajan and Storms, 1997). In addition to glycine, 0.1% w/v proline (as described in Courchesne and Magasanik, 1988) was also used in place of ammonium sulphate.

The effect of supplementing media with the C1-end products [adenine (40 μg/ml), histidine (20 μg/ml), methionine (20 μg/ml), serine (5 mM), and formate (10 mM)] on *GCV3-lacZ* expression were tested using SD, SD + 10 mM glycine or the medium using 250 mM glycine as the nitrogen source. The effects of carbon sources on *GCV3-lacZ* expression were tested with 2% glycerol/2% ethanol replacing 2% glucose. Supplements were added as needed except those required for plasmid selection (Sherman, 1991a;

Table 2. Yeast strains used in this study.

Strain	Genotype	Reference
3634	*MATa ura3 leu2::URA3*	Nagarajan and Storms, 1997
CY544	*MATα gcn4-2 bas1-2 bas2-2 ura3-52 leu2-3,112*	Devlin et al., 1991
CY614	Same as CY544 but transformed with YCp50	Devlin et al., 1991
CY615	Same as CY544 but transformed with YCp50 which has wild type *GCN4*	Devlin et al., 1991
CY945	Same as CY544 but transformed with *BAS2* cloned into YCp50	Devlin et al., 1991
5270	Same as CY544 but transformed with *BAS1* cloned into YCp50	This study
CY946	Same as CY544 but transformed with *BAS1* cloned into YCp50 and *BAS2* integrated into the chromosome	Devlin et al., 1991
PM38	*MATα ura3-52 leu2-3,112*	Minehart and Magasanik, 1991
PM81	*MATα ura3-52 leu2-3,112 gln3Δ6::URA3*	Minehart and Magasanik, 1991
S150-2B	*MATa leu2-3,112 his3 trp1-289 ura3-52*	Scott and Baker, 1993
HBY4	*MATa leu2-3,112 his3 trp1-289 ura3-52 gcr1 :: HIS3*	Scott and Baker, 1993
S288C	*MATα suc2 mal mel gal12 cup1*	Mortimer and Johnston, 1986

Ausubel et al., 1989). Solid media were prepared with 2% agar. All *S. cerevisiae* cultures were grown at 30°C.

The *E. coli* strains DH5α, MC1066 and XL-1 blue were used for plasmid manipulation, propagation and selection. The *E. coli* strains were grown in rich 2x YT medium [1% yeast extract (Difco), 1.6% tryptone (Difco), 0.5% NaCl]. Solid media were prepared with 2% agar. *E. coli* cells were grown at 37°C . Ampicillin was included at 100μg/ml for selecting plasmid transformants.

S. cerevisiae strain S150-2B was transformed with plasmid pRS313 (Sikorski and Hieter, 1989) to complement *his3* so that histidine supplementation of the medium was not required. Strains lacking general amino acid control and basal transcriptional control need histidine for growth, and therefore, media for studying the expression of *GCV3-lacZ* with strains CY614, CY615, CY945, CY946, 5270 were supplemented with histidine.

2.3. Radioactive tracer compounds, restriction enzymes and oligonucleotides:

[γ–^{32}P]ATP (Adenosine 5'-[γ–^{32}P]triphosphate)and [α–^{35}S]dATP (Deoxyadenosine 5'-[α–^{35}S]thiotriphosphate) were purchased from Amersham. Restriction endonucleases, DNA modifying enzymes and ribonucleases were purchased from MBI Fermentas, Promega, or Stratagene. Oligonucleotides used in this study were purchased from BioCorp Montreal (Table 3).

Table 3. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5' to 3')
p-GCV3	TTGTTTAGGGCATTGCCGGAGC
GH33	ATGGATCCCGTAACATTGTCGATGTGG
GCV232	CATCAAGCTTCACAACCATTACAT
GCV207	GATCAAGCTTACGCATGTGTAGGG
GCV186	GATCAAGCTTCAACTTGCAAATC
GCV176	GATCAAGCTTAAATCAATGTTTTATC
GCV161	GATCAAGCTTCTATCATTCTGTGCTA
GCV149	GATCAAGCTTGCTATAGGGACCTCG
GCV132	GATCAAGCTTATTTTGACACTTCCG
GCV117	GATCAAGCTTAAGGAGTCATTCAG
GCV107	GACTAAGCTTCAGCGGCGGAGTC
GCV83	GACTAAGCTTCTTTCTTCTATATATAC
5'-GCRE0	TT*T*A*G*ACTTCCGTAAGG*TC*T*A*ATTCAGCGGCGG*TC*T*A*ACTTTC
3'-GCRE0	AGT*T*A*GA*CCGCCGCTGAAT*T*A*GA*CCTTACGGAAGT*C*T*A*AAAATAG
5'-GCRE1	CGCAATTTT*T*A*G*ACTTCCGTAAGG
3'-GCRE1	CCTTACGGAAGT*C*T*A*AAAATTGCG
5'-GCRE2	TCCGTAAGG*TC*T*A*ATTCAGCGG
3'-GCRE2	CCGCTGAAT*T*A*GA*CCTTACGGA
5'-GCRE3	CAGCGGCGG*TC*T*A*ACTTTC
3'-GCRE3	GAAAGT*T*A*GA*CCGCCGCTG
5'GATA1	CAATGTTT*G*A*G*CTATCATTCTGTGC
3'GATA1	GCACAGAATGATAG*C*T*C*AAACATTG
5'CT-BOX	GTC*AA*CCTTCAACTTGCAAATCAATG
3'CT-BOX	CATTGATTTGCAAGTTG*AA*GGTTGAC
p-lacZ	AGCTGGCGAAAGGGGGATG

2.4. Transformation of *S. cerevisiae* and *E. coli*:

Yeast transformation was performed by the lithium acetate procedure with carrier DNA as previously described (Gietz and Woods. 1998). *E. coli* transformation was routinely carried out by heat-shock of $CaCl_2$- treated competent cells (Hanahan, 1983).

2.5. DNA isolation and manipulation:

S. cerevisiae genomic DNA was isolated essentially as described in Ausubel et al. (1989). The cells were lysed by treatment with Zymolyase 60,000 and SDS. Methods for the manipulation of DNA, including restriction endonuclease digestions and ligations, were performed according to Sambrook et al. (1989) unless otherwise indicated. DNA sequencing was performed using the dideoxynucleotide termination method and the USB "Sequenase Kit" or the Beckman CEQ 2000 Dye Terminator Cycle Sequencing Kit.

2.6. Primer extension:

i). RNA isolation:

Several precautions were followed to minimize RNA degradation by ribonucleases. Disposable gloves were worn when handling all materials. All solutions were prepared using DEPC (Diethyl Pyrocarbonate) treated H_2O except Tris buffer. All glassware was treated at 200°C for 4 hours. Unopened packages of microcentrifuge tubes and micropipet tips were ribonuclease-free and thus did not require prior treatment.

Plasmid pGCV3, which carries the complete *GCV3* gene, was introduced into the wild type yeast strain PM38. The transformant was grown overnight in 5 ml of SD. The overnight culture was used to inoculate 500 ml of the same medium and grown to an

OD_{600} of about 1. The cells were pelleted, washed with ice cold water and transferred to 2 litres of medium with 250 mM glycine as the sole nitrogen source. After growth to an OD_{600} of 0.6, the cells were harvested, pelleted and quickly placed into a mortar. Liquid nitrogen was poured over the cells and the cells ground with a pestle until the consistency was of a fine powder. Portions (100mg) of the powder were transferred to microcentrifuge tubes and frozen in liquid nitrogen. After the tubes were removed from liquid nitrogen, 1 ml of TriPure™ isolation reagent (Boehringer Mannheim), containing phenol and guanidine thiocyanate, and 400 µl of acid-washed 0.45-0.55mm glass beads (Sargent Welch) were added per tube. The mixture was vigorously vortexed for 1 min at room temperature. Cell debris was removed by centrifugation at 10,000 x g for 10 min and the aqueous phase was transferred to a new microcentrifuge tube. Contaminating proteins were removed as follows. An equal volume of phenol-chloroform (1:1) and 1/10 volume of 10% SDS were added, followed by vortexing for 1 mintute and centrifugation at 10,000 x g for 1 minute. The aqueous phase was then transferred to a new microcentrifuge tube and the phenol-chloroform with SDS treatment repeated until the interface became clear. An equal volume of chloroform was then added to the aqueous phase and the suspension was vortexed for 1 minute, centrifuged for 1 minute, and the aqueous phase was transferred to another microcentrifuge tube and treated once more with chloroform in the same fashion. The RNA was precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of anhydrous ethanol for 2 hours at –20°C. The precipitated RNA was pelleted by centrifugation for 15 minutes at 4°C in a microfuge. After the supernatant was discarded, the pellet was washed with 75% ethanol by gentle vortexing and centrifugation for 15 minutes at 4°C in a microfuge. The wash

was discarded and the pellet was dried in a centrifugal vacuum desiccator (SpeedVac, SVC100). Following purification, the RNA was dissolved in RNase-free water, and the quality and quantity of RNA was examined by formaldehyde denaturing agarose gel electrophoresis (Sambrook et al., 1989) and spectrophotometrically. RNA samples were stored at $-80^{o}C$ in an ultra cold freezer.

ii). Oligonucleotides purification and end-labeling:

Fifteen µl of oligo p-GCV3 (2 OD_{260} units) was mixed with an equal volume of formamide, heated at $55^{o}C$ for 5 minutes and loaded onto a 16 cm-long 19% polyacrylamide sequencing gel containing 7 M urea. Electrophoresis was carried out at 500 V and 7 mA until the oligonucleotide had migrated approximately two thirds of the length of the gel. The oligonucleotide band was excised from the gel and distributed to 3-4 microfuge tubes. One ml of elution buffer (0.1% SDS, 0.5 M ammonium acetate, 10 mM magnesium acetate) was added to each tube and the mixture was incubated at $37^{o}C$ overnight with shaking. After removal of acrylamide gel pieces by centrifuging at 12,000 x g for 5 min, the supernatants were pooled in a 5 ml syringe and passed through a Millex HV filter (Millipore, 0.45-micron pore size). The effluent was concentrated in a centrifugal vacuum desiccator (SpeedVac, SVC100) then treated with phenol-chloroform and ethanol preciptated. From this step onward care was taken to avoid RNase contamination. The pellet was dried and suspended in RNase-free water, quantified spectrophotometrically, adjusted to a concentration of 1 picomole/µl and stored at $-20^{o}C$.

End labeling was performed by incubating one picomole of purified primer p-GCV3 with 1µl of $[\gamma-^{32}P]$ATP (5000 Ci/mmole; 10 mCi/ml in aqueous solution) and T_4

polynucleotide kinase (10 units) in a volume of 20 µl kinase buffer [50 mM Tris-HCl (pH 7.6 at 25°C), 10 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 1 mM EDTA] at 37°C for 1 hour. The reaction was stopped by addition of 2 µl of 0.5 M EDTA (pH 8.0) followed by incubation at 65°C for 10 minutes. The efficiency of transfer of ^{32}P to the oligonucleotide and the specific activity of the labeled oligo were determined by comparing the quantity of labeled oligonucleotide and the free [γ–^{32}P]ATP after fractionation on a 19% polyacrylamide gel.

iii). Primer extension:

One µl of labeled primer p-GCV3 (containing 0.05 picomole primer) was mixed with about 10 µg of RNA, and RNase-free water was added to a volume of 11 µl. This mixture was heated at 75°C for 5 minutes, then chilled on ice for 10 minutes. Four µl of 5x reverse transcriptase buffer [250 mM Tris-HCl (pH 8.3), 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, 2.5 mM spermidine] and 2 µl of a 10 mM dNTP mixture were added to the above mixture, and incubated at 45.8°C for 2 minutes. 1 U of avian myeoblastosis virus reverse transcriptase (Promega) was added to the mixture and incubated at 45.8°C for 1 hour. The reaction was terminated by adding 20 µl of primer extension stop buffer (0.05% xylene cyanol FF, 0.05% bromophenol blue, 99% formamide, 10 mM EDTA, 10 mM NaOH). The RNA-DNA hybrids were denatured at 100°C for 2 min, chilled on ice for 10 min and electrophoresed on an 8% polyacrylamide-7 M urea sequencing gel at 1,700 V for 2 hours. The gel was exposed to X-ray film at –80°C.

