crl Mutants of *Saccharomyces cerevisiae* Resemble Both Mutants Affecting General Control of Amino Acid Biosynthesis and Omnipotent Translational Suppressor Mutants

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ABSTRACT

*C*ycloheximide *r*esistant *l*ethal (*crl*) mutants of *Saccharomyces cerevisiae*, defining 22 unlinked complementation groups, are unable to grow at 37°. They are also highly pleiotropic at their permissive temperature of 25°. The mutants are all unable to arrest at the G_1 stage of the cell cycle when grown to stationary phase or when starved for a single amino acid, though they do arrest at G1 when deprived of all nitrogen. The *crl* mutants are also hypersensitive to various amino acid analogs and to 3-aminotriazole. These mutants also "tighten" leaky auxotrophic mutations that permit wild-type cells to grow in the absence of the appropriate amino acid. All of these phenotypes are also exhibited by *gcn* mutants affecting general control of amino acid biosynthesis. In addition, the *crl* mutants are all hypersensitive to hygromycin B, an aminoglycoside antibiotic that stimulates translational misreading. The *crl* mutations also suppress one nonsense mutation which is phenotypically suppressed by hygromycin B. Many *crl* mutants are also osmotically sensitive. These are phenotypes which the *crl* mutations have in common with previously isolated omnipotent suppressors. We suggest that the the *crl* mutations all affect the fidelity of protein translation.

WE have recently reported the isolation of the *c*ycloheximide *r*esistant temperature sensitive *l*ethal (*crl*) mutations in *Saccharomyces cerevisiae* (McCusker and Haber 1988). The *crl* mutations were isolated as being resistant to the minimum inhibitory concentration of cycloheximide and then screened for temperature-sensitive (ts) lethality. There are 22 unlinked complementation groups of *crl* mutations, none of which appears to be allelic with any of 39 ts mutations which we have tested or with any of the known ribosomal protein mutations, cycloheximide-resistance loci or the omnipotent suppressors. In addition to the phenotypes by which they were identified, the *crl* mutations have many other phenotypes in common, ranging from a failure to arrest at the G_1 stage of the cell cycle under some starvation conditions to hypersensitivity both to amino acid analogs and to the protein synthesis inhibitors G418 and hygromycin B. The data presented in this paper suggests that all of these phenotypes may reflect alterations in protein synthesis, most probably in the fidelity of translation.

Several of these *crl* phenotypes are characteristic of *gcn* (general control of amino acid biosynthesis) mutations. The *gcn* mutations were identified by their hypersensitivity to amino acid analogs and to 3-aminotriazole (Schurch, Miozzari, and Hutter 1974; Wolfner *et al.* 1975; Penn, Galgoci and Greer 1983). A detailed molecular biological study of the role of *gcn* mutants has demonstrated that at least some of these genes act through the translational regulation mediated by a series of small open reading frames 5′ on the mRNA to the reading frame that encodes the *GCN4* protein (Hinnebusch 1984, 1985; Mueller and Hinnebusch 1986). The *GCN4* protein is a positive regulator of the transciption of many amino acid biosynthetic genes (Hinnebusch and Fink 1983; Hope and Struhl 1986). The failure to derepress the amino acid biosynthetic genes explains the hypersensitivity to amino acid analogs and also accounts for the failure of *gcn* mutants carrying a normally "leaky" amino acid auxotrophic mutation to grow in the absence of that amino acid (Wolfner *et al.* 1975; Neiderberger, Aebi and Hutter 1983). Less well understood is the observation that *gcn* mutants fail to arrest at the G_1 stage of the cell cycle when starved for an amino acid (Neiderberger, Aebi and Hutter 1983). The number of *gcn* mutations that have been described is large; there are at least nine known complementation groups (Greenberg *et al.* 1986). None of these mutants have been described as temperature-sensitive, but several *gcn* mutants have been shown to be nonessential by the creation of deletion mutations (A. Hinnebusch, personal communication).

Other phenotypes of the *crl* mutants have been described previously in characterizing omnipotent suppressor mutations. The omnipotent suppressors

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of *S. cerevisiae* were isolated on the basis of their ability to suppress different nonsense mutations (HAWTHORNE and LEUPOLD 1974; LIEBMAN and CAVENAGH 1980; ONO, STEWART and SHERMAN 1981; EUSTICE *et al.* 1986). In this regard they resemble the *ram* mutations in *Escherichia coli* (GORINI 1971). The *SUP46* gene product is either the structural gene for, or a modifier of, a ribosomal protein (ISHIGURO *et al.* 1981). The *SUP45* gene product, on the other hand is apparently not a ribosomal protein, although the wild type gene product is necessary for both wild-type translational fidelity and viability (HIMMELFARB, MAICAS and FRIESEN 1985). There may be other genes which affect translational fidelity which are not part of the 80S ribosome. In addition to their suppression of some nonsense mutations, the omnipotent suppressors have several other distinctive phenotypes; they are hypersensitive to aminoglycoside antibiotics that stimulate translational misreading (LIEBMAN and CAVENAGH 1980; HIMMELFARB, MAICAS and FRIESEN 1985; EUSTICE *et al.* 1986). Some omnipotent suppressor mutants are sensitive to increased osmotic pressure (LIEBMAN and CAVENAGH 1980); some are temperature sensitive (HIMMELFARB, MAICAS and FRIESEN 1985).

In this paper we show that the *crl* mutants share many of the phenotypes of both the *gcn* and omnipotent suppressor mutations that affect protein translation. Based on their shared properties with these other classes of mutants, we suggest that the *crl* mutants also affect the fidelity of protein translation. Based on parallel studies with wild-type cells we also present data suggesting that translational fidelity is strongly affected by increased temperature and osmotic pressure. This leads us to suggest that the ts lethality and osmotic sensitivity of the *crl* mutations is a consequence of increased translational misreading.

MATERIALS AND METHODS

Strains: All experiments were done in the Y55 genetic background using isogenic Y55 strains. The genotype of Y55 is *HO gal3 MAL1 SUC1*. All auxotrophic, ts lethal and drug-resistance mutations were isolated in the Y55 genetic background using Y55 or strains directly derived from Y55 (MCCUSKER and HABER 1988).

