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Expression of *mdr49* and *mdr65* multidrug resistance genes in larval tissues of *Drosophila melanogaster* under normal and stress conditions

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Abstract In situ expression of 2 multidrug resistance genes, *mdr49* and *mdr65*, of *Drosophila melanogaster* was examined in wild-type third instar larval tissues under physiological conditions and after heat shock or colchicine feeding. Expression of these 2 genes was also examined in tumorous tissues of lethal (2) giant larvae *l(2)gl*^{4} mutant larvae. These 2 *mdr* genes show similar constitutive expression in different larval tissues under physiological conditions. However, they are induced differentially by endogenous (tumorous growth) and exogenous stresses (colchcine feeding or heat shock): whereas heat shock and colchicine feeding induce *mdr49*, tumorous condition is accompanied by enhanced expression of *mdr49* and *mdr65* genes.

INTRODUCTION

A major impediment to most drug therapies is overexpression of the multidrug resistance genes, which makes cells or organisms resistant to a wide range of drugs (Beck 1990; Roninson 1992; Ling 1993, 1997). One of the most extensively studied mechanisms responsible for multidrug resistance phenotype is the overexpression of drug efflux pump proteins, the P-glycoproteins (PGP). PGPs are transmembrane glycoproteins of about 170 kDa belonging to a super family of adenosine triphosphate–binding cassette transporters (Juliano and Ling 1976). These proteins function in multidrug resistance by acting as drug efflux pumps to maintain the intracellular concentrations of drug below the cytotoxic levels. The PGP-coding multidrug resistance genes (*mdr*) have been identified in a wide range of species. In human and rodents, multiple *mdr* genes exist. The normal physiological function of PGPs in the absence of cytotoxic drugs is still not known because most of these genes were implicated in the development of multidrug-resistant phenotype. *mdr1* knockout mice are viable and fertile as long as they are not challenged with any drugs (Borst and Schinkel 1997). Expression of PGP on the secretory surfaces in a number of tissues including adrenal gland, kidney, liver, intestinal tract, uterine epithelium, etc, suggests a role either in transporting substances across the cell membrane or decreasing absorption from the surroundings (Thiebault et al 1987). Expression in the capillary endothelial cells of the brain, nerves, testis, and placenta suggests a role in keeping the toxins out of the system (Arceci et al 1988). In human and mouse, the expression of *mdr1* gene appears to be affected also by heat shock, heavy metals, differentiation-inducing agents, chemotherapeutics, hormones, and ultraviolet light (see Sukhai and Piquette-Miller 2000 for review).

Homology search in *Drosophila* led to identification of 3 *mdr* genes, named according to their chromosomal locations as *mdr49, mdr50,* and *mdr65* (Wu et al 1991; Gerrard et al 1993). The *Drosophila mdr* homologues share approximately 50% identity to mammalian homologues and 53% homology among themselves at the nucleotide level (Wu et al 1991; Gerrard et al 1993). The *mdr49* deletion mutants showed increased sensitivity to dietary colchicine, which did not affect expression of the *mdr65* gene (Wu et al 1991). It has also been shown that the *mdr65* gene is responsible for the alpha amanitin resistance found in a population of *Drosophila melanogaster* (Begun and Whitely 2000). In this study, expression of *mdr49*

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and *mdr65* in different tissues of late third instar larvae of *D melanogaster* was examined by in situ hybridization using gene specific riboprobes. The results show that although there are no tissue-specific differences in expression of these 2 genes under physiological conditions, heat shock induces only *mdr49* in all tissues, whereas colchicine feeding enhances *mdr49* expression in larval gut and brain tissues. On the other hand, malignant tumors in lethal (2) giant larvae *l(2)gl*^{4} homozygous larvae show enhanced levels of transcripts of both *mdr49* as well as *mdr65* genes.

RESULTS AND DISCUSSION

Expression of *Drosophila* mdr49 and mdr65 genes under physiological conditions and during stress

RNA-RNA in situ hybridizations were carried out to study the expression of mdr49 and mdr65 genes in different larval tissues under normal conditions and after heat shock. The study revealed a basal level of expression of *mdr49* (Fig 1A,F,K) as well as *mdr65* RNA (Fig 1A′,F′,K′) in the control larval tissues such as salivary glands, brain, and wing imaginal discs. The mdr transcripts were present only in the cytoplasm with little signal in the nucleus. The expression patterns of mdr49 and mdr65 transcripts in the different tissues were found to be similar with little tissue-specific differences.

