A Mammalian Homologue of GCN2 Protein Kinase Important for Translational Control by Phosphorylation of Eukaryotic Initiation Factor-2α

Ruchira Sood,* Amy C. Porter,* DeAnne Olsen,† Douglas R. Cavener† and Ronald C. Wek*

**Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202 and †Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235*

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ABSTRACT

A family of protein kinases regulates translation in response to different cellular stresses by phosphorylation of the α subunit of eukaryotic initiation factor-2 (eIF-2α). In yeast, an eIF-2α kinase, GCN2, functions in translational control in response to amino acid starvation. It is thought that uncharged tRNA that accumulates during amino acid limitation binds to sequences in GCN2 homologous to histidyl-tRNA synthetase (HisRS) enzymes, leading to enhanced kinase catalytic activity. Given that starvation for amino acids also stimulates phosphorylation of eIF-2α in mammalian cells, we searched for and identified a GCN2 homologue in mice. We cloned three different cDNAs encoding mouse GCN2 isoforms, derived from a single gene, that vary in their amino-terminal sequences. Like their yeast counterpart, the mouse GCN2 isoforms contain HisRS-related sequences juxtaposed to the kinase catalytic domain. While GCN2 mRNA was found in all mouse tissues examined, the isoforms appear to be differentially expressed. Mouse GCN2 expressed in yeast was found to inhibit growth by hyperphosphorylation of eIF-2α, requiring both the kinase catalytic domain and the HisRS-related sequences. Additionally, lysates prepared from yeast expressing mGCN2 were found to phosphorylate recombinant eIF-2α substrate. Mouse GCN2 activity in both the *in vivo* and *in vitro* assays required the presence of serine-51, the known regulatory phosphorylation site in eIF-2α. Together, our studies identify a new mammalian eIF-2α kinase, GCN2, that can mediate translational control.

An important mechanism regulating protein synthesis involves phosphorylation of the α subunit of eukaryotic initiation factor-2 (eIF-2; Samuel 1993; Wek 1994; Clemens 1996; de Haro *et al.* 1996; Hinnebusch 1996). eIF-2 forms a complex with Met-tRNA$_i^{Met}$ and GTP and, in conjunction with the small ribosomal subunit, participates in the selection of the start codon. During this translation initiation process the GTP associated with eIF-2 is hydrolyzed to GDP, facilitating the release of the initiation factor from the ribosome (Merrick and Hershey 1996). Phosphorylation of eIF-2α at residue serine-51 reduces the activity of the guanine exchange factor, eIF-2B, that recycles eIF-2-GDP to the GTP-bound form required for subsequent rounds of translation initiation. Two well-characterized protein kinases were found to inhibit general translation in mammalian cells in response to different stress conditions. RNA-dependent protein kinase (PKR) participates in the antiviral defense mechanism mediated by interferon and is proposed to function in the control of cell proliferation and apoptosis (Koromilas *et al.* 1992; Meurs *et al.* 1993; Barber *et al.* 1995; Proud 1995; Lee *et al.* 1997; Srivastava *et al.* 1998), and the heme-regulated inhibitor kinase (HRI) reduces translation initiation in erythroid tissues in response to hemin deprivation (Chen and London 1995).

In contrast to mammalian kinases PKR and HRI that repress global protein synthesis, the eIF-2α kinase in *Saccharomyces cerevisiae*, GCN2, enhances translation of a single species of mRNA, encoding GCN4 in response to starvation for amino acids (Wek 1994; Hinnebusch 1997). GCN4 belongs to the bZIP protein family and functions as a transcriptional activator by recruiting coactivator complexes to the RNA polymerase II holoenzyme (Hope and Struhl 1986; Vogt *et al.* 1987; Brandl and Struhl 1989; Hinnebusch 1992, 1997; Drysdale *et al.* 1998; Natarajan *et al.* 1998). Regulation of *GCN4* translation involves four short open reading frames (ORFs) located in the 5′-noncoding portion of the *GCN4* mRNA (Abastado *et al.* 1991; Hinnebusch 1997). When cells are not limited for amino acids, the upstream ORFs block translation of the *GCN4* coding sequences. In response to starvation for amino acids, GCN2 phosphorylation of eIF-2α leads to reduced eIF-2-GTP levels, thereby reducing the inhibitory effects of the upstream ORFs and allowing for elevated *GCN4* translation (Dever *et al.* 1992; Wek *et al.* 1995; Hinnebusch 1997). The degree of phosphorylation of eIF-2α by GCN2 during amino acid starvation appears to be important for delineating between a general or gene-specific translation response. Yeast cells expressing

Corresponding author: Ronald C. Wek, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Van Nuys Medical Science Bldg., Rm. 4067, 635 Barnhill Dr., Indianapolis, IN 46202-5122. E-mail: rwek@iupui.edu

mammalian PKR or activated mutants of GCN2 display a severe slow growth phenotype due to hyperphosphorylation of eIF-2α (Ramirez *et al.* 1992; Dever *et al.* 1993).

Activation of GCN2 protein kinase by amino acid limitation is mediated by a domain homologous to the entire sequence of histidyl-tRNA synthetase (HisRS) enzymes (Wek *et al.* 1989, 1995; Zhu *et al.* 1996). Uncharged tRNAs were shown to directly bind to the HisRS-related sequences and residue substitutions in this domain of GCN2 that block tRNA interaction were also found to abolish kinase function *in vitro* and *in vivo* (Wek *et al.* 1995; Zhu *et al.* 1996). Together, these findings suggest that the HisRS-related sequences of GCN2 bind multiple uncharged tRNAs that accumulate in cells starved for amino acids, leading to activation of the kinase and phosphorylation of eIF-2α. At the extreme carboxyl terminus of GCN2 is a second RNA-binding region that is required for association of GCN2 with ribosomes and is proposed to facilitate GCN2 dimerization (Ramirez *et al.* 1991; Qiu *et al.* 1998; Zhu and Wek 1998). This ribosomal context appears to be required for GCN2 stimulation of *GCN4* translation and is proposed to facilitate monitoring of uncharged tRNA levels in cells. Additional factors such as GCN1 and GCN20 that form a heterocomplex associated with ribosomes are required for high levels of eIF-2α phosphorylation by yeast GCN2, and may assist in directing tRNA to the HisRS-related domain (Marton *et al.* 1993, 1997; Vazques de Aldana *et al.* 1995).

Recent studies indicate that GCN2 protein kinase homologues function in translational control in *Drosophila melanogaster* and *Neurospora crassa* (Santoyo *et al.* 1997; Olsen *et al.* 1998; Sattlegger *et al.* 1998). Drosophila GCN2 (dGCN2) contains sequences similar to both kinase and HisRS-related domains of its yeast counterpart and functions as an eIF-2α kinase. Expression of dGCN2 mRNA is developmentally regulated and at later stages becomes restricted to the central nervous system (Santoyo *et al.* 1997; Olsen *et al.* 1998). The physiological function of dGCN2 and its role in regulating total or gene-specific translation are currently unclear.

Mammalian cells starved for amino acids were found to phosphorylate eIF-2α concomitant with reduced initiation of protein synthesis (Scorsone *et al.* 1987; Kimball *et al.* 1991; Pain 1994; Clemens 1996). This translational control was observed in a variety of cell types. However, the well-characterized mammalian eIF-2α kinases HRI and PKR are not regulated by amino acid limitation and neither contain sequences related to aminoacyl-tRNA synthetases, implicating the presence of an additional mammalian eIF-2α kinase (Clemens 1996).

In this report, we describe the identification and characterization of a mammalian homologue of yeast GCN2 (yGCN2). We have cloned three cDNAs encoding different isoforms of mouse GCN2 (mGCN2). The three isoforms share similar kinase and HisRS-related sequences but differ in their amino-terminal sequences. Northern blot analysis revealed expression of mGCN2 mRNA in all of the tissues examined. Interestingly, using reverse transcriptase (RT)-PCR analysis with oligonucleotide primers specific for each isoform, we found that the mGCN2α and mGCN2γ isoforms were restricted to a subset of the characterized tissues. Mouse GCN2 expressed in yeast was found to phosphorylate eIF-2α at serine-51 and mediate the translational control system. In addition, the kinase and HisRS-related sequences were important for mGCN2 activity. However, in contrast with yGCN2, the catalytic activity of mGCN2 when expressed in yeast was independent of the endogenous GCN1 protein. Taken together, our studies identify a new mammalian eIF-2α kinase, mGCN2, that can mediate translational control.

