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ORIGINAL ARTICLE

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A role for *Fringe* in segment morphogenesis but not segment formation in the grasshopper, *Schistocerca gregaria*

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Abstract Studies of somitogenesis in vertebrates have identified a number of genes that are regulated by a periodic oscillator that patterns the pre-somitic mesoderm. One of these genes, *hairy*, is homologous to a *Drosophila* segmentation gene that also shows periodic spatial expression. This, and the periodic expression of a zebrafish homologue of *hairy* during somitogenesis, has suggested that insect segmentation and vertebrate somitogenesis may use similar molecular mechanisms and possibly share a common origin. In chicks and mice expression of the *lunatic fringe* gene also oscillates in the presomitic mesoderm. *Fringe* encodes an extracellular protein that regulates Notch signalling. This, and the finding that mutations in Notch or its ligands disrupt somite patterning, suggests that Notch signalling plays an important role in vertebrate somitogenesis. Although Notch signalling is not known to play a role in the formation of segments in *Drosophila*, we reasoned that it might do so in other insects such as the grasshopper, where segment boundaries form between cells, not between syncytial nuclei as they do in *Drosophila*. Here we report the cloning of a single *fringe* gene from the grasshopper *Schistocerca*. We show that it is not detectably expressed in the forming trunk segments of the embryo until after segment boundaries have formed. We conclude that *fringe* is not part of the mechanism that makes segments in *Schistocerca*. Thereafter it is expressed in a pattern which shows that it is a downstream target of the segmentation machinery and suggests that it may play a role in segment morphogenesis. Like its Drosophila counterpart, *Schistocerca fringe* is also expressed in the eye, in rings in the legs, and during oogenesis, in follicle cells.

Key words Development · Evolution · Notch · Insect

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Introduction

Body segmentation is an important feature of both arthropods and vertebrates but has traditionally been viewed as a convergent character. Recent studies of the expression of homologues of *Drosophila* segmentation genes in vertebrates have been taken to suggest that the segmentation of these disparate organisms may have its origin in a segmented common ancestor (Holland and et al. 1997; Kimmel 1996; Muller et al. 1996; Palmeirim et al. 1997; Robertis 1997; Takke and Campos-Ortega 1999), and specifically that somitogenesis in vertebrates may use molecular mechanisms similar to those involved in insect segmentation. The strongest evidence for this is the involvement of homologues of the *Drosophila* pair-rule gene *hairy* in somitogenesis in chick (Palmeirim et al. 1997) and its apparent pair-rule-like expression pattern in zebrafish (Muller et al. 1996).

Somites form in vertebrates from the pre-somitic mesoderm on either side of the node, in sequence from anterior to posterior (reviewed in Jiang et al. 1998). In this tissue a molecular oscillator generates waves of transcription that pass anterior from the node through the presomitic mesoderm, with each wave becoming more compressed anteriorly until patterns of transcription stabilise in groups of cells that will form the next somite. The first molecule shown to be regulated by this oscillator was chick *hairy-1*, an HLH transcription factor of the *hairy/enhancer of split* class. Expression of *hairy* stabilises in cells that will form the posterior of each somite (Palmeirim et al. 1997).

A second gene, *lunatic fringe*, has recently been shown to be regulated by this oscillator (Forsberg et al. 1998; McGrew et al. 1998). *Lunatic fringe* belongs to one of three distinct families of vertebrate *fringe* genes encoding extracellular proteins that modulate *Notch* signalling. In chick and mouse *Lunatic fringe* is expressed in waves similar to, but out of phase with, those of *hairy*. Its transcription stabilises in cells that will form the anterior part of each somite, a pattern complementary to that of *hairy* homologues. Mice mutant for *lunatic fringe*

have somites of irregular size and shape, suggesting that *lunatic fringe* is an essential component of the segmentation machinery (Evrard et al. 1998; Zhang and Gridley 1998).

The activity of Fringe protein has been best studied in *Drosophila*, where the single *fringe* gene is involved, among other roles, in patterning the D/V compartment boundary of the *Drosophila* wing (Irvine and Wieschaus 1994; Klein and Martinez Arias 1998; Rauskolb et al. 1999). In this case the gene appears to modulate Notch interactions with its ligands in such a way as to produce asymmetric signalling across the compartment boundary (Panin et al. 1997). Fringe modulation of Notch signalling is probably direct (Fleming et al. 1997; Klein and Martinez Arias 1998) and may be through modification of glycosylation patterns on the Notch receptor (Yuan et al. 1997).