2.7. Promoter analysis:

i) PCR amplifications:

PCR (Saiki et al., 1988) reactions were performed with a Hybaid Thermal Reactor. Each PCR reaction contained 5 μl of 10x PCR buffer [100 mM Tris-HCl (pH 8.8), 500 mM KCl, 0.8% Nonidet P40], 2 μl of a 10 mM dNTP mix, 3 μl of 25 mM $MgCl_2$, 1 μl of 25 μM primer, 10 ng of DNA template and 1 U of Taq DNA polymerase. The final volume was 50 μl. The cycle parameters for amplifying the *GCV3* promoter with yeast genomic DNA (isolated from the wild-type strain S288C) as the template was as follows: 1 cycle of 95°C for 3 min; 35 cycles of 95°C for 30 seconds, 40°C for 30 seconds, and 72°C for 1 min; 1 cycle of 72°C for 1 min, and finally chilled at 4°C. *GCV3* sequence generated by using plasmid DNA as the template is the same as above except 25 amplification cycles were used.

ii). Plasmid construction:

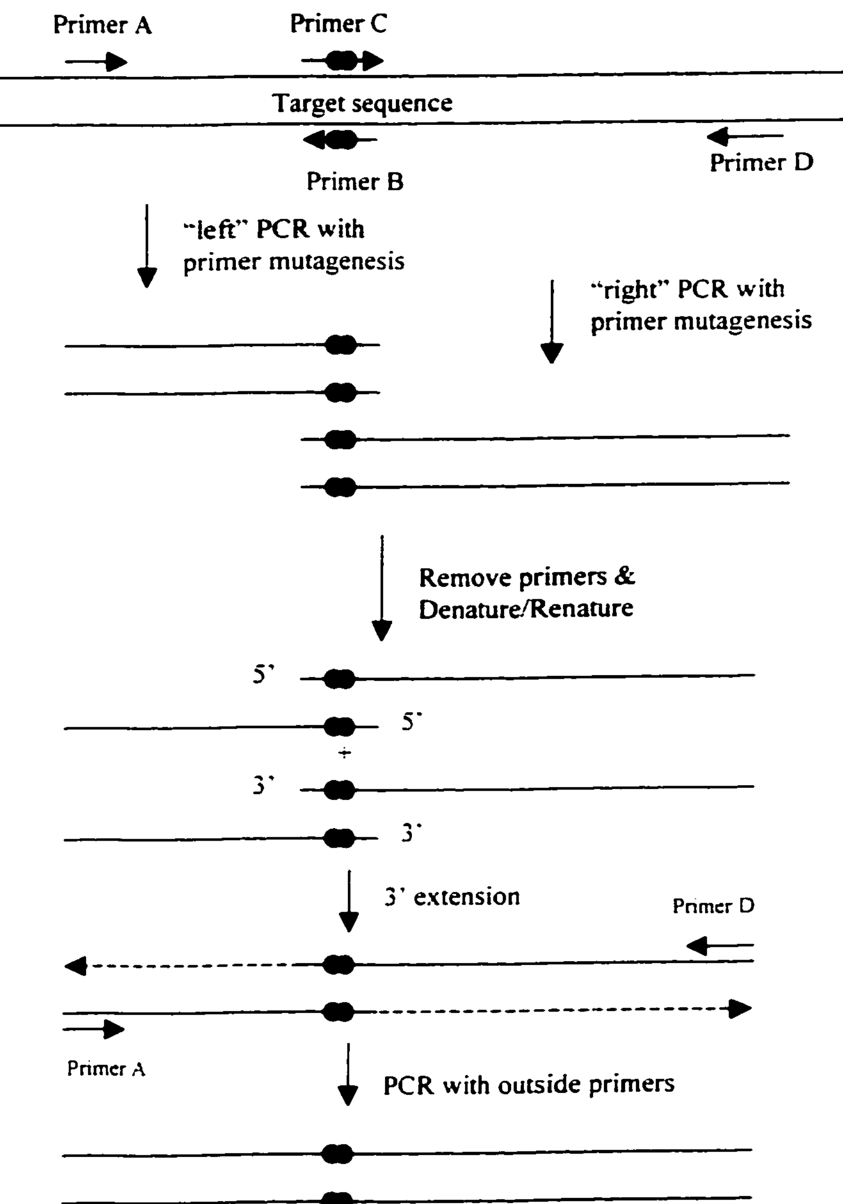
A set of *GCV3-lacZ* fusion plasmids were constructed. The backbone of a multicopy shuttle vector pRS3174 containing the *lacZ* reporter gene (Nagarajan and Storms, 1997) was prepared by digestion with *Hin*dIII and *Bam*HI.

Construct pGCV3-232 and all the deletion constructs depicted in Figure 7 (see results) were made as following. The common primer used to generate all these constructs was GH33, which contains two filler nucleotides, at the 5'end a *Bam*HI site from nucleotides 3-8 followed by a 19 base stretch that is identical to the sequence of the

antisense strand from nucleotides +8 through –11 (numbering throughout is relative to the first nucleotide of the start codon of *GCV3*). Each upstream primer consisted of two filler nucleotides at the 5' end, a *Hin*dIII site from nucleotides 5 to 10 followed by a 15 base stretch identical to various upstream regions of the coding strand beginning from –232, –207, –186, –176, –161, –149, –132, –117, –107, or –83 (oligos GCV232, GCV207, GCV186, GCV176, GCV161, GCV149, GCV132, GCV117, GCV107 and GCV83, respectively; Table 3). The template was genomic DNA from wild-type strain S288C for pGCV3-232 and pGCV3 (Nagarajan and Storms, 1997) for the deletion constructs. The ten PCR products, were digested with *Hin*dIII and *Bam*HI, and individually ligated with the *Hin*dIII and *Bam*HI digested pRS3174 backbone. The 5'end of constructs pGCV3-232 and pGCV3-Δ232 begins just after the stop codon of *PTA1* (O'Connor and Peebles, 1992).

GCV3 promoter derivatives harbouring point mutations were generated using the method of Higuchi (Higuchi, 1990; Figure 5). First, two primary PCR products that overlap in sequence are generated. Both primary PCR products contain the same mutations introduced as part of the PCR primers. These overlapping primary PCR products can be denatured and allowed to reanneal together, producing two possible heteroduplex products. Heteroduplexes that have recessed 3'ends can be extended by *Taq* DNA polymerase to produce a fragment that is the sum of the two overlapping products. A subsequent reamplification of this fragment with only the right- and left-most primers ("out-side" primers) results in the enrichment of the full-length, secondary product which contains the mutations away from the fragment ends.

Figure 5. The strategy for creating mutations in the full length promoter. This method introduces mutations into any part of a PCR product by combining two separate PCR products with overlapping sequences. Both primary PCR products contain the same mutation introduced as part of the PCR primers (B and C). Then, the two primary PCR products serve as overlapping templates for a second round of PCR amplification with primers A and D. ● represents the introduced mutations.


Primer A
Primer C
Target sequence
Primer B
Primer D
"left" PCR with
primer mutagenesis
"right" PCR with
primer mutagenesis
Remove primers &
Denature/Renature
5'
5'
+
3'
3'
3' extension
Primer D
Primer A
PCR with outside primers

pGCV3-232$_{GATA1M}$ was constructed as following. First, the *GCV3* promoter regions between −172 and +8 and the region between −232 and −147 were amplified by PCR using oligo pairs GH33 and 5'GATA1 and GCV232 and 3'GATA1, respectively, with pGCV3-232 as template. Next, the two primary PCR products were subjected to another round of PCR with outside primer GCV232 and GH33. The resulting fragment is identical to that of pGCV3-232 except for the mutations in the 5'-GATAA-3' sequence, indicated by the underlined and italicized letters in the sequences of oligos 3'GATA1 and 5'GATA1 in Table 3. After digesting with *Hin*dIII and *Bam*HI, the mutagenized PCR product was cloned into the backbone of vector pRS3174 prepared in the same way as described above.

Constructs pGCV3-232$_{GCRE1}$, pGCV3-232$_{GCRE2}$, pGCV3-232$_{GCRE3}$ and pGCV3-232$_{GCRE0}$ harbouring mutated versions of the GCRE were also constructed using the strategy outlined in Figure 5. Construct pGCV3-232$_{GCRE1}$ is identical to pGCV3-232 except for mutations in the upstream GCRE as indicated by the underlined and italicized letters in the sequence of oligos 3'GCRE1 and 5'GCRE1 (Table 3); pGCV3-232$_{GCRE2}$ is identical to pGCV3-232 except for mutations in the middle GCRE as indicated by the underlined and italicized letters in the sequence of oligos 3'GCRE2 and 5'GCRE2 (Table 3); pGCV3-232$_{GCRE3}$ is identical to pGCV3-232 except for mutations in the downstream GCRE as indicated by the underlined and italicized letters in the sequence of oligos 3'GCRE3 and 5'GCRE3 (Table 3) and pGCV3-232$_{GCRE0}$ is identical to pGCV3-232 except for mutation in all three GCREs as indicated by underlined and italicized letters in the sequence of oligos 3'GCRE0 and 5'GCRE0 (Table 3).

Plasmid pGCV3-232$_{CTBOXM}$ harbouring a mutated version of CT-box at –192 bp was generated by the two inside primers 5'CT-BOX and 3'CT-BOX and the two outside primers GCV232 and GH33. The PCR fragment was digested with *Hin*dIII and *Bam*HI, and immediately ligated with the *Hin*dIII and *Bam*HI digested pRS3174 backbone. The resulting construct is identical to pGCV3-232 except for the presence of a mutant CT-box sequence (indicated by the underlined and italicized letters in the sequence of oligos 3'CT-BOX and 5'CT-BOX in Table 3).

iii). Selection and identification of plasmid recombinants:

All the constructs described in the previous section were transferred into *E. coli* XL-1 blue competent cells. The cells were then plated on 2x YT plates with 100 μg/ml ampicillin and 200 μg/ml X-gal for selection and screening. After incubation at 37°C overnight, the blue colonies were selected for further identification.

PCR was used for further screening and identification of potential recombinant plasmids with primers which could amplify the entire inserts. Subsequently, the entire *GCV3* promoter region from two independent constructs for each plasmid was sequenced using primer p-lacZ.

vi). β-galactosidase assays:

β-galactosidase assays were performed essentially as described previously (Nagarajan and Storms, 1997). Four ml of mid log phase (OD_{600} at 0.2-0.25) cultures were harvested by centrifugation and immediately frozen in liquid nitrogen. The frozen cell pellets were either stored at –80°C or used immediately for β-galactosidase assays.

Cells were permeabilized by thawing, freezing in liquid nitrogen and resuspension in 1ml Z-buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol, (Miller, 1972)] containing 3% Brij35 (BDH diagnostics). The cell suspensions were allowed to equilibrate in this buffer for 5 minutes at 28°C. Then 0.2 ml of 4 mg/ml O-nitro-phenyl-β-D-galactopyranoside (ONPG) (Miller, 1972) was added. The reaction mixture was vortexed gently, and incubated at 28°C until a faint yellow color was detected. Assays were terminated by the addition of 0.5 ml of 1 M sodium bicarbonate. The samples were centrifuged to remove the cells and levels of O-nitro-phenyl production were measured spectrophotometrically at 420 nm. β-galactosidase activities were calculated for at least three independent transformants of each construct, performed in triplicate, and are presented as averages in Miller units (Miller, 1972).

