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Cloning and Characterization of a cDNA Encoding a Protein Synthesis Initiation Factor-2α (eIF-2α) Kinase from *Drosophila melanogaster*

HOMOLOGY TO YEAST GCN2 PROTEIN KINASE*

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Phosphorylation of the α subunit of the eukaryotic initiation factor 2 (eIF-2α) is one of the best-characterized mechanisms for downregulating protein synthesis in mammalian cells in response to various stress conditions. In *Drosophila*, such a regulatory mechanism has not been elucidated. We report the molecular cloning and characterization of DGCN2, a *Drosophila* eIF-2α kinase related to yeast GCN2 protein kinase. DGCN2 contains all of the 12 catalytic subdomains characteristic of eukaryotic Ser/Thr protein kinases and the conserved sequence of eIF-2α kinases in subdomain V. A large insert of 94 amino acids, which is characteristic of eIF-2α kinases, is also present between subdomains IV and V. It is particularly notable that DGCN2 possesses an amino acid sequence related to class II aminoacyl-tRNA synthetases, a unique feature of yeast GCN2 protein kinase. DGCN2 expression is developmentally regulated. During embryogenesis, *DGCN2* mRNA is dynamically expressed in several tissues. Interestingly, at later stages this expression becomes restricted to a few cells of the central nervous system. Affinity-purified antibodies, raised against a synthetic peptide based on the predicted DGCN2 sequence, specifically immunoprecipitated an eIF-2α kinase activity and recognized an ~175 kDa phosphoprotein in Western blots of *Drosophila* embryo extracts.

Protein synthesis is mainly regulated at the initiation of mRNA translation. The best-characterized mechanism of translational regulation in eukaryotes involves the phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF-2α)[1] (for review, see Refs. 1 and 2). Three distinct protein kinases regulate protein synthesis in eukaryotic cells by phosphorylating the α subunit of eIF-2 at serine 51 (3–4). They are two mammalian eIF-2α kinases, the heme-regulated inhibitor (HRI) and the double-stranded RNA-dependent kinase (PKR), and the yeast GCN2 (3–4). These kinases contain twelve conserved subdomains characteristic of all eukaryotic Ser/Thr protein kinases (5) and reveal similarities that make them distinguishable from other eukaryotic protein kinases. It is notable that eIF-2α kinases possess relatively large insert sequences between catalytic subdomains IV and V (3, 5). Even though the subdomain V is less conserved among the eukaryotic protein kinases (5), the known eIF-2α kinases share significant homology in this domain.

In contrast to the catalytic domains, the regulatory regions of the eIF-2α kinases are very different (6, 7). HRI from rabbit and rat contains two heme regulatory motifs, ACPYVM and RCPAQA, located within the HRI kinase domain and that are not found in either PKR or GCN2 (6, 7). PKR has been cloned from human, mouse, and rat cells (7). The PKR amino-terminal half contains three clusters of basic amino acids. The first two contain two divergent copies of a double-stranded RNA binding motif that are required for RNA binding (3, 8). Yeast GCN2 contains a 530-amino acid sequence related to histidyl-tRNA synthetases (HisRS) in the carboxyl-terminal region, adjacent to the kinase domain. This domain is required for GCN2 activation *in vivo* (4, 9).

Phosphorylation of eIF-2α prevents initiation of protein synthesis by sequestration of the guanine nucleotide exchange factor eIF-2B in an inactive complex (1). The phosphorylation of eIF-2α was first detected in rabbit reticulocyte lysates deprived of hemin. The absence of hemin results in the activation of HRI (1, 6). PKR, constitutively expressed in reticulocytes and inducible by interferon in other mammalian cells, is activated by low concentrations of double-stranded RNA in the presence of ATP (1, 8). The yeast GCN2 kinase is activated by amino acid deprivation (9).

Until recently, phosphorylation of eIF-2α was believed to nonspecifically down-regulate translation initiation, but recent results have suggested that it can up-regulate the synthesis of specific proteins (4, 7). *In vivo* and *in vitro* experiments indicated that the activity of the yeast GCN2 kinase is regulated

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U80223.

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[1] The abbreviations and trivial names used are: eIF-2α, the α subunit (38 kDa) of eukaryotic polypeptide chain initiation factor 2; HRI, heme-regulated inhibitor; PKR, double-stranded RNA-dependent kinase; GCN2, yeast general amino acid control eIF-2α protein kinase; DGCN2, *Drosophila* GCN2-like eIF-2α protein kinase; DGCN2-peptide, a synthetic peptide corresponding to residues 610–622 with a cysteine and a glycine residue in its NH_2-terminal end; anti-DGCN2 antibodies, affinity-purified anti-DGCN2-peptide antibodies; HisRS, histidyl-tRNA synthetase(s); ORF, open reading frame; CNS, central nervous system; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PCR, polymerase chain reaction.