Several other lines of evidence suggest that Notch signalling plays an important role in somitogenesis. In zebrafish Notch pathway proteins are expressed in the presomitic mesoderm during somitogenesis. Misexpression of these signalling components causes disruption in segmentation of the paraxial mesoderm but does not block differentiation of somitic muscle, suggesting that it affects patterning, not cell type specification (Takke and Campos-Ortega 1999). Similar defects are also present in mice mutant for *Notch* or *Delta* (Conlon et al. 1995; Hrabe de Angelis et al. 1997). Notch signalling has also been implicated in segmenting *Xenopus* (Jen and Gawantka et al. 1999).

Signalling through Notch has not been reported to play a major role in *Drosophila* segmentation. However, if Notch signalling and *fringe* are part of an ancestral segmentation pathway, it is possible that this pathway may have been masked by the highly derived nature of early *Drosophila* development, in which segment patterning precedes cellularisation and involves primarily the interactions of transcription factors between neighbouring nuclei in a syncytium. Examination of the role of *fringe* in basal insects may provide evidence for or against an ancestral role for Notch signalling in segmentation.

In this work we examine the expression of *fringe* in the Orthopteran *Schistocerca gregaria*. The Orthoptera occupy a more basal position in the insect lineage. *Schistocerca* displays an extreme form of short germband development, a mode of segmentation very different from that of *Drosophila*. In this insect all trunk segments are generated after cellularisation and gastrulation. Homologues of the *Drosophila* pair-rule genes *eve* and *ftz* are not expressed in pair-rule patterns, suggesting that the molecular mechanisms of segmentation in *Schistocerca* may differ from those characterised in *Drosophila* (Dawes et al. 1994; Patel et al. 1992).

Materials and methods

Cloning

Primers were designed using the codehop protocol (Rose et al. 1998) implemented at the codehop website (http://blocks.fhcrc.org/blockmkr/make_blocks.html). Primers were GGTTCTGCCA CGTGGATGAYGAYAAYTA (FNG5′) and GGCGCCGCCGGT NGCRAACCARA (FNG3′). Poly A^+ RNA was extracted from ovary and mixed embryonic stages using the Ambion polyA pure kit. First strand synthesis used random hexanucleotide primers and Expand Reverse transcriptase (Roche Diagnostics). The primers were first annealed at 25°C and then DNA synthesis was performed at 42°C for 1 h. Polymerase chain reaction (PCR) fragments were amplified from first-strand products using the following program. The reaction mixture, without nucleotides, was heated to 94°C for 4 min; this allowed the addition of 2 µl–5 mM nucleotide mix as a hot start. Three PCR cycles were then carried out with 67°C annealing, then three with 65°C annealing, then three with 62°C annealing and finally 30 cycles with 60°C annealing. The *Sgfringe* RT-PCR product was labelled with P^{32}-dCTP (NEN) using PCR, and used to screen a λ zap ovary cDNA library at high stringency. Probes for in situ hybridisation were prepared using Ambion megascript kits and digoxigenin (DIG)-11-UTP.

In situ hybridisation

In situ hybridisation on locust tissue was performed using a protocol modified from (Dawes et al. 1994). Embryos were dissected from eggs and fixed in PEM-FA (Patel et al. 1989a) for 45 min and washed in phosphate-buffered saline (PBS). Ovary tissue was dissected from adult females in PBS and fixed in PBS +4% formaldehyde for 45 min. The individual ovarioles were them dissected from the ovary mass, and the intima, a membrane surrounding each ovariole, was removed. Early eggs were pricked in PBS +4% formaldehyde and left in fix for 2 h. The chorion was then peeled off and the eggs washed in PBS. Ovary and egg tissue were washed in PBS plus 0.1% Tween 20 (PTw) and then treated with 5 µg/ml proteinase K for 1 h at room temperature. The ovaries were then washed in PTw treated with 0.2 N HCl for 15 min and rinsed in PTw.

Ovaries, embryos and eggs were prehybridised in hybridisation buffer (50% formamide, 5×SSC, 1×Denhart's solution, 1% blocking solution (Roche Diagnostics), 100 µg/ml heparin (Sigma), 1 mg/ml yeast tRNA (Sigma), 0.1% Tween-20, 0.1% CHAPS) for 2–6 h at room temperature and then incubated for 10 min at 65°C. The pre-hybridisation buffer was replaced with probe in hybridisation buffer, and the tissue hybridised overnight at 65°C. The tissue was washed for 10 min, 30 min and 1 h 15 min in hybridisation buffer at 55°C and then washed twice in 2×SSCP, 1% CHAPS. Bound DIG-labelled probe was then detected using Anti-DIG alkaline phosphatase FAB fragments (Roche Diagnostics) using the protocol of Dawes et al. (1994). Vector red staining was performed using a Vector Red staining kit (Vector Laboratories). Engrailed antibody staining was performed with the 4D9 cross reacting engrailed antibody using the protocol of Patel et al. (1989a). Photomicrographs were taken using an Axiophot microscope and camera. Confocal sections were taken and reconstructed using a Leica TCS SP confocal microscope and TCS NT software (Leica). Embryos were staged according to Bentley et al. (1979).