MATERIALS AND METHODS

Cloning of mGCN2 cDNA: The yeast GCN2 sequence was used as a query to search for related mammalian sequences in the GenBank database using the Blast program. Expressed sequence tags (ESTs) encoding portions of putative mammalian GCN2 homologues were obtained from Genome Systems (St. Louis, MO) and the sequences of the entire cDNA inserts were determined by the dideoxy method (Sanger *et al.* 1977). The cDNAs had an extended 3′-noncoding region that ended in poly(A) sequences. To identify the 5′-portion of the mouse GCN2 cDNA sequence, rapid amplification of cDNA 5′-end (5′-RACE) was carried out using a marathon mouse brain library (Clontech, Palo Alto, CA). DNA products obtained from the nested PCR reactions were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA), amplified in *Escherichia coli*, and analyzed by dideoxy sequencing. To ensure the fidelity of the PCR products, independent reactions were carried out and the sequences of these cDNAs were compared. Furthermore, during the course of cDNA isolation, additional EST sequences corresponding to GCN2 were identified and compared to our cDNA sequences.

Characterization of mGCN2 genomic DNA: A pBeloBACII library containing genomic DNA of ES cells derived from mouse strain 129SVJ was screened for the mGCN2 gene. A 2-kb DNA probe encoding the kinase portion of the mGCN2 cDNA was radiolabeled with ^{32}P by random-primed labeling and used in a hybridization screen of the BAC-based library (Genome Systems), and the isolated clone was further confirmed to contain the mGCN2 gene by Southern blot analysis and by direct dideoxy sequencing. The locations of introns were determined by comparing the cDNA and genomic sequences. While the lengths of the smaller introns were determined by direct sequencing, the size of larger introns was measured by PCR analysis using mouse genomic DNA template and oligonucleotides complementary to sequences flanking the intron boundaries.

Northern blot analysis: A Northern blot containing 2 μg per lane of poly(A)$^+$ RNA from different mouse tissues was purchased from Clontech. A 3-kb DNA fragment encoding sequences common to all three mouse GCN2 isoforms was radiolabeled with ^{32}P by random-primed labeling and used as a probe in northern blot analysis (Sambrook *et al.* 1989). Prehybridization and hybridization steps were carried out using ExpressHyb Hybridization Solution (Clontech) for 3 hr at 68°. The filter was washed with a solution containing 0.1× SSC and 0.1% SDS at 50°. Alternatively, a 2.0-kb DNA

probe encoding human β-actin was radiolabeled and used as a probe in the northern analysis using the same blot. Blots were exposed to X-ray film using an intensifying screen at −80°.

Analysis of mGCN2 isoform mRNA using RT-PCR: Poly(A)$^+$ RNA was purified from mouse brain, liver, skeletal muscle, and testis using a MicroPoly(A) Pure isolation kit (Ambion, Austin, TX). RT-PCR analysis was carried out using the Titan one tube system (Boehringer Mannheim, Indianapolis) with ~100 ng of mRNA from the indicated mouse tissue in a 50-μl volume reaction. RT was carried out for 30 min at 55° followed by heat inactivation of the reverse transcriptase and 35 cycles of PCR. DNA products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. The primers used for analysis of the mGCN2 isoforms included RCW150, ATGGAGGATGTCACACGAGCCAGGAGAG; RCW285, AAGTTGAGTCTGGTTGTTACACTGT; RCW244, ATACCCAGATGTAGTTCCCGAAA; and RCW201, GACCAG GTGGTACAGGGTT. Oligonucleotide RCW150 was the 3′-primer used in the RT-PCR analysis for each of the three mGCN2 isoforms. The 5′-primer used for RT-PCR analysis of mGCN2α was RCW285, complementary to sequences in the extended 5′-exon specific for the mGCN2α isoform. The 5′-primer RCW244 used for mGCN2β, was complementary to sequences contiguous between exons one and two of mGCN2β. RCW201, used in the RT-PCR analysis of mGCN2γ, was complementary to a portion of the 314-bp exon unique to mGCN2γ, located between exons one and two of mGCN2β. The control β-actin RT-PCR reaction was performed using the β-actin control amplimer primers set (Clontech) that flanks an intron in the β-actin gene.

Expression of mGCN2 in yeast: To express mGCN2 in yeast, a 5.2-kb *Kpn*I to *Xba*I fragment containing the mGCN2β cDNA with an amino-terminal Flag tag was inserted between the corresponding sites of pYES2 (Invitrogen), generating plasmid p587. p587 is a *URA3*-marked high copy number plasmid that contains the mGCN2 cDNA downstream of the galactose-inducible *GAL-CYC1* hybrid promoter. Mutant versions of this expression plasmid include p588 encoding mgcn2-K618M and p589 containing mgcn2-m2 with leucines substituted for Phe-1142 and Arg-1143. Plasmids p587, p588, p589, pYES2, p434 including human PKR in pEMBLyex4 vector (Cesareni and Murray 1987), or pC102-2 encoding yeast *GCN2* (Wek *et al.* 1990) were introduced into yeast strains H1894 (*MAT***a** *ura3-52 leu2-3 leu2-112 gcn2*Δ *trp1*Δ*-63*; Dever *et al.* 1993), J82 (*MAT***a** *ura3-52 leu2-3 leu2-112 gcn2*Δ *trp1*Δ*-63* p1098[*SUI2-S51A LEU2*]; Romano *et al.* 1998b), GP3299 (*MAT***a** *ura3-52 leu2-3 leu2-112 gcn2*Δ *trp1*Δ*-63 gcd2*Δ*::hisG*, pAV1033[*GCD2-K627T*, *TRP1*] containing the *GCD2-K627T* mutant allele; Pavitt *et al.* 1997), H2511 (*MAT***a** *ura3-52 ino1 gcn2*Δ), and H2683 (*MAT***a** *ura3-52 ino1 gcn2*Δ *gcn1*Δ; Vazques de Aldana *et al.* 1995). Plasmid-containing strains were selected for by uracil prototrophy.

Yeast transformants were either grown in patches on agar plates containing synthetic medium supplemented with 2% glucose, 2 mm leucine, 0.5 mm isoleucine, 0.5 mm valine, and 1 mm tryptophan (SD; Kaiser *et al.* 1994) or grown to saturation in liquid media of the same composition. After incubating the plates for 1 day at 30°, cell patches were replica-printed onto agar plates containing synthetic medium, 10% galactose, 2% raffinose (SGal) supplemented with 0.5 μg/ml sulfometuron methyl, and leucine (Wek *et al.* 1995). Alternatively, the saturated cultures were diluted to OD_{600} = 0.25 and 5 μl of each culture was spotted onto agar media plates. The agar plates were incubated for 4 days at 30° and photographed. For cell lysate preparation, yeast cultures were grown to saturation at 30° in SD and then induced overnight in SGal.

***In vitro* eIF-2α kinase assay:** Yeast cell lysates were prepared using glass beads and lysis buffer containing 1% Triton X-100, 25 mm Tris-HCl (pH 7.4), 300 mm NaCl, 1 mm $CaCl_2$, and protease inhibitors (1 μm pepstatin, 1 μm leupeptin, 0.15 μm aprotinin, and 100 μm phenylmethylsulfonyl fluoride). Equal amounts of yeast cell lysates were precleared by the addition of protein G-agarose (Boehringer Mannheim) and centrifugation at 4°. Anti-Flag M2 monoclonal antibody (Sigma, St. Louis) was added to the lysate, and mGCN2 was immunoprecipitated with protein G-agarose. The mGCN2 immunocomplex was rinsed twice with lysis buffer, followed by two washes with a low salt buffer [140 mm NaCl, 25 mm Tris-HCl (pH 7.4), 1 mm $CaCl_2$, and protease inhibitors] and a final wash with the kinase buffer [20 mm Tris-HCl (pH 7.9), 50 mm NaCl, 10 mm $MgCl_2$, 1 mm dithiothreitol, and protease inhibitors]. Immunoprecipitated mGCN2 was incubated in a 15-μl solution of kinase buffer, recombinant wild-type or S51A version of eIF-2α substrate, and 10 μCi [γ-^{32}P]ATP in a final concentration of 10 μm ATP at 30° for 10 min (Zhu *et al.* 1996). Reactions were terminated with the addition of an equal volume of 2× SDS-PAGE sample buffer, followed by heating at 95°. Phosphorylated proteins were separated by electrophoresis in a 12.5% SDS-polyacrylamide gel that was stained with Coomassie brilliant blue R250, dried, and visualized by autoradiography.

Immunoblot analysis of mGCN2 and eIF-2α: Lysates prepared from yeast cells were separated by electrophoresis in a SDS-polyacrylamide gel and transferred to nitrocellulose filters. Filters were blocked in a Tris-buffered saline (TBS) solution containing 5% nonfat dry milk. To measure Flag-mGCN2 protein levels, we incubated the filters in a TBS solution containing Anti-Flag M2 murine monoclonal antibody (Sigma). Filters were washed in TBS and Flag-mGCN2-antibody complex was detected using horseradish peroxidase-labeled anti-mouse secondary antibody and chemiluminescent substrate. Immunoblots measuring eIF-2α phosphorylation were carried out using cell lysates prepared with lysis buffer supplemented with 50 mm NaF and 40 mm β-glycerophosphate. Phosphorylated eIF-2α was visualized using affinity-purified antibody that specifically recognizes eIF-2α phosphorylation at serine-51 that was kindly provided by Dr. Gary Krause (Wayne State University; DeGracia *et al.* 1997). Total eIF-2α in yeast lysates was detected by immunoblot using rabbit polyclonal antibody prepared against a polyhistidine-tagged version of yeast eIF-2α expressed and purified from *E. coli*.

