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Expression Corner

Expression analysis of a family of developmentally-regulated cytosolic sulfotransferases (SULTs) in *Drosophila*

KHALID FAHMY[1,2] and STEFAN BAUMGARTNER[1]

[1]*Department of Experimental Medical Sciences, Lund University, Lund, Sweden*
[2]*Drosophila and Developmental Biology Lab, Department of Genetics, Ain Shams University, Shoubra Al-Khima, Cairo, Egypt*

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We have compared the amino acid sequence of all four cytosolic sulfotransferases (SULTs) in *Drosophila* and analyzed their spatial expression patterns during development. Three out of four SULTs show distinct expression activity during embryogenesis, while the 4th SULT shows expression only post-embryonically. *st1*, *st3* and *st4* are expressed in non-overlapping expression domains mainly confined to organs of the alimentary canal such as esophagus, malphigian tubules, hindgut, as well as in the tracheal system. All these organs are surrounded by the hemolymph suggesting that *Drosophila* SULTs exert their function in detoxification of substances upon influx from the hemolymph.

Stefan Baumgartner, Dept. of Experimental Medical Sciences, Lund University, BMC B12, SE-22184 Lund, Sweden. E-mail: Stefan.Baumgartner@med.lu.se

Sulfotransferases are enzymes that catalyze the sulfonation of many proteins, lipids and glycosaminoglycans, reviewed by Chapman et al. (2004). The large family can be subdivided into two sub-families, the membrane-bound sulfotransferases and the cytosolic sulfotransferases. The sub-family of membrane-bound sulfotransferases has been extensively studied and functional data from most members of this sub-family were reported from *Drosophila* up to humans (Nishihara 2010; Mizumoto et al. 2013). On the other hand, much less is known about the sub-family of cytosolic sulfotransferases. These enzymes have a predominant role in catalyzing the sulfonation of many xenobiotics, toxic compounds, bile acids, peptides, hormones and neurotransmitters (Lindsay et al. 2008). The sub-family of cytosolic sulfotransferases (referred to as SULTs) are found in genomes of a wide range of organisms, starting from bacteria, as evidenced by BLAST searches (Baumgartner unpubl.), to *Caenorhabditis elegans* (Hattori et al. 2006) and up to humans (Lindsay et al. 2008). Studies show that the more complex the biological system, the more SULTs are found in the genome. To this extent, *C. elegans* was shown to harbor a single SULT gene (Hattori et al. 2006), while humans have at least 13 family members (Lindsay et al. 2008). Here, we focus on a model system, *Drosophila melanogaster* which was shown to harbor four SULT genes (Hattori et al. 2008; McQuilton et al. 2012). Hattori et al. (2008) analyzed primarily the amino acid sequence and provided biochemical analyses showing that indeed they behave as true sulfotransferases. However, this report did not provide a deep analysis on the expression patterns of the four genes. Our data on the expression profiles provide evidence that all four SULTs are developmentally-regulated and show distinct expression patterns during *Drosophila* embryonic and larval stages. We also demonstrate that three out of four SULT genes show expression in many organs of the alimentary canals and air supply. All tissues with expression constitute tubular organs with a common theme as being exposed to the hemolymph, suggesting a function for detoxification of substances taken up from the hemolymph. Conversely, the 4th SULT does not show any embryonic expression, but rather shows its activity confined to the late stages of the *Drosophila* life cycle, the 3rd instar larval and pupal stage.

MATERIAL AND METHODS

PCR amplification of cDNAs from the four sulfotransferase (SULT) loci

st1 (CG5428): TCGCCCACTTGAGCCACCTAGCCA (forward primer) and ATATATGTACATATATCATCA CAG (reverse primer) were used to amplify a 1130 bp

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cDNA using an embryonic MARATHON cDNA library (Clontech) as template.

st3 (CG5431): ACGAACCTTATCGATAAGGACTGG (forward primer) and CTTAGTTTATGTCTACGTGT GCCT (reverse primer) were used to amplify a 1065 bp cDNA using an embryonic cDNA library (as above) as template.

st4 (CG67049): CACCAGCACCAGCAGCTGACAC TT (forward primer) and TATTCTGTTCCACCCACT CGTTGG (reverse primer) were used to amplify a 1125 bp cDNA using an embryonic cDNA library (as above) as template.

st2 (CG16733): GAACTCGAGGAGGACATACTTC GA (forward primer) and GAACCACGCATATGTCTC TCGGTC (reverse primer) were used to amplify a 910 bp cDNA using a third instar MARATHON library (Clontech) as template.

