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Mammalian eukaryotic initiation factor 2α kinases functionally substitute for GCN2 protein kinase in the GCN4 translational control mechanism of yeast

(phosphorylation/initiation factors/double-stranded RNA-dependent eIF-2α kinase/p68 kinase/heme-regulated eukaryotic initiation factor 2α kinase)

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ABSTRACT **Phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α) in *Saccharomyces cerevisiae* by the GCN2 protein kinase stimulates the translation of GCN4 mRNA. The protein kinases heme-regulated inhibitor of translation (HRI) and double-stranded RNA-dependent eIF-2α protein kinase (dsRNA-PK) inhibit initiation of translation in mammalian cells by phosphorylating Ser-51 of eIF-2α. We show that HRI and dsRNA-PK phosphorylate yeast eIF-2α *in vitro* and *in vivo* and functionally substitute for GCN2 protein to stimulate GCN4 translation in yeast. In addition, high-level expression of either mammalian kinase in yeast decreases the growth rate, a finding analogous to the inhibition of total protein synthesis by these kinases in mammalian cells. Phosphorylation of eIF-2α inhibits initiation in mammalian cells by sequestering eIF-2B, the factor required for exchange of GTP for GDP on eIF-2. Mutations in the *GCN3* gene, encoding a subunit of the yeast eIF-2B complex, eliminate the effects of HRI and dsRNA-PK on global and GCN4-specific translation in yeast. These results provide further *in vivo* evidence that phosphorylation of eIF-2α inhibits translation by impairing eIF-2B function and identify GCN3 as a regulatory subunit of eIF-2B. These results also suggest that GCN4 translational control will be a good model system to study how mammalian eIF-2α kinases are modulated by environmental signals and viral regulatory factors.**

Mammalian cells use translational control mechanisms for a rapid response to various types of stress. The protein kinases heme-regulated inhibitor of translation (HRI) and double-stranded RNA-dependent α subunit of eukaryotic initiation factor 2 (eIF-2α) kinase (dsRNA-PK; also referred to as p68 kinase, DAI, or dsI) are activated by heme-deprivation and virus infection, respectively, to inhibit total protein synthesis by phosphorylating Ser-51 of eIF-2α (for review, see refs. 1 and 2). Composed of three subunits, eIF-2 forms a ternary complex with GTP and charged initiator $tRNA_i^{Met}$ (Met-$tRNA_i^{Met}$) and functions to deliver the Met-$tRNA_i^{Met}$ to the ribosome during translation initiation. The GTP of the ternary complex is hydrolyzed to GDP during translation initiation, and eIF-2 is released from the ribosome as an eIF-2·GDP complex. A second factor, eIF-2B, is required to catalyze the exchange of GTP for GDP in the eIF-2·GDP complex. Phosphorylation of eIF-2α on Ser-51 inhibits translation initiation by sequestering eIF-2B (2, 3). This inhibition of eIF-2B activity diminishes the recycling of eIF-2·GDP to eIF-2·GTP, decreasing ternary complex formation and reducing the rate of initiation of translation.

In the yeast *Saccharomyces cerevisiae*, phosphorylation of eIF-2α by the protein kinase GCN2 mediates translational control of the *GCN4* gene. GCN4 is a transcriptional activator of amino acid biosynthetic genes that must be expressed at high levels to overcome the effects of amino acid starvation. GCN4 expression is regulated at the translational level by four short open reading frames (ORFs) in the GCN4 mRNA 5′ leader (4). Mutations in the α and β subunits of eIF-2 were shown to impair GCN4 translational control, and reductions in the level of eIF-2 activity were correlated with increased translation of GCN4 (4). GCN2, a protein kinase required for translational derepression of *GCN4* (4), was found to exhibit unique sequence similarity with the eIF-2α kinase dsRNA-PK (5). Cloning of the cDNA encoding HRI and the identification of its homology to GCN2 and dsRNA-PK gave strong impetus to the idea that GCN2 protein is also an eIF-2α kinase (6). We recently provided genetic and biochemical evidence supporting this model by showing that GCN2 stimulates GCN4 translation by phosphorylating eIF-2α on Ser-51 in amino acid-starved cells (7). We proposed that the consequent reduction in the availability of ternary complexes would allow ribosomes that have translated the first ORF in GCN4 mRNA to ignore the AUG codons at ORFs 2–4 and reinitiate translation at the GCN4 ORF instead (4, 7).

