Isolation of the Gene Encoding the *Drosophila melanogaster* Homolog of the *Saccharomyces cerevisiae GCN2* eIF-2α Kinase

DeAnne S. Olsen,*,[1] Barbara Jordan,*,[2] Dreeny Chen,*,[3] Ronald C. Wek† and Douglas R. Cavener*

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

Manuscript received October 30, 1997
Accepted for publication April 13, 1998

ABSTRACT

Genomic and cDNA clones homologous to the yeast *GCN2* eIF-2α kinase (*yGCN2*) were isolated from *Drosophila melanogaster*. The identity of the Drosophila *GCN2* (*dGCN2*) gene is supported by the unique combination of sequence encoding a protein kinase catalytic domain and a domain homologous to histidyl-tRNA synthetase and by the ability of *dGCN2* to complement a deletion mutant of the yeast *GCN2* gene. Complementation of Δ*gcn2* in yeast by *dGCN2* depends on the presence of the critical regulatory phosphorylation site (serine 51) of eIF-2α. *dGCN2* is composed of 10 exons encoding a protein of 1589 amino acids. *dGCN2* mRNA is expressed throughout Drosophila development and is particularly abundant at the earliest stages of embryogenesis. The *dGCN2* gene was cytogenetically and physically mapped to the right arm of the third chromosome at 100C3 in STS Dm2514. The discovery of *GCN2* in higher eukaryotes is somewhat unexpected given the marked differences between the amino acid biosynthetic pathways of yeast *vs.* Drosophila and other higher eukaryotes. Despite these differences, the presence of *GCN2* in Drosophila suggests at least partial conservation from yeast to multicellular organisms of the mechanisms responding to amino acid deprivation.

A major control point regulating eukaryotic protein synthesis is the phosphorylation of the α subunit of eukaryotic initiation factor-2 (eIF-2α) by a family of eIF-2α–specific kinases. This extensively studied process involves inhibition of a guanylate exchange factor, eIF-2B. eIF-2B is required to recycle eIF-2/GTP so that sufficient ternary complex (eIF-2/GTP/initiator tRNA-methionine) is present to initiate translation. The eIF-2α kinases have been cloned previously in both mammalian (PKR and HRI) and yeast (GCN2) systems. Each of these eIF-2α kinases originally were identified as affecting protein synthesis in response to physiological stress signals. PKR participates in the interferon-induced antiviral response and is thought to be activated by double-stranded RNA produced during viral infection. PKR has been implicated in a number of other processes including cellular growth, differentiation, oncogenesis, and apoptosis (Petryshyn *et al.* 1988; Li and Petryshyn 1991; Koromilias *et al.* 1992; Barber *et al.* 1995; Der *et al.* 1997; Lee *et al.* 1997; Williams 1997). HRI is activated in reticulocytes by low levels of hemin or by heat shock. These mammalian kinases, when active, are thought to effect a global repression of protein synthesis by ternary complex limitation (for reviews see Mathews 1990; Wek 1994; Chen and London 1995). The yeast eIF-2α kinase GCN2 is activated by amino acid starvation. Unlike the mammalian kinases, GCN2 derepresses the translation of a single mRNA encoding the transcription factor GCN4 without repressing global protein synthesis. GCN4 activates the transcription of more than 30 amino acid biosynthetic enzymes participating in multiple biosynthetic pathways. This transcriptional upregulation occurs in response to starvation for any one of at least 10 amino acids, and thus has been termed the "general control" of amino acid biosynthesis in yeast (for a review see Hinnebusch 1988).

Control of amino acid biosynthesis by eIF-2α phosphorylation has been studied extensively in yeast (for reviews see Wek 1994 and Hinnebusch 1996), and occurs by an elaborate mechanism involving the 5′-untranslated region (5′ UTR) of the *GCN4* mRNA. This unique leader contains four short upstream open reading frames (uORFs 1–4), whose ability to engage the ribosome in translational initiation and termination is critical for translational control. When amino acids are not limiting, after translating uORF 1, the 40S ribosomal subunits are thought to remain associated with the mRNA and reinitiate at one of the downstream uORFs 2, 3, or 4. In the example of uORF 4, the unique sequence context surrounding its stop codon causes strong translational termination in which the 40S ribosomal subunits dissociate from the mRNA. As a result, translation

Corresponding author: Douglas R. Cavener, Department of Molecular Biology, Vanderbilt University, Box 1820, Station B, Room SC2527, Nashville, TN 37235. E-mail: dcavener@ctrvax.vanderbilt.edu

[1] *Current address:* Laboratory of Eukaryotic Gene Regulation, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892.

[2] *Current address:* Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

[3] *Current address:* College of Medicine, Emory University, Atlanta, GA 30322.

at the authentic *GCN4*-coding sequence is inhibited. However, under conditions of amino acid starvation, yGCN2 is activated by the presence of increased levels of uncharged tRNA, leading to eIF-2α phosphorylation and a reduction in the amount of ternary complex available to initiate translation. GCN2 senses the level of uncharged tRNA via a domain closely related to histidyl-tRNA synthetases. Ternary complex limitation is thought to increase the length of time required for the scanning ribosome to reinitiate translation. Thus, 40S ribosomal subunits bypass the downstream uORFs 2, 3, and 4 and, instead, reinitiate at the authentic *GCN4* AUG. As a result, *GCN4* translation is derepressed.

A similar "cross-pathway" control system appears to exist in at least two other lower eukaryotes, *Neurospora crassa* and *Aspergillus nidulans*, as well as in higher plants (Guyer *et al.* 1995; Sachs 1996). Metazoans, however, cannot synthesize all 20 amino acids and obtain the 10 essential amino acids from their diet. This raises the question of how starvation for essential and nonessential amino acids may affect protein synthesis in higher eukaryotes.

Although it has been frequently speculated that an eIF-2α kinase may exist in higher eukaryotes to regulate a response to amino acid deprivation, attempts to identify biochemically a specific eIF-2α kinase have not been successful. We report the identification of an apparent homolog of yeast *GCN2* eIF-2α kinase in Drosophila, Drosophila *GCN2* (*dGCN2*). The presence of this enzyme in higher eukaryotes strongly suggests that similar pathways for controlling amino acid starvation may exist between lower and higher eukaryotes despite the difference in their capacities to synthesize amino acids.

MATERIALS AND METHODS

PCR cloning: Total RNA was isolated from developmentally staged Drosophila embryos, larvae, pupae, and adults (13 stages total) using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD) following reagent protocol. Up to 50 mg of tissue was homogenized by pellet pestle per sample. Poly(A)$^+$ RNA was isolated by the Oligotex mRNA Mini Kit (Qiagen, Chatsworth, CA) using 250 μg total RNA per sample. First-strand cDNA was synthesized by random hexamer priming using the Superscript II RT kit (GIBCO-BRL) from an amount of poly(A)$^+$ RNA equivalent to 5 μg of total RNA. Approximately 20 μl of cDNA was synthesized per sample. Two microliters of cDNA was used in PCR amplification using degenerate oligonucleotide primers (5′ primer: 5′ ATY CAA RAT GSA RYW SGY GA; 3′ primer: 5′ GGY TTS ARR TCR CGR TG) at a final concentration of 1 μm each in a 50-μl reaction with 1.5 mm $MgCl_2$. The PCR amplification profile included an initial denaturation at 94° for 5′ followed by a cycle of 94° for 15 sec, 43° for 30 sec, and 72° for 1 min, for a total of 30 cycles, followed by a final elongation at 72° for 7 min. A "hot-start" procedure was used by adding a cocktail containing the polymerase and dNTPs after the thermocycler reached 94°. Twenty microliters (40%) of each PCR reaction was visualized by agarose gel electrophoresis.

Screening of PCR products: Entire PCR reactions were cloned *en masse* into the TA cloning vector pT7Blue (Novagen, Inc., Madison, WI). Plasmid DNA (Qiagen) was isolated from the 310 colonies found to hybridize to both PCR primers, and the thymidine pattern was sequenced by Sequenase (U.S. Biochemical Co., Columbus, OH) using a modified annealing reaction. Sequencing was primed using a T7 promoter primer (5′ TAA TAC GAC TCA CTA TAG GG). Plasmids containing extremely short inserts and plasmids containing obviously duplicate sequences were eliminated. Some 125 plasmids containing apparently unique sequences were fully sequenced on one strand by T7 priming. Deduced amino acid sequences were subjected to a BLAST protein database similarity search using a Pam250 matrix.

Library screening: Some 10^6 pfus from a Drosophila Canton-S (18-hr embryonic) cDNA library in λgt10 (Clontech, Palo Alto, CA) were screened using a ^{32}P-labeled random primed probe (Decaprime II; Ambion) consisting of four tandem repeats of the original *GCN2*-like PCR product (derived from pTK334). Phage DNA was isolated using the Qiagen Lambda Maxi Kit, and inserts were released with *Eco*RI digestion and cloned into pBluescript (KS+). Subsequent sequence analysis indicated that library inserts were truncated internally at *Eco*RI sites during library construction. Full-length cDNA sequence thus was isolated by rapid amplification of cDNA ends (RACE; see below).

Some 10^6 pfus from a Drosophila Canton-S (0–12-hr embryonic) genomic library in λFIXII (Stratagene, La Jolla, CA) were also screened using the pTK334-derived probe. Phage DNA was isolated from positively screening clones (Lambda Maxi Kit; Qiagen); inserts were released with *Not*I digestion and cloned into pBluescript (KS+). The molecular map of these clones was determined by a combination of restriction digestion and Southern hybridization. A variety of smaller restriction fragments were subcloned and sequenced to analyze the sequence of these clones.

5′ and 3′ RACE: To isolate terminal cDNA sequence, RACE was performed on total RNA isolated from 2–4-hr Drosophila embryos. The 5′ RACE system for rapid amplification of cDNA ends (GIBCO-BRL) was used to isolate 5′ cDNA sequence. A *dGCN2*-specific primer (5′ GTG ACT ATG GGA TAC AC) was used to synthesize first-strand cDNA. PCR amplification was performed using a second, nested *dGCN2*-specific primer (5′ TAC TAA GGC ATC CAG GAC ACC G) with the Abridged Anchor Primer provided in the kit. The single ~1.5-kb PCR product was cloned into pT7Blue and sequenced.

3′ RACE was performed using a single anchored oligo(dt) primer (5′ CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TTV N) to synthesize first-strand cDNA. PCR amplification was performed using this same oligonucleotide as a 3′ PCR primer with a set of three staggered *dGCN2*-specific primers as 5′ primers (5′ GAT ACG ACT CTA TGC TGC ATG; 5′ AGC CAT TTG TCA GCC TTG; 5′ CCT CAA GAA GGA GAC CTT TG).