Hygromycin B-suppressible auxotrophic mutations: A total of 251 different auxotrophic mutations (different complementation groups or independent isolates in the same complementation group) were isolated as described in MCCUSKER and HABER (1988). These mutations were screened for phenotypic suppression by hygromycin B by the ability of strains carrying single auxotrophic mutations to grow when replica plated to minimal media containing 100–800 μg/l hygromycin B at 25, 30 and 37°. All *lys2* and *lys5* mutations were isolated in a separate selection and screen. CHATTOO *et al.* (1979b) have shown that 95% of the mutations isolated through the use of the α-aminoadipate selection are in *lys2* and only 5% in *lys5*. An interesting observation was that in spite of this strong bias in favor of *lys2* mutations we found only one hygromycin B suppressible *lys2* mutation while seven hygromycin B suppressible *lys5* mutations were found. Twenty-five mutants proved to be hygromycin B suppressible. These mutations, with the number of independent alleles in parentheses, were found in the following genes: *arg3* (3), *arg6* (1), *argB* (2), *leu1* (1), *lys2* (1), *lys5* (7), *met2* (5), *met4* (1), *met13* (2), *trp1* (1) and *ura2* (1) (MCCUSKER 1986).

The hygromycin B suppressible mutants were then classified as being UAG, UGA and UAA by their co-reversion with known nonsense mutations, *his4-580* (UAG), *his4-260* (UGA), *his4-39* (UAA) and *ade2-1* (UAA) or with other mutations isolated in Y55 that co-reverted with these known nonsense mutations. In each case, a hygromycin B suppressible mutation was crossed with strains carrying the known nonsense mutations and meiotic segregants carrying both mutants were then obtained by tetrad dissection. One UAG, two UGA, and 22 UAA mutants were identified. The reason for the large proportion of UAA mutations is not known but may reflect some degree of specificity in the translational misreading caused by hygromycin B. However, it should be noted that all three types of nonsense mutations have been described as being suppressible by hygromycin B (SINGH, URSIC and DAVIES 1979). Two mutations that co-reverted with a UAA mutation also showed some co-reversion with a UGA mutation; three other UAA mutations showed some co-reversion with a UAG mutation. the *lys5-4* mutation showed some co-reversion with certain UAA, UAG and UGA mutations.

Media: Both defined and rich media are described in the accompanying paper (MCCUSKER and HABER 1988). Hygromycin B supersensitivity was determined using YEPD + 100 or 200 μg//ml hygromycin B. Cryptopleurine resistance was determined using YEPD + 3 μg/ml cryptopleurine. Cryptopleurine hypersensitivity was determined using YEPD + 1 μg/ml cryptopleurine. Hypersensitivity to anisomycin was scored using YEPD + 50 or 20 μg/ml (a wild type strain grows poorly in the presence of 50 μg/ml anisomycin). All antibiotics were added after autoclaving.

Media containing amino acid analogs or the histidine synthesis inhibitor 3-aminotriazole contained 1.7 g/liter yeast nitrogen base without $(NH_4)_2SO_4$ and without amino acids and 2.5 g/liter urea instead of regular yeast nitrogen base. Urea, amino acid analogs and 3-aminotriazole were added after autoclaving. Only the necessary nutritional supplements were added to these media.

Osmotic remediability and sensitivity: Osmotic suppression of ts lethality was determined by the ability of cells to grow at 37° on YEPD containing glycerol (at concentrations ranging from 2.5 to 1.0 M) or KCl (at concentrations of 1.5 to 0.75 M). Cells were pregrown on YEPD at 25° and were replica plated to plates containing either glycerol or KCl at various concentrations. These plates were then incubated at 37° for 24 hr, scored and then re-replica plated to the same type of plate which was again incubated at 37° for 24 hr and was then scored. Osmotic sensitivity of the *crl* mutations was determined by the inability to grow on the same types of media at 25°.

RESULTS

***crl* mutants fail to arrest in G_1 under some starvation conditions:** All of the *crl* mutants also show an inability to arrest as single unbudded cells upon reaching stationary phase. This phenotype is more pronounced when the cells are grown on plates than

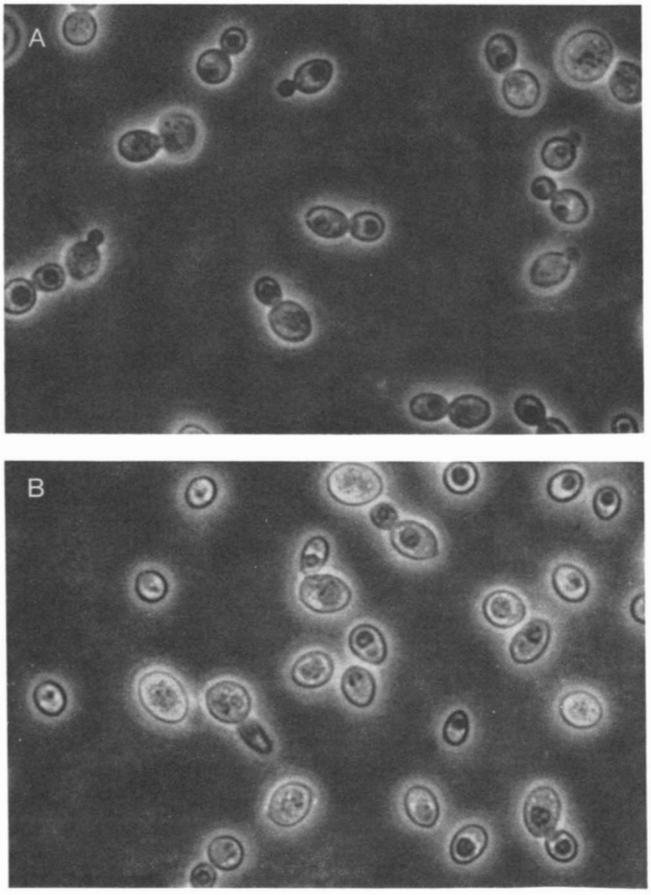

FIGURE 1.—A, Failure of *crl19* cells to arrest as single unbudded cells when grown to stationary phase. B, Stationary phase arrest of *crl3* cells, which are more nearly like the wild type in their behavior. In this case, approximately 10% of the cells fail to arrest as unbudded cells. G_1 arrest in wild-type cells is observed in greater than 98% of all cells (data not shown).

in liquid media, and some *crl* mutants are more severely affected than others. Two examples are shown in Figure 1.