Several factors are required, in addition to the protein kinase GCN2 and the subunits of eIF-2, for translational control of GCN4 expression (4). Recent work indicates that several of these factors are subunits of a high-molecular-weight complex that is the yeast equivalent of mammalian eIF-2B (8, 20). One of the constituents of this complex, GCN3, is dispensable for normal growth in yeast; however, strains lacking *GCN3* cannot increase GCN4 translation in response to amino acid starvation (4, 9).

We have expressed the mammalian eIF-2α kinases in yeast and shown that they will substitute for GCN2 to phosphorylate eIF-2α and stimulate *GCN4* expression. In addition, high-level expression of the mammalian kinases inhibits growth of yeast cells, analogous to the translational inhibition associated with activation of these kinases in mammalian cells. Both the stimulation of GCN4 translation and the growth inhibition caused by the mammalian kinases are eliminated by deletion of *GCN3*, identifying GCN3 as a

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Abbreviations: eIF-2α, α subunit of eukaryotic initiation factor 2; HRI, heme-regulated inhibitor of translation; dsRNA-PK, double-stranded RNA-dependent eIF-2α kinase; 3-AT, 3-aminotriazole; IEF, isoelectric focusing; ORF, open reading frame.

regulatory subunit of eIF-2B in yeast. Our findings establish GCN4 translational control as an excellent model system to study the regulation of mammalian eIF-2α kinases.

MATERIALS AND METHODS

Yeast Strains and Plasmids. The construction of yeast strain H1816 (a *ura3-52 leu2-3 leu2-112 trp1-Δ63 sui2Δ gcn2Δ*, p1108[*GCN4–lacZ, TRP1*] at *trp1-Δ63*, p1097[*SUI2, LEU2*]) was described (7). Strain H1817 is isogenic to strain H1816, except that p1097[*SUI2, LEU2*] was replaced with p1098[*SUI2–S51A, LEU2*]. The wild-type *GCN3* allele in strain H1644 [a derivative of H1643 (7) containing p1097 isogenic to H1816] was replaced with a *gcn3Δ* allele to create strain TD-F3-15, as follows. The *Hin*dIII fragment containing *GCN3* was eliminated from plasmid Ep235 (9), creating plasmid p1142. An *Eco*RI–*Bam*HI fragment from p1142 was inserted in the *URA3* integrating plasmid pRS306 (10), creating plasmid p1143. Strain H1644 was transformed with p1143 DNA digested with *Nru* I to direct plasmid integration to *GCN3*. Derivatives of the resulting transformants resistant to 5-fluoroorotic acid were isolated, and strains carrying only the *gcn3Δ* allele were identified by their inability to complement the 3-aminotriazole (3-AT)-sensitive phenotype of a *gcn3-101* strain. The chromosomal *GCN2* allele in strain TD-F3-15 was replaced by an unmarked *gcn2Δ* allele to create strain H2065, as described (7). Strains carrying both the *gcn2Δ* and *gcn3Δ* alleles were identified by their inability to complement the 3-AT-sensitive phenotype of *gcn2* mutants. The *gcn3-102* allele in strain H17 (*α gcn3-102 leu2-3 leu2-112 ura3-52*) was replaced with wild-type *GCN3*, creating strain H2064, by transforming strain H17 with plasmid pAH27 linearized with *Spe* I to direct plasmid integration to *GCN3*. pAH27 carries a wild-type *GCN3* allele on the *URA3* integrating plasmid YIp5. 5-Fluoroorotic acid-resistant derivatives of these transformants were isolated, and strains carrying the wild-type *GCN3* allele were identified by their 3-AT-resistant phenotype.

The wild-type *GCN2* gene was introduced into yeast on the low-copy-number plasmid p585 (5). The cDNAs encoding the wild-type mammalian kinases were introduced into yeast on plasmids p1246 (HRI) and p1420 (dsRNA-PK), derivatives of the high-copy-number vector pEMBLyex4, and were expressed under the control of a yeast *GAL–CYC1* hybrid promoter (11). The HRI cDNA (6) was subjected to site-directed mutagenesis to introduce an *Nco* I restriction site at the ATG initiation codon, as described (6), whereas the cDNA encoding dsRNA-PK (pBS-8.4R; ref. 12) was mutated to introduce an *Nde* I restriction site at the ATG initiation codon (13). Cleavage at these newly introduced restriction sites, followed by ligation to pEMBLyex4 at the *Sma* I site in the polylinker (11), inserted the kinase coding sequences (beginning with the ATG codon) in the 5′ UTR of the *GAL–CYC1* transcription unit ≈60–70 nt downstream from the predicted start site of transcription.

