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Casein Kinase II Mediates Multiple Phosphorylation of *Saccharomyces cerevisiae* eIF-2α (Encoded by *SUI2*), Which Is Required for Optimal eIF-2 Function in *S. cerevisiae*

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Previous studies have demonstrated that the α subunit of eukaryotic initiation factor 2 (eIF-2α), encoded by the *SUI2* gene in the yeast *Saccharomyces cerevisiae*, is phosphorylated at Ser-51 by the GCN2 kinase in response to general amino acid control. Here we describe that yeast eIF-2α is a constitutively phosphorylated protein species that is multiply phosphorylated by a GCN2-independent mechanism. $^{32}P_i$ labeling and isoelectric focusing analysis of a *SUI2*$^+$ Δ*gcn2* strain identifies eIF-2α as radiolabeled and a single isoelectric protein species. Treatment of *SUI2*$^+$ Δ*gcn2* strain extracts with phosphatase results in the identification of three additional isoelectric forms of eIF-2α that correspond to the stepwise removal of three phosphates from the protein. Mutational analysis of *SUI2* coupled with biochemical analysis of eIF-2α maps the sites to the carboxyl region of *SUI2* that correspond to Ser residues at amino acid positions 292, 294, and 301 that compose consensus casein kinase II sequences. $^{32}P_i$ labeling or isoelectric focusing analysis of eIF-2α from conditional casein kinase II mutants indicated that phosphorylation of eIF-2α is abolished or dephosphorylated forms of eIF-2α are detected when these strains are grown at the restrictive growth conditions. Furthermore, yeast casein kinase II phosphorylates recombinant wild-type eIF-2α protein in vitro but does not phosphorylate recombinant eIF-2α that contains Ser-to-Ala mutations at all three consensus casein kinase II sequences. These data strongly support the conclusion that casein kinase II directly phosphorylates eIF-2α at one or all of these Ser amino acids in vivo. Although substitution of *SUI2* genes mutated at these sites for the wild-type gene have no obvious effect on cell growth, one test that we have used appears to demonstrate that the inability to phosphorylate these sites has a physiological consequence on eIF-2 function in *S. cerevisiae*. Haploid strains constructed to contain Ser-to-Ala mutations at the consensus casein kinase II sequences in *SUI2* in combination with a mutated allele of either the *GCN2*, *GCN3*, or *GCD7* gene have synthetic growth defects. These genetic data appear to indicate that the modifications that we describe at the carboxyl end of the eIF-2α protein are required for optimal eIF-2 function in *S. cerevisiae*.

Eukaryotic translation initiation factor 2 (eIF-2) has been extensively characterized at the biochemical and genetic levels. Biochemical studies have established eIF-2 to be composed of three nonidentical subunits, α, β, and γ, that function during the early steps of translation initiation by forming a ternary complex with GTP and the initiator tRNA (reviewed in references 29 and 39). This complex then binds the 40S ribosomal subunit, which in turn binds the 5′ end of mRNA. According to the scanning model, this preinitiation complex scans the leader region until the first AUG codon is reached, whereupon translation begins (reviewed in references 33 and 34). Genetic studies from our laboratory have implicated eIF-2 to also play a role in ribosomal recognition of an AUG start codon. By reverting *his4*$^-$ initiator codon mutants, three unlinked genes, *sui1*, *sui2*, and *SUI3*, were identified that when mutated act in *trans* to restore *his4* expression (12). Characterization of the *SUI2* and *SUI3* genes showed that they encoded the α and β subunits of eIF-2, which are 42 and 58% identical in amino acid sequence to the human eIF-2α and -β proteins, respectively (17, 25). Further analysis demonstrated that suppressor mutations in these genes conferred the ability to the ribosome to initiate translation at a UUG codon in the early *his4* coding region by allowing a mismatched base pair interaction between the UUG codon and the initiator tRNA (17, 25, 63). The mutations in *sui2* and *SUI3* that confer suppression altered amino acids that are conserved at the same relative positions in the sequences of the human α and β proteins, respectively (11, 17, 25). These studies have demonstrated the functional significance of eIF-2 in mediating the general mechanism of start site selection in all eukaryotic organisms, in addition to its function in promoting ternary complex formation.

eIF-2 activity has been shown to be regulated in human cells in response to adverse growth conditions (reviewed in references 29 and 35). After translation initiation, eIF-2 is released as a GDP-bound form. This eIF-2:GDP binary complex, which is inactive in translation, is then converted to the eIF-2:GTP form in order for eIF-2 to be recycled into the initiation process. This conversion is catalyzed by the guanine nucleotide exchange factor, eIF-2B (32). A number of different stress conditions such as heme deprivation or viral infection induce the eIF-2α-specific kinases HCR (heme-controlled repressor) and DAI (double-stranded RNA-activated inhibitor), which phosphorylate eIF-2α at a Ser residue at amino acid position 51 (13, 38). The net effect of this phosphorylation event is that it blocks the ability of eIF-2 to be recycled from the inactive eIF-2:GDP form to the active eIF-2:GTP-bound form, presumably because the phosphorylated form of eIF-2α sequesters eIF-2B to inhibit the exchange reaction (51, 56). Inhibition of the exchange reaction therefore leads to accumulation of eIF-2 in the inactive GDP-bound form, which inhibits or down-regulates global protein synthesis.

In collaboration with the Hinnebusch laboratory, we have

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recently demonstrated that yeast eIF-2 activity is also regulated by phosphorylation of the α subunit at Ser-51 (21). The phosphorylation of eIF-2α at Ser-51 results in gene-specific regulation of *GCN4*, the positive transcriptional activator of amino acid biosynthetic genes subject to the general amino acid control system. *GCN4* expression is regulated at the level of translation initiation which is mediated by the GCN2 kinase. GCN2 kinase contains two recognizable amino acid sequence domains, one of which has homology with the histidinyl-tRNA synthetase domain; the other has homology with the mammalian eIF-2α kinases HCR and DAI (13, 15, 47, 59). Under amino acid starvation conditions, the GCN2 kinase is presumed to be activated by accumulated uncharged tRNA, which leads to phosphorylation of eIF-2α at the Ser-51 position (21, 47). In light of the biochemical studies of mammalian eIF-2, it is proposed that phosphorylation of eIF-2α in yeast cells by GCN2 results in inhibition and sequestration of the guanine nucleotide exchange factor that results in accumulation of eIF-2:GDP. This lowers the level of active eIF-2:GTP. However, in contrast to mammalian studies, this does not lead to significant down-regulation of global protein synthesis. Instead, lower levels of active eIF-2 result in an alteration in the reinitiation properties at the upstream open reading frames in the *GCN4* leader region, which leads to specific translation initiation at the *GCN4* start codon (1, 30, 40).

The involvement of the guanine nucleotide exchange factor, eIF-2B, in the yeast eIF-2 regulatory response has been recently borne out. Five genes, *GCD1*, *GCD2*, *GCD6*, *GCD7*, and *GCN3*, have been identified as regulators of the general amino acid control response and characterized to encode subunits of yeast eIF-2B (7, 8, 16). In addition, *GCN2*^{c} alleles that result in increased levels of eIF-2α phosphorylated at Ser-51 have been identified (47, 60). A number of these *GCN2*^{c} mutant strains have been found to grow poorly, presumably because the increase in eIF-2α phosphorylation further inhibits eIF-2B activity, which now affects the general translation initiation process. In fact, reversion analysis of the slow-growth phenotype associated with *GCN2*^{c} mutants has identified second-site mutations in eIF-2α which restore normal growth to these strains without affecting eIF-2α phosphorylation (62). These second-site eIF-2α mutants have also been shown to suppress a growth defect associated with a *gcn3*^{c} mutant, the proposed regulatory subunit of eIF-2B (28, 62). These genetic data appear to indicate that eIF-2α and eIF-2B might interact to mediate the translational regulation at *GCN4*.

Although a number of parallels exist between the human and yeast mechanisms of phosphorylation of eIF-2α and its effect on protein synthesis, there seems to be a major difference. The response in mammalian cells is believed to be directed at down-regulation of protein synthesis presumably to slow down or inhibit cellular growth in response to adverse growth conditions. In contrast, the yeast regulatory response would appear to be directed at improving the ability of yeast cells to grow by increasing the rate of transcription of amino acid biosynthetic genes in response to amino acid starvation. Only upon mutation of *GCN2* or subunits of yeast eIF-2B, which severely alter their function, is general protein synthesis impaired, as reflected in reduced growth rates. Therefore, an important question to address is whether there exist other mechanisms for controlling eIF-2 function in yeast cells.

In this report, we show that yeast eIF-2α is an in vitro and in vivo substrate for casein kinase II. In addition, genetic data presented appear to indicate that phosphorylation of eIF-2α in yeast cells by casein kinase II is necessary for optimal eIF-2 function in yeast cells.

MATERIALS AND METHODS

Yeast strains and genetic methods. The *Saccharomyces cerevisiae* strains used in this analysis and their complete genotypes are listed in Table 1. The standard genetic techniques and media used for these studies have been described elsewhere (55). The medium used for testing amino acid analog sensitivity has also been described previously (31).

Yeast strain LF80 was derived from yeast strain H2507 (Table 1) by plasmid shuffle of a *SUI2*$^+$ *LEU2 CEN4* plasmid, p975, for the resident *SUI2*$^+$ gene on the *URA3* plasmid pRS316 (plasmid p919 [21]), using 5-fluoroorotic acid medium (5). Yeast strain LF91 was derived from yeast strain H1643/pRS316 (Table 1) by selecting for loss of the resident *URA3* plasmid pRS316 on 5-fluoroorotic acid medium.

Construction of yeast strains containing *SUI2-S292A,S294A* (*SUI2-CK2A*) and *SUI2-S292A,S294A,S301A* (*SUI2-CK3A*) mutations. A 2.5-kb *Eco*RI-*Bam*HI DNA fragment containing the complete *SUI2* gene (17) was subcloned into the *Eco*RI and *Bam*HI sites of bacteriophage vector M13mp10 and used as a template for site-directed mutagenesis (64). We first attempted to introduce serine-to-alanine substitutions at all three putative casein kinase II phosphorylation sites, Ser-292, Ser-294, and Ser-301, in the *SUI2* coding region, using the 42-nucleotide mutagenic primer 5′-GTC GTC AGC CTC ATC CTC ATC GTC TTC AGC GTC AGC TCT ATT-3′ (this primer is the antisense of the *SUI2* coding region). Positive plaques were identified by hybridization with the respective ^{32}P-end-labeled oligonucleotide. However, DNA sequencing (53) of the positive candidates identified Ser-to-Ala substitutions only at amino acid positions 292 and 294. We assume that the inability to generate all three substitutions with this strategy was related to some problem associated with the oligonucleotide primer. Nevertheless, the two mutations in the *SUI2* gene that we obtained from this mutagenesis gave rise to the *SUI2-S292A,S294A* mutant allele (abbreviated *SUI2-CK2A*) that we used as part of our analysis. To construct the *SUI2-S292A,S294A,S301A* mutant allele (abbreviated *SUI2-CK3A*), a 25-nucleotide oligonucleotide, 5′-A CTC GTC GTC AGC CTC ATC CTC ATC-3′, was synthesized to contain a Ser-to-Ala substitution at amino acid position 301. Site-directed mutagenesis was performed with this oligonucleotide as the primer and the *SUI2-CK2A* mutant as the template. Positive plaques containing mutations within the *SUI2* gene were identified by hybridization, and the presence of all three Ser-to-Ala substitutions was confirmed by DNA sequencing (TCT to GCT at amino acid positions 292 and 294 and TCA to GCT at amino acid position 301).

