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Molecular cloning of a *Drosophila* diacylglycerol kinase gene that is expressed in the nervous system and muscle

(signal transduction/phosphatidylinositol metabolism)

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ABSTRACT **We have isolated a *Drosophila melanogaster* diacylglycerol kinase (DGK, EC 2.7.1.107) homologue by using a porcine DGK cDNA probe and we have characterized its structure and expression. The DGK cDNA has a single open reading frame that encodes 791 amino acids. The *Drosophila* and porcine DGKs share a similar carboxyl-terminal region, a putative catalytic domain, which is divided into two separate domains in *Drosophila*. The DGK gene was mapped to the cytogenetic position 43F1, and its DGK mRNA is abundant both in embryo and in adult fly. By *in situ* hybridization to sections of adult flies, we demonstrated that the mRNA is present predominantly in the nervous system and muscles, including compound eyes, brain cortex, fibrillar muscle, and tubular muscle. In a 10- to 11-hr embryo, the DGK gene is expressed abundantly in a limited number of cells in the procephalic region and in the ventral nerve cord. The pattern of temporal and spatial expression suggests that the DGK protein has an important function in the adult nervous system and muscle and during the development of the embryonic nervous system.**

In a variety of cell types, phosphatidylinositol 4,5-bisphosphate is hydrolyzed by the action of phosphatidylinositol-specific phospholipase C (PI-PLC) in response to external stimuli, generating two second messengers (1). They are inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (DG), which constitute a bifurcating signaling pathway (2).

Upon cell stimulation, diacylglycerol kinase (DGK, EC 2.7.1.107) initiates the resynthesis of phosphatidylinositols by converting the released DG to phosphatidic acid (3). Furthermore, this enzyme is thought to reduce the level of DG, resulting in the regulation of protein kinase C (PKC) activity. DGK activity is known to exist in a wide variety of tissues and organisms, from *Escherichia coli* to mammals. Recent biochemical studies revealed the existence of various DGK isoforms that differ from each other with respect to cellular localization, substrate specificity, and molecular weight (3–8). However, details of the structural and functional differences among the various types are not yet known. In mammalian cells, the 80-kDa species was initially purified from pig brain and later from thymus cytosol (4). Cloning of the cDNA of 80-kDa DGK recently revealed that the enzyme has E-F hand and cysteine-rich zinc-finger motifs (9, 10).

To elucidate the role of DGK both in the signaling pathway and during development of organisms, we cloned a *Drosophila melanogaster* DGK homologue, which has conserved domains and biochemical features essential to DGK protein function. Here we report the nucleotide and amino acid sequence of the DGK homologue and its spatial and temporal expression.* We previously reported that DGK activity is defective in a retinal degeneration mutant, *rdgA* (11). However, the DGK homologue reported here was mapped to a cytogenetic position different from *rdgA* and is thought to be a second DGK species that is expressed predominantly in the embryonic and adult stages. The DGK gene is expressed predominantly in the nervous system and muscle in an adult fly. In a 10- to 11-hr embryo, the DGK mRNA was detected abundantly in three clusters on each segment in the ventral nerve cord and in sensory organs in the procephalic region. These results suggest that the DGK protein plays important roles both in the adult neuromuscular system and during development of the embryonic nervous system.

MATERIALS AND METHODS

Nucleotide Sequence Analysis. A series of deletion plasmids were prepared by using the exonuclease III/mung bean nuclease and sequenced by the dideoxy chain termination method (12) with modified T7 DNA polymerase (13), using double-stranded templates and specific oligonucleotide primers.

Northern Blot Analysis. Poly(A)$^+$ mRNA was electrophoresed in a formaldehyde/1% agarose gel and blotted to a nylon membrane (14).

