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Expression of pair-rule gene homologues in a chelicerate: early patterning of the two-spotted spider mite *Tetranychus urticae*

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SUMMARY

Embryo segmentation has been studied extensively in the fruit fly, *Drosophila*. These studies have demonstrated that a mechanism acting with dual segment periodicity is required for correct patterning of the body plan in this insect, but the evolutionary origin of the mechanism, the pair-rule system, is unclear. We have examined the expression of the homologues of two *Drosophila* pair-rule genes, *runt* and *paired* (Pax Group III), in segmenting embryos of the two-spotted spider mite (*Tetranychus urticae* Koch). Spider mites are chelicerates, a group of arthropods that diverged from the lineage leading to *Drosophila* at least 520 million years ago. In *T. urticae*, the Pax Group III gene *Tu-pax3/7* was expressed during patterning of the prosoma, but not the opisthosoma, in a series of stripes which appear first in even numbered segments, and then in odd numbered segments. The mite *runt* homologue (*Tu-run*) in contrast was expressed early in a circular domains that resolved into a segmental pattern. The expression patterns of both of these genes also indicated they are regulated very differently from their *Drosophila* homologues. The expression pattern of *Tu-pax3/7* lends support to the possibility that a pair-rule patterning mechanism is active in the segmentation pathways of chelicerates.

Key words: Pair-rule, Chelicerate, Evolution, Parasegment, Segmentation, *runt*, *pax3/7*

INTRODUCTION

The origin of animal segmentation is an unresolved question in developmental biology. Three hypotheses are currently proposed for the evolution of segmentation in metameric metazoans. They include: independent evolution of segmentation in chordates, arthropods and annelids, independent evolution of segmentation in chordates and a shared mechanism of segmentation in protostome groups, and finally homologous segmentation across the Bilateria (Davis and Patel, 1999). In order to test these hypotheses it is necessary to determine the ancestral mechanisms of segmentation in major animal groups. Arthropods are a good clade in which to examine conserved mechanisms of segmentation owing to the wealth of classical embryological studies and recent molecular data on development in various taxa.

Studies of *Drosophila* development have provided details of the genetic interactions that underlie the segmentation of this insect. The anterior/posterior patterning process in *Drosophila* is initiated by the localized deployment of maternal proteins that trigger downstream genetic hierarchies, including the gap, pair-rule and segment-polarity classes of genes (St Johnston and Nüsslein-Volhard, 1992). Gradients of maternal transcription factors activate gap genes in non-periodic domains. Overlapping domains of gap genes activate pair-rule gene expression in domains that represent the first signs of segmentation. Pair-rule genes act as intermediates between the non-periodic expression of gap genes and the segmentally repeated expression of segment polarity genes. *Drosophila* embryos mutant for pair-rule genes exhibit pattern defects that affect adjacent segments in different ways. These genes thus regulate patterning with a dual segment, rather than segmental, periodicity. This system of defining repeated territories that undergo further subdivision (re-segmentation) led to the recent hypothesis that arthropod segments form by subdivision of primary segments (eosegments) into terminal segments (merosegments) (Minelli, 2001). This scenario places the pair-rule mechanism at the crux of arthropod segmentation, implying that some form of pair-rule 'logic' is shared by all arthropod groups.

Pair-rule genes were initially isolated in a *Drosophila* mutant screen for pattern formation genes (Nüsslein-Volhard and Wieschaus, 1980). The original mutant screen isolated seven genes that exhibit a pair-rule phenotype including *hairy* (*h*), *runt* (*run*), *even-skipped* (*eve*), *fushi tarazu* (*ftz*), *odd-paired* (*opa*), *odd-skipped* (*odd*), *paired* (*prd*) and *sloppy paired* (*slp*). Subsequently, additional genes have been isolated that produce pair-rule phenotypes when mutated (Tang et al., 2001; Baumgartner et al., 1994; Levine et al., 1994). These genes

come to be expressed in a canonical 'pair-rule' fashion, in seven stripes of cells that run across the embryo, associated with every second segment. In addition to the 'pair-rule' expression domains, many of these genes show secondary, segmental expression.

Studies of engrailed protein expression in insects, crustaceans (Patel et al., 1989) and chelicerates (Telford and Thomas, 1998) have implied that the segment polarity gene network is probably conserved across arthropods. This observation is supported by the expression of *wingless* in insects (Dearden and Akam, 2001; Nagy and Carroll, 1994) and crustaceans (Nulsen and Nagy, 1999). Computer modelling of the molecular interactions in the segment polarity network (von Dassow et al., 2000) have implied it is robust to changes in its activation conditions, possibly explaining its evolutionary conservation. Conservation of the pair-rule cascade has been more controversial, but recent studies provide evidence that it may be conserved in insects. Amongst holometabolous insects, evidence for pair-rule patterning has been found in coleopterans (Brown et al., 1994; Brown et al., 1997; Patel et al., 1994; Schroder et al., 2000), dipterans (Rohr et al., 1999), lepidopterans (Kraft and Jackle, 1994) and hymenopterans (Binner and Sander, 1997; Grbić and Strand, 1998). The only exceptions appear to be two derived parasitic wasps, that do not express a homologue of the Even-skipped protein in a diagnostic pair-rule pattern (Grbić et al., 1996; Grbić and Strand, 1998).