RESULTS

Identification and characterization of cDNAs encoding mouse GCN2 protein kinase: In both yeast and mammalian cells, starvation for amino acids was shown to increase the levels of eIF-2α phosphorylation. In yeast cells, this phosphorylation is mediated by the GCN2 protein kinase. To determine whether a similar kinase functions in mammalian cells, we used the yeast GCN2 sequence as a query to search the EST database in the GenBank using the Blast program. A human EST clone, accession no. R19609, was found to encode sequences related to yGCN2 from kinase subdomain IX to the motif 1 region of the HisRS-related sequence. A second cDNA clone, accession no. W57530, was identified from mouse that contained sequences highly similar to the HisRS-related domain of yGCN2. Sequencing of the insert of clone W57530 revealed an ORF 638 residues in length similar to the entire HisRS-related domain. This cDNA included a stop codon and 3′-noncoding se-

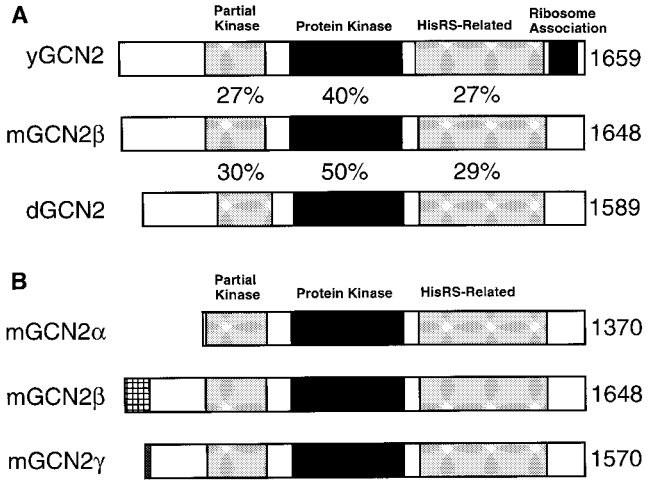

Figure 1.—GCN2 from yeast, mouse, and Drosophila contains a kinase catalytic domain juxtaposed to sequences homologous to histidyl-tRNA synthetases. (A) Sequences from yeast GCN2 (yGCN2), the mouse GCN2 β isoform (mGCN2β), and Drosophila GCN2 (dGCN2) are illustrated by boxes with the partial kinase, protein kinase, HisRS-related, and ribosome association domains indicated by shading. The percentage identity between mGCN2β and yeast or Drosophila GCN2 is indicated between each domain. (B) Comparison of mGCN2 isoforms α, β, and γ that differ in their amino terminal sequences. The length of each kinase is indicated to the right of the boxes. Note yGCN2 is shown as 1659 residues in length, 69 residues longer than previously reported (Wek *et al.* 1989).

quences ending with a poly(A) tract. To identify the 5′-portion of the mouse GCN2 sequence, we carried out multiple rounds of 5′-RACE using cDNAs derived from mouse brain mRNA. PCR products were cloned and characterized by dideoxy sequencing. To independently confirm 5′-RACE products, we carried out RT-PCR and generated cDNA products with identical length and sequence.

Interestingly, during the course of our characterization of the mGCN2 cDNAs, we found three variants that differed in their deduced amino-terminal sequences (Figure 1). As is discussed further below, these different cDNA forms, which we refer to as mGCN2 α, β, or γ, appear to be derived in part by splicing variations of the mGCN2 mRNA. Furthermore, the isoform mRNAs may have different transcriptional start sites. The mGCN2β cDNA sequence is 5230 bp in length and contains an ORF encoding a polypeptide 1648 residues in length with a predicted molecular weight of 186,000 (Figures 1 and 2). Sequences 5′ to this ORF contain an in-frame termination codon, indicating that the entire coding region was obtained in the mGCN2β cDNA. The 3′-noncoding region spans 199 nucleotides and includes a putative poly(A) recognition sequence (AATAAA; Figure 2). The mGCN2α and mGCN2γ cDNAs diverge from the β form at their 5′-ends, resulting in different predicted amino-terminal sequences (Figure 2). The mGCN2α cDNA contains a unique 587-bp sequence at its 5′-end compared with mGCN2β and γ. An in-frame termination codon occurs at the site of the splicing variation, resulting in a mGCN2α ORF that begins with the initiation codon at residue position 279 of the mGCN2β sequence (Figure 2). The predicted mGCN2α polypeptide is 1370 residues in length with a molecular weight of 155,000. The mGCN2γ appears to be a product of alternative splicing compared with mGCN2β. The mGCN2γ contains a 314-bp sequence corresponding to nucleotide positions 120–433 of mGCN2γ cDNA (Figure 2), inserted prior to nucleotide position 342 of the β isoform. This alters the predicted amino-terminal portion of the mGCN2γ cDNA such that the first 86 amino acid residues of mGCN2β are replaced with 8 residues unique to GCN2γ (Figure 2). The predicted mGCN2γ polypeptide is 1570 residues in length with a molecular weight of 178,000. We independently confirmed the identity and sequence of these three mGCN2 cDNA isoforms by RT-PCR using poly(A)$^+$ RNA prepared from mouse brain and a 3′-oligonucleotide primer derived from sequences encoding the HisRS-related domain, and 5′-primers with sequences unique to each of the mGCN2 isoform cDNAs. We generated PCR products of the predicted size for each isoform and after sequencing we found complete identities with the cDNAs derived from 5′-RACE.

Protein kinase and HisRS-related domains are conserved between yeast, mouse, and Drosophila GCN2: A defining feature of yGCN2 protein kinase is the HisRS-related sequences juxtaposed to the catalytic domain. Both regions are also found in all three isoforms of mGCN2 (Figures 1 and 2). The kinase catalytic domain of mGCN2 is 420 residues in length and shares many features previously described for yGCN2. The mGCN2 and yGCN2 share 40% identity in their catalytic domains, including 11 residues previously identified as being present among eIF-2α kinases but absent in the majority of other eukaryotic protein kinases (Figures 1–3). The kinase domain region of mGCN2 is 50% identical to that of dGCN2. mGCN2, like the yeast and Drosophila kinases, contains a large insert, 135 residues in length, between kinase subdomains IV and V. Excluding the insert sequence, the kinase domain of mGCN2 is 48% identical to that of yGCN2 and 58% identical to the catalytic region of dGCN2. In subdomain V, mGCN2 contains the sequence LYIQMEYCE that was found to be conserved among yGCN2 and other eIF-2α kinases. In kinase subdomain VIII, mGCN2 contains residues Thr-898 and Thr-903, aligning with known autophosphorylation sites of yGCN2 (Romano *et al.* 1998a), which may be important for activation of mouse GCN2 protein kinase. This pairwise comparison of GCN2 primary sequences from the three different organisms indicates that mouse GCN2 is most closely related to the Drosophila kinase.

The HisRS-related domain of the yGCN2 protein kinase shares sequence similarity with the class II amino-