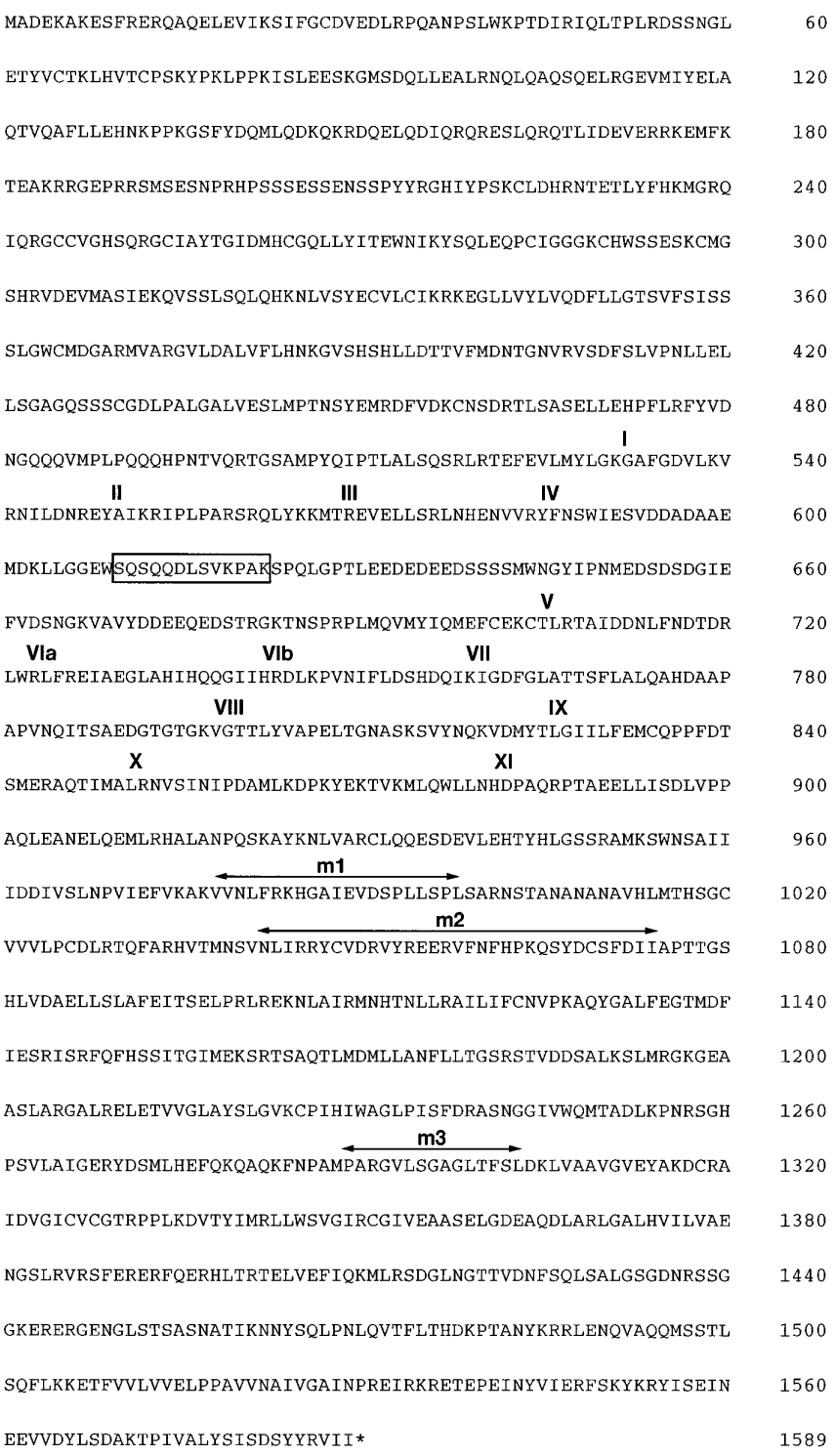

FIG. 1. **Amino acid sequence of the DGCN2 gene product deduced from the nucleotide sequence of *DGCN2* cDNA.** Amino acid numbering is shown to the *right*. Protein kinase subdomains I-XI according to Hanks and Hunter (5) are indicated by the *roman numeral* directly above the appropiate region. The conserved motifs, *m1*, *m2*, and *m3*, of the HisRS-related region of DGCN2 are also indicated. The *star* denotes the termination codon. Amino acid residue 1 is the putative initiator methionine. A synthetic peptide corresponding to amino acids 610–622 (*boxed* residues) with two additional residues (CG) in its NH_2-terminal end was conjugated to keyhole limpet hemocyanin and injected into rabbits to obtain anti-DGCN2 antibodies.

through the interaction of the HisRS-related domain with uncharged tRNAs that accumulate when cells are starving for amino acids. In this case, eIF-2α phosphorylation enhances the translation of *GCN4*, a transcriptional activator of genes involved in the biosynthesis of amino acids (4, 9, 10).

Previous studies have indicated that *in vitro* phosphorylation of *Drosophila* eIF-2α by reticulocyte HRI inhibits the exchange reaction catalyzed by mammalian eIF-2B (11). More recent results have provided evidence that translational regulation by phosphorylation of eIF-2α and sequestration of eIF-2B can operate in insect cells (12), suggesting that an endogenous eIF-2α kinase may be present in such cells. However, to date, no eIF-2α kinase activity has been detected in *Drosophila*. Here we report the cloning of a *Drosophila melanogaster* eIF-2α kinase (DGCN2). Amino acid sequence analysis indicates that DGCN2 is closely related to yeast GCN2 kinase.

EXPERIMENTAL PROCEDURES

PCR Amplification of Genomic DGCN2 Fragment—Genomic DNA was prepared from adult flies as described (13). Sense (5′-CTBYWYATYCARATG-3′) and antisense (5′-CCAAARTCWCCDATYTT-3′) degenerated oligonucleotide primers were synthesized based on regions of homology between all known eIF-2α kinases. Polymerase chain reaction was performed using each primer at 5 μM and 500 ng of genomic DNA.

