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Negative Regulatory Gene for General Control of Amino Acid Biosynthesis in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, many amino acid biosynthetic pathways are coregulated by a complex general control system: starvation for a single amino acid results in the derepression of amino acid biosynthetic genes in multiple pathways. Derepression of these genes is mediated by positive (*GCN*) and negative (*GCD*) regulatory genes. In this paper we describe the isolation and characterization of a previously unreported negative regulatory gene, *GCD3*. A *gcd3* mutation is recessive to wild type, confers resistance to multiple amino acid analogs, and results in overproduction and partially constitutive elevation of mRNA levels for amino acid biosynthetic genes. Furthermore, a *gcd3* mutation can overcome the derepression-deficient phenotype of mutations in the positive regulatory *GCN1*, *GCN2*, and *GCN3* genes. However, the *gcd3* mutation cannot overcome the derepression-deficient phenotype of a *gcn4* mutation, suggesting that *GCD3* acts as a negative regulator of the important *GCN4* gene. Northern blot analysis confirmed this conclusion, in that the steady-state levels of *GCN4* mRNA are greatly increased in a *gcd3* mutant. Thus, the negative regulatory gene *GCD3* plays a central role in derepression of amino acid biosynthetic genes.

In the yeast *Saccharomyces cerevisiae*, starvation for a single amino acid results in increased transcription of the biosynthetic genes for that amino acid, as well as the genes for other unrelated amino acid biosynthetic pathways (16, 21). This coregulation is referred to as general control of amino acid biosynthesis. The derepression is not coordinate; all the enzymes in a given pathway may not be derepressed, and those that are derepressed may be derepressed to varying extents (14). Similar regulation of amino acid biosynthesis occurs in other fungi, although these systems are more poorly characterized (1, 18).

The derepression of genes under general control is effected by an array of *cis*- and *trans*-acting regulatory elements. A 6-base-pair consensus sequence is repeated in the 5′ region of all genes thus far examined under general control, defining the recognition site for a positive regulatory factor which is required for derepression (4, 10, 23). There also exist transcribed open reading frames in the 5′ region of genes under general control which are needed to regulate the kinetics of the derepression response to amino acid starvation (7; B. Hauge and H. Greer, manuscript in preparation). In addition, five *trans*-acting regulatory genes that act formally as positive effectors for derepression have been identified, *GCN1*, *GCN2*, *GCN3*, *GCN4*, and *GCN5* (16, 17, 19, 25). The wild-type products of *GCN1*, *GCN3*, and *GCN4* are necessary for initiation of biosynthetic gene mRNA derepression, whereas the wild-type product of *GCN2*, itself under general control, is required for maintenance of elevated mRNA levels (17). *GCN2* has been shown to be transcriptionally regulated, whereas *GCN4* is translationally regulated (8, 17, 24). There are also *trans*-acting control genes which are formally negative regulators, acting to limit the expression of genes under general control. Two such genes, *GCD1* and *GCD2*, have been identified. A *gcd1* mutation causes constitutive levels of amino acid biosynthetic enzyme activity and temperature sensitivity for growth on rich medium (25). However, it now appears that *GCD1* may have a more basic metabolic role than general control regulation, in that a *gcd1* mutation decreases the rate of overall protein synthesis (D. Hill and K. Struhl, personal communication). A *gcd2* mutation results in constitutive levels of tryptophan biosynthetic enzyme activity (14); no further characterization has been reported. A mutation in another gene, *GEN*^{c}, affects arginine biosynthetic enzymes, but unlike *gcd1* or *gcd2*, enzyme activity is increased only under certain types of repressing growth conditions (13).

Overall, the role of negative regulation is more poorly characterized than that of positive regulation. Yet, it is clear that an understanding of the general control system requires a knowledge of all the major regulatory elements. Since the two *GCD* genes which have been reported to date are each represented by only a single allele, it seemed reasonable that other *GCD* loci might exist. We therefore undertook an extensive search for *gcd* mutations. In this paper we report the isolation of three new negative regulatory genes. One of these, designated *GCD3*, is characterized in detail. A *gcd3* mutation results in overproduction and partially constitutive derepression for multiple amino acid biosynthetic enzymes and has been shown to effect this response by increasing the steady-state mRNA levels of the corresponding genes. Epistasis data suggest that these increased mRNA levels are the result of the *GCD3* gene product being a negative regulator of the positive regulatory *GCN4* gene. This model was confirmed at the molecular level by showing that a *gcd3* mutation causes increased transcription of the *GCN4* gene. These results are the first indication that, in addition to being translationally regulated, a key positive controlling element, *GCN4*, is also transcriptionally regulated.

