THE GENERAL CONTROL OF AMINO ACID BIOSYNTHETIC GENES IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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I. AN OVERVIEW OF THE GENERAL AMINO ACID CONTROL

A complex regulatory system exists in the yeast *Saccharomyces cerevisiae* which coupled derepression of enzymes in multiple amino acid biosynthetic pathways to starvation for any one of a number of different amino acids. This cross-pathway regulation, known as the general amino acid control, governs the expression of at least 30 enzymes in seven different amino acid biosynthetic pathways. It has been shown in a number of cases that general control-mediated derepression of enzyme expression is brought about by an increase in the steady-state levels of the corresponding mRNAs, suggesting that the general control operates at the level of transcription of the structural genes which encode the coregulated enzymes. Mutational analyses of the regulatory regions of two such genes, *HIS4* and *HIS3,* have led to the identification of a *cis*-acting regulatory sequence, $^{5'}$TGACTC$^{3'}$, which mediates the transcriptional response of these two genes to amino acid starvation. The same DNA sequence is present in the 5′ noncoding regions of a number of other structural genes subject to the general control, making it likely that this sequence mediates the derepression of all genes whose expression is governed by the general control.

Regulatory mutations unlinked to the coregulated structural genes have been isolated which impair the derepression of amino acid biosynthetic enzymes subject to the general control. These mutations, known as *gcn*$^-$ (for general control nonderepressible), confer upon cells an increased sensitivity to a variety of culture conditions which inhibit amino acid biosynthesis. This phenotype indicates that the derepression of amino acid biosynthetic enzyme levels is an essential aspect of the adjustment of the yeast cell to amino acid-limiting growth conditions. Other mutations known as *gcd*$^-$ (for general control derepressed) have the opposite effect and lead to constitutive derepression of enzymes subject to the general control. In addition to their roles in the regulation of amino acid biosynthetic enzymes, it appears that the *GCD* gene products also carry out functions required for cell viability in the absence of amino acid starvation; however, the nature of these functions remains to be defined.

The phenotypes of the *gcn*$^-$ and *gcd*$^-$ regulatory mutants indicate that the *GCN*$^+$ and *GCD*$^+$ genes encode, respectively, positive and negative *trans*-acting effectors of structural genes subject to the general control. Genetic studies have suggested that these factors interact with one another at some level to regulate the levels of a single positive regulator of transcription encoded by the *GCN4* gene. This makes the *GCN4* product the most likely candidate for the transcriptional activator which binds to the nucleotide sequence identified as the site of positive control at structural genes subject to the general control. In fact,

biochemical evidence now exists that a specific interaction occurs between the *GCN4* protein and the TGACTC sequences. Another recent finding of great importance is that *GCN4* expression is regulated by the other GCN and *GCD trans*-acting factors in the general control at the translational level. These factors appear to act by modulating the effects of *cis*-acting negative regulatory sequences in the 5′ leader of *GCN4* mRNA in response to amino acid starvation. Thus, general amino acid control is a multifaceted regulatory mechanism that draws upon several different strategies for controlling gene expression.

General amino acid control has been discussed in two recent reviews[1,2] published within the last 3 years. As a result, the author has decided to consider in detail only the most recent findings and to limit discussion of earlier work to the essential background information needed to evaluate more recent results. For a more detailed treatment of the earlier work, the above-mentioned reviews should be consulted and are frequently cited below as sources of information on the more well-established aspects of the general amino acid control.

The present review begins with a summary of the conditions known to bring about derepression of enzymes subject to the general control and a brief discussion of the molecular nature of the starvation signal. The scope of the derepression response is considered next with special reference given to amino acid biosynthetic pathways in which both general and amino acid-specific controls operate. The roles of the general control *trans*-acting factors in derepression follow, with emphasis given to recent findings concerning the interactions which take place among these factors. This leads to a consideration of the various *cis*-acting regulatory signals identified at structural genes subject to the general control. Finally, prospects for future research on the general control system are presented.

II. THE DEREPRESSION SIGNAL

A. Starvation of Yeast for a Variety of Single Amino Acids Signals Derepression of The General Control

Derepression of amino acid biosynthetic enzymes subject to the general control can be observed experimentally in a variety of ways. One common approach is to grow wild-type cells in the presence of amino acid analogues, such as 3-aminotriazole or 5-methyltryptophan, which inhibit histidine and tryptophan biosynthesis, respectively. (3-Aminotriazole inhibits the activity of imidazoleglycerol-phosphate dehydratase,[3] the product of *HIS3*;[4] 5-methyltryptohan appears to act primarily by false feedback inhibition of anthranilate synthase, the first enzyme in the tryptophan pathway.[5,6]) As a typical example, Table 1, line 1, shows the derepression of the *HIS4* gene (measured using a *HIS4-lacZ* gene fusion) observed in a wild-type strain in response to 3-aminotriazole-induced histidine starvation.

An alternative to the use of amino acid analogues is to culture strains containing leaky auxotrophic mutations (bradytrophs) on minimal medium (e.g., References 10 and 11). General control-mediated derepression of enzymes in a pathway affected by a bradytrophic mutation is required in order for such mutants to grow on minimal medium.[12] Bradytrophic mutations which impair histidine, arginine, lysine, isoleucine-valine, leucine, serine, or phenylalanine biosynthesis all lead to derepression of enzymes subject to the general control.[10,11] The inhibitory effect of methionine and proline analogues on the growth of non-derepressible *gcn*$^-$ mutants further suggests that starvation for either of these amino acids also leads to derepression of the general control.[12] Thus, starvation for any single amino acid from a group of at least ten leads to derepression of enzymes subject to general amino acid control.

Derepression of enzymes subject to the general control has also been observed in wild-type cells grown on minimal media supplemented with certain amino acids. These culture conditions include (1) ornithine or lysine added in the absence of arginine; (2) a methionine supplement provided in the absence of valine; (3) a leucine supplement provided in the

Table 1
EXPRESSION OF *HIS4-lacZ*, *GCN4-lacZ*, AND *URA3-lacZ* FUSION GENES IN AMINO ACID-COMPLETE, MINIMAL, AND HISTIDINE STARVATION MEDIUM[a]

	Gene fusion	Transformat genotype	Units β-galactosidase: Amino acid-complete	Minimal	Minimal + 3-aminotriazole
1	*HIS4-lacZ*	Wild-type	60	275	2000
2		*gcn2-1*	20	200	160
3		*gcn3-102*	15	230	260
4		*gcd1-101*	600	2000	3000
5		*gcn4-101*	15	100	300
6	*GCN4-lacZ*	Wild-type	10	10	120
7		*gcn2-1*	1	2	2
8		*gcn3-102*	30	5	15
9		*gcd1-101*	200	300	350
10		*gcn4-101*	30	40	120
11		*gcn2-1 gcd1-101*		250	250
12		*gcn3-102 gcd1-101*		25	75
13		*gcn4-101 gcd1-101*		250	180
14	*GCN4-501-lacZ*[b]	Wild-type		600	500
15		*gcn2-1*		1000	2000
16		*gcn3-102*		400	600
17		*gcd1-101*		1600	1000
18		*gcn4-101*		900	1200
19	*URA3-lacZ*	Wild-type	25	80	70
20		*gcn2-1*	30	100	100
21		*gcn3-102*	15	50	125
22		*gcd1-101*	5	25	30
23		*gcn4-101*	40	80	120

[a] Unpublished data from A. Hinnebusch in which all three fusions were examined together in the same experiment. The values for the *HIS4-lacZ* and *URA3-lacZ* fusions are in good agreement with those reported by Lucchini et al.[7] Each of the fusion genes is integrated in single copy at the *URA3* locus in closely related yeast strains of the indicated genotype. The enzyme assays were conducted as described.[7] Composition of the complete medium is given in Sherman et al.[8] Cells were cultured in this medium and in minimal medium for 6 hr to achieve early logarithmic growth. 3-Aminotriazole was added to minimal medium at 10 m*M* and cells were cultured for 6 hr in this medium to bring about histidine starvation.

[b] *GCN4-501-lacZ* differs from *GCN4-lacZ* by the deletion of 240 base pairs from the 5′ leader region of the *GCN4* transcription unit. This deletion removes all four short open reading frames from the *GCN4* leader. *GCN4-501-lacZ* was described previously as *GCN4Δ1-lacZ*.[9]

absence of isoleucine or valine; and (4) isoleucine provided in the absence of leucine. The derepressing effect of these culture conditions is also revealed by the reduced growth rate, relative to the wild type, of nonderepressible *gcn*⁻ mutants grown on the same media.[11,13] By the latter criterion, it is expected that growth of wild-type cells on minimal medium supplemented with all of the amino acids except tryptophan or arginine also leads to derepression of the general control.[11,13] The following is an attempt to explain the derepressing effects of at least some of these culture conditions on the basis of regulatory interactions between different amino acid biosynthetic pathways.

For cases (1) and (2) above, it was shown that the indicated supplements directly induce significant reductions in the pool sizes of those amino acids which, when added together with the derepressing amino acid, can restore repression. For example, the internal arginine pool is reduced ninefold in the presence of excess ornithine,[11] suggesting that ornithine-induced derepression is a consequence of arginine starvation. This could be the result of specific repression of arginine biosynthetic enzymes known to be mediated by ornithine (reviewed in Reference 1). Similarly, the derepressing effect of a medium containing all of the amino acids except tryptophan is probably attributable to feedback inhibition by phenylalanine and tyrosine of the biosynthesis of chorismate, a common precursor of all three aromatic amino acids.[1] This seems likely because omission from the medium of tyrosine and phenylalanine in addition to tryptophan reverses the inhibitory effect of the medium upon the growth of *gcn*$^-$ mutants.[13] The derepressing effects of excess lecuine, isoleucine, and methionine are probably also attributable to feedback inhibition or repression of a biosynthetic step shared with those amino acids able to restore repression; however, at present, the precise regulatory targets of these derepressing amino acids cannot be identified with certainty.[1]

The derepressing effect of a lysine supplement in the absence of arginine (case (1) above) may require a different explanation. A lysine supplement reduces the size of the total arginine pool,[11] but no known regulatory link exists between these two pathways to explain this effect. However, an additional consequence of a lysine supplement is to increase the proportion of intracellular arginine and ornithine found in the cytoplasm compared to the vacuole. In cells grown in unsupplemented medium, the proportion of these amino acids located in the cytoplasmic compartment is very low (10%), whereas addition of lysine to the medium increases the figure to 30%.[14] It has been suggested that this repartitioning leads to the known arginine + ornithine-mediated specific repression of arginine biosynthetic enzymes to levels below those ordinarily found on medium lacking arginine, and this leads to an arginine deficiency.[14] An untested prediction of this explanation is that the derepressing effect of a lysine supplement should be eliminated in regulatory mutants (*argR*-) in which the specific arginine repression mechanism is impaired.

B. Uncharged tRNA Signals Derepression of the General Control

Although depletion of an amino acid pool by any of the means described above leads to general control derepression, it is likely that reduction of an amino acid pool is not the direct signal for derepression. This follows from the fact that derepression can be observed in the absence of amino acid starvation in *ils1*$^-$ mutants which contain a temperature-sensitive isoleucyl-tRNA synthetase.[15,16] At 36°, such strains cannot survive because protein synthesis is blocked. At lower temperatures, the *ils1-1* mutation is not lethal, but reductions in synthetase activity and the level of isoleucyl-tRNA are evident. These defects are accompanied by a derepression of enzymes in at least four amino acid biosynthetic pathways known to be subject to the general control, in spite of the fact that the free isoleucine and valine pools are not diminished in these conditions.[15,16] Similar behavior was observed in *mes1*- mutants which produce a temperature-sensitive methionyl-tRNA synthetase.[17] These findings suggest that a reduction in the level of tRNA aminoacylation is a more direct signal for derepression than the depletion of an amino acid pool. The *gcn1-1* mutation blocks general control derepression in an *ils1-1* synthetase mutant,[16] showing that the *GCN1* function is required for the normal response to this derepressing condition just as it is required for derepression in response to the depletion of an amino acid pool. Although the presence of uncharged tRNA is sufficient to trigger derepression, it is not known whether uncharged tRNA is the most proximal signal for derepression of the general control. It is possible that the level of one or more labile polypeptides is monitored instead as an indicator of the availability of amino acids for protein synthesis.

There are other conditions which also bring about a degree of derepression of enzymes subject to general amino acid control, but the underlying basis for these effects is not yet clear. Growth at 37° on either minimal or complete medium leads to derepression of two arginine biosynthetic enzymes subject to the general control and also results in the same increases in amino acid pool sizes which are characteristic of cross-pathway derepression.[18] In addition, certain temperature-sensitive lethal mutations lead to derepression of an arginine biosynthetic enzyme subject to the general control (arginosuccinate lyase) at the permissive temperature without the imposition of amino acid starvation.[18,19] At the nonpermissive temperature, these mutations also lead to arrest at specific points in the cell division cycle (*cdc*- mutants, see Reference 20). They include (1) *CDC19* (encodes pyruvate kinase) and *flx80*, both of which are blocked at the earliest point in the G1 phase of the cell cycle; (2) ts4572, ts8740 (both of which block RNA synthesis at the restrictive temperature), and *flx18*, all of which arrest cell division somewhat later in G1; and 3 *cdc16* which is blocked in nuclear division. In addition to these cdc^- mutations, certain mutations that lead to respiratory deficiency also bring about a degree of derepression of the general control. The nuclear *pet18* mutation (allelic to *tra5*;[12,21]) and ethidium bromide-induced mitochondrial Pet^- mutations partially derepress expression of *HIS4*, a gene which is subject to the general control, but do not affect *URA3* expression, a gene which is not governed by the general control.[22]

It is possible that growth at 37° and the derepressing cdc^- and pet^- mutations just mentioned interfere with amino acid biosynthesis, *tRNA* aminoacylation, or protein synthesis, and thus indirectly generate the signal for derepression of the general control. (It is useful to note in this connection that the derepressing isoleucyl-tRNA and methionyl-tRNA synthetase mutations discussed above also lead to G1 arrest at the restrictive temperature.[16,23]) For example, the derepression associated with a respiratory deficiency might be related to the fact that many amino acid biosynthetic pathways have some enzymes located inside the mitochondria and others located in the cytoplasm.[1] Perhaps an energized mitochondrial membrane is required for efficient shuttling of biosynthetic enzymes and/or intermediates between the cytoplasmic and mitochondrial compartments. An alternate explanation is that one or more of the *CDC* or *PET* gene products listed above plays a more direct role in the regulation of the structural genes subject to the general control. A determination of the epistasis relationships between the derepressing cdc^- and pet^- mutations and the known gcn^- and gcd^- general control regulatory mutations should help to decide between these alternatives (see Section IV).

C. Repression in Amino Acid-Complete Medium

A number of genes which derepress in response to amino acid starvation also exhibit a greater degree of repression in cells grown on amino acid-complete medium compared to cells cultured on unsupplemented minimal medium. For example, *HIS4* gene expression in a wild-type strain is four-to fivefold lower in cells grown on amino acid-complete medium compared to minimal medium (see Table 1, line 1). Kinetic analysis has shown that derepression of these genes occurs very rapidly in a shift from amino acid-complete to minimal medium, establishing a new steady-state level of derepression in less than 1 hr.[24] This phenomenon has generally been interpreted as a manifestation of the general amino acid control.[18,24-29] However, as discussed below, there are several reasons to suspect that the two derepression phenomena may be mechanistically distinct.