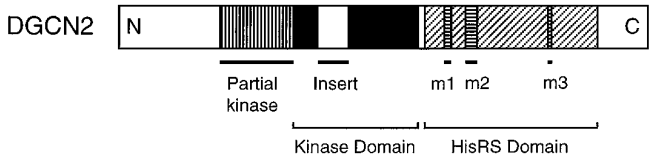

FIG. 2. **Schematic diagram of DGCN2 domain structure.** The 1589 amino acid long DGCN2 coding sequence is illustrated by the *larger box* (flanked by the *N*- and *C*-ends). The figure is drawn to scale. Highlighted domains include the "partial kinase" (*vertical dashed box*) that are related to subdomains III-XI of eukaryotic protein kinases, the eIF-2α kinase domain (*black boxes*) with an *open box* indicating a large insert of 94 amino acids between subdomains IV and V, and the HisRS domain (*stippled boxes*) that includes the three motifs (*m1*, *m2*, and *m3*) conserved among the class II aminoacyl-tRNA synthetases.

A "hot start" PCR was performed by adding the DNA polymerase after an initial denaturation step of 5 min at 95 °C and then 35 cycles consisting of 95 °C for 1 min, 37 °C for 2 min, and 72 °C for 2 min, followed by a final step of 72 °C for 10 min. The amplification was carried out in an Ericomp TwinBlock System (Ericomp, San Diego, CA). The reaction products were gel purified and subcloned into a pCRII vector using a TA cloning kit (Invitrogen).

cDNA Library Screening—A cDNA library in Uni-Zap XR, made from 2- to 14-h-old embryos (Stratagene), was screened according to standard procedures (14). 1×10^6 plaque-forming units were transferred to Hybond-C nitrocellulose membranes (Amersham Corp.). The 278-bp *DGCN2* genomic DNA fragment was radiolabeled with [α-^{32}P]dCTP using a random primed DNA labeling kit (Boehringer Mannheim). The membranes were prehybridized 2 h at 42 °C in a solution containing 5 × saline/sodium/phosphate/EDTA, 50% formamide, 0.1% SDS, 5 × Denhardt's solution, and 100 μg/ml denaturated salmon sperm DNA. Hybridization was carried out overnight at 42 °C in this solution containing a ^{32}P-labeled *DGCN2* probe (1×10^7 cpm/ml). The membranes were subjected to high stringency washings and processed for autoradiography. Positive phages were purified by successive rounds of screening. Insert cDNAs in positive phages were excised *in vivo* by plasmid rescue of a pBlueScript SK$^-$ phagemid, after superinfection with a helper phage, as recommended by the manufacturer (Stratagene). A second cDNA library in a λZAPII vector, made from 0- to 4-h-old embryos (Stratagene), was screened, as described above, using the 1131-bp *DGCN2* cDNA isolated from the first library.

DNA Sequencing and Analysis—The cDNA inserts were sequenced across both strands by the dideoxy chain termination method (15) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS and the Automatic Sequencer System 373A (Applied Biosystem). All primers for sequencing were from Isogen Bioscience (Amsterdam). Sequence homology searches were performed using BLAST (16) and FASTA (17) programs. Similarity and identity values were obtained by using the BESTFIT and GAP programs (Wisconsin Package, Genetics Computer Group, University of Wisconsin, Madison).

Rapid Amplification of cDNA 5′- and 3′-Ends (5′/3′ RACE)—1 μg of poly(A)$^+$ RNA of 0–12 h *Drosophila* Oregon R embryos, obtained according to Campuzano *et al.* (18), was reverse-transcribed using the Marathon cDNA amplification kit (CLONTECH) according to the instructions of the manufacturer. To avoid amplification of nonspecific products, "hot start" PCR was performed by adding TaqStart antibody (CLONTECH) to the DNA polymerase (Expand long template PCR system, Boehringer Mannheim). Amplifications were performed in a GeneAmp 2400 thermal cycler (Perkin-Elmer) as recommended by the Marathon kit protocol. The antisense primer (5′-ACATTCTCATGATTCAGCCGCGAAAGAAGC-3′), complementary to nucleotides 2033–2062 of *DGCN2* cDNA was used to amplify the 5′-end, in combination with the adaptor primer AP1 supplied with the Marathon kit. The sense primer (5′-ACTCACCGCTCTTATCCCCACTTTCCGCAA-3′) complementary to nucleotides 3289–3318 of *DGCN2* cDNA was used to amplify the 3′-end in combination with the adaptor primer AP1. The 5′ and 3′ RACE products were subcloned into the pCRII vector and sequenced across both strands. The sequence of three independent cDNA clones of each product were compared to detect errors that could have occurred during the reverse-transcription and the PCR amplification.

Northern Blot Analysis—Poly(A)$^+$ RNA was prepared as described (18). Five μg of poly(A)$^+$ RNA from each developmental stage were separated on a formaldehyde/agarose gel, transferred to nylon membrane, hybridized, and exposed as described (14).