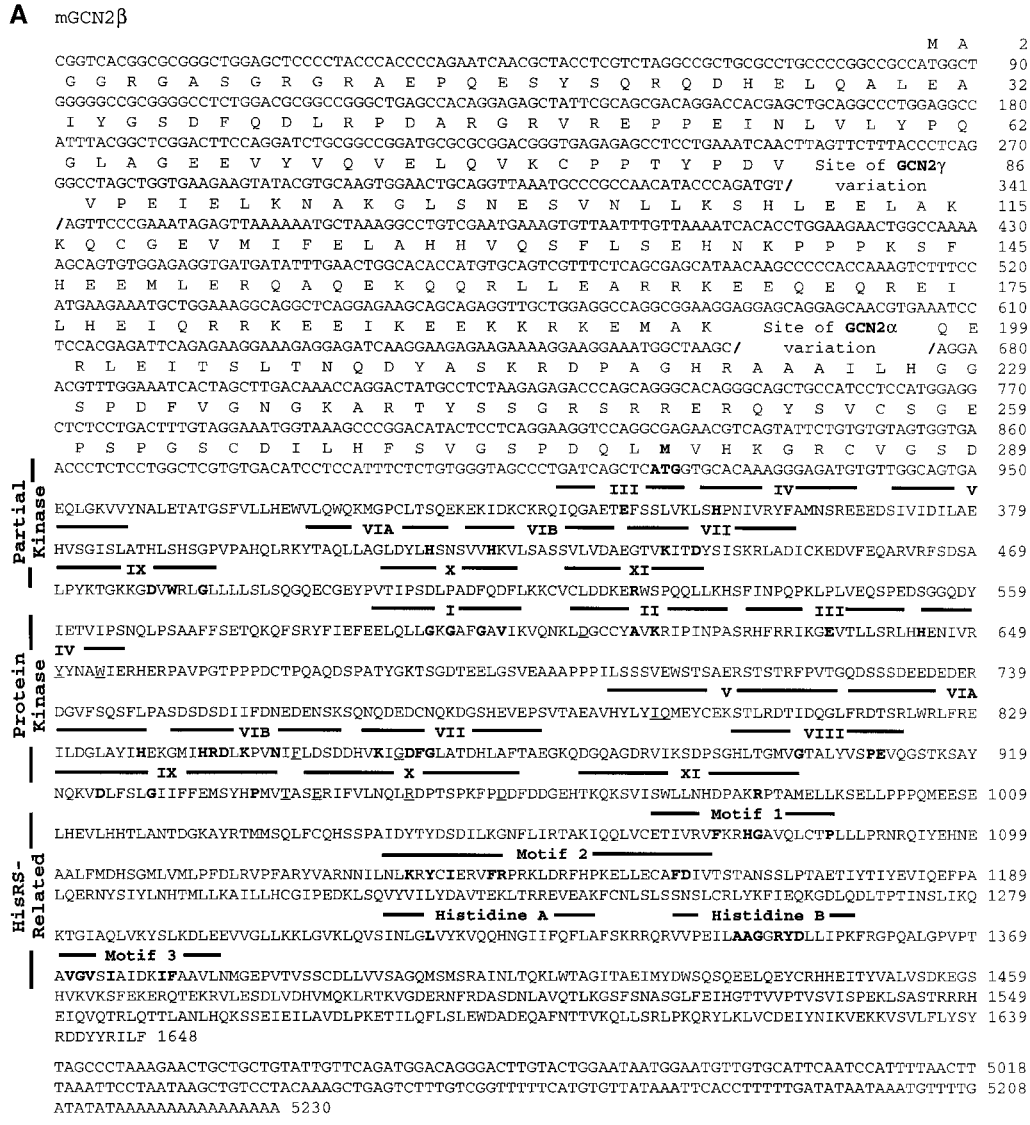

Figure 2.—The predicted sequences of the α, β, and γ isoforms of mGCN2. (A) The 1648-residue mGCN2β sequence is shown with the subdomains of the partial kinase, protein kinase, and HisRS-related domains highlighted above the sequence. Residues in bold in the kinase or HisRS-related regions of mGCN2β are those highly conserved between eukaryotic protein kinases or class II aminoacyl-tRNA synthetases, respectively (Moras 1992; Arnez *et al.*, 1995; Hanks and Hunter 1995). Underlined residues in the kinase catalytic sequences of mGCN2β are conserved among known eIF-2α kinases but not found in the majority of other protein kinases (Ramirez *et al.* 1992). Included with the mGCN2β sequences are the first 950 nucleotides of the mGCN2β cDNA with the initiation codon beginning at nucleotide position 85. Sites for variation for GCN2α and GCN2γ are indicated prior to nucleotide positions 342 and 677, respectively. Illustrated at the bottom is the termination codon, followed by a 199-nucleotide 3′-noncoding region of the GCN2β cDNA, including the polyadenylation sequences. (B) For mGCN2α, an extended 5′-exon results in a 587-nucleotide sequence being joined with mGCN2β sequences beginning with nucleotide position 677 (illustrated as the first mGCN2α exon in Figure 4). A termination codon is produced at the splicing junction, resulting in a predicted GCN2α polypeptide sequence beginning at the methionine codon, highlighted in bold letters, at nucleotide position 919 of mGCN2β. The 433-nucleotide sequence of the mGCN2γ cDNA is illustrated, along with the predicted initiation codon beginning at nucleotide position 411. The cDNA for mGCN2γ is derived from the insertion of a 314-bp exon, prior to nucleotide position 342 of mGCN2β. This additional exon unique to mGCN2γ corresponding to nucleotide positions 120 to 433 is inserted between the first and second exons of mGCN2β in Figure 4. The unique exon in mGCN2γ contains an in-frame termination codon at nucleotide position 393, resulting in the predicted ORF beginning with the methionine codon at nucleotide position 411. The first 124 nucleotides of mGCN2γ are identical with a portion of the first exon of mGCN2β as illustrated in Figure 4.


yGCN2
dGCN2
mGCN2
Partial Kinase
Protein Kinase
HisRS-related

acyl-tRNA synthetase family that contains 10 different synthetases including the bona fide HisRS enzymes (Moras 1992; Ramirez *et al.* 1992; Arnez *et al.* 1995; Wek *et al.* 1995). This class is defined by three motifs and these sequences are also found in mGCN2 (Figure 2). Motif 2 sequences bind directly to the acceptor stem of tRNAs and mutations in this region of yGCN2 were found to impair binding with uncharged tRNA *in vitro* and control of *GCN4* translation expression in cells starved for amino acids (Wek *et al.* 1995; Zhu *et al.* 1996). Additionally, the HisRS enzymes share two sequences, designated Histidine A and Histidine B, that were shown to facilitate substrate binding in the catalytic pocket of the HisRS enzyme from *E. coli* (Arnez *et al.* 1995). The Histidine B sequences, AAGGRYD, are highly conserved between the HisRS enzymes and GCN2 protein kinases from mouse, yeast, and Drosophila (Figure 3). By contrast, the Histidine A region in these GCN2 protein kinases is diverged from the HisRS enzymes and, as previously noted for yGCN2, the HisRS-related domain of mGCN2 would not be expected to function as an aminoacyl tRNA synthetase because it lacks key residues in the Histidine A region as well as in motif 3 (Arnez *et al.* 1995).

In addition to the catalytic and HisRS-related domains, yGCN2 contains amino-terminal sequences related to multiple subdomains of eukaryotic protein kinases (Figures 1–3). While this region of yGCN2 is required for *in vivo* and *in vitro* phosphorylation of eIF-2α, it is missing critical residues required for ATP binding and catalysis (Wek *et al.* 1989, 1990; Zhu *et al.* 1996). The mechanism by which the amino-terminal sequences, referred to here as the partial kinase domain, regulate the authentic kinase region of GCN2 is not known. The fact that the partial kinase domain is also conserved in each of the mouse GCN2 isoforms as well as in the Drosophila homologue further supports the idea that these amino-terminal sequences perform a regulatory role in GCN2 phosphorylation of eIF-2α.

A region rich in basic residues centered around 1560 was previously described to be important for yGCN2 association with ribosomes (Ramirez *et al.* 1991; Zhu and Wek 1998). This region was found to facilitate yGCN2 binding with double-stranded RNA, suggesting that interaction with rRNA is important for association with the translational machinery. Furthermore, the carboxyl terminus of yGCN2 is suggested to mediate kinase dimerization (Qiu *et al.* 1998). Comparisons between the GCN2 homologues indicate that the carboxyl-terminal sequences appear dissimilar, although both higher eukaryotes do contain clusters of basic residues (Figure 3).

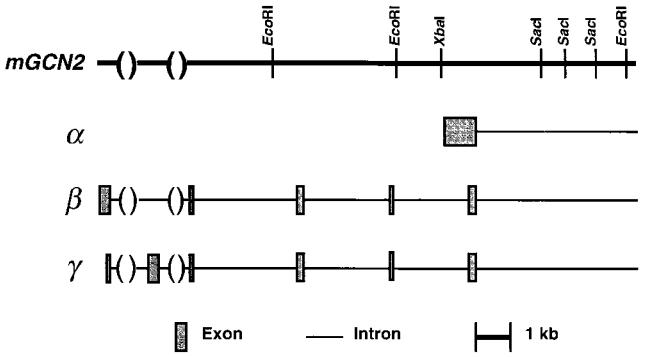

Figure 4.—Genomic organization of the 5′-end of the mGCN2 gene. Below the restriction map of the mGCN2 gene are the positions of exons for the mGCN2 α, β, and γ cDNAs. Exons are illustrated as gray boxes with lines representing introns. Parentheses indicate two introns of undefined length. The mGCN2α cDNA contains an extended exon at its 5′-end compared to mGCN2 β and γ isoforms. The mGCN2γ cDNA differs from mGCN2β by an additional exon between the first and second exons of mGCN2β.

Multiple forms of mouse GCN2 mRNA: To address the molecular basis for the different mGCN2 isoforms, we isolated the mGCN2 gene. Sequencing of the mGCN2 genomic DNA and PCR analysis using oligonucleotide primers derived from the cDNA suggests a pattern of exons contributing to unique 5′ regions in the mGCN2 mRNA isoforms (Figure 4). The 5′-end of the mGCN2α mRNA contains a long exon that is abbreviated in the β and γ transcripts. Elongation of this exon in GCN2α introduces multiple stop codons into the 5′-portion of the mRNA, thereby resulting in an ORF predicted to initiate just prior to the partial kinase domain (Figures 1 and 2). The mGCN2γ transcript contains an additional exon sequence inserted between the first and second exons of mGCN2β (Figure 4). This exon in isoform γ is spliced out along with adjacent intron sequences on either side to generate the mGCN2β mRNA.