First of all, complete medium repression of a given enzyme could be the result of an amino acid-specific repression system, several of which are known in yeast (reviewed in References 1, 2). Some of these amino acid-specific systems are activated by more than one amino acid and it is possible that as yet unidentified, multivalent repression systems mediate the complete medium repression of certain enzymes. In fact, in two instances it was shown that inactivation of an amino acid-specific repression mechanism can eliminate

the complete medium repression of a particular enzyme. One case deals with the arginine-specific repression of a number of arginine biosynthetic enzymes which are mediated by the *ARGR* genes. Growth of wild-type cells on undefined complete medium (*YPD*) or on minimal medium supplemented with 3% casamino acids leads to a repression of the ornithine carbamoyltransferase (*ARG3*) level two- to fourfold below its level in arginine-supplemented minimal medium. This result was interpreted as a superimposition of the general control upon the arginine-specific repression of *ARG3* expression. However, in an *argR*$^-$ mutant, in which the arginine-specific repression is impaired, there is no difference in the *ARG3* enzyme level between cells grown on arginine-supplemented minimal medium vs. amino acid-complete medium.[18] This suggests that the complete medium repression of *ARG3* expression is *ARGR* mediated. In a similar case, a *cis*-acting mutation which eliminates arginine-specific repression of one of the two subunits of carbamoylphosphate synthetase (*CPAI*) also eliminates the additional repressing effect of casamino acids upon *CPAI* expression beyond the level of repression exerted by arginine alone in wild-type cells.[25] (A complexity revealed in the latter study is that for some enzymes, casamino acids do not lead to nearly the same degree of repression as *YPD* medium. The use of these undefined media makes a comparative analysis of the findings difficult.)

A second problem with the idea that complete medium repression is an integral part of the general control response is that certain *gcn*$^-$ mutations substantially block derepression in response to amino acid starvation, but do not reduce the derepression ratio in a shift from amino acid-complete to minimal medium.[18,24,27,30] This is illustrated for the *HIS4* gene by the data shown in Table 1, lines 1 to 3 and 5. While *gcn*$^-$ mutations (particularly those in the *GCN4* gene) do lead to lower enzyme levels in cells grown on minimal medium, these mutations similarly reduce enzyme levels in cells cultured on amino acid-complete medium (References 7, 13, 24, 27 and Table 1). As a result, the derepression ratio in a shift from amino acid-complete to minimal medium in *gcn*$^-$ mutants is not diminished from that observed in wild-type cells. The fact that *gcn*$^-$ mutations impair derepression in response to severe amino acid starvation but do not prevent derepression in a shift from complete to minimal medium suggests that these two derepression phenomena may be mediated by different regulatory factors.

The third point deals with the level of repression of the *trans*-acting positive regulator of genes subject to the general control in cells grown on minimal medium. As will be discussed below, the *GCN4* product appears to be the direct activator of structural genes subject to the general control (see Section IV). As shown in Table 1, line 6, expression of a *GCN4-lacZ* fusion enzyme is substantially derepressed in wild-type cells in response to a histidine starvation — a response qualitatively identical to that of structural genes which are subject to *GCN4* positive control, e.g., *HIS4* (Table 1, line 1). This finding is consistent with the idea that derepression of genes subject to the general control is mediated by an increase in the level of the *GCN4* gene product in amino acid-starved cells. In contrast to the effect of amino acid starvation, *GCN4-lacZ* expression does not derepress in a shift from amino acid-complete medium to minimal medium (Table 1, line 6). This suggests that the derepression of structural genes observed in a complete medium to minimal medium shift is *GCN4*-independent. Of course, the activity of the *GCN4* protein could increase in such a shift even though its level of expression remains constant. However, taking all of the above into consideration, there is a strong possibility that the derepression response in a complete medium to minimal medium shift occurs by a different regulatory mechanism then is responsible for derepression in response to amino acid starvation. Until the matter can be resolved, it is advisable to measure derepression in a shift from minimal medium to amino acid starvation conditions when analyzing the general control response, since it is in these conditions that the requirement for the *GCN* factors is most clearly defined.

III. THE SCOPE OF THE DEREPRESSION RESPONSE

A. Amino Acid Biosynthetic Enzymes Which Are Subject to the General Control

Table 2 lists those amino acid biosynthetic pathways in which the expression of one or more enzymes is governed by the general control. Most of this information was obtained by the determination of enzyme levels after starvation of bradytrophs by growth in minimal medium or by culturing wild-type cells in the presence of an inhibitor of amino acid biosynthesis (see Section II). For each enzyme listed in Table 2, it was shown that derepression occurs in response to starvation for an amino acid whose biosynthesis does not involve that particular enzyme. This condition is required to distinguish between general amino acid control and amino acid-specific regulatory systems. An alternative approach has been to compare the expression of the enzyme in wild-type cells grown in the absence of amino acid starvation with that found under the same conditions in a strain containing a *gcd*- regulatory mutation, since *gcd*- mutations lead to derepression of enzymes subject to the general control even in the absence of a starvation signal. Using these approaches, 30 enzymes in seven different amino acid biosynthetic pathways have been shown to be subject to general amino acid control. The derepression ratios of these enzymes range from two- to tenfold, depending upon the enzyme in question and the derepression regime employed. (See Table 2 for complete references.)

Table 2 also indicates the enzymes for which derepression of the corresponding mRNA levels have been examined. These studies were made possible by the molecular cloning of the structural genes which encode these enzymes. The cloned DNAs provide radiolabeled probes for the homologous mRNAs and were used in RNA blot-hybridization experiments (Northern analysis) to compare the steady-state levels of the mRNAs present in cells grown under general control repressing vs. derepressing growth conditions. In each case, it was found that the same conditions which lead to derepression of enzyme activity also lead to an increase in the steady-state level of the corresponding mRNA. Furthermore, except in certain cases to be discussed below, the observed increases in enzyme and mRNA levels are of similar magnitude.[29] These results suggest that the derepression of enzyme levels mediated by the general control is brought about by an increase in the cellular concentration of the mRNA molecules which encode the regulated enzymes. In the case of the *TRP5* gene, the result of in vivo pulse-labeling of *TRP5* mRNA in repressing vs. derepressing growth conditions[36] further suggests that it is the rate of transcription initiation that increases in response to derepression of the general control. The mechanism of this transcriptional regulation will be considered in detail below.

B. Interplay Between Amino Acid-Specific Control and the General Amino Acid Control

With one exception (the *TRP1* product), all enzymes examined in the tryptophan, arginine, histidine, and lysine pathways are subject to the general control (Table 2). In the leucine and isoleucine-valine pathways, it has been reported that certain enzymes are subject to the general control whereas others are not. However, the failure to observe general control-mediated derepression is a negative result and, as discussed below, may have an alternative explanation.

Many arginine pathway enzymes are subject to arginine-specific repression in addition to the general control. It has been shown that starvation for an amino acid other than arginine does not lead to full derepression of *ARG* enzymes subject to arginine-specific repression unless the specific repression mechanism is inactivated by a mutation. In contrast, starvation of wild-type cells for arginine releases both repression mechanisms and leads to derepressed enzyme levels much higher than those observed in response to starvation for any other amino acid.[10,25,26,29,33] For two enzymes, ornithine carbamoyltransferase (encoded by *ARG3*), and a subunit of carbamoylphosphate synthetase (encoded by *CPA1*), in which arginine-specific

Table 2
ENZYMES SUBJECT TO THE GENERAL AMINO ACID CONTROL[a]

			Derepression in response to			
Pathway	**Enzyme**	**Genetic locus**	**Amino acid starvation**	***gcd***	**mRNA examined**	**Ref.**
Trp	Anthranilate synthase	*TRP2*	+	+		5, 10, 12, 31
	Anthranilate PR-transferase	*TRP4*	+	+		5, 31
	Indoleglycerol-P synthase	*TRP3*	+	+	+	5, 31, 41
	Tryptophan synthase	*TRP5*	+	+	+	31, 36
	PR-anthranilate isomerase	*TRP1*	−	−		31, 42
	α-IPM isomerase	*LEU1*	−	−		35
	β-IPM dehydrogenase	*LEU2*	−	−		35
Gln	Glutamine synthetase	*GLN1*	+	+		43
	NAD-GDH		+			10, 43
Glu	NADP-GDH	*GDH1*	−			10, 12
Met	Sulfite reductase	*MET10* *MET5* *MET18* *MET19* *MET20*	−			12
	Isoleucyl-tRNA synthetase	*ILS1*	+	+	+	37
	Fumarase		+			10
Trp	Anthranilate synthase	*TRP2*	+	+		5, 10, 12, 31
	Anthranilate PR-transferase	*TRP4*	+	+		5, 31
	Indoleglycerol-P synthase	*TRP3*	+	+	+	5, 31, 41
	Tryptophan synthase	*TRP5*	+	+	+	31, 36
	PR-anthranilate isomerase	*TRP1*	−	−	−	31, 42
Arg	Acetylglutamate synthase	*ARG2*	+			32
	Acetylglutamate kinase	*ARG6*	+			33
	Acetylglutamyl-P reductase	*ARG5*	+			33
	Acetylornithine amino transferase	*ARG8*	+			5
	Acetylornithine acetyltransferase	*ARG7*	+			32
	OTCase	*ARG3*	+	+	+	5, 10, 12, 18, 29, 33
	Arginosuccinate lyase	*ARG4*	+		+	10, 18, 27, 29
	Carbamoyl-P synthetase	*CPA1*	+		+	25, 26
		CPA2	+		+	25, 26, 29
His	ATP PR-transferase	*HIS1*	+		+	10, 12, 40
	PR-AMP cyclohydrase	*HIS4A*	+	+	+	12, 27, 29, 30, 39

Table 2 (continued)
ENZYMES SUBJECT TO THE GENERAL AMINO ACID CONTROL[a]

Pathway	Enzyme	Derepression in response to: Genetic locus	Amino acid starvation	gcd-	mRNA examined	Ref.
	Histidinol dehydrogenase	*HIS4C*	+	+	+	5, 12, 27, 29, 30,39
	Imidazoleglycerol-P dehydratase	*HIS3*	+	+	+	34, 38
	Histidinol-P aminotransferase	*HIS5*	+			5, 46
	Histidinol-P phosphatase	*HIS2*	+	+		5, 12
Lys	Homocitrate synthase		+			44
	Saccharopine dehydrogenase	*LYS1*	+			10, 44
	Saccharopine reductase	*LYS9* *LYS13* *LYS14*	+			44
	α-Aminoadipate reductase	*LYS2* *LYS5*	+	+		12, 44
Ilv	Transaminase B		+	+		10, 45
	Threonine deaminase	*ILV1*	+	+		45
	AHAS	*ILV2*	−			12, 45
Leu	α-IPM synthase	*LEU4* *LEU5*	+	+		35
	α-IPM isomerase	*LUE1*	−	−		35
	β-IPM dehydrogenase	*LEU2*	−	−		35
Gln	Glutamine synthetase	*GLN1*	+	+		43
	NAD-GDH		+			10, 43
Glt	NADP-GDH	*GDH1*	−			10, 12
Met	Sulfite reductase	*MET10* *MET5* *MET18* *MET19* *MET20*	−			12
	Isoleucyl-tRNA synthetase	*ILS1*	+	+	+	37
	Fumarase		+	−		10

[a] Adapted from Reference 1.

repression can partially block general control-mediated derepression,[10,25] it is interesting that the derepression block seems to apply only to the enzyme levels: the mRNAs which encode these enzymes derepress in response to starvation for a variety of amino acids to the same extent as that observed in an arginine starvation, in the presence or absence of functional arginine-specific repression.[26,29] This finding has lead to the idea that arginine-specific repression operates at the level of translation.[26,29] In this model, the elevated levels of *ARG3* and *CPA1* mRNAs produced in amino acid-starved cells are efficiently translated only in the event of arginine starvation. In cells starved for amino acids other than arginine, the level of arginine is presumed to be high enough even in the absence of an arginine supplement to block efficient translation of these mRNAs and thus override general control-mediated derepression.[10,18,25,26,29]

Seemingly at odds with the idea that the *ARGR* regulatory factors function at the trans-

lational level is the recent finding that two *cis*-dominant point mutations which substantially reduce arginine repression of *ARG3* map upstream from the normal 5′ end of *ARG3* mRNA.[47] A similar finding was made for a constitutively derepressing *cis*-dominant point mutation at *CAR1*,[48] a gene encoding a catabolic enzyme for which translational control has also been suggested on the basis of discrepancies between enzyme and mRNA levels.[29] In the latter case, it has been shown that the 5′ end of mature *CAR1* mRNA in the *cis*-dominant mutant is the same as in wild-type cells,[49] ruling out a change in the structure of the mRNA as the explanation for the altered regulation in this mutant. The fact that the dominant constitutive mutations map outside of the transcription units of these two genes is more consistent with a transcriptional rather then a translational regulatory mechanism for arginine-specific repression. Reconciling these apparently contradictory observations in the context of a translational model would seem to require that the *cis*-acting mutations lead to the production of transcripts which are less sensitive to arginine repression than are transcripts of the same sequence made in wild-type cells. At the moment, molecular models to explain this sort of regulatory mechanism are lacking. However, in spite of the uncertainties about the molecular details of arginine-specific repression, it seems clear that the pathway-specific response can significantly alter the outcome of general control-mediated derepression upon the expression of arginine biosynthetic enzymes subject to dual control.

A similar situation appears to exist in the regulation of lysine biosynthesis.[44] Addition of lysine to minimal medium leads to a repression of several lysine biosynthetic enzymes. Because a lysine supplement was not found to affect the expression of *ARG3*, it was suggested that the repressing effect of lysine is specific for *LYS* enzymes. In fact, an unlinked regulatory mutation known as *lys80*$^-$ was isolated which impairs lysine-mediated repression of *LYS* enzymes but does not affect *ARG3* expression. Interestingly, the derepressing effect of starvation for amino acids other than lysine on *LYS* enzyme expression is much clearer in *lys80*$^-$ cells than in wild-type cells. This is particularly true for saccharopine reductase which exhibits no derepression in response to arginine or isoleucine-valine starvation in a wild-type strain, but shows two- to fourfold derepression in a *lys80*$^-$ strain grown in the same conditions. By analogy with the arginine enzymes, it appears that the lysine-specific repression system can suppress the effects of general control-mediated derepression; however, in this case, the level of gene expression at which this suppression operates is completely unknown.

In view of the above considerations, it is noteworthy that in each of the pathways listed in Table 2 (except *Trp*) in which an enzyme has been reported to be insensitive to general control repression (Ilv, Leu, Gln, Met, Glu), there is evidence that a pathway-specific repression mechanism exists (see Reference 1). Thus, it is possible that in one or more of these cases, amino acid-specific repression mechanisms have obscured general control-mediated derepression in experiments conducted to detect cross-pathway regulation of these enzymes. For example, in the case of the *ILV2* gene product (AHAS), neither starvation for histidine nor a *gcd-* mutation was found to bring about derepression of enzyme activity;[12] however, starvation for isoleucine, valine, or leucine was reported to derepress *ILV2*.[50] Moreover, these three amino acids act in concert to repress the AHAS level below the value observed in cells grown on minimal medium (reviewed in Reference 1). Taken together, these observations could indicate either that *ILV2* is subject only to pathway-specific repression, or that *ILV2* is also subject to the general control, but that the effects of the latter can only be observed under conditions which also derepress the multivalent, amino acid-specific repression system, namely, starvation for leucine, isoleucine, or valine.

The same comments apply to sulfite reductase which is repressed by methionine and greatly derepressed by methionine starvation,[17] but is unaffected either by histidine starvation or by a *gcd1*$^-$ mutation. Likewise, leucine and threonine-specific repression of *LEU1* and *LEU2*[35,51,55] may suppress the cross-pathway regulation of these genes in response to star-

vation for other amino acids.[35] In each of these cases, by analogy with the results discussed above for the *ARG* and *LYS* pathways, it will be necessary to examine the response to amino acid starvation in a regulatory mutant in which amino acid-specific repression is abolished before it can be concluded that a particular enzyme is not subject to the general control.