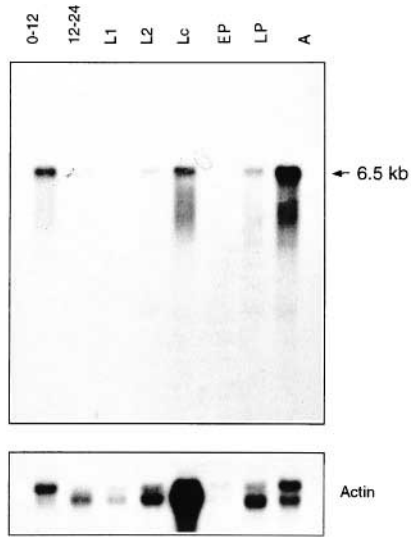

FIG. 3. **Developmental expression of DGCN2 transcripts.** A Northern blot of poly(A)$^+$ RNA (about 5 μg/lane) prepared from different stages of development was hybridized with *DGCN2* cDNAs that encompasses the entire open reading frame. The embryonic period was divided into two stages corresponding to hours 0–12 and 12–24 after egg laying. Other developmental stages include first instar larvae (24–48 h) (*L1*); second instar larvae (48–72 h) (*L2*); late crawling larvae (72–120 h) (*Lc*); early pupae (120–168 h) (*EP*); late pupae (168–216 h) (*LP*), and adult flies (*A*). RNA sizes were calculated from co-electrophoresed yeast and *Escherichia coli* ribosomal RNAs. The filter was rehybridized with a *Drosophila* actin probe to control for the amount of RNA loaded in each lane.

Whole-mount Embryo RNA in Situ Hybridization—Localization of RNA in whole-mount embryos with antisense digoxigenin-labeled probes was performed essentially as described (19).

Antibodies Against a Synthetic DGCN2 Peptide—Based on the *DGCN2* cDNA coding sequence, a synthetic peptide (CG-SQSQQDLSVKPAK) was made, corresponding to amino acids 610–622 (see Fig. 1) with two additional residues (CG) in its NH_2-terminal end. The peptide was synthesized by Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry using an Applied Biosystems 431A automated solid-phase synthesizer in the Protein Chemistry Facility of the Centro de Biología Molecular "Severo Ochoa." The purity of the peptide, monitored by reverse phase high performance liquid chromatography, was 95%, and peptide identity was confirmed by mass spectrometry. This peptide was coupled by the terminal cysteine residue to keyhole limpet hemocyanin (Calbiochem), and rabbits were immunized as described (20). Serum was purified by affinity chromatography by coupling synthetic peptide through the cysteine terminal residue to Sulfolink beads, according to the instructions of the manufacturer (Pierce). The specific IgGs were further purified by affinity chromatography on a protein A-Sepharose column as described (21). For simplicity, these affinity purified anti-DGCN2-peptide antibodies will be named in the future as anti-DGCN2 antibodies.

Immunoprecipitation and Drosophila eIF-2α Kinase Assay of Immune Complexes—One g of dechorionized 0- to 12-h-old *Drosophila* Oregon R embryos was suspended in 5 ml of lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 mM EDTA, 20 μg/ml DNAase, and 20 μg/ml RNAase. The embryos were homogenized with 20 strokes in a Dounce homogenizer (Type B pestle) and incubated 30 min on ice, and then the homogenate was centrifuged for 10 min at 4 °C, at 14,000 × *g*. The supernatant was precleared with 30 μl of protein A-Sepharose (Sigma) that was equilibrated in TBS buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 1% Nonidet P-40. After incubation for 1 h at 4 °C with continuous mixing, the supernatant was recovered and incubated with anti-DGCN2 antibodies (10 μg of IgG) for 1 h at 4 °C. In control experiments, the peptide immunogen (90 μg/ml) was also included in this incubation just before the antibody was added. Protein A-Sepharose (40 μl), equilibrated in TBS containing 2% bovine serum albumin and 1% Nonidet P-40, was then added, and the reaction mixture was incubated for 1 h at 4 °C with continuous mixing. The immunoprecipitates were washed three times with lysis buffer and