Results

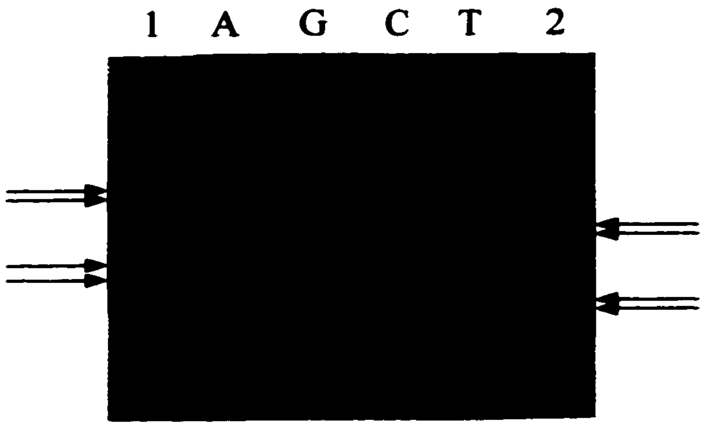
3.1. Determination of the transcription and translation initiation sites of *GCV3*.

To initiate the molecular characterization of *GCV3* expression, its transcription initiation sites were mapped by primer extension (Figure 6). The primer extension analysis identified multiple transcription initiation sites. Heterogeneity in transcription initiation position is common for yeast genes (McIntosh and Haynes, 1986; McIntosh et al., 1988; McNeil and Smith, 1986) and so our findings for *GCV3* are not unusual. There are three potential ATG codons that could be used to initiate translation of the *GCV3* ORF (Figure 6). Notably, the major transcription initiation sites are all located between the most upstream and the middle ATG.

S. cerevisiae mRNA leader regions show a strong bias for A nucleotides (~45%) and against G nucleotides (~12%). The preference is particularly striking at position –3 relative to the start codon where an A residue is present 75% of the time. In highly expressed genes this bias is even more striking and an A nucleotide occurs at position –3 100% of the time (Cigan and Donahue, 1987; Hamilton et al., 1987). In well over 50% of the genes examined A residues also occur at positions –1, –2, –5, –6, and –7. The consensus sequence before *S. cerevisiae* start codons was found to be 5'-(A/Y)(A/Y) A (A/Y) A (A/Y) **AAUG**-3' (Y: any pyrimidine; Figure 6; for a review, see Hinnebusch and Liebman, 1991). Also, the distribution of nucleotides downstream from the AUG codon is non-random: 28% of all genes contain a serine codon immediately following the initiation codon. Thr (10%), Leu (9.5%), Ala and Val (7.8% each), and Asn, Gly, and Pro

Figure 6. Mapping the 5' ends of *GCV3* mRNA by primer extension. A) Oligonucleotide p-GCV3, end labeled with ^{32}P, was hybridized to total cellular mRNA and extended with reverse transcriptase. The extension products were then resolved by polyacrylamide gel electrophoresis and detected by autoradiography. Lane 1, 1 μl of primer extension product. Lane 2, 2 μl of primer extension product. Lanes AGCT are the A, G, C and T sequencing reactions of the *GCV3* sense strand primed with primer p-GCV3. B) Nucleotide sequence of the same strand in the region surrounding the *GCV3* start codon. The arrows indicate the different transcription initiation sites. The region that p-GCV3 hybridizes to is underlined. The three potential start codons are also underlined. C) The alignment of the sequences upstream of the three potential translation start codons and the consensus sequence for *S. cerevisiae* mRNA sequences immediately upstream of start codon.

A).



B).

5'-

-37 -16-15 -11-10 -1 +1

GAAAGGGAATCTCCAC*ATG*AAGCATACCACATCGACAATGTTACGC

ACTACTAGACT*ATG*GACCACCCGC*ATG*CCCGCTGTGAGCAAATTGTT

TTTGAGAAACAGCTCCGGCAATGCCCTAAACAA-3'

C).

The consensus sequence of *S. cerevisiae*:	5'-(A/Y)(A/Y)A(A/Y)A(A/Y)AAUG
First AUG of *GCV3*:	5'- T C T C C A CAUG
Second AUG of *GCV3*:	5'- A T C G A C AAUG
Third AUG of *GCV3*:	5'- C T A G A C TAUG

(4% each) are the next most abundant penultimate aminoterminal residues (Cigan and Donahue, 1987).

Of the regions immediately upstream of the three potential *GCV3* start codons the sequence adjacent to the middle ATG, with A nucleotides present at positions –1, –3 and –7, and pyrimidines at –2, –5 and –6, most closely matches the consensus sequence (Figure 6). Further, the penultimate codon codes for leucine, the third most abundant amino acid at this position. Assuming that the first AUG encountered within the mRNA is used to initiate translation, the major transcription initiation sites are located at bp –16, –15, –11 and –10 relative to the A nucleotide of the first start codon and *GCV3* would encode a 170 amino acid residue protein with a predicted mass of 18,795 Da.

3.2. Glycine induces *GCV3* expression.

For our studies of *GCV3* regulation, we began by constructing plasmid pGCV3-232, which directs *lacZ* expression using the wild type *GCV3* promoter extending from nucleotide +3 to –232. To determine whether expression was induced by glycine, pGCV3-232 was introduced into three wild type yeast strains and the ability of these transformants to express β-galactosidase during growth in SD and SD + 10 mM glycine was determined. In these three wild type strains, *GCV3-lacZ* expression was induced three to five times by 10 mM glycine (Table 4), with expression with 10 mM glycine ranging from a high of 154 units in S150-2B to a low of 83 units in PM38.

Table 4. *GCV3* is induced by glycine.

Strain[a]	β-gal activity (Miller units)[b]	
	SD	**SD + 10 mM glycine**
3634 / pGCV3-232	30	102
PM38 / pGCV3-232	19	83
S150-2B / pGCV3-232	34	154

[a]pGCV3-232 expressed in strains 3634, PM38 and S150-2B (for genotypes, see Table 2) in SD and SD + 10 mM glycine media. pGCV3-232 harbours the full length wild type *GCV3* promoter. [b]β-galactosidase activity levels, presented in Miller units (Miller, 1972), were determined as described in the Materials and methods.

3.3. Localization of upstream information required for induction by glycine.

The upstream region important for *GCV3* expression in SD medium and induction by 10 mM glycine was localized using the promoter deletion plasmids depicted in Figure 7. Plasmids pGCV3-232, pGCV3-Δ232 and the nine promoter deletion constructs were introduced into yeast strain 3634 and their ability to express β-galactosidase was examined (Table 5). Transformants harboring plasmids pGCV3–Δ132 and all the longer fusions expressed significantly more β-galactosidase when grown in SD medium supplemented with 10 mM glycine than when growth was in SD medium. In contrast, the fusion genes with shorter promoter regions (pGCV3-Δ117, pGCV3-Δ107 and pGCV3-Δ83) directed significantly lower levels of expression in both SD and SD ÷ 10 mM glycine. pGCV3-Δ117 expressed only 1.7 times more activity when grown in SD + 10 mM glycine. Since pGCV3-Δ132 directed normal levels of expression in SD medium and was fully induced 10 mM glycine, information important for both basal expression and for the induction by 10 mM glycine must be located within 132 bp of the translation start codon.

3.4. *GCV3* is regulated by the nitrogen regulation system.

Nitrogen sources can be divided into two groups, the preferred and the non-preferred or poor nitrogen sources. In yeast, the nitrogen regulation system controls the expression of genes required for utilizing poor nitrogen sources. Yeast cells can utilize glycine as the sole nitrogen source. The glycine cleavage reactions, which are catalyzed by the GCS, are the only known way for cells to utilize glycine as a nitrogen source

Figure 7. The deletion promoter constructs. The ten constructs from pGCV3-232 through pGCV3-Δ83 were constructed as described in Section 2, Materials and Methods. The number after the dash in each plasmid name indicates the most upstream nucleotide relative to the first base of the translation start codon. * indicates a single mutation at –92 bp from the start codon (from A to C) that is present in all 10 constructs.

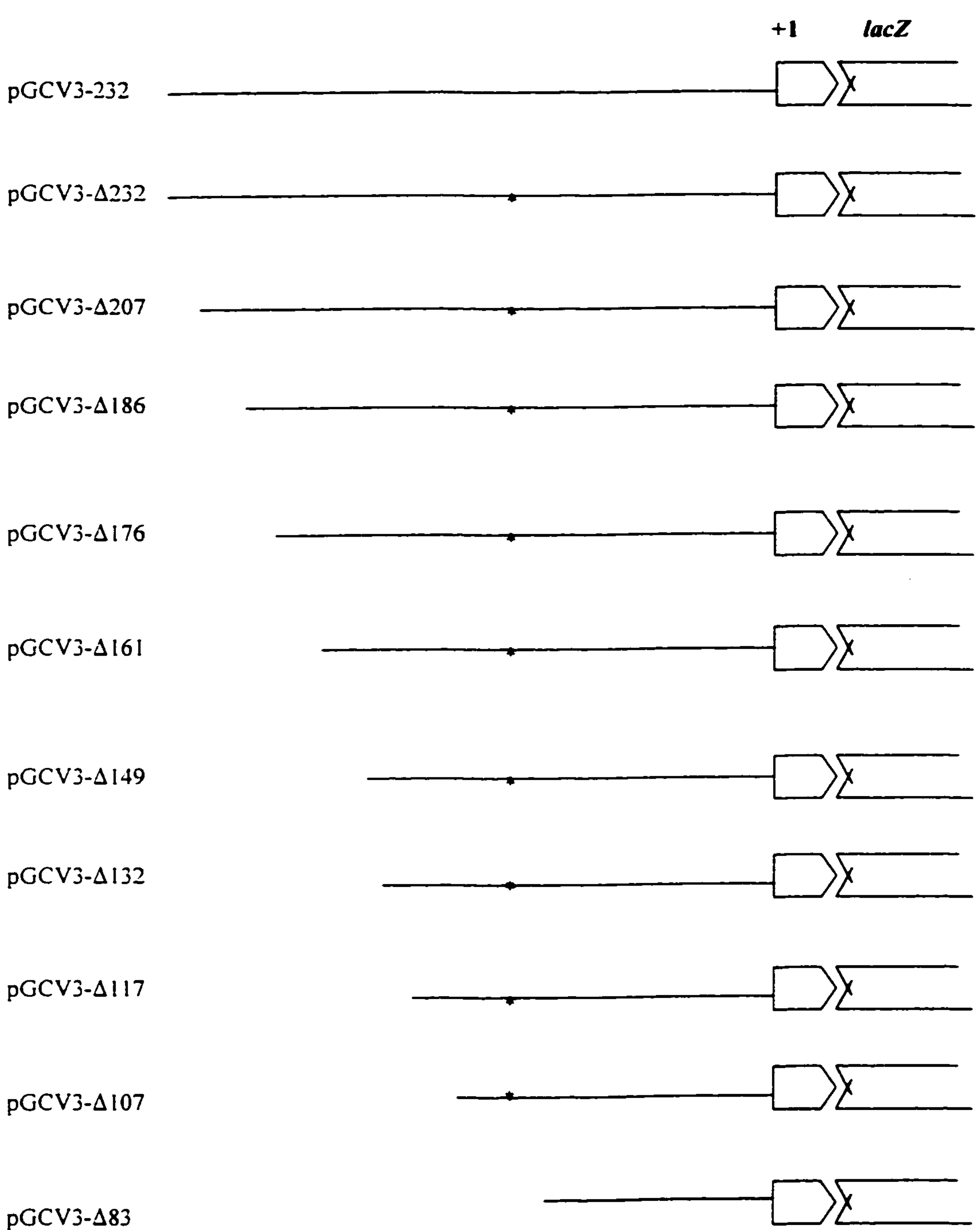

+1
lacZ
pGCV3-232
pGCV3-Δ232
pGCV3-Δ207
pGCV3-Δ186
pGCV3-Δ176
pGCV3-Δ161
pGCV3-Δ149
pGCV3-Δ132
pGCV3-Δ117
pGCV3-Δ107
pGCV3-Δ83

Table 5. Localization of the region required for induction by glycine.