Sequence analysis

Alignments were constructed using Clustal W (Thompson et al. 1994) for Macintosh. Neighbour-joining analysis was performed on aligned blocks of the protein using the procedure at the Blocks website (http://blocks.fhcrc.org/blockmkr/make_blocks.html). Data from this was used to draw neighbour-joining trees using the TreeView program (Page 1996). Maximum likelihood analysis was performed using the Puzzle program (Strimmer and von Haeseler 1996).

Results

Cloning

A 129-bp fragment of the *fringe* gene was amplified from *Schistocerca gregaria* embryonic cDNA using degenerate PCR. Seven independent clones of this fragment yielded identical sequence between the primers, suggesting that only a single *fringe* gene is expressed during embryogenesis in *Schistocerca*. We term the gene from which this fragment is derived *Sgfringe*. A 2.3-kb partial cDNA of this *Sgfringe* gene was recovered from an ovary cDNA library using the PCR fragment as a probe. This clone encodes 213 amino acids of the carboxyl terminus of the protein and a long 3′ untranslated region (Fig. 1A; the protein coding region of this clone is available at Genbank, accession number AF201829). Comparison of this protein (Fig. 1B) with the *Drosophila* and vertebrate *fringe* sequences demonstrates that the *Schistocerca* protein is most similar to that of *Drosophila*.

Conserved expression in eyes and limbs

Expression of the *Sgfringe* gene was analysed by in situ hybridisation using the *Sgfringe* cDNA as a probe. We first examined whether *Sgfringe* is expressed at sites where its homologue is known to be expressed in *Drosophila*, in the eye, and in the limbs. In both cases expression of the *S. gregaria* gene is consistent with a conserved developmental role, even though these tissues develop in the embryo of *Schistocerca*, whereas in *Drosophila*, their patterning is delayed until the larval period (Lecuit and Cohen 1997).

The thoracic and gnathal limbs of *S. gregaria* develop from the body wall of the embryo between 25% and 60% development. *Sgfringe* is expressed in each limb prior to the first appearance of joints. Rings of expression appear in the epithelium of each limb from 25% of development. Each ring forms separately, the most basal ring forming first. After the formation of the first two rings subsequent rings form quickly, apparently by intercalating between the first two rings.

The rings of *Sgfringe* expression appear earliest in the third limb where, by 40% development, seven or eight rings are visible. Most of these rings are located near folds in the limb bud epithelium. At the presumptive femur/tibia joint on the third leg, a joint that forms early and distinctively, expression of *Sgfringe* quickly becomes restricted to regions proximal to the invagination (Fig. 2B, C). This is not the case for all rings; in some cases the ring of *Sgfringe* expression is distal to the invagination of the epithelium. *Sgfringe* is also expressed in rings in the epithelia of the antenna, much as *fringe* in *Drosophila*, and in the gnathal appendages. As all of the appendages develop, *Sgfringe* is weakly expressed in mesodermal cells underlying the limb epithelia.

The expression of *Sgfringe* in the legs is similar to the expression of *fringe* in the limbs of *Drosophila*. Here *fringe* plays an important role in joint development (Bishop et al. 1999; de Celis et al. 1998; Rauskolb and Irvine 1999). *Fringe* expression first appears in *Drosophila* legs as rings in the imaginal disc around