The *crl* mutants also fail to arrest in G_1 in response to starvation for a single amino acid, produced by starving both *met13* and *trp5* derivatives of the *crl* mutants. There was a low proportion of unbudded cells and the high proportion of cells with different sized buds in the population (data not shown). In contrast, all of the *crl* mutants did show normal G_1 arrest in response to general nitrogen starvation as judged by the uniform population of unbudded cells (data not shown). Nitrogen starvation was produced by taking a log phase culture of a prototrophic strain growing in minimal defined medium, spinning the cells down, washing and resuspending the cells in fresh medium that did not contain any nitrogen source. The cells were examined under the microscope 24 hr later. The fact that the *crl* mutants arrest normally in response to nitrogen starvation shows that the failure to arrest in G_1 during amino acid starvation or at stationary phase is not due to a general inability to arrest in G_1.

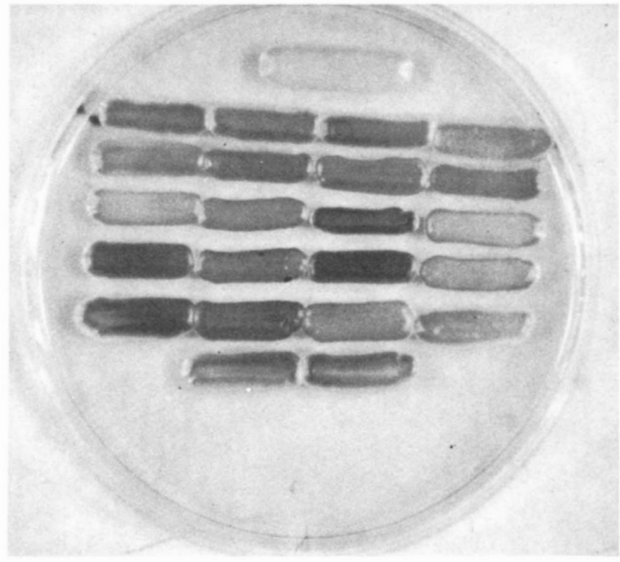

FIGURE 2.—Iodine:iodide vapor staining of wild-type and *crl* mutant strains after growth on YEPD at the permissive temperature of 25°. The *crl* strains are arranged numerically from the upper left hand corner from left to right (*i.e., crl 1, 3, 4, 5; 6, 7, 8, 9; 10, 11, 12, 13; 14, 15, 16, 17; 18, 19, 20, 21; 22, 23*). The wild-type control is the single streak at the top of the plate.

Accumulation of storage carbohydrates: The storage carbohydrates glycogen and trehalose are accumulated in response to starvation conditions (LILLIE and PRINGLE 1980). Because the *crl* mutants displayed an abnormal response to two different types of starvation conditions (stationary phase and amino acid starvation) we also asked if they differed from wild-type cells in their accumulation of storage carbohydrates. This was done by staining cells which had grown up to stationary phase on a YEPD plate by placing several drops of a concentrated iodine:iodide solution on the underside of the platecover. An example of the results can be seen in Figure 2; 18 of 22 of the *crl* mutants showed an abnormal overaccumulation of storage carbohydrates at 25°.

***crl* mutants share several phenotypes with *gcn* mutants:** Prototrophic derivatives of the *crl* mutants are all hypersensitive to amino acid analogs: triazolealanine, β-2-thienylalanine, β-chloroalanine and mimosine (Table 1). Amino acid analog sensitivity is also exhibited by *gcn* (general control non-derepressing) mutants which are sensitive to amino acid analogs because they fail to derepress the amino acid biosynthetic pathways in response to amino acid starvation (WOLFNER *et al.* 1975; LUCCHINI *et al.* 1984). In addition, *gcn* mutants are also hypersensitive to the histidine biosynthesis inhibitor 3-aminotriazole. Most, but not all, of the *crl* mutants are also more sensitive to 3-aminotriazole (Table 1). These results suggests that most *crl* mutants also fail to derepress the amino acid biosynthetic pathways to wildtype levels.

TABLE 1

***crl* mutations are hypersensitive to amino acid analogs and 3-aminotriazole**

			βCA		βTA		3AT		
	MIM	TAA							
crl	50	20	15	50	25	50	10	25	50
+	+	+	+	+	+	+	+	+	+
1	−	−	−	−	−	−	+/−	−	−
3	−	−	−	−	+	−	+/−	−	−
4	−	−	−	−	+	−	+/−	−	−
5	−	−	−	−	−	−	+	−	−
6	+	−	+/−	+/−/−	−	−	+/−	+/−	−
7	−	−	−	−	+	−	+/−	+/−	−
8	−	−	+	−	+	−	+	+	−
9	−	−	+	+/−/−	+	−	+	+	+
10	−	−	+/−	+/−/−	+	−	+	+	+
11	−	−	−	−	−	−	−	−	−
12	−	−	+	+/−/−	+	−	+	+	+
13	−	−	−	−	−	−	+/−	−	−
14	−	−	+/−	+/−/−	+	−	+	+	−
15	−	−	−	−	+	−	+	+/−	−
16	−	−	−	−	−	−	+/−	−	−
17	−	−	+	−	+	−	+	+/−	−
18	−	−	−	−	−	−	−	−	−
19	−	−	+/−	−	+	−	+	+/−	−
20	−	−	−	−	+	−	+	+	−
21	−	−	−	−	+	−	+	+	−
22	−	−	−	−	−	−	+	+/−	−
23	−	−	−	−	+	−	+	+	−

The growth of *crl* strains in the presence of amino acid analogs and the histidine synthesis inhibitor 3-aminotriazole. TAA, triazolealanine; βTA, β-2-thienylalanine; βCA, β-chloroalanine; MIM, L-mimosine; 3AT, 3-aminotriazole. The numbers given are the concentrations in μg/ml for the amino acid analogs and in mM for 3-aminotriazole. +, wild-type level of growth; +/+/−, slight inhibition of growth relative to wild type; +/−, strong inhibition of growth relative to wild type; +/−/−, very strong inhibition of growth relative to wild type; −, no growth.

TABLE 2

Growth of *crl* mutants carrying leaky auxotrophic mutations

crl	*lys2-9*	*trp5-24*	*met13-34*	*arg6-4*
Crl$^+$	+	+	+	+
1	−	−	+/−	+/+/−
3	+	+	+	+/+/−
4	+	−	+	+/−
5	−	−	+/−	+/+/−
6	−	−	+/−	+/+/−
7	+/−	−	+/−	+/−
9	+/−	+/−	+/−	+/+/−
10	+/−	+/−	+/+/−	+/+/−
11	−	−	+/−	+/+/−
12	+/−	+/−	+	+/+/−
13	+	+/−	+/−	+/−
14	+	+/−	+/−	+
15	+/−	+	+	+/−
16	+	+/−	+/−	+/−
17	−	+/−	+/−	+
18	+/−	+/−	+/−	+/+/−
19	+/−	+	+	+/+/−
20	−	−	+	+/−
21	+/−	−	+/−	+/+/−
22	−	−	+/−	+/+/−
23	−	−	+/−	+/+/−

Strains were constructed which contained the leaky auxotrophic mutations *arg6-4* and *lys2-9* and either *met13-34* and *trp5-24* or *met13-13* and *trp5-32*. Cells were grown at 25° on YEPD and then replica plated to plates lacking one amino acid. +, wild-type level of growth; +/+/−, slight inhibition of growth relative to wild type; +/−, strong inhibition of growth relative to wild type; −, no growth.

