Structural conservation of the salivary gland-specific slalom gene in the blowfly Lucilia sericata

Ali, Reda; Mellenthin, Katja; Fahmy, Khalid; Da Rocha Baez, Sol; Baumgartner, Stefan

Published in:
Development, Genes and Evolution

DOI:
10.1007/s00427-005-0010-2

2005

Structural conservation of the salivary gland-specific *slalom* gene in the blowfly *Lucilia sericata*.

Reda A. Ali%, Katja Mellenthin, Khalid Fahmy[+], Sol Da Rocha and Stefan Baumgartner#

Dept. of Exp. Medical Science, Lund University, BMC B13, S-22184 Lund, Sweden.

% present address: Dept. of Zoology, Faculty of Science, University of Assiut, Assiut, Egypt.

[+] present address: Dept. of Genetics, Faculty of Agriculture, Ains Shams University, Cairo, Egypt.

#corresponding author: Stefan.Baumgartner@medkem.lu.se

FAX 0046 46 2220899

Tel. 0046 46 2223893

3998 words, expected length: 4 pages

running title: conserved *slalom* gene in blowflies

Abstract

Glycosylation and sulfation are two of the essential post-translational modifications of proteins. The *slalom* gene encodes a PAPS transporter, a conserved protein found in organisms as diverse as plants and humans and required for sulfation of proteins. In *Drosophila, slalom* is exclusively expressed in salivary glands, which is unexpected, taken into account the general function for sulfation of proteins. In this paper, we present a detailed description of the *slalom* gene in a large insect, the blowfly *Lucilia sericata.* Our data demonstrates that the *slalom* gene structure, the protein and the expression pattern are highly conserved between *Lucilia* and *Drosophila*. *Lucilia slalom* promoter analysis, using transgenic *Drosophila*, demonstrates that the *Lucilia slalom* promoter can faithfully mimic the expression pattern of both *Lucilia* and *Drosophila slalom* in salivary glands. Taken together, this data shows the structure and the transcriptional cis-regulatory elements of the *slalom* gene to be unchanged during evolution, despite the 100 Mio. years of divergence between the two insects. Moreover, it suggests that the salivary gland-specific expression of *slalom* bears an important and conserved function for sulfation of specific macromolecules.

Keywords:

Lucilia sericata, *slalom*, PAPS transporter, conservation, salivary gland

Introduction

Glycosylation and sulfation are two of the essential post-translational modifications of proteins. Sulfation of proteins requires many different enzymes. The most common sulfate donor is 3'-phosphoadenosine 5'-phosphosulfate (PAPS; Robbins and Lipmann, 1957). PAPS is synthesized in the cytoplasm by PAPS-synthetase (Jullien et al., 1997) and is thereafter transported into the Golgi (Lyle et al., 1994), where it is incorporated into proteins. Transport activity is exerted by the PAPS transporter molecules which constitute a family of highly conserved proteins within the animal kingdom. In *Drosophila*, the *slalom* gene has been identified as the PAPS transporter homologue (Kamiyama et al., 2003; Luders et al., 2003), with one report also showing a functional characterization of *slalom* revealing an involvement in both *wingless* and *hedgehog* signaling, as well as dorsal-ventral axis determination (Luders et al., 2003). Apart from ubiquitous low level expression in the early embryo, the *slalom* gene is exclusively expressed in embryonic and larval salivary glands (Luders et al., 2003), however, the function of the strong expression in this tissue remains enigmatic thus far. According to their proposed biological function, PAPS transporters act in the Golgi apparatus to aid the transport of PAPS into this organelle. Consistent with this notion is the fact that both human and *Drosophila* Slalom show subcellular localization within the Golgi apparatus (Kamiyama et al., 2003; Luders et al., 2003).

Interestingly, there are two other *Drosophila* genes that show strong and exclusive expression in salivary glands: the PAPS synthetase gene (Jullien et al., 1997), and *pipe* (*pip*), a gene involved in dorsal-ventral axis formation encoding a putative heparan sulfate 2-O-sulfotransferase (Sen et al., 1998). The salivary gland specificity of all 3 genes demonstrate that these genes act together in a common pathway providing the sulfate donor for the modification of yet an unknown set of possibly secreted macromolecules. It is still unclear, however, what the nature of these macromolecules is, and which function they have during embryogenesis.