All four amplified fragments were sequenced to confirm their identities.

In situ hybridization

DNA of all four transcripts was labeled using random-primed synthesis and using high concentrations of random primers (Tautz and Pfeifle 1989). The procedure for *in situ* hybridization was carried out according to (Spirov et al. 2009; Tautz and Pfeifle 1989). An unrelated pKS plasmid, labeled using the same protocol, was used as a negative control.

RESULTS

A family of four cytosolic sulfotransferases (SULTs) in the Drosophila genome

In search for cytosolic sulfotransferases in the *Drosophila* genome, we performed TnBLAST searches using the protein sequence of a cytosolic sulfotransferase in *Caenorhabditis elegans*, Y113G7A.11 (Hattori et al. 2006). This led to the identification of four *Drosophila* paralogues, annotated *sulfotransferase 1* (*st1*; CG5428), *st3* (CG5431), *st4* (CG6704) and *st2* (CG16733) by the *Drosophila* sequencing consortium and FlybBase (BDGP) (Celniker et al. 2002; Hattori et al. 2008; McQuilton et al. 2012). Interestingly, *st1* and *st3* reside at chromosomal location 59F6 and appear as tandemly repeated genes, separated by about 500 bp of intergenic sequence. *st4* is located at 50C20-21 and *st2* at 85D4. All four genes code for proteins with similar sizes between 316 and 346 amino acids, with St4 constituting the largest and St2 the smallest protein of the family (Fig. 1). There are several regions where the homology is pronounced, one in the amino-terminal part and a conserved region in the middle part of the proteins (Fig. 1).

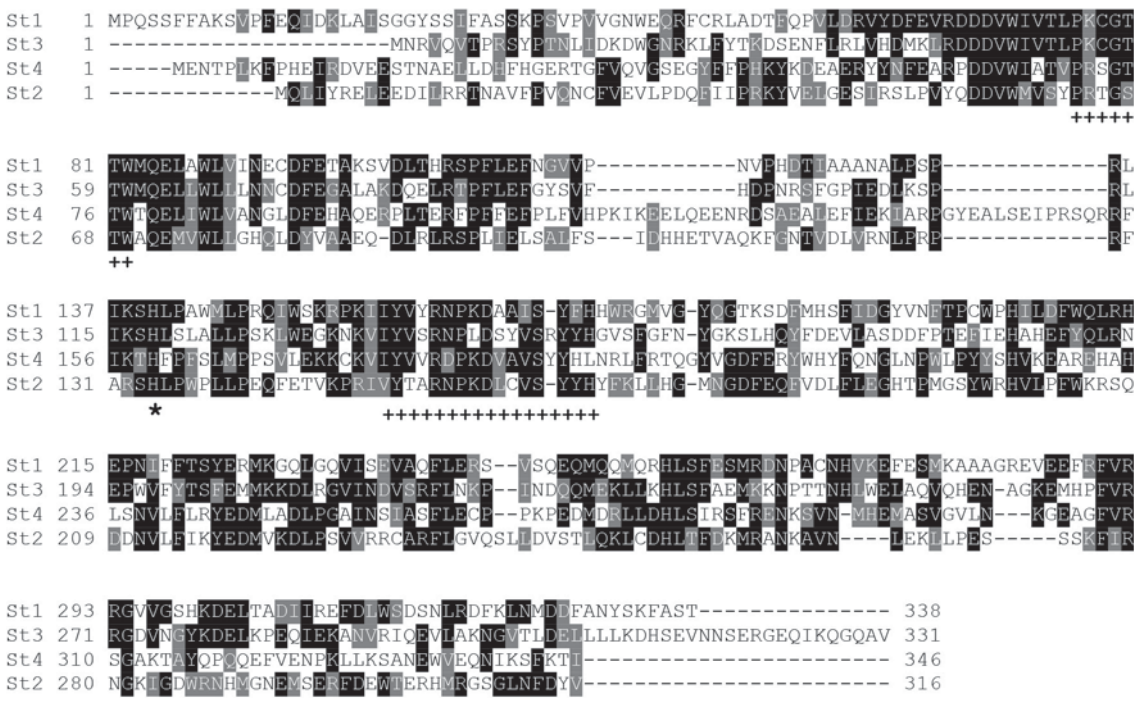

Fig. 1. Alignment of the four cytosolic sulfotranferases in *Drosophila.* Alignment (from top to bottom) of St1, St3, St4 and St2 protein sequences using CLUSTALW2 (Larkin et al. 2007) and BOXSHADE (ch.EMBnet.org). Black shading denotes identity in 50% of the cases or higher, gray shading denote similarities due to conservative exchanges. The asterisks denote the putative active site of the enzyme. Putative PAPS binding area are marked with +.