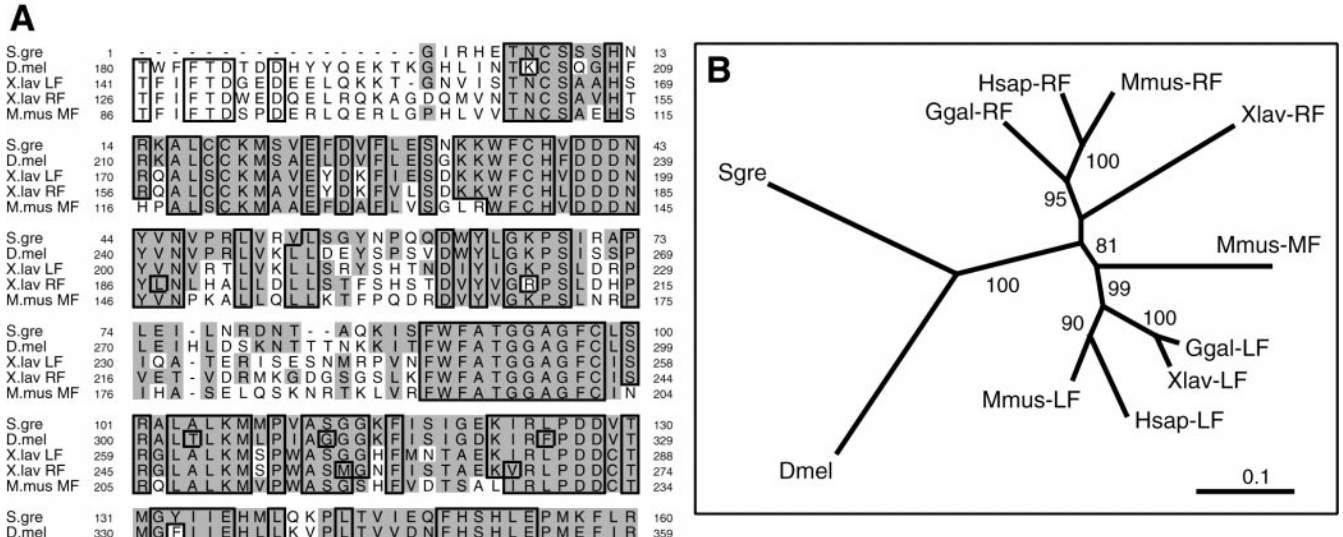

Fig. 1 **A** Clustal V alignment of *fringe* sequences including *Schistocerca fringe*. *S.Gre Schistocerca*; *D.mel Drosophila*; *X.Lav LF Xenopus lunatic fringe*; *X.lav RF Xenopus radical fringe*; *M.mus* mouse *manic fringe*; *boxes* identity; *shading* amino acid similarity. **B** Unrooted neighbour-joining tree of the segment of fringe sequence shown in **A**. *Xlav Xenopus*; *Mmus* mouse; *Hsap* human; *Ggal* chick; *LF Lunatic fringe*; *RF Radical fringe*; *MF Manic fringe*. *Scale bar* substitutions per site. This alignment was also examined with maximum likelihood, giving identical results