Plasmid[b]	β-gal activity (Miller units)[a]	
	SD	**SD + 10 mM glycine**
pGCV3-232	30	102
pGCV3-Δ232	5.7	37
pGCV3-Δ207	3	46
pGCV3-Δ186	5.4	79
pGCV3-Δ176	5.3	55
pGCV3-Δ161	4.7	74
pGCV3-Δ149	3.7	71
pGCV3-Δ132	7.7	57
pGCV3-Δ117	1.3	2.3
pGCV3-Δ107	0.3	0.1
pGCV3-83	0.2	0.1

[a]β-galactosidase activity levels, presented in Miller units (Miller, 1972), were determined as described in the Materials and methods. [b]Plasmids pGCV3-232 through pGCV3-83 are as in Figure 7.

(Freudenberg and Andreesen, 1989; Sinclair and Dawes, 1995; Walker and Oliver, 1986). Since *GCV3* encodes the GCS H-protein, we wanted to determine whether *GCV3* was regulated by the nitrogen regulation system. To assess this, pGCV3-232 was introduced into the wild-type strain PM38 and strain PM81, an isogenic derivative of PM38 defective in nitrogen regulation (*gln3*). The results in Table 6 demonstrate that *GCV3* expression is apparently regulated by the nitrogen regulation system. β-galactosidase expressed in the wild-type strain PM38 was 3 fold higher with the poor nitrogen source, proline (59 units), than with the "preferred" nitrogen source, ammonia (19 units). Expression with ammonia as the nitrogen source was essentially the same in PM38 (19 units) and PM81 (15 units). These results suggest that *GCV3* expression was activated by the nitrogen regulation system when growth was with the poor nitrogen source, proline.

Since the *gln3* defective strains are glutamine auxotrophs when utilizing poor nitrogen sources, it was necessary to include 15 mg/litre of glutamine when PM81 was grown with the poor nitrogen source, proline (Coffman et al., 1995). For comparison with PM81, *GCV3* expression during growth of PM38 with proline was also measured with glutamine supplementation. Expression in PM38 was repressed 3-fold by adding 15 mg/litre glutamine in the medium when proline was the nitrogen source. Apparently, glutamine, even at low concentration, repressed *GCV3* expression.

Many genes required for the utilization of nitrogen sources are regulated by Gln3p through its interaction with the upstream regulatory element UAS_{GATAA} (Bysani et al., 1991; Coffman et al., 1996; Blinder et al., 1996). The presence of a UAS_{GATAA} consensus sequence (bp −167 to −161) suggested the regulation of *GCV3* by the nitrogen regulation system was direct and involved the utilization of this GATAA site. To test this, the construct $pGCV3\text{-}232_{GATA1M}$ was introduced into strains PM38 and PM81. Construct

Table 6. *GCV3* is regulated by the nitrogen regulation system.

Strain[c]	β-gal activity (Miller units)[a]		
	ammonia	proline	proline + 15 mg/L glutamine
GLN3 / pGCV3-232	19	59	19
GLN3 / pGCV3-232_{GATA1M}	ND[b]	44	34
gln3 / pGCV3-232	15	ND[b]	27
gln3 / pGCV3-232_{GATA1M}	ND[b]	ND[b]	56

[a]Activity levels of β-galactosidase were determined as described in Table 4. [b]Not determined. [c]Wild-type (*GLN3*) and mutant (*gln3*) yeast strains used were PM38 and PM81 respectively. Construct pGCV3-232_{GATA1M} is identical to pGCV3-232 except for two mutations that were introduced into the *GCV3* promoter region at base pair -162 and -164. The two mutations change the potential GATA sequence from GATAA to GcTcA.

pGCV3-232$_{GATA1M}$ is identical to pGCV3-232 except for two transversion mutations that were introduced into the UAS$_{GATAA}$ consensus sequence at positions −162 bp and −164 bp (from GATAA to GcTcA). Expression of pGCV3-232 versus pGCV3-232$_{GATA1M}$ in the presence of different nitrogen sources showed that the UAS$_{GATAA}$ was important for regulating *GCV3* expression in response to changing the nitrogen source (Table 6). For example, in the wild type strain PM38 expression of the GcTcA mutant promoter (44 units) was lower than the wild-type promoter (59 units) in the presence of proline. With 15 mg/litre of glutamine and proline, the mutant promoter expressed 2-fold more activity (34 units) than the wild-type promoter (19 units). A similar effect was observed in PM81, as the mutant promoter was expressed at twice the levels observed with the wild type promoter (56 units versus 27 units). Since this was observed in both *GLN3* and *gln3* strains, there may be another protein which can utilize this same GATAA site to repress *GCV3* when 15 mg/litre glutamine was included when proline was the nitrogen source.

These results demonstrate that the GATAA sequence at −167 bp is used to regulate *GCV3* expression in response to growth with different nitrogen sources and that Gln3p is also involved. Furthermore, they suggest that another GATAA binding protein represses expression when glutamine at 15 mg/litre is included when proline is the nitrogen source.

3.5. Expression of *GCV3* is subject to the general amino acid control system.

Three sites that match the consensus sequence for general control response elements (GCREs, Mösch et al., 1990, 1991) are present in the *GCV3* promoter at bp −128, −113

and –97. Since the transcriptional activator Gcn4p can bind to DNA containing GCRE elements, the presence of these three sites suggested that Gcn4p regulated *GCV3* expression. Genes regulated by Gcn4p are induced by amino acid starvation, because cellular Gcn4p concentration increases in response to amino acid starvation (Struhl and Davis, 1981; Hinnebusch and Fink, 1983; Arndt and Fink, 1986). Imidazoleglycerolphosphate dehydratase, the product of the *HIS3* gene, is competitively inhibited by 3-amino 1,2,4-triazole (3-AT). It is therefore possible to cause histidine starvation by treating cells with 3-AT. Yeast cells starved for any one of several amino acids, including histidine, induce the expression of many genes because Gcn4p binds to GCRE sites present in their promoters (Kanazawa et al., 1988). A previous study showed that *GCV3* was regulated in a fashion similar to that observed with other genes subject to general amino acid control (Nagarajan and Storms, 1997). Data presented here (Table 7) confirmed these earlier results by showing that *GCV3* is induced by 3-AT. In addition, we performed a detailed mutational analysis of the promoter to locate and characterize the cis-acting region responsible for general amino acid control.

Expression from pGCV3-Δ232 in SD + 20 mM 3-AT was almost three times higher than in SD (Table 7). pGCV3-Δ132 and all the longer constructs were also induced by 3-AT. In contrast, pGCV3-Δ117 was not induced by 3-AT. Instead pGCV3-Δ117 expressed 2.7 units of β-galactosidase in SD without 3-AT versus only 0.7 units in SD with 3-AT. The upstream sequences for general amino acid control are, therefore, located within 132 bp of the start codon. Consistent with this, the region downstream of bp 132 contains three GCRE consensus sites present in the *GCV3* promoter region.

Table 7. *GCV3* is regulated by the general amino acid control system.

Plasmid[b,c]	β-gal activity (Miller units)[a]	
	SD	SD + 3-AT
pGCV3-Δ232	5.1	13
pGCV3-Δ207	4.7	16
pGCV3-Δ186	4.8	14
pGCV3-Δ176	5.3	14
pGCV3-Δ161	5	16
pGCV3-Δ149	5.3	15
pGCV3-Δ132	4.8	12
pGCV3-Δ117	2.7	0.7

[a]Activity levels of β-galactosidase were determined as described in Table 4. [b]The plasmids used were described in Figure 7. [c]General control responses were assessed in transformants of strain PM38. Amino acid starvation was induced by growth in the presence of 20 mM 3-amino 1,2,4-triazole (3-AT).

To determine whether the GCRE sites were used for general control and to assess their relative importance, a series of *GCV3-lacZ* fusion genes with mutations in the three potential GCRE sites were constructed (Figure 8). Expression by the wild-type promoter and the various mutant promoters showed that mutating the most upstream GCRE site altered *GCV3* expression in a very different fashion than mutating the other two sites. For example, when the upstream site was mutated, expression in SD increased from 19 to 42 units of β-galactosidase and marginally decreased in SD + 3-AT (from 64 to 54 units). This suggested that a protein that interacts with the upstream GCRE or sequences overlapping it repressed expression during growth on SD. In contrast, a transcriptional activator or activators apparently bind the two downstream sites and activate expression during growth in both SD and SD + 3-AT, since mutating either the middle or downstream site dramatically reduced expression in both of these growth conditions (from 19 to 1.2 units in SD and from 64 to 2.7 and 2.4 units, respectively, in SD + 3-AT). Since individually mutating the upstream, middle or downstream site reduced expression with 3-AT about 16%, 96% and 97% respectively, from the 64 units obtained with the wild-type promoter, all three GCRE elements appear to be used for general amino acid control, although the upstream element has a very minor role.

3.6. Gcr1p may regulate *GCV3*.

Because the GCS is localized in the mitochondria we wondered whether expression with glucose, a fermentable carbon source that represses mitochondrial function, would be different from expression with glycerol, a non-fermentable carbon source that induces mitochondrial function. To assess this, the activities of pGCV3-232 were tested in two

Figure 8. The elements in the *GCV3* promoter important for responding to 3-AT. Various plasmids were constructed harbouring mutations in the three GCRE sites as indicated. The three potential GCREs are located at –128 bp, –113 bp and –97 bp where numbering indicates the most upstream base of the GCRE site. pGCV3-232 harbours the wild-type *GCV3* promoter. The rest of the constructs are identical to pGCV3-232 except for mutations introduced into the GCREs. (☐ indicates presence of a wild type GCRE, whereas ☒ indicates a mutant GCRE). Expression of the wild-type and mutant promoters was measured in SD and SD + 20 mM 3-AT media.

GCRE1 -128bp · GCRE2 -113bp · GCRE3 -97bp

	β-gal activity	
	SD	SD+3-AT
pGCV3-232	19	64
pGCV3-232GCREM0	1.5	1.9
pGCV3-232GCREM1	42	54
pGCV3-232GCREM2	1.2	2.7
pGCV3-232GCREM3	1.2	2.4

different wild-type strains, 3634 and S150-2B (Table 8). Surprisingly, in strain 3634 *GCV3* expression with glucose as the carbon source was the same as with glycerol, whereas in strain S150-2B, *GCV3* expression was higher with glucose as the carbon source than with glycerol. *GCV3* expression therefore responds to the carbon source in some genetic backgrounds.

Since a potential binding site for the glycolytic regulator Gcr1p (Willett et al., 1993. Scott and Baker, 1993) is present at bp –191, Gcr1p may regulate *GCV3* expression. The presence of a potential Rap1p binding site at bp –210 further supports this possibility, since Gcr1p binding is usually dependent upon Rap1p binding to an adjacent site (Chambers et al., 1995; Tornow et al., 1993). To assess whether Gcr1p regulates *GCV3*, the activities of pGCV3-232 in the *GCR1* strain S150-2B and the isogenic *gcr1* strain HBY4 were compared (Table 9). Expression in SD medium was 5.5 times higher in the *GCR1* strain than in the *gcr1* strain. In the *gcr1* strain, the expression was essentially the same with glucose or glycerol and ethanol as the carbon source. These results indicate that Gcr1p is important for normal levels of expression in SD. Since 10 mM glycine induced expression in both strains, Gcr1p is not required for induction by 10 mM glycine.