Construction of full-length *dGCN2* cDNA: As the λgt10 cDNA clones were all truncated at two internal *Eco*RI sites, corresponding to nucleotide positions +1889 to +4151, it was necessary to construct full-length cDNA in segments. 5′ and 3′ RACE products did not overlap with the λgt10 cDNA clones, as, at that time, more 5′ and 3′ terminal sequence was known from genomic sequencing. Thus, PCR products overlapping with the λgt10 *Eco*RI fragment and corresponding to positions +204 to +1977 and from positions +3945 to +5520 were amplified using two sets of *dGCN2*-specific primers (nt 204–1977: 5′ AA CTG CAG1 TTG TGA AAT TGC ACA CCG and 5′ ATG GCG TAC TCT CGG TTG; nt 3945–5520: 5′ CAT TGC GGG AAC TTG AAA C and 5′ AA CTG CAG GCA AGA AAG TTC GGA TAC TTA ATC). In the primer sequences, the underlined *Pst*I sites were included for subsequent cloning purposes. *Pst*I does not cleave the *dGCN2* cDNA between posi-

tions +204 and +5520). PCR amplification was performed using Vent DNA polymerase (New England Biolabs, Beverly, MA) on oligo(dt)-primed, first-strand cDNA (Superscript) synthesized from 2–4-hr embryonic total RNA (GIBCO-BRL). The 5′ PCR product was digested with *Pst*I/*Eco*RI and inserted into similarly cut pBluescript(KS+), generating pCK423. The 3′ PCR product was cloned without restriction digestion into pT7Blue at the *Eco*RV/T-A cloning site, generating pTK424.

A 2.2-kb *Eco*RI fragment corresponding to the originally isolated *dGCN2* cDNA λgt10 clone was inserted into similarly cut pBluescript (KS+), generating pCK344. To remove the 3′ *Eco*RI site of pCK344, allowing easy insertion of the 5′ PCR product at the 5′ *Eco*RI site, pCK344 was digested with *Nde*I (a unique site within the *dGCN2* insert at +4009) and *Hin*dIII (a unique site in the vector that was not contained in the *dGCN2* insert). This *Nde*I-*Hin*dIII fragment was replaced with a 99-bp *Nde*I-*Hin*dIII fragment from pSP72 (Promega, Madison, WI), generating pCK422 with a unique *Eco*RI at the 5′ portion of the cDNA insert.

The 5′ PCR product, as a 1.8-kb *Pst*I/*Eco*RI fragment from pCK423, was inserted in frame into similarly cut pCK422, generating pCK428. The 3′ PCR product, as a 1.5-kb *Nde*I fragment of pTK424, was inserted in frame into *Nde*I-digested pCK428, generating pCK429. Thus, the entire *dGCN2*-coding sequence was contained in frame in pCK429 as a 5.3-kb *Pst*I fragment.

Yeast plasmid construction: *dGCN2* full-length coding sequence, corresponding to positions +204 to +5520, was inserted as a 5.3-kb *Pst*I fragment from pCK429 into similarly cut pEMBLyex4 (Cesareni and Murray 1987), representing a leader–leader fusion and generating pYK380. This fusion places *dGCN2* under the control of the *GAL1-CYC1* hybrid promoter.

An alternative *dGCN2* construct was generated that removed the entire *dGCN2* 5′ UTR and added an N-terminal polyhisitidine tag for applications not included in this manuscript. To generate pEK390, a 1404-bp *dGCN2* cDNA fragment was ligated into *Eco*RV/*Sal*I cut pET30b+ (Novagen), replacing positions 206–179 of pET30b+ with positions +357 to +1760 in the *dGCN2* cDNA and fusing the *dGCN2* coding sequence to the first 47 codons of the pET30b+ cloning/expression region. This fusion replaces the *dGCN2* 5′ UTR and the first 11 codons of the *dGCN2* coding sequence with the pET30b+ N-terminal coding sequence containing a six-histidine (6-His) tag immediately after the start codon. A 1397-bp *Xba*I fragment from pEK390, consisting of 38 nt of pET30b+ 5′ UTR through the fusion point to position +1576 in *dGCN2* cDNA, was ligated into similarly cut pCK429, replacing the 5′ terminus of wild-type *dGCN2* with the new leader and the polyhisitidine-tagged terminus from pET30b+ and generating pCK392.

A series of cloning steps was then performed to provide the appropriate *Hin*dIII ends for cloning this 6-His–tagged *dGCN2* into the yeast expression vector. The 5′ terminus of His-tagged *dGCN2* was removed from pCK392 by *Sac*I/*Eco*RV digestion, with the *Sac*I site lying with the multiple cloning site of the pBluescript vector upstream of the *Xba*I cloning site in pCK392, and the *Eco*RV site lying within the *dGCN2* cDNA at position +573. This 414-bp fragment was ligated into similarly cut pET30b+, generating pEK393. In pEK393, a *Kpn*I site lies 33 bp upstream of the original *Nru*I/*Eco*RV junction of pET30b+ and *dGCN2* coding sequences, and another *Kpn*I site lies 3′ to the *dGCN2* insert in pEK393. Thus, pEK393 was digested with *Kpn*I to remove most of the pET30b+/*dGCN2* insert, and an ~5.2-kb *Kpn*I fragment from pEK392 was inserted in its place. This *Kpn*I fragment corresponds to the identical 5′ *Kpn*I site upstream of the original fusion point and contains the entire *dGCN2* cDNA from +357 to +5520, flanked by a 3′ *Kpn*I site from the multiple cloning site in pEK392. Thus, pEK398 contains positions 384–206 of pET30b+ in a coding sequence fusion to *dGCN2* cDNA from +357 to +5520, flanked by *Hin*dIII sites. The full-length, his-tagged *dGCN2* cDNA was ligated as an ~5.5-kb *Hin*dIII fragment into similarly cut pEMBLyex4, representing a leader–leader fusion and placing the 6-his–*dGCN2* fusion under the control of the hybrid *GAL1-CYC1* yeast promoter and generating pYK399.

Cytogenetic localization of *dGCN2*: The chromosomal location of the *dGCN2* gene was determined by two methods, *in situ* hybridization of polytene chromosomes and analysis of an ordered P1 phage genomic Drosophila library. For *in situ* hybridization, salivary glands were dissected (in 45% acetic acid) from third instar Drosophila larvae fed on high glucose media with fresh yeast. Glands were squashed between coverslip and slide in 45% proprionic acid, clamped, and frozen in liquid nitrogen for 15–30 sec. After freezing, the coverslips were removed, the slides washed in 70% ethanol for 5 min and 95% ethanol for 5 plus 10 min, and then the slides were air dried. Before hybridization, slides were treated with the following set of washes: 2× SSC/70°/30 min, 70% ethanol/2× 10 min, 95% ethanol/5 min/air dried, 0.07 m NaOH/3 min, 70% ethanol/2× 5 min, and 95% ethanol/5 min/air dried.

A digoxygenin (DIG)-labeled probe corresponding to a 2.2-kb *Eco*RI cDNA restriction fragment (pCK344) was prepared using the DIG High Prime Labeling and Detection Starter Kit (Boehringer Mannheim, Indianapolis). Slides were prehybridized in 50 μl of 40% formamide, 6× SSC, 5× Denhardt's solution, and 100 μg/ml sheared, denatured salmon sperm DNA for 15 min at room temperature (RT). After prehybridization, slides were hybridized in 20 μl of a solution containing 45% formamide, 6× SSC, 5× Denhardt's solution, 10% dextran sulphate, and 20 ng DIG-incorporated probe for >16 hr at 42° in a closed environment. Posthybridization stringency washes consisted of 45% formamide/6× SSC/42°/2× 15 min, 2× SSC/RT/2× 5 min, and 0.2× SSC/50°/7 min. Probe hybridization was carried out following the kit protocol; the color reaction was allowed to continue for 16 hr. Hybridization was localized by microscopy, and the *dGCN2* gene was found to be positioned on the right arm of the third chromosome at band 100C.

Eight overlapping P1 clones known to correspond to 100C were obtained (DS00057, DS01453, DS02433, DS02413, DS-02816, DS03260, DS03999, and DS08061; Hugo Bellen, Baylor University School of Medicine, Houston). PCR amplification of crude plasmid DNA isolated from these eight cosmid clones using *dGCN2*-specific primers (5′ primer: 5′ GGA TAG AAA GTG TAG ATG ACG CAG; 3′ primer: 5′ GCC TTG CTG GTG AAT ATG CG) indicated that 26-13 contained *dGCN2* sequence. DNA from clones DS02413 and DS08061 was compared to genomic λFIXII library clones by Southern hybridization of restriction fragments to a ^{32}P-labeled *dGCN2* probe corresponding to a 0.93-kb *Eco*RI genomic fragment (derived from pCK353). Additional P1 clones—DS00737, DS02910, DS-05535, and DS07511—were obtained and subjected to colony hybridization to the 0.93-kb probe.

Developmental reverse transcription PCR: Total RNA was isolated by the RNeasy Total RNA kit (Qiagen) from developmentally staged flies. First-strand cDNA was synthesized by random hexamer priming using the Superscript II kit (GIBCO-BRL) using 5 μg of total RNA per 20 μl reaction. All optimization experiments were performed with a single sample of cDNA from one developmental stage, 11- to 13-hr embryonic. To distinguish definitively between cDNA and any contaminating genomic DNA, amplification was performed across introns 5 and 6 of *dGCN2* (65 and 64 nt in length,

respectively) using *dGCN2*-specific primers (5′ primer: 5′ GGA TAG AAA GTG TAG ATG ACG CAG; 3′ primer: 5′ GCC TTG CTG GTG AAT ATG CG). As an internal control, both for the amount of template cDNA per reaction and for the amount of polymerase activity per reaction, PCR primers used to amplify Drosophila *eIF-2α* cDNAs also were included in the PCR reaction (5′ primer: 5′ CGA AAA GTC CAA ATT GCC; 3′ primer: 5′ GGC GCG AAT GTG CTC AAT). These primers span intron 1 (248 nt) in *eIF-2α*. $MgCl_2$ and primer concentrations were optimized at 1.5 mm and 0.25 μm, respectively, and 2 μl of cDNA was used per reaction. Each PCR reaction was amplified for 30 cycles, and products were visualized by agarose gel electrophoresis followed by ethidium bromide staining.

After initial optimization, DNase I–treated (GIBCO-BRL) cDNA samples were serially diluted in twofold increments (1:2, 1:4, 1:8) yielding relative concentrations of 8×, 4×, 2×, and 1×, 8× being the undiluted cDNA sample. These diluted cDNA templates were amplified at a series of reduced cycle numbers (30, 25, 22, 20, and 18 cycles) to determine the number of cycles producing amplification within a linear range of reaction. PCR products were detected by Southern hybridization to internal oligonucleotide probes (GCN2 probe: 5′ TGC TCT TCG TCG TCG TAG AC; eIF-2α probe: 5′ GGA CAG CAC GTT CAC CAT). Hybridization was quantitated by PhosphorImager scanning (PhosphorImager 445SI; Molecular Dynamics, Sunnyvale, CA) using the IP Lab Gel software (Signal Analytics, Vienna, VA). The optimal number of cycles to produce linear amplification was determined to be 25 cycles.

Data regarding the developmental expression levels of *dGCN2* mRNA were obtained by PCR amplification of multiple cDNA samples isolated from several stages of Drosophila development. Optimal PCR conditions for linear amplification were used, PCR products were detected by oligonucleotide hybridization, and amount of hybridization was quantitated by PhosphorImager scanning. Levels of *dGCN2* amplification were normalized to those of *eIF-2α*.