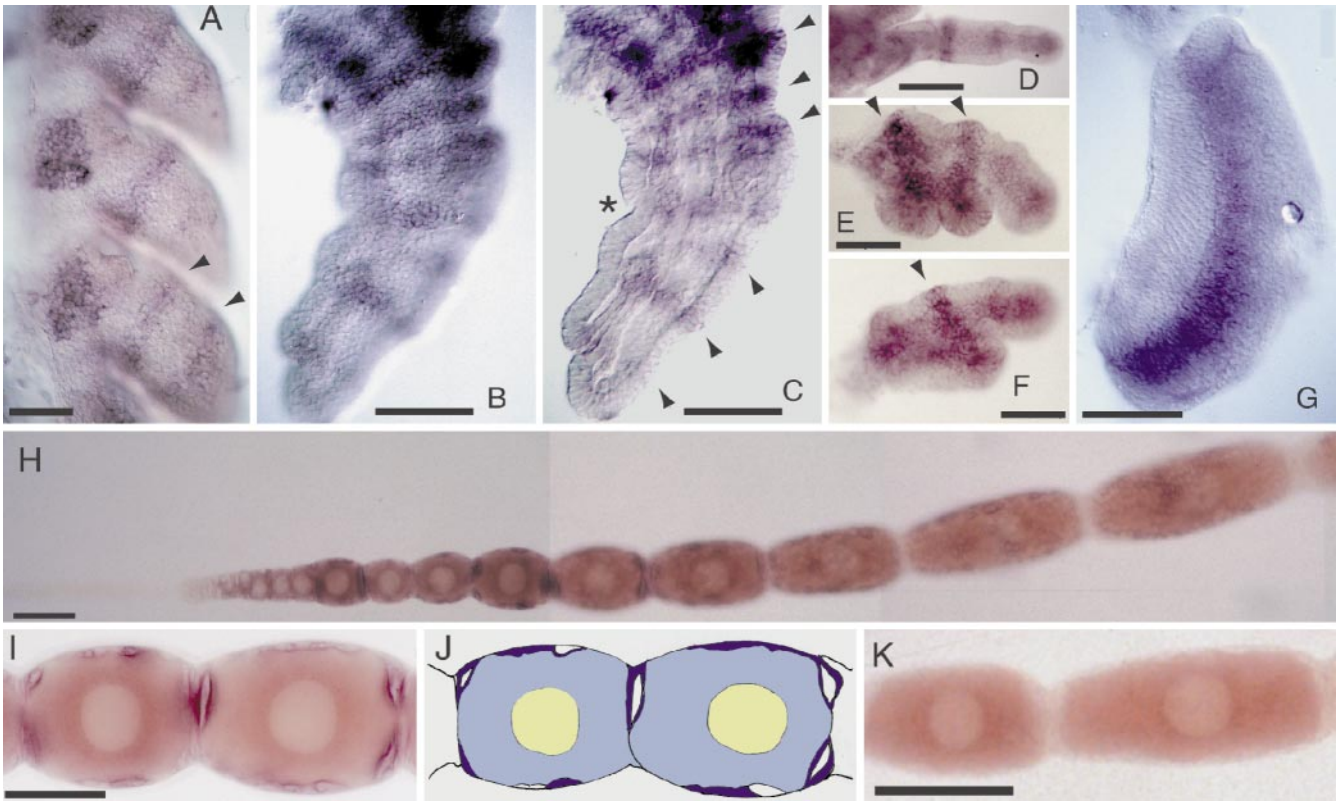

Fig. 2A–G *Sgfringe* RNA expression in the limbs and eye. *Scale bars* 100 µM. **A** *Sgfringe* expression in 30% leg buds. Expression appears as two distinct rings (*arrowheads*) Note also expression in the dorsal trunk epidermis at the base of the limb. **B**,**C** *Sgfringe* expression in the third leg, 40% of development (**B** surface view; **C** optical section) At least seven rings are visible. Expression appears around the infolding areas of the leg, particularly in the region just proximal to the forming femur/tibia joint (*starred*). **D** Antenna at 30%. The tip and three or four transverse bands express *Sgfringe*. **E**,**F** First maxilla (**E**) and second maxilla (**F**) at 30%. Rings of *Sgfringe* are visible as well as expression in mesodermal cells underlying the epithelium. **G** *Sgfringe* expression demarcates a boundary in the 45% *Schistocerca* eye. Organised ommatidia are visible in the anterior region of the eye. *Sgfringe* is expressed in developmentally younger tissue which has not yet been organised into ommatidia. **H–K** Expression of *Sgfringe* in the ovary. *Scale bars* 50 µM. **H** *Sgfringe* RNA in a single ovariole. The ovariole is made up of maturing oocytes surrounded by follicle cells. The youngest oocytes are to the left, oldest to the right. *Sgfringe* expression is restricted to the flattened follicle cells surrounding each oocyte. **I** Enlargement of follicle cells surrounding young oocytes. *Sgfringe* RNA is present only in follicle cells. **J** Cartoon of **I**. Flattened follicle cells (*dark blue* cytoplasm; *white* nuclei) enwrap the oocytes (*light blue*). *Sgfringe* RNA is present in the cytoplasm of the follicle cells. **K** Control ovariole of the same stage, hybridised with sense probe

joint forming regions (de Celis et al. 1998). As the leg everts and joint development proceeds, *fringe* becomes restricted to the proximal regions of each leg segment (Rauskolb and Irvine 1999).

In *Drosophila fringe* is expressed at two stages during eye development (Cho and Choi 1998; Dominguez and de Celis 1998). It is first expressed in the ventral half of the eye disc (Cho and Choi 1998; Dominguez and de Celis 1998), where it is required for establishment of ommatidial polarity. Later it is expressed just anterior to the morphogenetic furrow (Cho and Choi 1998). In *Schistocerca* the lack of markers makes it difficult to locate *Sgfringe* expression during the early stages of eye development. However, as the embryonic eye develops, a clear boundary becomes visible between tissue organised into ommatidia and the remaining area of unorganised tissue. *Sgfringe* is expressed in the unorganised part of the eye with a sharply defined limit of expression along the line where ommatidia become visible. We assume that this limit is developmentally equivalent to the morphogenetic furrow in *Drosophila*.

These regions of conserved expression between *Drosophila* and *Schistocerca* provide evidence that the cDNA we have cloned derives from a gene that is functionally equivalent to the known *fringe* gene of *Drosophila*.

Expression in the Ovary

Sgfringe is expressed in the somatic cells of the *Schistocerca* ovary (Fig. 2H). RNA first appears in the interstitial cells in the germarium, from which all follicle cells are derived. It remains detectable in all follicle cells until at least the end of vitellogenesis (Fig. 2I, J). In mature follicle cells *Sgfringe* mRNA is present near the basal membrane.