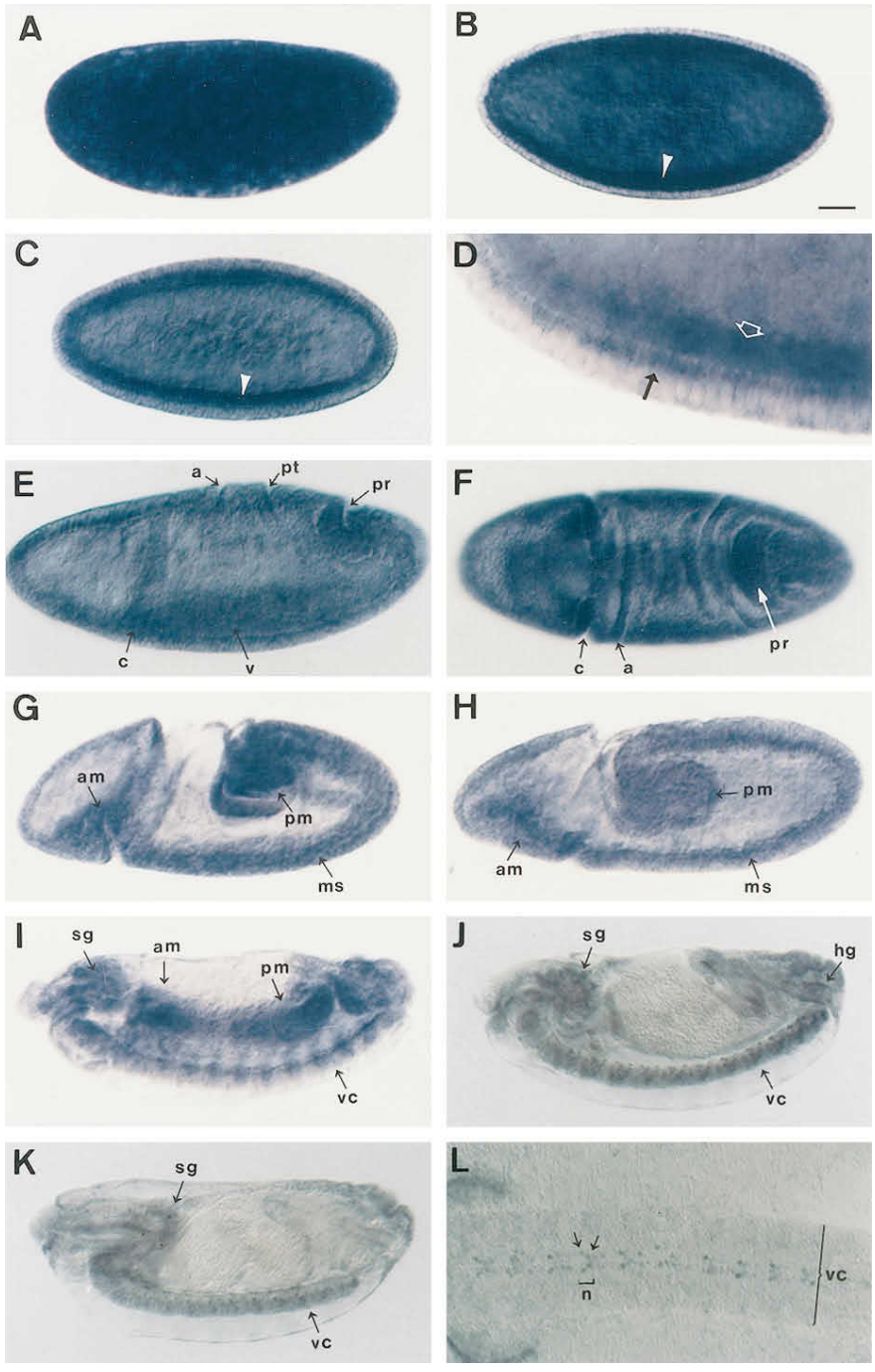

FIG. 4. **Expression of DGCN2 during embryogenesis.** Embryos were *in situ* hybridized with antisense *DGCN2* RNA probes. When appropriate, anterior is *left* and dorsal is *up*. Embryos in *panels E–K* are shown with a midsagittal plane of focus. *A*, stage 3 embryo (cellular blastoderm) containing high levels of mRNA (probably due to a maternal contribution). *B*, late stage 4 embryo (syncytial blastoderm). The mRNA concentrates in the cortex (*arrowhead*). *C*, stage 5 embryo. *DGCN2* transcripts accumulate in the cortex. *D*, high magnification of the embryo in *panel C* illustrates the localization of the mRNA in the cortex (*arrowhead*) and also in the cytoplasm of the cells (*arrow*). Shown is a stage 6 embryo with a midsagital plane of focus (*E*) or a dorsal view (*F*). Note the accumulation of the mRNA in the furrows (*a*, anterior; *pt*, posterior transversal; *c*, cephalic; *v*, ventral) and in the proctodeal primordium (*pr*). *G*, stage 8 (early) embryo. *DGCN2* mRNA is detected in the anterior and posterior midgut (*am* and *pm*, respectively) and in the mesoderm (*ms*). *H*, stage 9 embryo. The accumulation of mRNA in the mesoderm is now clearer. *I*, stage 12 embryo (shortened germ band). mRNA expression is now seen in the CNS, including the ventral cord (*vc*) and the brain primordia. *J*, stage 14 embryo. The expression in the midgut is now barely detectable. In the ventral cord (*vc*), many cells accumulate *DGCN2* transcripts. Note also the expression in the hindgut (*hg*). *K*, stage 16 embryo. Expression is preferentially detected in the nervous system in discrete groups of cells. *L*, dorsal view of the ventral cord (*vc*) of a dissected CNS of a stage 16 embryo showing the expression of *DGCN2* in two pairs of cells (*arrows*) per neuromer (*n*). Scale bar is 50 μm in *panels A–C* and *E–K*; 25 μm in *panel D*; and 15 μm in *panel L*.

three more times with 20 mM Tris-HCl, pH 7.5. The immunoprecipitates were then assayed for their ability to phosphorylate eIF-2. Assay mixtures (30 μl) containing 10 μl of pellet suspension were preincubated for 10 min at 30 °C in the presence of buffer containing 20 mM Tris-HCl, pH 7.5, 0.6 mg/ml bovine serum albumin, 50 μM ATP, and 5 mM $Mg(OAc)_2$. All samples were subsequently incubated for 20 min at 30 °C in the presence of purified rabbit reticulocyte eIF-2 (0.5 μg) as substrate and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). Incubations were terminated by the addition of SDS sample buffer, and samples were analyzed by electrophoresis on 7.5% SDS-PAGE as described (22). After the electrophoresis, the proteins were transferred to an Immobilon-P membrane (Millipore). The membrane was exposed to x-ray film for visualizing phosphorylated proteins and subsequent immunodetection with anti-DGCN2 antibodies.