The pEMBLyex4 plasmid is a high-copy-number yeast vector containing *URA3* and the *leu2-d* allele that complements *leu2* alleles by greatly increasing plasmid copy number (11). Moderate expression of the inserted kinase genes was achieved by selecting for complementation of a chromosomal *leu2* mutation on noninducing medium, whereas high-level overexpression was obtained by inducing the *GAL–CYC1* hybrid promoter on leucine-containing medium. A low-copy-number plasmid expressing dsRNA-PK (p1419) was constructed by transferring an *Apa* I–*Hin*dIII fragment containing the dsRNA-PK cDNA under the control of the *GAL–CYC1* promoter from p1420 to pRS316, a low-copy-number *URA3* plasmid (10).

Biochemical Methods. Yeast eIF-2 was purified as described (7); this information will be presented in detail elsewhere (20). For the *in vitro* kinase reactions, the wild-type and mutant yeast eIF-2 proteins were treated at 50°C for 5 min to reduce background phosphorylation by endogenous kinases. HRI was purified as described (14), and the HRI kinase reactions were done as described for GCN2 (7). dsRNA-PK purification from *Escherichia coli* and kinase reactions were performed as described (13). For the kinase reactions the indicated proteins (when present: 1.2 μg of eIF-2, 0.5 μg of HRI, and 0.25 μg of dsRNA-PK) were incubated with [γ-^{32}P]ATP, and the reaction products were separated by 10% SDS/PAGE followed by autoradiography.

Cell harvesting, β-galactosidase assays, and isoelectric focusing (IEF) gels were done as described (7). Whole-cell extracts were prepared from cells grown exponentially in synthetic minimal dextrose (SD) medium for ≈30 hr and then shifted for 12 hr to either SD medium or inducing conditions, as described in the legend to Table 1. The extracts prepared for the β-galactosidase assays presented in Table 1 were used for immunoblot analysis of kinase expression. Procedures for immunoblotting and detection of HRI (15) and dsRNA-PK (13) with monoclonal antibodies have been reported. Strains for the analysis of GCN2-dependent phosphorylation of eIF-2α were grown and harvested, as already described (7).

RESULTS

Mammalian eIF-2α Kinases Phosphorylate Yeast eIF-2α *in Vitro* and *in Vivo*. The amino acid sequences of the kinase domains of GCN2, HRI, and dsRNA-PK are highly homologous, suggesting that these proteins are members of a discrete subfamily of protein kinases (5, 6). These sequence conservations raised the possibility that the mammalian eIF-2α kinases could phosphorylate yeast eIF-2α. We first addressed this possibility by *in vitro* kinase reactions with purified HRI (Fig. 1*A*) or purified recombinant dsRNA-PK (Fig. 1*B*) and eIF-2 purified from yeast strains expressing either the wild-type α subunit or a mutant form in which Ser-51 was substituted with alanine. Both kinases specifically phosphorylated the α subunit of wild-type yeast eIF-2, but