The blowfly *Lucilia sericata* is part of the Calliphoridae family belonging to the Calyptratae group, which includes the house fly *Musca domestica*. These flies are about 100 million years diverged from the family of Drosophilidae (Beverley and Wilson, 1984). This evolutionary "small" distance is reflected in its similar body shape to *Drosophila*, although the size differences are quite obvious. Blowflies are characterized by the ability of their larvae to develop in animal flesh. Those flies, which are not invasive on live hosts, have been used therapeutically to remove dead tissue from wounds, and to promote wound healing. This medicinal use of maggots is increasing worldwide, due to its efficacy, safety and simplicity (Sherman et al., 2000). Moreover, *Lucilia sericata* is also an important tool for forensic entomology (Grassberger and Reiter, 2001).

In search for a salivary-gland specific promoter in *Lucilia*, we noticed the unique expression of the *Drosophila slalom* gene in this tissue, and an attempt was made to clone a *Lucilia* homologue of *slalom*. To learn more about the degree of conservation of *slalom* and if major changes have occurred during the 100 Mio years between the separation of the two insects, we extended our analysis and analyzed the gene structures, their promoters and their ability to confer salivary-gland specific expression. We demonstrate this gene to be highly conserved between *Lucilia* and *Drosophila*, and show that *slalom* is a valuable salivary gland marker. Moreover, our data suggest that the salivary gland expression bears an important function, which has been maintained during evolution for the sulfation of specific macromolecules.

Experimental procedures

Lucilia sericata stocks

A wild-type *Lucilia sericata* strain (kindly provided by Dr. Werner Rudin, Swiss Tropical Institute, Basel, Switzerland) which was collected in the surroundings of Basel (Switzerland), identified by a zoologist and shown to be effective in wound healing was chosen for all experiments. Flies were kept in 60 cm long and 30 cm thick Plexiglas tube-like containers and were fed with dry granular sugar supplemented with a separate mixture of sugar (66%), milk powder (33%) and yeast extract. Water was supplied in a small container and a fresh piece of liver was added every day. Flies were subject to day/night cycles (14h/10h) using a multispectrum lamp as light source.

Cloning of *Lucilia slalom*

Degenerated primers derived from the *Drosophila slalom* (*sll*) coding part were used to amplify initial fragments using an embryonic cDNA library or a first instar larval library from *Lucilia* using the Marathon kit (Clonetech). Based on the isolated sequence, new primers were used to amplify the 5' and the 3' ends of each gene. Additionally, a set of genomic libraries, digested with different enzymes and ligated with a unique linker primer was used to amplify the remaining parts of the gene (McGregor et al., 2001) (libraries kindly provided by Alistair McGregor, Liverpool, GB). At the end, a long cDNA spanning 5'UTR, coding part and 3'UTR was amplified to verify the sequence of the assembled *sll* cDNA. For the isolation of the 2.2 kb fragment encompassing the *slalom* promoter, an inverse PCR approach was chosen. Analysis of the core promoters was done using conventional web-based programs. The *Lucilia sericata slalom* cDNA sequence has accession number AY926574.

In situ hybridization and antibody staining.

Suitable DNA fragments were used to synthesizes probes using a modified DIG-labeling protocol as described (Tautz and Pfeifle, 1989). Treatment of embryos was essentially as

described, with the exception that the proteinase K digestion was at 100 µg/ml for 10 minutes, and postfixation steps were extended to 1 hour.

For antibody staining, a rabbit antiserum against a peptide sequence from *Drosophila* Slalom (Luders et al., 2003) was used. This antiserum does not show any cross-reactivity against *Lucilia* Slalom, despite the high degree of conservation.

***Drosophila* stocks.**

To test the *slalom* promoter activity, the pPTGAL vector was used (Sharma et al., 2002). This vector allows to test and to visualize the promoter activity by using βLacZ in a binary reporter system using GAL4 /UAS (Brand and Perrimon, 1993). A 2.2kb genomic fragment from *Lucilia sericata* and a 2.1kb genomic fragment from *Drosophila melanogaster*, both covering an area immediately upstream of the predicted transcription start site (Fig. 2B) was used and cloned into pPTGAL (Sharma et al., 2002) using conventional cloning methods. Germ-line transformation of w^{1118} was achieved using conventional techniques (Rubin and Spradling, 1982). Several independent integration lines on the autosomes were recovered. After crossing these lines to *UAS-βLacZ* which confers cytoplasmic β-galactosidase localization, the progeny was assayed for β-galactosidase activity as described (Bellen et al., 1989).