Yeast strains containing the *SUI2-CK2A* and *SUI2-CK3A* genes were constructed by first subcloning the respective 2.5-kb *Eco*RI-*Bam*HI DNA fragment into the *Eco*RI-*Bam*HI site of yeast *URA3*$^+$ integrating vector YIp5 (45), to yield plasmids p1883 and p1885, respectively. The presence of the corresponding mutations contained on plasmids p1883 and p1885 was confirmed by DNA sequencing. Plasmids were then linearized by restriction at the unique *Stu*I site in the *URA3* gene (50) and used to transform yeast strains LF80 and LF91 (Table 1) to Ura$^+$. Restriction at the *Stu*I site in *URA3* was used to target integration of the plasmid (42) at the *ura3-52* locus in strains LF80 and LF91. Both LF80 and LF91 contain a deletion of the chromosomal copy of the *SUI2* gene, and the wild-type gene is contained on plasmid p975 (21) as part of a 2.7-kb *Bam*HI DNA fragment (17) in the *LEU2 CEN4* vector pSB32 (49). Therefore, to generate yeast strains containing only the *SUI2-CK2A* or *SUI2-CK3A* genes, the resulting Ura$^+$ transformants were grown nonselectively in YEPD (yeast

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
117-1AR7	*MAT***a** *his4-303 ura3-52 ino1-13 SUI3-2* p[*his4*(AUU)-*lacZ URA3*$^+$]@*ura3-52*	25
118-1D	*MAT***a** *his4-303 leu2-3,112 ura3-52 ino1-13* p[*his4*(AUU)-*lacZ URA3*$^+$]@*ura3-52*	12
301-4D	*MAT***a** *his4-303 leu2-3,112 ura3-52 sui1-1*	63
327-2D	*MAT*α *his4-303 ura3-52 SUI3-2*	25
EKP1	*MAT***a**/*MAT*α *HIS4*$^+$/*his4-401 ura3-52*/*ura3-52 LEU2*$^+$/*leu2-3,112 INO1*$^+$/*ino1-13*	17
EP290	*MAT*α *his4-401 leu2-3,112 ura3-52 sui2*::*URA3*	This study
LF31-5A	*MAT***a** *his4-401 leu2-3,112 ura3-52 sui2*::*URA3*	This study
LF32-1C	*MAT***a** *his4-401 leu2-3,112 ura3-52 sui2*::*URA3*	This study
LF80	*MAT***a** *leu2-3,112 ura3-52* Δ*gcn2* Δ*sui2 trp1-Δ63* p[*SUI2*$^+$ *LEU2*$^+$ *CEN4*]	This study
LF81 and LF82	*MAT***a** *leu2-3,112 ura3-52* Δ*gcn2* Δ*sui2 trp1-Δ63* p[*SUI2*$^+$ *URA3*$^+$]@*ura3-52*	This study
LF83 and LF85	*MAT***a** *leu2-3,112 ura3-52* Δ*gcn2* Δ*sui2 trp1-Δ63* p[*SUI2-S292A,S294A URA3*$^+$]@*ura3-52*	This study
LF84 and LF86	*MAT***a** *leu2-3,112 ura3-52* Δ*gcn2* Δ*sui2 trp1-Δ63* p[*SUI2-S292A,S294A,S301A URA3*$^+$] @*ura3-52*	This study
LF91	*MAT***a** *ura3-52 leu2-3,112 trp1-Δ63* Δ*sui2* [*GCN4-lacZ TRP1*$^+$]@*trp1-Δ63* p[*SUI2*$^+$ *LEU2*$^+$ *CEN4*]	This study
LF92 and LF93	*MAT***a** *ura3-52 leu2-3,112 trp1-Δ63* Δ*sui2* [*GCN4-lacZ TRP1*$^+$]@*trp1-Δ63* p[*SUI2*$^+$ *URA3*$^+$]@*ura3-52*	This study
LF94 and LF96	*MAT***a** *ura3-52 leu2-3,112 trp1-Δ63* Δ*sui2* [*GCN4-lacZ TRP1*$^+$]@*trp1-Δ63* p[*SUI2-S292A,S294A URA3*$^+$]@*ura3-52*	This study
LF95 and LF97	*MAT***a** *ura3-52 leu2-3,112 trp1-Δ63* Δ*sui2* [*GCN4-lacZ TRP1*]@*trp1-Δ63* p[*SUI2-S292A,S294A,S301A URA3*$^+$]@*ura3-52*	This study
LF95-AUU	*MAT*α *his4-303 ura3-52 leu2-3,112* Δ*sui2* p[*SUI2-S292A,S294A,S301A URA3*$^+$]@*ura3-52*	This study
JC35-1a	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 cka1*::*HIS3 cka2*::*TRP1* p[*GAL1-CKA1 URA3*/*CEN4*]	43
YDH8	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 cka1*::*HIS3 cka2*::*TRP1* p[*cka2*^{ts2} *LEU2*$^+$ *CEN4*]	9; D. Hanna and C. V. C. Glover
H1333	*MAT*α *leu2-3,112 ura3-52 ino1* [*HIS4-lacZ*]@*ura3-52 gcn2*::*URA3*	28
H1373	*MAT*α *leu2-3,112 ura3-52 ino1* [*HIS4-lacZ*]@*ura3-52 GCN2*^{c}*-503*	A. Hinnebusch
H1402	*MAT*α *leu2-3,112 ura3-52 ino1* [*HIS4-lacZ*]@*ura3-52*	28
H1414	*MAT*α *leu2-3,112 ura3-52 ino1* [*HIS4-lacZ*]@*ura3-52 GCN2*^{c}*-501*	58
H1489	*MAT*α *leu2-3,112 ura3-52 ino1* [*HIS4-lacZ*]@*ura3-52 gcn3*^{c}*-R104K*	28
H1491	*MAT*α *leu2-3,112 ura3-52 ino1* [*HIS4-lacZ*]@*ura3-52 gcn3*^{c}*-A26T*	28
H1613	*MAT*α *leu2-3,112 ura3-52 ino1* [*HIS4-lacZ*]@*ura3-52 GCN2*^{c}*-E532K-E1522K*	47
H1643/pRS316	*MAT***a** *ura3-52 leu2-3,112 trp1-Δ63* Δ*sui2* [*GCN4-lacZ TRP1*$^+$]@*trp1-Δ63* p[*SUI2*$^+$ *LEU2*$^+$ *CEN4*] p[*URA3*$^+$ *CEN4*]	21
H1725	*MAT*α *leu2-3,112 ura3-52 gcd7-201*	8
H2507	*MAT*α *leu2-3,112 ura3-52* Δ*gcn2* Δ*sui2 trp1-Δ63* p[*SUI2*$^+$ *URA3*$^+$ *CEN4*]	T. Dever and A. Hinnebusch

extract, peptone, dextrose) liquid medium to allow loss of the *LEU2* plasmid, p975, that contains the wild-type *SUI2* gene. Cells were plated on YEPD medium and replica plated to synthetic dextrose plates lacking uracil and SD plates lacking leucine. Ura$^+$ Leu$^-$ colonies were selected as representative of each of the corresponding mutated *SUI2* genes having been substituted for the wild-type gene. The *SUI2-CK2A* and *SUI2-CK3A* strains derived from LF80 are LF83 and LF85, and LF84 and LF86, respectively (Table 1). The *SUI2-CK2A* and *SUI2-CK3A* strains derived from LF91 are LF94 and LF96, and LF95 and LF97, respectively (Table 1).

Isogenic strains containing the wild-type *SUI2* gene at *ura3-52* were constructed in the same fashion as described for the *SUI2-CK2A* and *SUI2-CK3A* strains, with one exception. The plasmid used for transformation, p615 (17), contained the wild-type *SUI2* gene as part of a 2.7-kb *Bam*HI DNA fragment ligated into the *Bam*HI site of YIp5. The 2.7-kb *Bam*HI fragment differs from the 2.5-kb *Eco*RI-*Bam*HI fragment by having approximately 200 additional contiguous base pairs in the upstream noncoding region of *SUI2* (17). Otherwise, the *SUI2* gene is transcribed in the same relative orientation as that of *URA3* in p615, p1883, and p1885. The *SUI2* wild-type strains derived from LF80 are LF81 and LF82 (Table 1). The *SUI2* wild-type strains derived from LF91 are LF92 and LF93 (Table 1). Southern blot analysis was performed with LF92, LF93, LF94, and LF95 and confirmed the presence of a single copy of the *SUI2*-containing plasmid integrated at the *ura3-52* locus in each of these strains (data not shown).

IEF. For the majority of the experiments, overnight yeast cultures (0.1 ml) were used to inoculate 100 ml of YEPD liquid medium and incubated at 30°C overnight to an optical density at 600 nm (OD_{600}) of 1.0. Cells were harvested by centrifugation for 10 min, and cell extracts were prepared as described previously (21) with the exception that the breaking buffer contained 40 mM 1,4-piperazinediethanesulfonic acid (PIPES; pH 6.0), 100 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 50 mM β-glycerophosphate (19a) and cells were broken with an equal volume of glass beads on a vortex blender. Protein concentration was determined by Bradford assay using Bio-Rad dye (6). Twenty-microgram aliquots of extracts were frozen and lyophilized to dryness and used for isoelectric focusing (IEF) analyses.

For the GCN2 kinase induction experiment presented in Fig. 4D, 0.1 ml of an overnight preculture of yeast strain LF95 (Table 1) was used to inoculate 100 ml of SD liquid medium and grown overnight to an OD_{600} of 1.0. The 100-ml culture was divided into two 50-ml cultures, and 3-aminotriazole (3-AT) was added to one of the 50-ml cultures at a final concentration of 10 mM (21). Both of the cultures were incubated for 4 h, at which time cells were harvested and extracts were prepared as described above.

For the temperature shift experiments presented in Fig. 5C, 0.5 ml of a 23°C-grown, overnight preculture of either yeast strain 118-1D or YDH8 (Table 1) was used to inoculate 100 ml of YEPD medium, and cultures were incubated at 23°C to an OD_{600} of 0.5. Each culture was then divided into two 50-ml cultures; one was incubated at 23°C for 2 h, and the other was incubated at 37°C for 2 h. At the end of the incubation period, cells were harvested and extracts were prepared as described above.

The vertical slab gel IEF system used for phosphorylation analysis of yeast eIF-2α is identical to the system described previously (21) except that the polarity of the electrophoresis was reversed. After the slab IEF gel was assembled in the electrophoresis apparatus, the bottom chamber was filled with 20 mM aspartic acid, and the upper chamber was filled with 50 mM histidine so that the anode was at the top and the cathode was at the bottom of the gel. We found that reversing the polarity of electrophoresis gave more reproducible resolution of the isoelectric species of eIF-2α. The focusing was carried out at a constant current of 2.5 mA for 23 h, with cooling at 15.5°C with a HAAK cooling device. After focusing, the gel was transferred to a nitrocellulose membrane for 1 h at 1 mA. The nitrocellulose membrane was then stained with Ponceau S to detect the quality of focusing. The membrane was then blocked with 4% nonfat milk in 50 mM Tris (pH 7.4) containing 150 mM NaCl (Tris-buffered saline) and blotted with a polyclonal antiserum raised against a TrpE-SUI2 protein fusion at a 1:200 dilution (17). The membrane was subsequently blotted with ^{125}I-protein A (Amersham) at a final concentration of 0.3 μCi/ml, washed with 0.5% Tween 20 in Tris-buffered saline, and exposed to X-ray film at −70°C for 24 h with an intensifying screen.

Phosphatase treatment. Potato acid phosphatase was purchased from Sigma. Phosphatase was reconstituted by centrifugation of 1 ml of the slurry and resuspending the pellet in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4) containing 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 50% glycerol at a final phosphatase concentration of 1 mg/ml (19). Yeast crude extracts analyzed by phosphatase treatment were prepared as described above with the exception that the phosphatase inhibitors β-glycerophosphate and sodium orthovanadate were not added to the buffers. Twenty micrograms of crude extract was incubated with 2 μg of the potato acid phosphatase at 37°C from 1 to 5 h to achieve a range of partial to nearly complete dephosphorylation of eIF-2α (19a). Reaction mixtures were then dried in a lyophilizer and analyzed by IEF as described above.