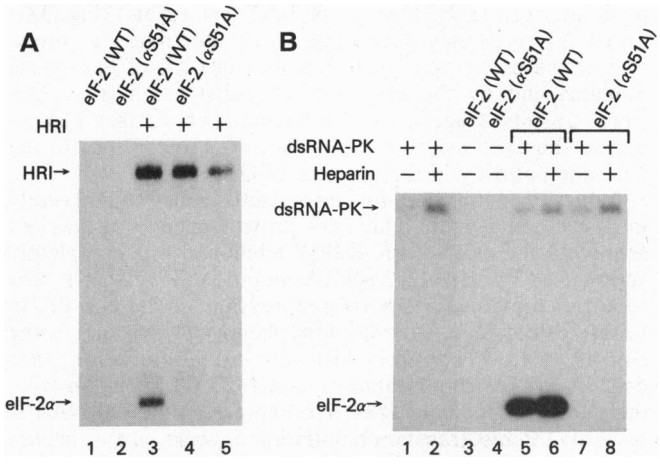

FIG. 1. Phosphorylation of α subunit of purified yeast eIF-2 by purified mammalian kinases. (*A*) *In vitro* kinase reactions with HRI. Kinase reactions were done without (lanes 1 and 2) or with (lanes 3–5) HRI purified from rabbit reticulocyte lysates. The reactions contained no yeast proteins (lane 5) or yeast eIF-2 purified from a wild-type strain (lanes 1 and 3) or from a strain expressing only the eIF-2α-S51A allele (lanes 2 and 4). (*B*) *In vitro* kinase reactions with dsRNA-PK. Reactions contained either no kinase (lanes 3 and 4) or recombinant human dsRNA-PK purified from *E. coli* (lanes 1, 2, and 5–8). The reactions also included yeast eIF-2 purified from a wild-type strain (lanes 3, 5, and 6) or a strain expressing the eIF-2α-S51A allele (lanes 4, 7, and 8) or no yeast proteins (lanes 1 and 2). Reactions were done without (−) or with (+) heparin, a kinase activator (13).

not the mutant α subunit, as expected if HRI and dsRNA-PK phosphorylate yeast eIF-2α on Ser-51 (Fig. 1*A*, lane 4 versus 3 and Fig. 1*B*, lanes 7 and 8 versus 5 and 6).

We tested whether the mammalian kinases would phosphorylate yeast eIF-2α when expressed in yeast cells under the control of a galactose-inducible *GAL* promoter. In yeast strains lacking GCN2, eIF-2α resolves in IEF gels as a single species that comigrates with the nonphosphorylatable eIF-2α–S51A protein, indicating that eIF-2α is not phosphorylated on Ser-51 in *gcn2*Δ mutants (7). When expression of the mammalian kinases was stimulated in a *gcn2*Δ strain by shifting cells from glucose to raffinose medium (HRI) or to raffinose plus galactose medium (dsRNA-PK), a hyperphosphorylated form of eIF-2α was seen that comigrated precisely with the species produced in wild-type yeast when GCN2 was activated by amino acid starvation (compare lanes 5–8 with lanes 1–4 in Fig. 2*C*). This hyperphosphorylated form was eliminated by mutation of either Ser-51 in eIF-2α or the essential conserved lysine residue in subdomain II of each kinase [Lys-199 in HRI (6); Lys-295 in dsRNA-PK (12)] (Fig. 2*A*, IEF, lanes 5 and 7 versus lane 3; Fig. 2*B*, IEF, lanes 4 and 6 versus lane 2). Immunoblotting experiments confirmed that expression of the mammalian kinases increased under the inducing growth conditions (Fig. 2*A* and *B*, Immunoblot); therefore, the lack of phosphorylation noted in the strains expressing the mutant kinases or eIF-2α-S51A cannot be attributed to diminished expression of the kinases.

Mammalian eIF-2α Kinases Functionally Substitute for GCN2. Having demonstrated that HRI and dsRNA-PK can phosphorylate yeast eIF-2α *in vitro* and *in vivo*, we tested whether the mammalian kinases could replace GCN2 and stimulate GCN4 translation in a *gcn2*Δ mutant. Strains lacking GCN2 fail to grow on medium containing the inhibitor of histidine biosynthesis 3-AT because they fail to increase GCN4 protein levels under these starvation conditions (4). Wild-type HRI conferred resistance to 3-AT in cells grown on glucose medium, as well as under inducing conditions on galactose medium (Fig. 3*A*, SD+3-AT, SGAL+3-AT). Expression of dsRNA-PK also conferred resistance to 3-AT on glucose medium; however, growth was inhibited on inducing medium, even in the absence of 3-AT (see below) (Fig. 3*A*). The 3-AT-resistance phenotypes were eliminated by mutation of the conserved lysine residues in each of the kinase domains and by the eIF-2α–S51A substitution (Fig. 3*A*). These results suggest that the mammalian kinases can increase the expression of *GCN4* and its target genes in the histidine pathway in the absence of GCN2.

Induction of dsRNA-PK on galactose medium severely impaired cell growth. This slow-growth phenotype was not seen with the dsRNA-PK-K295R allele and was completely suppressed by the eIF-2α-S51A mutation (Fig. 3*A*). It was reported previously that overexpression of dsRNA-PK in yeast results in a slow-growth phenotype and polysome run-off (16). Together with our demonstration that dsRNA-PK phosphorylates yeast eIF-2α on Ser-51 *in vivo*, these results indicate that overexpression of dsRNA-PK severely inhibits translation initiation in yeast, just as occurs when this kinase is activated in mammalian cells (1, 2). As discussed below, high-level expression of HRI in yeast led to a similar inhibition of cell growth.