Results and discussion

In an attempt to clone a salivary gland-specific promoter from the blowfly *Lucilia sericata*, we noticed that the *slalom* gene in *Drosophila* showed almost exclusive expression in salivary glands (Luders et al., 2003) and would therefore serve as a good candidate for isolating a salivary gland-specific gene from *Lucilia*. Slalom encodes a 3'-Phosphoadenosine 5'-Phosphosulfate (PAPS) transporter which represents an universal sulfuryl donor for the sulfation of proteins. Using degenerated primers from a central portion of the *Drosophila* Slalom coding region, we were able to amplify a 270 bp fragment from a *Lucilia sericata* cDNA library, with a high degree of similarity to *Drosophila* Slalom. Using a combination of subtracted DNA amplification (McGregor et al., 2001) and amplification of a cDNA library, we succeeded in establishing a cDNA that spanned the entire *Lucilia slalom* gene including a 5' untranslated region (UTR), and a 3'UTR incorporating a poly A tail (data not shown). Subsequently, using suitable primers from the 5' and 3' end of the assembled cDNA, we succeeded in amplifying a long cDNA, spanning all sequences previously isolated. This 2.1 kb cDNA contained an open reading frame (ORF) of 474 amino acids (Fig. 1) and verified the amino acid sequence derived from the different subfragments.

Analysis of the coding region of the Slalom protein showed that *Lucilia* Slalom revealed a high similarity to *Drosophila* Slalom, with 73% amino acid identity and 84% similarity (Fig. 1). The degree of identity was more pronounced at the C-terminal end. An extensive BLAST search was carried out, using the *Lucilia* Slalom protein as bait, which showed that PAPS transporter molecules exist in organisms from humans to plants (Fig. 1) with the molecules displaying a high degree of identity with each other. Again, the C-terminal part showed a higher degree of identity than the N-terminal part (Fig. 1). All insect PAPS transporter proteins were about 30-40 amino acids longer, due in part to an N-terminal extension absent in other PAPS transporters from other species (Fig. 1).

Using digoxygenin-labelled DNA probes and antibody staining, the spatial expression of the *slalom* gene was examined in both *Lucilia* and *Drosophila*. The embryology of *Lucilia* has been described in detail (Mellenthin, 2005) and developmental stages are referred to in this report. In *Lucilia*, *slalom* transcripts were first detected around stage 14 in the salivary gland placodes in a uniform pattern (Fig. 2A-D). As development proceeds and elongation of the salivary glands occurs, the transcripts persist in the cells (Fig. 2C, D) until the glands appear elongated before hatching (Fig. 2E). Only salivary gland expression was detected in *Lucilia* which correlates well to the observed unique expression of *Drosophila slalom* in this tissue (Luders et al., 2003; Fig. 2F), suggesting that the two genes are true orthologues. It also indicates that the enigmatic and strong expression in salivary glands must bear an important function which has been retained during evolution.

We also investigated to compare the gene structure of the *slalom* gene between different species. To this end, introns of the *Lucilia slalom* gene were subjected to PCR analysis using suitable primers to evaluate the size and phasing of intron/exon borders. For *Drosophila melanogaster slalom*, a suitable cDNA was compared to the genomic sequence, while in *Drosophila pseudoobsucra slalom*, a gene structure prediction program was used for analysis. As is evident from Fig. 3A, the *Lucilia slalom* gene organization is very similar to that of *Drosophila pseudoobscura* and *Drosophila melanogaster*, in regards to both intron/exon structure, length and phasing. Taking the two *Drosophila* genes as one reference, the *Lucilia* gene does not differ substantially. In particular, the 3' end of the gene shows a high degree of fidelity both in regard to intron/exon length and phasing of introns. In most cases, the phasing of introns relative to the ORF was retained, as was their size. There is a major discrepancy in the 5' end of the gene, here a shorter exon of 860 bp was observed as compared with the 1050 bp in the two *Drosophila* genes. Moreover, a long intron (1860bp) was detected, which is not present in the two *Drosophila* genes. The *Drosophila melanogaster* gene also harbors an intron within the predicted 5' UTR region, not observed in the *Lucilia* gene.