MATERIALS AND METHODS

Media and genetic and nucleic acid techniques. Yeast extract-peptone-glucose (YEP), minimal, minimal plus histidine (minimal-histidine), minimal plus all 20 amino acids (minimal-AA), minimal plus 10 mM 3-amino-1,2,4-triazole

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TABLE 1. Genotypes and phenotypes of His⁺ revertants[a]

Genotype	Min, 30°C	Min, 37°C	His, 37°C	YEP, 37°C	D/r[b]
his4C(Ts) *gcn*	−	−	+	+	NA
his4C(Ts) *gcn gcd*	+	−	+	+	r
HIS4C⁺ *gcn*	+	+	+	+	D
his4C(Ts) *GCN*⁺	+	−	+	+	D
HIS4C⁺ *gcn* leth(Ts)	+	−	−	−	D
his4C(Ts) *GCN*⁺ leth(Ts)	+	−	−	−	D
HIS4C⁺ *gcn* auxo(Ts)	+	−	−	+	D
his4C(Ts) *GCN*⁺ auxo(Ts)	+	−	−	+	D
his4C(Ts) *gcn* "*gcd1*"	+	−	−	−	r

[a] The genotype column lists the parent and the possible His⁺ revertants from mutant selections. leth(Ts) indicates a *ts* mutation in an essential gene; auxo(Ts) indicates a *ts* auxotrophic mutation; "*gcd1*" indicates a *ts* mutation similar to *gcd1*. The His⁺ revertants were replica plated to minimal plus uracil medium (min) at 30 and 37°C, minimal plus histidine plus uracil medium (his) at 37°C, and YEP medium at 37°C. +, growth; −, no growth.

[b] Dominance-recessiveness (D/r) was determined by mating the revertants to a *his4C-207*(Ts) *gcnX trp1-1* strain and testing the resultant diploids for growth on minimal medium at 30°C. (The *gcnX* mutation, *gcn1* to *gcn5*, used in a given cross was the same as the *gcn* mutation in the parent strain). D indicates dominance and r indicates recessiveness of the His⁺ phenotype. NA, Not applicable. Note that the desired class possesses a unique phenotype.

(minimal-3AT), and minimal proline (uses proline instead of NH_4^+ as a nitrogen source) media, as well as genetic procedures, have been described previously (6, 20). RNA was extracted, electrophoresed, blotted onto GeneScreen (New England Nuclear Corp., Boston, Mass.), and hybridized as described in references 2 and 16.

***gcd* mutant isolation.** Independent YEP cultures of *his4C-207*(Ts) *gcn*⁻ *ura3-52* or *his1-29*(Ts) *his4C-207*(Ts) *gcn*⁻ *ura3-52* were grown overnight at 30°C, washed, plated on minimal plus uracil medium (2×10^8 cells per plate), irradiated with UV light to 50% cell survival, and incubated for 4 days at 30°C to obtain His⁺ revertants. *gcd* mutants among the His⁺ revertants were identified by a series of growth, genetic, and molecular tests as described in the Results.

RESULTS

Mutant isolation. Mutations in negative regulatory elements required for repression of amino acid biosynthetic genes were isolated by reverting the His⁻ phenotype of *his4C-207*(Ts) *gcn*⁻ strains. *his4C-207*(Ts) is a temperature-sensitive (*ts*) lesion in the gene encoding histidinol dehydrogenase in the histidine biosynthetic pathway, resulting in severely reduced enzymatic activity at the nonpermissive temperature of 37°C and partially reduced activity at the permissive temperature of 30°C. *his4C-207*(Ts) mutants can grow on minimal medium at 30°C by derepressing their amino acid biosynthetic genes, thus compensating for the partially impaired *HIS4C* enzyme. However, if the *his4C-207*(Ts) strain also carries a mutation in any one of the five positive regulatory *GCN* genes, it will not be capable of derepression and thus will be unable to grow on minimal medium at 30°C. Using UV light mutagenesis, we isolated His⁺ revertants of *his4C*(Ts) *gcn*⁻ strains on minimal medium at 30°C which counteracted the *gcn* block by allowing constitutive expression of the *HIS4* gene.

