

Gene expression pattern

## Characterization of *Dir*: a putative potassium inward rectifying channel in *Drosophila*

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### Abstract

Potassium channels vary in their function and regulation, yet they maintain a number of important features – they are involved in the control of potassium flow, cell volume, cell membrane resting potential, cell excitability and hormone release. The potassium ( $K^+$ ) inward rectifier (Kir) superfamily of channels are potassium selective channels, that are sensitive to the concentration of  $K^+$  ions. They are termed inward rectifiers since they allow a much greater  $K^+$  influx than efflux. There are at least seven subfamilies of Kir channels, grouped according to sequence and functional similarities (Curr. Opin. Neurobiol. 5 (1995) 268; Annu. Rev. Physiol. 59 (1997) 171). While numerous Kir channels have been discovered in a variety of organisms, *Drosophila inward rectifier* (*Dir*) is the first putative inward rectifier to be studied in *Drosophila*. In fact, there are only three genes (including *Dir*) encoding putative inward rectifiers in the *Drosophila* genome. Though there are other known potassium channels in *Drosophila* such as *ether-a-go-go* and *shaker*, most are voltage-gated channels. As an important first step in characterizing Kir channels in *Drosophila*, we initiated studies on *Dir*. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Inwardly rectifying potassium channels; Kir; *Drosophila*; Kir6; *Dir*

### 1. Results and discussion

A cDNA corresponding to a predicted potassium inward rectifier gene (CG6747) located at 94E on the third chromosome was cloned from a 0–24 h *Drosophila* embryonic cDNA library (Fig. 1). We have named this gene *Dir*, for *Drosophila* inward rectifier. The protein contains several conserved domains found in other Kir channels, notably the M1 and M2 domains (Fig. 1). The predicted protein was also found to contain an exact match to the consensus-binding site (RSxpSxP) for 14-3-3 proteins (Fig. 1; Muslin et al., 1996). This result is significant because it suggests a possible regulatory mechanism for *Dir* function. The *Drosophila* potassium channel Slowpoke (Slo) was found to associate in a complex with, and was modulated by, a 14-3-3 protein (Zhou et al., 1999). Interestingly, the two binding sites in Slo do not match with the consensus sequence as well as the site in *Dir* does, yet the sites were found to be functionally significant.

Sequence analysis of *Dir* shows amino acid sequence similarity to Kir channels, with the greatest similarity (57% identity and 70% similarity in the conserved region)

to another predicted *Drosophila* inward rectifier, Irk2 (CG4370). Significant similarity (51% identity and 68% similarity in the conserved region) was also found with an uncharacterized predicted Kir from *Caenorhabditis elegans* (accession number GI1326273) (Fig. 2A).

Construction of a phylogenetic tree using the predicted conserved core region (bold amino acids in Fig. 1) places *Dir* relatively close to the Kir6 subfamily (Fig. 2B). The phylogenetic analysis confirms sequence analyses, indicating that *Dir*'s sequence is most closely related to the predicted *Drosophila* Irk2 and the *C. elegans* Kir. Hydrophobicity plots of *Dir* indicate a structure similar to known Kir channels, and one nearly identical to the *C. elegans* Kir (data not shown). The phylogenetic analysis was unable to place *Dir* into any known subfamily. The most closely related subfamily was Kir6, but there are three junctions between the Kir6 subfamily and *Dir*, indicating sequence divergence. Thus, it is likely that *Dir*, Irk2 and the predicted *C. elegans* Kir are members of a novel Kir subfamily, although this classification must await functional analyses.

Antisense RNA probes directed against the cDNA sequence were used for in situ hybridization to *Drosophila* tissues. *Drosophila* embryos show abundant, diffuse expression of *Dir* mRNA beginning with the earliest stages of embryogenesis, indicating that the gene product is mater-

