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Hexokinase like protein from *Drosophila melanogaster*.

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Summary

A genomic clone from *D. melanogaster*, when completely sequenced and analyzed, showed the presence of an uninterrupted coding sequence of 1398 bp coding for a 465 amino acid ORF. Although this protein showed overall 31% identity and 56% similarity to the mammalian HK1, critical glucose binding residues are altered in this protein. In mice, a moonlighting role has been assigned to HK1 testis specific isoform in the sperm egg interaction suggesting that the glucose binding ability of hexokinases can be co-opted for carbohydrate binding in the extracellular matrix of the egg. Conservation of such homologue in *Drosophila* raises very interesting possibilities about moonlighting roles of hexokinases during evolution.

Hexokinases ({ATP:d-hexose6-phosphotransferase}, EC2.7.1.1) are ubiquitous housekeeping enzymes catalyzing the phosphorylation of glucose by ATP, which occurs in all eukaryotic and prokaryotic cells as the first step in the utilization of glucose (Grossbard and Schimke, 1966; Knutsen *et al.*, 1969; Faulkner and Jones, 1976; Peters and Neet, 1977). Mammals show four hexokinase isoforms (Type I-IV) (Grossbard and Schimke, 1966). Apart from acting as a hexokinase, multiple moonlighting roles have been assigned to hexokinases. Rat brain HK-1 was shown to have autophosphorylating and protein kinase activity (Adams *et al.*, 1994), the yeast hexokinase pII was shown to involved in repression of SUC-II gene, which encodes for an enzyme involved in disaccharide metabolism (Piller *et al.*, 1998). Immunolocalization studies indicated the presence of testes specific HK-1 isoform on the mouse sperm surface suggesting its probable role in sperm and egg zona pellucida (ZP3) interaction (Kalab *et al.*, 1994; Travis *et al.*, 1998). Based on biochemical and genetic analysis, four isoforms of hexokinase from *D. melanogaster* have been reported (Murray and Ball, 1967; Cavener, 1980; Madhavan *et al.*, 1972; Voelker *et al.*, 1978). Using native gel electrophoresis, Murray and Ball (1967) have described a testes specific isoform Hex T. Hex A and Hex B isozymes are products of a single X-linked locus (8D/ X), which are largely expressed in adult thorax and larval muscle tissue, respectively, while Hex C (52 E/ 2R) enzyme is expressed in adult and larval fat body. Here, we report the sequence analysis of a 465 amino acid coding ORF, which, although it has homology to hexokinases, shows remarkable alteration in typical substrate binding domains.

A genomic clone from *D. melanogaster* genomic library EMBL3 was completely sequenced. Analysis of a 4509 bp region revealed presence of two genes separated by a short variable-length intergenic sequence of 41 bp (Figure 1). The first gene exhibits an uninterrupted coding sequence of 1398 bp, while the second gene contains a coding sequence of 1359 bp that is interrupted by a single intron of 88 bp. The sequence is deposited in EMBL database (Accession No. AJ 271350). This sequence was localized to 97B on the third polytene chromosome (Data not shown), which is consistent

with its localization reported in *Drosophila* Genome Project (Gadfly Gene CG54430) (Adams *et al.*, 2000) and is different from that of HexA (8D/X) and HexC (51B/2R).