To test if the CT-box located at –191bp was important for regulation by Gcr1p, expression of construct pGCV3-232_{CTBOXM} was examined. Construct pGCV3-232_{CTBOXM} is identical to pGCV3-232 except for mutations at positions –189 and –190 bp in the CT-box consensus sequence which changes it from 5'-CTTCC-3' to 5'-CaaCC-3'. The results in Table 9 show that mutating the CT-box did not alter levels of expression with glucose or glycerol significantly.

Table 8. Expression of *GCV3* in response to the fermentable carbon source glucose versus a non-fermentable carbon source.

Strain[b]	β-gal activity (Miller units)[a]	
	glucose	glycerol + ethanol
3634 / pGCV3-232	30	32
S150-2B / pGCV3-232	34	11

[a]Activity levels of β-galactosidase were determined as described in Table 4. [b]Strains 3634 and S150-2B harbouring plasmid pGCV3-232 were used.

Table 9. Gcr1p regulates *GCV3* expression.

Strain[b]	β-gal activity (Miller units)[a]		
	glucose	**glycerol + ethanol**	**glucose + 10 mM glycine**
GCR1 / pGCV3-232	34	11	154
gcr1 / pGCV3-232	6.2	5.3	48
GCR1 / pGCV3-232_{CTBOXM}	49	15	ND[c]

[a]Activity levels of β-galactosidase were determined as described in Table 4. [b]The wild type (*GCR1*) and mutant (*gcr1*) strains used were S150-2B and its derivative strain HBY4, respectively. pGCV3-232_{CTBOXM} is described in Materials and Methods. [c]Not determined.

3.7. *GCV3* is repressed by the C1-end products.

One of the products of glycine cleavage is MTHF (N^5, N^{10}-methylenetetrahydrofolate), a donor of one-carbon units used for biosynthesis of the C1-end products (formate, thymidylate, the purines, histidine, methionine, and serine). Yeast cells grown in SD + 10 mM glycine expressed about 37 units of β-galactosidase activity from pGCV3-Δ232, whereas they expressed only 7.7 units when the C1-end products were also included (Table 10). To locate the cis-acting information that responded to the C1-end products we compared expression from transformants of strain 3634 harbouring pGCV3-Δ232 and the promoter deletion constructs pGCV3-Δ207 through pGCV3-Δ117 in SD + 10 mM glycine and SD + 10 mM glycine supplemented with the C1-end products (Table 10).

Constructs pGCV3-Δ232 through pGCV3-Δ149 were all strongly repressed by the C1-end products. Their expression in medium without the C1-end products was about 6 times higher than when the C1-end products were present. Plasmid pGCV3-Δ132 expression was only 3.6 times higher in medium without the C1-end products. The behavior of pGCV3-Δ117 was very different. Its expression did not respond to the C1-end products. These results suggest that the cis-acting information that responds to the C1-end products is located between −149 bp and −117 bp.

We also tested whether *GCV3* expression in SD was subject to repression by the C1-end products. Transformants harbouring pGCV3-Δ232, pGCV3-Δ132 and pGCV3-Δ107 were grown in SD and SD + C1-end products. The results showed that *GCV3* was expressed at much lower levels when the C1-end products were present in SD medium. *GCV3* expression is therefore repressed by the C1-end products regardless of whether or

Table 10. The C1-end products repress *GCV3* expression.

Plasmid[b]	β-gal activity (Miller units)[a,d]	
	SD	**SD + 10 mM glycine**
pGCV3-Δ232	1.8 (x3.2)	7.7 (x4.8)
pGCV3-Δ207	ND[c]	7.5 (x6.1)
pGCV3-Δ186	ND[c]	10 (x7.9)
pGCV3-Δ176	ND[c]	9.1 (x6)
pGCV3-Δ161	ND[c]	10 (x7.4)
pGCV3-Δ149	ND[c]	11 (x6.4)
pGCV3-Δ132	3.3 (x2.3)	16 (x3.6)
pGCV3-Δ117	4.6 (x0.3)	2.2 (x1)

[a]Activity levels of β-galactosidase were determined as described in Table 4. Activity values presented represent levels of expression obtained when the C1-end products were present in the media indicated. [b]The plasmids used were described previously (Figure 7). [c]Not determined. [d]Numbers in brackets represent how many times higher the expression was in SD or SD + 10 mM glycine than in SD or SD + 10 mM glycine with C1-end products.

not glycine is present. Expression from pGCV3-Δ117 was different: its expression was three times higher during growth in SD supplemented with the C1-end products than it was during growth in SD. The results suggest that repression by the C1-end products in SD is similar to that in SD + 10 mM glycine and the cis-acting information required for the repression is located downstream of bp −149.

3.8. Bas1p but not Bas2p is important for *GCV3* expression.

Glutamine, glycine and 10-formyltetrahydrofolate are substrates required for *de novo* purine biosynthesis. In *S. cerevisiae* these three metabolites are synthesized by the enzymes glutamine synthetase, serine hydroxymethyl transferase and NAD-dependent 5,10-methylene tetrahydrofolate dehydrogenase. The genes coding for these three enzymes, *GLN1*, *SHM2* and *MTD1*, and several other genes encoding enzymes for purine biosynthesis are co-regulated by Bas1p and Bas2p (Denis and Daignan-Fornier, 1998). Several genes for histidine biosynthesis are also regulated by Bas1p and Bas2p (Arndt et al., 1987; Springer et al., 1996). The involvement of Bas1p and Bas2p in the regulation of both histidine and purine synthesis may reflect the fact that both the purine nucleotide ATP and glutamine are required for histidine biosynthesis. Since 10-formyltetrahydrofolate (generated from 5,10-methenylenetetrahydrofolate) is a metabolic product of glycine cleavage and genes regulated by Gcn4p are often co-regulated by Bas1p/Bas2p, we thought that Bas1p and Bas2p might also regulate *GCV3*.

To test whether Bas1p and Bas2p were important for *GCV3* expression we used the mutant strains CY614 (*gcn4 bas1 bas2*), CY615 (*GCN4 bas1 bas2*), CY945 (*gcn4 bas1 BAS2*), 5270 (*gcn4 BAS1 bas2*) and CY946 (*gcn4 BAS1 BAS2*), and the wild-type strain

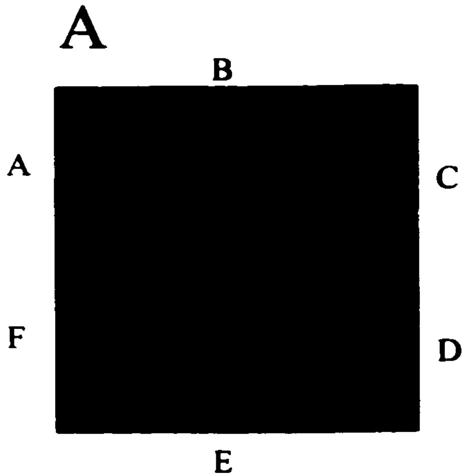
3634 (for complete genotypes, see Table 2). The genotypes of the various strains were verified by plating onto SD and SD + histidine. When grown on SD medium, strains CY614, CY945 and 5270 were unable to grow unless supplemented with histidine (Figure 9, Panel A and B, sectors B, D and E). This is consistent with previous results that found yeast required functional Gcn4p, or Bas1p and Bas2p for growth on SD (Devlin et al., 1991; Denis et al., 1998), but not on SD + histidine.

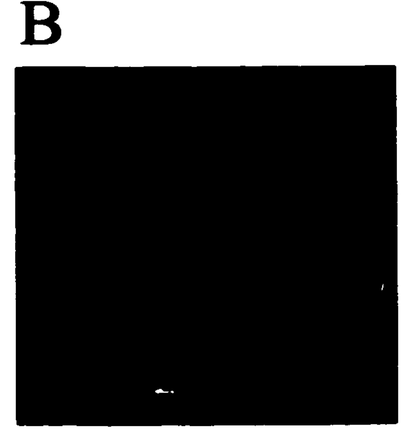
With glycine as the sole nitrogen source the wild-type strain and strain 5270 grew well, strains CY614 and CY945 were unable to grow, and strains CY615 and CY946 grew poorly (Figure 9, Panel C). Inability of the *gcn4 bas1 bas2* and *gcn4 bas1 BAS2* strains to grow with glycine as sole nitrogen source showed that Gcn4p and/or Bas1p were required for growth when glycine was the nitrogen source. Growth of strains CY615, 5270 and CY945 (Figure 9, Panel C), which express only Gcn4p, Bas1p or Bas2p respectively, showed that Bas1p supported essentially normal growth, that Bas2p could not support growth, and that Gcn4p supported modest growth. Strain CY946, which expresses functional Bas1p and Bas2p but not Gcn4p, was unable to grow as well as strain 5270 (*gcn4 BAS1 bas2*). This suggested that Bas2p negatively affected growth when glycine was the sole nitrogen source. That the *GCN4 bas1 bas2* strain grew, although slowly relative to the wild-type and the *gcn4 BAS1 bas2* strains, suggested that Gcn4p levels are elevated when this strain is grown on minimal medium with glycine as the nitrogen source. Perhaps for this strain, this growth condition mimics amino acid starvation.

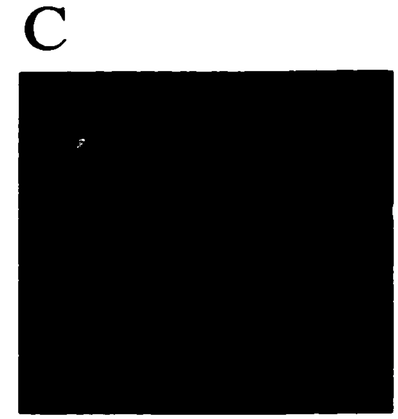
The poor growth observed in strains CY614, CY615, CY946 when glycine was the nitrogen source was rescued when the C1-end products were supplied (Figure 9, Panel D). The wild-type strain and 5270 (*gcn4 BAS1 bas2*) as well as CY615 (*GCN4 bas1*

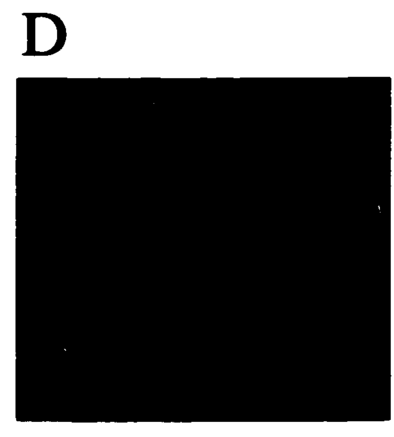
Figure. 9. Bas1p is required for growth with glycine as the sole nitrogen source.

Various strains were streaked for growth as follows: *plate sector A* (strain 3634, wild-type); *plate sector B* (CY614 *gcn4 basl bas2*); *plate sector C* (CY615 *GCN4 bas1 bas2*); *plate sector D* (CY946 *gcn4 bas1 BAS2*); *plate sector E* (5270 *gcn4 BAS1 bas2*); *plate sector F* (CY946 *gcn4 BAS1 BAS2*). Relative growth of the six strains on SD (Panel A), SD + histidine (Panel B), SD without ammonia + 250 mM glycine as sole nitrogen source + histidine (Panel C) and SD without ammonia + 250 mM glycine as sole nitrogen source plus the supplement of the C1-end products (Panel D).


A
B
A
C
F
D
E


B


C


D

bas2), grew better than all the other strains (CY614, CY945 and CY946). Even the triple mutant, CY614 (*gcn4 bas1 bas2*), grew better than it did on SD. Interestingly, CY945 (*gcn4 bas1 BAS2*) still grew poorly.

3.9. Induction by 10 mM glycine is Bas1p dependent.

Gcn4p or Bas1p was needed for growth of *S. cerevisiae* with glycine as the sole nitrogen source (Figure 9, Panel C). To learn whether this requirement was due to the role these transcription factors played in regulating the GCS, we compared pGCV3-232 expression in strains 5270, CY614, CY615, CY946, CY945 respectively during growth in SD and SD + 10 mM glycine. The results are presented in Table 11.