Among hemimetabolous insects, the expression patterns of pair-rule genes have been examined in grasshopper, earwig, cricket and cockroach. In the earwig, cricket and cockroach, an Eve homologous protein is not expressed in a pattern consistent with pair-rule function, though it is expressed in segmental stripes (Corley et al., 1999; Davis and Patel, 1999) raising the possibility that *eve* was expressed segmentally in the ancestors of insects. In grasshoppers neither *ftz* nor *eve* are expressed in stripes (Dawes et al., 1994; Patel et al., 1992). These findings led to the proposal that pair-rule patterning may have evolved only in holometabolous insects (French, 1996). Recent studies, however, have demonstrated that a Pax group III gene (PgIII), *pairberry 1 (pby1)*, is expressed in the grasshopper in a pattern consistent with a pair-rule function, indicating a pair-rule mechanism does function in the segmentation of this insect (Davis et al., 2001).

If pair-rule patterning is conserved in insects, is it present in more distant groups of arthropods? The expression patterns of three pair-rule gene homologues have been examined in the spider, *Cupiennius salei* (Damen et al., 2000). In this species, these genes are expressed during segmentation, but, owing to a lack of segmental markers, it is difficult to interpret the patterns seen. The expression pattern of a *fushi-taratzu* homologous gene has been examined in a mite (*Archegozetes longisetosus*), but it is not expressed in a pattern indicating a role in segmentation (Telford, 2000). To determine if pair-rule patterning is an ancient and conserved feature of arthropod development, we have examined the expression of two pair-rule genes in the spider mite *Tetranychus urticae.* Spider mites are chelicerates, an arthropod class that includes spiders, mites, scorpions and horseshoe crabs. Recent phylogenetic inferences imply that chelicerates are the sister group of myriapods, with insects and crustaceans forming a more distant clade (Cook et al., 2001; Giribet et al., 2001; Hwang et al., 2001). A fossil chelicerate, dated to the middle Cambrian (520-512 MYA) has been identified, demonstrating that the separation between the crustacean/insect clade and chelicerates is an ancient one (Briggs and Collins, 1988). Thus, the great evolutionary distance between spider mites and *Drosophila* implies that any developmental pathway we find common to both species is likely to be conserved and ancestral for all arthropods.

Here we describe the embryogenesis of *T. urticae* and analyse the expression of homologues of the *Drosophila* pair-rule genes *run* and *prd*. In *T. urticae*, a homologue of *prd* is expressed in stripes that appear first in even numbered segments, and then in odd numbered segments, implying that a pair-rule mechanism may underlie segmentation in this species. The early expression pattern of a *run* homologous gene, however, deviates greatly from the *Drosophila* pattern, being expressed in circular domains that delimit the limb primordia. These data imply that significant changes in the expression patterns of pair-rule homologous genes have evolved over 520 million years.

MATERIALS AND METHODS

Spider mite culture and embryo preparation

T. urticae were cultured at 25°C on broad bean plants in a growth chamber with 40-60% humidity and a 16-hour photoperiod. Spider mite embryos, nymphs and adults were rinsed off broad bean leaves and stems in 0.1% Tween. Embryos and young nymphs were separated from adults by sieving through 100- and 200-mesh sieves (Sigma). Adults remain in the 100-mesh sieve while embryos and young nymphs are collected from the 200-mesh sieve. Embryos were dechorionated with 50% bleach for 5 minutes, rinsed in tap water and fixed in PBS + 0.1% Tween + 4% formaldehyde for 15-30 minutes. Embryos were then washed in PTw (PBS + 0.1% Tween), and sonicated for 3 seconds in a aquasonic cleaning bath (VWR). Embryos were rinsed in PTw, re-fixed in PTw + 4% formaldehyde for 15 minutes and rinsed three times in PTw.

Embryo micro-injection

Spider mite embryos for micro-injection were individually picked off leaves using fine forceps under a dissecting microscope and placed in a drop of paraffin oil (Sigma) on a microscope slide. Slides were placed on a Zeiss Axiovert microscope. Embryos were steadied with negative pressure through a holding pipette. Holding pipettes were produced by the methods of Hogan et al. (Hogan et al., 1986) using a Narashige microforge. Embryos were injected with tetramethylrhodamine dextran (3×10^3 M_r, anionic, lysine fixable) using fine needles pulled from borosilicate glass on a Stutter needle puller. Embryos were left to recover for 30 minutes and then imaged using a Zeiss laser scanning confocal microscope.

Molecular cloning and sequence analysis

Spider mite poly(A)$^+$ RNA was extracted using a Quickprep mRNA purification kit (Amersham-Pharmacia Biotech). A directional cDNA library was produced from mixed embryonic-stage poly(A)$^+$ RNA using the Zap Express system (Stratagene). Random colonies were picked from mass-excised plasmid clones from this library. Plasmid DNA was extracted using a QIAprep 96 Turbo Miniprep Kit (Qiagen) on a Biomek 2000 Robot (Beckman). Clones were sequenced from their 5′ end using T3 primer. Sequencing was performed using Big Dye chemistry (ABI) on a Perkin Elmer 377 DNA sequencer.

Spider mite DNA was extracted using a QIAquick Kit (Qiagen). Degenerate PCR for *Tu-pax3/7* was performed using the methods of Davis et al. (Davis et al., 2001). *Tu-run* degenerate PCR was performed using the following primers: RCNRYNATGAARAAY-

CARGTNGC (runt 5′) and MRNTTYAAYGAYYTNMGNTTYGT-NGG (runt 3′). PCR products were cloned by ligation into a linearised Bluescript vector with terminal overhanging thymine residues.

Sequences were assembled using SeqMan from the DNASTAR suite of programs and homology assessed using translated BLAST (BlastX) searches (Altschul et al., 1990). Multiple alignments were created using Clustal X (Thompson et al., 1994), and Maximum likelihood analysis performed using TreePuzzle (Strimmer and von Haeseler, 1996).

Embryo staining

Antibody staining was performed as described previously (Patel, 1994), using an antibody raised against *Drosophila* Distal-less (Dll) described by Panganiban et al. (Panganiban et al., 1995).