It is known that promoters of human multidrug resistance genes harbor stress-responsive elements, which respond to heat shock, heavy metals, and cytostatic drugs (Chin et al 1990; Kioka et al 1992). To study expression of *Drosophila mdr* genes under heat stress, larvae were heat shocked at 37°C for 40 minutes and were either immediately dissected and different tissues were fixed or were allowed to recover from heat shock for different time periods at room temperature before tissue fixation. A 40-minute heat shock caused a significant increase in the expression of *mdr49* in different larval tissues (Fig 1B,G,L) when compared with control tissues (Fig 1A,F,K). Interestingly, levels of the mdr49 transcripts continued to remain increased even after 30 minutes (Fig 1C,H,M) or 60 minutes recovery (Fig 1D,I,N) from heat shock. However, the expression returned to basal non–heat shock levels after 90 minutes recovery (Fig 1E,J,O). The levels of mdr65 transcripts, on the other hand, remained comparable with the control tissues (Fig 1A′,F′,K′), after heat shock (Fig 1B′,G′,L′), or after recovery from heat shock for 30 minutes (Fig 1C′,H′,M′), 60 minutes (Fig 1D′,I′,N′), or 90 minutes (Fig 1E′,J′,O′).

The induction of the heat shock genes in eukaryotes by heat and other forms of stress are mediated by the heat shock transcription factor (HSF) (Fernandes et al 1995). HSF has been shown to be 1 of the major regulators of

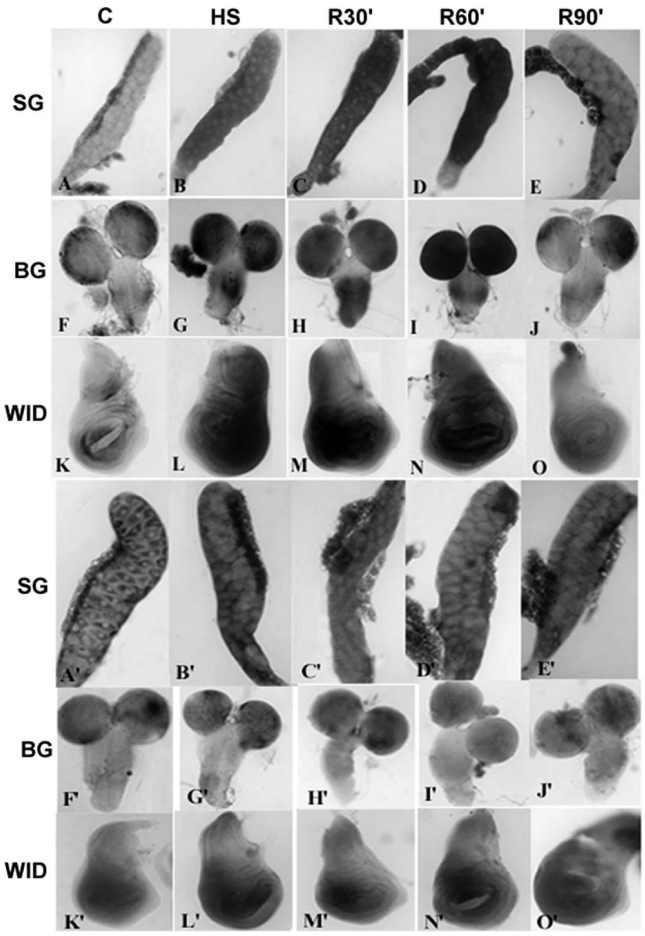

Fig 1. Expression of *mdr49* (A to O) and *mdr65* (A′ to O′) genes in wild-type late third instar larval tissues in control (column C) tissues (SG = salivary glands; BG = brain ganglia; WID = wing imaginal discs), after heat shock at 37°C 40 minutes (column HS), or after 30 minutes (column R30′), 60 minutes (column R60′), and 90 minutes (column R90′) recovery (at 22°C ± 1) from heat shock. The larvae were quickly dissected in Poels salt solution, PSS (Lakhotia and Tapadia 1998), and the tissues were fixed and processed for RNA-RNA in situ hybridization following Prasanth et al 2000. The *mdr49* and *mdr65* clones in pUC18 vector (received from Dr James Croop) were digested with EcoRI, and the 0.7 Kb and 1.7 Kb EcoRI fragments, corresponding to the 3′ ends of *mdr49* and *mdr65,* respectively, were subcloned in *pBS KS+* vector and named *pBSmdr49* and *pBSmdr65,* respectively. For obtaining antisense dig-labeled riboprobes, the *pBSmdr49* was digested with HindIII, whereas the *pBSmdr65* was digested with XhoI. The linearized fragments were used for in vitro transcription using digoxigenin-labeled uridine triphosphate (Transcription Kit, Roche, Germany) and T3 and T7 ribonucleic acid (RNA) polymerase for *pBSmdr49* and *pBSmdr65,* respectively. After in vitro transcription, the *pBSmdr65* probe was chopped by incubating it with 2× carbonate buffer at 65°C for 15 minutes, and the reaction was stopped with sodium acetate and acetic acid (Lehmann and Tautz 1994). This reduced the size of *pBSmdr65* probe to facilitate penetration in the tissues. Antisense riboprobes were hybridized to cellular RNA, and hybridization signal was detected using anti-DIG-alkaline phosphatase as described earlier (Prasanth et al 2000; Rajendra et al 2001). All the images in this and Figures 3 and 4 were collected on a Nikon E800 microscope equipped with the Nikon digital camera DXM1200.