The expression results obtained with these strains grown in SD medium can be summarized as follows. Expression from pGCV3-232 is at dramatically lower levels in the *gcn4* strain (CY946, *gcn4 BAS1 BAS2*) than in the wild type strain 3634. This shows that Gcn4p is required for normal levels of expression in SD. The results also show that Bas1p and /or Bas2p are important for expression in SD, since pGCV3-232 is expressed at much lower levels in strain CY614 (*gcn4 bas1 bas2*) than in strain CY946 (*gcn4 BAS1 BAS2*). Finally, the amount of GCV3-232 expression in strains 5270, CY614, CY945 and CY946 suggested that Bas1p but not Bas2p was important for expression in SD. Therefore, it is apparent that both Gcn4p and Bas1p are important for expression in SD.

In SD supplemented with 10 mM glycine, pGCV3-232 was expressed at high levels in strains 3634 (102 units), 5270 (84 units) and CY946 (64 units) and at very low levels in strains CY614 (1.4 units), CY615 (1.7 units) and CY945 (1.4 units). These results show that Bas1p can support high levels of expression when 10 mM glycine is present whereas Gcn4p and Bas2p cannot.

Table 11. Functional importance of Gcn4p, Bas1p and Bas2p for *GCV3* expression in SD medium and for induction by 10 mM glycine.

Strain genotype[b]	**β-gal activity (Miller units)**[a]	
	SD	**SD + 10 mM glycine**
GCN4 BAS1 BAS2 / pGCV3-232	30	102
gcn4 bas1 bas2 / pGCV3-232	0.64	1.4
GCN4 bas1 bas2 / pGCV3-232	1.3	1.7
gcn4 BAS1 bas2 / pGCV3-232	4.4	84
gcn4 bas1 BAS2 / pGCV3-232	0.75	1.4
gcn4 BAS1 BAS2 / pGCV3-232	2.1	64

[a]Activity levels of β-galactosidase were determined as described in Table 4. [b]pGCV3-232 expressed in strain 3634 (*GCN4 BAS1 BAS2*), strain CY614 (*gcn4 bas1 bas2*), strain CY615 (*GCN4 bas1 bas2*), strain 5270 (*gcn4 BAS1 bas2*), strain CY945 (*gcn4 bas1 BAS2*) and strain CY946 (*gcn4 BAS1 BAS2*).

3.10. Regulation of *GCV3* by the C1-end products is Bas1p dependent.

Earlier we found that the C1-end products repressed *GCV3* expression (Table 10). Once it was established that both Gcn4p and Bas1p were important for expression during growth in SD medium and that Bas1p was important for induction by 10mM glycine, we wanted to determine whether Gcn4p and/or Bas1p dependent expression was repressed by the C1-end products. To establish this, pGCV3-232 expression in strains CY614, 5270, CY945, CY946 and CY615 was compared during growth in SD + 10 mM glycine and SD + 10 mM glycine and the C1-end products. The C1-end products could only repress pGCV3-232 significantly in strains expressing functional Bas1p (Table 12). That is, expression in the *GCN4 BAS1 BAS2*, *gcn4 BAS1 BAS2* and *gcn4 BAS1 bas2* strains was about 3, 5 and 5 times higher, respectively, in SD + 10 mM glycine than when the C1-end products were also present. Strains lacking Bas1p (CY615, CY945 and CY614) expressed only 1.7, 1.4 and 1.4 units of β-galactosidase, respectively. These levels of expression were only 1.3 times higher than that observed when the C1-end products were also included. Since the modest repression observed in the absence of functional Bas1p was also obtained in strain CY614 (*gcn4 bas1 bas2*), neither Gcn4p nor Bas2p contributed to the modest repression obtained in the absence of Bas1p.

We also assessed the regulatory importance of Bas1p, Bas2p and Gcn4p when glycine was the sole nitrogen source (Table 13). Bas1p was required for elevated levels of expression. Gcn4p apparently enhanced expression in a strain that expressed Bas1p. Bas2p appeared to repress expression slightly. Expression of *GCV3* in strains (3634, CY615, 5270 and CY946) was lower when growth was in medium with glycine as the sole nitrogen source (Table 13) than in SD medium supplemented with 10 mM glycine

Table 12. Repression of *GCV3* expression by the C1-end products is Bas1p-dependent.

Strain genotype[c]	β-gal activity (Miller units)[a]	
	SD + C1-end products	SD + 10 mM glycine + C1-end products[d]
GCN4 BAS1 BAS2 / pGCV3-232	9.5 (x3)	39 (x2.6)
gcn4 bas1 bas2 / pGCV3-232	ND[b]	1.1 (x1.3)
GCN4 bas1 bas2 / pGCV3-232	ND[b]	1.3 (x1.3)
gcn4 BAS1 bas2 / pGCV3-232	ND[b]	18 (x4.7)
gcn4 bas1 BAS2 / pGCV3-232	ND[b]	1.1 (x1.3)
gcn4 BAS1 BAS2 / pGCV3-232	ND[b]	14 (x4.6)

[a]Activity levels of β-galactosidase were determined as described in Table 4. [b]Not determined. [c]The various strains used are described in Table 11. [d]Numbers in brackets represent how many times higher pGCV3-232 expression was in SD + 10 mM glycine than in SD + 10 mM glycine with the C1-end products (values indicated).

Table 13. Expression of *GCV3* is not repressed by the C1-end products when glycine is the sole nitrogen source.

Strain genotype[c]	β-gal activity (Miller units)[a]	
	glycine[b]	glycine + C1-end products
GCN4 BAS1 BAS2 / pGCV3-232	59	74
GCN4 bas1 bas2 / pGCV3-232	1.6	1.5
gcn4 BAS1 bas2 / pGCV3-232	36	47
gcn4 BAS1 BAS2 / pGCV3-232	31	30

[a]Activity levels of β-galactosidase were determined as described in Table 4. [B]250 mM glycine was used as the sole nitrogen source. [c]The various strains used are as in Table 11.

(Table 11). Interestingly, the C1-end products did not repress expression for any of the strains grown in medium with glycine as the sole nitrogen source, since expression either did not change significantly (for strains CY615 and CY946) or was modestly induced (3634, 5270).

3.11 The general control response elements play an important role in the regulation of *GCV3* by Bas1p.

The three GCREs located downstream of base pair −132 are potential Bas1p binding sites (Daignan-Fornier and Fink, 1992). To determine whether they were important for Bas1p-dependent regulation, constructs pGCV3-232_{GCRE1}, pGCV3-232_{GCRE2}, pGCV3-232_{GCRE3}, and pGCV3-232_{GCRE0} were introduced into strain 5270 (*gcn4 BAS1 bas2*). *GCV3* expression by the resulting transformants was tested under five different growth conditions (Table 14).

Individually mutating the upstream, middle and downstream GCRE caused pGCV3-232 expression in SD to increase about 2 times, decrease by 97% and decrease by 73% respectively. Mutating the middle and proximal GCRE, therefore, had the greatest effect on expression in SD. The upstream, middle and downstream elements were all also important for expression in SD + 10 mM glycine, since expression by constructs with these sites mutated was reduced by 50%, 99% and 96%, respectively. C1-end products in SD + 10 mM glycine reduced expression from the wild-type promoter as well as the promoter with a mutant upstream GCRE by close to 80%. The C1-end products repressed expression by the middle and proximal GCRE mutant promoters 60% and 80% respectively. When glycine was the nitrogen source, expression by the wild type

Table 14. Assessment of the functional importance of the three GCRE consensus elements for Bas1p-dependent expression.

Plasmid[b]	β-gal activity (Miller units)[a]				
	SD	SD+10 mM glycine	SD+10 mM glycine+C1-end products	glycine[c]	glycine + C1-end products
pGCV3-232	4.4	84	18	36	47
pGCV3-232$_{GCRE1}$	9.7	42	8.4	44	45
pGCV3-232$_{GCRE2}$	0.58	0.84	0.32	6.1	6.2
pGCV3-232$_{GCRE3}$	1.2	2.4	0.6	4.6	4.2
pGCV3-232$_{GCRE0}$	1.2	2.2	0.62	3.1	2.6

[a]Activity levels of β-galactosidase were determined as described in Table 4. [b]The various plasmids used are described in Figure 8. [c]250 mM glycine was used as the sole nitrogen source. These were assessed in transformants of strain 5270 (*gcn4 BAS1 bas2*).

promoter was higher than that in SD and lower than that in SD + 10 mM glycine. The level of activity expressed by the upstream GCRE promoter mutant in the presence of glycine as the nitrogen source was similar to that of the wild-type and no decrease was observed from activity in the presence of SD + 10 mM glycine. Mutations at the middle and downstream GCREs reduced promoter activity about 6 and 9-fold respectively, although they still respond to glycine. For high levels of expression in glycine as the nitrogen source, the middle and the proximal GCREs, but not the upstream GCRE, are important. The C1-end products did not repress expression when growth was with glycine as the nitrogen source. Previous experiments showed that Bas1p was required for the induction by glycine. These results show that the middle and proximal GCRE are important for Bas1p-dependent expression in SD, in SD + 10 mM glycine and when glycine is the nitrogen source. The upstream GCRE is possibly bound by Bas1p when the media is SD + glycine, but not when the media only contains glycine as the nitrogen source. A negative transcription factor probably binds to the upstream GCRE when cells are grown in SD.

Discussion

GCV3 encodes a 170 amino acid residue protein that has a predicted mass of 18,795 Da. Analysis performed by using the program psort II (κ-NN prediction, website: http://psort.nibb.ac.jp:8800/) predicts that the N-terminal 47 amino acid residues of this protein contain a mitochondrial targeting signal (95.7% probability). This is consistent with the known mitochondrial location of the glycine cleavage system. The expression of *GCV3* is strongly stimulated by glycine, the substrate of the glycine cleavage system. The C1-end products, which are indirect products of the GCS, negatively regulate *GCV3* expression. Bas1p is required for maintaining basal level transcription of *GCV3* and for the induced levels of *GCV3* expression observed when glycine is present in the media. Interestingly repression by the C1-end products is also Bas1p dependent. In response to amino acid starvation, *GCV3* expression is induced in a Gcn4p-dependent fashion. Since *GCV3* is also regulated in response to the availability of alternative nitrogen sources, it is also regulated by the nitrogen regulation system. Gcr1p, a transcription factor important for the expression of glycolytic genes, apparently activates the expression of *GCV3* in the presence of glucose. An unknown protein apparently represses expression in SD.

The intergenic region between the stop codon of *PTA1* (the gene immediately upstream of *GCV3*) and the *GCV3* start codon is 232 bp long. The information for responding to glycine, the C1-end products, and amino acid starvation are located within 132 bp of the *GCV3* start codon. This region harbours three potential GCREs. All three GCREs were found to be important for *GCV3* expression. The middle and proximal GCREs are critical for the basal expression of *GCV3*, induction by glycine and general

amino acid control. The upstream GCRE is apparently important for the repression of *GCV3* during growth in SD, for induction by 10 mM glycine, and induced expression by 3-AT. All three general control elements (GCREs) played significant roles in regulating expression in response to the availability of the C1-end products. I also found that the region upstream of bp –132 played a modest but significant regulatory role. A 5'-GATAA-3' sequence (the cis-acting regulatory element used by the nitrogen regulation system) at bp –167 is utilized by the nitrogen regulation system.