***In Situ* Hybridization to Adult Tissue Sections.** Probes for *in situ* hybridization were prepared from a recombinant plasmid constructed by subcloning the 2-kilobase (kb) *Xba* I fragment of no. 40 phage insert in the Bluescript II SK(−) vector (Stratagene). This does not carry such repetitive sequences as the opa repeat, and it contains the coding region homologous to the porcine DGK cDNA. Frozen sections (8 μm) of white (*w*) adult flies were cut on a cryostat and pretreated according to the methods of Hafen *et al.* (15). After pretreatment and air-drying, mild alkali hydrolysis of the RNA probe, overnight RNA·RNA hybridization at 50°C, posthybridization washes, and immunological detection of the hybridized probe were performed as described by Coen *et al.* (16).

Whole-Mount *in Situ* Hybridization to Embryos. Digoxigenin-labeled single-stranded probes were prepared, using the polymerase chain reaction, from the same recombinant plasmid as used for *in situ* hybridization to adult tissue sections. Whole-mount *in situ* hybridization was carried out as described by Tautz and Pfeifle (17).

Chromosome *in Situ* Hybridization. Chromosome squashes for hybridization were prepared as described by Engels *et al.* (18). The procedure for hybridization and post-hybridization was according to de Frutos *et al.* (19).

RESULTS

Cloning of a *Drosophila* DGK Gene and Its Genomic Organization. We screened a *Drosophila* genomic library with the

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Abbreviations: DGK, 1,2-diacylglycerol kinase; PI-PLC, phosphatidylinositol-specific phospholipase C; DG, 1,2-diacylglycerol; PKC, protein kinase C.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. D11120).

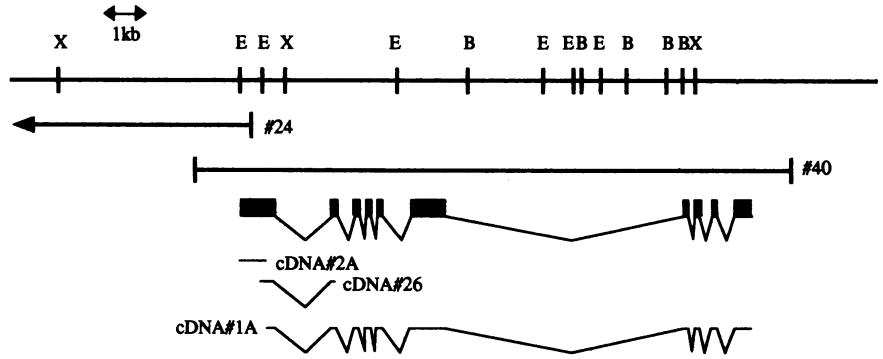


FIG. 1. Structure of the *Drosophila* DGK gene: Restriction map of the *Drosophila* DGK genomic region, genomic phages no. 24 and no. 40, and the composite cDNA. Restriction endonuclease sites: B, *Bam*HI; E, *Eco*RI; and X, *Xba* I. The nine introns shown interspersed in the composite cDNA sequence (lines) were located by comparison with the corresponding genomic sequence (data not shown). The three transcripts below the composite cDNA correspond to the cDNAs no. 1A, no. 2A, and no. 26.

porcine DGK cDNA (λDGK1) (9) under low-stringency conditions and isolated several positive clones. Since the pattern of hybridization of *Drosophila* DNA with phage designated no. 40 (Fig. 1) was identical to that of the Southern blot using the porcine DGK cDNA (data not shown), we concluded that clone no. 40 represents the genomic sequence highly homologous to the porcine DGK gene. Furthermore, we screened the *Drosophila* adult whole cDNA library with the no. 40 genomic fragment under high-stringency conditions, resulting in the isolation of six cDNA clones. We