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ATG TAC ATC TTG TTG TTC CGT TTC AGG TAC CGG CAG ACA CGT TTC AGT TCG CGG CGT GTT CGA AAA CGT GTG GTC TTC AAG CAC GAG GGC TGC AAC GTT
M Y I L L F R R R Y R Q T R F S S R R V R K R V V F K H E G C N V
GTG CAG GGA AAT GTG GCC AAG AGA CGG CGT CGC TAT CTG CAG/ GAC ATC TTC ACC ACC CTG GTC GAC GCC CAG TGG CGC TGG ACG CTG CTC GTC TTC GCC
V Q G N V A K R R R R Y L Q D I F T T L V D A Q W R W T L L V F A
GCC AGC TTC GTG TTC TCG TGG GCC TTC TTT GGA TTC ATC TGG TGG ATC ATC GCC TAC GCA CAC AAT GAT CTG GAG TAC ACC AAT CTC AAG AAC CAG TCA
A S F V F S W A F F G F I W W I I A Y
CCC GAT CTG GTG GCC AAC ATA ACG CAC ACG GTC TGC GTA ACA CAG GTC TCC AAT ATG ATG TCC GCC TTC CTG TAC TCC GTG GAA ACC CAG ACG ACG ATA
P D L V A N I T H T V C V T Q V S N M M S A F L Y S V E T Q T T I
GGC TAT GGT AAT CGC TAT GTG ACG GAG GAG TGC CCG GAG GCG ATA TTC ACG ATG TGC ATC CAG TGC ATC ACG GGT GTC TTC ATC CAG GCG TTC ATG GTG
G Y G N R Y V T E E C P E A I F T M C I Q C I T G V F I Q A F M V
GGC AIT GTG TTT GCC AAG TTG TCG CGT CCC AAG AAA CGT GCC CAG ACG CTG CTC TTC TCG CGC AAT GCT GTT ATC TGC CAC CGG GAT GGA GTT CCA TGC
G I V F A K L S R P K K R A Q T L L F S R N A V I C H R D G V P C
CTG ATG TTC CGT GTG GGA GAC ATG CGC AAG TCG CAC ATC ATC GAG GCC CAT GTG CGG GCC CAG ATC ATC CGC AAG AAG GTG ACA AAG GAG GGC GAG GTG
L M F R V G D M R K S H I E A H V R A Q I I R K K V T K E G E V
CTG CCC TTC TAC CAG CAG GAG TTG CAC ATC GGA GAT GGT GGA GAG GAT CGG ATG ATG TTC ATC TGG CCC ACT ACC ATA GTG CAC AAA AIT GAT AGG
L P F Y Q Q E L H I G A D G G E D R L M F I W P T T I V H K I D R
AAC AGT CCA CTG TAC ATG CTC TCC GCC TCC GAT ATG CTA AAG GAA CGC TTC GAA GTG GTG GTT ATG CTG G/AG GGC GTC ATC GAG TCC ACT GGC ATG ACC
N S P L Y M L S A S D M L K E R F E V V V M L E G V I E S T G M T
ACA CAG GCC AGA AGC AGC TAC TTG CCC TCG GAG GTG CTG TGG GGC CAT CGT TTC GTG AAT GTG GTG TCC TTC CGC AAG GAG ACC GGC GAA TAC CAG GTG
T Q A R S S Y L P S E V L W G H R F V V N V V S F R K E G T G E Y E V
GAC TAC ACG CTA TTC AAC AAC ACC TAC GAC GTG GAC ACT CCG CTG TGC AGC GCC AAG CAG CTG GAC GAG CTT AAG TCG GAG TAC TCG AAG AGC GCC AAG
D Y T L F N N T Y D V D T P L C S A K Q L D E L K S E Y S K S A K
TGT GTG AAT GCA CCC TTT GCG GAT CGC ACG CTC TCG GCC AGC TTG TTC CAG CGT ATT GGC TCC GCC GGC TCG GTG GAT CAT CTG GAT CCG GCT AGC GAC
C V N A P F A D R T L S A S L F Q R I A S A A S V D H L D P A S D
GAA TCG CTG GAC TCT GGC CGC CTG CAG ATA CGC TCC CAT TCC ATA CCC AAC GGC GTG CTG GCC AGC GAG CTG AGC CGC TGA ATA ACA ACA AGC ATG GCG
E S L D S G R L Q I R S H S I P N G V L A S E L S R *
TTC GTT CAC ATG GTG CCT GAC CAT CAC GAC GAC GGC GCC CAG CAT CCC CAC CTA TCC AGT ATT AAC GAG AAG ACC AAC TCC GGA AAT TCC ATA AAC AGC
AGC AAT TAC AGC AAT AAC AAT AGC CTG AGC ACG CTG ACC GGA GGA GGA AGT GCT GCC AGC GGA CCC GGA GGA GGC ATC ACC ATT GTG GCG TTC CGG GAA
CCA TGG CGG CGC TGG TGG AGG AGG AGG CGG CGG TAG ACA CAA GTC GAA TCC CCG CGG TCT TAG CAT CAA GCA GGA TCA GCT GCC CAT CGA TTC CAT
TTG TTG ATA TTA TTA AAT TGA GTT AGT CAG TTA TCT GAT GTC GTT GTA TTC TAG TTA AGC ATA GGC TCC CAA TAT ACA TAA TTC TAA CTC AAA CTA AGC
TAA GCG AAA TTC CAT GGA GAG CTC ATC TGT CAT AGC CAT AGA GCT GCT GCA GCT GTG AAC ATC CCT CAA AAA AAT CCC CCT ATC CTG CTG AAG TGG AGG
CAA TCT GAT TGT TAT CCA CCT ATC TAC TAT ACT TAC TAC TAA CCC TGA AAC GAT TGT ACA TGT TTA TGT CTA GGC TAA GCT GAT GAA TGA TTC ACG TTG
TTA GCT TTA GCT TTG TGT TGA ATT TAC TAT AGT TAA GTT ACA AAT TGA TAT TAA CGC AAA GTA AGA TGT GAG ACC CAG AAA CCA AAA TGC TGC AAC TTA
GAC AAC TAG ATA AGT CAT TAA ACG GCT CAC TTA ACA TGA AAT TTA TGC GAA GAC GCA GCG GAA TGG AAA ATT CCC TTT AAG TCG ATT ATT GTT TAA TAT
TAC TCA CTT CAA TGA GAT ATA AAG AAT GTA CAT TTT AAT CCA TAT ACT GTT TCA AAA ACA AGA GCT TCA ATT ATC ATA AGG AGT GGA CAA TAA AAT TGT
ATG CGT ATG TGA GAG TGA AAA AAA AAA AAA AAA AAA