The regulation of glutamine synthetase (*GLN1*) expression presents a different example of the interplay between general and amino acid-specific regulatory mechanisms with somewhat different consequences. This enzyme is subject to a dramatic repression by glutamine which is mediated, in part, by glutamine-inactivation of a positive regulator encoded by the *GLN3* gene.[43,56,57] In glutamine-free medium, in which *GLN1* is greatly derepressed, neither starvation for histidine nor a *gcd1*$^-$ mutation leads to any additional derepression of glutamine synthetase activity.[12,43] However, in glutamine-supplemented medium, in which the enzyme is repressed to a low level, both conditions result in derepresson of glutamine synthetase activity.[43] Thus, the effect of amino acid starvation is observed only when glutamine-specific repression is operating. (Note that this situation is the exact opposite of arginine and lysine-specific regulation in which derepression by the general control is most clearly observed when pathway-specific repression is removed.) To explain the regulatory pattern of *GLN1* expression, it has been suggested that glutamine synthetase is subject to the general control but that *GLN3*-mediated derepression of *GLN1* in glutamine-free medium is so great that an additional increase in *GLN1* enzyme expression in response to amino acid starvation is either impossible or undetectable.[43] It is not clear why this should be the case, especially since general control-mediated derepression of NAD-dependent glutamate dehydrogenase, another enzyme subject to *GLN3* positive control, is detectable either in the presence or absence of glutamine.[10,43] In fact, it appears that much of the starvation-induced derepression of glutamine synthetase may be the indirect consequence of the effect of general control derepression upon *GLN3* activity. This follows from the fact that derepression of glutamine synthetase in response to histidine starvation is significantly reduced by a *gln3*$^-$ mutation. Thus, derepression of *GLN1* in response to amino acid starvation is mediated, at least in part, by the glutamine-specific positive effector *GLN3*. The mechanism of *GLN3* activation in cells starved for amino acids other than glutamine is not known, but is presumed to involve a reduction in the effective glutamine pool.[43] The fact that a degree of glutamine synthetase derepression remains during histidine starvation of *gln3*$^-$ cells has been taken to indicate that a portion of the glutamine synthetase response to amino acid starvation is a direct effect of the general control.[43] However, because glutamine-specific repression also still remains in *gln3*$^-$ cells,[43] the possibility exists that this residual derepression is likewise an indirect effect of the consequences of general control derepression upon glutamine-specific regulation. In spite of the uncertainties regarding the molecular basis for the effect of amino acid starvation upon *GLN1* expression, these results are of great interest because they suggest that the general control can affect the impression of some enzymes indirectly by producing changes in amino acid pool sizes which, in turn, can influence amino acid-specific regulatory mechanisms.

C. General Control Derepression of Enzymes Within a Pathway Is Not Strictly Coordinate

The levels of some enzymes in amino acid biosynthetic pathways which are subject to the general control derepress more than others. This is most notable in the histidine[11,12] and lysine[44] pathways where the derepression ratios among enzymes in the same pathway can vary by as much as threefold. Slightly smaller differences in derepression ratios have been noted for enzymes in the arginine pathway.[29] For the lysine and arginine pathways, these figures refer to the situation in which pathway specific repression is inactive, and in both cases, the relative derepression ratios are affected somewhat by the imposition of the amino acid specific regulatory mechanisms which operate on these pathways.[10,29,44]

The full significance of differences in derepression ratios among enzymes in the same

pathway is not immediately obvious. One comment which generally applies is that enzymes which carry out rate-limiting steps in these pathways (e.g., *HIS1*, *CPAI*, *CPAII*, *TRP2*, and homocitrate synthase; (see Reference 1) also exhibit the greatest derepression ratios.[11,12,26,29,31] Obviously, this would tend to ensure that general control-mediated derepression leads to increased flux through the regulated pathways. However, other enzymes in these pathways are derepressed as much or even more so than the apparent rate-limiting steps and the necessity for these additional increases is unclear. Perhaps under starvation conditions the kinetic parameters of a pathway are altered from their values in nonstarved cells so that additional steps become rate-limiting.

D. Changes in Amino Acid Pools Are Associated with Derepression of the General Control

Derepression of the general control is associated with significant changes in the sizes of several different amino acid pools.[10,18] The greatest effects are seen in the tyrosine, phenylalanine, lysine, arginine, and histidine pools which exhibit increases ranging from two- to tenfold in response to starvation for any one of several different amino acids. The leucine and glutamate pools show less striking increases in the range of 50 to 100% and the effects on these pool sizes are not observed consistently. There are also indications that the tryptophan and methionine pools increase in size in response to starvation for other amino acids, although the small sizes of these pools have made their measurement difficult.[6,10,18] Thus, with the exception of isoleucine, valine, and perhaps also glutamine, the amino acid pools in starved cells increase in size for each of the pathways in which at least some of the enzymes undergo derepression in amino acid-starved cells. This observation is consistent with the idea that the derepression of enzyme levels leads to increased flux through the derepressed pathways. (The dramatic increases in the sizes of the phenylalanine and tyrosine pools in response to amino acid starvation may indicate that enzymes in these pathways are also subject to the general control. In fact, preliminary findings indicate that *ARO3* and *ARO4*, which encode isozymes that carry out the first step in aromatic amino acid biosynthesis, are both subject to the general control.[58])

The absence of a consistent increase in the leucine, isoleucine, and valine pools in cells starved for other amino acids is probably related to the above-mentioned fact that certain enzymes in the Leu and Ilv pathways fail to derepress under such starvation conditions. As suggested in Section II, pathway-specific repression mechanisms may suppress the response of these enzymes to derepression by the general control. By contrast, the increase in the glutamate pool observed in amino acid-starved cells may be the result of an increase in the activity of NAD-dependent glutamate dehydrogenase rather than increased synthesis of this enzyme, since no increase in the enzyme level has been observed in the same starvation conditions which lead to an increase in the size of the glutamate pool.[10,12]

It is not clear whether the substantial increases in the sizes of aromatic and basic amino acid pools which result from derepression of the general control have any role in the cells's adaptation to starvation for other amino acids. In other words, the significance of the cross-pathway character of the general control is not understood. One possibility is that the accumulation of these particular amino acids alters the partitioning of other amino acids in starved cells, producing an increase in the cytoplasmic fraction of the limiting amino acid(s) which can be utilized for protein synthesis. For example, it was noted in Section II that a lysine supplement can increase the cytoplasmic concentration of arginine and ornithine. Moreover, arginine and histidine supplements have the same effect on a number of different amino acid pools.[14] In these instances, the supplemented basic amino acids make up 50% or more of the total amino acids and are located predominantly in the vacuole. In histidine-starved cells, the concentrations of arginine, ornithine, and lysine also become very large and make up almost three fourths of the total vacuolar amino acid pool. By analogy with

the effects of basic amino acid supplements on amino acid partitioning, the naturally occurring vacuolar accumulation of basic amino acids in starved cells might result in larger cytoplasmic fractions for other amino acids. This could provide a mechanism for greater utilization of limiting amino acids in starvation conditions. However, it should be noted that many amino acid pools are either unchanged or actually decrease in their cytoplasmic proportions in amino acid-starved cells.[14] This seems inconsistent with the idea that altered partitioning of amino acid pools is the major physiological consequence of general control derepression, although it is possible that the repartitioning effect is more pronounced for these amino acids when they become the limiting amino acids.

An alternative explanation for the increases observed in multiple amino acid pools in cells starved for a single amino acid is that the overproduction of multiple basic and aromatic amino acids is of little consequence in the adaptation of the cell to starvation for other amino acids, and occurs simply because mechanisms to fully prevent it do not exist. If the overproduction of these multiple amino acids does not interfere with the response to starvation for a different amino acid, there would be little selection pressure to produce regulatory mechanisms to prevent cross-pathway derepression from occurring. In fact, a coupling of the derepression of many biosynthetic pathways to a single derepression signal (accumulation of uncharged tRNA) can be viewed as the simplest solution to the complex problem of increasing the biosynthetic capacity of multiple pathways, each of which contributes to the same macromolecular synthesis, when the end product of any one pathway becomes limiting. Such a system requires only a single set of *cis* and *trans*-acting regulatory elements to mediate the derepression of all biosynthetic enzymes which are subject to the common control. As will be discussed in the following sections, general amino acid control appears to be structured in exactly this fashion.

IV. *TRANS*-ACTING FACTORS IN THE GENERAL AMINO ACID CONTROL

A. Mutations in *GCN* Genes Lead to Nonderepressibility of the General Control

Mutations in multiple unlinked genes have been isolated which alter the regulation of enzymes subject to general amino acid control (Table 3). Based on their phenotypes, these mutations fall into two different classes. In one class are recessive mutations mapping in five different genes, known as *GCN* genes. These mutations lead to an increased sensitivity to a variety of culture conditions which inhibit amino acid biosynthesis. The phenotype of *gcn*$^-$ mutants is explained by the fact that a mutation in any one of the five *GCN* genes blocks the derepression of amino acid biosynthetic enzymes in amino acid-starved cells. The nonderepressibility of *HIS4* expression in *gcn*$^-$ cells when grown in histidine starvation conditions is shown in Table 1, lines 1 to 3 and 5. Because the *gcn*$^-$ mutations are recessive, this phenotype indicates that the *GCN* genes encode positive regulators of enzyme expression. For each of the *GCN* genes, it has been shown that a mutation in the gene blocks the derepression of at least two different amino acid biosynthetic pathways in response to starvation for at least two different amino acids (references given in Table 3). Moreover, the variety of amino acid analogues and amino acid imbalances to which all *gcn*$^-$ mutants are sensitive[12,13,27] is consistent with the idea that each of the *GCN* gene products promotes cross-pathway derepression in response to any amino acid starvation known to signal derepression in wild-type cells.

The derepression defect in *gcn*$^-$ mutants appears to be the result of nonderepressibility of the mRNAs which encode enzymes subject to the general control.[24,27] Although it has not been shown directly that the rate of transcription initiation at structural genes subject to the general control is reduced in *gcn*$^-$ cells, analyses of the *cis*-acting regulatory sequences required for the derepression of these genes suggest that transcription initiation is indeed the regulatory target of the *GCN* gene products (see Section V).

Table 3
TRANS-ACTING REGULATORY MUTATIONS OF THE GENERAL AMINO ACID CONTROL

Locus	Alleles: Current designation	Alleles: Previous designation	Phenotype	Genetic interactions: Epistatic to	Genetic interactions: Hypostatic to	Map position
GCN1	*gcn1-1* *gcn1-2* *gcn1-201*	*ndr1-1*(5) *ndr1-2*(5) *aas103-15*(27)	Nonderepressible (5, 31, 18, 11, 35, 27)	*ils1-1*(16)	*gcd1-101*(13) *gcd2-1*(58) Multi-copy *GCN4*$^+$(13) *GCN4Δ 1*(60) *GCN4-501*^{a}(62)	
GCN2	*gcn2-1* *gcn2-101* *gcn2-201*	*ndr2-1*(5) *aas1-1*(12) *aas102-15*(27)	Nonderepressible (5, 12, 6, 30, 27)		*gcd1-101*(12, 13) *gcd2-1*(31, 58) Multi-copy *GCN4*$^+$(13) *GCN4Δ 1*(60) *GCN4-501*(62)	IV,R (13)
GCN3	*gcn3-101* *gcn3-102* *gcn3-103*	*aas2-1*(12) *aas2-2*(13) *aas2-3*(13)	Nonderepressible (12, 13, 7)		*gcd2-1*(58) *Multi-copy GCN4*$^+$(13) *GCN4Δ 1*(60) *GCN4-501*(62)	XI,R (13)
GCN4	*gcn4-201* *gcn4-101* *gcn4-102* *gcn4-600*	*aas101*-1(27) *aas3-1*(13) *aas3-2*(13) *arg9*(13)	Nonderepressible (27, 13, 24, 7, 43, 61)	*gcd1-101*(13) *gcd2-1*(58)		V,R (13, 27)
	GCN4Δ 1(60) *GCN4-501*^{a}*(62)*		Constitutively derepressed	*gcn1*(60) *gcn2*(60, 62) *gcn3*(60)		
GCN5	*gcn5-201*	*aas104-1*(27)	Nonderepressible (27)			

GCD1	*gcd1-101*	*tra3-1*(12)	Constitutively derepressed (12, 30, 39, 37, 7, 43, 61)	*gcn1-2*(13) *gcn2-1*(13) *gcn2-101*(12, 13) *gcn3-102*(13) *gcn3-103*(13)	*gcn4-101*(13) *gcn4-102*(13) *gcn4-600*(13)	XV,R (59)
GCD2	*gcd2-1*	*cdr1-1*(31)	Constitutively derepressed (31, 35, 41, 7)	*gcn2-1*(31) *gcn1*(58) *gcn3*(58)	*gcn4*(58)	
GCD3	*gcd3-1*	*cdr2-1*(42)	Constitutively derepressed (42)	*gcn1*(58)	*gcn4*(58)	

[a] The *GCN4-501* allele is a deletion similar to *GCN4Δ1* in which sequences in the *GCN4* mRNA 5′ leader containing the four short open reading frames have been deleted. The end points of the deletions are the same as those described in Reference 9 for *GCN4Δ-lacZ*.

Whereas all *gcn*$^-$ mutations block derepression of enzyme levels in response to amino acid starvation, only *gcn4*$^-$ mutations lead to significant reductions in enzyme expression in cells grown in nonstarvation conditions. For example, the *gcn4-101* mutations lowers the level of arginosuccinate lyase (*ARG4*) activity five- to sixfold in cells grown on minimal medium.[19] This effect can be attributed to a reduction of similar magnitude in the steady-state level of *ARG4* mRNA observed in the same conditions.[27] The requirement for *GCN4* in maintaining proper levels of *ARG* enzymes in minimal medium is also indicated by the fact that *gcn4*$^-$ mutations lead to leaky arginine auxotrophy. (In fact, the first *gcn4*$^-$ mutation isolated was designated *arg9*.[13]) No other auxotrophy is apparent in *gcn4*$^-$ mutants, indicating a lesser requirement for *GCN4* in the expression of enzymes in other pathways in nonstarvation conditions. A three- to fourfold reduction in *HIS4* expression is observed in *gcn4*$^-$ cells relative to wild-type cells when both are grown in minimal medium,[7,27] (see Table 1, lines 1 and 5) but apparently the magnitude of this effect is not great enough to produce a histidine requirement. In contrast to the effects on *ARG4* and *HIS4* expression, a *gcn4*$^-$ mutation has little or no effect upon *TRP* enzyme expression in cells cultured in minimal medium.[58] These results indicate differing requirements for *GCN4* in maintaining the levels of different enzymes in the absence of amino acid starvation.

Mutations in *GCN* genes other than *GCN4* have lesser effects on enzyme expression in cells grown on minimal medium.[5,7,11,12,18,27,31,35] This is illustrated for the *HIS4* gene in Table 1, lines 1 to 3, and 5. Whereas the *gcn4-101* mutation leads to a threefold reduction in *HIS4-lacZ* expression on minimal medium, the *gcn2-1* and the *gcn3-102* mutations have less than a twofold effect in the same conditions. Consistent with these results is the fact that the growth rates of *gcn1*$^-$, *gcn2*$^-$, and *gcn3*$^-$ strains on minimal medium show little or no reduction relative to the growth rates of closely related wild-type strains cultured in the same conditions.[5,11,27] By contrast, the growth rate of *gcn4*$^-$ strains on minimal medium is generally twofold less than wild-type as the result of the leaky arginine auxotrophy associated with *gcn4*$^-$ mutations.[13,27]

The negative effect of *gcn4-* mutations on the levels of certain enzymes during growth in minimal medium suggests that *GCN4* positive regulatory function is required to maintain normal levels of gene expression in the absence of amino acid starvation, in addition to its role in the derepression of gene expression in response to starvation. However, this result could be interpreted differently as an indication that general control repression is not complete in minimal medium and that activation or derepression of the *GCN4* gene product is required in these "mild" starvation conditions to achieve proper levels of gene expression. There are certain observations at odds with the latter view. First, as already mentioned in Section II, the requirement for *GCN4* function is evident even in amino acid-complete medium (Table 1, line 5), suggesting that *GCN4* is needed for proper gene expression in all growth conditions. The second observation relates to genetic and molecular evidence (to be discussed below) which indicates that *GCN4* is the most direct of the positive regulators of structural genes which are subject to the general control and that the *GCN1*, *GCN2*, and *GCN3* products function indirectly as positive regulators of *GCN4* (see Figure 1). Given the fact that *gcn1*, *gcn2*, and *gcn3* mutations have little effect on gene expression and growth rate in cells growing on minimal medium, it follows that positive regulation of *GCN4* by the other *GCN* factors is not required for normal levels of enzyme expression in cells grown on minimal medium. This conclusion is supported by the finding shown in Table 1, line 6 that a *GCN4-lacZ* fusion protein is expressed at similar levels in cells grown on amino acid-complete vs. minimal medium, suggesting that *GCN4* is already fully repressed in cells grown on minimal medium. Taken together with the negative effect of *gcn4*$^-$ mutations on gene expression, these results suggest that a basal level of *GCN4* function is necessary for proper expression of genes subject to the general control in cells growing on either minimal medium or amino acid-complete medium, but that the *GCN1*, *GCN2*, *GCN3*-dependent mechanism for de-

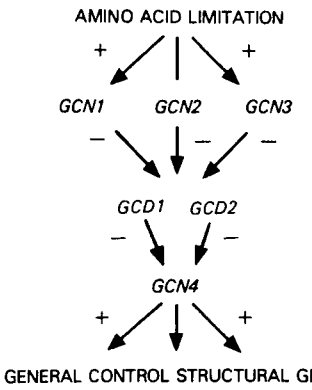

FIGURE 1. Hierarchy of *trans*-acting factors in the general amino acid control.[13] Activation is symbolized by a +; antagonism or repression by a –.

repression of *GCN4* is not required in either of these nonstarvation conditions. Positive regulation of *GCN4* by the other *GCN* factors is required only in response to amino acid starvation.