DIG-labelled probes were produced and in situ hybridisation carried out according to the methods of Dearden and Akam (Dearden and Akam, 2000). Images were collected using a Sony DXC-390P camera mounted on a Zeiss Axioplan II microscope and processed using Photoshop (Adobe).

RESULTS

Embryogenesis in *T. urticae*

T. urticae eggs and embryos were examined with DIC microscopy (Fig. 1). The transparent chorion of this species allows visualisation of development without prior preparation. Indeed, time-lapse photography can be used to make animated sequences of live embryos under the microscope.

Spider mite females lay a spherical, 150 μm egg with little internal morphology (Fig. 1A). Over the course of the first hour after egg laying (AEL), a central nucleus becomes visible. The egg then undergoes nine divisions, approximately one per hour, creating a blastoderm with a layer of cells surrounding a yolk filled centre (Fig. 1B-D; http://devbiol.zoo.uwo.ca/movies/smite_early_cleavages_mov.mov.). From the first division, cell membranes are visible between the nuclei.

The blastoderm remains static for 12-14 hours with no changes in morphology. A small swelling of blastoderm cells then appears, internally, on one side of the egg, which we take to be the ‘germ disc’ described for other mite embryos (reviewed by Anderson, 1973) (Fig. 1E). The germ disc starts as an ovoid swelling (Fig. 1E,F), and then flattens (Fig. 1G). Flattening of the germ disc is quickly followed by the appearance of leg primordia on both sides of the ventral midline (viewed from the anterior in Fig. 1H).

Leg buds and the prosoma region of the germ band appear rapidly and simultaneously (Fig. 1I-P; http://devbiol.zoo.uwo.ca/movies/smite_legs_growth_mov.mov.) approximately two hours after the appearance of the germ disc. The two halves of the germ band are separated by a small ventral sulcus, which quickly closes (Fig. 1M). Limb buds in the chelicera-bearing segment and the germ band in the opisthosoma appear 3-4 hours after formation of the germ band. Eyes become coloured and limbs grow on the chelicera, pedipalp and first three walking leg segments, becoming jointed and hirsute by 30 hours AEL. The fourth walking leg does not extend in embryonic stages. Hexapod larvae hatch approximately 39 hours AEL. Larvae undergo two moults, during which the fourth leg extends, before becoming reproductively active.

Early *T. urticae* embryos do not have a syncitial phase

To determine if the initial divisions of the embryo involve cytokinesis or are only nuclear (syncitial) we micro-injected tetramethylrhodamine dextran into 1-, 2-, 4- and 16-cell embryos and examined its distribution using a confocal

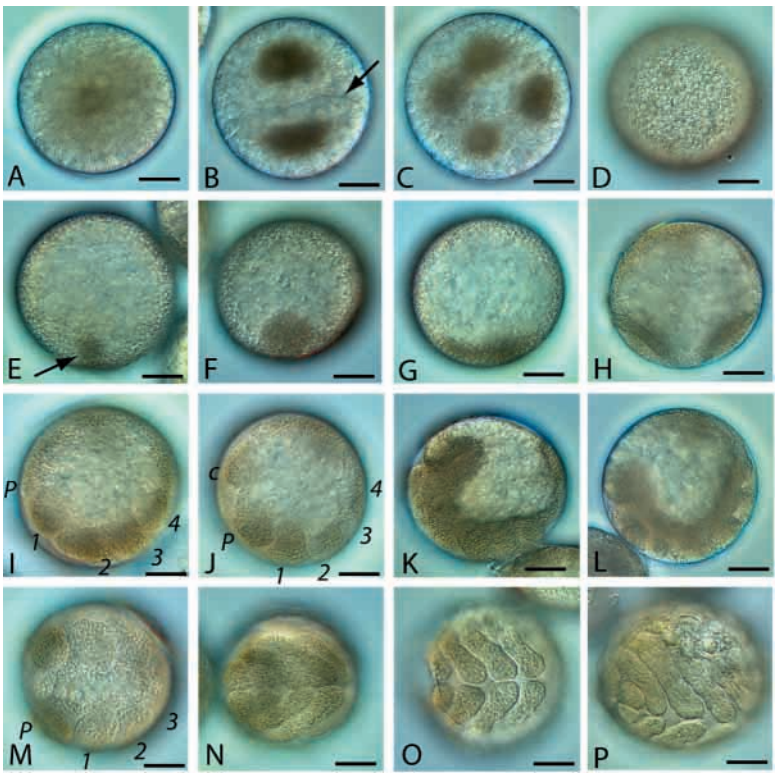

Fig. 1. Embryogenesis in the two-spotted spider mite. (A-D) Early cleavages. (A) Uncleaved egg. The nucleus is just starting to become visible in the centre of the egg. (B) First division, the egg nucleus (dark area) has divided. A clear membrane between the two blastomeres is visible (arrow). (C) Second division. (D) Blastoderm stage embryo. Nine divisions have taken place forming a cellular blastoderm. (E-F) Formation of the ‘germ disc’, and the germ band. (E) At 20-22 hours AEL a thickened portion of the germ band is visible (arrow), probably in ventral regions, which we interpret to be the germ disc. The germ disc starts as an ovoid swelling (E,F), and then flattens (G). Flattening of the germ disc is quickly followed by the appearance of leg primordia on both sides of the ventral midline (viewed from the anterior in H). (I-L) Formation of the germ band, limb primordia and limb outgrowth (lateral view). All embryos are viewed with anterior to the left and dorsal up. (I) The initial limb primordia form by 23 hours AEL. P, pedipalps; 1-4, walking legs. Primordia for the chelicera-bearing segment and the opisthosoma germ band become visible soon after. (J) C, chelicerae. (K,L) Limb buds grow and become jointed. (M-P) Formation of the germ band, limb primordia and limb growth (ventral view). All embryos are viewed from the ventral side, with anterior to the left. Embryos are of the same stage as those in I-L. Scale bars: 50 μm.

microscope (Fig. 2). Dextran molecules of this size will not move passively through a cell membrane (Grbić et al., 1996).