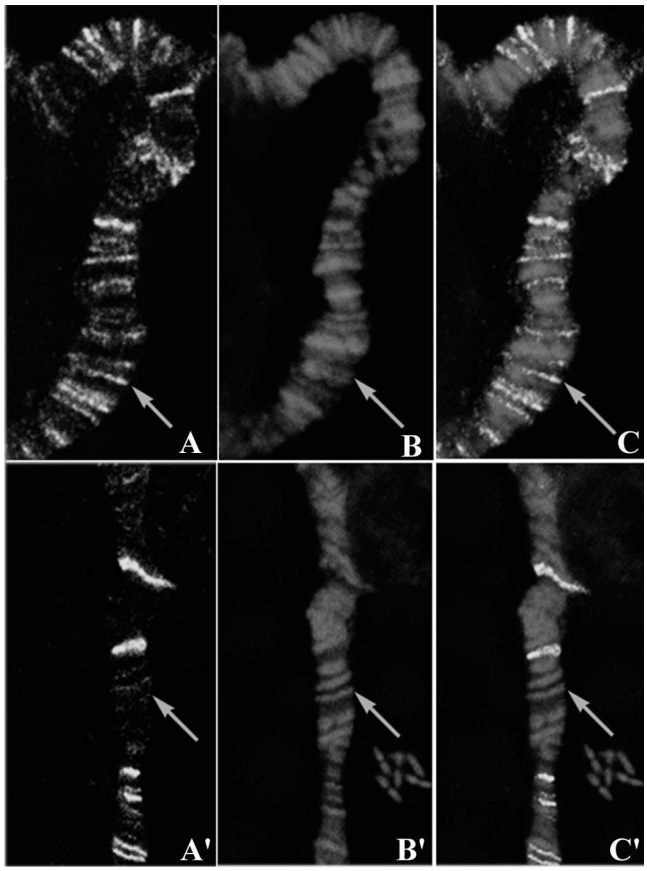

Fig 2. Heat shock factor (HSF) localization on salivary gland polytene chromosomes. Salivary glands were dissected from wild-type late third instar larvae, heat shocked for 30 minutes at 37°C. Squash preparations were prepared following Dangli and Bautz (1983) and processed for immunostaining using the anti-HSF antibody (1:50 dilution, kind gift from Dr Carl Wu) as described earlier (Prasanth et al 2000). The signal was detected using Alexa Fluor 488 anti-mouse antibody (1:200 dilution, Molecular Probes Inc). Polytene chromosomes were counter stained with propidium iodide (PI). Preparations were examined under BIO-RAD Radiance 2000 confocal microscope using 60× oil immersion objective. A and A′ show the antibody localization on 2R and 3L chromosomes, respectively; B, B′ are same chromosome regions counterstained with PI. C and C′ show the merged images. Arrow points to the 49E band in A, B, C and 65A band in A′, B′, C′.

human *mdr1* gene expression (Kim et al 1997); it has been further shown that the expression was reduced when HSF was antagonized using quercitin (Kim et al 1998). In order to examine the involvement of HSF in the upregulation of mdr49 gene after heat shock, polytene chromosomes were immunostained with anti-HSF antibody. HSF was indeed found to be present at *mdr49* gene locus on polytene chromosomes from heat shocked salivary glands (Fig 2A) suggesting that the increase in expression is most likely mediated via the HSF. However, HSF was not present at the 65A region, where mdr65 gene is located, either in control or in heat shocked salivary glands (Fig 2A′).