The ability of HRI and dsRNA-PK to confer resistance to 3-AT in a *gcn2*Δ mutant suggests that these kinases can stimulate translation of GCN4. To test this prediction, *GCN4–lacZ* expression was measured in *gcn2*Δ strains producing the mammalian kinases. We found that *GCN4–lacZ* expression was very low during exponential growth on noninducing medium, whereas growth on inducing medium led to an ≈6-fold stimulation of *GCN4* expression by HRI and a 4- to 5-fold stimulation by dsRNA-PK (Table 1, strains 1 and 6). This induction of *GCN4* depended on the wild-type kinase

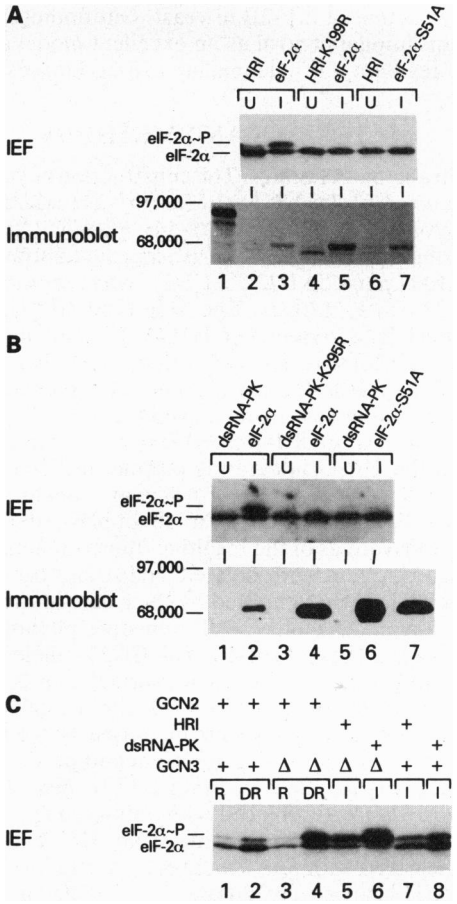

FIG. 2. IEF gel electrophoresis of eIF-2α and immunoblot analysis of kinase levels in yeast strains expressing the eIF-2α kinases HRI, dsRNA-PK, or GCN2. (*A*) Strains expressing HRI. eIF-2α phosphorylation was monitored in *gcn2*Δ strains expressing wild-type HRI (lanes 2, 3, 6, and 7) or mutant HRI-K199R (lanes 4 and 5) and either wild-type eIF-2α (lanes 2–5) or eIF-2α-S51A (lanes 6 and 7) grown under uninducing (U) or inducing (I) conditions, as described in the legend to Table 1. The small amount of phosphorylated eIF-2α in lane 2 can probably be attributed to low-level induction of HRI in glucose-grown cells that were approaching stationary phase (see text). Aligned below the IEF data are the results of immunoblot analysis of HRI in 50 μg of whole-cell extracts from the corresponding cultures. Lane 1 contains rabbit reticulocyte lysate and shows the intact HRI of 90 kDa. The HRI in the yeast extracts was truncated, and this truncation could not be prevented by including additional protease inhibitors or by rapid suspension of cells in cold trichloroacetic acid before cell lysis. The basal-level expression of the faster-migrating form of HRI (lane 4) varied nonsystematically in different experiments. (*B*) Strains expressing dsRNA-PK. eIF-2α phosphorylation was monitored in *gcn2*Δ strains expressing wild-type dsRNA-PK (lanes 1, 2, 5, and 6) or the mutant dsRNA-PK-K295R (lanes 3 and 4) and either eIF-2α (lanes 1–4) or eIF-2α-S51A (lanes 5 and 6) grown under uninducing (U) or inducing (I) conditions, as described in the legend to Table 1. Immunoblot analysis on 100 μg of extract from the corresponding cultures is aligned below the IEF data. Lane 7 contains a crude extract from Daudi cells overexpressing dsRNA-PK. The increased expression observed for the mutant kinases and the wild-type kinases in the eIF-2α-S51A strains suggests that the wild-type kinases inhibit their own translation. (*C*) IEF analysis of *gcn3*Δ strains. eIF-2α phosphorylation was monitored in *gcn2*Δ strains carrying either a wild-type *GCN3* allele (lanes 1, 2, 7, and 8) or a *gcn3*Δ allele (lanes 3–6). Lanes 1–4 are from cells that express wild-type GCN2 grown under nonstarvation conditions, where GCN4 expression is repressed (R), or under histidine starvation conditions (+ 3-AT), where GCN4 expression is derepressed (DR). The cultures used in lanes 5–8 were grown under inducing conditions, as described in the legend to Table 1, for the indicated kinase, HRI (lanes 5 and 7) or dsRNA-PK (lanes 6 and 8).