One other phenotype of the *gcn* mutants is a consequence of an inability to derepress the amino acid biosynthetic pathways: auxotrophic mutations which are "leaky" (*i.e.*, exhibit slow growth in the absence of an amino acid) in a wild-type strain are "tight" (allowing little or no growth) in the presence of a *gcn* mutant (NIEDERBERGER, MIOZZARI and HUTTER 1981; NIEDERBERGER, AEBI and HUTTER 1983). To test whether *crl* mutants had a similar effect on leaky auxotrophic mutations, a Crl$^+$ strain was constructed which contained four leaky auxotrophic mutations: *arg6-4 lys2-9 met13-14* and *trp5-24*. In a *Crl*$^+$ background *met13-34* grew much better at 25° than at 30°; all of the other mutations showed temperature-independent leakiness. This strain was crossed with one allele from each of the *crl* loci and segregants carrying both the *crl* mutation and the auxotrophic mutations were tested for their ability to grow in the absence of a single amino acid at 25°. The results are summarized in Table 2. Many of the *crl* mutant strains carrying one of the leaky amino acid auxotrophic mutations are completely unable to grow in the absence of that amino acid. This effect could be seen most clearly with the *lys2-9* and *trp5-24* mutations, lesser effects could be seen with the *arg6-4* and *met13-34* mutations.

The *crl* mutants are sensitive to some translational inhibitors: A single allele from each of the 22 *crl* complementation groups was used to determine their degree of resistance or sensitivity to protein synthesis inhibitors relative to wild-type cells (Table 3). Increased resistance was determined by the ability of a *crl* mutation to grow on media where a wild-type control could not grow (YEPD + 3 μg/ml cryptopleurine, 50 μg/ml anisomycin, 200 μg/ml G418 and 300 μg/ml hygromycin B). Increased sensitivity was determined by the inability of a *crl* mutation to grow on media where a wild-type control could grow (YEPD + 1 μg/ml cryptopleurine, 20 and 50 μg/ml anisomycin, 100 μg/ml G418, 100 μg/ml hygromycin B and 200 μg/ml hygromycin B). The same concentration of anisomycin was used to determine both resistance and sensitivity (a wild-type control strain grows poorly at the high concentration).

As shown in Table 3, all of the *crl* mutations showed increased sensitivity to hygromycin B which is known to stimulate translational misreading (SINGH, URSIC and DAVIES 1979). By way of contrast, only a few *crl* mutations show increased sensitivity to the structur-

TABLE 3

Antibiotic sensitivity of *crl* mutants *vs.* the wild-type strain

crl locus	Cryptopleurine[a]	Anisomycin[b]	G418[c]	Hygromycin B[d]
1		S		S
3		S		S
4		S		S
5			S	S*
6		S	S	S*
7		S		S
8	S	S		S
9				S*
10	R			S
11			S	S*
12	R		S	S
13	R	S	S	S*
14	R	S		S
15		S		S*
16				S
17	R	S		S*
18	S	S	S	S*
19	R	S		S
20	R	S		S
21	R			S*
22	S		S	S*
23				S*

Cells grown on YEPD plates at 25° were replica plated to YEPD plates containing various antibiotics in the concentrations indicated. Only differences from wild-type strains are indicated.

[a] Cryptopleurine: S = failure to grow on 1 μg/ml cryptopleurine; R = growth on 3 μg/ml cryptopleurine.

[b] Anisomycin: S = failure to grow on 50 μg/ml anisomycin.

[c] G418: S = failure to grow on 100 μg/ml G418.

[d] Hygromycin B: S = failure to grow on 200 μg/ml hygromycin B; S* = failure to grow on 100 μg/ml hygromycin B.

TABLE 4

Osmotic sensitivity of *crl* strains at 25°

crl locus	No. of alleles[a]	Growth at 25° in YEPD + 1.5 M KCl
1	6	−
3	16	+
4	1	+
	1	−
5	3	−
	3	+
6	10	+
	1	−
7	2	−
8	1	−
9	17	+
10	12	+
11	2	+
12	3	−
	1	+
13	1	−
14	1	+
15	3	−
16	1	−
17	3	+
18	2	+
	1	−
19	1	+
20	1	+
	1	−
21	2	+
22	1	+
23	1	+

Osmotic inhibition of growth at 25° was determined by replica plating cells from YEPD to plates containing 1.5 M KCl.

[a] The number of alleles within each *crl* complementation group which have a particular osmotic phenotype.

ally related antibiotic G418. Many of the *crl* mutations which show the most extreme sensitivity to hygromycin B show normal sensitivity to G418. The differences in the sensitivity of the *crl* mutants to G418 and hygromycin B appear to be correlated with the greater ability of hygromycin B to stimulate translational misreading. In a study of 25 nonsense mutations in Crl$^+$ cells, we found that G418 can suppress only a subset of the auxotrophic mutations suppressed by hygromycin B and does not suppress as strongly as hygromycin B (McCusker 1986). The fact that G418 promotes phenotypic suppression less efficiently then hygromycin B is interesting in view of the fact that the minimum inhibitory concentration for G418 is lower than that for hygromycin B in wild-type cells (Table 3).

The antibiotics kasugamycin, gentamycin, kanamycin, neomycin, spectinomycin and streptomycin had no effect on the growth of any of the *crl* mutations or the wild-type control (data not shown). All of these antibiotics were used at a concentration of 400 μg/ml in YEPD. The *crl* mutations therefore appear to differ from the *ags* mutations which show increased sensitivity to the aminoglycoside antibiotics hygromycin B, G418, gentamycin, neomycin and kanamycin (Ernst and Chan 1985). In addition, *ags1* is linked to *leu2* while none of the unmapped *crl* mutations are linked to *leu2* (McCusker and Haber 1988).