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Fig. 1. *Dir* cDNA sequence and predicted open reading frame. The *Dir* gene contains three predicted exons (not shown). The exon boundaries are indicated by a '/' in the nucleotide sequence in this figure. The conserved region of the protein that was used for amino acid alignments and to generate phylogenetic trees (see Fig. 2) is indicated in bold. The K<sup>+</sup> channel signature sequence is underlined. Transmembrane regions (M1 and M2), predicted from hydrophobicity plots and comparison with other Kir channel sequences, are shaded grey. The putative 14-3-3 binding site (RSxSxP) at the carboxy terminus is boxed. Single letter amino acid codes are used.

nally supplied (Fig. 3A). The ubiquitous expression continues through the remainder of embryogenesis and is accompanied by a marked enrichment in the salivary glands beginning at stage 12 (Fig. 3C). The salivary gland localization is maintained at high levels throughout the development of the glands.

We also examined the localization of *Dir* mRNA in the larval central nervous system and in the imaginal discs (Fig. 4). Examination of the larval brain and ventral ganglion shows several regions of hybridization. In imaginal discs, ubiquitous expression is detected in the eye-antennal, leg, and wing discs (Fig. 4).

Since every cell in an organism maintains membrane potentials and ion balance, ubiquitous expression of *Dir* in embryos and larvae is consistent with the role of Kir channels in determining the resting potential of both excitable and non-excitable cells (Jan and Jan, 1997). Specific cells, such as nerve cells and cells involved in fluid exchange, due to their specialized functions, require a greater concentration of ion channels (Hille, 1992). Thus, enriched expression

of *Dir* in the brain and in the salivary glands is consistent as well.

## 2. Experimental procedures

### 2.1. Isolation of cDNA

The cDNA for *dir* was isolated from a 0–24 h *Drosophila* embryonic cDNA library cloned into the pJG vector (kindly provided by Roger Brent and Russell Finley). The cDNA encoded most of the open reading frame (ORF) and the complete 3' untranslated region (UTR) of *Dir*. The putative initiation Met of *Dir* was determined from comparison of the partial cDNA sequence with the available genomic sequence and by comparison with homologues.

### 2.2. Sequence

Manual sequencing was performed with the Thermo Sequenase Kit (Amersham Pharmacia Biotech). Sequence



Decoration 'Decoration #1': Shade (with solid black) residues that match dir exactly.