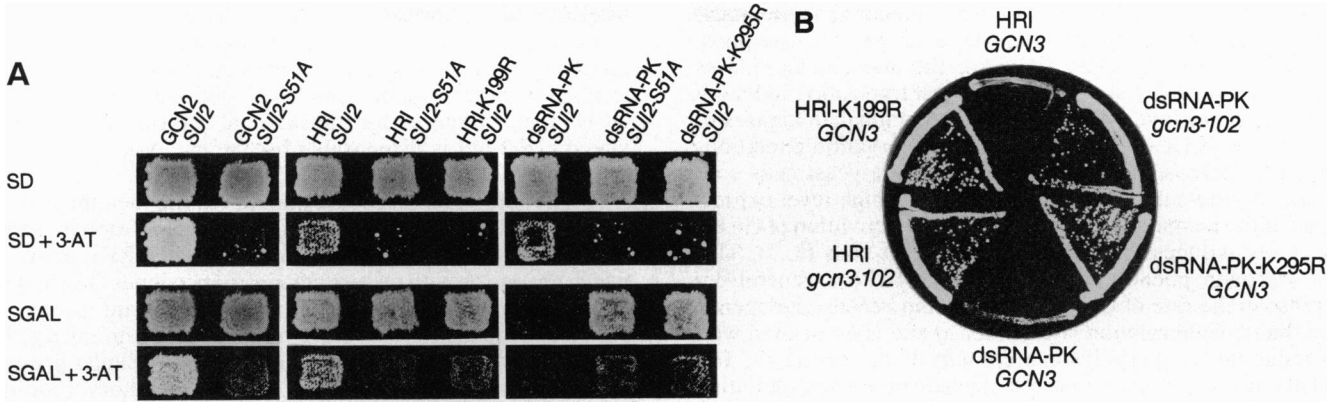

FIG. 3. Amino acid analogue sensitivity and growth-rate analysis of yeast strains expressing the eIF-2α kinases GCN2, HRI, or dsRNA-PK. (*A*) Plasmids, utilized in Fig. 2, encoding the indicated eIF-2α kinases were introduced into the isogenic *gcn2Δ* strains H1816 and H1817 expressing either wild-type eIF-2α or eIF-2α-S51A, respectively (the eIF-2α protein is encoded by the *SUI2* gene). Patches of transformants were grown to confluence on minimal SD plates and replica-plated to SD plates, SD plus 3-AT plates (30 mM), 10% galactose plates (SGAL), and SGAL plus 3-AT plates. Plates were incubated at 30°C for 3–4 days. (*B*) Plasmids encoding HRI and dsRNA-PK were introduced into the isogenic *GCN3 leu2* and *gcn3-102 leu2* strains H17 and H2064, respectively. The expression vector carries a defective *LEU2* gene, *leu2-d*, that leads to very high plasmid-copy-number in *leu2* strains grown on medium lacking leucine. Strains were streaked on SD plates and incubated 4 days at 30°C.

domains and on Ser-51 of eIF-2α (Table 1, strains 2, 3, 7, and 8), in accord with the requirements for growth on 3-AT medium (Fig. 3*A*). The low levels of *GCN4–lacZ* expression seen under noninducing conditions are ostensibly at odds with the 3-AT resistance on glucose medium shown in the replica-printing experiments of Fig. 3*A*. This discrepancy can be explained by noting that cells are in stationary phase before replica-plating and that both kinases were found to be partially induced in stationary-phase cells grown on liquid glucose medium (data not shown). Because the high-copy dsRNA-PK construct led to slow growth on galactose medium (Fig. 3*A*), we expressed this enzyme from a low-copy-number vector to minimize the inhibition of total protein synthesis. Table 1 shows (strains 9 and 10) that the low-copy dsRNA-PK construct produced somewhat greater stimulation of *GCN4* expression under inducing conditions (6- to 7-fold) compared with the high-copy construct. The induction of *GCN4* expression by the mammalian kinases is comparable in magnitude to that given by GCN2 when activated on these media by amino acid starvation (5- to 10-fold induction). Additional experiments showed that the induction of *GCN4* expression by the mammalian kinases occurred at the translational level because it required the small ORFs in the leader of *GCN4* mRNA (data not shown).