Osmotic sensitivity of *crl* mutations: Previous work has shown that the omnipotent suppressors are unable to grow on media supplemented to generate high osmotic pressure (Liebman and Cavenagh 1980). We wondered if the *crl* mutants, which like the omnipotent suppressors are sensitive to hygromycin B, were also osmotic pressure sensitive. The ability of the *crl* mutants to grow at their permissive temperature of 25° on media supplemented to generate high osmotic pressure was carried out as described in the Materials and Methods. The results are shown in Table 4. At least some alleles of 13 of the 22 *crl* complementation groups are unable to grow at 25° in the presence of 1.5 M KCl. Thus, many of the *crl* mutants share both the osmotic sensitivity and aminoglycoside antibiotic sensitivity exhibited by omnipotent suppressors.

Synergistic interactions of temperature, osmotic pressure and hygromycin B: In addition to their sensitivity to hygromycin B and osmotic pressure, the *crl* mutants are also unable to grow at 37°. One

TABLE 5

Interaction of high osmotic pressure and hygromycin B in wild type Y55 cells

A. Effect of temperature on sensitivity of Y55 to hygromycin B

Hygromycin B (μg/ml)	25°	30°	34°	37°
50	+	+	+	+
100	+	+	+	−
150	+/+/−	+/+/−	−	−
200	+/−	−	−	−
250	+/−/−	−	−	−
300	−	−	−	−

B. Effect of osmotic pressure on sensitivity of Y55 to hygromycin B

	30° YEPD	30° YEPD + 2.5 M glycerol
0	+	+
25	+	+/+/−
50	+	+/−/−
100	+	−
150	+/+/−	ND
200	−	−
300	−	−

Cells of the wild-type strain Y55 were grown on YEPD at 30° and were replica plated to YEPD plates supplemented with hygromycin B and/or with 2.5 M glycerol. These plates were incubated at the temperatures listed above for 2 days and were then scored for growth. A "+" indicates no effect on growth relative to a no drug control plate, "+/+/−" slight inhibition, "+/−/−" strong inhibition and "−" no growth. ND = not determined.

explanation for their temperature sensitivity would be that elevated temperature itself increases translational misreading and that a synergistic interaction between two or more conditions that decrease translational fidelity prevents cell growth. We therefore asked if increased temperature or increased osmotic pressure had a similar synergistic effect on hygromycin B sensitivity in wild-type cells. The results, summarized in Table 5, indicate that wild-type cells become much more sensitive to the antibiotic at 37° or in the presence of 2.5 M glycerol. It should be pointed out that the effect of temperature on hygromycin B sensitivity is not a general effect of elevated temperature on antibiotic sensitivity. A less severe drop is seen for the minimum inhibitory concentration of G418 (which has much less ability to phenotypically suppress nonsense mutations) from 200 μg/ml at 25° to 100 μg/ml at 37°. The minimum inhibitory concentration of cycloheximide was identical at the two temperatures. Consistent with the hypothesis that there is increased translational misreading at higher temperatures is the observation that some hygromycin B suppressible auxotrophic mutations that fail to grow at 25° exhibit a considerable amount of leaky growth at 37° in the absence of hygromycin B (McCusker 1986).

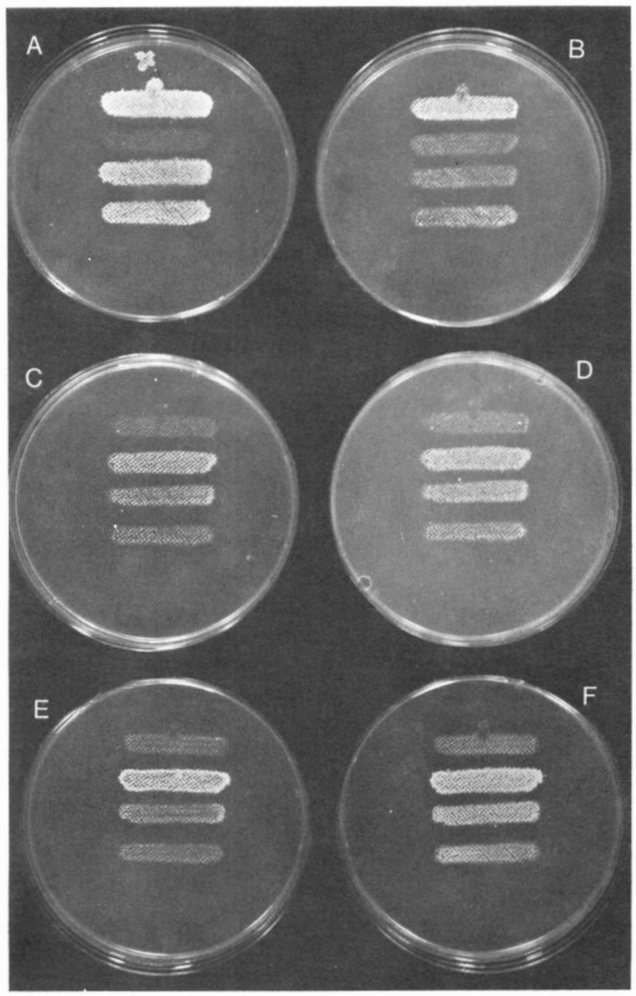

FIGURE 3.—Four strains (from the top of each plate: Crl$^+$, *crl3*, *crl3-2-8* and *crl3 SCL1-1*) carrying the *met13-2* mutation were grown on YEPD and replica plated to A, YEPD (37°); B, MIN-urea + methionine + 100 μg/ml β-thienylalanine (25°); C, methionine dropout medium (25°); D, minimal medium (25°); E, YEPD + 1 μg/ml cycloheximide (25°); and F, YEPD + 0.5 μg/ml cycloheximide (25°). Note that the extragenic suppressor *SCL1-1* and the intragenic suppressor *crl3-2-8* suppress the ts lethality of *crl3*. In addition these suppressors reduce the cycloheximide resistance and the suppression of *met13-2* by *crl3* without affecting the amino acid analog sensitivity.