RESULTS

Isolation of DGCN2 cDNA—Degenerate oligonucleotide primers were synthesized based on regions of homology between all previously known eIF-2α kinases. These primers correspond to conserved sequences L(F/H)IQM (kinase subdomain V) and KIGDFG (kinase subdomain VII). Despite the fact that subdomain VII is well conserved in all protein kinases, any one of the amplified products had to belong to an eIF-2α kinase gene because the subdomain V is specific to eIF-2α kinases (23). Following PCR with the indicated primers on genomic DNA, a product of 278 bp was subcloned and sequenced. The nucleotide and the amino acid analyses of this product revealed a gene fragment that showed an ORF (nucleotides 2397–2608) displaying 73.3% similarity to yeast GCN2. This amplified gene fragment contained an intron of 64 nucleotides interrupting the ORF. The intron boundaries presented the donor/acceptor splice signals (GT/AG) and were inserted after the position 2437 of the *DGCN2* cDNA (data not shown). Because of its potential authenticity, this gene fragment was used to screen a cDNA library from *Drosophila* embryos. From 1×10^6 phages,

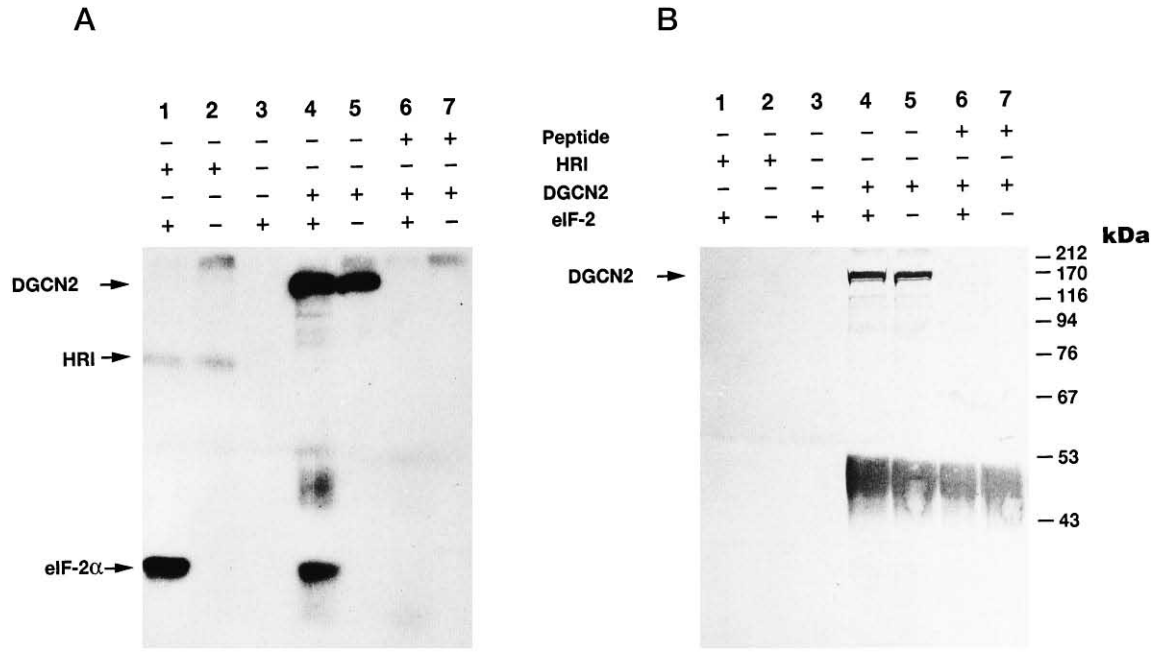

FIG. 5. **Phosphorylation of rabbit reticulocyte eIF-2α by DGCN2 immune complexes from *Drosophila* embryo extracts.** *A*, *in vitro* phosphorylation of eIF-2α by purified HRI from rabbit reticulocytes (20) (*lane 1*) as a control and by either immune complexes prepared from 0–12-h-old *Drosophila* embryo extracts immunoprecipitated with the anti-DGCN2 antibodies in the absence (*lanes 4* and *5*) or in the presence (*lanes 6* and *7*) of the competing peptide, as described under "Experimental Procedures." In *lanes 2, 5,* and *7*, the addition of eIF-2 was omitted. The purified eIF-2 preparation was free of endogenous kinase activity (*lane 3*). Proteins were separated by 7.5% SDS-PAGE and transferred to an Immobilon-P membrane, and this was followed by autoradiography to visualize phosphorylated proteins. Positions of phosphorylated DGCN2, HRI, and phosphorylated eIF-2α are indicated by *arrows*. *B*, the same membrane was probed with the anti-DGCN2 antibodies in a Western analysis. The position of phosphorylated DGCN2 is indicated by the *arrow*. The positions of molecular weight markers are indicated on the *right*.

one positive plaque (clone N1) was purified, excised into pBlue-Script SK$^-$ and sequenced. It contained an ORF of 1131 bp, that was 56% similar to yeast GCN2. The N1 cDNA was used to screen a second embryo cDNA library, leading to the isolation of three independent positives. One of the clones (S24), that overlapped 534 nucleotides with the 3′-N1 clone, contained an ORF of 1060 bp. This ORF shared homology with eIF-2α kinase catalytic domains and with motifs m1 and m2 of HisRS.