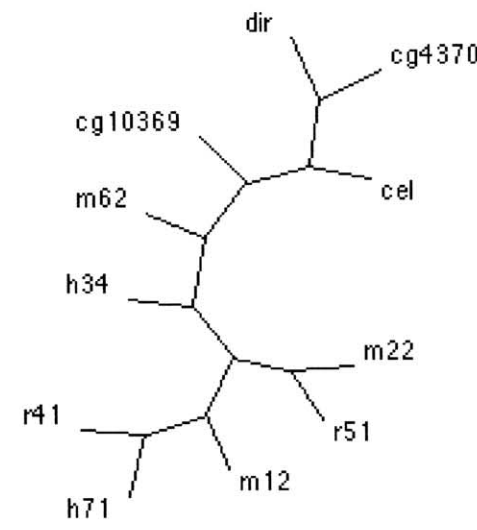


Fig. 2. Multiple sequence alignment (A) and phylogenetic analysis (B) of the conserved region of *Dir* and other Kir channel family members. Multiple sequence alignment was carried out with LASERGENE. Amino acids matching the consensus sequence are shaded. Phylogenetic analysis was performed using PROTML. The unrooted tree shown is the best statistically, of the probable trees determined. The labels in the tree and alignment represent the following: h7.1 (human Kir7.1, accession number G13150184), r4.1 (rat Kir4.1, accession number G1609668), m1.2 (mouse Kir1.2, accession number G13282371), r5.1 (rat Kir5.1, accession number G1609672), m2.2 (mouse Kir2.2, accession number G16624203), h3.4 (human Kir3.4, accession number G11352484), cg4370 (predicted *Drosophila* Irk2, accession numbers G17316093/7300988), *dir* (*Drosophila Dir*, accession number AY044166), cel (predicted *C. elegans* Kir, accession number G11326273), m6.2 (mouse Kir6.2, accession number G12493609), cg10369 (predicted *Drosophila* Irk3, accession numbers G17316108/7298465).

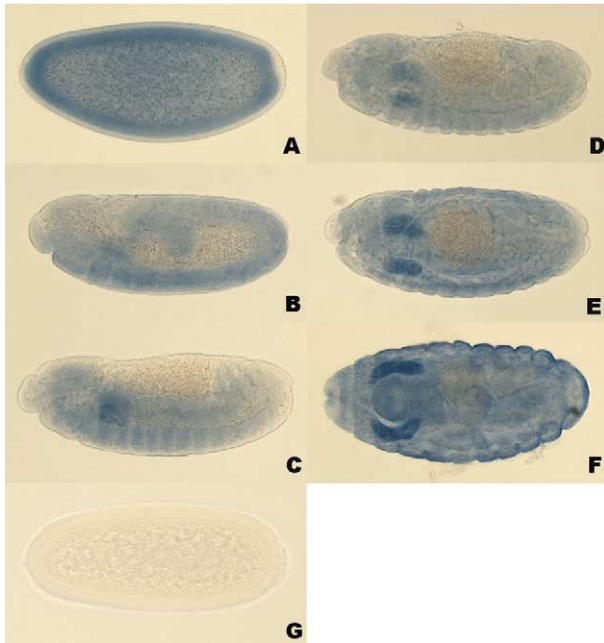


Fig. 3. Localization of *Dir* mRNA in *Drosophila w<sup>118</sup>* embryos. The images in panels (A–F) were obtained after hybridization with an antisense probe. Panel G depicts an embryo hybridized with the negative control sense probe. (A) Stage 5 embryo, lateral view (B) stage 10 embryo, lateral view (C) stage 12 embryo, lateral view (D) stage 13 embryo, lateral view (E) stage 14 embryo, dorsal view (F) stage 16 embryo, dorsal view. *Dir* is expressed abundantly and ubiquitously until stage 12 when enriched expression in the salivary glands becomes apparent. This enriched expression is maintained throughout the embryonic development of the salivary glands.