The *crl* mutations suppress a hygromycin B suppressible auxotrophic mutation: Previous work has shown that mutations which are suppressed by aminoglycoside antibiotics, which stimulate translational misreading, are almost entirely nonsense mutations (Palmer, Wilhelm and Sherman 1979; Singh, Ursic and Davies 1979; Chattoo *et al.* 1979a). In addition Chattoo *et al.* (1979a) have shown that all mutations suppressed by the omnipotent suppressor *SUP46* were also suppressed by the aminoglycoside antibiotic paromomycin and similarly that many mutations suppressed by paromomycin were also suppressed by *SUP46*. Therefore it seemed reasonable to ask if the *crl* mutants, which are hypersensitive to hygromycin

TABLE 6

Suppression of ts lethality of some *crl* mutants by high osmotic pressure

CRL locus	No. of alleles[a]	Growth at 37°			
		Glycerol (M)		KCl (M)	
		2.5	1.5	1.5	0.75
1	6	−	−	−	−
3	16	+	+	+	+
4	2	−	−	−	−
5	2	−	+/−	−	−
	1	−	−	−	−
	3	+	+	+	+
6	3	−	+	−	+
	1	−	+	−	+/−
	7	+	+	−	+
7	1	+	+	−	+
	1	−	+	−	+
8	1	−	−	−	−
9	17	−	+	−	+
10	12	+	+	+/−	+
11	1	−	−	−	−
	1	+	+	+/−	−
12	2	−	+	−	−
	1	−	−	−	−
	1	−	+	−	+
13	1	+	+	+/−	+
14	1	+	+	+/−	+
15	2	−	−	−	−
	1	−	+/−	−	−
16	1	+/−	+	−	+
17	2	+	+	−	+
	1	−	+	−	+/−
18	2	+	+	+	+
	1	−	+/−	−	+/−
19	1	−	+	−	+
20	2	−	+	−	−
21	1	−	+	−	+
	1	−	−	−	−
22	1	−	+	−	+
23	1	−	+	−	+

Osmotic pressure suppression of ts lethality at 37° was determined as described in the MATERIALS AND METHODS. All cells failed to grow at 37° in the absence of glycerol or KCl. A "+" indicates that there was strong growth; "+/−" indicates weak growth; "−" equals no growth.

[a] The number equals the number of alleles within each *crl* complementation group which have a particular osmotic phenotype.

B like the omnipotent suppressors, could suppress any auxotrophic mutations that are phenotypically suppressed by hygromycin B.

Each of 25 nonsense mutations listed in MATERIALS AND METHODS were crossed with one allele from each of the *crl* loci. Segregants carrying both the *crl* mutation and the hygromycin-suppressible auxotrophic mutation were tested for growth on the appropriate dropout media at both 25° and 30° to determine whether the *crl* mutation was capable of suppressing the auxotrophic mutation. Of all the mutations tested only one, *met13-2*, was suppressed. This mutation was suppressed by all of the *crl* mutations. An example is shown in Figure 3, C and D. This suppression was somewhat more pronounced at 30° and on minimal medium compared with methionine dropout medium. The *crl* mutations suppressed *met13-2* more strongly than hygromycin B (data not shown). The fact that only one hygromycin B suppressible auxotrophic mutation was suppressed by the *crl* mutations was unexpected, although ONO *et al.* (1986) recently reported a similar result for another omnipotent suppressor, *sup113-2*. Possible explanations for this result will be discussed later.

Both an intragenic and an extragenic suppressor of the ts growth defects of *crl3* have been isolated (MCCUSKER and HABER 1988). We have constructed *met13-2 crl3-2-8* and *met13-2 crl3 SCL1-1* strains and have compared their methionine requirement with *met13-2 crl3* and *met13-2 CRL*$^+$ strains. The results are also shown in Figure 3. Both the *crl3-2-8* and the *SCL1-1* mutation show reduced suppression of *met13-2* relative to *crl3*.

Osmotic remediability of *crl* mutations at 37°: Despite the fact that many *crl* mutants are osmotic sensitive at their permissive temperature, at least some alleles from 19 of the 22 complementation groups are able to grow at their nonpermisive temperature (37°) in the presence of high osmotic pressure (Table 6). Among these mutants are those that cannot grow on the same media at 25° (*e.g.*, *crl13* does not grow on YEPD + 1.5 M KCl at 25° but does grow on this media at 37°). Thus, like many other ts mutants (HAWTHORNE and FRIIS 1964; SINGH and SHERMAN 1975) many *crl* alleles are osmotic pressure remediable.

DISCUSSION

The *crl* mutations are a large class of pleiotropic mutations that were selected for resistance to the minimum inhibitory concentration of cycloheximide and then screened for ts growth (MCCUSKER and HABER 1988). Alleles from all 22 *crl* loci display properties of two other groups of mutants that have previously been described, the *gcn* mutants and omnipotent suppressor mutants. All of these mutants appear to affect aspects of protein translation.

***crl* mutants are similar to *gcn* mutants:** The *crl* mutants apparently affect the regulation of amino acid biosynthesis. All of the *crl* mutants have many phenotypes in common with the *gcn* mutants: failure to show proper arrest in response to amino acid starvation (NIEDERBERGER, AEBI and HUTTER 1983), hypersensitivity to amino acid analogs (WOLFNER *et al.* 1975), hypersensitivity to the histidine biosynthesis inhibitor 3-aminotriazole (WOLFNER *et al.* 1975) and finally the "tightening" of leaky amino acid auxotrophic mutations (NIEDERBERGER, AEBI and HUTTER

1983; NIEDERBERGER, MIOZZARI and HUTTER 1981; WOLFNER *et al.* 1975).

A major component in the regulation of amino acid biosynthesis by the *GCN* gene products appears to be mediated at the level of translation of 4 small open reading frames 5′ to the GCN4 coding region on the GCN4 mRNA (MUELLER and HINNEBUSCH 1986). MUELLER and HINNEBUSCH (1986) have hypothesized that translation of these small open reading frames reduces the initiation of translation of the *GCN4* protein and thus prevents the transcription of amino acid biosynthetic genes that are positively regulated by *GCN4*. The *gcn2*, *gcn3* and perhaps other *gcn* mutants appear to permit translation of the upstream open reading frames even under deprepressing conditions (HINNEBUSCH 1984, 1985; GREENBERG *et al.* 1986). Thus the level of amino acid biosynthetic genes remains low. Consistent with this picture of the effects of *crl* mutants, our preliminary results indicate that all of the *crl* mutants express a *HIS4-LACZ* fusion at lower than wildtype levels in response to amino acid starvation (J. H. MCCUSKER, unpublished observations). Further indication of alterations in amino acid biosynthesis comes from our preliminary studies of amino acid incorporation; we have found that many *crl* mutants may have reduced pools of at least some amino acids (our unpublished observations). The failure to express high levels of the amino acid biosynthetic genes may account not only for the sensitivity of *gcn* (and *crl*) mutants to amino acid analogs, but may also explain why the *crl* mutants have a limited spectrum of omnipotent suppression (see below).