Response of *mdr49* and *mdr65* to colchicine

One of the substrates transported by PGP is colchicine, and it has been widely used in isolating drug-resistant cell lines. Expression of *mdr49* and *mdr65* in response to dietary colchicine was examined to assess the potential role of these genes against colchicines toxicity. *Drosophila* larvae were fed on food containing nontoxic concentration (10 μM) of colchicine (Wu et al 1991), and the levels of mdr49 and mdr65 transcripts in larval tissues were examined by RNA-RNA in situ hybridization with the *mdr49* and *mdr65* riboprobes, respectively. Larvae fed on colchicine food showed increased expression of *mdr49* in brain (Fig 3B) and gut (Fig 3F) compared with those not fed on colchicine (Fig 3A,E). The mdr65 riboprobe did not reveal any difference in expression of *mdr65* gene between the colchicine-fed larval brain and gut (Fig 3D,H, respectively) and control larval brain and gut (Fig 3C,G, respectively). Imaginal discs and salivary glands did not show enhanced expression under these conditions with either of the probes (not shown).

Overexpression of *mdr* genes in *Drosophila l(2)gl*^{4} mutant

The *mdr* genes have been shown to overexpress in mammalian tumor cells without any previous exposure to drugs (Chin et al 1992). It is postulated that the pathways leading to the tumor formation may somehow also trigger the *mdr* expression (Lee et al 1994). To assess whether the *Drosophila mdr* genes are also upregulated in response to tumor progression, larval tissues from wild-type and tumor suppressor mutant, lethal(2) giant larvae (*l(2)gl*^{4}), were immunostained with anti-Mdr antibody. The *l(2)gl*^{4} mutation results in malignant transformation of late larval imaginal discs and larval brain (Mechler et al 1985). Compared with wild type (Fig 4A,G), the *l(2)gl*^{4} tumorous brain and imaginal discs (Fig 4B,H) showed enhanced levels of Mdr protein. RNA-RNA in situ hybridization with *mdr49* and *mdr65* riboprobes showed that, unlike heat shock and colchicine feeding, both *mdr49* (Fig 4D,J) and *mdr65* genes (Fig 4F,L) were overexpressed in *l(2)gl*^{4} larval tissues when compared with the expression of *mdr49* (Fig 4C,I) and *mdr65* (Fig 4E,K) genes in wild-type tissues.

Results of this study show that unlike in mouse, which shows tissue-specific expression of different *mdr* genes (Croop et al 1989; Hsu et al 1989), the *mdr49* and *mdr65* genes of *Drosophila* do not show any tissue specificity in their developmental expression. It needs to be examined further whether the third *mdr* gene (*mdr50,* Gerrard et al 1993) of *D melanogaster* shows a tissue-specific expression.

It is significant that nonphysiological conditions, such as heat shock, colchicine feeding, or tumorous develop-