B. Mutations in *GCD* Genes Lead to Constitutive Derepression of the General Control

A second class of regulatory mutations known as *gcd*$^-$ lead to a phenotype which is opposite that of the *gcn*$^-$ mutants. Recessive mutations in *GCD* genes result in constitutive derepression of enzymes subject to the general control (e.g., Table 1, line 4). Thus, *gcd*$^-$ mutations bypass the starvation signal normally required for the derepression response. Mutations in three different *GCD* genes have been described in some detail (see Table 3) and it now appears that additional *GCD* loci exist.[58,63] The *gcd1-101* mutation leads to derepression of enzyme levels even in amino acid-complete medium,[12,30,37] indicating that the constitutive derepression observed in this mutant is not the result of a leaky amino acid auxotrophy. It has also been reported that levels of all aminoacylated tRNAs are normal in a *gcd1-101* strain,[64] ruling out a defect in tRNA charging as the explanation for the constitutive derepression observed in *gcd1-101* cells. A similar conclusion is suggested for *GCD2* on the basis of genetic data: unlike the *ils1-1* tRNA synthetase mutation, the constitutive derepression associated with a *gcd2*$^-$ mutation is not suppressible by a *gcn1*$^-$ mutation.[16,58] This argues that *GCD2* functions at a step beyond the detection of a deficiency in aminocylated tRNA. The *gcd1-101* allele has been shown to bring about constitutive derepression of both *HIS4* and *ILS1* steady-state mRNA levels.[37,39] This suggests that, like the *GCN* factors, *GCD1* exerts its effect on the expression of genes subject to general control at the level of their transcription. It is interesting that most *gcd*$^-$ alleles are pleiotropic and also reduce cell viability.[58,63] In fact, *GCD1* is known to be an essential gene.[11,64] The implications of the pleiotrophy of the *gcd*$^-$ class of regulatory mutations will be addressed further below.

C. Interactions Between *gcn*- and *gcd*- Mutations

The regulatory phenotypes of *gcn*$^-$ and *gcd*$^-$ double mutants (Table 2) provide important insights into the roles of the different *trans*-acting factors in the general control. The *gcd1-101* mutation suppresses the amino acid sensitivity associated with mutations in *GCN1*, *GCN2*, and *GCN3*.[12,13] The same interaction has been demonstrated between a *gcd2*$^-$ mutation and *gcn1*$^-$, *gcn2*$^-$, and *gcn3*$^-$ mutations.[31,58]

These findings suggest that the positive regulatory functions of *GCN1*, *GCN2*, and *GCN3*

are dispensible when either *GCD1* or *GCD2* is defective. Such behavior is consistent with the idea that *GCD1* and *GCD2* function at the same stage in the regulatory mechanism. Moreover, it suggests that the role of the *GCN1*, *GCN2*, and *GCN3* factors may be to antagonize the repressing function(s) exerted by the *GCD* factors in amino acid-starved cells. In contrast to the genetic interactions just described, *gcn4*$^-$ *gcd1*$^-$, and *gcn4*$^-$ *gcd2*$^-$ double mutants exhibit the nonderepressible phenotype associated with *gcn4*$^-$ single mutants.[13,58] This indicates that *GCN4* is a more direct positive regulator of gene expression than the other *GCN* factors and that its function is required for derepression regardless of the degree of repression exerted by the *GCD* factors. Because it would seem necessary to repress the positive regulatory function of *GCN4* in the absence of amino acid starvation, it was suggested that the level of *GCN4* activity is negatively regulated by *GCD1*.[13] These regulatory interactions are summarized schematically in Figure 1.

Although the model in Figure 1 explains most of the available genetic data, it is possible that no direct interaction exists between the *GCN* and *GCD* factors, either at the level of the gene products or gene expression, and, instead, these factors modulate the derepression of enzyme synthesis by independent mechanisms. Moreover, it is possible that additional *GCN* factors exist which act either in concert with *GCN4* or more directly than *GCN4* to bring about derepression of gene expression in amino acid-starved cells. For example, the position of *GCN5*[27] in the regulatory scheme is not yet known. In addition, even the most exhaustive attempt at the isolation of *gcn*$^-$ mutants failed to identify new *gcn3*$^-$ alleles[27] so that the *gcn*$^-$ class of mutations may not yet be saturated. As already mentioned, the total number of *GCD* genes is not yet known. Finally, it is possible that other important interactions exist between the known regulatory factors in addition to those depicted in Figure 1. For example, Driscoll Penn et al.[24] have reported that transcription of *GCN2* increases in response to amino acid starvation and that this response is dependent upon the *GCN4* gene product.

D. Regulatory Consequences of Increased *GCN* Gene Dosage

One line of evidence in favor of the regulatory interactions proposed in Figure 1 derives from the regulatory effects of the cloned *GCN* genes when present in the cell on multi-copy plasmids. The *GCN*$^+$ genes were isolated from transformants of the corresponding *gcn*$^-$ strains in which the amino acid analogue sensitivity of the mutants was complemented by plasmids from a wild-type yeast DNA library.[13,24] Interestingly, plasmids containing sequences derived from more than one genomic locus were isolated in this fashion and it was necessary to identify from among the multiple cloned sequences the bona fide *GCN*$^+$ genes. This was accomplished by testing each cloned DNA sequences for its ability to direct integration of nonreplicating yeast plasmid to the locus of the *gcn*$^-$ mutation which the cloned sequence complements.[13]

The mechanism whereby DNA sequences other than the true *GCN*$^+$ genes can complement the amino acid analogue sensitivity of *gcn*$^-$ mutants when present in multi-copy remains unknown. However, an important related observation is that the *GCN4*$^+$ gene in multi-copy partially complements the amino acid analogue sensitivity of *gcn1*$^-$, *gcn2*$^-$ and *gcn3*$^-$ mutations as well as mutations in *GCN4*.[13] Similarly, *HIS4-lacZ* expression is partially derepressed in transformants containing multi-copy *GCN4*$^+$ in the absence of both amino acid starvation and the *GCN2* and *GCN3* gene products.[7] These effects are not observed when the same *GCN4*$^+$ fragment is present on a low-copy, centromere-containing plasmid.[7,65] Nor do they occur in transformants containing either the *GCN2*$^+$ or the *GCN3*$^+$ genes in multi-copy.[7,13] These findings suggest that overexpression of *GCN4* by virtue of increased gene dosage can partially bypass both the starvation signal and the functions of *GCN1*, *GCN2*, and *GCN3* to bring about derepression of structural genes subject to the general control. In contrast, overexpression of any one of the other three *GCN* genes is not sufficient

to derepress enzyme synthesis in nonstarved cells. This interpretation is consistent with the idea embodied in Figure 1 that *GCN4* encodes the most direct regulator in the general control and that its activity is stimulated or its expression derepressed by the combined action of the other *GCN* factors in response to amino acid starvation.[7,13]

E. Regulation of *GCN4* Expression Examined Using a *GCN4-lacZ* Fusion

A *GCN4-lacZ* translational gene fusion has been employed as a model system to study the effects of *gcn*$^-$ and *gcd*$^-$ mutations on the expression of *GCN4*. The results of this approach suggest that the broad outlines of the model shown in Figure 1 are correct and that the regulation of *GCN4* function occurs, at least in part, at the level of *GCN4* gene expression.[9,60,66] Table 1, line 6 shows that histidine starvation leads to a tenfold derepression of *GCN4-lacZ* enzyme activity in wild-type cells. This result suggests that the expression of *GCN4* is itself derepressed by amino acid starvation.[9,60] Table 1 also shows that a mutation in either *GCN2* or *GCN3* blocks efficient expression of *GCN-lacZ* enzyme activity in histadine-starved cells (lines 7 and 8) supporting the idea that *GCN2* and *GCN3* are positive regulators of *GCN4* (Figure 1). By contrast, a mutation in *GCD1* leads to constitutive derepression of fusion enzyme activity at levels 20 to 30-fold higher than the wild-type repressed level (Table 1, line 9), in agreement with the idea that *GCD1* is a repressor of *GCN4*. A chromosomal *gcn4*$^-$ mutation does not appear to block derepression of *GCN4-lacZ* enzyme activity (Table 1, line 10), suggesting that *GCN4* does not act as a positive regulator of its own expression.[66]

As mentioned above, the *gcd1-101* mutation suppresses the Gcn$^-$ phenotype of *gcn1*$^-$, *gcn2*$^-$, and *gcn3*$^-$ mutations but does not suppress *gcn4*$^-$ mutations. The expression of the *GCN4*$^+$*-lacZ* fusion enzyme in *gcd1*$^-$ *gcn2*$^-$ and *gcd1*$^-$ *gcn3*$^-$ double mutants suggests that the epistasis observed in both of these strains can probably be accounted for at the level of *GCN4* expression. In each of the double mutants, the *gcd1-101* mutation leads to significant increases in *GCN4-lacZ* enzyme expression. This is most dramatic for the *gcn2-1* mutation where fusion enzyme activity in the double mutant is 50 to 100-fold higher than in the *gcn2*- single mutant (Table 1, lines 7 and 11). In this instance, *gcd1-101* epistasis appears to be complete. Although suppression of *gcn3-102* is much less complete (a fivefold increase; Table 1, lines 8 and 12), the fusion enzyme level in the histidine-starved *gcn3-102 gcd1-101* double mutant (70 units) is similar to the derepressed level observed in wild-type cells (100 units). The fact that the *gcd1-101* mutation makes *GCN2* and *GCN3* at least partially dispensible for efficient expression of *GCN4-lacZ* enzyme activity in amino acid-starved cells is consistent with the idea that these gene products act indirectly as positive regulators of *GCN4* by antagonism of *GCD1*-mediated repression. The *gcd1-101* mutation also leads to constitutive derepression of fusion enzyme activity in the *gcn4-101 gcd1-101* double mutant (Table 1, lines 10 and 13); however, the phenotype of this strain remains Gcn$^-$ because the *GCN4* product in these cells is defective.[57]

F. Translational Control in the Regulation of *GCN4* Expression

When *GCN4*$^+$*-lacZ* fusion mRNA levels were examined in parallel with the expression of the fusion enzyme, it was found that the regulation of *GCN4-lacZ* enzyme synthesis shown in Table 1 cannot be fully accounted for at the level of transcription. Histidine starvation leads to a modest derepression of the fusion transcript in wild-type cells, in parallel with the increase observed in the fusion enzyme level in these conditions.[9,60,66] Surprisingly, the fusion transcript also derepresses in histidine-starved *gcn2-1* and *gcn3-102* mutants, reaching steady-state levels significantly higher than those found in similarly treated wild-type cells. This occurs despite the fact that derepression of the fusion enzyme is blocked in these mutants.[9,66] This behavior is illustrated for *gcn2-1* strain in Figure 2. Such data suggest that the inefficient expression of *GCN4-lacZ* enzyme in *gcn2*$^-$ and *gcn3*$^-$ strains is not the

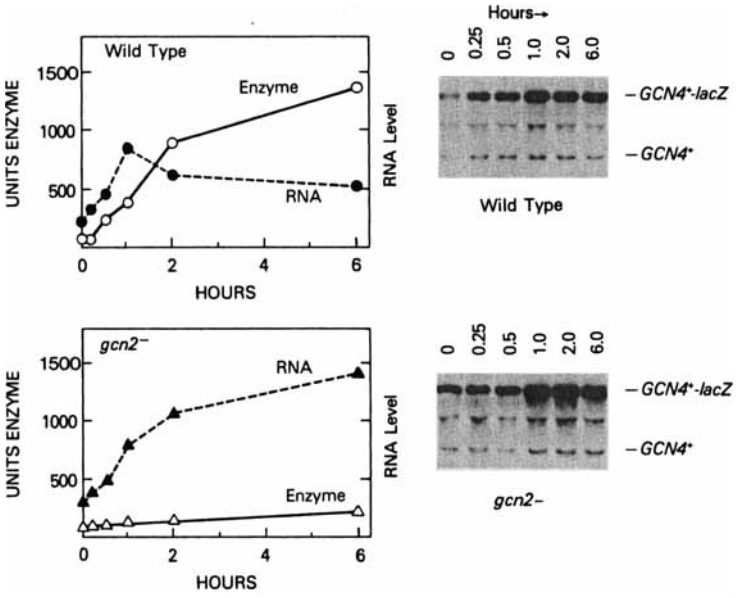

FIGURE 2. Time-course of *GCN4-lacZ* expression on a multi-copy episome in wild-type and *gcn2-1* transformants.[66] Cells were cultured in minimal medium to early logarithmic growth and at time = zero 3-aminotriazole was added to 10 m*M*. At the indicated times, cells were harvested and assayed for levels of β-galactosidase activity and *GCN4-lacZ* mRNA. The latter was measured by densitometry of the accompanying RNA blots probed with radiolabeled *GCN4* plus *lacZ* DNA.

result of a transcriptional defect; rather, it appears that translation of *GCN4-lacZ* mRNA is much less efficient in amino acid-starved *gcn2*$^-$ and *gcn3*$^-$ mutants than in similarly treated wild-type cells. This, in turn, suggests that the *GCN2* and *GCN3* gene products act to stimulate translation of *GCN4*$^+$ mRNA in response to amino acid starvation.

The derepression pattern of wild-type *GCN4* mRNA is qualitatively identical to that just described for the *GCN4-lacZ* fusion transcript;[24,66] however, at least in one study,[66] the depression ratio of the fusion transcript was found to be considerably greater than that of *GCN4*$^+$ mRNA. The underlying basis for this difference in derepression ratio is not known with any certainty, but most probably is the result of a destabilizing effect of the inserted *lacZ* sequences upon the fusion transcript in nonstarved cells. Neither is it known what regulatory factors are responsible for the transcriptional modulation of *GCN4* transcripts in response to amino acid starvation. Surprisingly, when transformants containing the *GCN4*$^+$ *lacZ* fusion are cultured in minimal medium containing galactose instead of dextrose as the sole carbon source, the large derepression ratio of the fusion transcript in amino acid-starved *gcn*$^-$ mutants is eliminated, yet the regulation of fusion enzyme synthesis in galactose-grown cells is unchanged from the pattern observed in dextrose-grown cells.[66] This result is illustrated for the *gcn2-1* mutation in Figure 3B. Taken together with the results shown in Figure 2, these data suggest that transcriptional regulation plays a relatively minor role in *GCN4* regulation and that translational control is the predominant mechanism whereby *GCN4* expression is regulated by the *GCN2* gene product.