Complementation of a yeast GCN2 null mutation: pYK380 or pYK399, pEMBLyex4, pRS316 (Sikorski and Hieter 1989), and p722 (Wek *et al.* 1990) were introduced into the yeast strain H1894 (*MAT***a** *gcn2Δ ura3-52 leu2-3 leu2-112 trp1-Δ63*; Vazquez de Aldana *et al.* 1994), and pEMBLyex4 was introduced into H1896 (*MAT***a** *GCN2 ura3-52 leu2-3 leu2-112 trp1-Δ63 Δsui2 GCN4-lacZ* p1097 [*SUI2 LEU2*] (Wek *et al.* 1995) using standard yeast transformation procedures, and was grown on SD/-ura minimal media. In addition, pYK399 and pEMBLyex4 were introduced into yeast strains H1816 (*MAT***a** *gcn2Δ ura3-52 leu2-3 leu2-112 trp1-Δ63 Δsui2 GCN4-lacZ* [*SUI2-LEU2*] and H1817 (*MAT***a** *gcn2Δ ura3-52 leu2-3 leu2-112 trp1-Δ63 Δsui2 GCN4-lacZ* [*SUI2 - S51A LEU2*] (Dever *et al.* 1992). To determine complementation of the yeast GCN2 starvation response, yeast transformants were plated on starvation-inducing media. Plating was performed by two alternative methods: by plating cells grown in liquid culture or by replica-plating cells from agar plates. For liquid culture plating using strain H1894, transformants were grown overnight at 30° in SD-ura liquid media, and titers were diluted to an OD_{600} of 5×10^{-2}. Three 10-fold serial dilutions were prepared, representing titers of 5×10^{-3}, 5×10^{-4}, and 5×10^{-5}. For strains H1816 and H1817, titers of overnight cultures were diluted to an OD_{600} of 5×10^{-1}, and three sevenfold serial dilutions were prepared, representing titers of 7×10^{-2}, 1×10^{-2}, and 1.4×10^{-3}. These dilutions were plated by pronged replica plator on a series of growth media including SD/-ura, SD/-ura/-his/+10 mm 3-aminotriazole (3-AT), SGal/raf/-ura, and SGal/raf/-ura/-his/+10 mm 3-AT. Galactose and raffinose were included at 10 and 2% concentrations, respectively. Unless otherwise noted, synthetic media were supplemented with histidine, tryptophan, lysine, leucine, and adenine. Yeast were incubated at 30°, and growth was observed for time of growth, number of colonies, and size of colonies. For replica plating from agar plates, synthetic medium was supplemented with 10% galactose, 2% raffinose, all amino acids except histidine, and 3-AT at the indicated concentrations. Yeast were incubated at 30° and observed for time and extent of growth.

RESULTS

Isolation of Drosophila GCN2 (*dGCN2*) cDNA: To identify and isolate potential eIF-2α kinases in *Drosophila melanogaster*, we used a PCR-based strategy. Degenerate PCR primers were designed against kinase subdomains V and VIB using the sequences of the known eIF-2α kinases (see underlined residues in Figure 2). Kinase subdomain V contains residues that are more unique to eIF-2α kinases, while subdomain VIB is highly conserved among serine-threonine kinases as distinguished from tyrosine kinases. These primers were used to amplify potential eIF-2α kinase sequences present in cDNA populations derived from 13 different developmental stages of *D. melanogaster*. PCR amplification was performed under conditions of low stringency, yielding numerous products and thus necessitating several rounds of secondary screening. The deduced amino acid sequences of 125 clones were subjected to a comparison of known protein sequences via a BLAST protein database search.

As expected, several different protein kinases were identified among the PCR-selected cDNA clones. These corresponded to several known Drosophila protein kinases and to potentially new Drosophila homologs of other kinases. Approximately one-half of the kinase clones were essentially identical and shared 56% amino acid sequence identity with yeast GCN2 within the 157-bp amplified region corresponding to kinase subdomains V–VI (Figures 1 and 2). No other potential eIF-2α kinases were identified despite the fact that the PCR primers match the other two eIF-2α kinases, PKR and HRI, better than they match most of the other protein kinase sequences isolated. As our screen was not biased toward a single eIF-2α kinase or developmental stage, it appears that if other Drosophila eIF-2α kinases exist, they are not expressed under normal growth conditions at levels detectable by our PCR strategy.

To facilitate the isolation of a full-length cDNA sequence of the putative *dGCN2* gene, we screened a cDNA library prepared from 18-hr embryonic cDNA using the GCN2-like PCR product as a probe. Several cDNA clones were identified and found to contain the same sequence as the initial PCR products, as well as substantial flanking sequence homologous to *yGCN2*. All clones, however, were truncated internally at two *Eco*RI sites (2.2 kb in cDNA), presumably because of faulty methylation protection during the construction of this library by Clontech. We therefore performed RACE to isolate the 5′ and 3′ termini of *dGCN2* cDNA. Embryonic total RNA was used as the template for cDNA

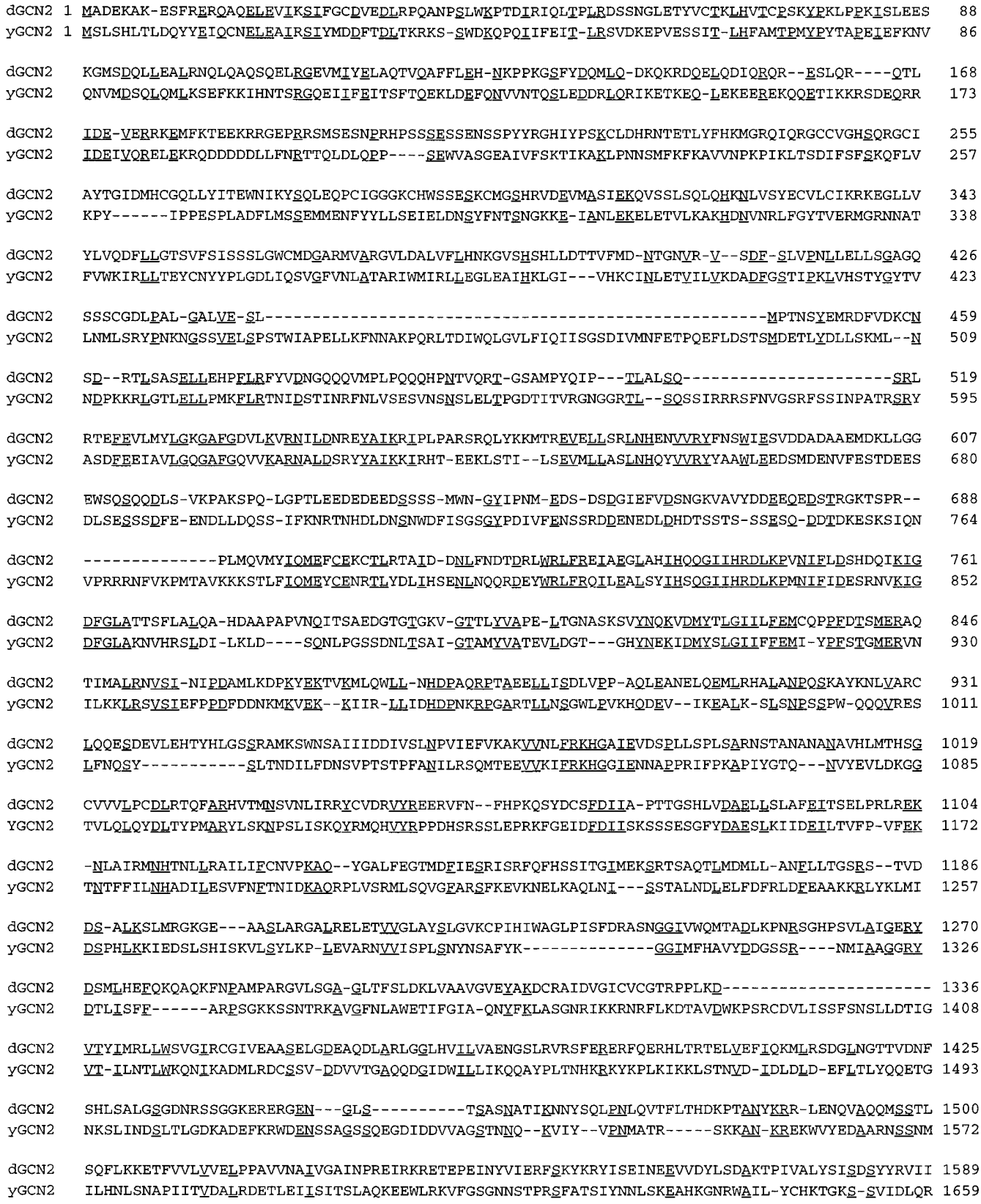

Figure 1.—Alignment of the amino acid sequence of Drosophila and yeast GCN2. Identities are underlined. The positions of conserved kinase subdomains found in the upstream kinase-like domain (lowercase roman numerals) and in the catalytic domain (uppercase roman numerals) are indicated. Also shown are positions of conserved motifs in the HisRS-related domain and the position of the ribosome association domain identified in yGCN2.

synthesis as *dGCN2* mRNA is expressed well at this stage (Figure 7 and data not shown). 5′ RACE identified a single 5′ terminus encoding the 5′ UTR and apparent N terminus of the *dGCN2*-coding region. 3′ RACE identified two 3′ termini from each of three staggered *dGCN2*-specific primers used for PCR amplification. Sequence analysis indicated that the ends are colinear, one extending 189 nt further than the other. Potential polyadenylation signals exist for each 3′ end.

Sequence analysis of the putative *dGCN2* cDNA predicts an mRNA of 5749 nt for the longer 3′ end (5559 nt for the shorter 3′ end) and a coding sequence of 1589 amino acids. The 5′ end of *dGCN2* contains 71 residues that do not correspond well to the published sequence of yeast GCN2 (Wek *et al.* 1989). However, a search of GenBank revealed an unpublished *yGCN2* sequence containing 69 additional N-terminal amino acids (1659 residues total) that align well to the putative amino terminus of *dGCN2* (Figure 1). We therefore argue that these two sequences comprise the authentic and complete GCN2 coding sequences in yeast and Drosophila. The 5′ UTR of *dGCN2* is predicted to have a length of 325 nt, while the 3′ UTR predicts a length of 654 nt for the longer and 464 nt for the shorter *dGCN2* mRNA. The start codon context, AGAAAUGG, is consistent with the consensus context found for Drosophila mRNAs (Cavener and Ray 1991).

***dGCN2* contains the kinase catalytic domain and histidyl-tRNA synthetase-like domain found in yeast GCN2:** Sequence analysis of *dGCN2* indicates the presence of the kinase catalytic domain conserved among all kinases (Figure 2), corresponding approximately to amino acids 517–912 within the *dGCN2* coding sequence. The *dGCN2* kinase domain contains all 11 conserved subdomains identified by Hanks *et al.* (1988) and Hanks and Hunter (1995). Within this catalytic motif, *dGCN2* contains an insert region located between kinase subdomains IV and V (Ramirez *et al.* 1992). This insert, found among all eIF-2α kinases, varies greatly between family members in both length and sequence. The *dGCN2* eIF-2α kinase insert is similar in size to that of yeast GCN2. In this region, *dGCN2*, as well as the other eIF-2α kinases, contains clusters of serine residues interspersed with clusters of acidic residues.

Within the kinase domain, *dGCN2* is most similar to *yGCN2* (36% identity) and HRI (34% identity) and somewhat less similar to PKR (25% identity). Some 59 positions in the catalytic domain are conserved among the seven eIF-2α kinase sequences (Figure 2), including 14 positions known to be nearly invariant among all kinases (Ramirez *et al.* 1992). At the junction of domains V and VI, five contiguous residues (WRLFR, residues 722–726) are unique to *dGCN2* and *yGCN2*, as compared to the other eIF-2α kinases.