In vivo $^{32}P_i$ labeling experiments. Preparation of phosphate-depleted YEPD liquid medium has been described previously (48). The phosphate-depleted YEPGal medium was identical to that of YEPD with the exception that galactose was substituted for glucose as a carbon source. The wild-type control strain 118-1D and the Δ*gcn2* strain H1333 (Table 1) were grown at 30°C in phosphate-depleted YEPD medium overnight. The preculture was then diluted 1:100 in 50 ml of the same medium and grown to an OD_{600} of 0.5. Five milliliters of each culture was then incubated for 2 h in the presence of 0.5 mCi of $^{32}P_i$ (ICN). Crude extracts were prepared, and labeled eIF-2α was immunoprecipitated and analyzed by Western blotting (immunoblotting) and autoradiography as previously described (17).

For the analysis of yeast strain JC35-1a (Table 1), which contains the *CKA1* gene under the control of the *GAL1* promoter, cells were grown overnight in phosphate-depleted YEPGal medium. The culture was divided in half, and one of the two cultures was further incubated in the same medium. The other culture was washed and resuspended in phosphate-depleted YEPD and grown overnight. Each preculture was then diluted 1:100 in 50 ml of the respective medium to an OD_{600} of 0.5. Five milliliters of each culture was then incubated for another 2 h in the presence of 0.5 mCi of $^{32}P_i$, and labeled eIF-2α protein was analyzed as described above.

For analysis of the temperature-sensitive *cka2* strain YDH8 (Table 1), an overnight preculture was diluted 1:100 in 50 ml of YEPD medium and further grown at 23°C to an OD_{600} of 0.5. One 5-ml aliquot was incubated with $^{32}P_i$ at 23°C for 2 h. A second 5-ml aliquot was first incubated at 42°C for 5 min, after which 0.5 mCi of label was added and the culture was incubated for 2 h at the nonpermissive growth temperature of 37°C.

The *SUI2* allele containing a deletion of the consensus casein kinase II phosphorylation sequences was constructed by insertion of the *URA3*$^+$ gene in the carboxyl region of the *SUI2* wild-type gene. Plasmid p628 contains the *SUI2* gene on a 2.7-kb *Bam*HI DNA fragment in the *Bam*HI site of pBR322 (17) and was used for the disruption experiment. A 1.1-kb *Hin*dIII DNA fragment containing the *URA3*$^+$ gene which was adapted with *Bam*HI ends (17) was ligated into the *Bgl*II restriction site located in the carboxyl end of the *SUI2* coding region (17) contained on plasmid p628. The resulting plasmid, p692, is similar to p693 (17) with the exception that the inserted *URA3*$^+$ gene in plasmid p692 is predicted to be transcribed in the same direction as the *SUI2* gene. This plasmid was cut with restriction enzyme *Bam*HI and used to transform the yeast diploid strain EKP1 (Table 1) to Ura$^+$ to replace the *SUI2*::*URA3* allele for the wild-type allele (42). The resulting *Ura*$^+$ transformants were sporulated, and four viable ascospores were obtained. The *URA3* insertion at *SUI2* in this orientation (referred to as *sui2*-Δ*CK3*) does not lead to cell lethality. This contrasts with the observation made with the converse disruption allele, whereby the transcription of the *URA3* gene inserted at the *Bgl*II site converged with *SUI2* transcription and resulted in cell lethality in haploid strains (17). However, the *sui2*-Δ*CK3* allele does confer a poor growth property when present as the sole copy of *SUI2* in haploid yeast strains. As a result of insertion of the *URA3* gene at this position in the coding region, the last 14 amino acids of *SUI2* are no longer contiguous with the *SUI2* reading frame inclusive of the consensus casein kinase II phosphorylation sequences. Instead, 31 nonspecific amino acids are inframe downstream from the *Bgl*II restriction site and are derived from read-through into the *URA3* insertion. The *sui2*-Δ*CK3*-encoded protein is still detectable by Western blotting using our anti-SUI2 antiserum (17). In addition, this protein still comigrates with the wild-type protein on sodium dodecylsulfate (SDS)-polyacrylamide gels, as it is not significantly different in its calculated M_r value. The in vivo labeling conditions for the *sui2*-Δ*CK3* strains EP290, LF31-5A, and LF32-1C (Table 1) were identical to those described above for yeast strain 118-1D with the exception that 5-ml cultures were incubated with radioactive label for 4 h instead of 2 h because of the slow-growth phenotype of the *sui2*-Δ*CK3* strains.

In vitro kinase assay. The eIF-2α expression vector PJLA603 was used to prepare recombinant wild-type eIF-2α protein from *Escherichia coli* (kindly provided by John McCarthy). It is a derivative of the λ expression vector PJLA501 (54). PJLA603 contains the complete coding region of the wild-type *SUI2* gene under the control of the λ promoters p_L and p_R, which is inducible by a temperature-sensitive allele of the λ repressor gene *cIts*857. To obtain recombinant eIF-2α protein containing Ser-to-Ala changes at all three of the consensus casein kinase II sequences, the downstream region

of the *SUI2-CK3A* gene was substituted for the downstream region of the *SUI2*$^+$ gene contained on PJLA603. The *Pvu*II-*Bam*HI DNA fragment from plasmid p1885 containing the *SUI2-CK3A* gene was substituted for the *Pvu*II-*Bam*HI DNA fragment contained in PJLA603 to yield the expression vector PJLA603-CK3A. The *Pvu*II-*Bam*HI DNA fragment contained in PJLA603-CK3A corresponds to the approximate carboxyl half of the coding region and the 3′ noncoding region of the *SUI2* gene (17). Thus, not only does PJLA603-CK3A differ from PJLA603 with respect to the Ser-to-Ala mutations, but the 3′ noncoding region of PJLA603 contains *E. coli* termination sequences (54). Presumably because of this difference in the constructs, we found that the level of SUI2-CK3A protein expressed in *E. coli* was over an order of magnitude lower than the level of expressed wild-type protein as determined by Western blotting. Therefore, to maintain constant amounts of the respective eIF-2α proteins in the in vitro kinase reactions, different amounts of recombinant wild-type (0.5 μg of total protein) and mutant (10 μg of total protein) protein extracts needed to be added to each assay (see Fig. 6). Nevertheless, for comparisons, the total amount of protein extract in the assay was always held constant, when needed, by addition of *E. coli* extracts derived from the parent strain containing no expression vector.

E. coli JA200 (F$^+$ *thr-1 leuB6 ΔtrpE63 recA56 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1* λ$^-$ *supE44*) was the host strain for the eIF-2α expression vectors and was obtained from the *E. coli* stock center at Yale University. JA200 was used as the host strain because it harbors a deletion of the *trpE* gene. This was important because our anti-SUI2 antiserum was generated from a TrpE-SUI2 fusion protein and cross-reacts with both TrpE and SUI2 proteins, which have similar apparent M_r values (17, 25). Thus, the use of JA200 as a host strain for expression of eIF-2α protein allowed us to specifically probe eIF-2α by Western blotting of *E. coli* extracts and eliminated any interference in these assays by detection of TrpE as a comigrating and cross-reactive protein.

JA200 cells containing the expression vectors PJLA603 and PJLA603-CK3A were grown in 100-ml cultures of LB medium containing ampicillin (50 μg/ml) and induced (3) to express eIF-2α. Cells were harvested by centrifugation and resuspended in lysis buffer containing 30 mM Tris-HCl (pH 8.0), 2 mM EDTA, 5% glycerol, 10 mM $MgCl_2$, 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 50 mM β-glycerophosphate at a cell/buffer ratio of 1 g/5 ml. Cells were frozen by addition of liquid nitrogen, thawed, and then lysed in a French press. Extracts were cleared by centrifugation at 23,400 × *g* for 15 min, and the supernatant (soluble fraction) was collected and used as the source for eIF-2α protein. Total protein amount was quantitated by the Bradford assay (6). The conditions for the kinase reaction were identical to those described previously (27, 44) with the exception that *E. coli* extracts (10 μg of total protein) were the substrate source for the kinase, and bovine serum albumin (BSA; 15 μg) was added to maintain the total protein amount at 25 μg in a 28-μl reaction mix. Reactions were incubated for 15 min at 25°C in the presence of [γ-^{32}P]ATP (10 μCi/ml, 1 μCi per reaction; Amersham). The yeast casein kinase II (400 μg/ml) was kindly provided by Claiborne Glover, and approximately 40 ng of casein kinase II protein was added to each reaction. This enzyme represents a homogeneously purified preparation from yeast cells that is composed of four protein subunits and is the same preparation shown in Fig. 5, lane 6, of reference 4. The kinase activity is inhibited in excess of 98% by heparin, an inhibitor of casein kinase II activity (26a, 44). Reactions were stopped by the addition of 1 μl of 100 mM unlabeled ATP and 1 μl of 0.5 M EDTA, and eIF-2α protein was immunoprecipitated with anti-SUI2 antiserum and detected by Western blot analysis and autoradiography as described previously (17).

Genetic analysis of *SUI2-CK2A* and *SUI2-CK3A* mutants. Yeast strains LF92 (*SUI2*$^+$), LF94 (*SUI2-CK2A*), and LF95 (*SUI2-CK3A*) were each analyzed in genetic crosses with yeast strains H1402 (*GCN2*$^+$), H1373 (*GCN2*^{c}*-E752K*), H1414 (*GCN2*^{c}*-E532K*), H1489 (*gcn3*^{c}*-R104K*), H1491 (*gcn3*^{c}*-A26T*), H1613 (*GCN2*^{c}*-E532K-E1522K*), and H1725 (*gcd7-201*). Meiotic products which contained the general control mutation were identified by their slow-growth phenotype and/or by their ability to grow after replica plating to 3-AT and 5-fluorotryptophan (5-FT) media. Meiotic products containing the corresponding *SUI2* gene were identified as Ura$^+$ colonies indicative of the *SUI2* allele being integrated at the *ura3-52* locus as part of the YIp5 plasmid. LF92, LF94, and LF96 contain a deletion of the chromosomal copy of the *SUI2* gene. Therefore, from these crosses, four spore meiotic products can be generated only if the two spores containing the *SUI2* deletion have the corresponding *SUI2* allele derived from LF92, LF94, or LF95 at the *ura3-52* locus as indicated by the Ura$^+$ phenotype. Therefore, to be certain that meiotic products contained only the *SUI2* allele of interest in combination with a mutation in the general control gene, Ura$^+$ haploid strains with slow-growth or 3-AT-resistant, 5-FT-resistant phenotype were picked from four spore tetrads for further analysis. Differences in growth properties between haploid strains containing the two mutant alleles and the parent strains were assayed by streaking out each strain on YEPD plates and comparing colony sizes after incubation at 30°C. In addition, five independently constructed *SUI2*$^+$ strains, LF93, LF98, LF99, LF100, and LF101 (not listed in Table 1), and independently constructed *SUI2-CK2A* and *SUI2-CK3A* strains, LF96 and LF97, respectively (Table 1), were analyzed in crosses with H1491 and H1725 and gave results identical to those reported in Fig. 7.

Aside from the above crosses, the *SUI2-CK2A* strain, LF96, was analyzed in crosses with H1373 (*GCN2*^{c}*-E752K*); the *SUI2-CK3A* strains, LF95 and LF97, were analyzed in a cross with 327-2D (*SUI3-2*). In addition, LF95-AUU (*SUI2-CK3A*), an ascospore derivative of LF95, was analyzed in crosses with yeast strain 301-4D (*sui1-1*) and a Ura$^-$ derivative of yeast strain 117-1AR7 (*SUI3-2*) obtained from 5-fluoroorotic acid selection.