The intron/exon structure of the human *slalom* gene was analyzed to compare it to that of the insect genes. As in *Drosophila*, the human *slalom* gene (PAPST1; (Kamiyama et al., 2003) is quite compact, and the mRNA spans only 3.3 kb of the genomic region (data not shown). However, the human *slalom* intron/exon structure does not show any resemblance to that of insect *slalom*, in particular with respect to the conserved carboxy-terminus (Fig. 3A). Instead, the carboxy-terminal 310 amino acids of human Slalom are all encoded in one large exon, and introns are only detected in the amino-terminal third of the coding sequence (data not shown).

We isolated and sequenced a 2.2 kb putative promoter fragment from *Lucilia slalom* (Fig. 3B) and compared the proximal 500 bp of this fragment to the proximal 500 bp of the *Drosophila slalom* promoter. A predicted core promoter region was to a large extent overlapping, and the predicted transcription start site was noted only 10 bp apart from each other. The *Lucilia* promoter contains a putative TATA box at around –30 (TATAAT), while the *Drosophila* counterpart does not show any TATA motif present. Several conserved transcription factor binding sites were observed, e. g. two *hunchback* binding sites, one immediately in front of the core promoter and one about 200 bp distal from that (Fig. 3B). However, neither *Lucilia* nor *Drosophila hunchback* show strong transcription in salivary glands (McGregor et al., 2001) which casts some doubt on the functionality of these two sites.

To further test the functionality of *Lucilia* and *Drosophila slalom* promoters, we cloned the 2.2 kb promoter fragment from *Lucilia* and a 2.1 kb promoter fragment from *Drosophila* into the pPTGAL vector (Sharma et al., 2002). This vector allows one to test and visualize the promoter activity by utilizing LacZ in a binary reporter system using GAL4 /UAS (Brand and Perrimon, 1993). As is evident in Figs. 3C-H, both insect promoter fragments mimic faithfully the *slalom* transcription pattern in the salivary glands of embryos and of first and third instar larval stages, thereby suggesting all enhancer elements are contained within each of the two promoter fragments. Remarkably, the *Lucilia* promoter appears to be functional in *Drosophila*, showing cross-functionality in both temporal and

spatial expression. This shows the promoter region of the *slalom* gene to be functionally conserved, and that the *slalom* promoter is a very specific tool to drive salivary gland expression. Moreover, the data suggest that the salivary gland expression of *slalom* has an important function which raises some issues on its function in this tissue.

As far as the expression of the human *slalom* gene is concerned, there is a good correlation that secretory organs show high levels of *slalom* expression, in particular pancreas and mammary gland, and somewhat less pronounced, also in salivary glands (Kamiyama et al., 2003). Moreover, human Slalom shows subcellular localization in the Golgi membrane too (Kamiyama et al., 2003), suggesting that also in humans, a set of macromolecules is a substrate for sulfation which subsequently becomes secreted.

The data presented here demonstrates that *Lucilia* and *Drosophila* are very similar, both in gene structure, promoter function and expression pattern of the *slalom* gene, despite their apparent size difference and evolutionary divergence about 100 Mio. years ago. Similar such behavior has been observed whilst analyzing the function of the *wingless* gene in *Lucilia*, although some evolutionary adaptations were observed for this gene (Mellenthin, 2005). Owing to the fact that the *slalom* gene product is instrumental for sulfation of proteins, there seems to be ample selection pressure during evolution to precisely maintain its appearance and function, in particular in salivary glands. It is for this reason that we today look upon a protein which exhibits well-conserved features between both fruitflies and blowflies.

Acknowledgements

S.B. would like to thank the Vetenskapsrådet (Grant 621-2003-3408), the Swedish Cancer Foundation (Grant 4714-B03-02XBB), the "Nilsson-Ehle Fonden" and the "Erik Philip-Sörensen Stiftelse for främjande av genetisk och humanistiks vetenskaplig forskning" for support. R. A. and K. F. would like to thank the "Swedish Institute", the "Stiftelsen Wenner Grenska Samfundet", "Crafoordska Stiftelsen" and "Anna Greta Kock Stiftelse" for support. We thank Alistair McGregor for the subtraction genomic library and Dr. Werner Rudin (Swiss Tropical Institute, Basel, Switzerland) for providing us with *Lucilia sericata* flies. We also wish to thank Darren Cleare and Mikael Fahlman for critical reading of the manuscript..

Figure legends

Figure 1. Comparison of PAPS transporter proteins.