GCN2 protein kinase homologues in other higher eukaryotes: In addition to GCN2 protein kinases in yeast, Drosophila, and mouse, a search of the GenBank database using the Blast program revealed that there are putative GCN2 homologues in humans and rat. In

Figure 3.—Comparison of GCN2 sequences from mouse, yeast, and Drosophila. GCN2 sequences were compared pairwise using the Blast and BestFit programs and manually incorporated into this multiple alignment. Boxes indicate identical residues between the GCN2 sequences. Gaps, indicated by dashes, were incorporated to maximize the alignment. Positions of the partial kinase, protein kinase, and HisRS-related sequences are illustrated to the right of the figure. The positions of the aligned residues are indicated on the left side of the alignment. Note the yGCN2 is shown as 1659 residues in length, 69 residues longer than previously reported (Wek *et al.* 1989). The asterisk highlights the residues in mGCN2 that have been substituted to generate the mgcn2-K618M and mgcn2-m2 mutant versions.

humans, there are numerous EST entries with >80% identity to mouse GCN2. These human EST entries span portions of the entire length of mGCN2. In addition to R19609, a clone mentioned earlier that overlaps part of the kinase catalytic and HisRS-related sequences, AA230276 is contiguous with the partial and full kinase sequences. In the HisRS-related domain, human clone AA316106 is 93% identical with sequences centered around motif 2 and AA376437 contains the motif 2 and the Histidine B regions. EST clone AA926944 is the only entry including homology with amino-terminal sequences of mGCN2, matching sequences from residues 19 to 157 of mGCN2β. As this sequence overlaps sections of variable splicing for mGCN2, this EST entry indicates the presence of the GCN2β isoform in humans.

A comparison of cDNA sequences derived from rat also supports the presence of a GCN2 homologue. Rat EST AI23468 is >90% identical with mGCN2 sequences centered around subdomain I of the kinase domain and clone AI69164 encodes sequences related to the extreme carboxyl terminus of mGCN2. In *Caenorhabditis elegans*, a genomic clone y81G3, accession no. AL021152, contains sequences highly similar to both the kinase and HisRS-related domains of GCN2. A cDNA entry yk323a9 encodes a portion of the kinase domain of the putative *C. elegans* GCN2, including sequences from subdomains V to VII.

GCN2 mRNA is expressed in a variety of mouse tissues: Similar amounts of poly(A)$^+$ RNA extracted from different mouse tissues were analyzed by Northern blot for mGCN2 transcript levels. A radiolabeled DNA probe containing sequences common to all three mGCN2 isoforms was found to hybridize to a transcript ~5.5 kb in length in each of the eight tissues analyzed (Figure 5). Interestingly, mRNA from skeletal muscle contained a transcript species >10 kb that also hybridized to the mGCN2 probe. Following this analysis, actin mRNA levels were measured (Figure 5) and used to normalize the total mRNA levels present in each lane. The level of mGCN2 transcript in brain, liver, kidney, and testis was found to be greatest, threefold higher when compared to that in lung. Lower amounts of mGCN2 mRNA were found in heart and spleen. Human ESTs encoding GCN2 were derived from a wide variety of tissues including prostate, uterus, and neuroepithelium in addition to those tissues found to contain mGCN2 mRNA. Taken together, mGCN2 transcripts are found in a wide variety of mammalian tissues, although their relative levels vary between different cell types.

To address whether the mGCN2 isoforms are differentially expressed in mouse tissues, we prepared poly(A)$^+$ RNA from brain, liver, skeletal muscle, and testis and carried out RT-PCR using 5′-oligonucleotide primers specific for each isoform in combination with a 3′-primer common to α, β, and γ transcripts (see materials and methods). DNA products synthesized by RT-PCR were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining (Figure 6). In the example of GCN2β, a DNA product with a predicted length of 555 bp was synthesized in the reactions using mRNA purified from each of the mouse organs, indicating that the GCN2β transcript is expressed in all four tissue types. By comparison, similar RT-PCR analysis for mGCN2α indicated that there was expression in brain as well as detectable levels in testis and liver. The mGCN2γ mRNA was found to be expressed only in brain and testis. A control RT-PCR reaction using β-actin primers produced similar amounts of 840-bp product in all four tissues analyzed. The absence of any other larger-sized RT-PCR products with the β-actin primers indicates that the poly(A)$^+$ preparation consists of only fully spliced mRNAs. These results indicate that mGCN2 isoform

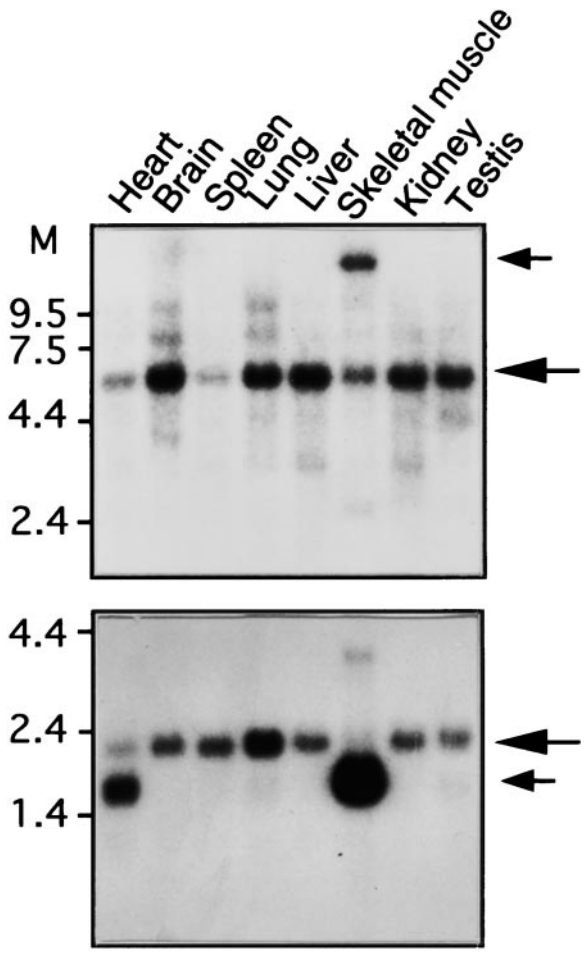

Figure 5.—GCN2 is expressed in many different mouse tissues. A northern blot containing poly(A)$^+$ RNA purified from the indicated mouse tissues was hybridized with a ^{32}P-labeled DNA probe encoding sequences common to all three isoforms of mGCN2. The mGCN2 transcripts were visualized by autoradiography (top). The large arrow indicates an mGCN2 transcript 5.5 kb in length and the small arrow highlights a transcript >10 kb found in skeletal muscle. (Bottom) An autoradiogram derived from a similar hybridization analysis using the identical northern blot and a radiolabeled probe encoding β-actin. Sizes of RNA standards included in the blot are illustrated to the left.

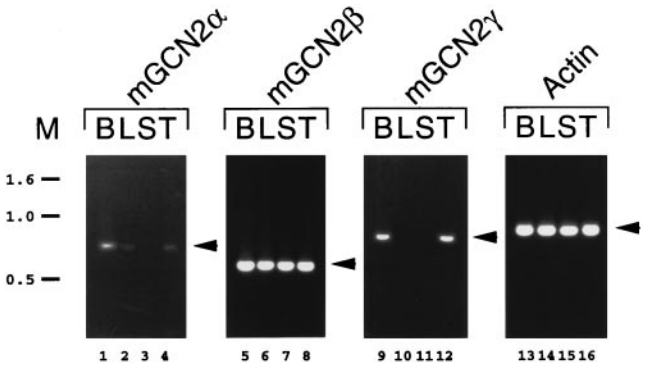

Figure 6.—GCN2 isoforms are differentially expressed in mouse tissues. RT-PCR analysis was carried out using poly(A)$^+$ RNA purified from mouse brain (B), liver (L), skeletal muscle (S), and testis (T). Unique 5′-oligonucleotide primers complementary to mGCN2 α, β, or γ mRNA were used in combination with a 3′-primer common to all three isoforms (see materials and methods). Lanes 1–4 are RT-PCR products amplified from mGCN2α mRNA derived from the indicated mouse tissue. The amplified DNA fragment is 700 bp, consistent with that predicted from the mGCN2α cDNA. This amplified DNA represents three exons in the mGCN2 gene. To detect amplified DNA in the liver and testis samples, 10 out of 50 μl RT-PCR reaction was loaded into lanes 2–4, while lane 1 contained 1 μl from a 50-μl RT-PCR reaction. Lanes 5–8 contain RT-PCR DNA products amplified from mGCN2β mRNA. RT-PCR products derived from mGCN2γ are present in lanes 9–12. The mGCN2γ-amplified DNA is 765 bp in length and mGCN2β is 555 bp in length. The sizes of both mGCN2β and γ DNA products are consistent with that predicted by the cDNA sequences and equal volumes were loaded into the mGCN2β- and mGCN2γ-derived lanes. The amplified DNAs from mGCN2β and mGCN2γ span seven exons in the mGCN2 gene. Lanes 13–16 contain equal volumes of RT-PCR reactions performed using control β-actin primers with mRNA from all four tissues. The β-actin RT-PCR product is 840 bp and spans an intron junction in the β-actin gene. Arrows indicate the DNA fragments amplified for each isoform. M, markers in kbp.

mRNAs have varied patterns of expression among different mouse tissues. Given that the three isoform cDNAs have distinct sequences at their 5′-ends, these expression variations may be the result of different transcriptional start sites as well as alternative splicing.