4.1. Nitrogen regulation system and *GCV3* expression.

The nitrogen regulation system is used to regulate genes required for utilization of various poor nitrogen sources. It is not unexpected that *GCV3* is regulated by the nitrogen regulation system since the glycine cleavage system is required for utilizing glycine as the nitrogen source. There are at least 5 known transcription factors in the nitrogen regulation system. Four of them belong to the family of GATA binding protein. They regulate genes by utilizing the cis-acting sequence 5'-GATAA-3' (Coffman et al., 1996; 1997; Courchesne and Magasanik, 1988; Cunningham et al., 1994; Cunningham and Cooper, 1991,1993; Minehart and Magasanik, 1991; Rowen et al., 1997; Stanbrough and Magasanik, 1996). This upstream element appears as a single site in some nitrogen regulation system genes and in multiple copies in others. The number of sites and their relative locations and orientations are important factors that determine their activity (Cunningham and Cooper, 1993; Cunningham et al., 1994; Stanbrough and Magasanik, 1996; Rowen et al., 1997). In the *GCV3* promoter, there is one potential GATA sequence located at bp –167. Mutations in this GATA sequence affected the activity of the *GCV3*

promoter in both *GLN3* and *gln3* strains (Table 6). Therefore this GATA sequence is utilized by the nitrogen regulation system.

Gene regulation by the nitrogen regulation system is however quite complicated, since the nitrogen regulation system regulates not only the genes required for the utilization of poor nitrogen sources, but also the GATA proteins themselves. Among the GATA binding proteins, only Gln3p is not regulated by a GATA binding protein, and it is required for activating the other known GATA proteins (Coffman et al., 1997). Thus, *GCV3* is not only regulated by *GLN3*, but it could be regulated directly and indirectly by all members of transcription factors in the GATA family.

Gln3p may not directly activate *GCV3* expression in proline media since expression of a GATAA mutant promoter in the *gln3* strain was as high as the wild-type promoter in *GLN3* strain. So far, the known positive-acting GATA binding proteins are Gln3p and Nil1p. Thus, we suspect that it is Nil1p which activates the promoter in the presence of proline. The slightly lower expression of the mutant promoter compared to the wild-type promoter in PM38 when proline was used as the nitrogen source may be due to the GATA mutations introduced into the promoter abolishing Gln3p binding to the GATA sequence (Bysani et al., 1991). Thus, the mutations may not have the same effect on the binding of the other GATA proteins as they do on Gln3p.

In the presence of preferred nitrogen sources such as ammonia, expression in the *GLN3* (PM38) and *gln3* (PM81) strains was essentially the same. Gln3p is apparently not required for expression in the presence of ammonia. In the presence of proline, the GATA mutant promoter had slightly lower activity than the wild-type promoter in PM38. Addition of glutamine to the proline medium caused decreased expression of the wild-

type promoter. However, the GATA mutation could overcome repression by glutamine. Since this is also observed in a *gln3* (PM81) strain, repression by glutamine is not directly affected by Gln3p. We suspect that there is another protein which represses the promoter by binding to the same GATA sequence. This repressor's level or activity is probably elevated by Gln3p since expression in PM81 (*gln3*) was higher than that in PM38 with additional glutamine in the proline medium. The known negative factors in the GATA family are Dal80p and Deh1p. Only Deh1p can utilize one GATA site to repress gene expression in the presence of glutamine (Coffman et al., 1997; Rowen et al., 1997). Thus, the repression by additional glutamine in proline may be due to Deh1p, whose principal role is to prevent activation of transcription by Nil1p by blocking the access of Nil1p to the GATA sequence (Rowen et al., 1997).

Since both the wild-type promoter and the mutant promoter had higher activities in the *gln3* strain than they did in the *GLN3* strain, Gln3p seems to act as a repressor of *GCV3* in the glutamine and proline mixed medium. It is reported that Ure2p, another protein in nitrogen regulation system, inactives Gln3p by modifying Gln3p when ammonia or high intracellular glutamine is present (Courchesne and Magasanik, 1988; Stanbrough et al., 1995). The inactive Gln3p could compete with Nil1p to repress *GCV3* expression.

The mechanisms by which the nitrogen regulation system affects GCV3 expression remain to be elucidated. Further studies of the regulation of *GCV3* by the nitrogen regulation system are required in order to gain more insight and information about nitrogen regulation of this promoter.

4.2. *GCV3* regulation by Gcr1p.

Analysis of the *GCV3* promoter region found that there are two potential CT-boxes (5'-CTTCC-3') located at −191 bp and −123 bp, respectively. It is believed that Gcr1p activates the "glycolytic" genes in the presence of glucose by binding to the CT-box (Baker, 1991; Nishi et al., 1995; Huie and Baker, 1996; Velmurugan et al., 1997). However, a single CT-box has very weak UAS activity (Uemura et al., 1997). During growth on fermentable carbon sources, the majority of cellular glycine comes from serine generated from 3-phosphoglycerate, an intermediate of glycolysis. During growth on non-fermentable carbon sources such as ethanol and acetate, "gluconeogenesis" provides 3-phosphoglycerate for serine and glycine biosynthesis (Ulane and Ogur, 1972). Genes involved in gluconeogenesis do not utilize Gcr1p to activate their transcription.

GCV3 expression is not significantly different during growth with glucose or glycerol as the carbon source in strain 3634. These results suggest that *GCV3* expression is not affected by whether a fermentable or non-fermentable carbon source is used. Gcr1p could still contribute to *GCV3* expression in the presence of glucose. However, in the absence of glucose other regulation mechanisms would compensate for the reduced role of Gcr1p.

Based on the results observed from strain 3634 (Table 8), it is not clear that Gcr1p regulates *GCV3* expression. Nonetheless, results obtained with the isogenic strains S150-2B (*GCR1*) and HBY4 (*gcr1*) (Table 9) suggest Gcr1p does play a role in *GCV3* expression. The different behaviors observed from strains 3634 and S150-2B may be caused by their different genetic backgrounds.

The CT-box element can have strong UAS activity under some conditions. For example, when a Rap1p binding site is adjacent to a single CT-box (5 bp apart), Gcr1p and Rap1p can cooperatively activate gene expression (Drazinic et al., 1996). Multiple tandem CT-boxes (five CT-boxes) can also have strong UAS activity (Drazinic et al., 1996; Lopez et al., 1998; Zeng et al., 1997). The *GCV3* promoter has a potential Rap1p binding site (at –209 bp) about 10 bp upstream from the CT-box at –191. Mutating the CT-box at –191 bp did not affect significantly expression. There are two possible explanations for this observation. First, the CT-box sequence at –191 bp is 5'-GTCTTCCTTCA-3'. The mutations were introduced as 5'-GTCaaCCTTCA -3' and the consensus sequence for Gcr1p binding is 5'-CTTCC-3'. Gcr1p binds to the CT-box element with high affinity but low specificity (Huie et al., 1992). The C-terminal half of Gcr1p, which includes the domain required for DNA binding to the CT-box *in vitro*, can be removed without affecting *GCR1*-dependent transcription of the glycolytic gene *ADH1* and the translational component genes *TEF1* and *TEF2* (Tornow et al., 1993). Thus, the mutations we introduced into the CT-box at –191 bp may not have affected the ability of Gcr1p to activate transcription. Secondly, there is a second potential CT-box at –123 bp (5'-CACTTCCGT-3'). However, we could not identify a Rap1p site adjacent this CT-box. Nevertheless, it may be worthwhile to assess the consequences on *GCV3* expression of mutating this site.

4.3. *GCV3* is subject to general amino acid control.

GCV3 is also regulated by the general amino acid control system, and the required cis-acting information is within 132 bp upstream of the start codon (Table 7 and Figure

8). Gcn4p regulates gene expression by binding to GCREs. Among the three potential GCREs in the *GCV3* promoter, the middle and the proximal GCRE are the most important for regulation by Gcn4p (Figure 8). Mutating either of these two elements dramatically reduced expression in SD and severely impaired expression in response to 3-AT. In contrast mutating the GCRE at –128 bp increased expression 2-fold in SD but did not significantly alter induction by 3-AT. When all the three GCRE elements were mutated, the general amino acid control was abolished. However, expression in SD was not reduced as dramatically in the triple mutant as when only the central or the proximal GCRE was altered. These results show that the central GCRE and the proximal GCRE are critical for both expression in SD and regulation by general control, whereas the distal GCRE is important for expression in SD but less important than the middle and proximal GCREs for general control. Even though the distal GCRE seems unimportant for general control, deletion construct pGCV3-Δ117 containing the middle and proximal GCREs did not respond to elevated Gcn4p levels (Table 7), but still responsed to glycine induction (1.8-fold increase in response to the induction by 10 mM glycine, Table 5). Since mutating the distal GCRE caused expression in SD to increase significantly, the wild-type site may be utilized to repress *GCV3* during growth in SD.

4.4. The regulation of *GCV3* in response to glycine and C1-end products.

4.4.1. *GCV3* expression is induced by glycine and repressed by the C1-end products.

Since substrate-dependent transcription activation is not unusual, it was not unexpected that *GCV3* was induced by glycine (Table 4 and Table 11). The C1-units are essential for a variety of anabolic processes that yield necessary cellular components including purines, pyrimidines and some amino acids. The C1-end products are indirectly products of glycine cleavage. It has been found in *S. cerevisiae* that the genes coding for purine and histidine biosynthesis are repressed by excess adenine (one of the C1-end products, Appling and Rabinowitz . 1985; Giani, et al., 1991). The C1-end products repress *GCV3* expression (Nagarajan and Storms, 1997; this study Table 10). Whether this repression is due to adenine present in the C1-end product mixture used here or the C1-end products in general needs to be futher defined.

4.4.2. Induction by glycine and repression by the C1-end products are Bas1p-dependent.

In *S. cerevisiae*, genes coding for purine and histidine biosynthesis are regulated by Bas1p and Bas2p (Tice-Baldwin et al., 1989; Daignan-Fornier and Fink, 1992; Rolfes et al., 1997). Glutamine, glycine and 10-formyltetrahydrofolate are consumed during *de novo* purine biosynthesis. *GLN1* (the gene required for synthesis of glutamine), *SHM2* (the gene required for synthesis of glycine and C1-units) and *MTD1* (the gene required for synthesis of 10-formyl tetrahydrofolate) are regulated by Bas1p and Bas2p (Denis and

Daignan-Fornier, 1998). Excess adenine represses several purine biosynthesis genes and this effect is believed to occur at the level of the interaction of Bas1p and Bas2p (Zhang et al., 1997). In this study, the C1-end products supplemented in media included adenine. Bas1p and Bas2p both bind DNA and cooperatively activate transcription (Daignan-Fornier and Fink, 1992). The DNA sequence that Bas2p recognizes has not been clearly defined. It has been found that simultaneous binding of the Bas1p-Bas2p complex unmasks a transcriptional activation function of Bas1p (Zhang et al., 1997). As a complex with Bas1p, the DNA binding activity of Bas2p was not needed for its ability to support transcription (Zhang et al., 1997).

Previously, Bas1p was reported to be able to activate transcription only with Bas2p. Recently, Denis and Daignan-Fornier (1998) found that the expression of *SHM2-lacZ* was essentially the same in a *BAS1 bas2* strain and a *BAS1 BAS2* strain in the presence of adenine. However, the expression of *SHM2-lacZ* was much lower in a *BAS1 bas2* strain than in a *BAS1 BAS2* strain in the absence of adenine. The Bas1p-dependent expression was not repressed by adenine like the Bas1p/Bas2p-dependent expression. They suggested that Bas1p may activate transcription by interacting with another protein partner other than Bas2p. Here, we also showed that without Bas2p, Bas1p can support *GCV3* expression in SD, SD + 10 mM glycine and 250 mM glycine as the nitrogen source (Tables 11 and 13). Repression by the C1-end products is not only observed in the *BAS1 BAS2* strain but also in the *BAS1 bas2* strain. Without Bas1p, *GCV3* expression was at a very low level in all the growth conditions tested (Tables 11 and 13). *bas1* defective strains could not grow well on the plate with glycine as the nitrogen source (Figure 9, Panel C). Furthermore, Bas1p supported better growth with glycine as the nitrogen

source and higher levels of *GCV3* expression in all tested growth conditions in the absence of Bas2p. Induction of *GCV3* expression apparently is Bas1p-dependent and does not require Bas2p. Bas2p may repress *GCV3* expression.