Dextran injected into a 1-cell embryo diffused rapidly to fill the entire egg (data not shown) demonstrating that egg cytoplasm is not a barrier to diffusion of this molecule. Two-cell embryos injected with dextran initially showed fluorescent signal only in the injected cell. Forty minutes after injection, however, both cells were equally labelled (Fig. 2A-C). After micro-injection into 4-cell or 16-cell embryos, dextran was localised in the injected cell and no leakage to other cells was observed, even after 1-hour incubation (Fig. 2D-I). These data imply that the spider mite embryo forms partial cell membranes at the 2-cell stage, and complete ones by the 4-cell stage.

Tu-run: cloning and sequence analysis

To determine if a pair-rule gene mechanism underlies segmentation in the two-spotted spider mite, we cloned a *T. urticae* homologue of *Drosophila runt*. Two clones with homology to *Drosophila runt* were identified in an EST screen of embryonic stage cDNA (4,000 ESTs sequenced). These clones were found to contain, where overlapping, an identical sequence. Further sequencing and assembly of the EST screen sequences demonstrated that one clone contained an apparently full-length open reading frame of a *runt*-like gene. This sequence has an 849 bp open reading frame with an upstream stop codon 21 bp from the putative start codon.

To ascertain if this clone represents the only *runt* homologue in the spider mite genome, PCR using degenerate primers designed to amplify the conserved *runt* domain was performed on spider mite genomic DNA. The single resulting PCR fragment was cloned and 18 colonies were sequenced. All colonies contained a sequence identical to a region in both *runt*-like clones from the EST screen, indicating that they derive from the same gene. We designate this gene *Tu-run*.

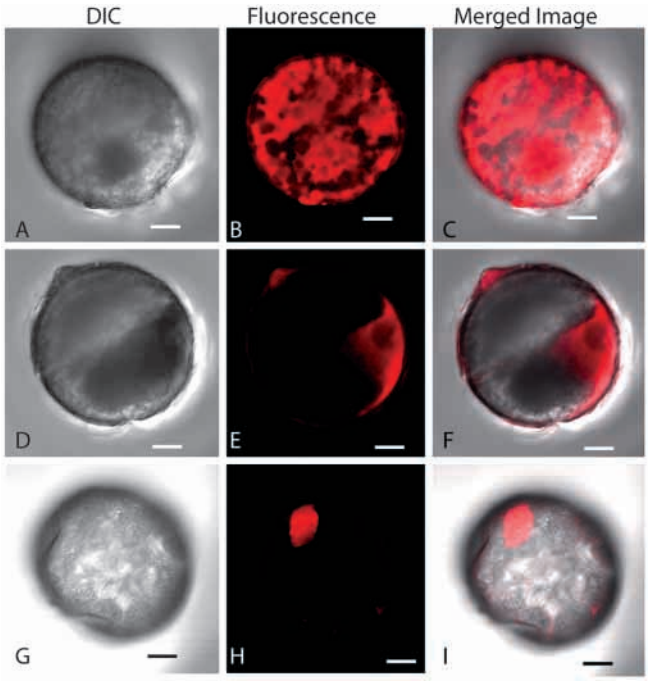

Fig. 2. Dextran injections. (A-C) A two-cell stage embryo 60 minutes after injection with tetramethylrhodamine dextran into a single blastomere. Dye has diffused into both blastomeres. (D-F) A four-cell stage embryo 60 minutes after injection with tetramethylrhodamine dextran. Dye remains localised in the injected blastomere. (G-H) A 16-cell stage embryo 60 minutes after injection with tetramethylrhodamine dextran. Dye remains localised in the injected blastomere. Scale bars: 20 μm.

We performed maximum likelihood phylogenetic analysis on a multiple alignment of the most conserved region of various *runt*-like proteins including *Tu-run* (Fig. 3A). This analysis demonstrated that the *Tu-run* protein falls into a clade containing both *Drosophila* and spider (*C. salei*) *runt*-like sequences, to the exclusion of vertebrate sequences (Fig. 3C).

Tu-run RNA is expressed in circular domains that resolve into segmental stripes

Tu-run transcription is first detectable in blastoderm stage embryos, at 23 hours AEL (Fig. 4). The RNA is distributed in five bilaterally paired rings of cells (3-4 cells wide) in the ventral regions of the embryo. These rings appear rapidly and simultaneously, and are paired across the ventral midline of the embryo (Fig. 4A). At 25 hours AEL, as limb-buds in the prosoma (excluding the chelicera buds which form later) become visible, the rings of *Tu-run*-expressing cells surround each limb bud. As the limb-bud grows, expression becomes undetectable in the cells of the posterior half of the ring, leaving a curved stripe of cells expressing *Tu-run* just anterior to the limb-bud. As the chelicera limb-bud becomes visible, expression is detected in a stripe of cells directly anterior to it. *Tu-run* is also detected in three rapidly forming stripes in the opisthosoma. At this stage, the embryo contains nine stripes of *Tu-run*-expressing cells (Fig. 4B). Expression is also detected in a diffuse group of cells in the head of the embryo, anterior to the chelicerae. By 30 hours AEL, the stripes of expression in epidermal cells becomes undetectable and *Tu-run* RNA appears in segmentally repeated groups of cells in the nervous system (Fig. 4C). This expression persists until hatching.