Alignment (from top to bottom) of human PAPS transporter (deduced from AB106538.1), murine PAPS transporter (deduced from AF414190), *Drosophila melanogaster slalom* (Kamiyama et al., 2003; Luders et al., 2003), *Drosophila pseudoobscura slalom* (assembled from contig 3681-434, courtesy of Baylor College of Medicine, USA), *Lucilia sericata slalom* (this work), *Arabidopsis thaliana* PAPS transporter (assembled from NM125366.3) and *Caenorhabditis elegans* PAPS transporter (assembled from NM171463). Black shading denotes identity in more than 50 % of the cases, gray shading denote similarities due to conservative exchanges. Note that the carboxy terminus shows a higher degree of identity, consistent with the higher degree of identity within the gene structure in the carboxy terminus (Figure 3A).

Figure 2. Spatial expression of the *Lucilia sericata* and *Drosophila slalom* genes.

(A) and (B, high magnification) stage 14 (stages are those defined in (Mellenthin, 2005) *Lucilia* embryo, horizontal view, *slalom* staining is visible in the salivary gland placode. (C) and (D, high magnification) late stage 14 *Lucilia* embryo, staining is in the entire salivary gland. (E) high magnification of a stage 16 *Lucilia* embryo, the salivary glands are now elongated and still show specific *slalom* expression. (F) stage 16 *Drosophila* embryo, staining using an anti-*Drosophila* Slalom antiserum reveals the protein in salivary glands.

Figure 3. Comparison of the gene structure and the promoter of *Drosophila melanogaster*, *Drosophila pseudoobscura* and *Lucilia sericata slalom* gene.

A) Predicted *slalom* gene structure from three different insects, *Drosophila pseudoobscura* (Dp), *Drosophila melanogaster* (Dm) and *Lucilia sericata* (Ls). Distances are drawn to scale with the exception of a larger intron in the 5' part of the *Lucilia slalom* mRNA. Above the

intron/exon structure, the phasing of the open reading frame relative to the intron/exon borders are shown, together with the corresponding amino acids shown immediately below. Below the gene structure, distances in nucleotides are shown as figures. Note that the intron/exon-structure and -phasing is better conserved in the carboxy-terminus part of the mRNA which corresponds well with the higher degree of identity in the carboxy terminus among all PAPS transporter proteins (Figure 1).

B) Analysis of about 500 bp of the *Lucilia sericata* (Ls) and *Drosophila melanogaster* (Dm) *slalom* promoter. Nucleotide identities are indicated shaded in black. Predicted core promoter regions are underlined, the predicted transcription start site is indicated with arrows. Transcription factor binding sites are indicated where they show up independently for both promoters.

(C) a stage 15 embryo (stages are those from (Campos-Ortega and Hartenstein, 1985) from the cross *2.1.kb Dm-sll GAL4 > UAS-βLacZ* showing specific expression in salivary glands. (D) a stage 15 embryo from the cross *2.2kb Ls-sll GAL4 > UAS-βLacZ* showing specific expression in salivary glands. (E) first instar larva of the cross *2.1.kb Dm-sll GAL4 > UAS-βLacZ* showing specific expression in salivary glands. Additional staining in the posterior part is an artefact due to pricking of the larvae to ensure penetration of the substrate. (F) first instar larva of the cross *2.2kb Ls-sll GAL4 > UAS-βLacZ* showing specific expression in salivary glands. Additional staining in the posterior part is an artefact due to pricking of the larvae to ensure penetration of the substrate. (G) isolated salivary glands and anterior head parts from a third instar larva from the cross *2.1.kb Dm-sll GAL4 > UAS-βLacZ* showing specific expression in salivary glands. (H) isolated salivary glands from third instar larvae from the cross *2.2.kb Ls-sll GAL4 > UAS-βLacZ* showing specific expression in salivary glands.

References

Bellen, H. J., O'Kane, C. J., Wilson, C., Grossniklaus, U., Pearson, R. K., and Gehring, W. J. (1989). P-element-mediated enhancer detection: a versatile method to study development in Drosophila. Genes Dev *3*, 1288-1300.

Beverley, S. M., and Wilson, A. C. (1984). Molecular evolution in Drosophila and the higher Diptera II. A time scale for fly evolution. In J Mol Evol, pp. 1-13.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Campos-Ortega, J. A., and Hartenstein, V. (1985). The Embryonic Development of Drosophila melanogaster, Springer, Berlin).