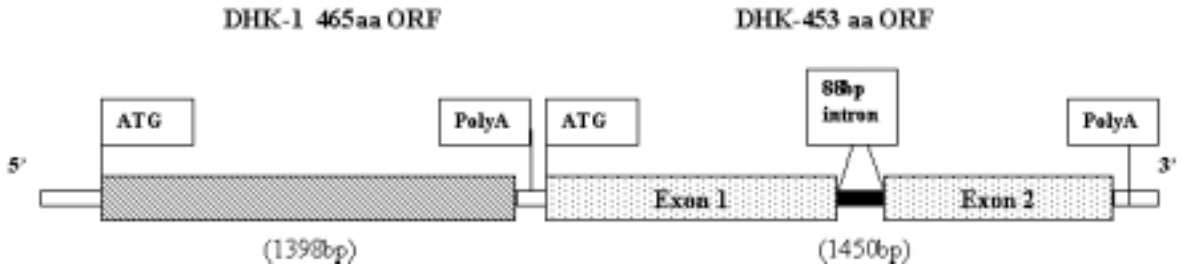

Figure 1. Genomic structure of the 97B-DHK genes. The box indicates the first gene encoding a 465 aa ORF. The second gene is represented by with two exons separated by 88 bp intron sequence coding together for a 453 aa ORF.

The 465 aa long protein (DHK-465) encoded by the first gene (ORF-1) has a molecular mass of 50.2 kDa and theoretical pI of 5.99. BLAST analysis of this ORF showed homology to known hexokinases. It showed 31% identity and 56% similarity with human HK-I isoform. It also showed 32% identity and 55% similarity with Hex C, 36% identity and 62% similarity with Hex A from *Drosophila*. The two other ORF's (ORF-2, ORF-3) together form a complete protein of 453 amino acids (DHK-453) with a calculated molecular mass of 49 kDa and theoretical pI of 8.56. This protein also showed homology to known hexokinases. It showed 41% identity and 59% similarity with human HK-1; 56% identity and 64% similarity with Hex A; 46% identity and 73% similarity with Hex C from *Drosophila.* Remarkably, DHK-465 and DHK-453 shared only 36% identity and 60% similarity with each other.

Further, glucose and ATP binding domains of DHK-465 and DHK-453 were compared with *Drosophila* hexokinases (Hex A and Hex C), mammalian and yeast hexokinase sequences (Figure 2A and B). DHK-465 sequence shows the presence of glucose binding domain at 138-165 aa and ATP binding domain at 68-100 aa residues, respectively. In DHK-453, glucose binding domain resides at amino acid position 133-161 and ATP binding domain resides at position 63-83, respectively. 8 major residues in the glucose binding domain are altered in DHK-465 protein (Figure 2A). Site directed mutagenesis of yeast hexokinase (Y-HKA) demonstrated the importance of Ser158 and Phe160, which are critical for glucose binding (Kraakman *et al.,* 1999). These residues are altered in DHK-465 with alanine and tyrosine, respectively (Figure 2A). Whereas, DHK-453 sequence shows the conservation in these residues. In addition to the involvement of the amino acid residues of glucose binding domain, site directed mutagenesis of human HK-1, demonstrated that Asp657, Glu708 and Glu742 residues were involved in glucose binding and catalysis (Arora *et al.,* 1991). Mutation in Glu708 of HK-1 resulted in 50-fold increase in the km for glucose. Asp657 and Glu708 of HK-1 are altered to Val and Asp respectively in DHK-465. Whereas, Glu742 is conserved at 286 position in DHK-465. All these residues are conserved in DHK-453 sequence (Table 1).

Out of 13 residues important in the ATP binding domain, DHK-465 shows alteration in 5 residues (Figure 2B). Arg539 of human HK-1 has been shown to be essential for catalysis as it stabilizes the transition state product ADP-HK (Zeng *et al.,* 1998). It is conserved in DHK465 at Arg83. Mutation of Gly534 affected ATP binding of HK-1 (Zeng *et al.,* 1998). This residue is altered in DHK-465 with a methionine. Earlier studies from the site directed mutagenesis of human brain HK-1 have

shown that Asp532 in the ATP binding domain interacts with Mg2+ ion and Thr680 stabilize the γ–phosphoryl group of ATP, and their interactions are important for the stabilization of the transition state (Zeng *et al.,* 1996). Both Asp532 and Thr680 are replaced by glutamate and serine in DHK-465, respectively, (Figure 2B and Table 1). Mutations in Gly862 and Gly679 in human HK-1 has a significant influence on the binding affinity of ATP (Zeng *et al.,* 1998). These residues are conserved in DHK-465. Most of the ATP binding residues are well conserved in DHK-453 (Figure 2B and Table 1) as well as other *Drosophila* hexokinases, Hex C (AF2347469 and AJ309864) and Hex A (AAF46507, AAG23047) (Data not shown).