Mouse GCN2 expressed in yeast inhibits growth by hyperphosphorylation of eIF-2α at serine-51: In response to amino acid limitation in yeast, GCN2 protein kinase stimulates *GCN4* translational expression and the expression of genes subject to its transcriptional control. Loss of *GCN2* function renders cells growth-sensitive to the chemical inhibitor sulfometuron methyl (SM) that blocks the synthesis of the isoleucine-valine biosynthetic pathway (Figure 7A; Wek *et al.* 1995). Previously, it was shown that expression of human PKR in *gcn2*Δ yeast cells leads to hyperphosphorylation of eIF-2α, resulting in impaired translation initiation and a slow growth phenotype (Feng *et al.* 1992; Dever *et al.* 1993; Romano *et al.* 1995, 1998a; Zhu *et al.* 1997). To determine whether mGCN2 can function in yeast deleted for its endogenous GCN2 activity, we expressed a Flag-tagged version of mGCN2β in the yeast strain H1894 (*gcn2*Δ). Cells expressing either mGCN2 or PKR had a severe growth defect (Figure 7A). By contrast, strain H1894 transformed with either vector alone or plasmid-encoded yGCN2 showed no growth defect in the galactose-containing media. All of the H1894 transformants grew to similar levels in synthetic medium containing glucose. In parallel with these growth studies, we measured the *in vivo* levels of eIF-2α phosphorylation using a polyclonal antibody specific to eIF-2α phosphorylated at serine-51 by an immunoblot assay. Levels of eIF-2α phosphorylation in H1894 cells containing mGCN2 were similar to that measured for the strain expressing PKR (Figure 7B). No phosphorylation of eIF-2α was detected in H1894 containing vector alone. Similar steady-state levels of eIF-2α protein were present in each of the lysate preparations as judged by immunoblot using a polyclonal antibody that recognizes both phosphorylated and nonphosphorylated forms of eIF-2α.

To determine whether mGCN2 control of translation in yeast requires the serine-51 phosphorylation site of eIF-2α, we expressed the mGCN2 cDNA in strain J82 (Δ*gcn2 SUI2-S51A*) that is isogenic to H1894 and contains an alanine substitution for serine-51 in eIF-2α. Expression of either mGCN2 or PKR kinases in J82 failed to cause a slow growth phenotype in SGal medium (Figure 7A). The eIF-2α-S51A also blocked the ability of yGCN2 to provide growth resistance in the presence of SM. Therefore, mGCN2 like the other eIF-2α kinase family members requires the regulatory site, serine-51, in eIF-2α for translational control. Furthermore, Pavitt *et al.* (1997) reported that certain mutations in *GCD2*, encoding the δ subunit of eIF-2B, rendered the guanine nucleotide exchange factor less sensitive to inhibition by phosphorylated eIF-2α. We expressed mGCN2 or human PKR in strain GP3299 (*gcn2*Δ *GCD2-K627T*) and found that the eIF-2Bδ mutation relieved the slow growth phenotype in the galactose-inducing medium (Figure 7A). Interestingly, GP3299 cells containing mGCN2 or PKR were growth-resistant on SGal medium supplemented with SM, consistent with the idea that these cells achieved sufficient reduction in eIF-2B activity to stimulate *GCN4* translational expression. These results taken together indicate that mGCN2 is an eIF-2α kinase that can control translation initiation through inhibition of eIF-2B.

Mutations in the kinase domain or HisRS region impair the activity of mGCN2 expressed in yeast: Previous studies described that mutations in either the kinase catalytic region or the HisRS-related domain of yGCN2 blocked its ability to phosphorylate eIF-2α and stimulate *GCN4* translation. To delineate the importance of these sequences in mGCN2 function, we constructed two mutants, mgcn2-K618M, substituted for the invariant lysine in the kinase subdomain II, and mgcn2-m2, altered for conserved residues in motif 2 of the HisRS-related do-

main that were previously shown to be important for yGCN2 association with uncharged tRNA and stimulation of *GCN4* expression in response to amino acid starvation. Both Flag-tagged mutant versions of mGCN2β were expressed in yeast strain H1894 using a galactose-inducible promoter and assessed for growth as described above. No growth defect was detected in cells expressing either mGCN2 mutant grown in SGal medium, suggesting the absence of eIF-2α hyperphosphorylation as found for wild-type mGCN2 cells (Figure 7A). However, the cells expressing mgcn2-m2 were observed to grow in the galactose medium containing SM, indicating that the mgcn2-m2 mutant kinase can phosphorylate eIF-2α to levels sufficient to stimulate expression of GCN4 and its target genes. By comparison there was no growth resistance in the cells containing vector alone. As expected, the SM resistance associated with mgcn2-m2 was lost in J82 and GP3299 containing *SUI2-S51A* or *GCD2-K627T*, respectively. The mutant versions of mGCN2β were present in the yeast cells at levels similar to that measured for wild-type kinase, as judged by an immunoblot (Figure 8).

To measure the *in vitro* eIF-2α kinase activity, lysates were prepared from the yeast strains expressing wild-type or mutant mGCN2 protein kinases or containing vector alone. The mGCN2 proteins were immunoprecipitated using Flag monoclonal antibody and protein G-agarose and incubated with recombinant yeast eIF-2α and [γ-^{32}P]ATP. Radiolabeled proteins were separated by SDS-PAGE and visualized by autoradiography. In the reaction sample containing wild-type mGCN2, we found phosphorylation of both the kinase and eIF-2α substrate (Figure 8). Phosphorylation was specific for serine-51 because we detected no phosphorylation in a similar kinase assay carried out with the eIF-2α-S51A mutant substrate. The mgcn2-K618M was defective for both autophosphorylation and eIF-2α phosphorylation. In the case of mgcn2-m2, we found lower levels of autophosphorylation compared to wild-type kinase with eIF-2α phosphorylation that was detected only in ex-