Like yeast GCN2 (Wek *et al.* 1990), Drosophila GCN2 contains an HisRS-related domain, corresponding approximately to amino acids 949–1447. Drosophila and yeast GCN2 share 17% identity with this region. The presence of this domain in combination with a protein kinase domain is a unique characteristic of GCN2 not found in PKR, HRI, or any other protein kinases. An alignment of the HisRS-related domain of *dGCN2* with that of yeast GCN2 and the histidyl-tRNA synthetases of human, *Saccharomyces cerevisiae*, and *Escherichia coli* (Figure 3) indicates that *dGCN2* contains all three conserved functional motifs found in class II aminoacyl tRNA synthetases (Cusack *et al.* 1991; Ramirez *et al.* 1992; Delarue and Moras 1993; Arnez *et al.* 1995). Although *dGCN2* matches the majority of consensus sequences in motifs 1–3, both Drosophila and yeast GCN2 are missing key residues, particularly the arginine in motif 3 (R311 in *E. coli*) present in HisRSs. This residue is important for HisRS enzymatic function (Arnez *et al.* 1995). In addition two HisRS-specific domains that participate in forming the histidine-binding pocket, histidyl-tRNA synthetases contain the histidine A and B regions (Arnez *et al.* 1995). Yeast and Drosophila GCN2 proteins do not contain the histidine A sequence, but do contain the histidine B sequence (Figure 3; residues 1265–1277 in *dGCN2*). As the HisRS-related domains of both *dGCN2* and *yGCN2* lack the histidine A region and important residues in the motif 3 sequence, we predict they also lack enzymatic activity. Aminoacyl-tRNA–binding activity, however, is not precluded by this prediction. In fact, it has been shown that yGCN2 can bind uncharged tRNA, and that mutations in motif 2 impair tRNA binding. Such binding is thought to be a critical activation function for yeast GCN2 (Wek *et al.* 1995).

Drosophila and yeast GCN2 contain a large N terminus (approximately residues 1–516 in *dGCN2*), the highest degree of conservation lying within the first few hundred residues (Figure 1). This region does not correspond well to any other known proteins, and its function in yGCN2 has not been determined. Downstream of this region (beginning at approximately residue 243 in *dGCN2* and 260 in *yGCN2*), both GCN2s contain an additional protein kinase–like sequence. However, this protein kinase domain lacks a number of residues invariant or highly conserved among active protein kinases (Hanks and Hunter 1995). It is therefore highly unlikely that this domain encodes an active protein kinase; we therefore have termed this region the degenerate-protein-kinase domain. Using extensive independent alignments of Drosophila and yeast GCN2, we have determined which subdomains are present in each degenerate kinase domain. We conclude that the Drosophila protein contains subdomains I–VIII and XI but lacks clear homology to subdomains IX–X, while the yeast protein contains subdomains III–XI. It is interesting to note that these degenerate kinase domains are very poorly conserved between Drosophila and yeast GCN2. Each is considerably more similar to other subclasses of protein kinases than they are to each other.

During the preparation of this manuscript, another

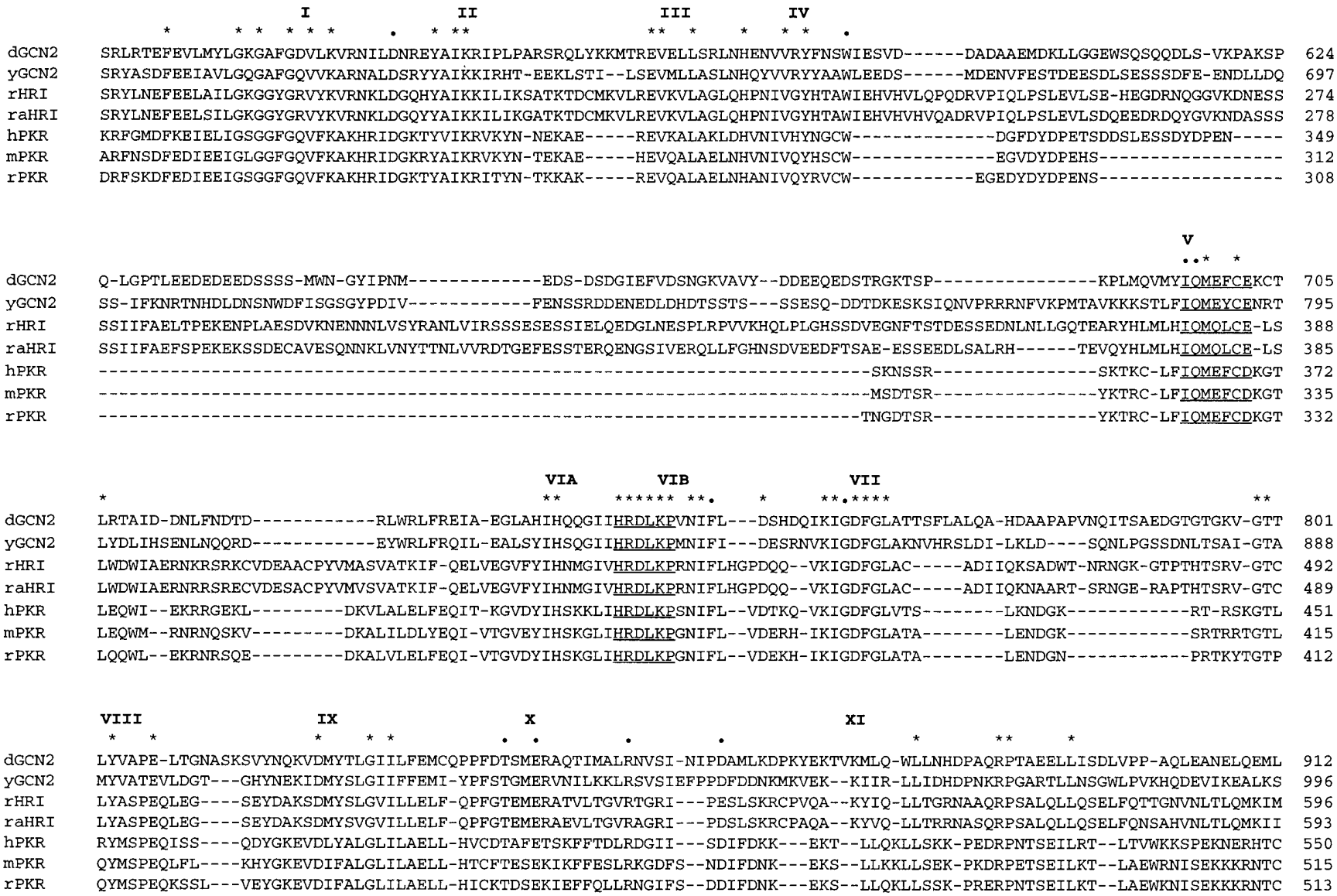

Figure 2.—Alignment of the kinase catalytic domain of *dGCN2* to other eIF-2α kinases. Conserved kinase subdomains are indicated by roman numerals. Asterisks indicate residues conserved among all seven eIF-2α kinases; solid dots indicate residues nearly unique to and conserved among eIF-2α kinases; yGCN2, yeast GCN2; rHRI, rat HRI; raHRI, rabbit HRI; hPKR, human PKR; mPKR, mouse PKR; rPKR, rat PKR.

```
                                        <-----Motif 1------>
consensus                               * ...*..+G*. .*..P**
yHisRS      KLQVSLKTPK GTKDWADSDM VIREAIFSTL SGLFKKHGGV TIDTPVFELR EILAGKYGED S----KLIYN LEDQGGELCS LRYDLTVPFA RYVAMNNI--   123
hHisRS      KQKFVLKTPK GTRDYSPRQM AVREKVFDVI IRCFKRHGAE VIDTPVFELK EILAGKYGED S----LKIYD LKDQGGELLS LRYDLTVPFA RYLAMNKL--   144
bHisRS      -MAKNIQAIR GMNDYLPGET AIWQRIEGTL KNVLGSYGYS EIRLPIVEQT PLFKRAIGEV TDVVEKEMYT FEDRNGDSLT LRPEGTAGCV RAGIEHGLLY    99
yGCN2HisRS  SLTNDILFDN SVPTSTPFAN ILRSQMTEEV VKIFRKHGGI ENNAPPRI-- FPKAPIYGTQ ------NVYE VLDKGGTVLQ LQYDLTYPMA RYLSKNPSLI  1109
dGCN2HisRS  SRAMKSWNSA IIIDDIVSLN PVIEFVKAKV VNLFRKHGAI EVDSPLLS-- PLSARNSTAN ---ANANAVH LMTHSGCVVV LPCDLRTQFA RHVTMNSVNL  1043
                                        .    .  ...   .  ...

                <-------------Motif 2------------->
consensus       +**.*. ..*R.-.... ...+*.-F.. .-*.**
yHisRS      -QSIKRYHIA KVYRRDQPAM TKGRMREFYQ CDFDVA--GT FESMVPDSEC LSILVEGLTS LGI--KD-FK IKLNHRKILD GIFQIAGVKD EDVRKISSAV   217
hHisRS      -TNIKRYHIA KVYRRDNPAM TRGRYREFYQ CDFDIA--GN FDPMIPDAEC LKIMCEILSS LQI--GD-FL VKVNDRRILD GMFAICGVSD SKFRTICSSV   238
bHisRS      NQEQRLWYIG PMFRHERPQ- -KGRYRQFHQ LGCEVF--GL QGPDI-DAEL IMLTARWWRA LGISEH---- VTLELNSI-G SLEARANYRD ALVAFLEQHK   199
yGCN2HisRS  S---KQYRMQ HVYRPPDHSR SSLEPRKFGE IDFDIISKSS SESGFYDAES LKIIDEILTV FPVFEKTNTF FILNHADILE SVFNFTNI-D KAQRPLVSRM  1205
dGCN2HisRS  I---RRYCVD RVYREERVFN --FHPKQSYD CSFDIIA-PT TGSHLVDAEL LSLAFEITSE LPRLREKNLA IRMNHTNLLR AILIFCNV-P KAQ--YGALF  1134
                .  .  .    ..  .        .  .      .  ..

                                                                                                                    <---
yHisRS      -DKLD-KSPW EAVKKEMTEE KGQSEETADK IGEY---VKL NGSLKEIHAV LSADANITS- -NEKAKQGLD DI-ATLMKYT EA--FDIDSF ISFDLSLARG   307
hHisRS      -DKLD-KVSW EEVKNEMVGE KGLAPEVADR IGDY---VQQ HGGVSLVEQL L-QDPKLSQ- -NKQALEGLG DL-KLLFEYL TL--FGIDDK ISFDLSLARG   327
bHisRS      -EKLD----- EDCKRRMYTN -------PLR V--------L DSKNPEVQAL LNDAPALGDY LDEESREHFA GLCKLLESAG IA--YTVNQR ------LVRG   258
yGCN2HisRS  LSQVGFARSF KEVKNELKAQ LNISSTALND LELFDFRLDF EAAKKRLYKL MIDSPHL--- --KKIEDSLS HISKVL-SYL KPLEVARNVV ISP-LSNYNS  1298
dGCN2HisRS  EGTMDFIESR ISRFQFHSSI TGIMEKSRTS AQTLMDMLLA NFLLTGSRST VDDSALK--- --SLMRGKGE AASLAR-GAL RELETVVGLA YSL-GVKCPI  1227

                                                                                <-----Motif 3----->
            A -->                                          <--- B --->                 #..*G* G*G*ER**** *
yHisRS      LDYYTGLIYE VVTSASAPPE NASELKKKAK SAEDASEFVG VGSIAAGGRY DNLVNMF--- ---SEASGKK STQIP-CVGI SFGVERIFSL IKQ-------   393
hHisRS      LDYYL----- ------QTPA QAGETGVIYE AVLE---PLG VGSVAAGGRY DGLVGMF--- ----DPKGRK ---VP-CVGL SIGVERIFSI VEQ-------   395
bHisRS      LDYYNRTVFE WVTNS----- ---------- -------LGS QGTVCAGGRY DGLVEQL--- ------GGRA ---TP-AVGF AMGLERLVLL V-Q-------   309
yGCN2HisRS  AFYKGGIMFH AVYDDGS--- ---------- ---------S RNMIAAGGRY DTLISFF--- ---ARPSGKK SSNTRKAVGF NLAWETIFGI A-QNYFKLAS  1369
dGCN2HisRS  HIWALPISFD RASNGGIVWQ MTADLKPNR- -------SGH PSVLAIGERY DSMLHEFQKQ AQKFNPAMPA RGVLSGA-GL TFSLDKLVAA VGVEYAKDCR  1319
                                                                                .   ..  . .  .... .

yHisRS      --RINS-STTI KPTATQVF-- VMAFGGGKD- WTGYLPERMK VTKQLWDAGI EAEYVYKAKA NP-RKQFDTTK KAGCHIAVIL GKEEYLEG-- -----KLR   479
hHisRS      --RLEALEEKI RTTETQV--- LVASA----- QKKLLEERLK LVSELWDAGI KAELLYKKNP KL-LNQLQYCE EAGIPLVAII GEQELKDG-- -----VIK   477
bHisRS      --AVNPEF--- KADPVVDIY- LVASGADTSQ AAMALAERLR --DEL--PGV KL-MTNHGGG NF-KKQFARAD KWGARVAVVL GESEVANG-- -----TAV   390
yGCN2HisRS  GNRIKKRNRFL KDTAVDWKPS RCDVLISSFS NSLLDTIGVT ILNTLWKQNI KADML-RDCS SV-DDVVTGAQ QDGIDWILLI KQQAYPLTNH KRKYKPLK  1467
dGCN2HisRS  AIDVGICVCGT RPPLKD--- ---------- ---------VTYIMRLLWSVGI RCGIV-EAAS ELGDEAQDLAR LGGLHVILVA ENGSLRVRSF ERERFQER  1397

yHisRS      VKRLG--QEFAD DDGELVS--- -AADIVPIVQ EKLSQIHEDG LNEVTRLIKG L*     526
hHisRS      LRSVTSREEVDV RREDLV---- --EEIKRRTG QPLCIC*--- ---------- --     509
bHisRS      VKDLRSGEQTAV AQDSV----- -AAHLRTLLG *--------- ---------- --     416
yGCN2HisRS  IKKLSTNVDIDL DLDEFLTLYQ QETGNKSLIN DSLTLGDKAD EFKRWD--EN --    1517
dGCN2HisRS  HLTRTELVEFIQ KMLRSDGLNG TTVDNFSHLS ALGSGDNRSS GGKERERGEN --    1449
```