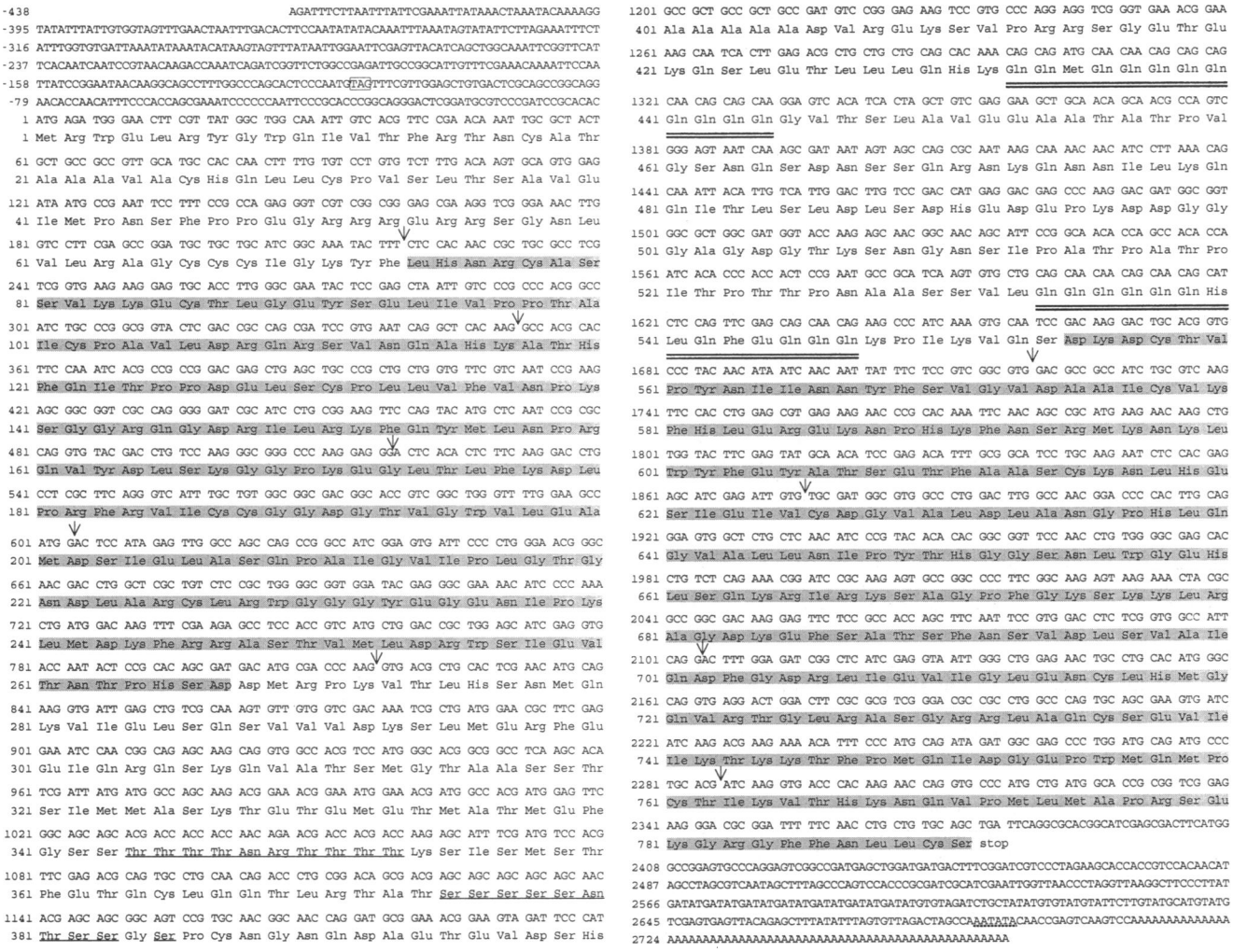


FIG. 2. Nucleotide and deduced amino acid sequences of the composite cDNA derived from the cDNAs no. 1A, no. 2A, and no. 26. A single open reading frame of 2373 nucleotides encodes a protein of 791 amino acids. An AATATA potential polyadenylylation signal is located at nucleotide position 2688–2693. The in-frame termination codon that precedes the translation initiation site is boxed in position −144. The two regions homologous to the porcine DGK are light shaded. The stretches of single amino acid residues are indicated by underlines (threonine and serine residues) and double underlines (glutamine residues). Intron positions are marked by arrows. Comparison of nucleotide sequences between DGK cDNAs (Oregon R *Drosophila* strains) and genome (Canton S *Drosophila* strain) revealed 16 differences in the coding region. Eight of them are silent and do not result in amino acid changes (C → T at position 660; T → C at 1515; A → G at 1608; C → T at 1647; T → C at 1959; C → T at 1980; A → G at 2214; G → A at 2274). Six of them are nucleotide substitutions accompanied by amino acid changes (G → T at 1104, Gln → His; A → G at 1163, Asn → Ser; A → T at 1298, Gln → Leu; G → T at 1504, Ala → Ser; C → T at 1556, Thr → Ile; G → A at 2021, Gly → Asp). Two insertions are found in the genome, CTG (Leu) between 1287 and 1288; CAACAGCAGCAG (Gln-Gln-Gln-Gln) between 1317 and 1318. The latter insertion increases the number of residues in the first glutamine repeat from 12 to 16.

sequenced these cDNA clones and about 9 kb of the corresponding genomic region except for a part of the sixth intron. The two clones having the largest cDNA inserts, phage no. 1A and no. 2A, were mapped in relation to the genomic region (Fig. 1). This transcript is found to correspond to 10 exons. The sequences at the intron borders conform strictly to the consensus sequences of splice donor and acceptor sites (20) (data not shown). These two cDNA clones were adjacent to each other at the *Eco*RI site in the genomic region but did not overlap. To obtain an overlapping clone, cDNA clone no. 26 was amplified from poly(A)$^+$ RNA of an adult fly with polymerase chain reaction using specific primers based on the sequence of these two clones. The cDNA sequence derived from the three overlapping clones was shown to contain a single open reading frame that encodes 791 amino acids (Fig. 2). The sequence surrounding the putative start codon, ACACATG, satisfies the proposed *Drosophila* translation initiation sequence, $^{C}_{A}AA^{A}_{C}ATG$ (21).