None of the *crl* mutants appears to be allelic with any of the previously described *gcn* mutants. This conclusion is based on the different map positions found for *crl* and *gcn* mutants (though not all of the *gcn* mutants have been mapped). Moreover, none of the *gcn* mutants have been described as being temperature sensitive and several have been deleted without affecting cell viability. There are two possible explanation as to why *crl* mutations have not been picked up in the many screens done for *gcn* mutations. The first is that we have used a different genetic background. The second possible explanation is that we employed very different selection and screening procedures. Indeed, GREENBERG *et al.* (1986), screening for new *gcn* mutants by their interactions with the *gcd5* mutant, isolated an entirely different spectrum of *gcn* mutants than had previously been described.

The storage carbohydrates glycogen and trehalose are accumulated in response to starvation conditions (LILLIE and PRINGLE 1980). We have found that most of the *crl* mutants show an abnormal accumulation of storage carbohydrates at 25°. These are two possible explanations for the storage defect; either the *crl* mutants are unable to utilize the storage carbohydrates or the *crl* mutants accumulate storage carbohydrates under inappropriate conditions, *i.e.*, they behave as if they were starving under conditions where the wild type is not. An inability to utilize storage carbohydrates might explain the reduction in sporulation efficiency of many of the *crl* mutants (MCCUSKER and HABER 1988). It has been shown that glycogen is degraded in sporulating cells at the time of ascospore formation (KANE and ROTH 1974). There is, however, no obvious correlation between the iodine staining of the *crl* mutants on YEPD and sporulation efficiency. An inability to utilize glycogen could explain why the *crl* mutants fail to arrest in G_1 in stationary phase.

Two mutants have been described previously which display defects in stationary phase arrest: *ard1* (*a*rrest *d*efective; WHITEWAY and SZOSTAK 1985) and $RAS2^{val19}$ (TODA *et al.* 1985). Like the *crl* mutants, both *ard1* and $RAS2^{val19}$ mutants do not show proper G_1 arrest in stationary phase. The *crl* mutants differ substantially from the *ard1* and $RAS2^{val19}$ mutations, as these two mutants appear to prevent the initiation of meiosis while the *crl* mutations appear to affect a much later stage of spore formation. *ard1* and $RAS2^{val19}$ mutants fail to arrest in G_1 in response to nitrogen starvation, but the *crl* mutants show normal G_1 arrest (which probably explains why they are able to initiate sporulation). In addition the *ard1* and $RAS2^{val19}$ mutants fail to accumulate storage carbohydrates while the *crl* mutants show hyperaccumulation. The effect of the *ard1* and $RAS2^{val19}$ mutations on the general control of amino acid biosynthesis, one of the best studied starvation responses, has not been reported.

Similarity of *crl* mutants to omnipotent suppressors: The *crl* mutants confer hypersensitivity to hygromycin B, an aminoglycoside antibiotic which stimulates translational misreading (SINGH, URSIC and DAVIES 1979). In addition, all of the *crl* mutants suppress an auxotrophic mutation which is suppressed by hygromycin B. The *crl* mutations do not show a uniform response to the three other protein synthesis inhibitors which were tested: cryptopleurine, anisomycin and G418. One class of mutations has been described previously which displays an increased sensitivity to antibiotics which stimulate translational misreading: the omnipotent suppressors (LIEBMAN and CAVENAGH 1980; HIMMELFARB, MAICAS and FRIESEN 1985; EUSTICE *et al.* 1986). The omnipotent suppressors were selected on the basis of their ability to suppress all three classes of nonsense mutations (HAWTHORNE and LEUPOLD 1974; LIEBMAN and CAVENAGH 1980; ONO *et al.* 1984; EUSTICE *et al.* 1986), although it is clear that they do not suppress all nonsense mutations (CHATTOO *et al.* 1979a). Some alleles of the omnipotent suppressors have been

described as being temperature sensitive lethal (HIMMELFARB, MAICAS and FRIESEN 1985). Unfortunately, no description of the ts lethal phenotype has been given; *crl* mutants arrest after several cell divisions as budded cells with a single nucleus (MCCUSKER and HABER 1988).

In addition to their inability to grow at 37°, we have found that many of the *crl* mutations are also unable to grow at the permissive temperature of 25° at high osmotic pressure. We suggest that these conditional growth phenotypes may be reflections of increased mistranslation caused both by the mutants and by the elevated temperature. The deleterious effects of mistranslation would then be exacerbated by increased osmotic pressure. Consistent with this idea, we have been able to produce a phenocopy of the *crl* hypersensitivity to hygromycin B in a wild type strain either by the addition of glycerol to the medium or by elevating temperature. In addition, we have found that several hygromycin B suppressible auxotrophic mutations show a considerable amount of growth at 37° in a Crl^+ strain in the absence of hygromycin B (MCCUSKER 1986). The way in which mistranslation may act to block cell growth is not known in yeast, but DAVIS, CHEN and TAI (1986) have shown that streptomycin-induced translational misreading in *E. coli* produces membrane damage. Thus, the osmotic sensitivity of omnipotent suppressors, the *crl* mutants and [and also the tRNA nonsense suppressors strains (SINGH 1977)], as well as of wild-type strains in the presence of hygromycin B, might all be due to translational misreading leading to membrane damage.

We do not wish to suggest that all of these interactions between various mutants and environmental conditions are exactly equivalent; for example, wild-type strains are able to grow at high temperature on high osmotic medium. Moreover, although increased temperature could produce a phenocopy of *crl* mutants with respect to antibiotic sensitivity, growth of wild-type cells at 37° in the presence of the minimum inhibitory concentration of hygromycin B did not lead to the uniform large budded population arrest phenotype of *crl* mutants (data not shown). Nevertheless, it is clear that most combinations of two mutant and/or environmental conditions that affect translational fidelity cause an arrest of cell growth.

Suppression of *met13-2* by the *crl* mutations and the limited spectrum of suppression: The most significant phenotype of the omnipotent suppressors is the basis for their selection, the suppression of nonsense mutations by increased translational misreading (ONO, STEWART and SHERMAN 1981; MASUREKAR *et al.* 1981; EUSTICE *et al.* 1986). Previous work has also shown that mutations which are suppressible by the aminoglycoside antibiotic paromomycin are frequently suppressed by the omnipotent suppressor *SUP46* and that all mutations suppressed by *SUP46* were phenotypically suppressed by paromomycin (CHATTOO *et al.* 1979a). We have isolated a large number of mutations which are phenotypically suppressed by hygromycin B in a Crl^+ strain. Consistent with previous results (SINGH, URSIC and DAVIES 1979), we have shown that these are nonsense mutations. When 25 of these hygromycin B-suppressible mutations were crossed with one allele from each of the *crl* loci, the somewhat surprising result was that all of the *crl* mutations were capable of suppressing only one of the hygromycin B-suppressible auxotrophic mutations tested.