Figure 3.—Alignment of the HisRS-related domain of *dGCN2* to other HisRSs. *dGCN2* HisRS-related domain aligned to yGCN2 HisRS-related domain and the HisRSs of yeast (yHisRS), human (hHisRS), and *E. coli* (bHisRS). Positions of the three conserved motifs are indicated. Consensus between the three HisRSs is indicated above alignment. Asterisks indicate hydrophobic (FYWIMVLA); pound sign indicates small (PGST); +, positively charged (HRK); −, negatively charged (DENQ). Solid dots below alignment, *dGCN2* fits consensus.

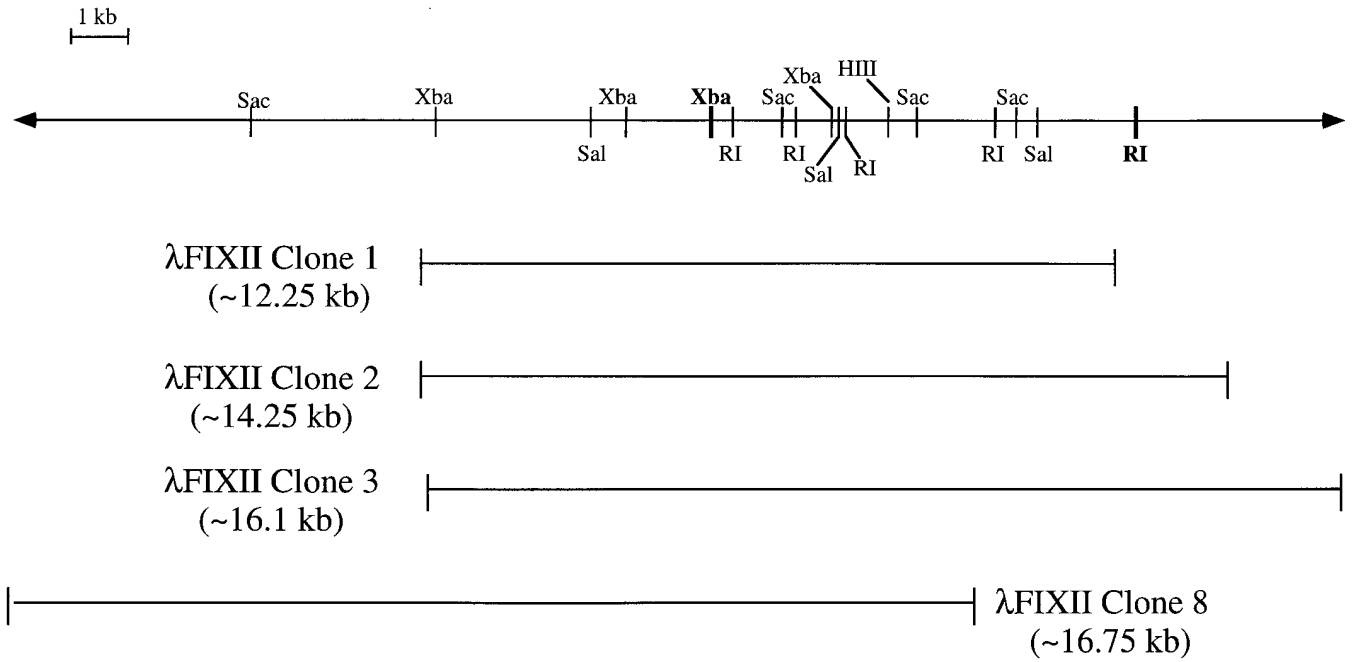

Figure 4.—Restriction map of the 24-kb genomic region surrounding the *dGCN2* locus. (Top) restriction sites. (Bottom) relative locations of four independent genomic λFIXII clones. *dGCN2* coding sequence is contained between the *Xba*I and *Eco*RI sites in bold. Sac, *Sac*I; Xba, *Xba*I; Sal, *Sal*I; RI, *Eco*RI; HIII, *Hin*dIII.

report of the isolation of *D. melanogaster* GCN2 cDNA was published (Santoyo *et al.* 1997). We note several differences in sequence at the nucleotide level, including a number of synonymous substitutions, two amino acid changes, and a 23-nt insertion within the 3′ UTR. These differences may represent polymorphisms between Drosophila strains.

Isolation of the Drosophila GCN2 gene: To determine the gene structure of *dGCN2*, we screened a genomic λFIXII library and isolated four independent clones containing genomic DNA inserts of ~12.3, 14.3, 16.1, and 16.8 kb. Our restriction mapping of these clones indicated that they are overlapping and cover a 24-kb region on the genome (Figure 4). Subsequent cloning and sequencing of restriction fragments indicated that all four clones contained sequence corresponding to the *dGCN2* cDNA.

A comparison of cDNA and genomic DNA sequences (Figure 5) shows that the *dGCN2* mRNA is interrupted by nine small introns ranging in size from 53 to 104 nt, which is consistent with the relatively small size of many Drosophila introns (Mount 1993). The coding sequence spans all 10 exons, with the putative start codon (the first in-frame methionine) in exon 1 and the putative stop codon (the first in-frame stop codon) in exon 10. The 5′ UTR of *dGCN2* contains three upstream AUG codons with ORFs ranging in length from one to 23 amino acids. One of these uORFs overlaps the N-terminal *dGCN2*-coding sequence.

Cytogenetic localization of the *dGCN2* gene: We determined the chromosomal location of the *dGCN2* gene by two methods, *in situ* hybridization of polytene chromosomes and analysis of an ordered P1 phage library. *In situ* hybridization using a DIG-labeled probe corresponding to the 2.2-kb *Eco*RI cDNA library fragment placed *dGCN2* on the right arm of chromosome 3 in the

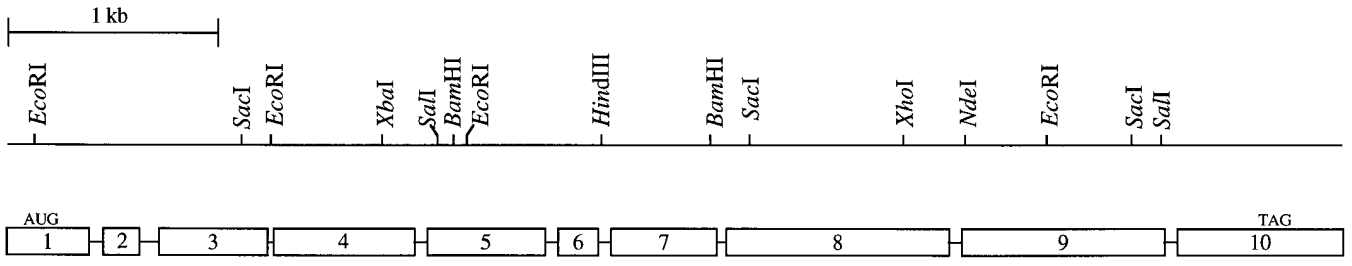

Figure 5.—Restriction map of the *dGCN2* transcript. (Top) restriction sites. (Bottom) *dGCN2* exons 1–10 indicated by boxes, separated by lines representing introns 1–9. Relative positions of the translational start (AUG) and stop (TAG) codons are shown.

region of 100C-D (data not shown). Southern hybridization analysis of genomic Drosophila DNA (data not shown) indicated that *dGCN2* is a single-copy gene, consistent with the presence of a single hybridization signal on salivary gland chromosomes. The chromosomal location of *dGCN2* was further delineated by mapping *dGCN2* to an array of P1 phage genomic clones in the 100C-D region (Figure 6). *dGCN2* maps to 100C3 at a position corresponding to STS Dm2514 near the *Gprk-2* locus.

Developmental expression pattern of *dGCN2* mRNA: Analysis of the *dGCN2* cDNA sequence predicts an mRNA of 5.5–5.7 kb, which is consistent with our findings by Northern analysis. Using two nonoverlapping *dGCN2* cRNA probes, we detected a single RNA species of ~6.0 kb throughout development at fairly low levels of expression, with higher expression in early embryos, midstage pupae, and female adults (data not shown).