Figure 3B also illustrates that in galactose medium the *gcd1-101* mutation leads to constitutive derepression of the *GCN4-lacZ* fusion enzyme level without making any significant change in the fusion transcript level. This result suggests that *GCD1* functions as a translational repressor of *GCN4*.[66] In contrast to the results in galactose-grown cells shown in Figure 3B, the *gcd1-101* mutation results in substantial derepression of the *GCN4-lacZ*

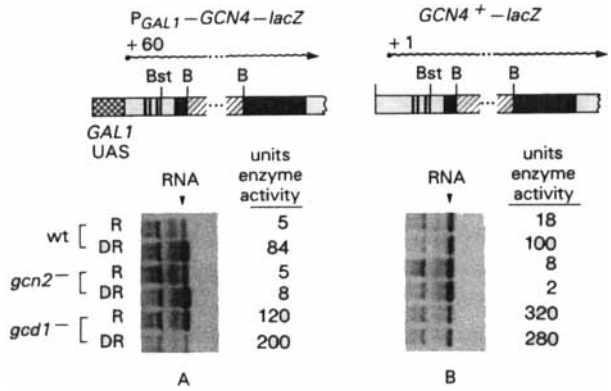

FIGURE 3. Steady-state levels of fusion transcript and fusion enzyme made from P_{GAL1}-*GCN4-lacZ* and *GCN4*$^+$-*lacZ* on single-copy episomes in transformants cultured on galactose-containing medium.[66] The upper portion of each panel shows a schematic of the fusion gene examined below. The wavy lines represent the transcription units determined by nuclease protection mapping.[9,66] The solid regions designate *GCN4* coding sequences (including the four small open reading frames in the mRNA 5′ leader). The hatched segment is *lacZ*, the double-hatched segment is the *GAL1* UAS. On the far lower left are the relevant gentoypes of the three pairs of transformants (wt = wild-type) containing P_{GAL1}-*GCN4-lacZ* (panel A) and *GCN4-lacZ* (panel B). For each transformant, data from cells grown on minimal medium (repressing conditions, R) and on minimal medium containing 10 m*M* 3-amino-triazole (derepressing, DR) are shown consecutively. In the RNA blot analyses, *lacZ* sequences were used as the probe. Migration of RNA is from right to left; the arrows mark the fusion transcripts.

transcript level when dextrose is the sole carbon source; nevertheless, the degree of derepression of the fusion enzyme level is five- to tenfold greater than the corresponding increase in the fusion transcript level in these conditions.[66] This discrepancy is at least consistent with the idea that the *gcd1-101* mutation leads to an increase in the translational efficiency of the fusion transcript in the absence of amino acid starvation. Also supporting this conclusion is the fact that the *gcd1-101* mutation increases the level of *GCN4-lacZ* enzyme activity in amino acid-starved *gcd1-101 gcn2-1* and *gcd1-101 gcn3-102* fusion transcript relative to its value in similarly treated *gcn*$^-$ single mutants. Thus, the suppression of the *gcn2*$^-$ and *gcn3*$^-$ mutations by the *gcd1-101* mutation appears to occur, at least in part, at the level of *GCN4* translation.[66]

G. Sequences in *GCN4* mRNA Repress its Translation in the Absence of Amino Acid Starvation

A second line of evidence that *GCN4* expression is regulated translationally has to do with the unusual sequence organization of *GCN4* mRNA. A translationally open reading frame of 800 nucleotides is located at the 3′ end of a 1500 nucleotide transcription unit which maps to the *GCN4* region.[9,60] Deletion analysis and marker rescue experiments indicate that this transcription unit spans the *GCN4* functional unit.[9] No processing of the 1.5 kb *GCN4* transcript has been detected by nuclease protection experiments either in wild-type cells, in the presence or absence of amino acid starvation,[9,60] or in *gcn2*$^-$ and *gcd1*$^-$ mutants grown in the same conditions.[66] These findings suggest that the *GCN4* protein is translated from a mRNA containing 600 nucleotides of 5′ leader. Moreover, there are four consecutive short open reading frames spanning a 250 nucleotide segment in the middle of the *GCN4*

mRNA 5′ leader, each of which consists of an AUG codon followed by one or two sense codons plus a termination codon.[9,60] This sequence organization is unusual because in yeast, as in other eukaryotes, the 5′ proximal AUG codon in a mRNA molecule generally functions as the translation initiation codon.[67,68] Moreover, it has been shown for a yeast transcript[67,69] and for several different mammalian mRNAs[70-73,90] that AUG codons introduced upstream from the normal translation initiation codon can substantially inhibit translation of downstream coding sequences.

These considerations suggest the possibility that the short upstream open reading frames in *GCN4* mRNA provide a mechanism with which to modulate the efficiency of translation of the downstream *GCN4* protein coding sequences. In support of this idea is the finding that two very similar deletions of 240 and 280 nucleotides, which remove all four short open reading frames from the leader of *GCN4* mRNA (*GCN4Δ1* and *GCN4-501*, Table 3) suppress the amino acid analogue sensitivity of *gcn1*$^-$, *gcn2*$^-$ and *gcn3*$^-$ mutations and lead to constitutive derepression of genes subject to the general control.[60,62] By analogy with the suppression of the same *gcn*$^-$ mutations in transformants containing multiple copies of the *GCN4+* genes,[13] this result suggests that deletion of the leader sequences results in an increased level of *GCN4* expression, thus bypassing the positive regulatory functions of the other *GCN* factors. This is consistent with the idea that the *GCN1*, *GCN2*, and *GCN3* products positively regulate *GCN4* by antagonism of the negative regulatory sequences present in the *GCN4* mRNA 5′ leader. No change in the steady-state level of *GCN4* mRNA was detected in response to a deletion of the leader sequences, suggesting that the inferred increase in *GCN4* expression is brought about at the translational level.[60]

The same conclusions were reached more directly by analyzing the effects of these deletions upon expression of the *GCN4-lacZ* gene fusion. The deletions lead to high constitutive expression of the fusion enzyme at levels even greater than those observed for the wild-type fusion gene in amino acid-starved cells (compare in Table 1, lines 6 to 10 with lines 14 to 18). At least in wild-type transformants, this increased expression of fusion enzyme activity is associated with little or no change in the level of the fusion transcript,[9,60,66] thus suggesting that the leader sequences act to repress translation of *GCN4* mRNA in nonstarved cells. The 240 base pair leader deletion in *GCN4-501-lacZ* bypasses the requirement for both *GCN2*$^+$ and *GCN3*$^+$ for efficient expression of the fusion enzyme activity (Table 1, lines 15 and 16). Although this deletion leads to as yet unexplained increases in the level of the fusion transcript in *gcn2-1* and *gcn3-102* cells, it is nevertheless clear that the greatly enhanced expression of fusion enzyme activity which results from the leader deletion in these *gcn*$^-$ cells occurs largely by an increase in the translational efficiency of the fusion transcript.[9,66]

In a *gcd1*$^-$ mutant, in which constitutive derepression of the fusion gene with the wild-type leader is already observed (Figure 3B), the 240 base pair leader deletion leads to even higher levels of fusion enzyme activity (Table 1, compare lines 9 and 17); however, examination of the fusion transcript levels in these conditions suggests that the leader deletion results in only a slight additional increase in the translational efficiency of the fusion transcript.[66] This result implies that the leader sequences have little effect upon translation of *GCN4* mRNA in the absence of *GCD1*$^+$. In other words, the negative regulatory effect of the *GCN4* leader appears to be mediated by the *trans*-acting *GCD1* product. The genetic interactions between the *gcn*$^-$ and *gcd*$^-$ mutations described above suggest that the role of *GCN1*, *GCN2* and *GCN3* in reversing the repressing effect of the leader sequences is indirect and involves antagonism of the translational repression exerted by *GCD1*.

H. Translational Regulation of *GCN4* is Independent of the *GCN4* Promoter

An important prediction of the hypothesis that *GCN4* is translationally controlled is that the regulation of its expression should be largely independent of the *GCN4* promoter. There is now evidence that this is indeed the case.[66] Insertion of the upstream transcriptional

activation site (UAS) of the *GAL1* gene 53 base pairs upstream from the 5′ end of the *GCN4* transcription unit results in the production of a novel set of *GCN4* transcripts which are missing the first 60 nucleotides of *GCN4*$^+$ mRNA but still contain the regulatory sequences in the *GCN4* leader. The *GAL1*- promoted transcripts are present in cells grown on galactose but are undetectable in dextrose-grown cells. Since *GCN4* mRNA is normally produced both in dextrose and galactose grown cells, these results, combined with the novel 5′ ends, indicate that transcription of the *GAL1-GCN4* hybrid gene is under the control of the *GAL1* UAS. The effect of replacing the *GCN4* transcriptional regulatory signals with those of the *GAL1* gene upon expression of *GCN4-lacZ* enzyme activity was examined in different regulatory mutants (see Figure 3A). As might be expected, there are differences observed in the levels of the *GCN4-lacZ* fusion transcript promoted by the *GAL1* UAS compared to the wild-type fusion gene examined in the same conditions (compare Figure 3A with Figure 3B); however, the patterns of enzyme synthesis directed by the two fusion contructs are qualitatively similar. In both cases, the expression of fusion enzyme is derepressed in amino acid-starved wild-type cells, nonderepressible in *gcn2-1* cells and constitutively derepressed in *gcd1-101* cells. Moreover, as in the case of the wild-type fusion gene, the steady-state fusion transcript levels from the *GAL1*-promoted fusion construct do not correlate with fusion enzyme levels, again requiring a translational mechanism to fully account for the observed pattern of fusion enzyme synthesis (Figure 3A). These results indicate that the regulation of *GCN4* expression is largely independent of its promoter, consistent with the idea that translational control predominates in the regulation of *GCN4* expression.

I. Possible Mechanisms for Translational Control of *GCN4*

The effects of the *GCN4* leader deletions described above suggest certain possibilities for translational regulation of *GCN4* expression. As mentioned earlier, the insertion of a short open reading frame into the 5′ leader of a eukaryotic mRNA generally reduces the translational efficiency of the transcript.[67,69,72,73] This effect is consistent with the "scanning" mechanism of translation initiation in eukaryotes[68] which proposes that the initiation complex must first associate with the 5′ end of a mRNA molecule and then translocate downstream until an AUG codon suitable for initation is encountered. According to this model, efficient translation of a downstream coding sequence in a eukaryotic polycistronic mRNA cannot be initiated directly, and requires reinitiation by ribosomes (or 40S subunits) which remain attached to the transcript after translation of upstream coding sequences is complete.[72] In this view, the observed inhibitory effect of upstream open reading frames upon translation of downstream coding sequences is attributed to the relative inefficiency of reinitiation.[72,73]

If the scanning mechanism applies to translational initiation on *GCN4* mRNA, the four upstream AUG codons will be encountered prior to initiation at the AUG codon for the protein coding sequences. Assuming that the upstream AUG codons are recognized as initiation sites, then multiple reinitiation events will be required for expression of the *GCN4* protein. If in nonstarvation conditions reinitation downstream is inefficient following initiation at the upstream AUG codons, this could account for the repressing effects of the leader sequences on *GCN4* expression. Inefficient reinitiation could occur either because translation is arrested at the upstream open reading frames, thus blocking continued scanning downstream, or because reinitiation at the protein coding sequences is unlikely following termination at the upstream open reading frames. Depending upon which mechanism operates, amino acid starvation and the *GCN1*, *GCN2*, and *GCN3* products might then be expected to promote more efficient reinitiation either by releasing the block to translation of the upstream open reading frames, thus allowing continued scanning downstream, or by promoting more efficient reinitiation following termination at the upstream open reading frames. Alternatively, these factors could function to suppress initiation at the upstream AUG codons in starvation conditions without affecting initiation at the protein coding sequences. Which-

ever mechanism is involved, the genetic evidence suggests that the positive functions of these *GCN* factors are indirect and involve antagonism of *GCD1*. Clearly, additional genetic and molecular data are needed to understand at the biochemical level how the *trans*-acting factors in this system modulate the translational repression exerted by sequences in the *GCN4* leader and thereby couple the starvation signal to increased expression of the *GCN4* protein.

Assuming that the upstream open reading frames contribute to *GCN4* regulation, it seems unlikely that the codons represented in these short coding sequences have any special significance. Aside from the initiating methionine codons, only codons for alanine, cysteine, tyrosine, proline, and phenylalanine are represnted in the short open reading frames.[54,56] Three of the seven nonmethionine codons are among preferred codons found in very efficiently expressed yeast genes,[74] whereas one of the proline codons is used rarely in yeast but also appears once in the *GCN4* coding sequences downstream. The limited codon representation in the short open reading frames would seem to preclude models for *GCN4* regulation involving translational stalling at "hungry codons" in the upstream open reading frames in amino acid-starved cells, since starvation for other amino acids not encoded in this region (e.g., histidine and tryptophan) are known to elicit efficient derepression of *GCN4*.

Given that the *GCN* and *GCD* gene products regulate the translation of *GCN4* mRNA, is it possible that these factors are normal components of the translational apparatus involved in the expression of other proteins in addition to *GCN4*? This question raises the matter of the specificity of *gcn*$^-$ and *gcd*$^-$ mutations for the regulation of amino acid biosynthetic genes. Arguing in favor of the specificity of *gcn*$^-$ and *gcd*$^-$ mutations is the fact that there are numerous genes whose expression shows little response to these mutations, in the presence or absence of amino acid starvation. Among these are *TRP1*,[31,42] *LEU2*,[35] *GLN1* in glutamine-free medium,[12,43] *GDH1*,[12] *MAL2*,[12] and *URA3*.[7,9,27] The first four of these genes encode enzymes in amino acid biosynthesis and their expression could still be subject to the general control but exhibit little response to general control regulatory mutations because of the overriding effects of pathway-specific repression mechanisms (see Section III). In contrast, the insensitivity of *MAL2* and *URA3* to general control regulatory mutations is a good indication that *gcn*$^-$ and *gcd*$^-$ mutations are specific to amino acid biosynthetic enzyme expression. (The relative insensitivity of *URA3* expression to amino acid starvation and general control regulatory mutations is illustrated in Table 1, lines 19 to 23). Seemingly at odds with this conclusion is the observation that galactose induction of a *GAL1-lacZ* fusion enzyme is significantly lower in a *gcn2-1* strain than in wild-type cells when amino acid starvation is imposed.[66] However, the *gcd1-101* mutation has little effect on *GAL1* induction,[66] suggesting that the negative effect of *gcn2*$^-$ mutation on *GAL1* expression may be the result of a severely depleted amino acid pool expected to occur in a nonderepressible strain grown in starvation conditions. (The consequent low level of the corresponding aminoacylated tRNA could reduce the overall efficiency of translation simply by limiting one of the substrates for protein synthesis.) Since the induction of *MAL2* and the expression of *URA3* are not altered in the same conditions, such as effect would necessarily depend on other factors, such as the abundance of the transcript or its inherant translational efficiency. (In fact, the nonderepressibility of the *GCN4-lacZ* fusion in amino acid-starved *gcn2*$^-$ and *gcn3*$^-$ mutants could be partly the result of depleted amino acid pools in these conditions. However, in this instance the argument is circular because the depletion of amino acid pools is presumed to be the direct result of the nonderepressibility of *GCN4* expression in *gcn2*$^-$ and *gcn3*$^-$ mutants.)

Even if the expression of most non-amino acid biosynthetic genes is found to be relatively insensitive to *gcn*$^-$ and *gcd*$^-$ mutations, this would not necessarily exclude the possibility that *GCN* and *GCD* factors function as general components of the translational apparatus. It is possible that the existing *gcn*$^-$ and *gcd*$^-$ alleles are leaky mutations and that *GCN4*

mRNA, because of its novel polycistronic organization, is more sensitive to the effects of these mutations than most other transcripts. In fact, it is known that the *gcd1-101* allele is a leaky mutation, because deletion of *GCD1* is lethal.[64]

J. The Essential Function of *GCD1*

If, as suggested above, general control *trans*-acting factors which regulate *GCN4* expression are components of the protein synthesis apparatus, it might be expected that mutations which completely inactivate their function would be lethal. As just mentioned, this is the case for *GCD1;* although the underlying basis for the lethality associated with a *gcd1*$^-$ null allele is not known. The *gcd1-101* mutation does significantly reduce the in vivo rate of total protein synthesis at the restrictive temperature,[12] but RNA and DNA synthesis are also impaired in these conditions and it is not clear which of these defects in macromolecular synthesis, if any, is the direct cause of lethality in *gcd1-101* cells.