A. Putative Substrate binding sites of DHK-465 and DHK-453 were compared with *Drosophila* HKs (HexA; HexC), mammalian (Human HK-1) and yeast hexokinases (Y-HKA; Y-HKB). The important residues are marked with **'*'**

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DHK-465     138 LPLGIAFAFTLKKLALDVGILVSWTKEF
DHK-453     133 LPLGFTFSFPLQQQGLSKGILVAWTKGF
HexA        181 LPLGFTFSFPLRQLGLTKGILETWTKGF
HexC         92 LPLGFTFSFPCVQLGLKEGILVRWTKGF
H-HK-1      138 MPLGFTFSFPCQQTSLDAGILITWTKGF
Y-HKA       219 LPLGFTFSYPASQNKINEGILQRWTKGF
Y-HKB       129 IPLGFTFSFPASQNKINEGILQRWTKGF
                ****  *  *   *  *   *       *
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B. Core sequence of ATP binding domain of other hexokinases was compared with putative DHK-465 and DHK-453 domains. Highly conserved amino acids in this domain are marked with **'*'**

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DHK-465      73  LALEMMPTNCRIMLV
DHK-453      68  LALDLGGSNFRVLLV
HexA        155  LALDLGGTNFRVLLI
HexC         65  LALDLGGTNFRVLLV
H-HK-1       71  LALDLGGTNFRVLLV
Y-HKA        83  LAIDLGGTNLRVVLV
Y-HKB        82  LAIDLGGTNLRVVLV
                 ******  *****
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Figure 2. Comparison of putative substrate binding domain and ATP binding domain of DHK with other hexokinases.

Thus, although all these observations suggest that the DHK-465 shows overall similarity to hexokinases, the amino acid residues crucial for substrate binding and catalysis are altered in this protein suggesting that this protein has much less efficiency to act as hexokinase. DHK-453 on the other hand shows conservation of all residues significant for substrate binding and catalysis representing a typical hexokinase. Recent findings suggest that somatic hexokinases may have additional locations and different functions within cells (Adams *et al.,* 1994; Piller *et al.,* 1998; Kalab *et al.,* 1994; Travis *et al.,* 1998). A sperm specific HK-1 isoform in mouse and humans has been attributed a role in sperm and zona pellucida (ZP) binding (Kalab *et al.,* 1994; Travis *et al.,* 1998). It is therefore likely that the

Table1. The comparison of amino acid residues important in substrate binding and catalysis between Human brain HK1 and DHKs.

	Human Brain-HK-1	DHK-465	DHK-453
Glucose binding residues	¶Asp657	Val199	¶Asp194
	¶Ser603	Ala145	¶Ser140
	¶Glu708	Asp252	¶Glu247
	*Glu742	*Glu286	*Glu281
	¶Thr658	Gly200	¶Thr195
ATP-binding and catalytic residues	¶Asp531	Glu76	¶Asp71
	¶Thr680	Ser222	¶Thr217
	¶Arg539	Arg83	¶Arg78
	¶Gly534	Met78	¶Gly73
	*Gly862	*Gly402	*Gly397
	♣Gly679	♣Gly221	Val216

♣ Identical residues between HK-1 and DHK-465; * Identical residues between HK-1, DHK-465 and DHK-453; ¶ Identical residues between HK-1 and DHK-453.

glucose-binding ability of hexokinases is co-opted for carbohydrate binding in the extracellular matrix of the egg (Travis *et al.*, 1998). The alterations in substrate binding and catalytic residues critical for a hexokinase in DHK-465 raises further interests about the probable functions associated with this protein in *Drosophila*.

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