Grassberger, M., and Reiter, C. (2001). Effect of temperature on Lucilia sericata (Diptera: Calliphoridae) development with special reference to the isomegalen- and isomorphen-diagram. Forensic Sci Int *120*, 32-36.

Jullien, D., Crozatier, M., and Kas, E. (1997). cDNA sequence and expression pattern of the Drosophila melanogaster PAPS synthetase gene: a new salivary gland marker. Mech Dev *68*, 179-186.

Kamiyama, S., Suda, T., Ueda, R., Suzuki, M., Okubo, R., Kikuchi, N., Chiba, Y., Goto, S., Toyoda, H., Saigo, K., *et al.* (2003). Molecular cloning and identification of 3'-phosphoadenosine 5'-phosphosulfate transporter. J Biol Chem *278*, 25958-25963.

Luders, F., Segawa, H., Stein, D., Selva, E. M., Perrimon, N., Turco, S. J., and Hacker, U. (2003). Slalom encodes an adenosine 3'-phosphate 5'-phosphosulfate transporter essential for development in Drosophila. Embo J *22*, 3635-3644.

Lyle, S., Stanczak, J., Ng, K., and Schwartz, N. B. (1994). Rat chondrosarcoma ATP sulfurylase and adenosine 5'-phosphosulfate kinase reside on a single bifunctional protein. Biochemistry *33*, 5920-5925.

McGregor, A. P., Shaw, P. J., and Dover, G. A. (2001). Sequence and expression of the hunchback gene in Lucilia sericata: a comparison with other Dipterans. Dev Genes Evol *211*, 315-318.

Mellenthin, K., Fahmy, K., Ali, R., Hunding, A. DaRocha, S. and Baumgartner, S. (2005). Wingless signalling in a large insect, the blowfly Lucilia sericata: a beautiful example of evolutionary developmental biology.

Robbins, P. W., and Lipmann, F. (1957). Isolation and identification of active sulfate. J Biol Chem *229*, 837-851.

Rubin, G. M., and Spradling, A. C. (1982). Genetic transformation of Drosophila with transposable element vectors. Science *218*, 348-353.

Sen, J., Goltz, J. S., Stevens, L., and Stein, D. (1998). Spatially restricted expression of pipe in the Drosophila egg chamber defines embryonic dorsal-ventral polarity. Cell *95*, 471-481.

Sharma, Y., Cheung, U., Larsen, E. W., and Eberl, D. F. (2002). PPTGAL, a convenient Gal4 P-element vector for testing expression of enhancer fragments in drosophila. Genesis *34*, 115-118.

Sherman, R. A., Hall, M. J., and Thomas, S. (2000). Medicinal maggots: an ancient remedy for some contemporary afflictions. Annu Rev Entomol *45*, 55-81.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. Chromosoma *98*, 81-85.