Structure of the *Drosophila* DGK Gene. An alignment of the *Drosophila* homologue and the porcine DGK gene is shown in Fig. 3*a*. These genes share a similar carboxyl-terminal domain, which is thought to be the catalytic domain of DGK. However, the most striking feature of the *Drosophila* DGK protein is that the homologous sequences are divided into two clusters. The first homologous region is located closer to the amino-terminal region of the *Drosophila* sequence (residues 74–267; Fig. 3*b*), and the second is at the carboxyl-terminal region (residues 555–790; Fig. 3*c*), the latter being located at a comparable position in the *Drosophila* and porcine DGKs. *Drosophila* DGK protein has an ≈50% amino acid identity with the porcine homologue in each of the two homologous regions. The homology of these regions is even more striking if one takes into account the fact that many of the differences between the *Drosophila* and porcine proteins represent conservative amino acid changes. In this case, the similarities of the first and second domains would be about 76% and 72%, respectively. The characteristic amino-terminal domain of the porcine DGK, which contains E-F hand and cysteine-rich zinc finger motifs, does not exist in the *Drosophila* DGK. There are several stretches of repeated single amino acid residues in the central domain that do not align with the porcine DGK.

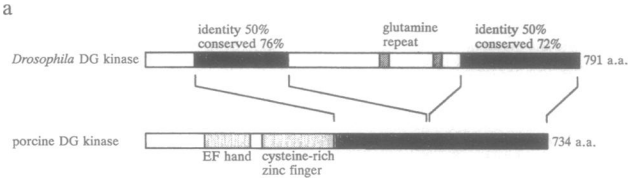
Mapping of the *Drosophila* DGK Gene. To determine the cytogenetic location of the DGK gene, a 9-kb digoxigenin-labeled *Xba* I genomic fragment of clone no. 40 was used as a probe for *in situ* hybridization to polytene chromosomes of larval salivary glands. This probe hybridized to a single site that was identified as position 43F1 on chromosome 2 (data not shown). This genetic locus is different from that of *rdgA* (*rdgA* maps at position 8A4-C6 on chromosome X).