Negative regulatory *GCD* mutations were initially identified by replica plating the His⁺ mutants to (i) minimal medium at 30°C (to confirm suppression of the *gcn* mutation, (ii) minimal medium at 37°C (to identify unwanted *his4C*(Ts) to *HIS4C*⁺ revertants), (iii) minimal-histidine medium at 37°C (to confirm that no additional *ts* auxotrophic mutation had been isolated), and (iv) YEP (rich) medium at 37°C (to identify *ts* mutations in essential genes, e.g., *gcd1*-like or cell-division-cycle mutations). Dominance-recessiveness tests were also performed. Table 1 shows the possible classes of revertants resulting from single and double mutational events.

The overwhelming majority of His⁺ mutants from a *his4C*(Ts) *gcn*⁻ strain are revertants of *his4C*(Ts) to *HIS4C*⁺. To circumvent this problem, selections were also performed with *his1-29*(Ts) *his4C-207*(Ts) *gcn*⁻ parental strains [reversion of either *his*(Ts) mutation alone would not allow growth]. In addition, to maximize recovery of the broadest possible spectrum of *gcd* mutations, various parental strains were used for both the single and double *his*(Ts) selections, each of which carried a mutation in one of the five different *GCN* genes. The results of the selections are shown in Table 2.

The desired *gcd* mutants are extremely rare. Of a total of 3.1×10^{11} cells used in the selections, only three confirmed *gcd* mutants were isolated. One of these mutants, J21, was derived from a *his4C-207*(Ts) *gcn1-15* parental strain (PLM295-3A) and had acquired a mutation in a previously unidentified gene, designated *GCD3*. The other *gcd* mutations will be described elsewhere.

Single-double mutation. To determine whether the Gcd⁻ phenotype in J21 [*his4C-207*(Ts) *gcn1-15* Gcd⁻] resulted from a single or double mutational event, it was mated to a wild-type strain (cross SKL44). Meiotic analysis of 27 tetrads indicated that five spores did not grow on minimal medium at 30°C [*his4C*(Ts) *gcn1* GCD⁺]. If the Gcd⁻ phenotype was due to a single unlinked nuclear gene, 14 such His⁻ spores would have been expected (one-eighth of the total). Either one mutation, loosely linked to *HIS4C* or *GCN1*, or two unlinked mutations, either one of which alone would allow a *his4C*(Ts) *gcn1* spore to grow on minimal medium at 30°C, would result in the observed lowered recovery of His⁻ spores. To distinguish between these possibilities, several of the *his4C*(Ts) *gcn1* Gcd⁻ spores from the SKL44 cross were themselves crossed to a wild-type strain. If only one loosely linked mutation was present in the original isolate, then all these new crosses should give about 1/16th His⁻ spores [*his4C*(Ts) *gcn1*], the frequency seen in the SKL44 cross. If two mutations were present in the original isolate, then cross SKL44 would have produced some spores with one and some with two mutations. The spores with one mutation should give rise to 1/8th His⁻ spores; the spores with two mutations should give 1/16th His⁻ spores. The results indicated that the original J21 mutant contains two unlinked mutations. For one of the spores from the original cross, SKL44-3D, that was tested in this way (in cross PLM637), 36 tetrads were examined, and of the 29 *his4C*(Ts) *gcn1* spores obtained, 15 grew and 14 did not grow on minimal medium at 30°C. These results clearly indicate that SKL44-3D carries only one regulatory mutation which is capable by itself of suppressing *gcn1*. We designated this mutation *gcd3-201*. SKL44-3D was used for all subsequent analyses.