In addition to a general reduction in the rates of macromolecular syntheses,[12] the *gcd1-101* mutation also leads to G1 arrest at the restrictive temperature, and as expected for a G1-blocked cell cycle mutant, homozygous *gcd1-101* diploids fail to sporulate at 36°.[75] Based on the Cdc$^-$ phenotype of *gcd1-101,* it has been suggested that *GCD1* might function as a cell cycle regulatory factor which serves to coordinate the cell division cycle with amino acid availability.[12] However, it should be noted that a variety of metabolic stresses can lead to G1 arrest. For example, inactivation of an aminoacyl tRNA synthetase can have this effect,[16,23] as does starvation for many different essential nutrients.[20] It is believed that these conditions substantially reduce, but do not immediately block, protein synthesis. As a result, new rounds of cell division are inhibited but existing rounds of the cell cycle can be completed, leading to an accumulation of cells in the G1 phase.[20] This behavior is in contrast to the effect of a cyclohexamide treatment which results in sudden inhibition of protein synthesis and a complete block to any further progression through the cell cycle.[23] A defect in an essential translational factor might be expected to produce an effect similar to that of cyclohexamide treatment, perhaps arguing against the idea that *GCD1* is a general translational factor. However, a leaky defect in such a factor might leave intact a residual level of protein synthesis sufficient to permit existing rounds of the cell cycle to be completed and thus lead to G1 accumulation.

Whatever the essential function of *GCD1,* there is suggestive evidence that certain *GCN* factors, most notably *GCN3,* are also involved in this function. Certain alleles of *GCN3* and *GCN4* were originally isolated as suppressors of the temperature-sensitive phenotype of *gcd1-101.*[13] The level of suppression has proven to be significantly greater for *gcn3*$^-$ alleles isolated in this way than for the *gcn4*$^-$ suppressors of *gcd1-101,* but in neither case is the suppression complete.[62] In contrast to these suppressive interactions, another allele of *GCN3, gcn3-101,* is lethal in combination with *gcd1-101.*[13] The latter *gcn3*$^-$ allele was isolated on the basis of its associated amino acid analogue sensitivity. Thus, some *gcn3*$^-$ alleles are able to suppress, whereas others exacerbate the conditional lethality associated with the *gcd1-101* mutation. Assuming that *gcd1-101* encodes a temperature sensitive *GCD1* protein, the *gcn3*$^-$ suppressors of *gcd1-101* might encode proteins that physically interact with and stabilize a thermolabile *gcd1-101* gene product. Such a mechanism would explain the allele specificity of these interactions.

Regardless of whether they suppress or exacerbate the *gcd1-101* growth defect, all *gcn3-* alleles are at least partially suppressed by *gcd1-101* with respect to the regulation of amino acid biosynthetic genes. This suggests that the growth defect in *gcd1*$^-$ cells is not due primarily to the overproduction of the *GCN4* gene product expected to occur in *gcd1*$^-$ mutants. This conclusion is also consistent with the fact that *gcn4*$^-$ alleles which are only partial suppressors of the growth defect in *gcd1-101* cells completely inactivate *GCN4* regulatory function.[13] Clearly, the full significance of the pleiotropy of the *gcd1-101* mutation

and that of other *gcd*$^-$ mutations currently under investigation[58,63] is not known. While it seems certain that the *GCD* factors carry out essential functions in the cell in addition to their effects upon the expression of amino acid biosynthetic enzymes, the relationship between these two functions remains to be determined.

V. *cis*-ACTING REGULATORY ELEMENTS IN THE GENERAL AMINO ACID CONTROL

A. A Repeated Hexanucleotide Sequence Functions as a Site for Positive Regulation of Genes Subject to the General Control

Genes which encode enzymes whose expression is governed by the general control are unlinked in the yeast genome. The fact that these unlinked genes are coregulated at the transcriptional level (see Section III) suggests that there exist homologous DNA sequences at which these genes mediate their common transcriptional response to amino acid starvation. There is now good evidence, obtained primarily from mutational analyses of the *HIS4* and *HIS3* genes, that a short nucleotide sequence found repeated upstream of many genes subject to the general control functions as a *cis*-acting positive regulatory element in their derepression in response to amino acid starvation.

Expression of a *HIS4-lacZ* gene fusion containing 670 base pairs of *HIS4* 5′ noncoding DNA and only the first 90 base pairs of the *HIS4* transcription unit was shown to be subject to general amino acid control.[30] This result localized the *HIS4* regulatory sequences to within the −670 to +90 interval with respect to the 5′ end of the transcription unit at +1. The precise location of the *cis*-acting *HIS4* regulatory sequences within this region was determined subsequently by deletion analysis.[7,39] Deletions with a 5′ end point at −588 and 3′ end points between positions −235 and −138 reduce the efficiency of *HIS4* expression in nonstarved wild-type cells, but do not block derepression of *HIS4* expression in response to either amino acid starvation[7] or the *gcd1-101* mutation[39] (see Figure 4A). In contrast, a deletion to position −136 abolishes derepression of *HIS4*. The latter result suggests that a sequence in the vicinity of −136 is important for *HIS4* regulation. In fact, the hexanucleotide sequence T-G-A-C-T-C at −136 to −131 is also present at two positions in the −235 to −138 interval shown to be required for a wild-type level of *HIS4* expression. Furthermore, when revertants of *his4Δ-136* were isolated in which derepression of *HIS4* expression had been restored, it was found that a copy of the short repeated sequence had also been restored by either of two different single base pair substitutions (see Figure 4A). In one class of revertants, a base substitution occurred at the −136 deletion junction to restore the T residue normally found at position −136 (the first T of the T-G-A-C-T-C sequence). In the second class of revertants, a substitution occurred 20 base pairs downstream from −136, changing the wild-type sequence at positions −119 to −114 from T-C-A-G-T-C to -T-G-A-G-T-C. Note that the new sequence differs from the wild-type −136 repeat at only one position. (In fact, this substitution also generates a perfect copy of the T-G-A-C-T-C sequence on the opposite DNA strand (Figure 4A).) As discussed below, the hexanucleotide appears to function bidirectionally.) In a third class of revertants, multiple mutations occurred at the deletion junction which do not restore a copy of the repeat. In this class of revertants, the basal level of *HIS4* expression is elevated relative to *his4Δ-136;* however, general control derepression of *HIS4* expression was not found to be restored.[7,39] Taken together, these results strongly suggest that at least one copy of the short repeat is necessary for general control-mediated derepression of *HIS4* expression. Moreover, they indicate that the short repeat functions as a *cis*-acting site for positive regulation of *HIS4* transcription. (The 5′ end of *HIS4* mRNA is unaffected by the *HIS4* deletions and their suppressor mutations,[39] demonstrating that the regulatory elements affected by these mutations influence the efficiency of *HIS4* transcription but do not influence the site of transcription initiation.)

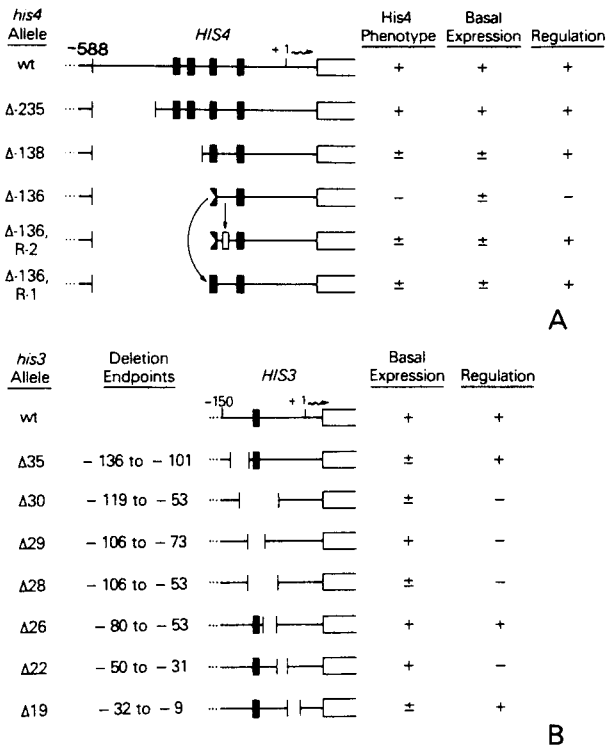

FIGURE 4. Schematic representation of the results of deletion analyses of the regulatory regions at (A) *HIS4*[7,39] and (B) *HIS3*.[38] The open boxes represent the coding sequences of the two genes; the thin horizontal lines are the 5′ noncoding sequences; the solid boxes represent the approximate positions of the T-G-A-C-T-C sequence motif. +1 marks the 5′ end of each transcript and the vertical hash marks indicate the deletion junctions in the different deletion alleles. The 3′ end points of the *his4* deletions are given by the allele names; the 5′ junction is −588 for each *his4* deletion. In both cases, the deletion alleles were used to replace the wild-type alleles in the yeast chromosome and the effects of the deletions on the level of basal expression and regulation were examined in vivo. The effects of these mutations are summarized qualitatively as: + (similar to the wild-type), +/− (partially impaired with respect to wild-type but still functional), or − (virtually nonfunctional). The last two constructs in panel A represent His+/− revertants (R-2 and R-1) of *his4Δ−136*. The arrows indicate the positions of the substitution mutations that gave rise to these revertants. The open box for Δ-136, R-2 designates an inverted copy of the repeat.

Deletion analysis of the *HIS3* 5′ noncoding region is consistent with the idea that a copy of the T-G-A-C-T-C sequence found at position −96 at *HIS3* plays an important role in the regulation of this gene as well. *his3Δ28*, a deletion which removes the sequences between positions −53 and −106, abolishes *HIS3* derepression in amino acid-starved cells without affecting the basal level of *HIS3* expression in cells grown on minimal medium (Figure 4B). Smaller deletions which remove subsets of the sequence deleted by Δ28, but which leave the T-G-A-C-T-C sequence at position −96 intact, do not affect *HIS3* derepression (Figure 4B). Taken together, these deletions localize a regulatory element to a 20 base pair segment containing the −96 copy of the T-G-A-C-T-C sequence at *HIS3*.[38]

In addition to this regulatory element, deletions in *HIS3* 5′ noncoding sequences located

further downstream also confer a nonderepressible phenotype, suggesting that a second *HIS3* regulatory element may be located between positions −33 and −52 (Figure 4B).[38] No sequence with obvious similarity to the T-G-A-C-T-C element exists in this region. (The closest matches to the T-G-A-C-T-C sequence are two T-G-A-N-T sequences immediately flanking the downstream regulatory region.) This suggests the possibility that more than one sequence element is required for the derepression of *HIS3*. However, it should be noted that the *HIS3* downstream regulatory element overlaps a sequence element needed for proper *HIS3* expression in the absence of amino acid starvation.[76] Thus, it is possible that deletions in this region destroy the *HIS3* promoter and result in an alternative mechanism for transcription initiation, the efficiency of which is no longer governed by the upstream regulatory element containing the T-G-A-C-T-C sequence. (It is not known where the 5′ end of *HIS3* mRNA maps for these deletion alleles. This information would be useful for assessing the likelihood of an alternative transcription initiation mechanism in these promoter mutants.)

Preliminary results of a deletion analysis on the *ARG3* gene indicate that an upstream region located between −282 and −364 is critical for derepression of *ARG3* expression in response to arginine starvation. This region contains two T-G-A-C-T-C sequence motifs arranged in opposite orientation, consistent with the idea that the short repeat is also involved in the regulation of *ARG3*.[47] In addition, two copies of the T-G-A-C-T motif reside within a segment of the 5′ noncoding DNA known to be required for normal expression of the *HIS5* gene.[46]

The T-G-A-C-T-C sequence is present in the 5′ noncoding regions of a number of other yeast genes known to be subject to the general control: *TRP5*,[36] *HIS1*,[40] *ARG4*,[77] *TRP2*,[78] *TRP3*,[41,78] *ILV1*,[79] and *CPA1*.[80] In most of these cases, there is at least one exact copy of the sequence T-G-A-C-T-C located within 200 base pairs of the 5′ end of the transcription unit; however, if one also considers the pentanucleotide T-G-A-C-T, each of the above mentioned genes (with the exception of *HIS3*) has multiple copies of the sequence motif on one strand or the other in the 5′ noncoding region (see Figure 5). (As discussed below, the repeat appears to function in an orientation-independent manner, so that inverted copies of the repeat have been considered in the present tally of repeat occurrences.) By contrast, this sequence motif is generally not found in the 5′ noncoding regions of other yeast genes,[40] suggesting that its presence in multiple copies upstream of genes subject to the general control is related to their coregulation. (The repeat has also been observed upstream from *LEU1*[54] and *LEU2*[82] which encode leucine biosynthetic enzymes which fail to derepress in response to starvation for other amino acids. In view of the evidence just described and the additional findings reviewed below which strongly implicate the repeat in the general control response, the presence of multiple copies of this sequence upstream from these *LEU* genes may be an indication that their expression is subject to the general control, but that their response is suppressed by pathway-specific regulation [see Section III].)

B. The T-G-A-C-T-C Sequence is Sufficient to Confer General Control Upon A Heterologous Yeast Promoter

The results of the deletion analyses described above suggest that a copy of the T-G-A-C-T-C sequence motif is necessary for derepression of the structural genes subject to the general control. The results of a different experimental strategy suggest that this short sequence may also be sufficient for general control derepression.[61] In this approach, a variety of small *HIS4* fragments containing different numbers of the *HIS4* T-G-A-C-T-C sequence and different amounts of *HIS4* DNA sequences flanking the repeats were used to replace the normal upstream regulatory region of the *CYC1* gene (Figure 6). The downstream *CYC1* promoter element which specifies the sites of transcription initiation at this gene were left intact in these constructions. Expression of *CYC1* does not normally respond to amino acid