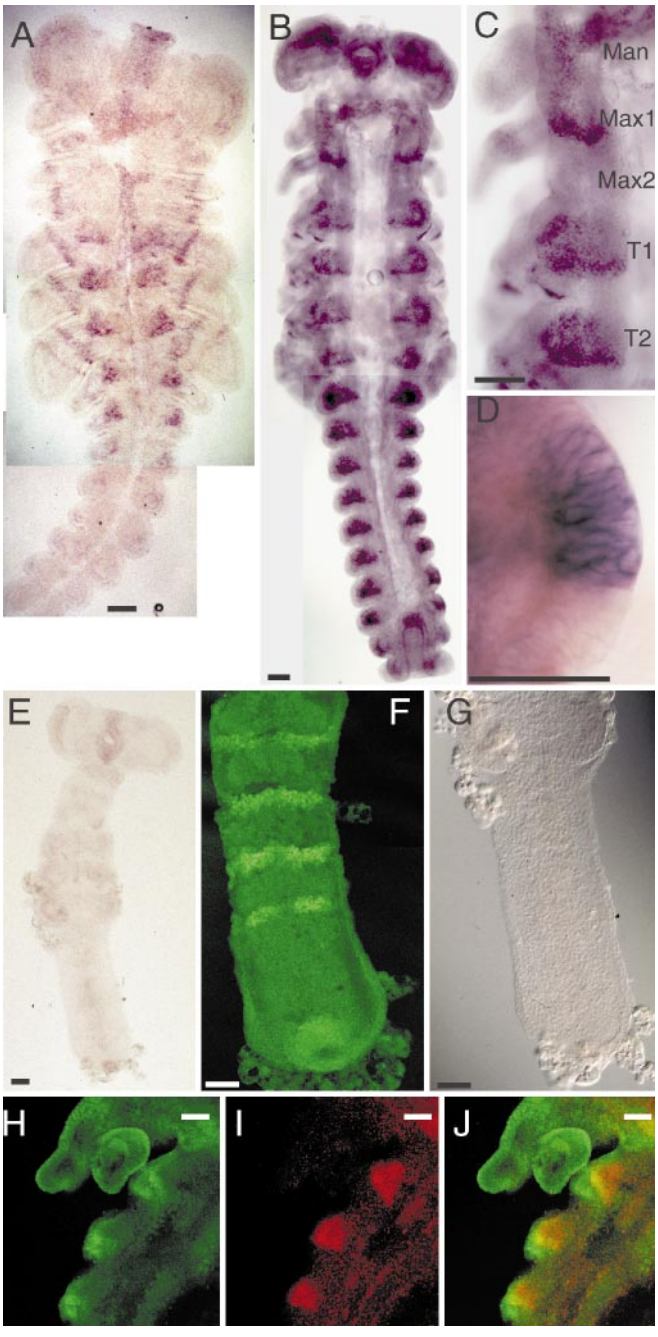

Fig. 3A–J *Sgfringe* RNA expression in the trunk epidermis, *Scale bars* 100 µM. **A** At 30% of development. *Sgfringe* RNA is detectable in segmentally repeated patches of cells in the dorsal epidermis from T1 to A3. Expression in the limbs is also visible. **B** At 35% development. In the dorsal epidermis, *Sgfringe* expression now extends to all segments of the abdomen. In thoracic regions the expressing cells form a wedge shape with a sharply defined posterior boundary. The stoma and proctodeum are also stained. **C** Enlargement of the three gnathal segments and the thoracic segments. *Sgfringe* expression is absent from two of the gnathal segments. RNA is only present in the first maxillary segment. *Man* Mandibular; *Max1* 1st maxilla; *Max2* 2nd maxilla; *T1* 1st thoracic; T2 2nd thoracic. **D** Enlargement of the sixth abdominal segment; anterior *up*. *Sgfringe* is expressed in the anterior of the segment with a sharp boundary to the posterior. **F** Embryo at 25% of development stained for *engrailed* protein. Segmental stripes are visible as far back as A3. This specimen was also hybridised for *Sgfringe* RNA (*red*), but no localised signal is visible. **E**,**G** Embryo from the same pod (within 1% development) hybridised for *Sgfringe* RNA only. Under these conditions very weak signal can be detected in the proctodeum, stomodeum, the thoracic limb buds and the midline. **H–J** Embryo at 40% of development. **H** *Engrailed* expression (*green*) in the dorsal epidermis of segments T3 (+limb), A1 (+pleuropod), A2 and A3 at 40% of development. **I** *Sgfringe* expression (*red*). **J** Merged image. *Sgfringe* expression overlaps with *engrailed* in the dorsal epidermis

Sgfringe is expressed after but not during segment formation

Sgfringe is first expressed in the trunk epidermis of *Schistocerca* embryos at 30% development in wedge-shaped patches of cells in the anterior dorsal part of each thoracic segment. From 30% to 35% development these patches appear in the abdominal segments in anterior to posterior sequence. By 35% development every thoracic and abdominal segment has a patch of *Sgfringe* expressing cells in the anterior dorsal epidermis although the shapes of these patches differ somewhat in thoracic and abdominal regions. Expression in the gnathal region shows a different periodicity. Neither the mandibular nor the second maxillary segment expresses *Sgfringe* in the dorsal epidermis, but the first maxillary segment expresses a stripe of *Sgfringe* (Fig. 3C).