Dominance-recessiveness. A dominance-recessiveness test was performed on SKL44-3D [*his4C-207*(Ts) *gcn1-15 gcd3-201*]. This strain was mated with *his4C-207*(Ts) *gcn1-15*, and the resulting diploids were tested for growth on minimal medium at 30°C. As expected for a mutation in a negative regulatory gene, the *gcd3-201* mutation is recessive.

2:2 segregation. SKL44-3D [*his4C-207*(Ts) *gcn1-15*

TABLE 2. His⁺ revertants recovered from selections[a]

gcn	No. of cells screened	his4C(Ts) gcn screens (His⁺ revertant genotypes)						his1(Ts) his4C(Ts) gcn screens	
		gcd⁻	HIS4C⁺	GCN⁺	HIS4C⁺ leth(Ts) and GCN⁺ leth(Ts)	HIS4C⁺ auxo(Ts) and GCN⁺ auxo(Ts)	"gcd1"	No. of cells screened	Total His⁺
1	4.8×10^{10}	1[b]	2.7×10^4	39	10	30	0	2.6×10^{10}	1[c]
2	7.6×10^{10}	3[d]	4.0×10^4	33	71	173	0	1.0×10^{10}	1[e]
3	8.0×10^9	0	4.0×10^3	80[f]	18[f]	9[f]	0	1.3×10^{10}	0
4	5.5×10^{10}	0	2.7×10^{10}	25[f]	65[f]	83[f]	0	1.0×10^{10}	0
5	4.0×10^{10}	0	7.0×10^4	10^4	ND[g]	ND[g]	0	2.0×10^{10}	3[h]

[a] The genotypes of the different classes of His⁺ revertants from the *his4C-207*(Ts) *gcn⁻* screens and the total number of His⁺ revertants from the *his1-29*(Ts) *his4C-207*(Ts) *gcn⁻* screens are shown. The *gcn* column indicates which *gcn* mutation is present in the different parental strains. The His⁺ revertant genotypes columns list the single or double mutational event in the *his4C*(Ts) *gcn* parental strains, e.g., the complete genotype of *HIS4C⁺* leth(Ts) is *HIS4C⁺* leth(Ts) *gcn*. leth(Ts) indicates a *ts* mutation in an essential gene; auxo(Ts) indicates a *ts* auxotrophic mutation. The "*gcd1*" column represents mutants with a *gcd1*-like phenotype, i.e., recessive for growth on minimal medium at 30°C and *ts* for growth on rich (YEP) medium at 37°C. The genotypes were assigned on the basis of growth and dominance-recessiveness as described in Table 1.

[b] This mutant is *gcd3-201* and is described in this paper.

[c] This mutant is recessive, but was not analyzed further because it is unable to sporulate.

[d] Two of these mutants represent *gcd4-201* and *gcd4-202* and are described in reference 22. The third mutant is a His⁺ revertant that has not been well characterized.

[e] This mutant is a double reversion event of *his1-29*(Ts) to *HIS1⁺* and *his4C-207*(Ts) to *HIS4⁺*.

[f] The genotypes of these revertants were confirmed by random spore analysis in crosses to wild type. (The genotypes of the GCN⁺ revertants not tested by random spore analysis were inferred from complementation and growth tests.)

[g] ND, Not determined.

[h] These three mutations are each unlinked to *HIS1*, *HIS4*, or *GCN5*, are recessive, and will be described elsewhere.

gcd3-201] was mated to *his4C-207*(Ts) *gcn1-15* (cross PLM746). Of 15 tetrads analyzed, each had two His⁺ [*his4C*(Ts) *gcn1 gcd3*] and two His⁻ [*his4C*(Ts) *gcn1*] spores. Thus, *GCD3* segregates as a single nuclear gene.