The degree of identity among the *Drosophila* members is around 40%, while the degree of identity between invertebrate and vertebrate SULTs is around 30%, with St1 showing highest degree of homology. Interestingly, *st3* shows alternative splicing generating at least 4 transcripts according to FlyBase (McQuilton et al. 2012), in contrast to *st1*, *st2* and *st4* which all encode single transcription units.

Expression of the four SULTs during Drosophila development

We employed *in situ* hybridization to detect the spatial transcript patterns of the four sulfotransferase gene in *Drosophila* whole mount embryos. *st1* is expressed ubiquitously in the early developing embryo at low levels (Fig. 2A) up to stage 14, stages are according to Campos-Ortega and Hartenstein (1985), while no maternal contribution was detected (unpubl.). At stage 15, transcripts becomes highly concentrated in the pharynx, esophagus, proventriculus and hindgut (Fig. 2B, D, E), but are also enriched at segmental folds (Fig. 2C, F). Anteriorly, there are some patches of cells that express *st1* (Fig. 2D). This pattern persist until the larva hatches. No transcripts were detected in 3rd instar larval imaginal discs (unpubl.), although FlyBase reports a modest expression during 2nd instar larval and the pupal stages.

st3 shows a quite similar temporal expression profile, suggesting a common gene regulation. There is no transcription detected before stage 14 (data not shown). At stage 14, transcripts are detected in the malphigian tubules (Fig. 2G, I, J, K, L), in the proventriculus (Fig. 2G, H) and in a series of unidentified cells with bilateral symmetry above the pharynx (Fig. 2G, H, I, J). At later stages (stage 16), expression is mainly confined to the malphigian tubules (Fig. 2K, L).

st4 shows no maternal nor early embryonic expression (data not shown) and the first signs of expression are low level ubiquitous expression after stage 12 (Fig. 2M). At stage 13, however, there is conspicuous expression in the pharynx and the esophagus region (Fig. 2N), as well as strong expression in the posterior spiracles, and also in the main trunk of the tracheal system, the dorsal trunk. Notably, the tracheal expression develops from posterior to the anterior (Fig. 2O, P), as observed in other instances (Castillejo-Lopez et al. 2004). During subsequent stages, the dorsal trunk expression becomes more pronounced in a posterior-anterior manner (Fig. 2O). At stage 15 up to 17, the dorsal trunk, the dorsal branches, the transverse connectives as well as the visceral branches show expression (Fig. 2Q), while anteriorly, two bilateral groups of cells, presumably sensory organ precursors, show strong expression (Fig. 2P, R, arrows). Post-embryonically, FlyBase (McQuilton et al. 2012) reported moderate levels of expression during 2nd instar larval and pupal stages.

st2 is quite unique among the three other sulfotransferases in that it does not show any embryonic expression (unpubl.), confirmed by micro-array data from FlyBase (Wilson et al. 2008). Its expression starts during 3rd instar larval development and shows more or less ubiquitous expression in several discs such as the wing disc (Fig. 2S), the eye disc (Fig. 2T) or the leg disc (Fig. 2U) with some elevated levels of expression in the presumptive wing margin (Fig. 2S, arrow) or in tissues posterior to the morphogenetic furrow in eye discs (Fig. 2T, arrow). To note, according to FlyBase, the expression levels of *st2* are predicted to be highest in 2-days old pupae where transcripts are sharply upregulated, to about 60-fold compared to the larval stages.

DISCUSSION

Our expression analysis clearly shows that each SULT has a distinct expression pattern and there is hardly any overlapping expression domain between SULTs (Fig. 2). This applies even for the closely related *st1* and *st3* genes which are arranged in tandem on the chromosome and where shared regulatory elements could enable similar expression patterns.