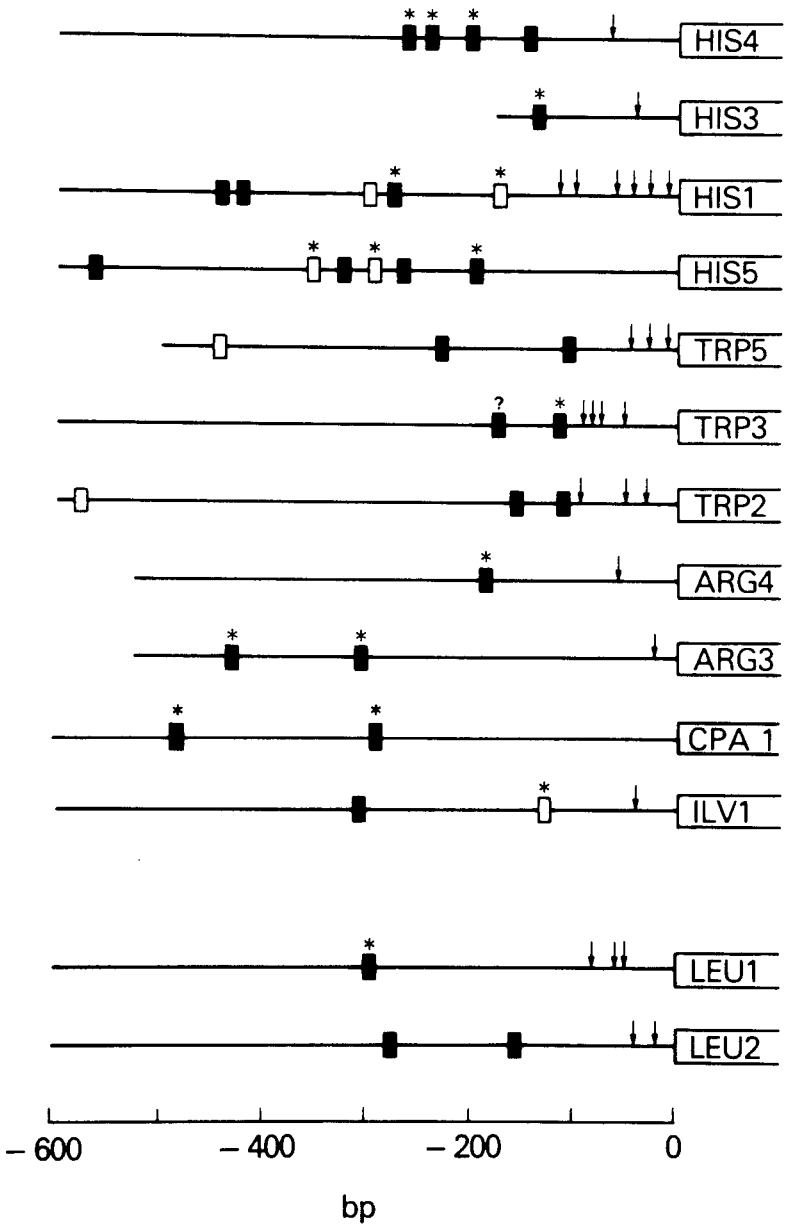

FIGURE 5. Schematic representation of the location of the T-G-A-C-T-C sequence motif upstream from structural genes subject to the general control. The coding sequences at each gene are shown as open boxes labeled with the gene name; the thin lines are the 5′ noncoding sequences and the arrows indicate the 5′ ends of the stable transcripts. The repeat is shown by solid boxes when oriented in the direction of transcription and as open boxes when oriented in the opposite orientation. Repeats marked by asterisks are perfect matches to the hexanucleotide repeat; those lacking asterisks contain only the higly conserved T-G-A-C-T pentanucleotide core. The repeat marked with a ? at *TRP3* is found at a position of discrepancy between the two published sequences of *TRP3*. "bp" stands for base pairs. (Note that *LEU1* and *LEU2* have not been shown to be subject to the general control. In fact, the available evidence is to the contrary; however, the matter may not have been settled conclusively; see Section III.) The sequence information was derived from the following sources: *HIS4*,[81] *HIS3*,[76] HIS1[40], *HIS5*,[46] *TRP5*,[36] *TRP3*,[41,78] *TRP2*,[78] *ARG4*,[77] *ARG3*,[47] *CPA1*,[80] *ILV1*,[79] *LEU1*,[54] *LEU2*,[81]

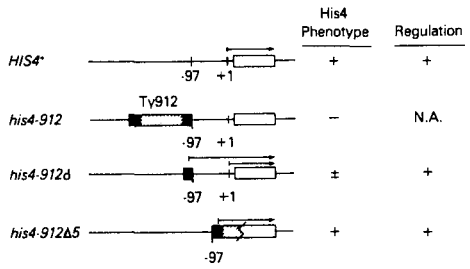

FIGURE 6. Summary of selected *HIS4-CYC1* promoter fusions on autonomously replicating plasmids in transformants of three different genotypes: wild-type (wt), *gcn4-101*, and *gcd1-101*.[61] The constructs are shown schematically on the left. The thin horizontal lines represent the 5′ noncoding regions of the *CYC1* gene (wavy) or the *HIS4* gene (straight) located upstream from *CYC1-lacZ* or *HIS4-lacZ* translational fusions, respectively. The arrows mark the start sites of transcription. (Only the three most abundant 5′ ends of *CYC1* are indicated.) The solid boxes symbolize the *HIS4* T-G-A-C-T-C repeat. The open boxes at the junctions between *CYC1* and *HIS4* 5′ noncoding sequences are the *XhoI* molecular linkers used in the constructions. The first construct shown is the *CYC1*$^+$ promoter, the second is a truncated derivative of the latter missing the upstream activation site (UAS). *HIS4-lacZ* is shown next, followed by five promoter fusions, in which *HIS4* fragments of different sizes were used to replace the upstream region of the *CYC1* promoter. The *HIS4* fragments used in the first four promoter fusions contain 169, 66, 25, and 14 bp of *HIS4* 5′ noncoding DNA. The fifth construct contains two tandem copies of the 14 base pairs fragment shown at the bottom. On the right are the levels of β-galactosidase activity directed in vivo by the various constructs in different growth conditions and in different strains. R (repressed) refers to growth of transformants in minimal medium; DR to growth in histidine starvation conditions (10 m*M* 3-aminotrizaole).

starvation. By contrast, every *HIS4-CYC1* hybrid promoter (except one) which contains at least one copy of the T-G-A-C-T-C sequence exhibits some degree of derepression in response to histidine starvation (Figure 6). This derepression response is mediated by the general control because *gcn4*$^-$ and *gcd1*$^-$ mutations affect the expression of the *HIS4-CYC1* hybrid promoters in the same manner observed for the *HIS4*$^+$ gene, i.e., low constitutive expression in *gcn4*$^-$ cells and high constitutive expression in *gcd1*$^-$ cells. Each of the *HIS4-CYC1* constructs directs a set of regulated transcripts with a 5′ end pattern characteristic of *CYC1*, showing that the *HIS4* sequences regulate the amount of transcription but do not influence the sites of transcription initiation.

Two pairs of the *HIS4* fragments analyzed in this fashion are only 14 and 24 base pairs in length and, in effect, isolate from the surrounding *HIS4* sequences the −136 copy and the upstream pair of T-G-A-C-T-C repeats, respectively. The fact that both of these fragments confer general control derepression and yet share no obvious sequence homology other than the T-G-A-C-T-C motif strongly suggests that the T-G-A-C-T-C element is sufficient to confer a general control response. (Ostensibly at odds with this conclusion is the finding that an even smaller seven base pair synthetic fragment containing a consensus T-G-A-C-T-C sequence does not confer general control derepression upon *CYC1*.[61] However, in view of the weight of evidence supporting the importance of the repeat, this result probably indicates that certain sequence contexts can interfere with the regulatory function of the T-G-A-C-T-C sequence.) The fact that both the upstream pair of T-G-A-C-T-C sequences and the −136 copy of the repeat each confer general control-mediated derepression upon *CYC1* expression demonstrates a degree of functional redundancy among the *HIS4* T-G-A-C-T-C elements. It is not known whether the fourth, most proximally located copy of the repeat at *HIS4* is functional.

There are several other interesting features of the *HIS4-CYC1* hybrid promoters. First, two tandemly arranged copies of the 14 base pairs fragment bearing the −136 *HIS4* repeat confer about a tenfold greater level of derepressed expression than a single copy of the same fragment (Figure 6). This suggests that multiple copies of the repeat can increase the degree

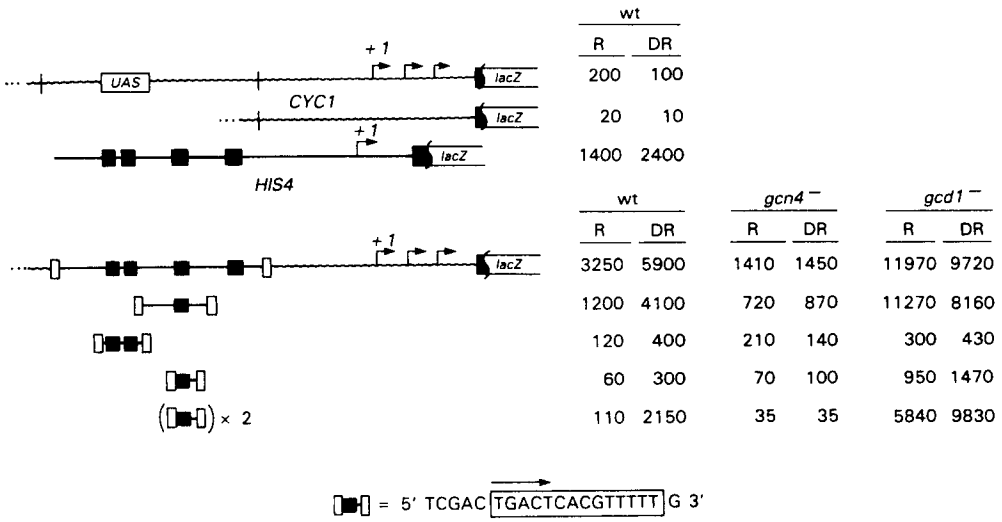

FIGURE 7. General control of transcription from Ty sequences inserted at *HIS4*. The *HIS4* coding sequences are shown as the open rectangles; the arrows represent the transcripts produced by these alleles. +1 marks the 5′ end of *HIS4* mRNA. See text for additional details. (Adapted from Silverman, S. J. and Fink, G. R., *Mol. Cell. Biol.*, 4, 1246, 1984.)

of derepression. However, it appears likely that the number of copies of the repeat is not the only determinant of efficient derepression because the 25 base pairs fragment which contains two closely spaced copies of the repeat functions much less efficiently than the tandem copies of the 14 base pairs fragment (Figure 6). Thus, sequences flanking the T-G-A-C-T-C motif are likely to influence the efficiency of derepressed expression.[61]

A second point of interest is the finding that the *HIS4* fragments function nearly as well in either orientation with respect to the direction of *CYC1* transcription, even though the repeat sequence itself has no obvious symmetry. This suggests that the repeat mediates an event which has bidirectional consequences. It is also evident from these hybrid promoters that the precise spacing between the *HIS4* upstream regulatory sequences and the downstream *CYC1* promoter element is not a critical factor in determining the regulatory function of the upstream sequences.[61] Both bidirectionality and the degree of position independence exhibited by the *HIS4* repeats are characteristics which suggest a similarity between the general control regulatory sequences and the enhancer elements of mammalian genes.[83]

C. *HIS4* Regulatory Sequences Can Control Transcription from a Ty Element

The relative position independence of the *HIS4* regulatory sequences is also illustrated by the characteristics of certain insertion mutations at *HIS4*. The *his4-912* allele contains a large insertion of a transposable element of the Ty class (Ty912) at position −97 (see Figure 7). This insertion abolishes transcription of the *HIS4* gene and leads to a His4⁻ phenotype. *his4-912δ* is a cold-sensitive His4⁺ revertant of *his4-912* in which only the 333 base pairs terminal direct repeat of Ty912 (the delta sequence) remains inserted at position −97.[84-86] In *his4-912δ* strains, a low level of *HIS4* mRNA with the normal 5′ end (position +1) is observed and expression of this transcript is subject to the general control (Figure 7).[87] Recalling that the *HIS4* regulatory sequences are located upstream from the Ty insertion site, this result indicates that the *HIS4* regulatory sequences can modulate transcription from the normal *HIS4* initiation site from a distance >300 base pairs further upstream from their normal location in the *HIS4*⁺ gene. A larger *HIS4* transcript is also observed in *his4-912δ* strains,

with a 5′ end located in the delta sequence,[88] and expression of this transcript is also subject to the general control.[87] Thus, as in the case of the *HIS4-CYC1* promoter fusions discussed above, this result shows that the *HIS4* upstream regulatory region can confer general control upon a heterologous promoter element — in this case, one located in a Ty element. A related situation exists for another class of *his4-912* revertants in which the beginning of the Ty element is fused to the middle of the *HIS4* gene, deleting all of the Ty and *HIS4* DNA sequences in between (Figure 7).[84,85] (This fusion, known as *his4-912Δ5*, retains the coding sequences for the histidinol dehydrogenase activity of the multifunctional *HIS4* polypeptide (*HIS4C* function) which permits growth on histidinol.) Like *his4-912δ*, the 5′ end of the *his4-912Δ5* transcript is located in the delta sequence and expression of this fusion transcript is subject to the general control.[87] *his4-912Δ5* is also noteworthy because this allele lacks all *HIS4* 5′ noncoding DNA downstream from the position of the original Ty912 insertion at −97. The fact that the *his4-912Δ5* transcript is subject to the general control indicates that all sequences downstream from position −97 at *HIS4* are dispensible for the general control response.

D. The *GCN4* Protein Binds Specifically to the T-G-A-C-T-C Sequence

Biochemical evidence for a specific interaction between the *GCN4* protein and the TGACTC sequence in the promoters of the *HIS3*[91] and *HIS4*[92] genes has been obtained very recently. In these studies, radiolabeled *GCN4* protein was synthesized by in vitro translation of *GCN4* transcripts produced in vitro using SP6 RNA polymerase. The *GCN4* protein thus produced was shown to bind tightly to promoter-containing fragments from *HIS3*, *ARG4*, *TRP5*, and *HIS4*, but to have low affinity for promoter fragments of five yeast genes not subject to the general control (*URA3*, *TRP1*, *GAL1,10*, and *DED1*).[91] Using an electrophoretic mobility-shift assay, the binding site at the *HIS3* gene was mapped precisely to the −104 to −83 interval in the 5′ noncoding region. This interval contains a copy of TGACTC (−99 to −94) and is also required for general control-mediated derepression of *HIS3* expression.[38] The DNAse I "footprinting" technique allowed even finer mapping of the *HIS3* binding site to a 10 base pairs region from −102 to −92 in which the TGACTC sequence is centered.[91] A *GCN4* binding site at *HIS4* was also mapped by DNAse I footprinting to the TGACTC sequence located at −136,[92] a copy of the repeat known to play a critical role in *HIS4* regulation. Specific binding to the other copies of the TGACTC motif at *HIS3* and *HIS4* has not been detected suggesting that these sites have lower affinity for *GCN4*. Since several of these other copies are perfect matches with TGACTC, it appears that sequences flanking the repeat are important determinants of the binding reaction. The same conclusion was reached from genetic analysis of the *HIS4* regulatory region (Section V.B). The nature of these sequence context effects and the functional significance of TGACTC copies with low binding affinity for GCN4 remain to be determined. The demonstration of a sequence-specific interaction between the *GCN4* protein and the TGACTC sequence represents an important biochemical confirmation of one of the predictions of the genetic model for general amino acid control.

Another observation of interest is the existence of a protein detected in yeast lysates which exhibits tight binding to a region partially overlapping the *HIS4* TGACTC at −136 and extending further upstream. This protein, which has now been extensively purified, is present in *gcn4*$^-$ cells and in wild-type cells plus or minus starvation.[92] Determination of the significance for *HIS4* regulation of this potentially very interesting binding protein awaits genetic analysis of its coding sequences.

E. Upstream Open Reading Frames at *HIS4* and *LEU2* are Dispensable for Normal Regulation

In addition to the T-G-A-C-T-C sequence, a second structural homology shared by some

genes whose expression is subject to the general control is the presence of one or more short open reading frames in the 5′ noncoding region. These elements have been noted in the *HIS4*,[81] *TRP5*,[36] *HIS3*,[89] *HIS1*,[40] *ARG4*,[77] *ILV1*,[79] and *CPA1*[80] genes. It has been suggested that at least some of these sequences may play a role in the regulation of the adjacant amino acid biosynthetic genes.[89] One observation at odds with this idea is that a deletion which removes all but the very 3′ ends of two different short open reading frames upstream from *HIS4* has no detectable effect upon either the efficiency of *HIS4* expression in nonstarved cells or the kinetics and extent of derepression of *HIS4* in response to amino acid starvation.[7,39] Similarly, deletions which remove the 3′ end of an upstream open reading frame *HIS3* (some of which also shift the reading frame of the upstream coding region) do not appear to affect derepression of *HIS3* expression.[38] These results make it unlikely that the upstream open reading frames at *HIS4* and *HIS3* are critical elements in the regulation of these genes by the general control. However, it remains possible that they function in some other capacity in regulating the expression of these genes.