To determine quantitatively the expression of *dGCN2* mRNA throughout development, we performed reverse transcription PCR. After optimizing PCR conditions to ensure amplification within a linear range of reaction, we amplified developmentally staged Drosophila cDNA from embryogenesis and the first larval instar. PCR amplification of serially diluted cDNA was performed across two small introns within the kinase domain to distinguish amplification of cDNA from any residual genomic DNA. PCR products were visualized by hybridization to an internal oligonucleotide probe and quantitated by PhosphorImager scanning. *eIF-2α* primers also were included in each PCR reaction to provide an internal control for both the amount of cDNA and the efficiency of amplification in each sample.

Relatively high levels of *dGCN2* mRNA were detected in very early embryogenesis (0–1-hr), a time before the onset of zygotic transcription (Figure 7; Anderson and Lengyel 1981). Between 1 and 6 hr, *dGCN2* mRNA expression rapidly drops, increasing again slightly during the first larval instar. The high expression level of *dGCN2* mRNA early in embryogenesis strongly suggests that *dGCN2* may be a maternally deposited mRNA, and thus may play an important role early in development. Further RT-PCR analysis detected *dGCN2* mRNA expression throughout development (data not shown), consistent with our Northern analysis.

Drosophila GCN2 functionally complements a yeast *GCN2*-null mutation: To provide functional evidence for the identity of *dGCN2*, we tested whether *dGCN2* could genetically complement a yeast GCN2 mutant. We intro-

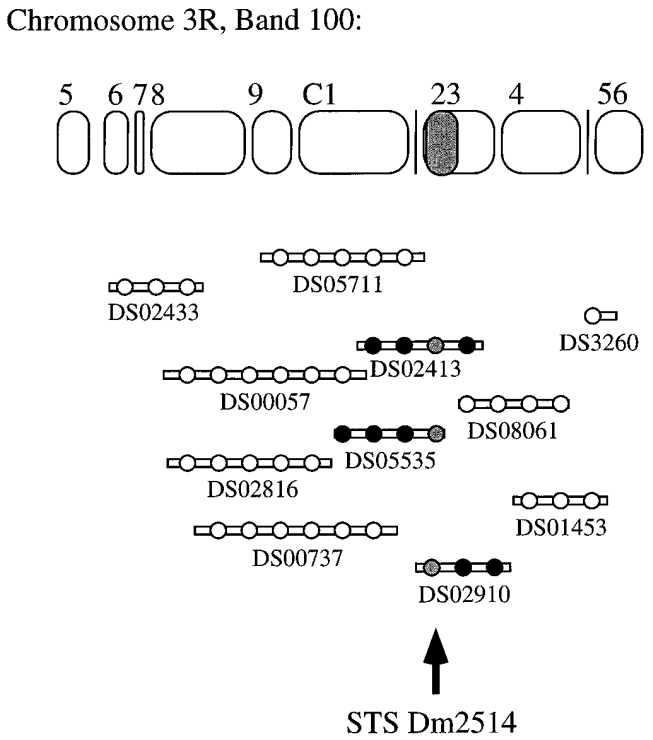

Figure 6.—Physical map of *dGCN2* to arrayed P1 phage genomic clones in band 100 C. (Top) schematic of chromosomal banding pattern. (Bottom) relative positions of P1 clones. Open circles indicate clones negative for *dGCN2*. Closed, dark circles indicate clones positive for *dGCN2*. Closed, grey circles indicate position of STS 2514 to which *dGCN2* maps by comparison of positive and negative clones.

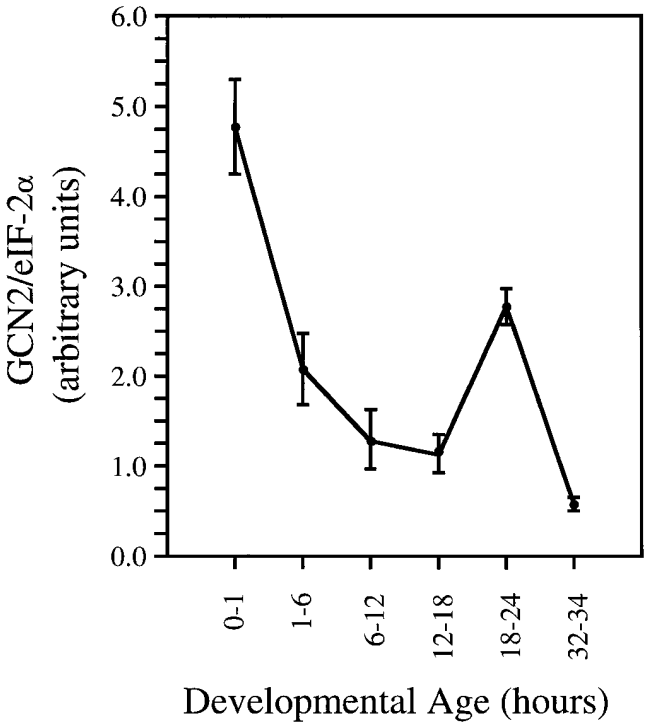

Figure 7.—Quantitative RT-PCR analysis of *dGCN2* mRNA expression during early development. Values for *dGCN2* or eIF-2α RT-PCR products were quantitated by PhosphorImager for each of four serial twofold dilutions of cDNA template. The amount of *dGCN2* relative to eIF-2α for each dilution was determined by dividing *dGCN2* by eIF-2α. The average of these four ratios was determined and plotted on the *y* axis. Standard deviation bars are included. *x* axis, different cDNA samples ranging in developmental age, as indicated.

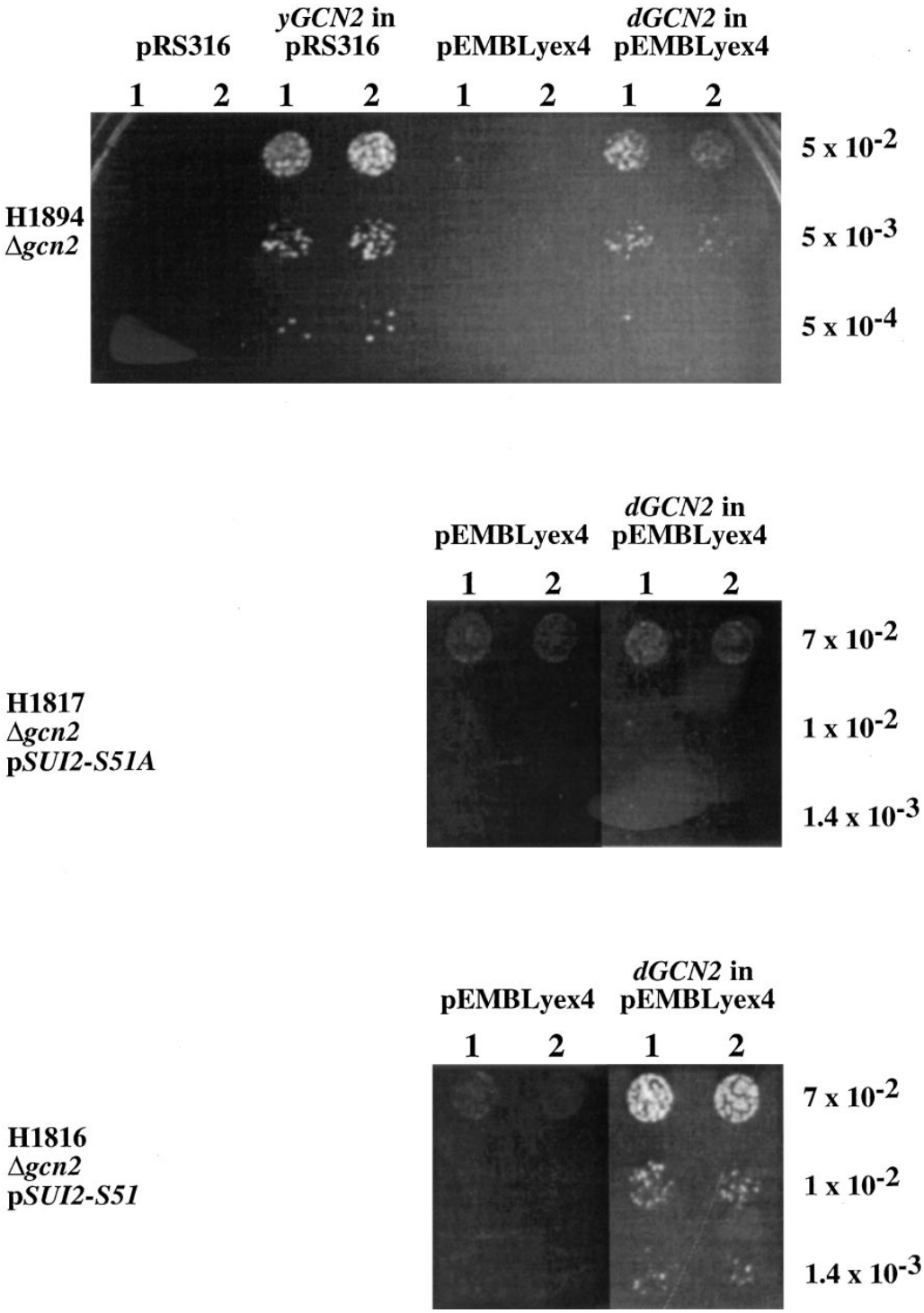

Figure 8.—Expression of *dGCN2* in yeast cells deleted for endogenous *GCN2* kinase stimulates the general amino acid control pathway in response to histidine starvation and is dependent on *SUI2 Ser51*. Dilutions of overnight liquid yeast cultures were replica plated onto agar plates containing SGal media lacking histidine and supplemented with 10 mm 3-AT. Plates were incubated for 5 days at 30° and photographed. (*Top*) *dGCN2* was expressed using a galactose-inducible promoter in strain H1894 and compared to H1894 containing vector pEMBLyex4, wild-type *GCN2* (*yGCN2*), or vector pRS316. Two independent transformants were used per plasmid (lanes 1 and 2). Overnight liquid cultures were diluted to concentrations of 5×10^{-2}, 5×10^{-3}, and 5×10^{-4} OD_{600} units. (Middle and bottom) *dGCN2* was expressed as in top panel in strains H1816 and H1817, respectively, and compared to H1816 and H1817 containing vector pEMBLyex4. Two independent transformants were used per plasmid (lanes 1 and 2). Overnight liquid cultures were diluted to concentrations of 7×10^{-2}, 10^{-2}, and 1.4×10^{-3} OD_{600} units.

duced *dGCN2* as two different constructs, pYK380 and pYK399, expressed from the galactose-inducible *GAL1-CYC1* hybrid promoter into the GCN2-deleted yeast strain H1894. Endogenous GCN2 expression is required for yeast growth in the presence of the drug 3-AT, which inhibits imidazole glycerol phosphate dehydratase in the histidine biosynthetic pathway (Sachs 1996; Figure 8). When we expressed Drosophila GCN2 in H1894 (pYK380 or pYK399), the eIF-2α kinase restored growth in response to the histidine starvation conditions as compared to vector alone (Figure 8; data not shown). These results were determined independently by both the Cavener and Wek laboratories.

To determine the dependence of the complementation phenotype on the presence of the regulatory serine 51 (Ser51) phosphorylation site in eIF-2α, we introduced *dGCN2* (pYK399) into the GCN2-deleted yeast strains H1816 and H1817. Both strains have eIF-2α, en-

coded by the *SUI2* gene, replaced in plasmid form, which in strain H1817 contains a serine to alanine point mutation at Serine 51. *dGCN2* was only able to restore growth under histidine starvation conditions in the H1816 strains containing wild-type *SUI2*. This same result has been shown for yeast *GCN2* (*yGCN2*; Dever *et al.* 1992, 1993).