Northern Blot Analysis. Using DGK cDNA clone no. 17B (nucleotides 671–2189), which contained no poly(A) tract, we measured the size and level of mRNA for DGK in various developmental stages (data not shown). Northern blot analysis revealed the existence of two equally abundant transcripts of ≈6 and 3.5 kb in adult head. Only the 6-kb mRNA could be detected in the embryo and only the 3.5-kb mRNA was found in the adult body. No expression could be observed during larval stages. This developmental profile shows that the expression of this DGK mRNA is regulated in a tissue- and stage-specific manner. The sequencing analysis of all seven cDNA clones did not detect nucleotide differences that might have resulted from an alternative splicing. A genomic fragment located downstream of the polyadenylylation site of the composite cDNA was used as a probe for Northern blot analysis; it hybridized only to the 6-kb transcript (data not shown), suggesting that the length of 3′-untranslated region may be responsible for the difference in size between the two transcripts.

Tissue Localization of the DGK mRNA. The spatial localization of the DGK transcripts was investigated more precisely by *in situ* hybridization to adult tissue sections. Single-stranded antisense RNA probes were used, with sense strands as a negative control. The gene was expressed abundantly in the adult head (Fig. 4*a*), where the highest hybridization signal was in the retina and in the brain cortex. In contrast to the cortex, the central neuropil region showed only background hybridization. In adult horizontal sections, strong hybridizations were also observed in the indirect flight muscles and in the jump muscles (Fig. 4*c*).

Whole-mount *in situ* hybridization to stage 14 embryos (10:20–11:20) revealed that the DGK transcript exists predominantly in several groups of cells in the procephalic region and the central nervous system. In the procephalic region, strong signals were detected on both sides of the dorsolateral foregut wall (Fig. 5*a*). This expression continues to the hatching stage. At the stage 17, these cells were adjacent to the dorsal pharyngeal musculature (Fig. 5*b*). The location corresponds to the larval photoreceptor organ described by Bolwig (22) in *Musca*. Furthermore, a strong hybridization was also detected in three different regions of

a



b

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Drosophila DG kinase  74' LHNRCASSVKKECTLGEYSELIVPPTAICPAVL--DRQR
                          •|• | ••• •|| |  • |•||••| |•||   •|
porcine DG kinase    306" IHDDCLPAMGHECDCGLLRDHILPPSSIYPSVLASGQER

111' SVNQ-AHKAT-HFQITPPDEL-------SCPLLVFVNPKSGGRQGDRILRKFQYMLNPRQ
      |  • |•|  •••••• |       • |||||||||||•||•|•| ||||•|||||
345" KVSKTSQKTTDDLNLSTSEALRIDPVSNTHPLLVFVNPKSGGKQGERVLWKFQYLLNPRQ

162' VYDLSKGGPKEGLTLFKDLPRFRVICCGGDGTVGWVLEAMDSIELASQPAIGVIPLGTGN
     |••| | ||  ||  |•••| •|•• ||||||||||•||••|  •|•  |•••|•||||||
405" VFNLLKDGPEPGLRFFREVPDYRILVCGGDGTVGWILETIDKANLPFVPPVALPLGTGN

222' DLARCLRWGGGYBGENIPKLMDKFRRASTVMLDRWSIEVTNTPHSD
     ||||||||||||||•|• |••     •  | •||||•||  •  ••
465" DLARCLRWGGGYBGQNLGKILKDLEASKVVHMDRWSVEVI-PQQTE
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c

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Drosophila DG kinase  555' DKDCTVPYNIINNYPSVGVDAAICVKPHLEREKNPHKF
                           •|  •|||••|||||||•||||•|  •|||• ||| | ||
porcine DG kinase     510" EKSDPVPPQIINNYFSIGVDASIAHRFHIMREKYPEKF

593' NSRMKNKLWYFEYATSETFAASCKNLHESIEI-VCDGVALDLANGPHLQGVALLNIPYTH
     ||||||||||||•||||•  ••|| | ||• • •| | •|||•| • |•|•|•|||| ||
548" NSRMKNKLWYFEFATSESIFSTCKKLEESLTVEIC-GKPLDLSNLS-LEGIAVLNIPSTH