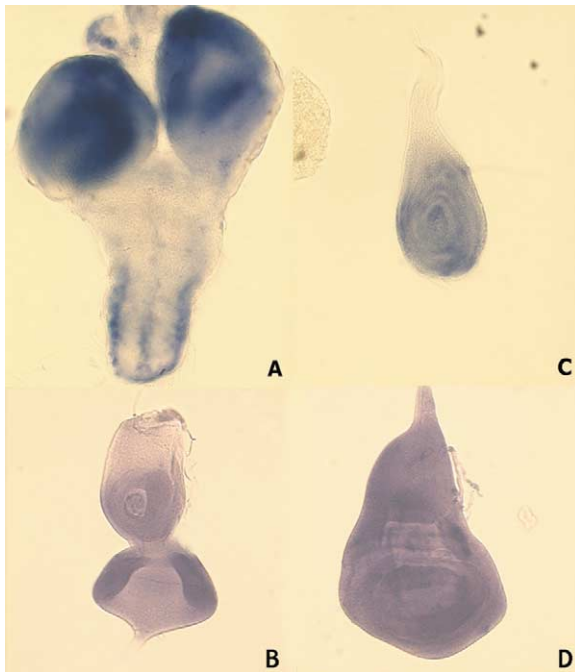


Fig. 4. Expression of *Dir* in larval brain and imaginal discs. There is enriched expression in the (A) larval brain lobes and stem and uniform expression in the (B) eye antennal, (C) leg and (D) wing discs.

was compared to genomic sequence data available from the Berkeley *Drosophila* Genome Project (<http://www.fruit-fly.org>) and from the Celera genome project. Upon sequencing our cDNA, we noted a discrepancy in the 3' end. We found that our *Dir* cDNA and the genomic DNA sequences from both genome projects were in agreement. However, the annotated gene CG6747 contained an additional G residue, which shifted the reading frame after the 14-3-3 binding motif and resulted in a longer carboxy terminus. This discrepancy is not yet resolved, and may still reflect alternative splicing of the gene.

### 2.3. Homology and phylogenetic analysis

Multiple sequence alignments were carried out with LASERGENE (DNASTAR, Madison, WI). Phylogenetic trees were constructed using the amino acid sequence of the conserved core region (spanning from amino acid 21 to 249 of *Dir* and including M1 and M2) of a number of known and predicted Kir channels using PROTML and NUCML programs in the MOLPHY package (version 2.3, Adachi and Hasegawa, 1996), using the JTT model (Jones et al., 1992). Differing tree topologies were compared using the Kishino–Hasegawa test (Kishino and Hasegawa, 1989) at a 95% confidence interval. The trees were visualized using TREEVIEW. Hydropathic plots were constructed with the Weizmann Institute program ([http://bioinfo.weizmann.ac.il/hyd-bin/plotfft\\_hydroph.pl](http://bioinfo.weizmann.ac.il/hyd-bin/plotfft_hydroph.pl)) using the Kyte–Doolittle method of calculating hydrophobicity. The PROSITE database was searched for known protein domains (<http://expasy.cbr.nrc.ca/cgi-bin/scanprosite/>).

### 2.4. In situ hybridization

In situ hybridization of *w<sup>1118</sup>* tissue was performed as described in Lehmann and Tautz (1994), using both sense and antisense probes. The sense probe showed no hybridization in embryos or imaginal discs.

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### References

- Adachi, J., Hasegawa, M., 1996. MOLPHY: programs for molecular phylogenetics based on maximum likelihood, version 2.3, Institute of Statistical Mathematics, Tokyo.
- Hille, B., 1992. Ionic Channels of Excitable Membranes, 2nd ed. Sinauer, Sunderland, MA.

- Jan, L.Y., Jan, Y.N., 1997. Cloned potassium channels from eukaryotes and prokaryotes. *Annu. Rev. Neurosci.* 20, 91–123.
- Jones, D.T., Tayler, W.R., Thornton, J.M., et al., 1992. The rapid generation of mutation data matrices from protein sequences. *Comput. Appl. Biosci.* 8, 275–282.
- Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Mol. Evol.* 29, 170–179.
- Lehmann, R., Tautz, D., 1994. In situ hybridization to RNA. *Methods Cell Biol.* 44, 575–598.
- Muslin, A.J., Tanner, J.W., Allen, P.M., Shaw, A.S., 1996. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* 84 (6), 889–897.
- Zhou, Y., Schopperle, W.M., Murrey, H., Jaramillo, A., Dagan, D., Griffith, L.C., Levitan, I.B., 1999. A dynamically regulated 14-3-3, Slob, and Slowpoke potassium channel complex in *Drosophila* presynaptic nerve terminals. *Neuron* 22 (4), 809–818.