Linkage. If the His⁺ phenotype of the original mutant had resulted from a reversion event at the *his4C-207*(Ts) or *gcn1-15* locus, no His⁻ [*his4C*(Ts) *gcn1*] spores would have been recovered in crosses SKL44 or PLM637. Two additional crosses were analyzed to confirm the nonlinkage of *gcd3-201* to *HIS4* and *GCN1*. In cross PLM748, SKL44-3D [*his4C-207*(Ts) *gcn1-15 gcd3-201*] was mated to a strain carrying *his4C-207*(Ts). Half of the spores from this cross are *his4C*(Ts) *gcn1*. If *gcd3* is unlinked to *gcn1*, then half of the *his4C*(Ts) *gcn1* spores will be *gcd3* (His⁺ phenotype) and half will be *GCD3⁺* (His⁻); linkage would give only His⁺ spores. Nine tetrads were analyzed, and of 18 *his4C*(Ts) *gcn1* spores, 9 were His⁺ and 9 were His⁻. Thus, the *GCD3* gene is not linked to the *GCN1* gene. In an analogous cross, PLM747, in which SKL44-3D [*his4C-207*(Ts) *gcn1-15 gcd3-201*] was crossed to a strain carrying *gcn1-15*, it was shown that *GCD3* is unlinked to *HIS4C* [of the 16 tetrads analyzed, 32 spores were *his4C*(Ts) *gcn1*, and of these, 13 were His⁺ and 19 were His⁻].

Allelism. *GCD3* is not allelic to *GCD1* or *GCD2*. *GCD1* has previously been mapped by others to chromosome XV (15). We have shown by the *spo11* mapping technique (12) that *GCD3* is not on chromosome XV. No rearrangements are apparent in either strain. Consistent with *GCD1* and *GCD3* being different genes is that *GCD1* is an essential gene (the *gcd1* mutation is temperature sensitive for growth on rich medium, and a disruption of *GCD1* is lethal); in addition, *gcd1* has been shown to affect overall protein synthesis (25; K. Struhl, personal communication). *GCD3* does not share any of these properties and must therefore be a different gene. *GCD3* was shown to be different from *GCD2* by analysis of a cross between a *his4C-207*(Ts) *gcn1-15 gcd3-201* strain and a *gcd2-1* strain (cross PLM756). Twenty-six tetrads yielded 25 *his4C*(Ts) *gcn1* spores. If *gcd3* and *gcd2* are nonallelic, one-fourth of these spores should be His⁻. If they are allelic, then all the spores should be His⁺. The results gave 20 His⁺ spores and 5 His⁻ spores, indicating that *gcd3* and *gcd2* are mutations in different genes.

Epistasis. *gcd3-201* was isolated because of its ability to overcome the *gcn1-15* phenotype (inability to derepress). To better understand the interaction of *gcd3* with other general control regulatory genes, we crossed SKL44-3D [*his4C-207*(Ts) *gcn1-15 gcd3-201*] to a set of strains, each carrying a mutation in *GCN2*, *GCN3*, or *GCN4*. The *his4C*(Ts) *gcnX* spores in each of these crosses were identified by complementation. Half of these spores carry *gcd3*. If *gcd3* is epistatic to a particular *gcn⁻* mutation, then the *his4C*(Ts) *gcnX gcd3* spores will be His⁺; if it is not epistatic they will be His⁻. Analysis of 40 tetrads from each cross indicated that *gcd3* is epistatic to *gcn2* and *gcn3*, i.e., a *gcd3 gcnX* strain behaves like a *gcd3* strain. However, *gcn4* is epistatic to *gcd3* [analysis of 10 *his4C*(Ts) *gcn4* spores indicated that they were all His⁻]. Thus, *GCD3* plays a more direct role in the general control system than *GCN1*, *GCN2*, or *GCN3*, but a less direct role than *GCN4*.

Amino acid analog resistance. Wild-type cells overcome amino acid analog-induced starvation by derepressing the enzymes for amino acid biosynthesis. A mutation in one of the positive regulatory *GCN* genes prevents this derepression. Analog sensitivity was tested in SKL44-3D [*his4C-207*(Ts) *gcn1-15 gcd3-201*] to determine whether the presence of a *gcd3* mutation counteracts the analog sensitivity caused by the *gcn1* mutation. SKL44-3D was considerably more resistant than the parent to analogs of tryptophan, methionine, arginine, and histidine (Fig. 1). In fact, SKL44-3D was even more resistant than the *GCN⁺* wild-type control strain for most of these analogs. Analysis of spores from a cross with SKL44-3D as one of the parents indicated that the analog resistance cosegregates with the *gcd3*-dependent His⁺ phenotype. Furthermore, strains carrying only the *gcd3-201* mutation (wild type for all other markers), obtained from cross PLM637, are also more resistant to amino acid analogs than a wild-type strain (data not shown). These composite results suggest that the *gcd3* mutation causes increased levels of enzymes in multiple amino acid biosynthetic pathways, strongly implicating the *GCD3* gene in the general control system.