To test whether the timing of *Sgfringe* expression in *S. gregaria* is consistent with a role in generating segments in the embryo, we examined embryos double-stained for *Sgfringe* RNA and Engrailed, a protein which marks forming segment boundaries. Engrailed protein stripes form in the abdomen in an anterior to posterior sequence after 25–30% of development (Patel et al. 1989b). *Sgfringe* RNA is not detectable in the epidermis (Fig. 3E, G) prior to or concurrently with the initial expression of *engrailed* in each segment (Fig. 3F). It first appears in the thoracic segments after Engrailed stripes have formed in the entire abdomen and after the segments are demarcated by bulges in the epidermis. This implies that *Sgfringe* does not act to generate segments in *S. gregaria* but is probably activated in the dorsal epidermis downstream of the segmentation cascade.

After 30–40% of development the expression of *Sgfringe* in the dorsal epidermis is restricted to the anterior of each segment. The anterior limit of expression is diffuse, while the posterior limit is sharply defined, suggesting that it might juxtapose with the expression of *engrailed* in posterior regions. However, double labelling demonstrates that *Sgfringe* expression overlaps with the domain of *engrailed* expression by a few cells (Fig. 3H, I).

Sgfringe expression and segment morphogenesis

The limits of *Sgfringe* expression are coincident with a morphological change in the abdominal epidermis. Between 30 and 50% of development the epithelium of

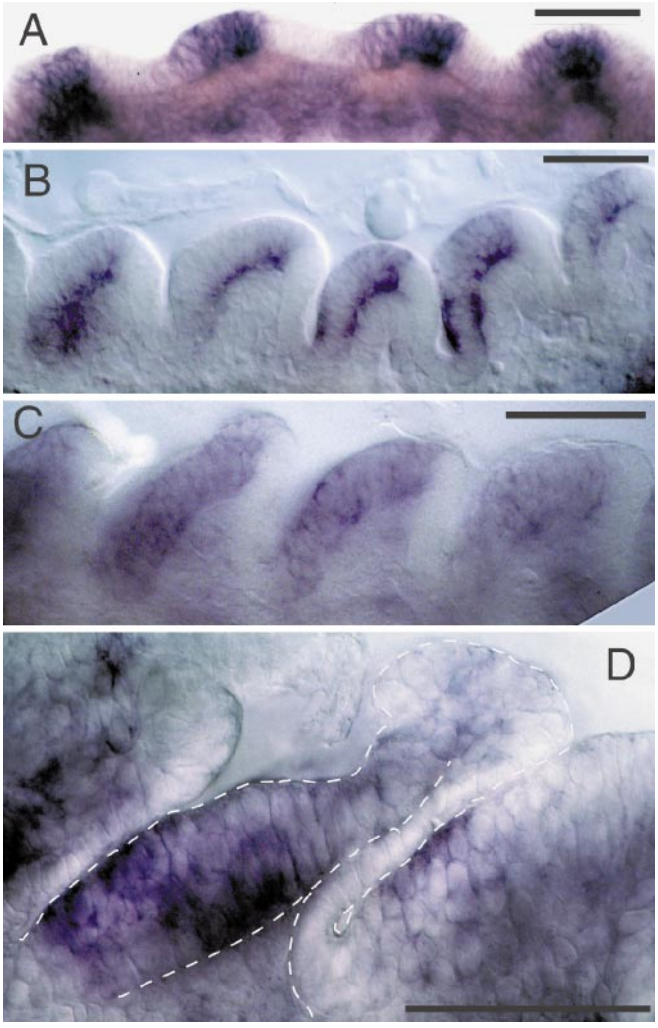

Fig. 4A–C Time series of *Sgfringe* RNA expression in the dorsal abdominal epidermis. **A** At 35% development. **B** At 40% development. **C** At 45% development. The posterior boundary of *Sgfringe* expression closely mirrors the boundary between tergite and intersegmental membrane as the epidermis undergoes morphological change. Cells expressing *Sgfringe* elongate at later stages and form the thickened tergite structure. **D** Close up of folding abdominal segment at 55% of development; *dashed line* edges. *Sgfringe* RNA is expressed in the tergite but is absent from the intersegmental membrane. *Scale bars* 100 µM