To understand the distribution of *Tu-run* RNA, we co-stained embryos for *Tu-run* RNA and Distal-less protein (Dll). Dll is an evolutionarily conserved marker for limb-bud fate (Panganiban et al., 1997). Both the expression pattern and function of this gene are conserved in the spider *C. salei* (Schoppmeier and Damen, 2001). Dll protein is first detected in five paired oval domains of cells, in ventral regions of the germ band (not shown). These domains mark the forming limb buds. As development proceeds, a domain of expression becomes visible in the anterior region, marking the chelicera limb bud (Fig. 4J). As the legs become fully formed, Dll protein is initially present in all cells of the limb (Fig. 4K), but then becomes restricted to a ring of cells in the proximal region of the limbs, and a broad domain at the distal tip (Fig. 4L). In the segment containing the fourth walking leg, it is restricted to a circular patch of cells, slightly dorsal to the proximal edge of the other limbs, until that limb extends in larval stages. Dll protein is also present in segmentally reiterated cells in the nervous system (asterisk in Fig. 4F).

Dll protein expression appears slightly before *Tu-run* RNA. The rings of *Tu-run*-expressing cells abut and encircle the cells initially expressing Dll protein (Fig. 4D and G). The initial five paired rings of *Tu-run* expression thus mark cells surrounding,