mRNA levels. Blots of RNA extracted from wild type, the parental strain PLM295-3A [*his4C-207*(Ts) *gcn1-15*], the original mutant isolate J21 [*his4C-207*(Ts) *gcn1-15* Gcd⁻],

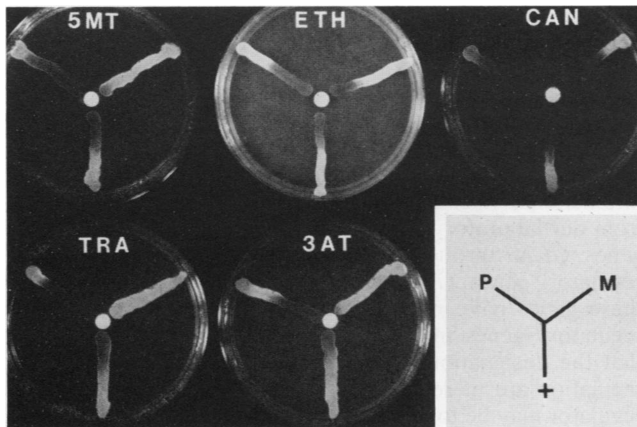

FIG. 1. YEP cultures were washed and striped onto minimal proline plus uracil plus histidine medium. A 10-μl sample of 50 mM 5-methyltryptophan (5MT), 20 μl of 10-mg/ml ethionine (ETH), 20 μl of 6-mg/ml canavanine (CAN), 40 μl of 50 mM triazole alanine (TRA), and 40 μl of 1 M 3-amino-1,2,4-triazole (3AT) solutions were each pipetted onto a Sensi-disc in the center of a petri plate and incubated for 2 days at 30°C. The distance from the disk to the end of the zone of growth inhibition indicates the degree of analog sensitivity. +, Wild-type S288C (*MAT***a**); P, parent PLM295-3A [*MAT***a** *his4C-207*(Ts) *gcn1-15 ura3-52*]; M, mutant SKL44-3D [*MAT***a** *his4C-207*(Ts) *gcn1-15 ura3-52 gcd3-201*].

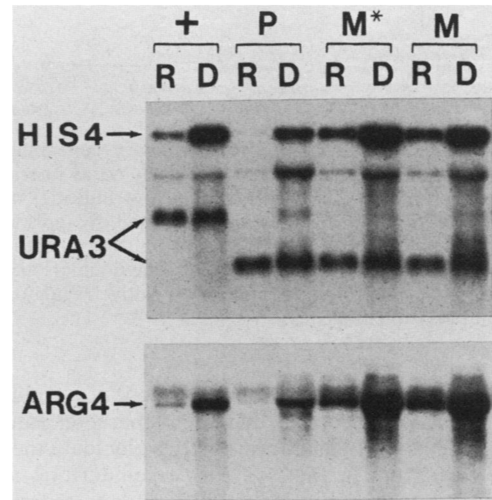

FIG. 2. RNA was prepared from cell cultures grown at 30°C under repressing conditions (R) in minimal-AA plus uracil medium and derepressing conditions (D) in minimal-3AT plus uracil medium, electrophoresed, blotted, and hybridized with a ^{32}P-labeled probe containing the yeast *HIS4* and *URA3* genes (pBH21B; B. Hauge and H. Greer, unpublished data). This same blot was dehybridized and rehybridized with a ^{32}P-labeled probe containing another gene under general control, the yeast *ARG4* gene (pYe511 [3,13]). +, Wild-type S288C (*MAT***a**); P, parent PLM295-3A [*MAT***a***his4C-207*(Ts) *gcn1-15 ura3-52*]; M*, original mutant isolate J21 [*MAT***a** *his4C-207*(Ts) *gcn1-15 ura3-52* Gcd$^-$]; M, mutant SKL44-3D [*MAT***a** *his4C-207*(Ts) *gcn1-15 ura3-52 gcd3-201*]. The extent of hybridization with the *URA3* gene was used to normalize the amount of RNA within each lane. The *ura3-52* mutation, present in the P, M*, and M strains, is a rearrangement (M. Rose, personal communication), and thus the *URA3* RNA runs at a different position when compared with +. The unidentified band in the top panel corresponds to *HIS4* degradation products which comigrate with the 18S rRNA band.