Amino acid sequence analyses of N1 and S24 cDNA clones revealed that they lacked the full coding sequence. To get the missing 5′- and 3′-ends, additional sequences were obtained by 5′ and 3′ RACE experiments producing two additional cDNA clones, AR5 and DR3, of 2062 and 2433 bp, respectively. Taken together, all the overlapping *DGCN2* cDNA clones, (AR5, N1, S24, DR3) span a total of 5721 nucleotides (GenBank™ accession number U80223). The resulting full-length cDNA displays a large ORF of 4767 bp (nucleotides 318–5084) flanked by 317 bp of a 5′ non-coding sequence and 637 bp of a 3′ non-coding sequence, which contains a putative polyadenylation signal (AATAAA, nucleotides 5679–5684). The first methionine codon of the ORF (ATG, nucleotides 318–320) is likely to represent the translational start since it matches the *Drosophila* consensus for translational initiators at positions -2, -1, +4, and +5 (24). Moreover, in-frame termination codons are present upstream from the initiation codon, suggesting that this is indeed the authentic start site. The full-length *DGCN2* cDNA encodes for a protein of 1589 amino acids (Fig. 1), with a predicted molecular mass of 178.7 kDa. Note that yeast GCN2 is a 1590-amino acid protein with a molecular mass of about 180 kDa (9). Southern blot analysis of *Drosophila* genomic DNA revealed that *DGCN2* is present as a single copy gene (data not shown).

Conserved Protein Kinase and HisRS Domains in DGCN2 cDNA—The eIF-2α kinase domain of DGCN2 (residues 523–898) contains the 12 conserved catalytic subdomains of Ser/Thr protein kinases (Fig. 1). This domain, as in HRI and yeast GCN2, possesses a large (94 residues) insert between subdomains IV and V (Fig. 2). DGCN2 kinase domain is more closely related to the rat PKR (there are 40.2% identities and 61.3% similarities) and the yeast GCN2 (39.4% identities and 60.3% similarities) although it shares high homology with all of the known eIF-2α kinases (37 to 40% identities and 56 to 61% similarities).

Downstream from the eIF-2α kinase domain, DGCN2 contains a 519 amino acid sequence (residues 919–1437) related to histidyl-tRNA synthetases (HisRS) (Fig. 2). HisRS enzymes share three relatively short sequence motifs (m1, m2, and m3) (Fig. 1; Ref. 25). Like yeast GCN2 (26), the highest degree of similarity between DGCN2 and HisRS occurs in the roughly 360 amino acids stretching from motif 1 to motif 3 (47 to 50% similarities and 22 to 24% identities).

In addition, the amino-terminal portion of DGCN2 contains a sequence (residues 304–518) related to subdomains III to XI of eukaryotic protein kinases (5). The partial kinase segment (Fig. 2) is comparable with that observed in yeast GCN2 kinase, although in the case of yeast, the sequences in the amino-terminal segment are only related to subdomains VIb to XI (10). Taken together, these data indicate that DGCN2 is very closely related to the yeast GCN2 kinase.

Developmental Expression of DGCN2 Transcripts—Northern blot analysis of poly(A)$^+$ RNA isolated from different developmental stages revealed a unique transcript of approximately 6.5 kb. The transcript is expressed throughout development and with two major peaks of accumulation, one during early embryogenesis and the second in the adult stage (Fig. 3).

To determine the embryonic transcriptional regulation of the *DGCN2* gene, we performed *in situ* hybridization on embryos with antisense digoxigenin-labeled probes. Fig. 4 depicts the

spatio-temporal pattern of *DGCN2* expression in embryonic stages classified according to Campos-Ortega and Hartenstein (27). *DGCN2* mRNA can be first detected at high levels during stages 2 and 3 prior to cellularization of blastoderm nuclei (Fig. 4*A*). Later, at the stage of syncytial blastoderm (late 4), the mRNA concentrates peripherally in the so-called cortex (Fig. 4*B*). By stage 5, when the cellularization is complete, the concentration of the mRNA in the cortex is more evident (Fig. 4, *C* and *D*). By the onset of gastrulation, *DGCN2* expression concentrates in several areas of cell movement, including all the furrows and the proctodeal primordium (Fig. 4, *E* and *F*). During the stages of germ band elongation, the mRNA accumulates in the primordia of the gut (anterior and posterior) and in the mesoderm (Fig. 4, *G* and *H*). This mesodermal staining diffuses during stages 11 and 12, corresponding to the reorganization of this mesodermal germ layer. By the end of the stage 12, the mRNA accumulates in the anterior and posterior midgut as well as in areas of the primitive brain (supraesophageal ganglion) and in the central nervous system (CNS) (Fig. 4I). During stage 13 and extending into later stages, accumulation of *DGCN2* transcripts are seen throughout the CNS including the supraesophageal ganglia and the ventral cord. Although by stage 14 where many cells of the ventral cord express *DGCN2* transcripts (Fig. 4J), in later stages, this expression becomes restricted to a few cells (four per neuromer) that probably correspond to neurons (Fig. 4, *K* and *L*).

Characterization of an eIF-2α Kinase Activity from Drosophila Embryos—To determine that DGCN2 is an eIF-2α kinase, we immunoprecipitated DGCN2 from 0- to 12-h-old *Drosophila* embryo extracts by using the anti-DGCN2 antibodies. The isolated immune complexes were incubated with [γ-^{32}P]ATP in the presence or absence of highly purified rabbit reticulocyte eIF-2 as a substrate, and the radiolabeled products were analyzed by SDS-PAGE and Western blot as described under "Experimental Procedures." In control experiments, the reticulocyte heme-reversible HRI (20) was incubated in the presence (Fig. 5, *A* and *B*, *lane 1*) or absence (*lane 2*) of eIF-2. In addition to the eIF-2α phosphorylation, the phosphorylated HRI was observed. Note that the eIF-2 preparation is free of endogenous protein kinases (Fig. 5*A*, *lane 3*). As expected, the anti-DGCN2 antibodies did not recognize either HRI (Fig. 5*B*, *lanes 1* and *2*) or eIF-2 (*lane 3*).