RESULTS

GCN2-independent phosphorylation of yeast eIF-2α. Previous studies have demonstrated that the α subunit of eIF-2 in yeast cells is a phosphoprotein (17, 48). In collaboration with the Hinnebusch laboratory, we have shown that the GCN2 protein kinase phosphorylates the Ser-51 position of yeast eIF-2α in vivo in response to amino acid starvation (21). IEF gel electrophoresis detects two isoelectric species of eIF-2α, the more acidic form increasing in relative abundance in response to amino acid starvation signals. This acidic species can be eliminated by deletion of the *GCN2* gene in yeast cells, which yields only the single more basic species as the detectable form of eIF-2α (21).

To determine whether this latter species of eIF-2α is also a phosphoprotein in yeast cells, we incubated a *GCN2* deletion and *GCN2* wild-type strain with $^{32}P_i$. Extracts were then prepared, and eIF-2α was immunoprecipitated and resolved on SDS-polyacrylamide gels. As shown in Fig. 1A (lanes 1 and 2), Western blot analysis identifies comparable amounts of

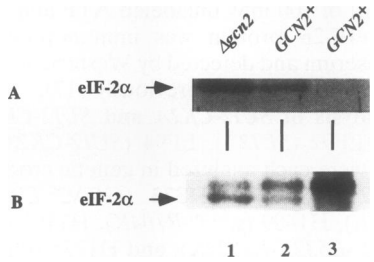

FIG. 1. Western blot and $^{32}P_i$ labeling analysis of eIF-2α from *GCN2*$^+$ and Δ*gcn2* yeast strains. Protein extracts were prepared from yeast strains 118-1D (*GCN2*$^+$) and H1333 (Δ*gcn2*), which were grown in phosphate-depleted YEPD liquid medium in the presence of $^{32}P_i$. eIF-2α was immunoprecipitated with antiserum raised against a TrpE-SUI2 fusion protein and identified by Western blotting with the same antiserum (A). Western blots were then subjected to autoradiography (B) to determine if eIF-2α was ^{32}P labeled. Lanes 1 and 2, labeling experiment with the Δ*gcn2* and *GCN2*$^+$ strains, respectively. The experiment presented in lane 3 is identical with the experiment presented in lane 2 with the exception that preimmune sera were substituted for the anti-SUI2 antisera in the immunoprecipitation step. Arrows mark positions of eIF-2α as detected by Western blotting (A) and autoradiography (B). The upper band observed by autoradiography represents a contaminating ^{32}P-labeled protein species that does not correspond to the α subunit of eIF-2 as indicated by our preimmune control (lane 3). In addition, this band is not always observed in these in vivo labeling experiments (see Fig. 3, lane 1).

eIF-2α immunoprecipitated from these strains. Exposure of the Western blot to X-ray film reveals the eIF-2α protein derived from both strains to be labeled with ^{32}P (Fig. 1B, lanes 1 and 2). This phosphoprotein is clearly related to eIF-2α, as substitution of preimmune antiserum for anti-eIF-2α antiserum neither identifies a comigrating protein by Western blot analysis (Fig. 1A, lane 3) nor identifies a comigrating phosphoprotein (Fig. 1B, lane 3) after autoradiography. The observation that eIF-2α is still phosphorylated in a *GCN2* deletion strain suggests that another kinase or other kinases phosphorylate this protein in vivo.