and SKL44-3D [*his4C-207*(Ts) *gcn1-15 gcd3-201*] grown under repressing and derepressing conditions were hybridized with a probe containing the *HIS4* and *URA3* sequences. (The *URA3* gene is not under general control.) The steady-state *HIS4* mRNA levels under both repressing and derepressing conditions were greatly increased in the *gcd3* strains as compared with the parent (Fig. 2); they were also increased (although to a lesser extent) when compared with the wild-type control strain (which has the additional advantage of being wild type for the positive regulator *GCN1*). (It should be noted that the mutant effects are superimposed on the parent strain which itself shows some derepression.) Steady-state mRNA levels were also determined for a gene in the arginine biosynthetic pathway known to be under general control, *ARG4*. An even more dramatic effect was seen under both repressing and derepressing conditions for the *ARG4* mRNA levels than is seen for *HIS4*. Thus, *gcd3* is a mutation which affects the regulation of at least two amino acid biosynthetic genes under general control by significantly elevating their steady-state mRNA levels. Since the increased mRNA levels are more pronounced under repressing conditions than under derepressing conditions, the *gcd3* mutation, in addition to causing overproduction, is also conferring a partially constitutive phenotype on the strains.

***GCN4* transcription.** Blots of RNA extracted from the wild type, the parental strain PLM295-3A [*his4C-207*(Ts) *gcn1-15*], the original mutant isolate J21 [*his4C-207*(Ts) *gcn1-15* Gcd$^-$], and SKL44-3D [*his4C-207*(Ts) *gcn1-15 gcd3-201*] grown under repressing and derepressing conditions were hybridized with a probe containing the *GCN4* gene (Fig. 3). A clear derepression effect for *GNC4* mRNA was observed in the parental [*gcn1-15 his4C-207*(Ts)] strain. The *gcd3* mutant strain, compared with the parental strain, showed a marked increase in the *GCN4* mRNA levels under both repressing conditions (approximately fourfold) and derepressing conditions (approximately twofold). It should be noted that these effects parallel those ultimately seen for the *HIS4* mRNA levels in the same *gcd3* mutant (a greater increase under repressing conditions) (Fig. 2). Thus, *GCD3* regulates the transcription of the most direct-acting positive regulatory gene thus far identified, *GCN4*. In addition, it is possible that the previously documented translational regulation of *GCN4* under derepressing conditions (8, 24) may be superimposed on this *GCD3*-dependent transcriptional regulation.

DISCUSSION

To date, the contribution of negative regulation in the general control of amino acid biosynthesis has been poorly

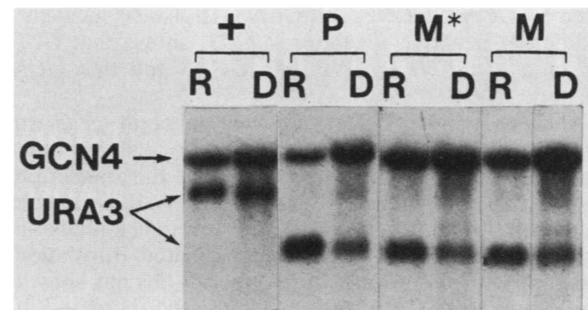

FIG. 3. RNA preparation, Northern blot analysis, and strains are the same as described in the legend to Fig. 2. Hybridization was with a probe containing the *GCN4* and *URA3* genes (pYeGCN4; M. Penn and H. Greer, unpublished data).