A number of explanations are possible for the limited spectrum of suppression of *crl* mutants. Some specificity of suppression has been demonstrated for paromomycin and the omnipotent suppressor *SUP46*. Only some nonsense mutations are phenotypically suppressed by paromomycin and only a subset of these paromomycin-suppressible mutations are suppressed by *SUP46* (CHATTOO *et al.* 1979a). One extreme example of a restricted spectrum of suppression is exhibited by *sup113-2*, which is also capable of suppressing only one nonsense mutation (ONO *et al.* 1986). The reason for the restricted spectrum of suppression of omnipotent suppressors is not known, but the context of the mutation which is being suppressed may be important. Context specific differences in the efficiency of suppression by tRNA suppressors have been demonstrated in *E. coli* and *Salmonella typhimurium* (MILLER and ALBERTINI 1983; BOSSI 1983).

One explanation for the limited spectrum of suppression by *crl* strains assumes that the *gcn*-like phenotypes of the *crl* mutants reflect an inability to derepress the amino acid biosynthetic genes in response to amino acid limitation. It is quite likely that growth of a strain carrying a suppressible amino acid auxotrophic mutation is dependent both on translational misreading and upon the ability to derepress the gene containing the mutation. If the *crl* mutants are unable to derepress the amino acid biosynthetic gene the level of enzyme activity may be too low to support growth even though partial suppression had occurred. We feel it is significant that all of the *crl met13-2* double mutants show more growth on minimal medium (no added amino acids) as compared to methionine dropout medium. This result suggests that the presence of high concentrations of other amino acids in the methionine dropout medium reduces the transcription of *met13* and therefore reduces the suppression of *met13-2*. All but one of the 25 nonsense mutations used in our study were in amino acid biosynthetic genes.

These results have significance with regard to the isolation of mutations which affect translational fidelity. Every selection for omnipotent suppressors

has used the suppression of nonsense mutations in amino acid biosynthetic genes as at least part of the selection (HAWTHORNE and LEUPOLD 1974; LIEBMAN and CAVENAGH 1980; ONO *et al.* 1984; EUSTICE *et al.* 1986). If it is true that many mutations which affect translational fidelity have a *gcn*-like phenotype, then all of these selections have been seriously biased *i.e.*, mutations which have a severe effect on translational fidelity and general control of amino acid biosynthesis would not be isolated. To the best of our knowledge, the omnipotent suppressors have not been tested for defects in the general control of amino acid biosynthesis. These results suggest that a more general screen for omnipotent suppressors should be based on the suppression of nonsense mutations in a gene such as galactokinase that is not affected by *gcn*-like mutants.

What are the *crl* genes? Based on their similarities with omnipotent suppressors and to *gcn* mutations and to their altered sensitivity to both the protein synthesis inhibitors cycloheximide and hygromycin B, it seems most likely that the 22 *crl* complementation groups all affect protein synthesis. One additional consideration is that we have found that most of the combinations of *crl* double mutants which were tested are lethal (J. H. MCCUSKER, unpublished observation). The fact that two different extragenic suppressors of the ts phenotypes of a specific *crl* mutant show only partial suppression of only two of the other *crl* mutations (MCCUSKER and HABER 1988) argues against the idea that all of the *CRL* genes are part of a single pathway, but does not preclude the possibility that they all affect ribosome function. There are at least two possible ways that the *crl* mutations could affect protein synthesis.

The first of these possibilities is that all of the *crl* loci are structural genes for ribosomal proteins. The argument against this hypothesis is that it is difficult to imagine how mutations in 22 different ribosomal protein genes could be so similar phenotypically. On the other hand the *crl* mutations were isolated through the use of a very narrow selection which could account for the phenotypic similarities. Very few ribosomal protein genes have been mapped, so that the fact that none of the *crl* mutants are allelic with these few r-protein genes is not significant.

The second possible explanation of the role of the *crl* gene products is that they constitute a one or more pathways that affect ribosome function. Ribosome function might be affected by modification of ribosomal RNA, ribosomal protein(s) or other translation factors. Alternatively, ribosome function could be influenced by the presence or absence of a small molecule analogous to the regulators ppGpp and pppGpp in *E. coli*. Indeed, there are many similarities between the *crl* mutations and the *relA* mutation in *E. coli*. In the *relA* (relaxation of stringent control) mutant this regulation and the accumulation of ppGpp and pppGpp is impaired (GALLANT 1979). A *relA* mutant loses viability much more rapidly upon starvation for a single amino acid. In contrast, there is no difference between *relA*$^+$ and *relA* strains when they are subjected to NH_4^+ starvation (HECKER *et al.* 1986). All of the *crl* mutants differ in a similar way to these two types of starvation; abnormal arrest in response to amino acid starvation and normal arrest in response to nitrogen starvation. In addition to their role in the regulation of some genes, the *relA*$^+$ gene appears to be necessary for the maintenance of translational accuracy when the cell is starved for an amino acid. *relA* mutants show a high level of translational misreading when they are starved for an amino acid (GALLANT 1979; GALLANT *et al.* 1982; WEISS and GALLANT 1986). This includes the suppression of nonsense mutations (GALLANT *et al.* 1982). Like the *relA* mutants, all of the *crl* mutants exhibit reduced translational fidelity as seen by their increased sensitivity to hygromycin B and suppression of a nonsense mutation.

The compounds ppGpp and pppGpp have not been found in yeast; however, starvation for an amino acid has been reported to result in a decrease in the amount of a major phosphorylated compound (LUSBY and MCLAUGHLIN 1980). Yeast has been shown to have a true stringent response (WARNER and GORENSTEIN 1978). It is possible that the *crl* mutants may elicit a spectrum of relaxed responses under inappropriate conditions.

Recently we have cloned *CRL13* (J. H. MCCUSKER, D. HILL and J. E. HABER, unpublished observations) and have shown it to lie within 1 kb of the *GCD1* gene that is also involved in translational regulation of *GCN4*. Analysis of this cloned gene should help establish more clearly the role of *CRL* genes in protein synthesis and in regulation of both amino acid biosynthesis and cell cycle arrest.

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