DISCUSSION

GCN2 is the only known protein kinase that contains a modified histidyl-tRNA synthetase-related domain. We have identified a protein kinase in *D. melanogaster* (Drosophila GCN2) that contains this unique signature. Drosophila GCN2 also shows a higher degree of similarity to yeast GCN2 than to any other protein sequence in the current databases. These two facts clearly establish that we have identified the Drosophila homolog of yeast GCN2. In addition, the ability of *dGCN2* to complement a mutation of *yGCN2* under conditions of amino acid deprivation, as well as the dependence of that complementation on serine 51 of eIF2α, argue that it is functionally homologous.

Drosophila GCN2 is 35% identical to *yGCN2* within the catalytic domain and contains all 11 conserved subdomains and all residues known to be highly conserved among kinases. *dGCN2* also contains all residues known to be particularly conserved among eIF-2α kinases (Ramirez *et al.* 1992). Clusters of amino acids in subdomains V–VII are shared among all seven eIF-2α kinases. Each of these regions contains residues nearly unique to eIF-2α kinases in addition to residues conserved among serine/threonine and tyrosine kinases (Ramirez *et al.* 1992). The residues unique to eIF-2α kinases in these regions are IQ (IQM), F (HRDLKPF), and G (KIGDFGL). Subdomain V participates in linking the N- and C-terminal lobes of the catalytic domain to form the catalytic cleft, while subdomains VI–VII compose part of the catalytic core. Subdomain VIB, for example, is involved in substrate recognition, and the sequence HRDLKP is consistent with serine/threonine recognition (Hanks *et al.* 1988; Hanks and Hunter 1995).

Between kinase subdomains IV and V, all eIF-2α kinases contain an additional domain typically not present in other protein kinases. This domain, denoted the eIF-2α kinase insert region, varies greatly in length and in sequence between PKR, HRI, and GCN2 (Ramirez *et al.* 1992; Wek 1994). Within this region, rat and rabbit HRI and Drosophila and yeast GCN2 contain stretches of serines in combination with highly acidic sequences. While these sequences do not align perfectly, the conservation of the general composition of this region may be significant. The location of the eIF-2α kinase insert region within a three-dimensional structure has been predicted by Ramirez *et al.* (1992), based on a sequence alignment with cyclic AMP–dependent protein kinase, to be in the deep catalytic cleft created by a smaller N-terminal lobe and a larger C-terminal lobe (Knighton *et al.* 1991). The N-terminal lobe contains the ATP binding site, while the C-terminal lobe contains sites involved in peptide binding and catalysis. Within this structure, the eIF-2α kinase insert region is predicted to form a loop that extends down into the catalytic cleft from the N-terminal lobe (Hanks *et al.* 1988; Knighton *et al.* 1991; Ramirez *et al.* 1992; Hanks and Hunter 1995).

Like *yGCN2*, *dGCN2* contains the three conserved motifs found in class II aminoacyl tRNA synthetases within its HisRS-related domain (Cusack *et al.* 1991; Ramirez *et al.* 1992; Blechynden *et al.* 1996). The HisRS-related domain in yeast GCN2 has been shown to bind uncharged tRNA and is required for the activation of yeast GCN2 by amino acid starvation (Wek *et al.* 1995). The inclusion of a HisRS-related domain in *dGCN2* suggests that this kinase similarly may be activated by amino acid starvation. An additional stretch of unique amino acid conservation between yeast and Drosophila GCN2 lying outside of the functional motifs or HisRS-specific regions corresponds to residues 1127–1129 (KAQ). The functional significance of this region is unknown; however, one constitutively active *yGCN2* mutation in yeast (GCN2^c, A1128G) has been identified within this sequence (Ramirez *et al.* 1992).

The presence of the degenerate protein kinase domain in the N terminus of both Drosophila and yeast GCN2 suggests an important functional role for this domain. Because this domain lies directly N-terminal to the active kinase domain in both *dGCN2* and *yGCN2*, and because both of these degenerate domains contain the subdomain involved in substrate recognition, it is possible that the truncated kinase domain may be involved in helping to recruit the eIF-2α substrate to GCN2. Alternatively, this domain may assist in catalysis and/or oligomerization of GCN2. However, the lack of critical residues in catalytic subdomain II in both yeast and Drosophila GCN2, as well as other critical residues, suggests that the degenerate kinase domain does not play a catalytic role. An in-frame deletion of residues 84–490 or of residues 391–466 (numbering based on 1659 total residues) in yeast GCN2 inhibits GCN2 activity in both *in vivo* and *in vitro* assays (Wek *et al.* 1989, 1990). The larger deletion completely removes the upstream kinase-like domain and more than 150 amino acids immediately upstream of the kinase-like domain, whereas the smaller deletion removes domains VIB-IX. These sequences are thus critical for GCN2 kinase activity. It remains to be seen which regions of this deletion are essential for GCN2 function. Another region C-terminal to the HisRS-related domain has been shown to be necessary for ribosome association in yGCN2 (Ramirez *et al.* 1991). Mutations of lysine residues in this region in particular abolish ribosome binding (Zhu and Wek 1998). Furthermore, one GCN2^c mutation in this region, R1488K, adds an additional lysine (Ramirez *et*

al. 1992). Thus, lysines appear to be critical for ribosomal association. *dGCN2* contains lysines in this region but does not align well to *yGCN2*. It is therefore unclear whether *dGCN2* contains an active ribosome association domain.

We have shown by genetic complementation that *dGCN2* can restore growth to Δ*gcn2* yeast under conditions of amino acid deprivation (3-AT treatment), and that this restoration depends on the regulatory phosphorylation site (Ser51) in eIF2α. This result implies that *dGCN2* replaces *yGCN2* in the GCN2/GCN4 pathway to upregulate the expression of HIS3, whose product is inhibited by 3-AT. Cell growth is thereby restored, as increased expression of HIS3 is able to titer out the negative effects of 3-AT. Therefore, we predict that *dGCN2* is activated by amino acid deprivation to phosphorylate eIF-2α, inhibit eIF-2B exchange activity, and derepress *GCN4* translation. Although PKR and HRI can complement *yGCN2* mutations as well (Dever *et al.* 1993), both apparently are constitutively active and do not respond to amino acid deprivation. Overexpression of PKR and HRI in yeast results in a slow growth phenotype under nonstarvation conditions, induced by hyperphosphorylation of eIF-2α and a presumed inhibition of total protein synthesis (Dever *et al.* 1993). This is consistent with their well-established global effects in mammalian cells (Mathews 1990; Wek 1994; Chen and London 1995). Wild-type yGCN2, on the other hand, selectively affects *GCN4* translation without an effect on global protein synthesis (Hinnebusch 1996). Furthermore, mutants of *yGCN2* that are constitutively active negatively affect growth under normal nutrition conditions because of eIF-2α hyperphosphorylation, although not to the same extent as wild-type PKR or HRI (Ramirez *et al.* 1992). In contrast to these constitutively active kinases, we have found that overexpression of *dGCN2* in Δ*gcn2* yeast by the galactose-inducible promoter does not inhibit growth in nonstarved cells (data not shown), and that galactose induction of *dGCN2* expression in Δ*gcn2* yeast is required to restore growth under starvation conditions (Figure 8). Although an alternative possibility is that *dGCN2* protein and/or mRNA is unstable in yeast, and that only a small amount of active *dGCN2*p is present to phosphorylate eIF-2α, we predict that *dGCN2* must be activated by amino acid starvation to restore growth to Δ*gcn2* yeast.

dGCN2's ability to restore growth to starved Δ*gcn2* yeast combined with the presence of the HisRS-related domain leads us to speculate that *dGCN2* will participate in a similar amino acid–sensing pathway in Drosophila. An outstanding question is whether starvation for essential amino acids affects protein synthesis differently than starvation for nonessential amino acids in higher eukaryotes. Starvation for both essential and nonessential amino acids has been shown to inhibit total protein synthesis (Rannels *et al.* 1978; Lofgren and Thompson 1979; Ogilvie *et al.* 1979; Austin *et al.* 1982; Flaim *et al.* 1982; Austin and Clemens 1984; Everson *et al.* 1989; Kimball *et al.* 1989, 1991; Pain 1994; Laine *et al.* 1996) in addition to upregulating the expression of at least one amino acid biosynthetic enyzme, asparagine synthetase (AS). AS mRNA levels in rat FAO hepatoma cells (Hutson and Kilberg 1994) or HeLa cells (Gong *et al.* 1991) are increased in response to starvation for essential amino acids or when Chinese hamster ovary cells are deficient for leucyl-, methionyl, and lysyl-tRNA synthetases (Andrulis *et al.* 1979; Laine *et al.* 1996). The response of higher eukaryotes to deprivation of nonessential amino acids also appears to be similar to the yeast general control system. Expression of AS mRNA in rat FAO hepatoma cells is also elevated dramatically under deprivation of asparagine or other nonessential amino acids (Hutson and Kilberg 1994). In addition, increased AS mRNA expression is seen in BHK ts11 hamster cells deprived of functional asparaginyl-tRNA synthetase (Gong *et al.* 1991).

Whether the regulatory mechanism(s) mediating starvation responses differ for essential *vs.* nonessential amino acids in animal cells is unknown. The global downregulation of mammalian protein synthesis observed in response to starvation for an essential amino acid appears to result from an abrogation of eIF-2B activity caused by a large increase in eIF-2α phosphorylation (Everson *et al.* 1989; Kimball *et al.* 1989; Kimball and Jefferson 1994). The upregulation of biosynthetic enzyme expression in response to starvation for a variety of amino acids, by comparison to the yeast general control system, may also be regulated by an inhibition of eIF-2B mediated by eIF-2α phosphorylation. The similarities in these two mechanisms suggests that similar, but not necessarily identical, pathways may exist in higher eukaryotes for regulating starvation responses of essential and nonessential amino acids.

We foresee at least two possibilities regarding amino acid deprivation in Drosophila: that amino acid deprivation may be strictly dependent on diet or that amino acid deprivation may be part of the normal developmental program. These alternatives are not necessarily mutually exclusive: GCN2 may be operating in both situations to regulate amino acid biosynthesis and protein synthesis. The latter possibility is suggested by the fact that *dGCN2* mRNA is expressed during early development at a nonfeeding stage (Figure 7). Genetic evidence suggests that eIF-2α phosphorylation can affect viability and developmental rate under normal growth conditions (Qu *et al.* 1997). This implies a potentially important role for *dGCN2* during early development. If *dGCN2* does play a developmental role, then its control of amino acid metabolism may be an important component of development. During nonfeeding stages, such as metamorphosis, when there is no dietary input of protein, the amino acids necessary for protein synthesis must be obtained either from free stored amino acids or from the breakdown of larval protein. At such times, it is

possible that one or more amino acids may drop below a critical concentration threshold and thus activate *dGCN2*.

An interesting alternative is that *dGCN2* may respond to additional signals that are not necessarily related to amino acid biosynthesis. There are large domains in the N terminus of Drosophila and yeast GCN2 with unknown functions and no obvious similarity to any known protein in the current databases. These regions may include additional activation domains capable of responding to ligands other than those involved in amino acid deprivation.