Amino acid sequence comparison between the eIF-2α subunits from yeast, *Artemia*, and human cells. Biochemical studies have shown that the α subunit of yeast and *Artemia* eIF-2 can be phosphorylated by mammalian casein kinase II, whereas the human homolog is not phosphorylated by this kinase in vitro (37). In light of these in vitro data, we compared the amino acid sequences of the yeast (17), *Artemia* (22), and human (26) eIF-2α proteins to determine if the eIF-2α protein derived from the former two organisms might contain consensus casein kinase II phosphorylation sequences (SEE, SDD, SED, or SDE [46]) which are not present in the human sequence. As shown in Fig. 2, both yeast and *Artemia* eIF-2α contain consensus casein kinase II phosphorylation sequences located in the carboxyl end of the protein. The yeast protein has two sites which match well to consensus sequences beginning with Ser-294 (SED) and Ser-301 (SDD) and a possible third sequence that begins with Ser-292 (SD) that could be phosphorylated by casein kinase II (46). The *Artemia* protein contains four consensus casein kinase II phosphorylation sequences beginning with Ser-304 (SEE), Ser-309 (SEE), Ser-313 (SDE), and Ser-328 (SEE). Aside from these consensus sequences being common to the carboxyl end of each protein, there is no apparent extended alignment of the amino acids in the yeast and *Artemia* consensus sequences (Fig. 2). In contrast, consensus casein kinase II phosphorylation sites are not present in the human eIF-2α protein (Fig. 2). These comparative data appear to match well with the in vitro casein kinase labeling data for the yeast, *Artemia*, and human proteins (22, 37) and could suggest that casein kinase II phosphorylation of yeast eIF-2α is related to the GCN2-independent phosphorylation of eIF-2α that we see in our in vivo labeling experiment (Fig. 1).

```
          1                                                  50
Yeast     MS-TSHCRFYENKYPEIDDIVMVNVQQIAEMGAYVKLLEYDNIEGMILLSE
Artemia   .P-L.-..Y.AE.F..TE.V...K.RS..D.....N....DD.........
Human     .PGL.-..F.QH.F..VE.V...N.RS..E.....S....NN.........

         51                                                   99
Yeast     LSRRRIRSIQKLIRVGKND-VAVVLRVDKEKGYIDLSKRRVSSEDIIKC-E
Artemia   .........N....I.KTEP.-..I....D............A.DID..T.
Human     .........N....I.RNEC.-..I....E............P.EAI..-.

        100                                                  141
Yeast     EKYQKSKTVHSILRYCAEKFQI----PLEELY-KTIAWPLSRKFGH----A
Artemia   -.FS.A.A.N....HV.FMLDYQTNEQ..E.YQK.-..LFDK.AKRQS-A.
Human     D.FT.S.T.Y....HV.EVLEYTKDEQ..S.FQR.-..VFDD.YKRPGYG.

        142                                                  182
Yeast     YEAFKLSIIDETVWEGIEPPS-KD--VLD------E-LKNYISKRLTPQAV
Artemia   .E...QAVK.----------..LL.ECG..--EKTK.T.L.Q.KQK......
Human     .D...HAVS.----------..IL.--S..LNEDER.V.I.N.NRR......

        183                                                  231
Yeast     KIRADVEVSCFSYEGIDAIKDALKSAED-MSTEQMQVKVKLVAAPLYVL-T
Artemia   ....YV.CS.YG....N.V.R..R-.GMSV...EIPI.IN.V.S.IF.IN.
Human     ....DI.VA.YG....D.V.E..R-.GLNC...NMPI.IN.I.P.RY.M-.

        232                                                  280
Yeast     -TQALDKQKGIEQLESAIEKITEVIT-KYGGVCNITMPPKAVTATEDAELQ
Artemia   H.--QEKED.MKA.TL.LKA.E.AAE-.E..TFRIKSA..V..K-E.-.AE
Human     -.TTLERTE.LSV.SQ.MAV.K.KIEE.R.-VFNVQME..V..DT-.-.TG

        281                                  304
Yeast     -AL-LESKELDNR----SD-SEDDEDESDDE
Artemia   H.RQM.LA.QESRQVAGD.-S.EE.--SEEGSDEEDDEKKENGVKESEEEEWGLA
Human     L.RQM.RL.RENAEVDGD.DA.EM.AKAED
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FIG. 2. Amino acid sequence alignment of eIF-2α proteins from yeast, *Artemia*, and human cells. The amino acid sequences of the yeast, *Artemia*, and human eIF-2α proteins were aligned from the methionine encoded by the AUG start codon in all three genes. The amino acid numbers presented pertain only to the yeast protein sequence. Identical amino acids in all three sequences are presented as boldface letters in the yeast sequence and followed by dots in the corresponding position of the *Artemia* and human proteins. Hyphens represent gaps in the sequences which were introduced to achieve the optimal alignment. The serine residues of the consensus casein kinase II sequences in the yeast and *Artemia* proteins are underlined.

Mutations of the casein kinase II phosphorylation sites abolish the constitutive phosphorylated form of eIF-2α. As a first attempt to determine if the consensus casein kinase II phosphorylation sequences that we observed in the carboxyl end of the *SUI2* coding region were relevant to the mechanism of phosphorylation of yeast eIF-2α, we constructed a *SUI2* allele which when expressed in yeast cells would be deleted of these consensus sequences. This allele was constructed by insertion of the *URA3* gene at a convenient restriction site in the carboxyl end of the *SUI2* gene. As a result of this insertion, the last 14 amino acids would not be contiguous with the *SUI2* gene inclusive of the three consensus casein kinase II sequences. This allele, *sui2-ΔCK3*, was substituted for the wild-type gene in yeast cells, and the ability to detect eIF-2α as a phosphoprotein was assessed after labeling of cells with $^{32}P_i$. The Western blot in Fig. 3A shows eIF-2α immunoprecipitated from a *SUI2*$^+$ strain (lane 1) and three different *sui2-ΔCK3* strains (lanes 2 to 4). Autoradiography of this Western blot (Fig. 3B) shows that eIF-2α obtained from the *SUI2*$^+$ strain is detected as a ^{32}P-labeled protein (lane 1), whereas eIF-2α derived from the *sui2-ΔCK3* strains is not detected as a ^{32}P-labeled protein (lanes 2 to 4). This finding suggests that either some or all of these consensus casein kinase II sequences are relevant phosphorylation sites in vivo.

To gain further insight into the importance of these se-

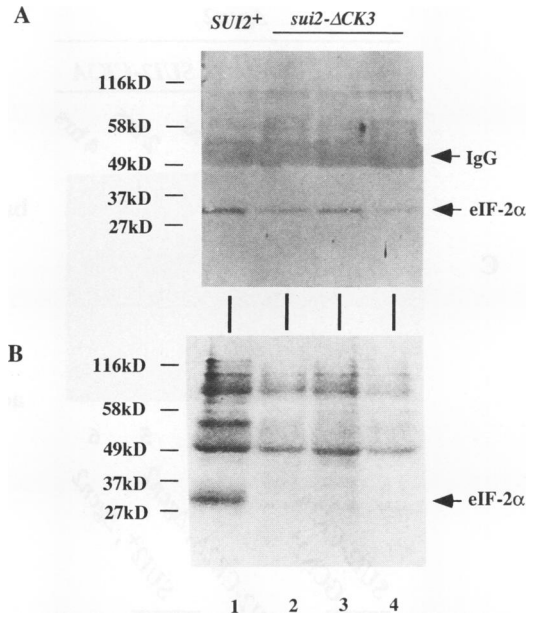
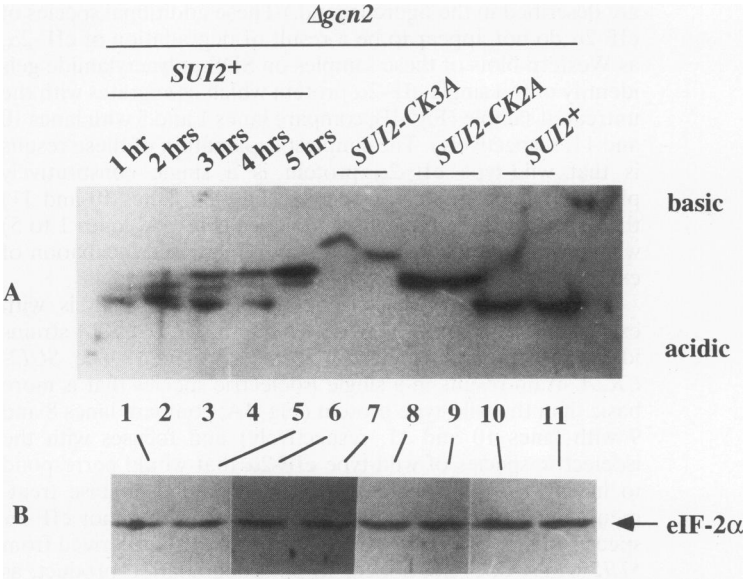


FIG. 3. Western blotting and $^{32}P_i$ labeling of eIF-2α from *SUI2*$^+$ and *sui2-ΔCK3* strains. Protein extracts were prepared from the *SUI2*$^+$ yeast strain 118-1D (lane 1) and the *sui2-ΔCK3* strains EP290 (lane 2), LF31-5A (lane 3), and LF32-1C (lane 4), which were grown in phosphate-depleted YEPD liquid medium in the presence of $^{32}P_i$. eIF-2α was immunoprecipitated with antiserum raised against a TrpE-SUI2 fusion protein and identified by Western blotting with the same antiserum (A). Western blots were then subject to autoradiography (B) to determine if eIF-2α was ^{32}P labeled. Arrows mark positions of the heavy chain of immunoglobulin G (IgG) from our polyclonal rabbit antiserum and eIF-2α as detected by Western blot (A) or eIF-2α as detected by autoradiography (B). The relative positions of standard protein molecular weight markers which were coelectrophoresed are noted for both the Western blot and the autoradiograph.

quences, we mutated the Ser codons which correspond to part of these consensus sites to Ala codons by site-directed mutagenesis. Two different *SUI2* alleles were constructed. The *SUI2-CK2A* allele contains Ser-to-Ala substitutions at amino acid positions 292 and 294 (Fig. 2) and is predicted to abolish phosphorylation at two of the three consensus sites. The *SUI2-CK3A* allele contains Ser-to-Ala substitutions at amino acid positions 292, 294, and 301 (Fig. 2) and is predicted to eliminate phosphorylation at all three consensus sites. Each allele was substituted for the wild-type copy of *SUI2* in a *GCN2* deletion strain, and the phosphorylated state of eIF-2α produced from these strains was assessed on IEF gels in comparison with eIF-2α produced from the parent strain before and after incubation with phosphatase. As shown in Fig. 4A, the *GCN2* deletion strain (Δ*gcn2*) containing the *SUI2*$^+$ gene produces a single isoelectric species of eIF-2α (lanes 10 and 11). Treatment of *SUI2*$^+$ Δ*gcn2* extracts with phosphatase for increasing periods of time results in three additional isoelectric species of eIF-2α detected by IEF (Fig. 4A, lanes 1 to 5) that are more basic than the species observed in the untreated samples (lanes 10 and 11). (The isoelectric species that corresponds to one phosphate removed after phosphatase treatment focuses closely to the most acidic wild-type species [Fig. 4A, lanes 1 and 2] and is difficult to detect under these phosphatase conditions. A better illustration of this species relative to eIF-2α produced from *SUI2-CK2A* is shown in Fig. 4D [compare lanes 3 and 4], using different phosphatase conditions that are described in the figure legend.) These additional species of eIF-2α do not appear to be a result of degradation of eIF-2α, as Western blots of these samples on SDS-polyacrylamide gels identify only a single eIF-2α protein which comigrates with the untreated sample (Fig. 4B; compare lanes 1 and 5 with lanes 10 and 11, respectively). The simplest explanation of these results is that wild-type eIF-2α protein is a single constitutively phosphorylated isoelectric species (Fig. 4A, lanes 10 and 11) that contains three phosphate moieties (Fig. 4A, lanes 1 to 5) which are sequentially removed upon increased incubation of extracts with phosphatase.

In contrast to these results, the identical analysis with extracts derived from the *SUI2-CK2A* and *SUI2-CK3A* strains identified different isoelectric forms of eIF-2α. The *SUI2-CK2A* strain results in a single isoelectric species that is more basic than the wild-type protein (Fig. 4A; compare lanes 8 and 9 with lanes 10 and 11, respectively) and focuses with the isoelectric species of wild-type eIF-2α that would correspond to having two phosphates removed after phosphatase treatment (Fig. 4A; compare lanes 8 and 9 with the minor eIF-2α species in lane 5). The isoelectric form of eIF-2α derived from *SUI2-CK2A* does not appear to be a degradation product, as Western blots of these samples on SDS-polyacrylamide gels identifies only a single eIF-2α protein that comigrates with the wild-type protein (Fig. 4B; compare lanes 8 and 9 with lanes 10 and 11, respectively). A simple interpretation of these results is that the Ser-to-Ala mutations at amino acid positions 292 and 294 in the *SUI2-CK2A* allele eliminate the ability of eIF-2α to be phosphorylated at each position, and hence it gives rise to an isoelectric form of eIF-2α with only one phosphate. This was further tested by treatment of the extract with phosphatase. As shown in Fig. 4C (lanes 1 to 3), phosphatase treatment of the extract derived from the *SUI2-CK2A* strain resulted in production of a single, more basic isoelectric form of eIF-2α, consistent with the notion that a single phosphate resides on this mutant form of the protein.

The identical analysis using a *SUI2-CK3A* Δ*gcn2* strain identifies a single isoelectric form of eIF-2α that is the most basic of all species resolved by IEF, even more basic than the most basic wild-type protein identified after extended phosphatase treatment (Fig. 4A; compare lanes 6 and 7 with lane 5). This isoelectric form of eIF-2α derived from *SUI2-CK3A* also appears not to be a degradation product, as Western blots of these samples on SDS-polyacrylamide gels identifies only a single eIF-2α protein that comigrates with the wild-type protein (Fig. 4B; compare lanes 6 and 7 with lanes 10 and 11, respectively). A simple interpretation of these data is that the Ser-to-Ala mutations at amino acid positions 292, 294, and 301 in the *SUI2-CK3A* allele eliminate the ability of eIF-2α to be phosphorylated at each position. This gives rise to an isoelectric form of eIF-2α with no phosphates. We therefore tested this interpretation by treating extracts prepared from a *SUI2-CK3A* strain with phosphatase and resolving the eIF-2α species by IEF. In contrast to the *SUI2-CK2A* analysis, phosphatase treatment of the extract derived from the *SUI2-CK3A* strain (Fig. 4C, lanes 4 to 6) does not produce an additional dephosphorylated species of eIF-2α. This finding indicates that the three mutations introduced abolish the ability of eIF-2α to be phosphorylated by a GCN2-independent mechanism. We do not understand why eIF-2α produced from *SUI2-CK3A* focuses aberrantly in comparison with fully dephosphorylated eIF-2α derived from either a *SUI2*$^+$ strain (Fig. 4A; compare lanes 6 and 7 with the major eIF-2α species in lane 5) or a *SUI2-CK2A* strain (Fig. 4C; compare lanes 4 to 6 with the more basic eIF-2α species in lanes 2 and 3, respectively). However, we assume that the aberrant focusing of eIF-2α derived from