652' GGSNLWGEHLSQKRIRKSAGPFGKSKKLRAGDKEFSATSFNSVDLSVAIQDFGDRLIEVI
     |||||||•     ••• |   • • •   •   •• |  • | •|  •||•
606" GGSNLWGD------TKRPHG---DIHGINQALGAMAKVITDPDILKTCVPDLSDTRLEVV

712' GLENCLHMGQVRTGLRASGRRLAQCSEVIIKTKKTFPMQIDGEPWMQMPCTIKVTHKNQV
     |||  • |||• | |• •|•||| |||•  •| || |||||||||| |||||•||•||•
657" GLEGAIEMGQIYTKLKNAGHRLAKCSEITFHTTKTLPMQIDGEPWMQTPCTIKITHRNQM

772' PMLMAPRSEKGRGFFNLLC
     ||••| • ••  ||  ||
717" PMLVGPPP-RSSNFFGFLC
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FIG. 3. Homology between the *Drosophila* and porcine DGKs. (*a*) Schematic comparison between the *Drosophila* and porcine DGKs. The dark shaded boxes indicate homologous regions. The extent of amino acid identity and total conservation between the two regions of homology are indicated. Glutamine repeat (*Drosophila*), E-F hand (porcine), and cysteine-rich zinc-finger motif (porcine) are marked. a.a., amino acids. (*b* and *c*) Detailed alignment of the *Drosophila* and porcine DGK sequences. Vertical lines indicate identical amino acids and single dots represent conservatively changed amino acids. Conservative substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; and F, Y, and W.

each segment of the ventral nerve cord (Fig. 5*c*). These signals were observed only at stage 14 and disappeared later, unlike the expression in the procephalic region. By stage 14, anterior and posterior commissures become clearly separated from each other, resulting in the appearance of the ladder-like central nervous system. The strongest signals could be observed in three or four midline cells located ventral to each posterior commissure at this stage (Fig. 5*d*). These cells are distinct from midline precursors, because midline precursors are located between and within the transverse commissures. It is not clear whether these cells correspond to the ventral unpaired median (VUM) neurons known as the identified pioneer neurons (23). In addition, the DGK mRNA was also expressed abundantly in a bilateral pair of cells located on the most lateral border of the ventral cord, at the entry point of the axon tracts from sensory organs (Fig. 5*e*). Other signals were observed just lateral to the longitudinal connectives, at the cross-point of the anterior commissures, but they were weaker than the expression in the cells described above (Fig. 5*d*).

DISCUSSION

The *Drosophila* DGK homologue and the porcine DGK gene product share homologous amino acid sequences at the carboxyl-terminal domain. This is consistent with the idea that the region is necessary for the DGK catalytic activity. Furthermore, this region is divided into two in *Drosophila*. This suggests that each part may be responsible for different functions necessary for the catalytic activities. In the homologous regions, significant similarity was detected at several blocks: residues 132–162, 188–199, 214–237, 560–577, 585–609, 639–659, and 743–774. These sequences may represent functionally important domains. The porcine and human DGKs contain two putative ATP-binding sites, one of which is within the first cysteine-rich zinc-finger motif. The *Drosophila* DGK has only one putative ATP-binding site in the carboxyl-terminal region, and lysine (Lys) in the sequence Gly-Xaa-Gly-Xaa-Xaa-Gly- - - -Lys is replaced by serine (Ser) at residue 249. At present it is not established whether this ATP-binding motif constitutes a true ATP-binding site. In fact, the activity of porcine DGK is not affected by the amino acid substitution of Lys for arginine (Arg) when assayed after transfection of COS cells with cDNA expression vectors (H.