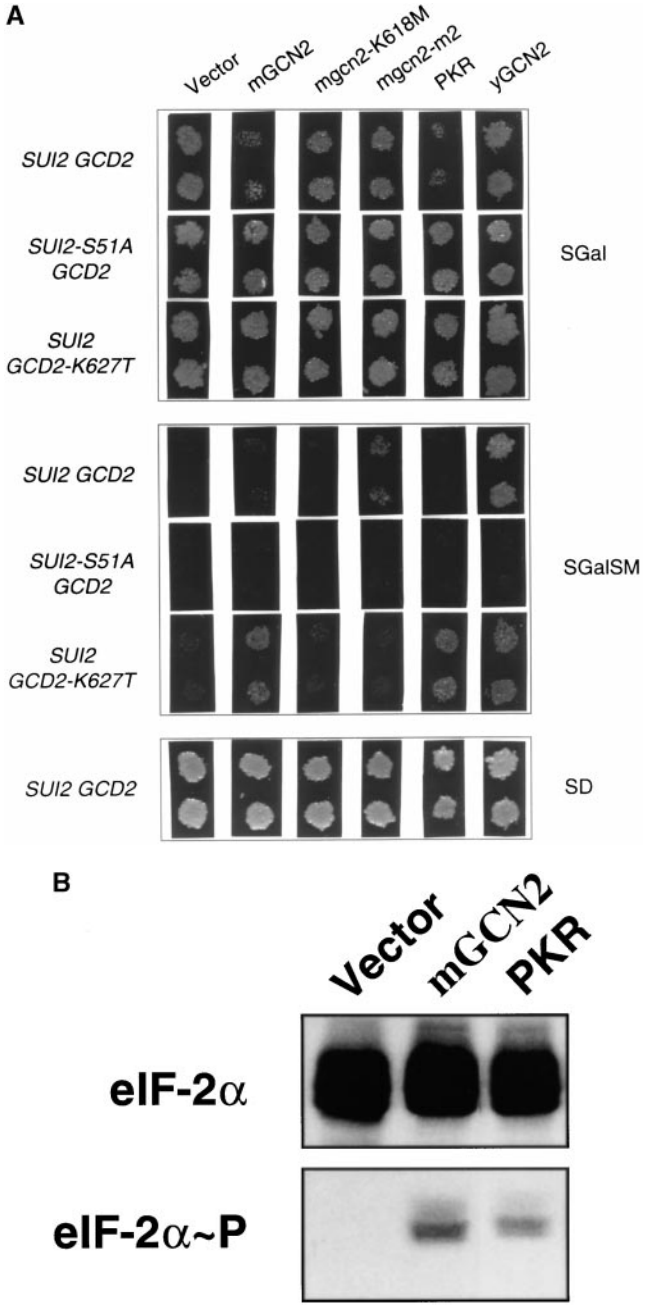

Figure 7.—Expression of mGCN2 in yeast produces a slow growth phenotype due to hyperphosphorylation of eIF-2α. (A) Wild-type mGCN2, mgcn2-K618M, mgcn2-m2, and PKR were expressed from a galactose-inducible promoter in yeast strains lacking the endogenous eIF-2α kinase, yGCN2. The isogenic yeast strains used included H1894 (*gcn2*Δ *SUI2 GCD2*), J82 (*gcn2*Δ *SUI2-S51A GCD2*), and GP3299 (*gcn2*Δ *SUI2 GCD2-K627T*). *SUI2* encodes eIF-2α and the mutant allele *SUI2-S51A* encodes a product that is blocked for phosphorylation by an eIF-2α kinase (Cigan *et al.* 1989; Dever *et al.* 1992). *GCD2* encodes the δ subunit of eIF-2B and the *GCD2-K627T* mutant renders eIF-2B less sensitive to inhibition by phosphorylated eIF-2 (Pavitt *et al.* 1997). Yeast cells were patched and printed onto agar plates containing synthetic medium of glucose (SD), or galactose (SGal), or galactose supplemented with an inhibitor of the pathway for biosynthesis of branched-chain amino acids, sulfometuron methyl (SGalSM). Agar plates were incubated for 4 days at 30° and photographed. While expression of mGCN2 and PKR in H1894 significantly reduced growth in the galactose-inducing medium, this growth defect was alleviated when mGCN2 was either mutated or expressed in J82 and GP3299 host strains containing *SUI2-S51A* and *GCD2-K627T*, respectively. Expression of mgcn2-m2 allowed for growth of the H1894 yeast cell in SGalSM media similar to that observed with yGCN2, indicating that there were low levels of eIF-2α kinase activity in the galactose-inducing condition (see Figure 8). The expression of mGCN2, PKR, and yGCN2 was also able to impart growth resistance to the GP3299 strain. (B) Lysates were prepared from growth-inhibited mGCN2- and PKR-expressing cells as well as from cells containing vector alone. The lysates were analyzed by immunoblot using an antibody that specifically recognizes eIF-2α phosphorylated at serine-51 (B, bottom), or with an antibody that recognizes total eIF-2α protein (B, top).

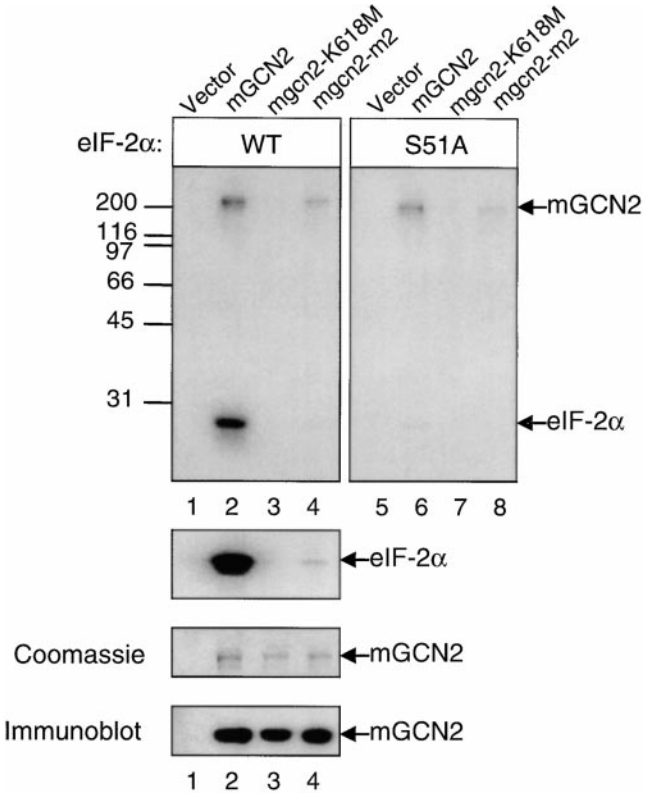

Figure 8.—Mouse GCN2 phosphorylates recombinant eIF-2α substrate at serine-51. Lysates were prepared from yeast cells containing Flag-tagged wild-type and mutant versions of mGCN2 or vector alone. Mouse GCN2 was immunoprecipitated using a monoclonal antibody specific to the Flag epitope and incubated with [γ-^{32}P]ATP and recombinant eIF-2α. Radiolabeled proteins were separated by SDS-PAGE, followed by autoradiography. Kinase reactions represented in lanes 1–4 contained wild-type eIF-2α substrate (WT) and lanes 5–8 included mutant eIF-2α-S51A (S51A). Phosphorylated mGCN2 and eIF-2α are indicated by arrows. The autoradiogram was exposed for a longer time to visualize the substrate phosphorylation by the mutant mgcn2-m2 kinase (second panel from top). The amounts of mGCN2 proteins in the kinase assays were measured by Coomassie staining. Equal expression of the wild-type and mutant versions was further confirmed by immunoblot analysis with an antibody that detects the Flag epitope. Protein markers are listed in kilodaltons.

tended exposures of the X-ray film (Figure 8). This low level of kinase activity would be consistent with the ability of mgcn2-m2 to facilitate growth resistance to SM under galactose-inducing conditions. Similar levels of wild-type and the mutant versions of mGCN2 were included in the immunoprecipitation kinase assay, as measured by Coomassie staining and Flag immunoblot. We conclude that the functions of both the kinase and HisRS-related domains of mGCN2 are important for eIF-2α kinase activity and *in vivo* translational control.

mGCN2 functions in yeast in the absence of GCN1, a factor essential for yGCN2 stimulation of general control by eIF-2α phosphorylation: Ancillary proteins, such as GCN1, were described to be essential for yGCN2 phosphorylation of eIF-2α in response to amino acid starvation. It was proposed that ribosome-associated GCN1 protein is involved in the interaction of yGCN2 with uncharged tRNA (Marton *et al.* 1993). In addition to yeast, a GCN1 homologue is also represented in humans, suggesting a conserved function among diverse eukaryotes (Marton *et al.* 1997). We addressed whether *GCN1* was required for mGCN2 control of translation in yeast by introducing the mGCN2β and mgcn2-m2 cDNAs expressed from the galactose-inducible promoter in strain H2683 (*gcn2*Δ *gcn1*Δ) or an isogenic strain H2511 (*gcn2*Δ) encoding wild-type GCN1. The slow growth phenotype associated with expression of mGCN2 was observed in cells grown in galactose-containing medium independent of *GCN1* function (Figure 9). Additionally, the growth of mgcn2-m2-containing cells in SGalSM was also found to be similar in the presence or absence of *GCN1*. By comparison, deletion of *GCN1* blocked the ability of yGCN2 to stimulate the general amino acid control pathway, leading to growth sensitivity to SM. These results suggest that at least in the yeast model system, the eIF-2α kinase activity of mGCN2 functions by a mechanism independent of GCN1. Elevated expression levels of mGCN2 may also contribute, in part, to the *in vivo* eIF-2α kinase activity that is independent of GCN1. However, the GCN1 independent phenotype is consistent with the observation that there is no sequence similarity between the ribosome association domain located in the extreme carboxyl terminus of yGCN2 and the sequences in mGCN2.