A single labeled polypeptide with a molecular mass of about 175 kDa was produced by DGCN2 immune complexes (Fig. 5*A*, *lane 5*). This phosphopolypeptide was recognized by the anti-DGCN2 antibodies in Western assays (Fig. 5*B*, *lanes 4* and *5*). More importantly, DGCN2 immune complexes phosphorylated the α subunit of the eIF-2 (Fig. 5*A*, *lane 4*). The immunoprecipitation was specific because it was prevented by addition of the peptide immunogen in the immunoprecipitation assay (Fig. 5, *A* and *B*, *lanes 6* and *7*). All together, our results indicate that DGCN2 is, indeed, an eIF-2α kinase of *Drosophila melanogaster* and is phosphorylated in the immune complexes. This reaction may occur by autophosphorylation as in yeast GCN2 (28).

DISCUSSION

Inactivation of the eIF-2 function by phosphorylation of its α subunit is the best-characterized mechanism for regulating total protein synthesis in mammalian cells (1, 2). The regulation of *GCN4* expression in yeast represents gene-specific translational control by phosphorylation of eIF-2α. This unique response depends on four short upstream ORFs in the leader sequence of *GCN4* mRNA (9). As mentioned above, previous studies provided evidence that protein synthesis in insect cells can be regulated by phosphorylation of eIF-2α and inhibition of eIF-2B activity (11, 12).

We have cloned a *Drosophila* eIF-2α kinase through a PCR-based strategy. The data reported here strongly suggest that we have succeeded in characterizing the first homologue of yeast GCN2 from *Drosophila melanogaster*.

The amino-terminal portion of yeast GCN2 contains a sequence related to subdomains VIb to XI of eukaryotic protein kinases. Similarly, DGCN2 contains a sequence, in the amino-terminal region, related to subdomains III to XI of eukaryotic protein kinases (5). It is especially noteworthy that this partial kinase domain of DGCN2 (Fig. 2) displays a significant homology to mammalian Raf proto-oncogene and to yeast BCK1, which are involved in the mitogen-activated protein kinase signaling cascades in vertebrates and *Saccharomyces cerevisiae*, respectively (29). Our finding raises the possibility that DGCN2 may also play additional roles in the signal transduction pathways. In this respect, it has been reported that interleukin 3 stimulates protein synthesis by regulating PKR (30) and that PKR is autophosphorylated *in vivo* in response to platelet-derived growth factor (31).

DGCN2 mRNA is dynamically expressed during embryogenesis. During gastrulation, high levels are detected in the furrows, active areas of cell rearrangements during which cells change their neighbors and environments, allowing interactions and inductive processes between various regions of the developing body (32). Since these morphogenetic movements represent the first morphological manifestation of cell fate and differentiation programs, the localized expression of *DGCN2* in these areas may indicate a putative role in some of these processes, for example, helping to differentiate the three different germ layers of mesoderm, endoderm, and ectoderm. In fact, the early expression in the mesoderm suggests that *DGCN2* could be involved in determining mesoderm germ layer identity during early stages of development.

At late stages of embryogenesis, during the formation of the axon scaffold, *DGCN2* expression concentrates in the nervous system. Interestingly, when the commissures are almost completely formed, this expression is selectively restricted to a few cells of the ventral cord (two pairs of cells per neuromer) that probably correspond to neurons. This surprising selectivity is also consistent with the idea that DGCN2 might be involved in determining neural cell identity for these cells.

Anti-DGCN2 antibodies specifically immunoprecipitated a polypeptide of about 175 kDa from *Drosophila* embryo extracts that specifically phosphorylated the α subunit of eIF-2 (Fig. 5*A*). This polypeptide is a phosphoprotein and is identified by Western blotting with the antibodies (Fig. 5B). The same results were obtained previously when yeast GCN2 immune complexes were used (28, 33).

Collectively, our results indicate that DGCN2 is a *Drosophila* eIF-2α kinase homologue of the yeast GCN2 kinase. In the future, it will be important to know whether uncharged tRNA or other activators act as a signal to regulate DGCN2. The fact that HRI, as well as PKR, functionally substituted for GCN2 in the *GCN4* translational control of yeast (34) may suggest that DGCN2 will play a similar role. It is noteworthy that amino acid starvation or a defective aminoacyl-tRNA synthetase leads to increased levels of eIF-2α phosphorylation in mammalian cells (35) although the kinase responsible for that has yet to be identified. It will also be interesting to see whether translation of any *Drosophila* embryonic gene is modulated by a system of short upstream ORFs, like that present at *GCN4*, in response to modest changes in the level of eIF-2α phosphorylation.

Several recent studies suggest a role for eIF-2α phosphorylation in the control of cell growth and differentiation (7). Although they mostly concern PKR, it will be of great interest to determine whether DGCN2 is involved in the translational

control of mRNAs that encode key growth regulating proteins and to determine whether eIF-2α phosphorylation has any role in cell-cycle control and cellular differentiation in *Drosophila*.

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