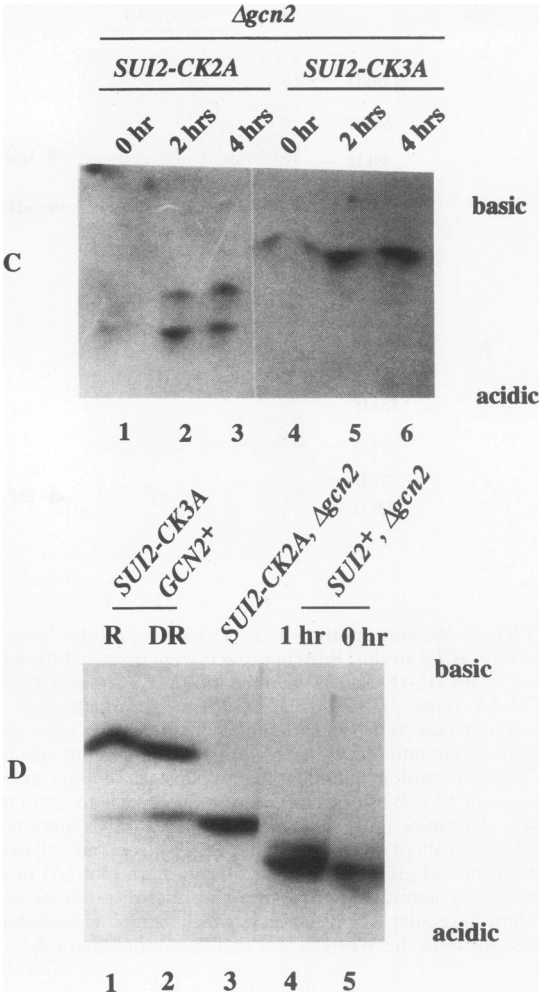


FIG. 4. IEF analysis of eIF-2α proteins from *SUI2*$^+$, *SUI2-CK2A*, and *SUI2-CK3A* yeast strains. Yeast cell extracts (20 μg) were analyzed by IEF on a vertical slab gel system. eIF-2α was detected by Western blotting with a polyclonal antiserum raised against a TrpE-SUI2 fusion protein. The basic and acidic ends of each gel are indicated. (A) Aliquots of protein extract prepared from yeast strain H1333 (Δ*gcn2 SUI2*$^+$) were treated with potato acid phosphatase for increasing lengths of time, as indicated, and resolved by IEF (lanes 1 to 5). The resulting isoelectric species of eIF-2α were compared with those from phosphatase-untreated extracts derived from the Δ*gcn2 SUI2-CK3A* yeast strains LF84 (lane 6) and LF86 (lane 7), the Δ*gcn2 SUI2-CK2A* yeast strains LF83 (lane 8) and LF85 (lane 9), and the Δ*gcn2 SUI2*$^+$ yeast strains LF81 (lane 10) and LF82 (lane 11). (B) Duplicate sets of phosphatase-treated (lanes 1 and 5) and untreated (lanes 6 to 11) extracts shown in panel A were analyzed by Western blotting using SDS-polyacrylamide gels. The position of eIF-2α is indicated with an arrow. (C) Protein extracts were prepared from the Δ*gcn2 SUI2-CK2A* strain LF83 (lanes 1 to 3) and the Δ*gcn2 SUI2-CK3A* strain LF84 (lanes 4 to 6), and eIF-2α was resolved by IEF before (0 h) and after treatment with phosphatase for increasing lengths of time (2 h and 4 h). (D) The *GCN2*$^+$ *SUI2-CK3A* yeast strain LF95 was grown in either SD medium (lane 1, R [repressed]) or SD medium containing 3-AT (lane 2, DR [derepressed]). Whole cell extracts were prepared, and eIF-2α was resolved by IEF and compared with eIF-2α derived from extracts of yeast strain LF83 (Δ*gcn2 SUI2-CK2A*) and strain H1333 (Δ*gcn2 SUI2*$^+$) before and after treatment with phosphatase (lane 3 and lanes 5 and 4, respectively). For the phosphatase-treated sample shown in lane 4, the phosphatase inhibitors sodium orthovanadate (0.1 mM) and β-glycerophosphate (50 mM) were added to the extract. This allowed us to significantly decrease the rate of eIF-2α dephosphorylation by the phosphatase and more clearly resolve the isoelectric form of eIF-2α that corresponds to removal of one phosphate. The basis for this experiment is that this isoelectric species was not clearly resolved by using alternative phosphatase conditions (see Fig. 4A, lanes 1 to 5).

SUI2-CK3A is due to some difference between having Ala as opposed to Ser at these positions and not some proteolytic form of the protein that is not detectable on SDS-polyacrylamide gels. The basis for this assumption is that if we perform IEF analysis with extracts derived from a *SUI2-CK3A GCN2*$^+$ strain before and after induction of the GCN2 kinase, we can identify the Ser-51-phosphorylated form of this mutant eIF-2α protein (Fig. 4D, lanes 1 and 2). This protein focuses at the same relative position as that of eIF-2α derived from a *SUI2-CK2A* Δ*gcn2* strain which also represents eIF-2α that contains a single phosphate moiety (Fig. 4D, lane 3).

Conditional casein kinase II mutants alter eIF-2α phosphorylation in vivo. Our mutational studies suggest that eIF-2α is constitutively phosphorylated at three Ser residues in the carboxyl end of the protein. In light of the fact that these positions correspond to parts of consensus casein kinase II phosphorylation sites, we tested whether alterations in the expression or activity of casein kinase II would alter the ability to detect wild-type eIF-2α as a phosphoprotein. It has been demonstrated that the α catalytic subunit of yeast casein kinase II is encoded by two duplicated genes, *CKA1* and *CKA2* (14, 43). The amino acid sequences of these two genes are distinct, but the genes are functionally interchangeable (14, 43, 44). Either one of the two genes when expressed in yeast cells can fully support cell growth, but lethality occurs when both genes are deleted. Two different conditional mutants of casein kinase II activity were used in this analysis. One mutant, JC35-1a, contains null alleles of the chromosomal genes, *CKA1* and *CKA2*, and a plasmid containing the *GAL1* promoter inserted upstream of the *CKA1* coding region (43). This allows *CKA1* expression to be turned off by switching yeast from galactose to glucose as a carbon source. The second mutant strain we used, YDH8, contains null alleles of the chromosomal *CKA1* and *CKA2* genes and a plasmid which contains a mutated allele of the *CKA2* gene (*cka2*^{ts}) that confers a temperature-sensitive growth defect (9, 27a). Therefore, casein kinase II activity can

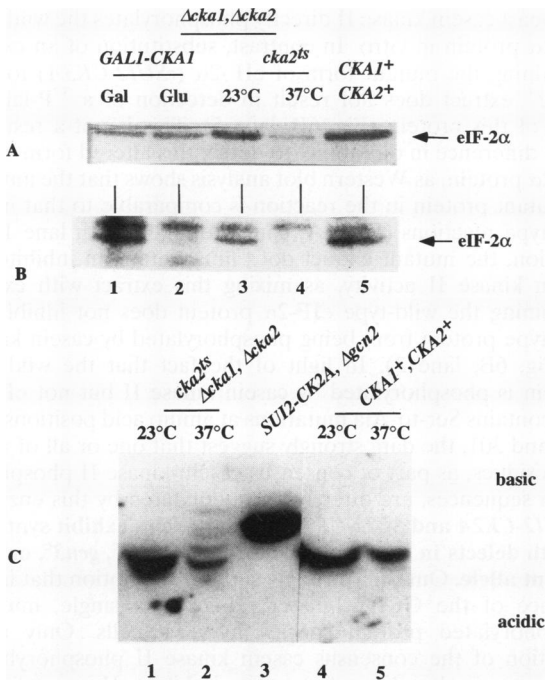


FIG. 5. $^{32}P_i$ labeling and IEF analysis of eIF-2α from conditional casein kinase II mutant strains. Five-milliliter aliquots of yeast strain JC35-1a (*GAL1-CKA1*) either grown in phosphate-depleted YEPGal medium (lane 1, Gal) or after having been shifted to phosphate-depleted YEPD medium (lane 2, Glu) were incubated in the presence of $^{32}P_i$. Five-milliliter aliquots of yeast strain YDH8 (*cka2^{ts}*) grown in phosphate-depleted YEPD medium either at the permissive temperature, 23°C (lane 3), or after having been shifted to the restrictive temperature, 37°C (lane 4), were incubated in the presence of $^{32}P_i$. Whole cell extracts were prepared, and eIF-2α was immunoprecipitated with polyclonal antiserum against yeast eIF-2α and detected by Western blotting with the same antiserum (A). Western blots were then subjected to autoradiography (B) to determine if eIF-2α was ^{32}P labeled. Lane 5, ^{32}P labeling experiment using the wild-type yeast strain 118-1D. The positions of eIF-2α as detected by Western blotting and autoradiography are indicated with arrows. The upper band observed by autoradiography represents a contaminating ^{32}P-labeled protein species that does not correspond to the α subunit of eIF-2 as indicated by our preimmune control (Fig. 1, lane 3). In addition, this band is not always observed in these in vivo labeling experiments (see Fig. 3, lane 1). (C) Protein extracts were prepared from yeast strain YDH8 (*cka2^{ts}*) either grown at the permissive temperature, 23°C (lane 1), or after having been shifted to the nonpermissive temperature, 37°C (lane 2), and eIF-2α was analyzed by IEF. The basic and acidic ends of the IEF gel are noted. Lane 3, extract prepared from yeast strain LF83 (*Δgcn2 SUI2-CK2A*). Lanes 4 and 5, extract prepared from the wild-type yeast strain 118-1D grown at 23 and 37°C, respectively.

be reduced in vivo by shifting this strain to the nonpermissive growth temperature.

Two different assays were used to determine the effects of altering the expression of the α subunit of casein kinase II or its activity on eIF-2α phosphorylation. The first assay determined the ability of eIF-2α to be labeled with $^{32}P_i$ after each of the conditional casein kinase II mutants was shifted to the nonpermissive growth conditions. As shown in Fig. 5A, Western blot analysis of eIF-2α shows that the levels of this protein are comparable when immunoprecipitated from extracts derived from the *GAL1-CKA1* strain either grown in galactose or shifted to glucose medium (lanes 1 and 2). Autoradiography of the Western blot shows that eIF-2α obtained from the cells grown in galactose medium is readily detected as a ^{32}P-labeled protein (Fig. 5B, lane 1). In contrast, eIF-2α obtained from cells which had been shifted to glucose medium was not readily detected as a ^{32}P-labeled protein (Fig. 5B, lane 2). Thus, reducing the level of *CKA1* expression and presumably the level of casein kinase II in the cell correlates with reduced phosphorylation of eIF-2α. A similar result was obtained for the *cka2^{ts}* strain. eIF-2α is readily detected as a ^{32}P-labeled protein when this strain is grown at the permissive temperature but is not readily detected as a ^{32}P-labeled protein after this strain has been shifted to the restrictive temperature (Fig. 5B, lanes 3 and 4, respectively). However, this experiment is less compelling, as the level of eIF-2α protein observed by Western blotting is decreased after the temperature shift (Fig. 5A; compare lane 3 with lane 4).

To gain better insight into the effects of the *cka2^{ts}* mutant on eIF-2α phosphorylation, we used a second assay. IEF gels were used to identify the isoelectric species of eIF-2α in extracts of *cka2^{ts}* cells grown at the permissive temperature or after the shift to the nonpermissive temperature. As shown in Fig. 5C, the *cka2^{ts}* strain grown at the permissive temperature produces a single isoelectric species of eIF-2α that focuses at the same position as eIF-2α derived from a *CKA1$^+$ CKA2$^+$* wild-type strain (compare lane 1 with lane 4). In contrast, shifting the *cka2^{ts}* strain to the nonpermissive growth condition results in multiple isoelectric species of eIF-2α (Fig. 5C, lane 2). Aside from the major acidic species observed with the wild-type strain, we detect a number of basic isoelectric species of eIF-2α that are not apparent in an extract of *cka2^{ts}* grown at the permissive temperature or in an extract derived from a *CKA1$^+$ CKA2$^+$* strain grown at 37°C (Fig. 5C, lanes 1 and 5, respectively). The relative focusing pattern of these basic species closely resemble the relative pattern of species obtained after phosphatase treatment of extracts from *SUI2$^+$* strain (Fig. 4A, lanes 1 to 5) and focus at a relative position similar to that of SUI2-CK2A (Fig. 5C; compare lanes 2 and 3). A simple interpretation of these data is that after the temperature shift, casein kinase II activity is inactivated in the *cka2^{ts}* mutant. This leads to either newly synthesized eIF-2α protein being inefficiently phosphorylated and/or eIF-2α synthesized prior to the temperature shift being dephosphorylated by phosphatases in vivo to generate the collection of basic isoelectric species observed in Fig. 5C (lane 2). Therefore, the *GAL1-CKA1* and *cka2^{ts}* studies strongly support the notion that alterations in the expression of casein kinase II or changes in its in vivo activity modify the phosphorylation state of eIF-2α. These studies complement our mutational studies of the Ser residues in the consensus casein kinase II sequences and collectively suggest that casein kinase II either phosphorylates eIF-2α at multiple sites in the protein or mediates the ability of eIF-2α to be multiply phosphorylated perhaps by activating other kinases or inhibiting phosphatase activities in vivo.

Recombinant eIF-2α is phosphorylated directly by casein kinase II in vitro. To gain direct evidence that the yeast casein kinase II enzyme will phosphorylate yeast eIF-2α at the proposed consensus sequences, we incubated yeast casein kinase II with recombinant forms of eIF-2α protein synthesized in *E. coli*. Two different recombinant forms of eIF-2α were assayed for the ability to be phosphorylated by casein kinase II: eIF-2α synthesized from an expression vector containing the intact coding region from the wild-type *SUI2* gene, and eIF-2α synthesized from an expression vector containing the intact coding region from the *SUI2-CK3A* allele. For these experiments, extracts prepared from the corresponding *E. coli* strains were incubated with casein kinase II and [γ-^{32}P]ATP. eIF-2α was then immunoprecipitated from the reaction mix-