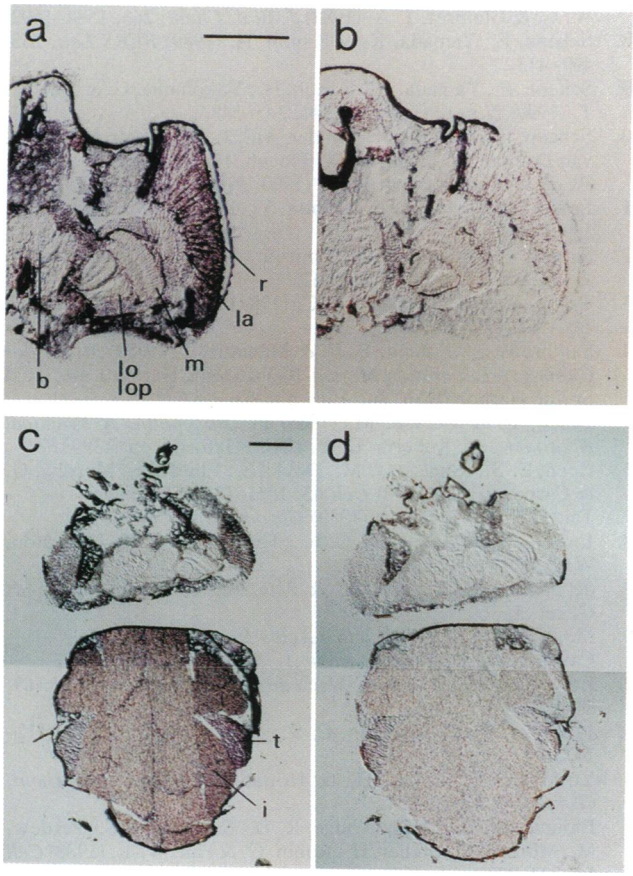

FIG. 4. *In situ* hybridization of the DGK gene to tissue sections. Cryostat sections of adult *Drosophila* were hybridized with single-stranded digoxigenin-labeled antisense RNA. (*a* and *b*) Photographs of adult head section with antisense-stranded probe (*a*), and with the corresponding sense strand (*b*). The identified regions of the head section are brain (b), lamina (la), lobula (lo), lobula plate (lop), medulla (m), and retina (r). (*c* and *d*) Horizontal section of adult flies hybridized with antisense (*c*) and sense-stranded probe (*d*). The identified regions of the thorax section are jump muscle (tergal depressor of the trochanter muscle) (t) and indirect flight muscle (i). (Scale bars, 200 μm.)