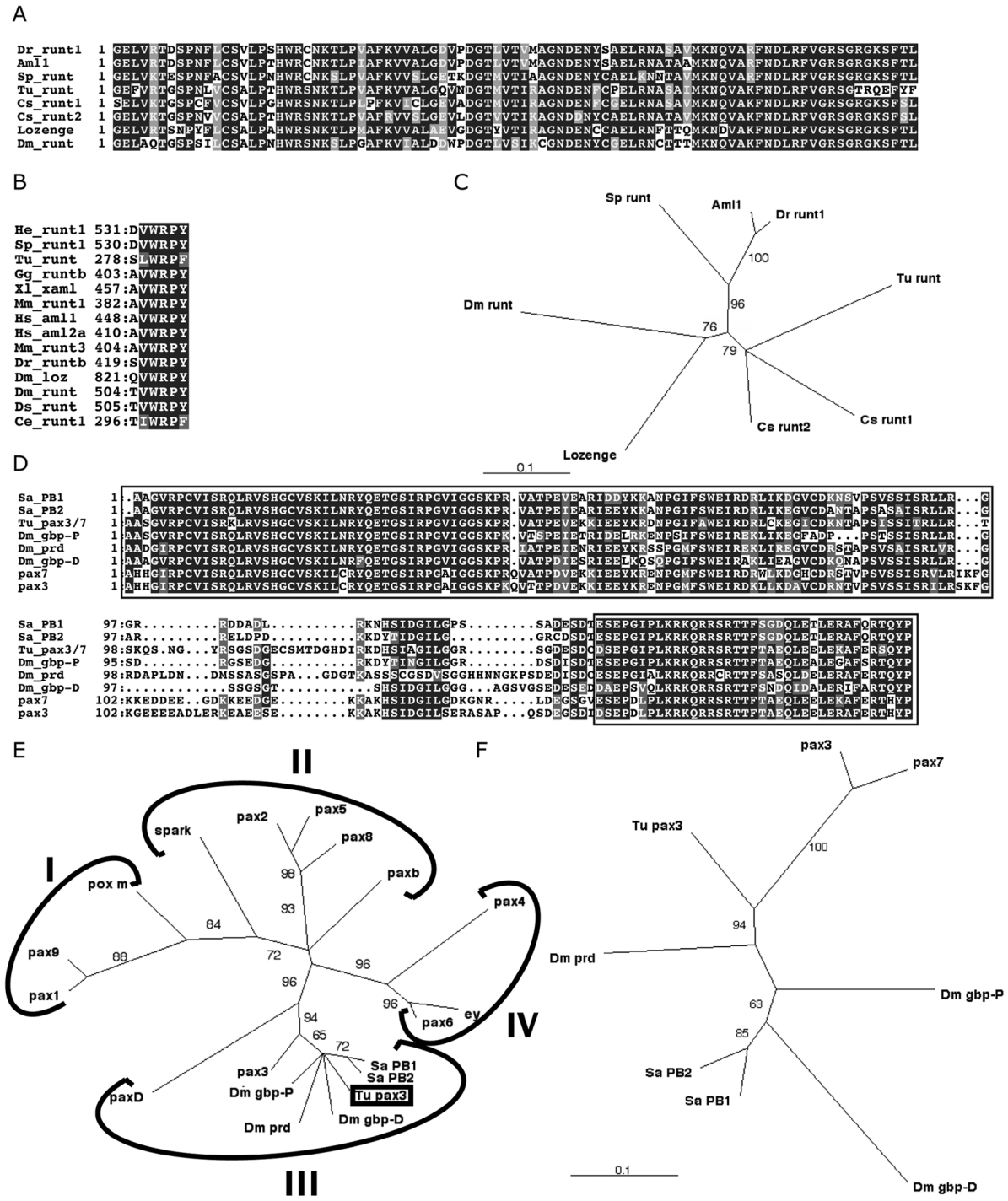

Fig. 3. Sequence analysis. (A) Multiple sequence alignment of the most conserved domain of *runt*-like proteins including *Tu-run*. Dr_Runt1, *Danio rerio* runt; Aml1, human AML1 protein; Sp_runt, *Strongylocentrotus purpuratus;* Cs_runt 1 and 2, *Cupiennius salei* runt1 and 2; Lozenge, *Drosophila melanogaster* loz; Dm_runt *Drosophila melanogaster* runt. (B) Multiple alignment of the C-terminal VWRPY motif of Runt-like proteins. He_Runt1, *Heliocidaris erythrogramma*; Gg_runtb, chick runtB; Xl-aml1, *Xenopus laevis* AML-like protein 1; Mm_runt1 and 3, mouse runt 1 and 3; Hs-aml1 and 2a, human AML-like proteins 1 and 2a; Dr_runtb, *Danio rerio* runt b; Dm_loz, *Drosophila melanogaster* lozenge; Ds_runt, *Drosophila simulans* runt; Ce_runt1, *Caenorhabditis elegans* runt1. (C) Unrooted maximum likelihood cladogram of the multiple alignment shown in A. (D) Amino acid sequence alignment of the cloned region of *Tu-pax3/7* with other PgIII genes. Boxed region shows the amino acids used for the phylogenetic analysis shown in F. Sa_PB1 and 2, *Schistocerca americana* Pairberry 1 and 2; Dm_prd, Dm_gbp-D, Dm_gbp-P, *Drosophila melanogaster* paired, gooseberry paired distal and gooseberry paired proximal respectively; pax 7 and 3, mouse pax7 and 3. (E) Unrooted maximum likelihood cladogram of the region of the alignment shown in D including representatives of all pax group genes. Pax1-9, mouse Pax proteins; pox m, pox messo; spark, sparkling; ey, eyeless proteins from *Drosophila melanogaster*; paxD and paxb from *Acropora millepora*. (F) Unrooted maximum likelihood cladogram of the boxed region of the alignment in D. *Tu-run* GenBank accession number, AY148194; *Tu-pax3/7* GenBank accession number: AY148194.

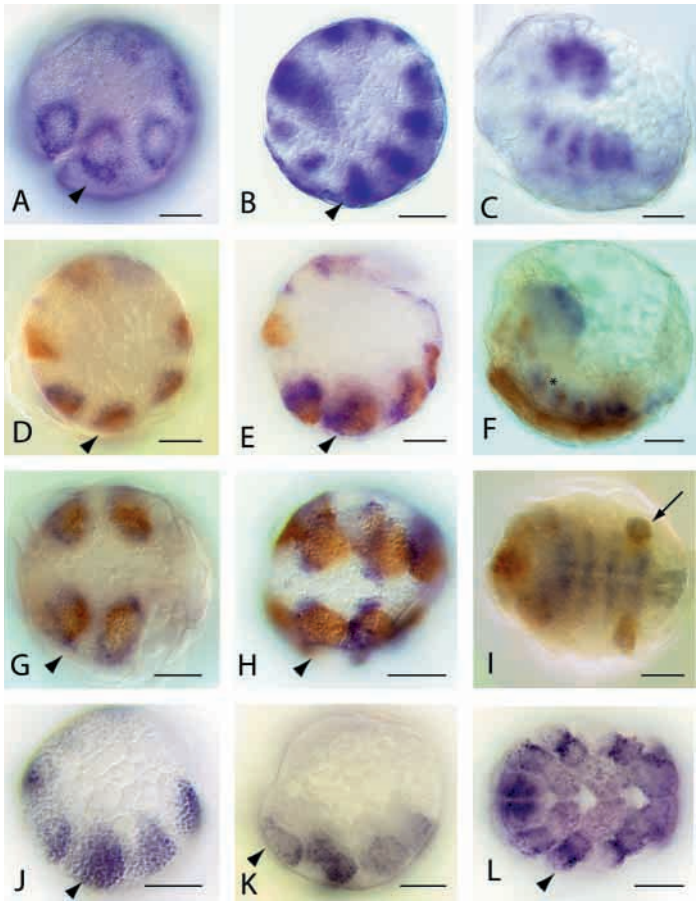

Fig. 4. Expression of *Tu-run* and Dll in spider mite embryos. (A-C) Detection of *Tu-run* using in situ hybridisation. Embryos are oriented with dorsal up and anterior to the left. Arrowhead indicates the first walking leg segment in all panels. (A) *Tu-run* RNA is first detected in five pairs of rings in ventral regions of the embryo (blue). (B) The posterior side of each ring of cells loses detectable *Tu-run* expression, forming stripes across the ventral midline. (C) Expression of *Tu-run* becomes undetectable in stripes in the epidermis, and segmental stripes appear in the nervous system. (D-F) Lateral view of embryos hybridised with an RNA probe for *Tu-run* RNA (blue), and stained with an antibody that detects Dll protein (brown). Embryos are oriented with dorsal up and anterior to the left. Dll protein marks the developing limb buds. (D) The rings of cells expressing *Tu-run* RNA entirely surround cells expressing Dll protein. (E) As *Tu-run* comes to be expressed in stripes of cells, these stripes lie directly anterior to Dll-expressing cells. (F) As epidermal expression of *Tu-run* RNA becomes undetectable, both Dll protein (asterisk) and *Tu-run* RNA are expressed in cells in the nervous system. (G-I) Ventral view of embryos hybridised with an RNA probe for *Tu-run* RNA (blue), and stained with an antibody that detects Dll protein (brown). Embryos are oriented looking down on the ventral surface with anterior to the left. (G) Ventral view of *Tu-run* RNA surrounding Dll protein-expressing cells. (H) Stripes of *Tu-run* RNA-expressing cells lie directly anterior to cells expressing Dll protein. (I) Ventral view of the central nervous system showing *Tu-run* RNA-expressing cells. Arrow marks the fourth walking leg primordium. (J-L) Spider mite embryos stained for Dll protein (black). Embryos are oriented with dorsal up and anterior to the left except L where the embryo is oriented looking down on the ventral surface. Distal-less is initially expressed only in limb buds. (J) Expression of Dll protein in the chelicerae, pedipalp limb buds and the four walking legs. Dll expression in the fourth walking leg is out of focus. (K) Expression of Dll in the limbs as they grow. (L) At the late germband stage, Dll is expressed in a ring of cells in proximal regions of the limb, and a broad domain of cells at the distal tip. Scale bars: 50 μm.