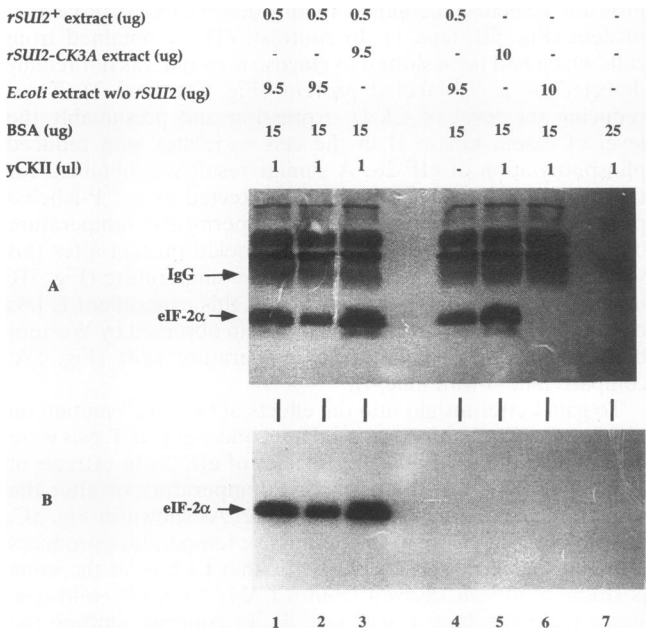


FIG. 6. In vitro casein kinase II phosphorylation assay. The *SUI2*$^+$ and *SUI2-CK3A* gene products were overexpressed in *E. coli* JA200 (Δ*trpE*) as part of a bacteriophage λ expression vector. The soluble fraction of bacterial extracts was incubated with yeast casein kinase II (yCKII; approximately 40 ng of total protein) and [γ-^{32}P]ATP in the presence of BSA. eIF-2α was immunoprecipitated from the reaction mixtures by using polyclonal antiserum directed against a TrpE-SUI2 fusion and identified by Western blotting with the same antiserum (A). Western blots were then subjected to autoradiography (B) to determine if eIF-2α was ^{32}P labeled. Because of differences in the construction of the *SUI2-CK3A* and *SUI2*$^+$ expression vectors (see Materials and Methods), the amount of eIF-2α protein in the *rSUI2-CK3A* extract was significantly less than that of eIF-2α protein in the *rSUI2*$^+$ extract as determined by Western blotting. Therefore, 10 μg of total protein from the *rSUI2-CK3A* extract (lane 5) and 0.5 μg of total protein from the *rSUI2*$^+$ extract (lane 1) were added to the respective reaction mixtures to provide equivalent amounts of eIF-2α as a potential substrate for casein kinase II. To compensate for the difference in the amount of protein added, 9.5 μg of a soluble extract from *E. coli* JA200 without an *rSUI2* construct was added to the wild-type reaction (lane 1). The positions of eIF-2α as detected by Western blotting and autoradiography are indicated with arrows. Lane 2, same as lane 1 but only one-third of the sample was loaded on the SDS-polyacrylamide gel; lane 3, labeling reaction containing both *rSUI2*$^+$ (0.5 μg of total protein) and *rSUI2-CK3A* (9.5 μg of total protein) extracts; lanes 4, 6, and 7, control reactions lacking casein kinase II, *rSUI2* protein, and *E. coli* extract, respectively.

tures with anti-SUI2 antiserum and identified by Western blotting (Fig. 6A). Western blots were then exposed to X-ray film to determine if eIF-2α was labeled with ^{32}P (Fig. 6B).

As shown in Fig. 6, incubation of extracts containing wild-type eIF-2α (*rSUI2*$^+$) protein with casein kinase II results in the ability to detect a single ^{32}P-labeled protein species (Fig. 6B, lanes 1 and 2) that comigrates with eIF-2α as detected by Western blot analysis (Fig. 6A, lanes 1 and 2). Identical reaction mixtures that lack casein kinase II or contain extract from the *E. coli* parent strain lacking the *SUI2*$^+$ expression vector do not result in the identification of a ^{32}P-labeled protein (Fig. 6B, lanes 4 and 6). These data demonstrate that the ability to detect this ^{32}P-protein is dependent not only on yeast casein kinase II but also on the presence of the recombinant eIF-2α in the *E. coli* extract. This finding establishes that yeast casein kinase II directly phosphorylates the wild-type eIF-2α protein in vitro. In contrast, substitution of an extract containing the mutant form of eIF-2α (*rSUI2-CK3A*) for the *rSUI2*$^+$ extract does not result in detection of a ^{32}P-labeled form of this protein (Fig. 6B, lane 5). This is not a result of some difference in our ability to detect this altered form of the eIF-2α protein, as Western blot analysis shows that the amount of mutant protein in the reaction is comparable to that in the wild-type reactions (Fig. 6A; compare lane 5 with lane 1). In addition, the mutant extract does not contain an inhibitor of casein kinase II activity, as mixing this extract with extract containing the wild-type eIF-2α protein does not inhibit the wild-type protein from being phosphorylated by casein kinase II (Fig. 6B, lane 3). In light of the fact that the wild-type protein is phosphorylated by casein kinase II but not eIF-2α that contains Ser-to-Ala mutations at amino acid positions 292, 294, and 301, the data strongly suggest that one or all of these Ser residues, as part of consensus casein kinase II phosphorylation sequences, are directly phosphorylated by this enzyme.

***SUI2-CK2A* and *SUI2-CK3A* mutant alleles exhibit synthetic growth defects in combination with the *GCN2*^{c}, *gcn3*^{c}, or *gcd7* mutant allele.** Our data strongly support the notion that in the absence of the GCN2 kinase, eIF-2α is a single, multiply phosphorylated protein species in yeast cells. Only upon mutation of the consensus casein kinase II phosphorylation sequences or alteration of yeast casein kinase II expression or activity are alternative isoelectric forms of this protein detected in vivo that correspond to dephosphorylated forms of the protein. An important question to address is whether this constitutively phosphorylated form of eIF-2α is of any physiological or biochemical importance to eIF-2 function in yeast cells.

As an attempt to gain some initial insight into the physiological significance of these phosphorylation events, we assayed a number of properties of the mutant *SUI2-CK2A* and *SUI2-CK3A* strains. The assays that we used were based on observations made previously with other types of mutations in the *SUI2* gene. As previously mentioned, the *SUI2* gene in yeast cells was originally identified on the basis of its ability to suppress a non-AUG start codon in the *HIS4* coding region (12, 17). Aside from suppression of a non-AUG start codon, a number of *sui2* suppressor genes also confer poor growth properties and elicit the general amino acid control response (12, 61). We therefore tested the *SUI2-CK2A* and *SUI2-CK3A* strains for similar types of properties. As shown in Fig. 7, *SUI2-CK2A* and *SUI2-CK3A* strains do not demonstrate any defect in growth properties compared with a *SUI2* wild-type strain (compare *SUI2-CK2A* and *SUI2-CK3A* with the wild type). In addition, these strains do not suppress a non-AUG codon mutation at *HIS4*, nor do they alter the general amino acid control response as determined by growth on 3-AT and 5-FT media (data not shown).

Another property of *sui2* suppressor mutants is that when analyzed by tetrad analysis in crosses with *sui1* or *SUI3* suppressor strains, haploid meiotic products were found to be lethal when *sui2* mutant alleles were present in combination with either of the two other suppressor alleles (12). As previously mentioned, the *sui1* and *SUI3* genes were also identified as suppressors of non-AUG codons. The *SUI3* gene product encodes the β subunit of eIF-2 (25). The *sui1* gene product, whose specific function has not yet been identified, has been implicated genetically to be involved in the start site selection process and presumably functions at this step in conjunction with eIF-2 (63). In light of the related functions, suppressor mutations in either of two of these three genes must result in defective translation initiation in haploid yeast that

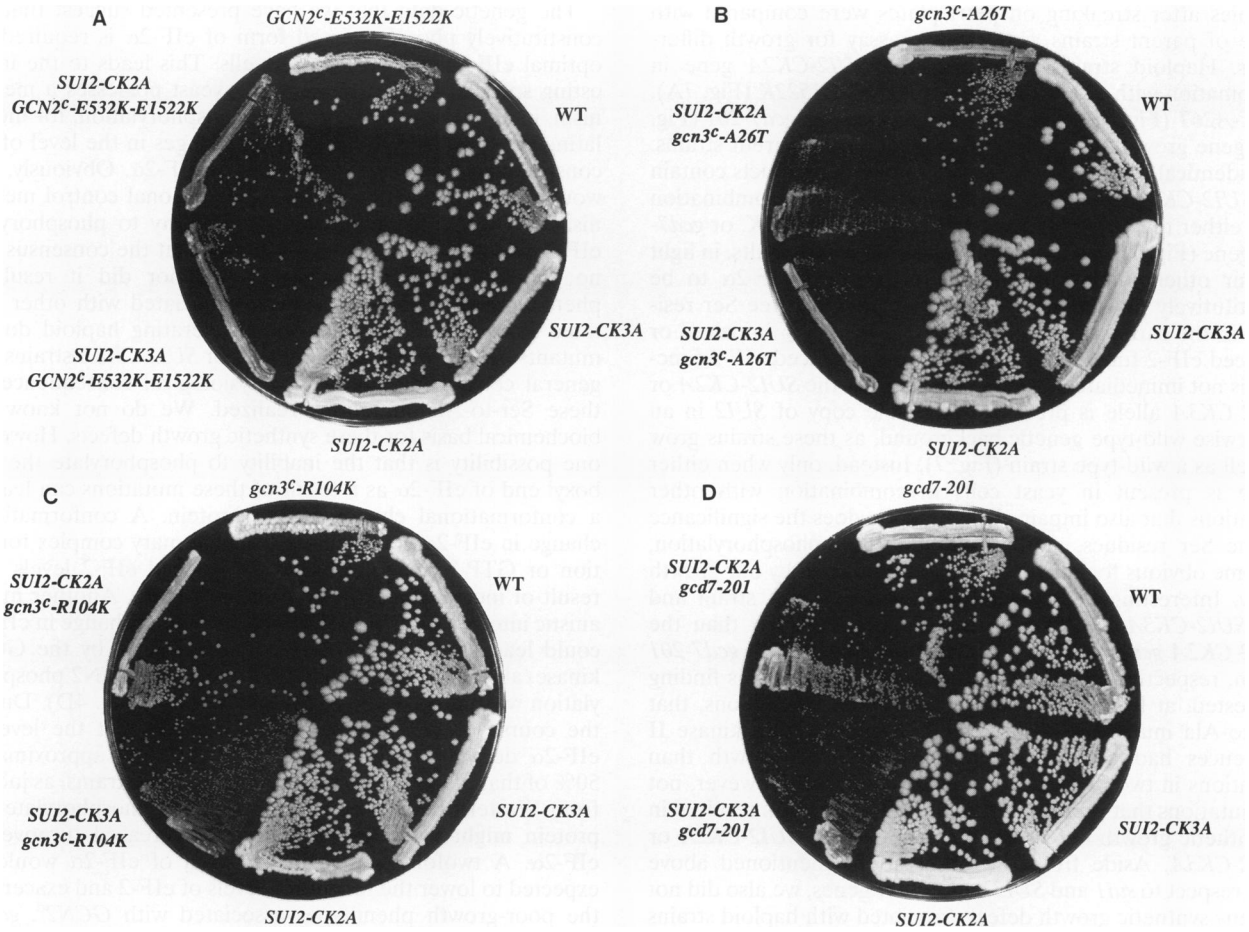


FIG. 7. Growth assay. Yeast strains LF94 and LF95, containing the *SUI2-CK2A* and *SUI2-CK3A* alleles, respectively, were each crossed to the general amino acid control mutants H1613 (*GCN2c-E532K-E1522K*), H1491 (*gcn3c-A26T*), H1489 (*gcn3c-R104K*), and H1725 (*gcd7-201*) and analyzed by tetrad analysis. Haploid meiotic products containing the *SUI2-CK2A* gene and the *SUI2-CK3A* gene in combination with each of the general control mutant alleles, *SUI2-CK2A GCN2c-E532K-E1522K* and *SUI2-CK3A GCN2c-E532K-E1522K* (A), *SUI2-CK2A gcn3c-A26T* and *SUI2-CK3A gcn3c-A26T* (B), *SUI2-CK2A gcn3c-R104K* and *SUI2-CK3A gcn3c-R104K* (C), and *SUI2-CK2A gcd7-201* and *SUI2-CK3A gcd7-201*. (D) were identified and streaked on YEPD medium. The colony sizes of these double mutants are compared with those of the wild-type (WT) yeast strain H1402 and the parent strains used for the crosses LF94, LF95, H1613, H1491, H1489, and H1725 after 3 days of incubation at 30°C.

results in cell lethality. To determine if the mutations in *SUI2-CK2A* and *SUI2-CK3A* strains conferred impaired translation initiation properties in combination with either *sui1* or *SUI3* suppressor mutations, we crossed these strains and analyzed the meiotic products for apparent growth defects. In summary, we did not detect a synthetic lethal phenotype or a synthetic growth phenotype associated with haploid meiotic products deduced to contain either of these mutant *SUI2* alleles in combination with the *sui1* or *SUI3* suppressor gene (data not shown). In addition, there was no apparent alteration in the ability of the *sui1* or *SUI3* suppressor gene to suppress a non-AUG start codon at *his4* when *SUI2-CK2A* or *SUI2-CK3A* was the sole copy of *SUI2* in these haploid strains (data not shown).

The last test that we used to determine whether mutations in the casein kinase II sequences had any effect on eIF-2 function in yeast cells was based on other genetic observations that have been made with *SUI2* mutations in combination with mutated genes identified as regulators of the general amino acid control system. One observation concerns suppressive effects of *SUI2* mutations when in combination with specific *GCN2* mutant alleles. A number of GCN2 kinase mutants have been characterized for the ability to hyperphosphorylate the Ser-51 position of eIF-2α in yeast cells. These *GCN2c* alleles can confer severe growth defects, as hyperphosphorylation of eIF-2α at Ser-51 leads to down-regulation of global protein synthesis. Therefore, a Ser-to-Ala mutation at Ser-51 in the *SUI2* gene (21), as well as other types of selected mutations in *SUI2* (62), is capable of suppressing this severe growth defect by either preventing GCN2 kinase phosphorylation of eIF-2α or negating the ability of the Ser-51-phosphorylated form of eIF-2α to interact with eIF-2B. Conversely, a *sui2* suppressor mutation has also been shown to confer a synthetic lethal or growth defect when present in a haploid strain with a *GCN3* mutation (61). This latter gene is believed to encode the regulatory subunit of eIF-2B (28, 62).

We therefore crossed a *SUI2-CK2A* strain and a *SUI2-CK3A* strain to yeast strains containing either mutated forms of the GCN2 kinase gene, *GCN2c-E532K*, *GCN2c-E752K*, and *GCN2c-E532K-E1522K*, or mutated forms of subunits of eIF-2B, *gcn3c-A26T*, *gcn3c-R104K*, and *gcd7-201*. Meiotic products containing the *SUI2-CK2A* or *SUI2-CK3A* allele in combination with each of the general control mutant alleles were identified. The abilities of these haploid strains to form

colonies after streaking on petri plates were compared with those of parent strains as a relative assay for growth differences. Haploid strains containing the *SUI2-CK2A* gene in combination with either the *GCN2^c-E532K-E1522K* (Fig. 7A), *gcn3^c-A26T* (Fig. 7B), *gcn3^c-R104K* (Fig. 7C), or *gcd7-201* (Fig. 7D) gene grow more poorly than either of the parent strains. The identical result is obtained when meiotic products contain the *SUI2-CK3A* allele as the sole copy of *SUI2* in combination with either the *GCN2^c-E532K-E1522K*, *gcn3^c-R104K*, or *gcd7-201* gene (Fig. 7). A simple explanation of these results, in light of our other studies, is that the inability of eIF-2α to be constitutively phosphorylated at either two or three Ser residues in the carboxyl end of the protein results in impaired or reduced eIF-2 function. This impaired or reduced eIF-2 function is not immediately obvious when either the *SUI2-CK2A* or *SUI2-CK3A* allele is present as the sole copy of *SUI2* in an otherwise wild-type genetic background, as these strains grow as well as a wild-type strain (Fig. 7). Instead, only when either allele is present in yeast cells in combination with other mutations that also impair eIF-2 function does the significance of the Ser residues, and presumably their phosphorylation, become obvious for eIF-2 function as measured by our growth assay. Interestingly, the *SUI2-CK3A gcn3^c-R104K* strain and the *SUI2-CK3A gcd7-201* strain grew more poorly than the *SUI2-CK2A gcn3^c-R104K* strain and the *SUI2-CK2A gcd7-201* strain, respectively (Fig. 7C and D, respectively). This finding suggested, at least for these mutant gene combinations, that Ser-to-Ala mutations at all three consensus casein kinase II sequences had a more deleterious effect on growth than mutations in two of these consensus sequences. However, not all mutations that presumably impair eIF-2 function resulted in a synthetic growth defect in combination with *SUI2-CK2A* or *SUI2-CK3A*. Aside from the observations mentioned above with respect to *sui1* and *SUI3* suppressor genes, we also did not see any synthetic growth defect associated with haploid strains containing either the *SUI2-CK2A* or the *SUI2-CK3A* allele in combination with either the *GCN2^c-E532K* or the *GCN2^c-E752K* mutant allele (data not shown).