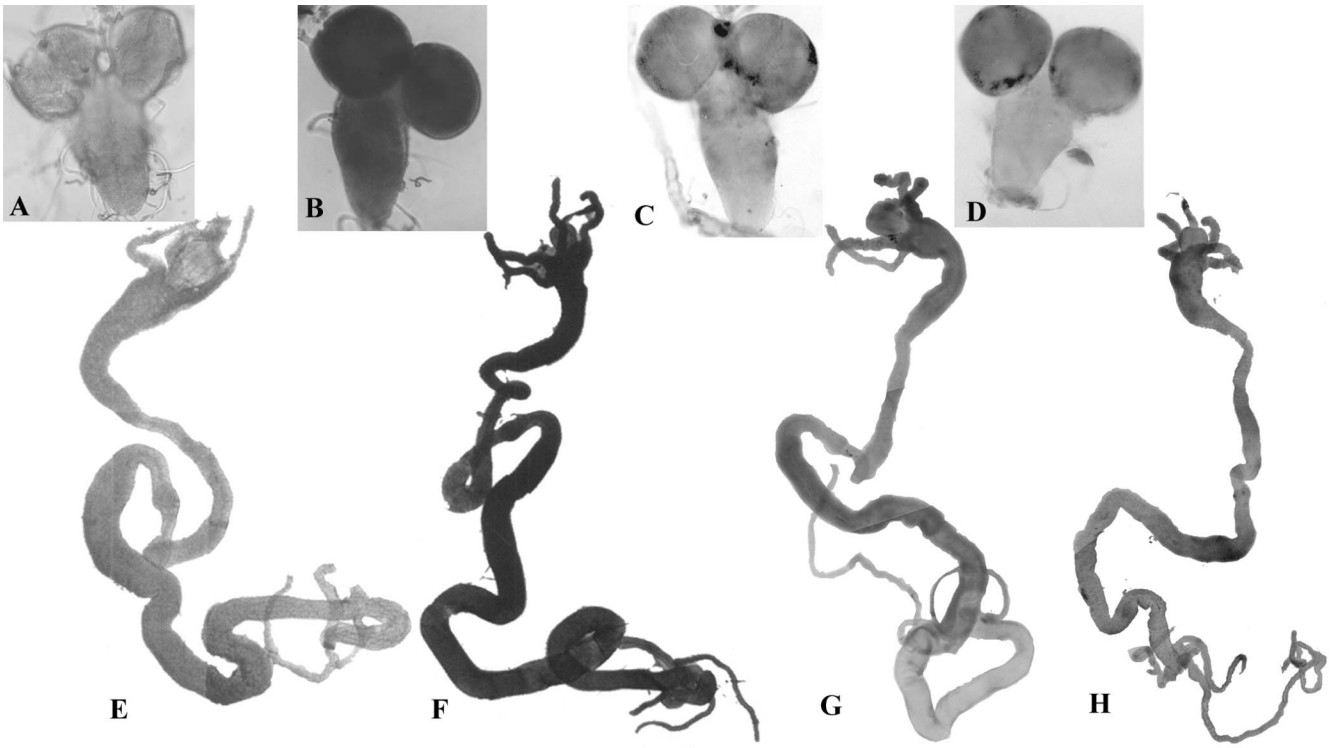

Fig 3. Expression of *mdr49* gene is enhanced upon colchicine feeding. Wild-type flies were allowed to lay eggs on food containing 10 M colchicine, and the larvae were grown on the same food. This concentration although not lethal slows the development. Whole organ RNA-RNA in situ was carried out using antisense riboprobe. There was an elevated level of mdr49 transcripts in brain (B) and gut (F) of colchicine-fed larvae when compared with control brain (A) and gut (E). Mdr65 riboprobe did not show any enhanced expression in brain (D) and gut (H) of colchicine-fed larvae when compared with control brain (C) and gut (G).

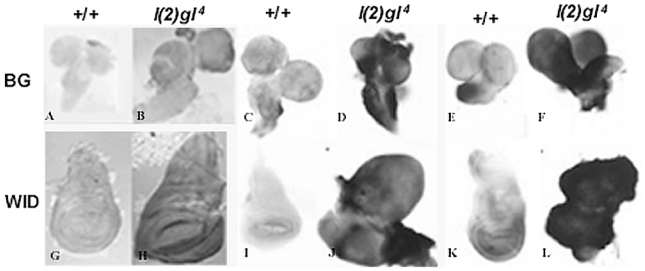

Fig 4. Expression of P-glycoprotein (PGP) in wild-type and tumorous tissues of *l(2)gl*^{4} larvae. Immunostaining with PGP antibody (A,B,G,H) shows that compared with the wild-type (+/+) brain (A) and wing imaginal disc (G), the tumorous brain (B) and wing imaginal disc (H) from *l(2)gl*^{4} mutant larvae show enhanced expression of PGP. RNA-RNA in situ hybridization with mdr49 (C,D,I,J) or mdr65 (E,F,K,L) riboprobe to brain (BG) and wing imaginal disc (WID) of +/+ and *l(2)gl*^{4} larval tissues reveals enhanced expression of both mdr49 (D,J) and mdr65 (F,L) transcript levels in *l(2)gl*^{4} larval tissues. For whole organ immunostaining of the larval tissues, the PGP antibody (kind gift from Dr J. Croop) was used at 1:200 dilution and its binding was chromogenically detected using horseradish peroxidase–labeled anti-rabbit antibody as described earlier (Prasanth et al 2000).

ment, evoke differential induction of the 2 *mdr* genes analyzed in this study. It has been shown in human cell lines and tissues that *mdr* genes use alternative promoters under different stimuli (Ueda et al 1997). Similarly distinct nuclear protein binding sites in the promoter of murine *mdr* gene have also been identified (Yu et al 1993). Analysis of promoter regions of the *mdr* genes of *Drosophila* may reflect differences in the regulation of these genes.

Thus, the *Drosophila* Mdr proteins have physiological roles as transporters for various endogenous compounds and also protect the organism against cytotoxic compounds and environmental insults. Because these functions are similar to those of the vertebrate Mdr protein, *Drosophila* provides a genetically amenable system to elucidate the complex regulation of *mdr* genes.

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