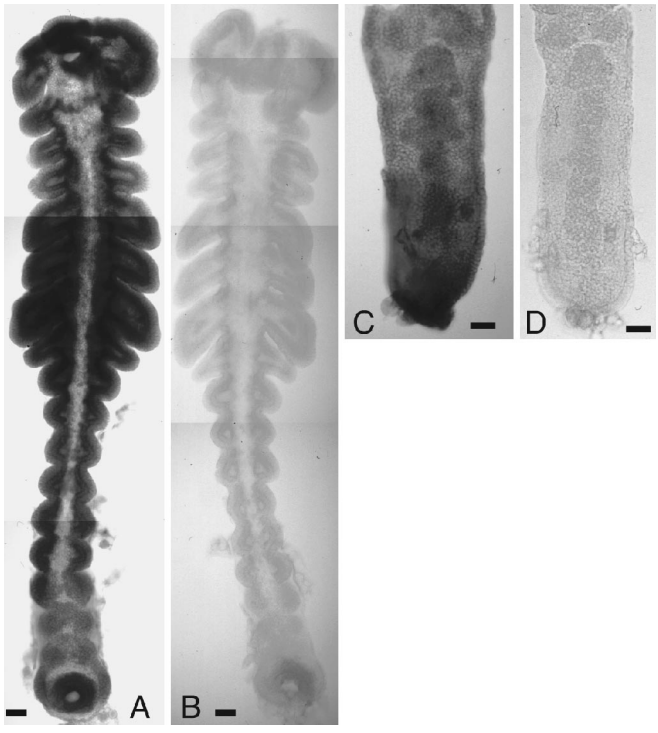

Fig. 5. In situ hybridisation with a 300-bp fragment of the *Schistocerca Notch* gene (**A**,**C**) and with a sense control (**B**,**D**). **A**,**B** Embryos at 30% development. **C**,**D** 25% abdomens. *Scale bars* 100 µM. *Notch* RNA appears to be ubiquitously distributed. *Schistocerca Notch* sequence is deposited in Genbank, accession number AF221597

Discussion

Both sequence and conserved patterns of expression suggests that the gene we have isolated is the orthologue of the single *fringe* gene known in *Drosophila*. We see no evidence that there are distinct representatives in *Schistocerca* of the three vertebrate *fringe* gene families, which probably arose in the chordate lineage after the divergence of the common ancestor of vertebrates and arthropods.

the abdomen invaginates in the posterior part of each segment, forming the thin flexible intersegmental membrane on the interior and leaving the anterior epidermis of each segment to form the thickened, sclerotised tergite. The posterior boundary of *Sgfringe* expression is closely associated with this change in cell behaviour (Fig. 4). Cells expressing *Sgfringe* form the thickened tergite. They become more columnar and organise into a broad trapezoid shape. Cells not expressing *Sgfringe* remain as a thin epithelium that connects the tergites of each segment.

The expression of *Sgfringe* is lost from the abdominal segments by 60% of development. In the three thoracic segments it continues to be expressed at the edges of the forming tergites until 60% development (data not shown).

Segmental expression

Sgfringe is expressed in a segmentally reiterated pattern during embryogenesis. However, the timing of this expression is not consistent with a role in segmentation. *Engrailed* expression in *Schistocerca* is the only marker yet discovered that demarcates each segment before the morphology of the segments is apparent (Patel et al. 1989a). If *Sgfringe* is involved with the patterning of segments we would expect it to be expressed before, or concurrently with, the onset of *engrailed* expression in each segment. No *Sgfringe* expression is detectable at this stage.

The later expression in the anterior part of each segment overlaps with, rather than abuts, *engrailed* express-

ing cells. In *Drosophila* the boundary between *engrailed* expressing and non-expressing cells is an early lineage restriction and the primary boundary that establishes segments (the parasegment boundary). The failure of *Sgfringe* expression to respect this boundary further suggests that its regulation lies downstream of the processes that first establish segment boundaries in *Schistocerca*.

Neither the expression of *fringe* in *S. gregaria* nor in *Drosophila* suggests a role for this gene in the formation of segments. As *fringe* acts to modulate *Notch* signalling in *Drosophila* and vertebrates, these observations suggest that *Notch* signalling is not part of the mechanism that makes segments in short germ band insects, although they do not rule this out entirely. The role of *fringe* in chick and mouse somitogenesis is thought to be that of restricting *Notch* signalling to specific parts of the pre-somitic mesoderm (Evrard et al. 1998; Zhang and Gridley 1998) Restriction of *Notch* signalling could also be achieved by localised expression of *Notch* ligands or the *Notch* receptor. We have cloned *Notch* from *Schistocerca*, but in situ hybridisation suggests that it is uniformly expressed during segment formation (Fig. 5), as it is in *Drosophila*. However, the expression of *Notch* ligands has not yet been examined.

The timing and location of *Sgfringe* expression in the central region of each segment is consistent with a role in defining the boundary between the tergite and the inter-segmental membrane. We suggest that *Notch* signalling may be involved in this process, and that in *Schistocerca*, as in the *Drosophila* wing disc, the boundary between *fringe* expressing and non-expressing cells creates asymmetric activation of *Notch* by its ligands. This signalling event could trigger the morphogenetic movements involved in forming intersegmental invaginations, a role analogous to that proposed for *fringe* in *Drosophila* leg development (de Celis et al. 1998) and somitogenesis in vertebrates (Jiang et al. 1998). Thus if there are common mechanisms of segmentation between insects and vertebrates, they may lie in the control of morphogenesis rather than in the establishment of segmental boundaries.

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