As far as the function of the four *Drosophila* SULTs is concerned, there are currently no functional data from any model system nor from patient data available that might help to elucidate the role of the SULTs and to give an answer as to why they reveal such distinct expression patterns. Biochemical studies were performed showing that *Drosophila* SULTs are true sulfotransferases and exhibit strong sulfating activity (Hattori et al. 2008), although their function remains elusive.

There is a strong correlation between the expression profiles between fly and human SULTs. For example, many human SULTs are expressed in liver, fetal kidney, gastrointestinal tract and lung (Lindsay et al. 2008). Particularly the latter three expression sites are reminiscent of the expression patterns of *st1, 3* and *4* in tubular organs such as esophagus, malpighian tubules, hindgut or tracheae (Fig. 2).

A quest for possible functions of *st1*, *st3* and *st4* may converge to the notion that all three genes are expressed in tissues of the alimentary canal that are exposed to the hemolymph. These tissues have the capacity to absorbs solutes, water and wastes from the surrounding hemolymph. The function of the SULTs in these tissue could be ascribed to detoxificate substances in the cytosol upon influx from the hemolymph. As far as *st2* is concerned, this SULT shows expression not related to the other three members. A phylogenetic comparison also shows that *st2* does not assemble within the clade of the other

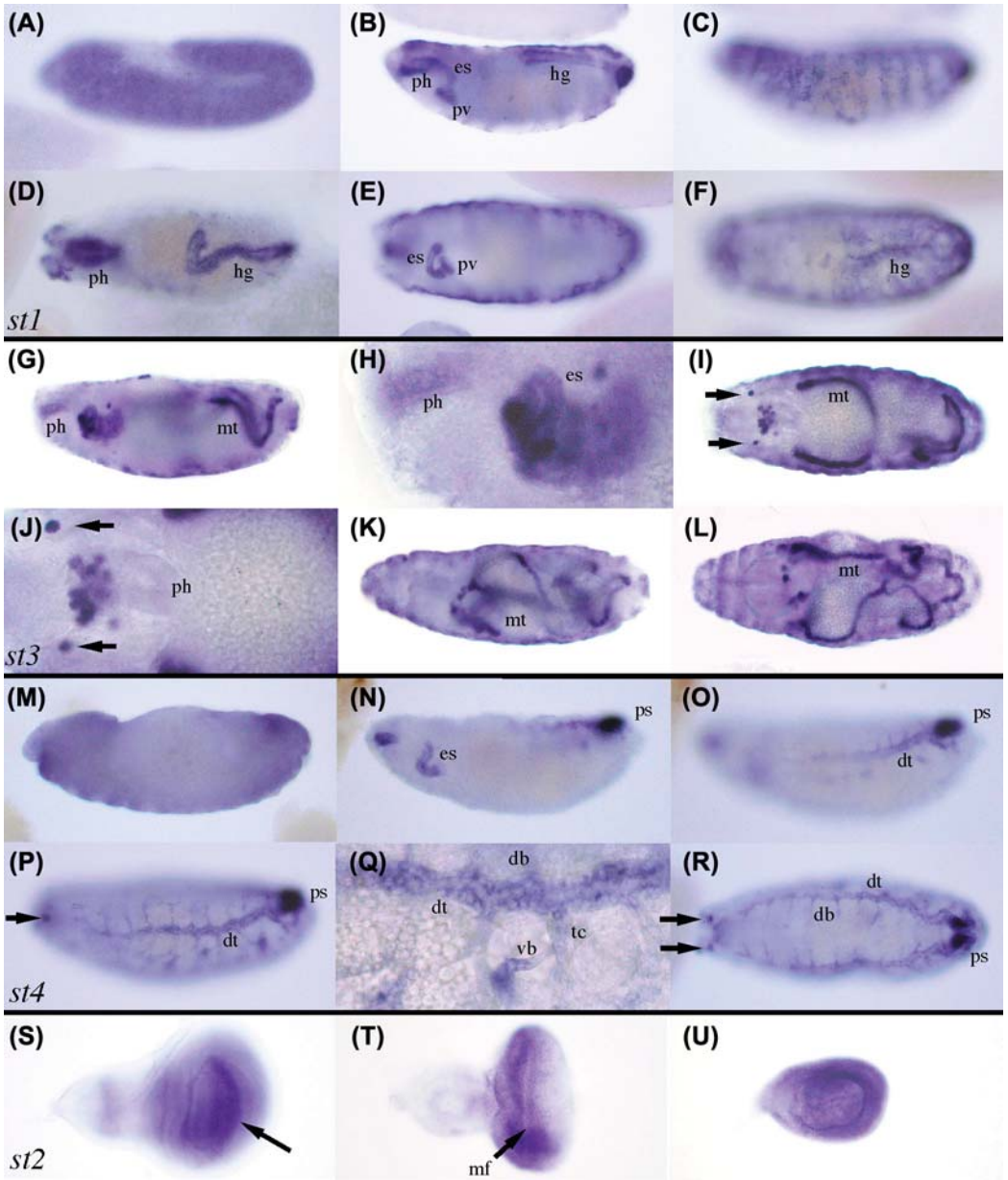

Fig. 2A–U. Expression analysis of the four SULTs during embryonic and post-embryonic development of *Drosophila*. (**A**–**F**) expression of *st1*, (**G**–**L**) expression of *st3*, (**M**–**R**) expression of *st4*, (**S**–**U**) expression of *st2*. (**A**) a stage 11 embryo, all stages are according to Campos-Ortega and Hartenstein (1985), *st1* expression is ubiquitous. (**B**) sagittal and (**C**) surface view of an identical stage 15 embryo, expression is in the pharynx, esophagus, proventriculus, hindgut and at segmental grooves. (**D**) dorsal view of a stage 15 embryo, expression is in a few anterior cells, the pharynx and the hindgut. (**E**) mid-frontal and (**F**) dorsal surface view of an identical stage 16 embryo, expression is still in pharynx, esophagus, hindgut and segmental grooves. (**G**) and (**H**, magnification from **G**) stage 14 embryo, *st3* expression is in the proventriculus, esophagus and the malpighian tubules. (**I**) and (**J**, magnification from **I**) stage 14 embryo, mid-frontal