Table 1. Regulation of GCN4 expression in *gcn2Δ* yeast strains expressing HRI or dsRNA-PK

Strain	Mammalian eIF-2α kinase	Relevant regulatory alleles	GCN4–lacZ expression, units U*	I†
1	hc HRI	*SUI2, GCN3*	11	66
2	hc HRI-K199R	*SUI2, GCN3*	7	10
3	hc HRI	*SUI2-S51A, GCN3*	10	11
4	hc HRI	*SUI2, gcn3Δ*	8	17
5	hc HRI-K199R	*SUI2, gcn3Δ*	9	10
6	hc dsRNA-PK	*SUI2, GCN3*	7	32
7	hc dsRNA-PK-K295R	*SUI2, GCN3*	6	6
8	hc dsRNA-PK	*SUI2-S51A, GCN3*	8	8
9	lc dsRNA-PK	*SUI2, GCN3*	8	53
10	lc dsRNA-PK	*SUI2-S51A, GCN3*	5	8

The indicated kinases were expressed from a *GAL* promoter in the isogenic series of *gcn2Δ* strains H1816 (*SUI2, GCN3*), H1817 (*SUI2-S51A, GCN3*), or H2065 (*SUI2, gcn3Δ*) (*SUI2* encodes yeast eIF-2α). β-Galactosidase activities expressed from an integrated wild-type *GCN4–lacZ* fusion were measured in whole-cell extracts and are the averages of results from two or more independent transformants; SEMs were 20% or less. hc, High-copy-number plasmid derived from pEMBLyex4; lc, low-copy-number plasmid derived from vector pRS316 (10). Cells were grown exponentially in SD medium for ≈30 hr and then shifted to either SD medium or inducing medium and harvested after 12 hr.

*U, uninducing conditions: glucose-containing SD medium in which the *GAL* promoter is repressed.

†I, inducing conditions: SR medium containing 2% raffinose for HRI, and SGR medium containing 10% galactose and 2% raffinose for dsRNA-PK, in which the *GAL* promoter is functional.

Mutation of the GCN3 Subunit of Yeast eIF-2B Suppresses the Effects of Phosphorylation of eIF-2α. Functional substitution of the mammalian kinases for GCN2 enabled us to study how phosphorylation of eIF-2α regulates yeast translation. The GCN3 protein is a subunit of the yeast eIF-2B complex and is not essential for cell viability (4, 8, 20). In strains deleted for *GCN3*, expression of *GCN4* does not increase in response to activation of the GCN2 protein kinase by amino acid starvation (4, 9). Expression of both HRI and dsRNA-PK from plasmids maintained at very high-copy numbers led to slow growth on glucose medium in a wild-type *GCN3* strain; however, this slow-growth phenotype was not seen in an isogenic strain carrying the *gcn3-102* allele (Fig. 3*B*). A deletion of *GCN3* had the same effect as *gcn3-102* in overcoming the toxic effect of dsRNA-PK on cell growth (data not shown), and it also eliminated the ability of HRI to stimulate *GCN4* expression (Table 1, strains 4 and 5). However, the *gcn3Δ* mutation did not reduce the ability of mammalian kinases (or GCN2) to phosphorylate eIF-2α *in vivo* (Fig. 2*C*). These results lend strong support to the idea that GCN3 is a regulatory subunit of eIF-2B that mediates the inhibitory effect of eIF-2α phosphorylation on translation initiation.