DISCUSSION

We have characterized yeast eIF-2α as multiply phosphorylated by a GCN2-independent mechanism. Three different criteria have been used to establish that this phosphorylation of eIF-2α is mediated by casein kinase II. (i) Ser-to-Ala mutations in either of two or all three of the Ser residues that compose the three casein kinase II consensus sequences in *SUI2* result in altered isoelectric forms of eIF-2α. These data, in light of the IEF analysis of phosphatase-treated *SUI2$^+$* extracts, are consistent with each of these three Ser residues being phosphorylated in vivo and potential target sites for the casein kinase II enzyme. (ii) Conditional casein kinase II mutants give rise to more basic isoelectric species of eIF-2α or lose the ability to phosphorylate eIF-2α after being shifted to restrictive growth conditions. This result is consistent with eIF-2α being an in vivo substrate for casein kinase II. (iii) Purified yeast casein kinase II will phosphorylate wild-type recombinant eIF-2α in vitro but will not phosphorylate recombinant eIF-2α that contains Ser-to-Ala substitutions at the three casein kinase II consensus sequences. Although we have not established that each of the three Ser amino acids is directly phosphorylated by casein kinase II in vitro, these data complement our in vivo studies and strongly suggest that the constitutively phosphorylated eIF-2α species that we detect in yeast cells, at least in part, is a direct consequence of casein kinase II phosphorylation.

The genetic data that we have presented suggest that the constitutively phosphorylated form of eIF-2α is required for optimal eIF-2 function in yeast cells. This leads to the interesting speculation that *S. cerevisiae* yeast possesses a mechanism, in addition to GCN2 kinase phosphorylation, for modulating the level of eIF-2 activity by changes in the level of the constitutively phosphorylated form of eIF-2α. Obviously, this would not appear to be a major translational control mechanism, as we have shown that the inability to phosphorylate eIF-2α as a result of Ser-to-Ala changes at the consensus had no detectable effect on cell growth, nor did it result in phenotypes that have been found associated with other mutated forms of *SUI2*. Only upon generating haploid double mutants between either *SUI2-CK2A* or *SUI2-CK3A* strains and general control mutants were physiological consequences of these Ser-to-Ala mutations realized. We do not know the biochemical basis for these synthetic growth defects. However, one possibility is that the inability to phosphorylate the carboxyl end of eIF-2α as a result of these mutations can lead to a conformational change in the protein. A conformational change in eIF-2α could alter efficient ternary complex formation or GTP exchange or lead to reduced eIF-2 levels as a result of increased turnover of the α subunit. Another mechanistic interpretation is that a conformational change in eIF-2α could lead to hyperphosphorylation of Ser-51 by the GCN2 kinase, although this was not apparent when GCN2 phosphorylation was induced in a *SUI2-CK3A* strain (Fig. 4D). During the course of our analysis, we did notice that the level of eIF-2α derived from *SUI2-CK3A* strains was approximately 50% of that of eIF-2α derived from wild-type strains, as judged from Western blots. Thus, the inability to phosphorylate this protein might result in instability or increased turnover of eIF-2α. A twofold change in the level of eIF-2α would be expected to lower the functional levels of eIF-2 and exacerbate the poor-growth phenotypes associated with *GCN2^c*, *gcn3^c*, and *gcd7* mutations that are conferred by inhibition of eIF-2 function in yeast cells (8, 28, 47). However, the significance of this observation is unclear, as the level of eIF-2α from *SUI2-CK2A* strains did not appear to be reduced relative to eIF-2α produced from a wild-type strain. Nevertheless, the *SUI2-CK2A* allele also resulted in a synthetic growth defect when combined with these general control mutations (Fig. 7).

Alternatively, the genetic data could represent an allele- or gene-specific effect that would imply a specific mechanistic consequence for the inability to phosphorylate eIF-2α by casein kinase II. The general control mutants for which synthetic growth defects were observed in combination with *SUI2-CK2A* and *SUI2-CK3A* all have in common an expected inhibitory effect on eIF-2B activity that mediates the eIF-2 nucleotide exchange reaction. Therefore, the inability to phosphorylate eIF-2α might contribute further to this inhibition. One possibility is that this is an indirect effect. A conformational change in eIF-2α might reduce its interaction with eIF-2B and lower the efficiency of eIF-2B-mediated exchange. Alternatively, the phosphorylated carboxyl region of eIF-2α normally interacts with eIF-2B during the exchange reaction, and the dephosphorylated form of the protein does not fully support an interaction. Although this latter point cannot be ruled out, recent suppression studies of *GCN2^c* strains have suggested that a conserved region of the eIF-2α protein sequence immediately downstream from Ser-51 is the relevant region of eIF-2α that interacts with eIF-2B (62). Future biochemical studies would prove useful in clarifying these mechanistic interpretations. However, it is important to emphasize that exacerbation of the very poor growth properties associated with the *GCN2^c*, *gcn3^c*, and *gcd7* mutants that we

used in this analysis may prove to be the most reliable and sensitive test for detecting modest alterations in eIF-2 function, especially as it relates to identifying significant and physiological relevant effects for these mutations in eIF-2α.

It is important to note that whereas yeast eIF-2α possesses casein kinase II consensus sequences and serves as a substrate for casein kinase II phosphorylation, human eIF-2α lacks such consensus sequences (Fig. 2) and is not phosphorylated by the mammalian casein kinase II enzyme (37). Thus, casein kinase II phosphorylation of eIF-2α appears to occur only in *S. cerevisiae*, and probably *Artemia* sp., which also contains consensus casein kinase II sequences in the carboxyl end of the protein and is also phosphorylated by this enzyme in vitro (22, 37). In contrast, the mechanism of Ser-51 phosphorylation by eIF-2α kinases is conserved in all eukaryotic organisms (15, 20). However, as previously mentioned, despite this mechanistic conservation, phosphorylation of Ser-51 in mammalian cells appears to be directed at down-regulation or inhibition of global protein synthesis in response to different stress conditions, whereas in yeast cells, its primary function is gene-specific translational regulation of *GCN4* expression. The net effect in yeast cells is to up-regulate amino acid biosynthesis in response to amino acid starvation. Perhaps under severe stress conditions when growth of yeast cannot be adjusted by changes in *GCN4* expression, alterations in casein kinase II activity play a role in decreasing eIF-2 function and contribute to inhibition of global protein synthesis. In fact, changes in the phosphorylation state of yeast eIF-2α have been monitored previously under a number of adverse growth conditions (48). One condition, severe carbon source starvation, was associated with a gross change in the phosphorylated state of eIF-2α that would be consistent with loss or a reduction of casein kinase II phosphorylation of eIF-2α. However, other stress conditions such as heat shock, poor carbon source, and nitrogen starvation did not result in a change in the phosphorylation state of eIF-2α. These latter studies, however, may be limited, as the strain used for these analyses was $GCN2^+$, and therefore it was not clear if eIF-2α phosphorylation at Ser-51 could have been induced under some of these growth conditions.

Although casein kinase II would appear not to modify human eIF-2α, it has been implicated to modify other mammalian translation initiation factors, namely, the β subunit of eIF-2 and the 83-kDa subunit of eIF-2B, ε (18, 23, 24). In addition, the modification of the ε subunit of eIF-2B by casein kinase II results in an increase in eIF-2B activity. Conversely, dephosphorylation reduced eIF-2B activity by a factor of 5. Thus, there is precedent not only that casein kinase II can utilize translation factors as in vitro substrates but also that the absence of such modifications can lead to lowered activity. Our data, however, are unique in that they establish that eIF-2α is an in vivo substrate and that there is a potential physiological consequence for translation associated with the unphosphorylated form of the protein.

Casein kinase II is a Ser/Thr kinase, and a wide variety of substrates that function in transcription, translation, cell cycle control, metabolism, and cytoskeleton structure have been identified in mammalian cells (reviewed in references 10 and 46). Its involvement in cell cycle regulation is suggested by the fact that its activity is stimulated in transformed cells or in response to growth factors, and a number of cell cycle-related or growth-promoting proteins have been found to be modified by casein kinase II or to modify casein kinase II activity (2, 10, 36, 41, 46, 52, 57). In contrast, little is known about the regulation of casein kinase II activity or its potential substrates in yeast cells. Duplicated genes, *CKA1* and *CKA2*, encoding the α catalytic subunit of casein kinase II have been isolated, and deletion of both copies results in cell inviability, indicating that casein kinase II phosphorylation is essential for cell growth (14, 43). Cells deprived of both *CKA1* and *CKA2* have been observed to become enlarged and exhibit a pseudomycelial morphology (43), suggesting a cell cycle-related function for casein kinase II in yeast cells. Aside from eIF-2α as presented here, topoisomerase II is the only other natural substrate for casein kinase II that has been identified in yeast cells (9). Interestingly, the modification also occurs at the extreme C terminus, similar to the case for eIF-2α. If casein kinase II activity is regulated in a cell cycle-dependent manner in yeast cells, then cell cycle arrest would represent a stress condition whereby global protein synthesis needs to be turned off. It would be important to determine if other translation factors such as the yeast counterpart of the mammalian ε subunit of eIF-2B, as mentioned above, are also phosphorylated by casein kinase II and whether alterations in their activity can affect eIF-2B function in yeast cells. It is conceivable that global protein synthesis in yeast cells may be down-regulated under extreme stress conditions by a concerted change in the phosphorylation and subsequent activity of a number of translation factors. The identification of casein kinase II-mediated phosphorylation of eIF-2α could shed some light on such a translational control mechanism aside from determining the physiological relevance of casein kinase II phosphorylation in yeast cells.

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