section, expression is in the malpighian tubules, a group of cells surrounding the esophagus, and two anterior cells (arrows in **I**, **J**). (**K**) sagittal and (**L**) horizontal view of stage 16 embryos. *st3* expression is mainly confined to malpighian tubules. (**M**) stage 13 embryos showing *st4* expression ubiquitously throughout the embryo. (**N**) sagittal and (**O**) surface view of an identical stage 14 embryo, expression is in the pharynx, esophagus and the dorsal trunk. Note the expression in the tracheae evolving from posterior to the anterior. (**P**) and (**Q**, magnification from **P**) stage 16 embryo showing expression in tracheae. Anteriorly, two unidentified bilateral cells shows expression (arrow in **P**). (**R**) Stage 16 embryo, horizontal view, the whole tracheal tree shows expression. In addition, the two unidentified cells at the lateral anterior show expression (arrows). (**S**) wing disc, *st2* reveals rather uniform expression with higher levels in cells of the presumptive wing margin. (**T**) eye-antennal disc, only the eye disc shows expression anterior to the morphogenetic furrow. (**U**) leg disc with uniform expression. Abbreviations: ph - pharynx, es - esophagus, pv - proventriculus, hg - hindgut, mt - malpighian tubules, ps - posterior spiracles, dt - dorsal trunk, db - dorsal branch, tc - transverse connective, vb - visceral branch, mf - morphogenetic furrow.

three enzymes (Hattori et al. 2008) suggesting a specialized function. Moreover, St2 was the only enzyme that could not be solubilized upon expression in bacteria (Hattori et al. 2008), compared to the other three enzymes, pointing to a distinct secondary structure.

Recently, a model was put forward how SULTs select their substrates upon influx into cells. Further, the model should explain which mechanism may provide specificity despite the fact that the cytosol is flooded with possible other substrates. This model is summarized as the "molecular pore hypothesis" (Cook et al. 2012, 2013). SULTs were shown to harbour a cap with an active site with affinity to both the nucleotide – and acceptor-binding pockets. Upon nucleotide binding, the cap is closed, thereby opening a small pore at the acceptor binding site. This small pore is crucial for the specificity in sorting xenobiotic substances away from homeostatic substances. Taken this model as basis, it is likely that this mechanism also holds true for SULTs in *Drosophila*, given the high degree of conservation and their documented role as true sulfotransferases.

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