DISCUSSION

Characterization of a mammalian homologue of GCN2 protein kinase: The protein kinase GCN2 was initially cloned from the yeast *S. cerevisiae* as an activator of the general amino acid control pathway in response to amino acid starvation. Previously, it was reported that nutrient deprivation in mammalian cells also alters translation initiation by elevating phosphorylation of eIF-2α. By analogy with the yeast system, this has led to the speculation that a homologue of GCN2 may carry out translational control in mammals (Clemens 1996). In this report, we identified a mammalian homologue of the GCN2 protein kinase. We cloned cDNAs for three different isoforms of mGCN2-encoding polypeptides with juxtaposed eIF-2α kinase and HisRS-related domains characteristic of the yeast GCN2 (Figure 1). Expression of mGCN2 controls translation in the yeast system by phosphorylation of eIF-2α (Figure 7). Both *in vitro* and *in vivo* analyses showed a requirement for the serine-51 regulatory site of eIF-2α for mGCN2 function. The kinase and HisRS-related domains of mGCN2 were found to be important for activity. These studies indicate that mGCN2 is a new mammalian eIF-2α kinase important for regulation of translation initiation. Additionally, conservation of the kinase catalytic domain and regula-

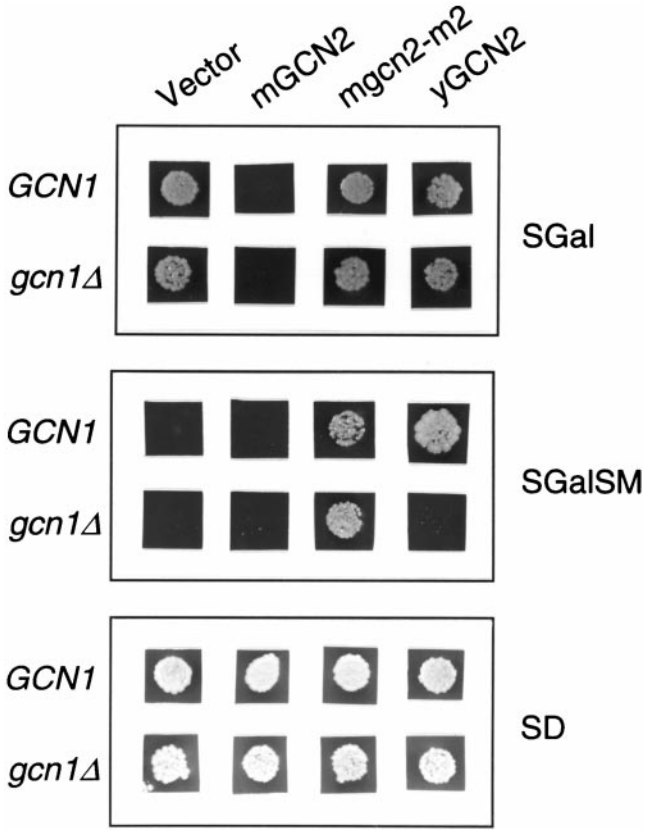

Figure 9.—Mouse GCN2 functions in the yeast translational control system independent of GCN1 protein. Plasmids encoding mGCN2, mgcn2-m2, and yGCN2 were introduced into the isogenic yeast strains H2683 (*gcn2*Δ *gcn1*Δ) and H2511(*gcn2*Δ). *GCN1* encodes a ribosome-associated protein that is essential for yGCN2 phosphorylation of eIF-2α and is thought to be important for yGCN2 interaction with uncharged tRNA. Yeast cultures were spotted onto agar plates containing synthetic medium supplemented with glucose (SD), or galactose (SGal), or galactose and sulfometuron methyl (SGalSM). Agar plates were incubated for 4 days at 30° and photographed. Expression of mGCN2 inhibited cell growth in galactose-inducing media even in the absence of *GCN1*. Yeast cells expressing the partially defective mgcn2-m2 version were found to grow in the SGalSM media independent of *GCN1*. In contrast, growth of yGCN2-containing cells required the presence of the GCN1 protein.

tory sequences between the yeast and mouse homologues suggests common mechanisms control eIF-2α phosphorylation by the GCN2 protein kinase. Together with PKR, HRI, and the recent characterization of pancreatic eIF-2α kinase (PEK; Shi *et al.* 1998; Harding *et al.* 1999), there are currently four characterized mammalian kinases that control translation initiation by phosphorylation of serine-51 in eIF-2α. While the catalytic domains of these eIF-2α kinases share sequence and structural features distinct from other eukaryotic protein kinases, their flanking regulatory sequences are dissimilar, providing for regulation by different cellular stress signals.

Activation of mammalian eIF-2α kinases by cellular stress: The mRNAs encoding α, β, and γ isoforms of mGCN2 were found to be differentially expressed in a variety of mouse tissues, including brain, liver, skeletal muscle, and testis (Figures 5 and 6). Phosphorylation of eIF-2α and inhibition of protein synthesis was observed in many of these cell types in response to different stresses (Clemens 1996). Notably, impaired amino acid metabolism by starvation, chemical inhibition, or temperature-sensitive aminoacyl-tRNA synthetase mutants elicits phosphorylation of eIF-2α and impaired initiation of translation (Pollard *et al.* 1989; Kimball *et al.* 1991; Pain 1994; Clemens 1996). Given the well-characterized role of yGCN2 in the general amino acid control pathway, it is likely that mammalian GCN2 responds to this starvation signal. However, yGCN2 was also observed to phosphorylate eIF-2α in response to purine limitation by a mechanism involving the HisRS-related sequences (Rolfes and Hinnebusch 1993). Thus, GCN2 protein kinase appears to be regulated by multiple starvation signals in yeast and suggests a role for a more generalized nutrient regulation of mammalian GCN2. This activation by different nutrient limitations may indicate that GCN2 protein kinase interacts with other regulatory ligands in addition to uncharged tRNA; alternatively, starvation for nutrients such as purines may contribute to elevated uncharged tRNA levels.

Role of eIF-2α kinases in the regulation of general and gene-specific translation: In the yeast model system, GCN2 phosphorylation of eIF-2α during amino acid limitation delays translation reinitiation but does not impact general protein synthesis (Wek 1994; Hinnebusch 1997). It is postulated that the level of eIF-2α phosphorylation that occurs during starvation leads to a modest decrease in the activity of this initiation factor and reinitiation may be particularly sensitive to reductions in the levels of eIF-2 ternary complex (Dever *et al.* 1992; Wek 1994; Hinnebusch 1997). Consistent with the idea that the level of eIF-2α phosphorylation can delineate between general or gene-specific translation, yeast cells expressing constitutively active mutants of GCN2 or high levels of human PKR were found to hyperphosphorylate eIF-2α and suffer a slow growth phenotype due to reduced protein synthesis (Feng *et al.* 1992; Ramirez *et al.* 1992; Dever *et al.* 1993; Romano *et al.* 1995; Zhu *et al.* 1997). Also contributing to the degree to which eIF-2-GTP levels are reduced by phosphorylation are the cellular levels of this initiation factor relative to the guanine exchange factor, eIF-2B (Clemens 1996).

Given our finding of a mammalian homologue for GCN2 protein kinase, it is inviting to speculate that mammalian cells may also stimulate gene-specific translation in response to phosphorylation of eIF-2α. Although a mammalian homologue of GCN4 has not yet been found, a different regulatory protein may be translationally controlled through multiple upstream ORFs in its mRNA. Numerous mammalian genes have been

reported to have increased transcriptional expression in response to amino acid limitation (Phojanpelto and Holtta 1990; Gong *et al.* 1991; Guerrini *et al.* 1993; Kilberg *et al.* 1994; McGivan and Pastor-Anglada 1994; Bruhat *et al.* 1997). Notably, asparagine synthetase, a gene regulated in the general amino acid control pathway in yeast, has elevated transcription in mammalian cells starved for one of several different amino acids (Gong *et al.* 1991; Guerrini *et al.* 1993; Hutson and Kilberg 1994). Furthermore, Andrulis *et al.* (1979) observed that elevated uncharged tRNA in CHO cells not limited for amino acids facilitated increased transcription of the asparagine synthetase gene. This was a true general control response as described in yeast because asparagine synthetase expression was elevated in mutant CHO containing temperature-sensitive mutations in asparaginyl-, leucyl-, methionyl-, or lysyl-aminoacyl-tRNA synthetases that were transferred from permissive to restrictive conditions. Sequences in the asparagine synthetase promoter from nucleotides −164 to +44 were found to be sufficient for induced gene expression. Included within this region is an amino acid response element (AARE) suggested to associate with a transcriptional activator protein (Guerrini *et al.* 1993). The identity of this regulatory protein and the role of amino acid limitation on its function awaits further study.

Amino acid starvation also stimulates the expression of CHOP and calreticulin (Bruhat *et al.* 1997; Heal and McGivan 1998). These proteins are important for facilitating proper protein folding in the endoplasmic reticulum in response to a variety of different cellular stresses that, in addition to starvation for amino acids, include glucose limitation and depletion of calcium stores. Many of the stress signals that trigger this unfolded protein response were also observed to impair translation by phosphorylation of eIF-2α. Recent work on PEK suggests that eIF-2α kinases can function as part of a larger coordinated response linking the protein synthetic and folding pathways.

In closing, GCN2 protein kinase has been identified in eukaryotic organisms, from yeast to mammals. In each of the GCN2 homologues, there is conservation of the three central regions: partial kinase, kinase catalytic, and HisRS-related sequences. We identified three isoforms of mGCN2 that result in predicted mGCN2 polypeptides with different amino-terminal sequences. While the function of the sequences in the amino terminus of GCN2 is not yet understood, the fact that the mGCN2 isoforms are differentially expressed in mouse tissues suggests a role for these sequences in the regulation of kinase activity in response to different stress conditions. An understanding of the mechanisms modulating GCN2 activity in mammalian tissues and the role of this eIF-2α kinase in the control of general and gene-specific translation awaits future genetic and biochemical analyses.

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