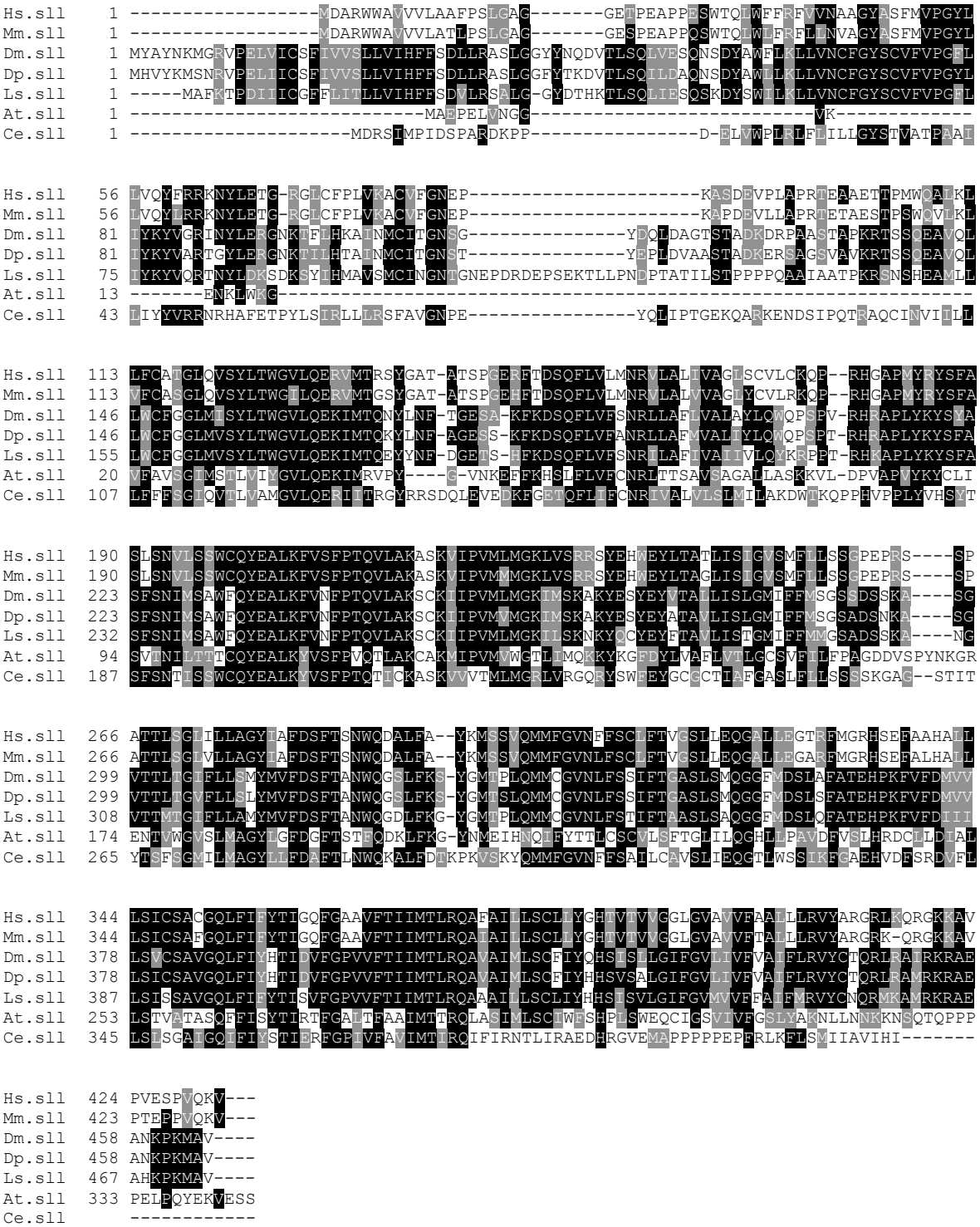
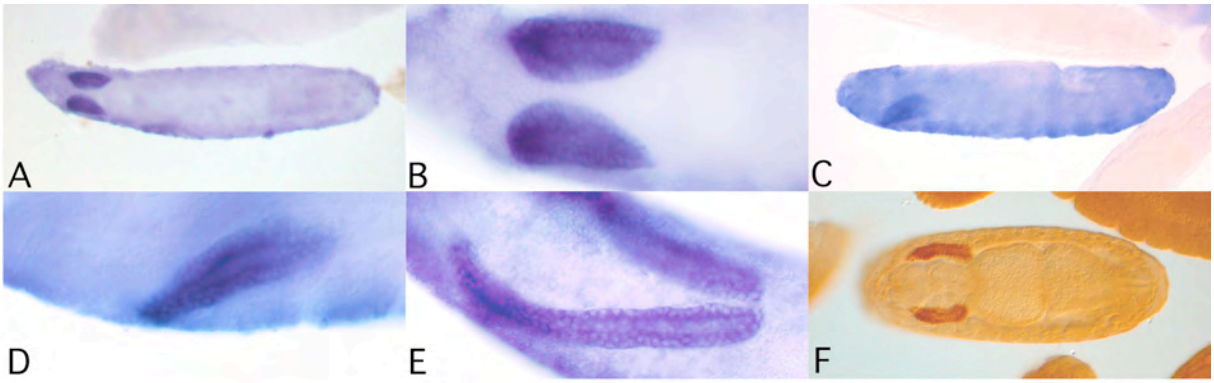
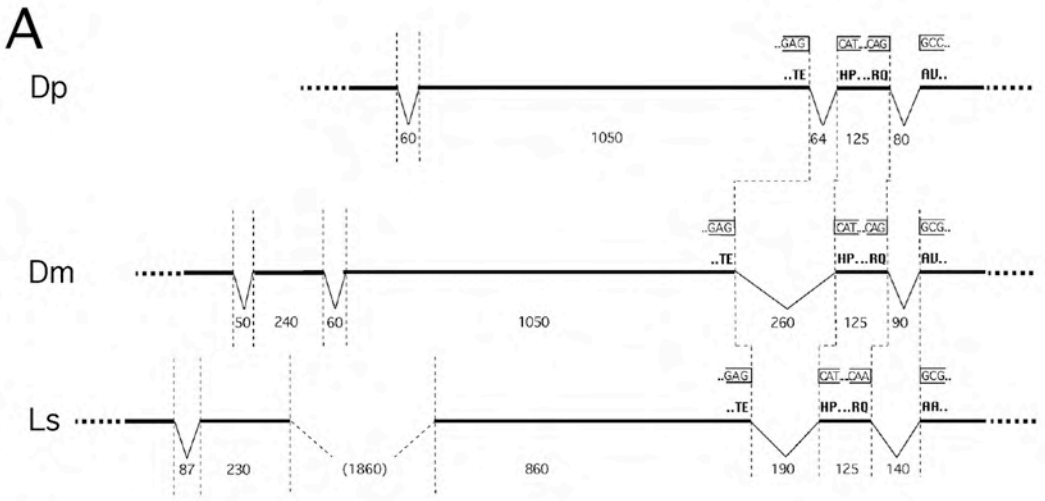
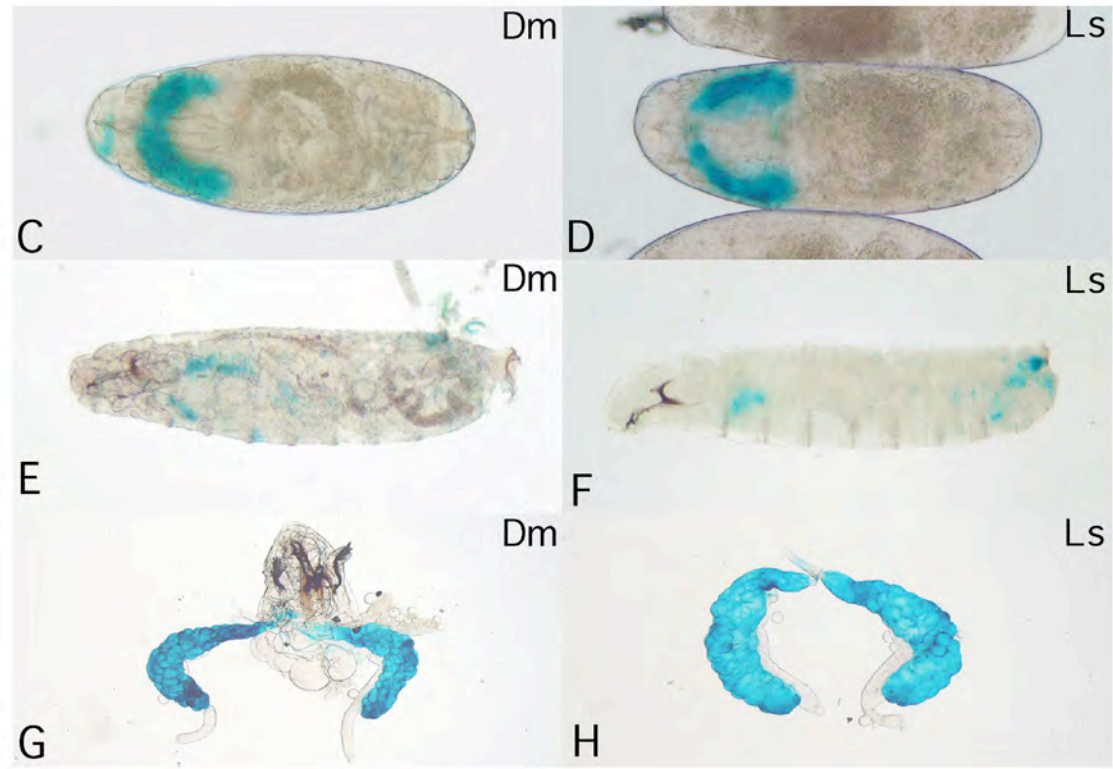


Fig. 1 Ali et al.


A
B
C
D
E
F


A
Dp
Dm
Ls
GAG
CAT
CAG
GCC
..TE
HP...RQ
AV..
GCG..
AA..
60
1050
64
125
80
50
240
60
1050
260
125
90
87
230
(1860)
860
190
125
140


B
Ls-sll
Dm-sll
hb/Oct-1/HNF-1
hb/HNF3/ATF
predicted core promoter


Dm
Ls
C
D
E
F
G
H