and just outside, the limb bud in the pedipalp-bearing segment and the four walking limb segments. A ring of *Tu-run* does not form around the chelicera limb bud, only a stripe anterior to it, as it forms later in development. Cells that express *Tu-run* RNA do not initially express detectable levels of Dll protein. As Dll expression initially spreads posteriorward, apparently by recruitment of cells to the limb bud, cells in the posterior of the *Tu-run*-expressing oval begin to express Dll. Expression of *Tu-run* RNA rapidly becomes undetectable in cells that express Dll protein. As the limb buds extend, *Tu-run* RNA becomes restricted to a stripe just anterior to, and abutting, the expression domain of Dll in the limb (Fig. 4E and H). By 30 hours AEL, *Tu-run* is no longer expressed in stripes of epidermal cells. Co-expression of Dll and *Tu-run* has not been observed (Fig. 4F and I).

Tu-pax3/7: cloning and sequence analysis

Degenerate PCR was used to amplify sequences homologous to Pax Group III (PgIII) genes. Twenty clones were isolated containing an identical sequence with homology to both *Drosophila prd* and *gsb*. We designate the gene from which this sequence derives *Tu-pax3/7*.

We performed maximum likelihood analysis on a multiple alignment of the protein sequences containing the most conserved regions of Pax-type homeoprotein sequences (Fig. 3D). Cladograms derived from this analysis show *Tu-pax3/7* to be most closely related to other PgIII genes (Fig. 3E). Phylogenetic analysis of an amino acid alignment of PgIII homologues containing the paired domain and one end of the extended homeobox motif (boxed in Fig. 3D), implies that *Tu-pax3/7* forms a clade with the *pax3* and *pax7* genes from mouse (Fig. 3F) but not insect PgIII genes.

Stripes of cells expressing *Tu-pax3/7* RNA do not form in anterior-posterior sequence in the prosoma

Distribution of *Tu-pax3/7* mRNA was determined using in situ hybridisation to whole-mount embryos (Figs 5, 6). *Tu-pax3/7* RNA is first detected in three stripes of cells in the ventral regions of blastoderm stage embryos (Fig. 5A,B, 19 hours AEL). The two most anterior stripes meet each other at their tips and the posterior stripe is slightly thinner and does not meet the anterior two. *Tu-pax3/7* expression in these stripes appears quickly and simultaneously. After examining over 1000 embryos hybridised for *Tu-pax3/7* RNA, no intact embryos were found that contained only one or two stripes of cells expressing *Tu-pax3/7* RNA.

Very quickly after the first three stripes of *Tu-pax3/7*-expressing cells have appeared, a fourth stripe of cells, between the two most anterior stripes, begins to express *Tu-pax3/7* RNA (Fig. 5C,D). The stripe is initially one to two cells wide, but quickly becomes as broad (3-4 cells) as the initial three stripes (Fig. 5E,F). Soon after the fourth stripe becomes visible, a fifth stripe, 1-2 cell wide, begins to express *Tu-pax3/7* between the two posterior most stripes (Fig. 5G,H). Expression in this fifth stripe then widens to 3-4 cells (Fig. 5I,J). In both of the secondary stripes, a lateral focus of cells first starts expressing

Tu-pax3/7, and expression spreads around the ventral surface of the embryo.

Dll protein expression becomes detectable only after all five stripes of *Tu-pax3/7* are present. Dll expression appears in oval domains in each limb-bearing segment. By correlating the early expression of Dll protein with the stripes of cells expressing *Tu-pax3/7* RNA, we can identify the stripes as they form. The three initial stripes of cells expressing detectable levels of *Tu-pax3/7* RNA are cells that will underlie the pedipalp limb bud, the second walking leg limb bud, and the fourth walking leg limb bud. The next stripe to appear underlies the first walking leg limb bud, and the fifth stripe underlies the third walking leg limb bud. The appearance of the first five stripes of *Tu-pax3/7* expression is consistent with a segmental pattern with pair-rule modulation.

The domains of Dll expression (Fig. 6A-D) overlap the anterior edge of the stripes of *Tu-pax3/7*-expressing cells. As the limb buds develop, Dll expression spreads posteriorwards, first overlapping the entire *Tu-pax3/7* stripe, and then extending beyond it (Fig. 6E,I). *Tu-pax3/7*-expressing cells that come to express Dll, immediately lose detectable *Tu-pax3/7* expression. As Dll expression spreads across the stripe, *Tu-pax3/7* RNA is only detectable in a square block of cells, ventral to the limb bud (Fig. 6F,J).

Tu-pax3/7 RNA becomes undetectable in the epidermis by 30 hours AEL and becomes visible in the nervous system at around the same time (Fig. 6H,L). Segmentally repeated groups of cells in the central nervous system express *Tu-pax3/7*, appearing as a broad stripe of cells in each segment, broken at the ventral midline (Fig. 6H,L). *Tu-pax3/7* is expressed in limb joints in just hatched nymphs (data not shown).

Tu-pax3/7 RNA is expressed in segmental stripes in the opisthosoma

As Dll expression begins to spread across the stripes of cells expressing *Tu-pax3/7*, a stripe of *Tu-pax3/7* RNA-expressing cells appears in the posterior of the germ band (Fig. 6M). This 2- to 3-cell wide stripe becomes broken at the ventral midline, and two more stripes appear posterior to it, one after another (Fig. 6N,O). These stripes of cells mark the forming opisthosoma segments. Without segmental markers, it is difficult to interpret the pattern in which these stripes are forming. However, we have never seen (in over 1000 embryos) a stripe forming between two already formed ones in the opisthosoma. This implies that *Tu-pax3/7* RNA expression is not modulated in a pair-rule manner in the opisthosoma. Stripes of cells expressing *Tu-pax3/7* RNA form in the central nervous system underlying the opisthosoma segments (Fig. 6P) in late embryos.