DISCUSSION

We have shown that the two known mammalian eIF-2α kinases, HRI and dsRNA-PK, can functionally substitute for the *S. cerevisiae* protein kinase GCN2 by phosphorylating eIF-2α and stimulating translation of GCN4 mRNA in yeast strains that lack GCN2. These results strongly suggest that phosphorylation of eIF-2α is the primary function of GCN2 in translational control of *GCN4* expression, and they pro-

vide additional *in vivo* and *in vitro* evidence that the mammalian kinases phosphorylate eIF-2α on Ser-51. The phosphorylation of eIF-2α by GCN2 or the mammalian kinases affects both GCN4-specific and general translation initiation in yeast. Low-level expression of the mammalian kinases or activation of GCN2 under amino acid starvation conditions leads to increased *GCN4* expression while yeast cells continue to grow and divide (4). In contrast, high-level expression of the mammalian kinases or genetic activation of GCN2 severely reduces the growth rate of yeast cells (5, 7). This slow-growth phenotype can be attributed to a general decrease in the rate of translation initiation because it depends on the phosphorylation site in eIF-2α and is associated with a reduction in the polysome content of the cell (5, 7, 16). These results indicate that the mechanism for the inhibition of total protein synthesis by phosphorylation of eIF-2α has been highly conserved between yeast and mammals.

In mammalian cells, activities of the eIF-2α kinases are modulated by environmental signals and viral regulatory factors (1, 2, 17). The functional substitution of the mammalian eIF-2α kinases for GCN2 should allow us to use GCN4 translational control as a genetic tool to study the regulation of these kinases in yeast. Thus far, we have seen no differences in the level of GCN4 induction by HRI or dsRNA-PK when the levels of heme or uncharged tRNA are varied in yeast cells; however, the insensitivity of HRI to heme supplementation or deprivation in yeast may be attributable to the fact that most kinase molecules were truncated at the N terminus (Fig. 2*A*, Immunoblot, and unpublished observations). Further experiments are required to determine whether the effects of the mammalian eIF-2α kinases on GCN4 translation in yeast depend on endogenous activators or arise simply from high-level expression of the enzymes.

The ability of the mammalian kinases to functionally substitute for GCN2 in yeast cells allowed us to explore the role of GCN3 in translational control by phosphorylation of eIF-2α. Recent biochemical studies (8, 20) have shown that GCN3 is a subunit of the yeast equivalent of eIF-2B, the guanine nucleotide exchange factor for eIF-2. Surprisingly, deletion of *GCN3* has no effect on yeast cell growth under normal conditions and is required only for induction of *GCN4* translation under amino acid starvation conditions (4). Recently, it was shown that GCN3 is required for the slow-growth phenotype associated with genetically activated forms of GCN2 (5). In reticulocyte lysates, phosphorylated eIF-2 sequesters eIF-2B in eIF-2$\approx$*P*·eIF-2B complexes that are inactive for guanine nucleotide exchange on eIF-2 (2, 3). Our results provide further *in vivo* evidence that phosphorylation of eIF-2α inhibits translation by impairing eIF-2B activity. Also our results identify GCN3 as a regulatory subunit of eIF-2B that mediates the inhibitory effect of phosphorylated eIF-2 on eIF-2B. Perhaps GCN3 directly interacts with phosphorylated eIF-2α and stabilizes the formation of the inactive eIF-2$\approx$*P*·eIF-2B complexes.

One prediction of this model is that GCN3 should be required for translational control whether eIF-2α is being phosphorylated by GCN2, HRI, or dsRNA-PK. This prediction was borne out by the experiments shown in Fig. 3*B* and Table 1. An alternative model would be that GCN3 is required for the kinases to phosphorylate eIF-2α. At odds with this second possibility, deletion of *GCN3* led to even higher levels of eIF-2α phosphorylation than were seen in wild-type cells (Fig. 2*C*). This latter finding suggests that deletion of *GCN3* destroys a mechanism that limits the level of eIF-2α phosphorylation in yeast. Perhaps the expression or activation of the kinases or an opposing phosphatase is regulated by phosphorylation of eIF-2α. It will be interesting to learn whether mammalian eIF-2B contains a subunit like GCN3 that is required for translational control by phosphorylated eIF-2 but is dispensable for the catalytic function of eIF-2B.

Finally, our results provide impetus to the idea that mammalian eIF-2α kinases may stimulate the translation in mammalian cells of mRNAs containing upstream ORFs, many of which encode growth factors and protooncogenes (18). It was recently shown that overexpression of mutant forms of dsRNA-PK leads to oncogenic transformation in mice (19). Perhaps the role of dsRNA-PK in regulating cellular growth stems in part from the effect of eIF-2α phosphorylation on translational control of one or more protooncogenes.

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