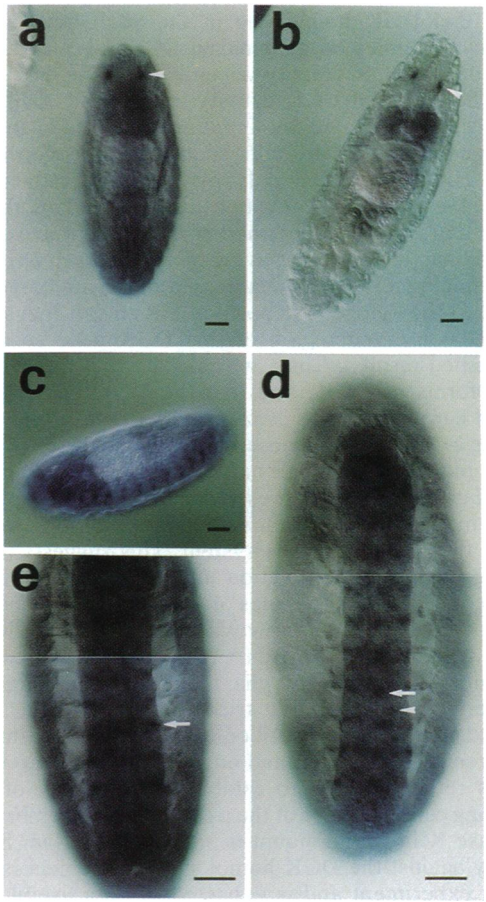

FIG. 5. Whole-mount *in situ* hybridization to embryos. Cells expressing the DGK mRNA were localized in whole-mount embryos by using a digoxigenin-labeled probe. Antisense probe was used in all photographs. (*a* and *b*) Dorsal views of whole-mount *in situ* hybridization of a stage 14 embryo (*a*) and a stage 17 embryo (*b*). Anterior is up. Arrowheads indicate hybridization signals in the procephalic region. (*c*) Ventrolateral view of a stage 14 embryo. Anterior is left. The strong expression could be observed in three different classes of cells of each segment in the ventral nerve cord. (*d* and *e*) Higher magnification of the central nervous system of a stage 14 embryo at different ventrodorsal levels. (*d*) Arrow marks the midline cells that abundantly express the DGK mRNA. Arrowhead indicates weaker signals located on either lateral side of anterior commissures, closely associated with the longitudinal connectives. (*e*) Arrow indicates a bilateral pair of cells located on the most lateral border of the ventral cord. (Scale bars, 50 μm.)

Kanoh, personal communication). These facts suggest that the ATP-binding site of DGK protein may have properties different from those of binding sites found in other protein kinases and ATPases.

In situ hybridization of the DGK probe to adult head cryosections shows that the DGK gene is abundantly expressed in the retina. In stage 14–17 embryos, the DGK mRNA is abundantly expressed in the larval photoreceptor organ. The studies on visual mutant no receptor potential A (*norpA*) have revealed that phosphatidylinositol metabolism plays a central role in phototransduction in the *Drosophila* eye (24–27). The expression of the DGK gene in early embryonic and adult photoreceptors raises the possibility that this DGK protein may phosphorylate the DG that is released by the action of photoreceptor-specific PI-PLC (the product of the *norpA* gene) in response to light. It is necessary as a next step to analyze the possible visual function of this DGK homologue by its ectopic photoreceptor-specific expression using *P*-element-mediated germ-line transformation. The DGK activity is drastically reduced in the head in the retinal degeneration mutant, *rdgA*. This seems to be contradictory to the expression of this DGK homologue in the adult head. But the biochemical studies on mammalian DGK revealed that estimation of the DGK activity depends upon the assay conditions. The defect of DGK activity in the head of *rdgA* was originally demonstrated by Inoue *et al.* (11). If the DGK homologue reported here corresponds to an isoform whose activity can be detected only by the more refined assay method, the above contradiction disappears. It is therefore possible that *rdgA* encodes another DGK isozyme expressed in the retina. In fact, we have isolated another *Drosophila* DGK homologue, which is expressed in the adult eye and is mapped to the cytogenetic position 8BC (*rdgA* locus), using the most conserved region of this DGK gene as a probe (unpublished results). Thus, we consider that there are multiple isoforms of DGK, and that the DGK homologue we reported here is the DGK isozyme other than *rdgA*.

Strong expression was observed in three groups of cells of each segment of the ventral nerve cord in stage 14 embryos. These data show the expression pattern of phosphatidylinositol-metabolizing enzyme during the embryonic development of the central nervous system. This suggests that this DGK homologue may function during nervous system development. To clarify the roles of the DGK in the embryonic stage, it is important to know whether the cells in the three clusters where the DGK mRNA is abundantly expressed are the identified neurons or glia that have been previously characterized in detail. We tried to recognize the several identified neurons, using antibodies that detected fushi tarazu (*ftz*), even skipped (*eve*), and engrailed (*en*) gene products, but it is rather difficult to establish the identity of DGK-expressing cells, because of the treatment of the embryo with proteinase K in whole-mount *in situ* hybridization. Specific antibody against the DGK homologue will be necessary for such an experiment and is expected to be a useful tool to characterize the expression pattern of the embryonic nervous system more clearly.

To investigate further the biological function of the DGK gene, in particular the physiological role of the DG–PKC cascade, it is essential to isolate a mutant of the DGK gene and analyze its phenotype. Furthermore, it may be possible to transform *Drosophila* with a DNA sequence coding for the DGK protein under the control of tissue-specific promoters or enhancers for artificial expression of the DGK activity in ectopic cells. If ectopically tissue-specific expression of the DGK gene alters cellular DG metabolism, the examination of such a transformant for phenotype in physiology, morphology, and behavior will provide a powerful means to genetically dissect the role of the DG–PKC cascade.

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