When glycine was the nitrogen source, the C1-end products did not repress expression (Table 13). This implies that the effect of C1-end products on the regulation of *GCV3* is complex. Further studies to determine the mechanism of Bas1p activation of the transcription of *GCV3* and how C1-end products represses *GCV3* expression are also needed.

4. 4. 3. Regulation related to the GCREs.

Bas1p recognition sites include the GCRE core sequence (Daignan-Fornier and Fink, 1992; Springer et al., 1996). In the *GCV3* promoter there are three potential GCREs. These three GCREs were found to be important for expression in SD and general amino acid control (Figure 8). The expression in strain *gcn4 BAS1 bas2* showed mutating these GCREs changed *GCV3* expression (Table 14).

4.4.3.1. The middle and proximal GCREs are critical for induction by 10mM glycine.

The promoter region that was required for induction by 10mM glycine was found to be localized to within 132 base pairs of the start codon (Table 5) and Bas1p-dependent (Table 11). Individually mutating the GCRE sites showed that the middle and proximal GCREs were very important for the Bas1p-dependent expression obtained in SD and SD + 10 mM glycine (Table 14).

4.4.3.2. The distal GCRE plays a minor role for induction by 10mM glycine and repression during growth in SD.

The distal GCRE may not be important for Gcn4p-dependent expression of *GCV3* since mutations at this site had little effect on the expression in response to elevated Gcn4p. When glycine served as the nitrogen source, mutation at this GCRE also showed no effect on expression. However, mutating this GCRE did reduce the expression of *GCV3* in SD + 10 mM glycine, but increased it in SD. Thus, we suspect this GCRE is bound by a repressor during growth in SD and by an activator in SD + 10 mM glycine.

4.4.3.3. Bas1p-dependent expression.

Bas1p was required for expression in SD, SD + 10 mM glycine and glycine as the sole nitrogen source (Tables 11 and 13). Repression by the C1-end products is also dependent on Bas1p (Table 12). GCREs present in the *GCV3* promoter were also important for the regulation (Table 14).

The distal GCRE is apparently bound by a repressor in SD (Figure 8 and Table 14). Glycine or amino acid starvation may change the binding affinity of this repressor and allow a transcription activator to bind to the distal GCRE. The middle and proximal GCREs are required for the activation of transcription.

The results in Table 11 and Table 14 suggest that induction by 10 mM glycine involves Bas1p binding to the distal GCRE instead of the putative repressor (Table 11). Glycine or 3-AT probably changes the affinity of this repressor binding to this site (Figure 8, Table 11). The elevated expression caused by 3-AT was apparently dependent on

Gcn4p binding to the two downstream GCREs and the repressor leaving the distal GCRE (Figure 8). The two GCREs close to the start codon are probably used to support basal transcription (Figure 8 and Table 14).

The distances between the core sequences for the three GCREs are 9 and 10 bp. Since individually mutating the proximal and middle GCREs severely impaired *GCV3* expression in strain 5270 (*gcn4 BAS1 bas2*) (Table 14), we suspect that Bas1p binding to these two GCREs activates transcription. Repression by the C1-end products in SD and SD + 10 mM glycine apparently results from interference with Bas1p binding.

4.5. *GCV3* expression and glycine and C1-unit metabolism.

Glycine is consumed in the biosynthesis of serine, the purines, porphyrin, by protein synthesis and degradation by the glycine cleavage system. Glycine catabolized by the glycine cleavage system generates ammonia (a nitrogen source), N^5N^{10}-methylenetetrahydrofolate (which provides the active C1 unit for thymidylate, purine and amino acid sbiosynthesis) and NADH. This is the only known pathway which completely degrades glycine. In *S. cerevisiae*, the glycine cleavage system is required for utilizing glycine as the sole nitrogen source (Sinclair and Dawes, 1995; Sinclair et al., 1996) and for utilizing glycine as the only source of C1-units in *shm1shm2* strains (Nagarajan, Ph. D. thesis). Regulation of GCS activity provides a mechanism to co-ordinate glycine metabolism with C1-metabolism and nitrogen utilization.

10 mM glycine dramatically stimulated *GCV3* expression (Table 4) and the C1-end products repressed expression (Table 10). This suggested that the activity of the GCS is dependent on the intracellular concentration of glycine and the availability of C1 units.

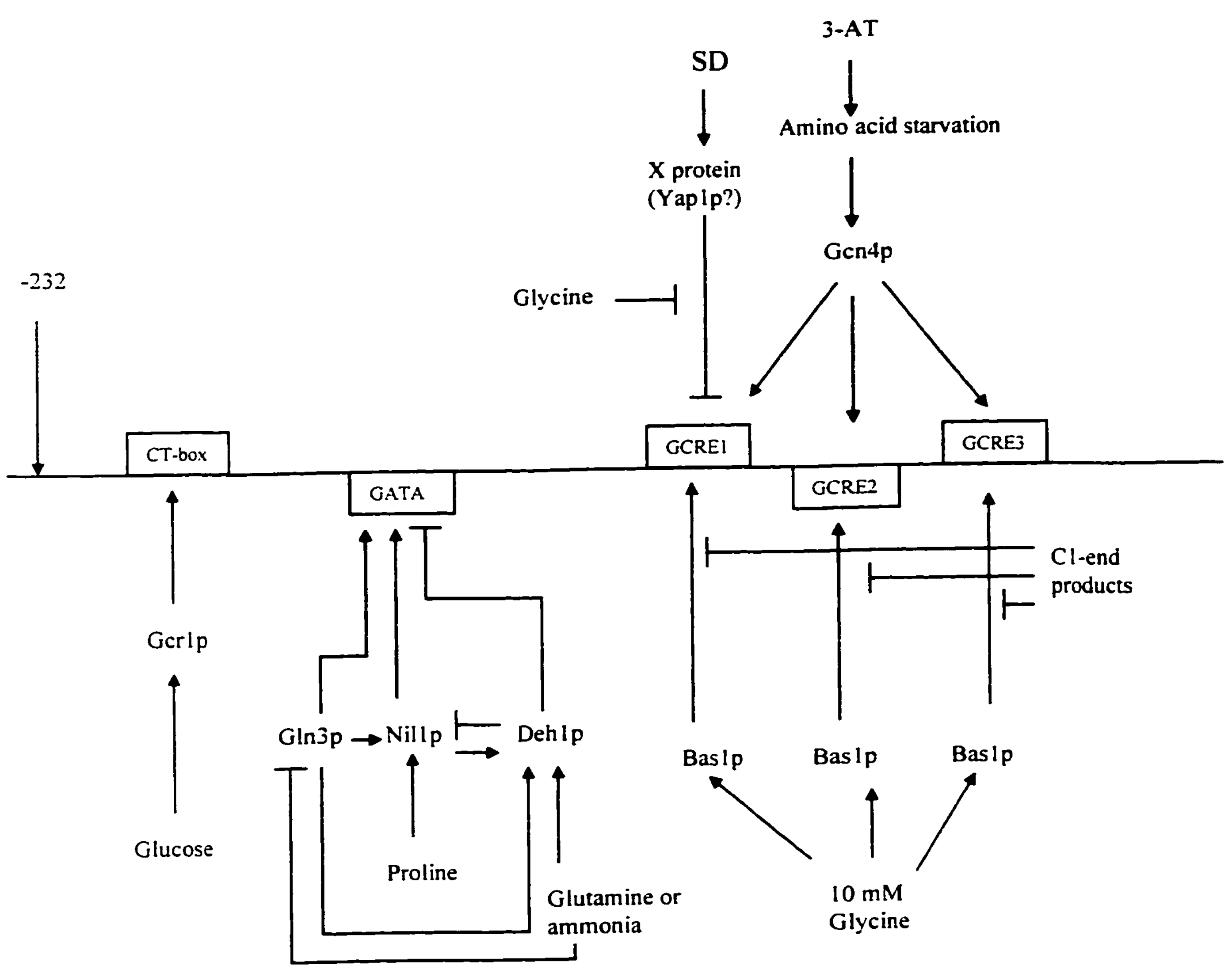
There are two major pathways for glycine biosynthesis in yeast: one pathway originates from an intermediate of glucose breakdown by glycolysis (3-phospho-D-glyceric acid or PGA) and proceeds via phosphorylated intermediates, and a second pathway starts with glyoxylate, a product of the anaplerotic glyoxylate cycle, that is necessary for gluconeogenesis. The latter pathway proceeds via non-phosphorylated intermediates and is the major one during growth on non-fermentable carbon sources such as ethanol and acetate. During growth on glucose, the enzymes for the "gluconeogenesis" pathway are repressed by glucose and synthesis of glycine proceeds mainly via PGA, the "glycolytic" pathway (Ulane and Ogur, 1972). The "glycolytic" pathway generates glycine directly from serine and also generates the active C1-unit, N^5N^{10}-methylenetetrahydrofolate. This reversible reaction takes place in both the cytosolic and mitochondrial compartments and is catalyzed by the enzyme glycine hydroxymethyltransferase. The mitochondrial and cytoplasmic versions of the enzymes are encoded by the *SHM1* and *SHM2* genes respectively (Barlowe and Appling, 1990; Pasternack et al., 1992). The cytosolic reaction accounts for about 75% of the intracellular C1-units generated during growth in minimal medium. The mitochondrial reaction, together with the reaction catalyzed by the glycine cleavage system, accounts for the synthesis of the remaining 25% (Pasternack et al., 1994). C1-units are required for biosynthesis of the C1-end products such as serine, methionine, histidine, formate, thymidylate, adenine and SAM (Mudd and Cantoni, 1964). Thus, the reaction catalyzed by *SHM1* and *SHM2* gene products, provides most of the intracellular glycine. Since excess glycine inhibits cell growth (Nagarajan, Ph. D. thesis; this study, Figure 9, Panel C), the activation of the GCS by glycine makes it possible for the cell to prevent glycine toxicity by reducing the concentration of

intracellular glycine. The C1-end products rescued cell growth in excess glycine condition (Figure 9, Panel D) and repressed *GCV3* expression (Table 10). This suggests that the C1-end products somehow reduce the intracellular levels of glycine by slowing down the synthesis of C1 units derived from serine. The toxicity of glycine may be due to high concentrations of glycine inhibiting cytoplasmic production of C1 units and /or the transportation of C1 units out of mitochondria. The net effect being that cells are deprived of C1 units. Since the slow growth of cells with glycine as the nitrogen source was significantly increased by supplementing the C1-end products, yeast cannot effectively make one or all of the C1-end products when glycine is the sole nitrogen source.

4.6. Overview of *GCV3* regulation.

GCV3 expression was found to be regulated by glycine and the C1-end products. *GCV3* was found to be regulated by Gcn4p, Bas1p, Gcr1p, Gln3p. Bas2p and an unknown protein are implicated as factors that repress expression. A model summarizing the regulatory mechanism controlling *GCV3* expression is presented in Figure 10.

Figure 10. Model depicting the regulation of *GCV3* in *S. cerevisiae*. Arrows indicate positive regulation. Closed bars indicate negative regulation. The CT-box is located at −191 bp relative to the translation start codon. The GATA sequence is at −162 bp. The GCREs are at −128 bp, −114 bp and −98 bp respectively. X protein is an unknown protein assumed to be responsible for repression of *GCV3* in this study.


3-AT
SD
Amino acid starvation
X protein
(Yap1p?)
Gcn4p
-232
Glycine
CT-box
GATA
GCRE1
GCRE2
GCRE3
C1-end
products
Gcr1p
Gln3p
Nil1p
Deh1p
Bas1p
Bas1p
Bas1p
Glucose
Proline
Glutamine or
ammonia
10 mM
Glycine

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