This work was supported by a National Science Foundation grant MCB 9304983 (D.R.C.), by National Institutes of Health grants GM 34170 (D.R.C.) and GM 49164 (R.C.W.), and by a Vanderbilt University Dissertation Enhancement Grant (D.S.O.). We thank Steve Hanks for help in PCR primer design and Hugo Bellen for supplying the Drosophila P1 genomic clones.

LITERATURE CITED

Anderson, K. V., and J. A. Lengyel, 1981 Changing rates of DNA and RNA synthesis in Drosophila embryos. Dev. Biol. **82:** 127–138.

Andrulis, I. L., G. W. Hatfield, and S. M. Arfin, 1979 Asparaginyl-tRNA aminoacylation levels and asparagine synthetase expression in cultured Chinese hamster ovary cells. J. Biol. Chem. **254:** 10629–10633.

Arnez, J. G., D. C. Harris, A. Mitschler, B. Rees, C. S. Francklyn *et al.*, 1995 Crystal structure of histidyl-tRNA synthetase from *Escherichia coli* complexed with histidyl-adenylate. EMBO J. **14:** 4143–4155.

Austin, S. A., and M. J. Clemens, 1984 Stimulation of protein synthesis by lysine analogues in lysine-deprived Ehrlich ascites tumour cells. Biochim. Biophys. Acta **804:** 16–22.

Austin, S. A., V. M. Pain, J. A. Lewis and M. J. Clemens, 1982 Investigation of the role of uncharged tRNA in the regulation of polypeptide chain initiation by amino acid starvation in cultured mammalian cells: a reappraisal. Eur. J. Biochem. **122:** 519–526.

Barber, G. N., R. Jagus, E. F. Meurs, A. G. Hovanessian and M. G. Katze, 1995 Molecular mechanisms responsible for malignant transformation by regulatory and catalytic domain variants of the interferon-induced enzyme RNA-dependent protein kinase. J. Biol. Chem. **270:** 17423–17428.

Blechynden, L. M., C. M. Lawson and M. J. Garlepp, 1996 Sequence and polymorphism analysis of the murine gene encoding histidyl-tRNA synthetase. Gene **178:** 151–156.

Cavener, D. R., and S. C. Ray, 1991 Eukaryotic start and stop translation sites. Nucleic Acids Res. **19:** 3185–3192.

Cesareni, G., and J. A. H. Murray, 1987 Plasmid vectors carrying the replication origin of filamentous single-stranded phages, pp. 135–154 in *Genetic Engineering: Principles and Methods*, Vol. 9, edited by J. K. Setlow. Plenum Press, New York.

Chen, J.-J., and I. M. London, 1995 Regulation of protein synthesis by heme-regulated eIF-2α kinase. Trends Biochem. Sci. **20:** 105–108.

Cusack, S., M. Hartlein and R. Leberman, 1991 Sequence, structural and evolutionary relationships between class 2 aminoacyl-tRNA synthetases. Nucleic Acids Res. **19:** 3489–3498.

Delarue, M., and D. Moras, 1993 The aminoacyl-tRNA synthetase family: modules at work. BioEssays **15:** 675–687.

Der, S. D., Y. L. Yang, C. Weismann and B. R. Williams, 1997 A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis. Proc. Natl. Acad. Sci. USA **94:** 3279–3283.

Dever, T. E., L. Feng, R. C. Wek, A. M. Cigan, T. F. Donohue *et al.*, 1992 Phosphorylation of initiation factor 2a by protein kinase GCN2 mediates gene-specific translational control in yeast. Cell **68:** 585–596.

Dever, T. E., J. J. Chen, G. N. Barber, A. M. Cigan, L. Feng *et al.*, 1993 Mammalian eukaryotic initiation factor 2 alpha kinases functionally substitute for GCN2 protein kinase in the GCN4 translational control mechanism of yeast. Proc. Natl. Acad. Sci. USA **90:** 4616–4620.

Everson, W.V., K. E. Flaim, D. M. Susco, S. R. Kimball and L. S. Jefferson, 1989 Effect of amino acid deprivation on initiation of protein synthesis in rat hepatocytes. Am. J. Physiol. **256:** 18–27.

Flaim, K. E., D. E. Peavy, W. V. Everson and L. S. Jefferson, 1982 The role of amino acids in the regulation of protein synthesis in perfused rat liver. I. Reduction in rates of synthesis resulting from amino acid deprivation and recovery during flow-through perfusion. J. Biol. Chem. **257:** 2932–2938.

Gong, S. S., L. Guerrini and C. Basilico, 1991 Regulation of asparagine synthetase gene expression by amino acid starvation. Mol. Cell. Biol. **11:** 6059–6066.

Guyer, D., D. Patton and E. Ward, 1995 Evidence for cross-pathway regulation of metabolic gene expression in plants. Proc. Natl. Acad. Sci. USA **92:** 4997–5000.

Hanks, S. K., and T. Hunter, 1995 The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J. **9:** 576–596.

Hanks, S. K., A. M. Quinn and T. Hunter, 1988 The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:** 42–52.

Hinnebusch, A., 1988 Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **52:** 248–273.

Hinnebusch, A. G., 1996 Translational control of *GCN4*: gene-specific regulation by phosphorylation of eIF2, pp. 199–244 in *Translational Control*, edited by J. W. B. Hershey, M. B. Mathews and N. Sonenberg. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hutson, R. G., and M. S. Kilberg, 1994 Cloning of rat asparagine synthetase and specificity of the amino acid-dependent control of its mRNA content. Biochem. J. **304:** 745–750.

Kimball, S. R., and L. S. Jefferson, 1994 Mechanisms of translational control in liver and skeletal muscle. Biochimie **76:** 729–36.

Kimball, S. R., W. V. Everson, K. E. Flaim and L. S. Jefferson, 1989 Initiation of protein synthesis in a cell-free system prepared from rat hepatocytes. Am. J. Physiol. **256:** 28–34.

Kimball, S. R., D. A. Antonetti, R. M. Brawley and L. S. Jefferson, 1991 Mechanism of inhibition of peptide chain initiation by amino acid deprivation in perfused rat liver. J. Biol. Chem. **266:** 1969–1976.

Knighton, D. R., J. Zhend, L. F. T. Eyck, N. Xuong, S. S. Taylor *et al.*, 1991 Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science **253:** 407–414.

Koromilias, A. E., S. Roy, G. N. Barber, M. G. Katze and N. Sonenberg, 1992 Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. J. Biol. Chem. **267:** 23092–23098.

Laine, R. O., R. G. Hutson and M. S. Kilberg, 1996 Eukaryotic gene expression: metabolite control by amino acids. Prog. Nucleic Acids Res. Mol. Biol. **53:** 219–248.

Lee, S. B., D. Rodriguez, J. R. Rodriguez and M. Esteban, 1997 The apoptosis pathway triggered by the interferon-induced protein kinase PKR requires the third basic domain, initiates upstream of Bcl-2, and involves ICE-like proteases. Virology **231:** 81–88.

Li, J., and R. A. Petryshyn, 1991 Activation of the double-stranded RNA-dependent eIF-2α kinase by cellular RNA from 3T3-F442A cells. Eur. J. Biochem. **195:** 41–48.

Lofgren, D. J., and L. H. Thompson, 1979 Relationship between histidyl-tRNA level and protein synthesis rate in wild-type and mutant Chinese hamster ovary cells. J. Cell. Physiol. **99:** 303–312.

Mathews, M. B., 1990 Control of translation in adenovirus-infected cells. Enzyme **44:** 250–264.

Mount, S. M., 1993 Messenger RNA splicing signals in Drosophila genes, pp. 333–358 in *An Atlas of Drosophila Genes*, edited by G. Maroni. Oxford University Press, New York.

Ogilvie, A., U. Huschka and W. Kersten, 1979 Control of protein synthesis in mammalian cells by aminoacylation of transfer ribonucleic acid. Biochim. Biophys. Acta **565:** 293–304.

Pain, V. M., 1994 Translational control during amino acid starvation. Biochimie **76:** 718–728.

Petryshyn, R., J. J. Chen and I. M. London, 1988 Detection of activated double-stranded RNA-dependent protein kinase in 3T3-F442A cells. Proc. Natl. Acad. Sci. USA **85:** 1427–1431.

Qu, S., S. E. Perlaky, E. L. Organ, D. Crawford and D. R. Cavener, 1997 Mutations at the Ser50 residue of translation factor eIF-2α dominantly affect developmental rate, body weight, and viability of *Drosophila melanogaster*. Gene Express. **6:** 349–360.

Ramirez, M., R. C. Wek and A. G. Hinnebusch, 1991 Ribosome association of GCN2 protein kinase, a translational activator of the GCN4 gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 3027–3036.

Ramirez, M., R. C. Wek, C. R. Vazquez de Aldana, B. M. Jackson, B. Freeman *et al.*, 1992 Mutations activating the yeast eIF-2 alpha kinase GCN2: isolation of alleles altering the domain related to histidyl-tRNA synthetases. Mol. Cell. Biol. **12:** 5801–5815.

Rannels, D. E., A. E. Pegg, S. R. Rannels and L. S. Jefferson, 1978 Effect of starvation on initiation of protein synthesis in skeletal muscle and heart. Am. J. Physiol. **235:** E126–E133.

Sachs, M. S., 1996 General and cross-pathway controls of amino acid biosynthesis, pp. 315–345 in *The Mycota III: Biochemistry and Molecular Biology*, edited by R. Brambl and G. A. Marzluf. Springer-Verlag, Berlin.

Santoyo, J., J. Alcade, R. Mendez, D. Pulido and C. Deharo, 1997 Cloning and characterization of a cDNA encoding a protein synthesis initiation factor-2α (eIF-2α) kinase from *Drosophila melanogaster*. J. Biol. Chem. **272:** 12544–12550.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designated for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Vazquez de Aldana, C. R., R. C. Wek, P. S. Segundo, A. G. Truesdell and A. G. Hinnebusch, 1994 Multicopy tRNA genes functionally suppress mutations in yeast eIF-2 alpha kinase GCN2: evidence for separate pathways coupling GCN4 expression to uncharged tRNA. Mol. Cell. Biol. **14:** 7920–7932.

Wek, R. C., 1994 eIF-2 kinases: regulators of general and gene-specific translation initiation. Trends Biochem. Sci. **19:** 491–496.

Wek, R. C., B. M. Jackson and A. G. Hinnebusch, 1989 Juxtaposition of domains homologous to protein kinases and histidyl-tRNA synthetases in GCN2 protein suggests a mechanism for coupling GCN4 expression to amino acid availability. Proc. Natl. Acad. Sci. USA **86:** 4579–4583.

Wek, R. C., M. Ramirez, B. M. Jackson and A. G. Hinnebusch, 1990 Identification of positive-acting domains in GCN2 protein kinase required for translational activation of GCN4 expression. Mol. Cell. Biol. **10:** 2820–2831.

Wek, S. A., S. Zhu and R. C. Wek, 1995 The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. Mol. Cell. Biol. **15:** 4497–4506.

Williams, B. R., 1997 Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. Biochem. Soc. Trans. **25:** 509–513.

Zhu, S., and R. C. Wek, 1998 Ribosome-binding domain of eukaryotic initiation factor-2 kinase GCN2 facilitates translational control. J. Biol. Chem. **273:** 1808–1818.

Communicating editor: V. G. Finnerty