FIG. 4. Model of general control regulatory gene interaction. Dashed arrows indicate negative control of *GCD3* as inferred from epistasis experiments. The light solid arrow indicates negative control of *GCN4* by *GCD3* as demonstrated by epistasis and Northern blot experiments. The bold arrow indicates that the *GCN4* mRNA is translationally regulated (8, 24). The double solid arrows show that the *GCN4* protein is formally a positive regulator for the amino acid biosynthetic genes.

understood. Only a few negative regulatory mutations (gcd^-) have been reported, and their characterization has not been extensive. In an effort to better elucidate the role of negative regulators in this system, we undertook a large-scale search for such mutations. All previous searches had used analog resistance as a selection for *gcd* mutations; we used an alternate approach in the hope of recovering mutations in previously unidentified negative regulatory genes. One of the mutations we isolated, *gcd3*, is described in detail.

The *gcd3* mutation was selected on the basis of its ability to effect greatly increased *HIS4C* gene expression and thus to counteract a *gcn1* block to derepression. The *gcd3* mutation also results in increased resistance to analogs of histidine, methionine, arginine, and tryptophan. Thus, as expected of a regulatory mutation in the general control system, *gcd3* acts on unrelated amino acid biosynthetic pathways. Northern blot analysis of *gcd3* strains confirmed the general nature of its action and showed that the increase of amino acid biosynthetic gene activity is the result of overproduction and a partially constitutive pattern of elevated mRNA levels.

A general framework for the interaction of *GCD3* with other general control regulatory genes is provided by epistasis data. Double mutants of *gcd3* and *gcn1*, *gcn2*, or *gcn3* possess the derepressed phenotype of *gcd3*. Thus, the wild-type gene products of *GCN1*, *GCN2*, and *GCN3* are not necessary for derepression when *GCD3* is mutationally inactivated. These results suggest a more direct role in the general control circuitry for *GCD3* than for *GCN1*, *GCN2*, or *GCN3*. On the other hand, *gcd3 gcn4* double mutants possess the repressed phenotype of a *gcn4* strain. Therefore, *GCN4* acts more directly than does *GCD3*. Both the epistasis data and mutant phenotypes are consistent with a model in which the positive regulator *GCN4* is negatively regulated by *GCD3*, while *GCD3* is itself negatively regulated by *GCN1*, *GCN2*, and *GCN3*. (Epistasis analysis with a different negative regulator, *GCD1*, shows that *GCD1* is epistatic to *GCN1*, *GCN2*, and *GCN3* and that *GCN4* is epistatic to *GCD1* [9].)

Analysis of the *GCN4* message level in *gcd3* strains confirmed the role of *GCD3* as a negative regulator of *GCN4* and showed this regulation to occur at the transcriptional level. In a *gcd3* strain, the levels of *GCN4* RNA are clearly increased. In addition to this transcriptional regulation of *GCN4* by *GCD3*, *GCN4* is also regulated translationally. Previous work in this and other laboratories has shown that translation of *GCN4* mRNA is greatly increased under derepressing conditions (8, 24). The *GCN4* protein, in turn, is required for derepression of the amino acid biosynthetic genes. It should be noted that not only is *GCN4* the most direct-acting regulatory gene thus far identified, but, in addition, its mutant phenotype is much more severe than mutations in other *GCN* genes (16, 17). Furthermore, *GCN4* has been shown to bind to the TGACTC consensus sequence common to amino acid general control genes (11). A composite model of all these relationships is depicted in Fig. 4.

Clearly, this model must be viewed as representing a subset of a much more complex pattern of regulatory gene interactions. Published data and the more recent screens from our laboratory have identified nine positive regulatory genes, *GCN1* through *GCN9* (5, 16, 19, 25) and five negative regulatory genes, *GCD1* through *GCD5* (13, 22, 25). Many of these genes have not been analyzed in detail; also, other regulatory genes may exist. Another important proviso is that the designations in the model of positive or negative regulation are merely formal. The direct role of a positive regulator may be to negatively regulate a negative regulator, as, for example, appears to be the case for *GCN1*, *GCN2*, and *GCN3*.

In summary, the interaction of *GCD3* with *GCN4* revealed a key point of control for amino acid biosynthesis. Similar characterization of other general control regulatory genes should allow a more comprehensive understanding of this regulatory system.

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