The presence of a short open reading frame rich in leucine codons has also been noted upstream from the *LEU2* gene, with the suggestion that it might play a role in the leucine-specific repression of *LEU2*.[81] However, such an element does not occur at the coregulated *LEU1* gene.[54] Furthermore, the 3′ two thirds of the *LEU2* open reading frame can be deleted without any apparent loss of leucine-specific repression. Other *LEU2* promoter mutations which remove the 5′ end of the short open reading frame also retain leucine repression, albeit to a lesser degree. By contrast, a 6 base pairs deletion in a palindromic sequence around position −150, which leaves the upstream open reading frame completely intact, dramatically reduces the derepressed level of *LEU2* expression and completely abolishes leucine repression.[53] Because this small deletion reduces the derepressed level of *LEU2* expression so extensively (30-fold), it is not certain that the deleted sequences play a direct role in leucine repression. Nevertheless, taking all of the above into consderation, it seems unlikely that the *LEU2* upstream open reading frame plays an important role in leucine-mediated repression of this gene.

F. Sequences Required for Efficient Gene Expression in Nonstarvation Conditions

The results discussed so far make it clear that the T-G-A-C-T-C sequence is an importrant *cis*-acting regulatory element for general control-mediated derepression under conditions of amino acid starvation. By contrast, the sequence determinants which maintain efficient expression in the absence of amino acid starvation are less well defined. As already mentioned, *HIS4* deletions which remove sequences between positions −235 and −173 substantially reduce the level of *HIS4* expression in cells grown in minimal medium without impairing derepression in response to amino acid starvation[7,39] (Figure 4). This suggests that a promoter element required for efficient *HIS4* expression in nonstarvation conditions may be located in the −235 to −173 interval. However, a *HIS4-CYC1* hybrid promoter which carries only the *HIS4* sequences in this upstream interval exhibits very inefficient expression in nonstarved cells compared to hybrid promoters which also contain *HIS4* sequences located further downstream (Figure 6).[61] This result suggests that the −235 to −173 sequences by themselves do not constitute a strong activation element but probably work in conjunction with sequences further downstream to determine promoter strength in nonstarved cells. Since T-G-A-C-T-C sequences are found in both the −235 to −173 interval and further downstream in the *HIS4* regulatory region, it seems possible that efficient expression in the absence of amino acid starvation involves multiple copies of the short repeat. This possibility is supported by the fact that most of the *HIS4* expression in nonstarvation conditions is *GCN4* dependent (see Section IV above). An alternative explanation is that there are other sequences located between the upstream and downstream repeats which determine *HIS4* promoter strength. Clearly, point mutations in the region between and surrounding the repeats are

needed to fully identify the determinants of *HIS4* promoter efficiency in nonstarvation conditions.

Deletion analysis of *HIS3* suggests that sequences located upstream from the −97 copy of the T-G-A-C-T-C sequence between positions −155 and −107 are required for normal *HIS3* expression in nonstarved cells, but are not essential for *HIS3* derepression in starvation conditions. This region is very A + T rich and exhibits only weak homology with the T-G-A-C-T-C motif.[38] This suggests that there are sequence elements in addition to the T-G-A-C-T-C element which determine the efficiency of *HIS3* expression in the absence of amino acid starvation. It is not known to what extent, if any, this *HIS3* promoter activity is *GCN4*-dependent.

Deletion analyses of both *HIS3* and *HIS4* have also identified an important downstream promoter element required for normal expression in the presence or absence of amino acid starvation. This region maps between −53 and −34 at *HIS3*[76] and between −85 and −47 at *HIS4*.[39] In both genes, these regions contain T-A-T-A sequences similar to those found just upstream from mammalian transcription units. These sequences may carry out in yeast the same function defined for such elements in mammalian cells, namely, determination of the initiation site of transcription; however, at present there is no direct evidence to support this idea.

VI. CROSS-PATHWAY CONTROL IN OTHER FUNGI AND IN BACTERIA

A general control of amino acid biosynthetic enzyme expression similar to that observed in *Saccharomyces* has also been detected in two other fungi in the Ascomycete class, *Neurospora* and *Aspergillus*. In *Neurospora crassa*, starvation on minimal medium for histidine, tryptophan, arginine, isoleucine, or valine leads to two- to tenfold derepression of various enzymes in the histidine, tryptophan, arginine, and lysine biosynthetic pathways.[93-96] This derepression of enzymes in multiple nonlimiting pathways cannot be reversed by addition of the nonlimiting amino acids, and it occurs in the presence of large intracellular pools for these amino acids.[93,94] Thus as in *Saccharomyces*, cross-pathway control in *Neurospora* is not the result of indirect starvation for multiple amino acids induced by starvation for a single amino acid. A set of observations similar to those just enumerated for *Neurospora* has also been reported for *Aspergillus nidulans*.[97]

In *Neurospora*, it has also been possible to isolate nonderepressible *gcn*-like mutations.[95,98] To date, these mutations map to a single locus known as *cpc-1*. Like *gcn* mutations in yeast, *cpc-1*$^-$ alleles block derepression of all enzymes which normally derepress in response to starvation, but have little or no effect on enzymes not subject to cross-pathway control.[95] Another interesting similarity between *cpc-1* mutations and general control regulatory mutations in yeast is that *cpc-1* strains have a slow-growth phenotype even in nonstarvation conditions.[95,98] In fact, one allele is lethal in an otherwise wild-type background and requires the presence of an unlinked mutation *slo* for viability. Moreover, the *slo* mutation itself is pleiotropic, resulting in sterility when separated from the *cpc-1* mutation.[99] A recent analysis of the patterns of total protein synthesis by in vitro translation of mRNA populations extracted from a *cpc-1* mutant and wild-type reveals that amino acid starvation leads to a striking shift in the pattern of gene expression, affecting as much as 20% of the total mRNA population, and that *cpc-1*$^+$ function is required at some level for this response.[100] The pleiotropy of the *cpc-1* mutation is reminiscent of the lethality associated with the *gcd1* mutations in yeast, a phenotype which the *GCN* products also influence.[12,13] In both systems, it remains to be determined whether the growth defects are the result of a perturbation of amino acid biosynthesis or whether the regulatory gene products are involved in some other essential function. It will be of great interest to compare the molecular mechanism of *CPC-1* positive regulation in *Neurospora* with that of the *GCN* functions in yeast.

There are also indications that a general control of amino acid biosynthesis operates in enteric bacteria and is mediated by the unusual nucleotide guanosine tetraphosphate. However, in these organisms, the primary effects of cross-pathway control are observed when cells are shifted from amino acid-complete medium to media lacking one or more amino acids. In such shifts, *his* operon expression has been found to derepress severalfold even in the presence of exogenous histidine and independent of the *his* attenuator. This derepression response is dependent upon the *relA* gene product.[101,102] *RelA* encodes a ribosomal-associated protein which carries out the biosynthesis of guanosine tetraphosphate[103,104] and in vivo levels of guanosine tetraphosphate increase in response to a shift-down.[102,103] Moreover, it is known that guanosine tetraphosphate stimulates transcription of certain amino acid biosynthetic operons in vitro.[101,105] These findings are consistent with the idea that guanosine tetraphosphate mediates a general increase in the expression of multiple amino acid biosynthetic operons in response to depletion of one or more amino acids from the growth medium.[101]

The general control in bacteria differs from that in yeast in that nearly maximal derepression of the *his* operon occurs at guanosine tetraphosphate levels found in cells growing on minimal medium. While more severe starvation increases guanosine tetraphosphate levels further, there is little additional derepression of *his* operon expression attributable to the general control mechanism.[101,102] Derepression in response to this more severe starvation is primarily the result of the operon-specific attenuator mechanism.[106] By contrast, when yeast growing in minimal medium are starved for an amino acid, substantial cross-pathway derepression occurs beyond that observed in a shift-down from amino acid-complete to minimal medium.

One feature which yeast and bacteria share is that cross-pathway derepression in both systems is mediated by a low level of aminoacylation of tRNA. In bacteria, this signal is detected by the *relA* gene product as an idling of protein synthesis by virtue of its close association with the peptide elongation reactions on the ribosome.[107] While it is not expected that any of the *GCN* factors in yeast synthesize guanosine tetraphosphate in response to starvation,[108] it will be of interest to dètermine whether one or more of the *GCN* products detect the starvation signal as a result of a close association with the protein synthetic machinery.

VII. SUMMARY AND PROSPECTS FOR FUTURE RESEARCH

The expression of a large number of unlinked genes encoding enzymes in multiple amino acid biosynthetic pathways in yeast is coupled to starvation for any one of a number of different amino acids. At present, 30 enzymes in seven different pathways are known to be subject to general amino acid control. Because the outcome of general control-mediated derepression of a particular enzyme can be modified by pathway-specific repression, it is possible that the general control extends to an even greater number of amino acid biosynthetic enzymes than is currently recognized. To resolve this question, it will be necessary to examine the starvation response of those enzymes subject to pathway-specific repression under conditions in which these pathway-specific controls are inactivated. The molecular details of the interplay between amino acid-specific and general control repression mechanisms in determining the expression of genes subject to dual control is likely to be a fascinating aspect of future research on the regulation of amino acid biosynthesis in yeast.

It is important to ascertain whether the repression of amino acid biosynthetic enzymes observed in cells grown on amino acid-complete medium vs. minimal medium is a manifestation of the general amino acid control, or instead, is indicative of an independent repression system. The existing *gcn*$^-$ mutations block derepression in response to starvation but do not appear to block derepression in a shift-down from amino acid-complete medium to minimal medium. This suggests that the two derepression phenomena may be distinct. However, it is possible that the latter results from leakiness of the existing *gcn*$^-$ alleles.

Since the *GCN* genes have now been isolated, it should be possible to generate null alleles of these genes and determine the effects of such mutations upon complete medium repression. It would also be useful to isolate mutations which completely block derepression in a shift from amino acid-complete to minimal medium and then examine the effects of such mutations upon the derepression response to amino acid starvation.

General control derepression takes place at the level of transcription of the structural genes which encode the regulated enzymes and it is mediated by both *cis*-acting and *trans*-acting regulatory elements. An important *cis*-acting element is the T-G-A-C-T-C sequence found nontandemly repeated upstream from many of the co-regulated structural genes. This sequence element acts as a site for positive regulation of transcription at these genes and appears to be the binding site for the *trans*-acting positive regulator encoded by *GCN4*. Unfortunately, an in vitro transcription system for yeast RNA polymerase II is not yet available so that a functional assay for a yeast transcription factor cannot be coupled to the analysis of a DNA-protein interaction. Nevertheless, demonstration of a specific interaction between the *GCN4* protein and the regulatory sequences in the promoters of those genes subject to its control is a valuable biochemical confirmation of the genetic model for general amino acid control.

Mutational analyses of the regulatory regions of the *HIS4* and *HIS3* genes have shown that these are complicated elements which may contain multiple signals for transcriptional regulation in addition to the T-G-A-C-T-C sequences. It is important to characterize the roles of the different elements present in the regulatory regions of these and other genes subject to the general control and to determine how they interact with the T-G-A-C-T-C sequences to determine promoter strength in different growth conditions. A related aspect of this problem is that the 5′ noncoding regions of different genes subject to general control exhibit considerable variation in the number of T-G-A-C-T-C repeats, the spacing of the repeats, the distance of the repeats from the transcription initiation sites, and the nature of the DNA sequences that surround the repeats. These variations are likely to be responsible for the differences in derepression ratio observed among genes coregulated by the general control. A fine-structure analysis of several such genes should provide insight into the significance of these structural variations.

A combination of genetic and molecular analysis has shown that the level of *GCN4* activity in yeast cells is modulated by a variety of positive and negative effectors in response to amino acid starvation. This regulation appears to operate primarily at the level of *GCN4* gene expression. The other known *GCN* gene products stimulate *GCN4* expression in amino acid-starved cells, whereas *GCD* factors act as negative effectors of *GCN4*. Genetic analysis of the regulatory genes suggests that the *GCD* factors act more directly than the *GCN* products to regulate *GCN4* and that the role of the indirect *GCN* factors (*GCN1*, *GCN2*, and *GCN3*) is antagonism or repression of the *GCD* products in amino acid-starved cells. Unexpectedly, at least some of the regulators of *GCN4* seem to operate at the level of *GCN4* translation and do so by mediating the effects of a *cis*-acting negative regulatory region present in the 5′ leader of *GCN4* mRNA. This region includes four small, consecutive open reading frames. By analogy with the behavior of similar coding sequences introduced into the 5′ leaders of other eukaryotic genes, the short open reading frames at *GCN4* may act to reduce the efficiency of translation initiation at the *GCN4* coding sequences downstream. The identification of the precise nucleotides in the *GCN4* leader which are involved in the translational control of *GCN4* is an important undertaking for the future. It should help to clarify the mechanism of the negative regulatory effect of the leader sequences on *GCN4* expression and may serve to define binding sites for *trans*-acting factors which mediate the regulatory function of these sequences.

Molecular analysis of the *trans*-acting factors should help to identify the precise roles these molecules play in the regulation of *GCN4*. Since general control derepression is brought

about by a depletion of aminoacylated tRNA and because the general control *trans*-acting factors operate by modulating the translational efficiency of *GCN4* mRNA, it is possible that these factors are components of the translational apparatus. This might explain the pleiotropy of *gcd*$^-$ mutations, which lead to varying degrees of growth inhibition in addition to their effects on gene expression. The *gcd1-101* mutation is a conditional lethal and leads to G1 arrest at the restrictive temperature. It is important to learn whether the essential function carried out by *GCD1* is involved specifically in coordinating the status of amino acid availability with the decision to enter the cell cycle, or instead, merely reflects the role this factor plays in the expression of essential cellular proteins.

One of the more puzzling characteristics of the general control is its cross-pathway character. It is not known whether the increase in size of the nonlimiting amino acid pools which occurs in response to starvation for a single amino acid promotes increased synthesis and/or utilization of the limiting amino acid. If increasing the size of multiple amino acid pools has important physiological consequences, it should be possible to isolate mutations which interfere with the putative regulatory consequences of this effect. Such mutations should impair the adaptation to single amino acid starvation without interfering with the derepression of enzyme levels. An alternative explanation for the cross-pathway character of the general control is that the increase in pool sizes of nonlimiting amino acids is inconsequential, occurring simply because a single set of regulatory elements control the expression of all genes subject to the general control and no mechanism exists to fully suppress the derepression of pathways unaffected by starvation. In this view, all mutations which impair adaptation to amino acid starvation should exert their effects at the level of gene expression.

In conclusion, much has been learned about the mechanism whereby the levels of multiple amino acid biosynthetic enzymes derepress in amino acid-starved yeast cells. Both *cis* and *trans*-acting regulatory elements which function to generate this response have been identified and the general outline of how their interaction is regulated by amino acid availability has been defined. It will be of great value to further elucidate the molecular details of this regulatory system for several reasons. First, thanks to the ease of genetic analysis in yeast, general amino acid control now represents one of the best characterized examples in a eukaryotic organism of the coordinate regulation of unlinked genes — a phenomenon central to the understanding of the molecular mechanism of differentiation in higher eukaryotes. Second, analysis of the general control offers an opportunity to study the interesting problem of how regulatory molecules are themselves regulated by changes in the environment. Third, it is possible that the general control system is involved in the regulation of the cell division cycle in response to nutrient limitation. Finally, given the prominent role of translational control in the regulation of *GCN4*, it seems likely that by studying the general control we will learn as much about the details of protein synthesis in yeast as the regulation of amino acid biosynthesis.

ADDENDUM

Since the time this manuscript was written it was demonstrated conclusively that the upstream open reading frames are critical regulatory sequences in the *GCN4* mRNA leader. This was shown by making point mutations in the AUG codons of all four of these small coding sequences. The AUG mutations reveal interesting differences among the upstream open reading frames in their effects on *GCN4* expression and regulation. It now appears that interactions among the functionally distinct leader open reading frames provide the basis for translational control and that *trans*-acting *GCD* and *GCN* factors modulate these interactions in response to amino acid availability.[109] Four new *GCN* genes have been identified by genetic analysis. It is suggested that the new *GCN* genes regulate *GCN4* expression more directly than *GCN1* - *GCN5* and that two of these genes control *GCN4* at the transcriptional

level.[110] Finally, there is now direct evidence that the TATA sequence at $^{-}63$ in the *HIS4* promoter is required for transcription initiation at position $^{-}1$; however, sequences at the mRNA start site also contribute to the specificity of transcription initiation at *HIS4*.[111]

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