DISCUSSION

We have examined early patterning of the chelicerate, *T. urticae*, in order to ascertain the generality of pair-rule patterning in arthropod early development. We have found that in the prosoma the *Tu-pax3/7* gene is modulated in a manner that may reflect a pair-rule patterning mechanism, though this is not seen in the opisthosoma. Despite this, the early patterns of both *Tu-pax3/7* and *Tu-run* expression are very different from that of their homologues in *Drosophila*.

T. urticae embryogenesis

Despite being the second largest group of animals, the developmental genetics of chelicerates are poorly understood. The main obstacle for future progress in this field is the lack of a chelicerate model organism. The analysis of early patterning in chelicerates has proved difficult so far because of the inaccessibility of early embryonic stages (Damen et al., 1998; Telford and Thomas, 1998). *T. urticae* is a good

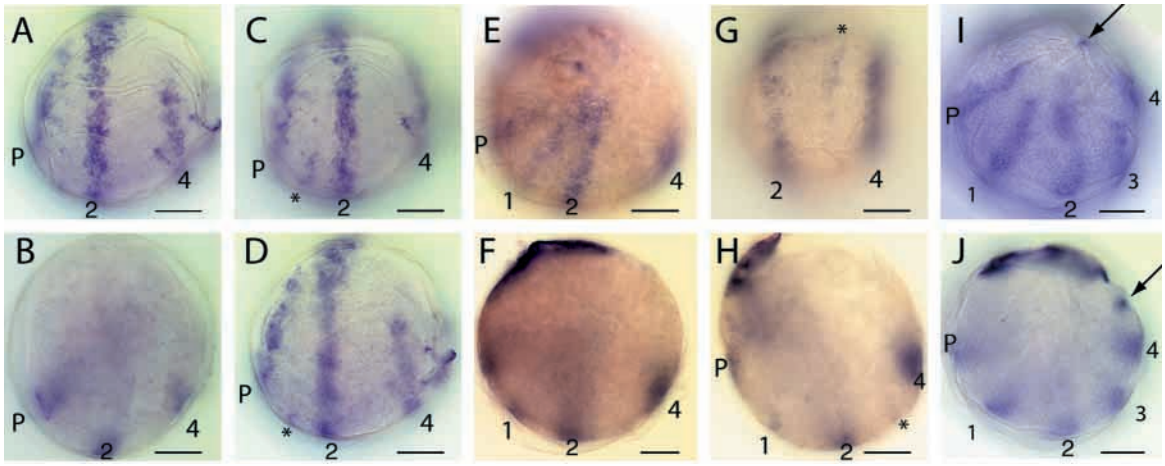

Fig. 5. Expression of *Tu-pax3/7* in blastoderm stage embryos. (A,C,E,G,I) Surface view of blastoderm stage embryos hybridised for *Tu-pax3/7* RNA. (B,D,F,H,J) Deeper focal plane of blastoderm stage embryos hybridised for *Tu-pax3/7* RNA. (A,B) *Tu-pax3/7* RNA is first expressed in three stripes of cells across the ventral surface, corresponding to the pedipalp segment, the second walking leg and the fourth walking leg. (C,D) Expression of *Tu-pax3/7* RNA becomes apparent in a stripe in the first walking leg segment (asterisks). (E,F) Embryo with four detectable stripes of *Tu-pax3/7*-expressing cells, corresponding to the pedipalps, the first and second walking legs and the fourth walking leg. (G,H) Expression of *Tu-pax3/7* RNA becomes apparent in a stripe in the third walking leg segment (asterisks). (I,J) Embryo with five detectable stripes of *Tu-pax3/7* expression, corresponding to all the segments of the prosoma except the chelicera segment. A small stripe is also forming posterior to the fourth walking leg segment (arrows). *Tu-pax3/7* expression is starting to disappear from cells in the centre of each stripe in the prosoma. Scale bars: 50 μm. P, pedipalp; 1-4, walking legs. Anterior to the left, dorsal up.

candidate organism for a model chelicerate. *T. urticae* completes its embryonic development in 39 hours and its full development from egg to adult is less than 7 days (Rao et al., 1996). In contrast, the predatory spider *C. salei* has a nine-month development time. *T. urticae* has small eggs (150 μm) that are surrounded by a transparent chorion, allowing easy visualisation of embryonic development. Its rapid generation time, simple diet (bean plants), and the organisation of its genome on three chromosomes (Oliver, 1971), also make *T. urticae* an ideal candidate for genetic studies. Recent studies have further indicated that *T. urticae* has a smaller genome (0.08 pg/ haploid genome) than *Drosophila* (0.18 pg), or even *C. elegans* (0.09 pg) (T. R. Gregory and M. G., unpublished).

Early embryogenesis in *T. urticae* does not include an early syncitial phase. Chelicerates exhibit both syncitial and total cleavage patterns (Anderson, 1973; Hafiz, 1935). The first nine cleavage divisions occur over 9 hours and result in the formation of a blastoderm surrounding a yolky interior. Germ band formation is reminiscent of that of intermediate germ band insects. In both intermediate germ band insects and spider mites, the anterior segments of the germ band are formed early and almost simultaneously, and the trunk regions form later, in anterior to posterior sequence.

Recent studies of chelicerate segmentation, using Hox genes as markers, homologised the chelicerate prosoma with insect head segments. In this model, the chelicera segment corresponds to the insect antennal segment, pedipalps to intercalary segment, and walking legs to mandibular, maxillary, labial and first thoracic segments (Damen et al., 1998; Telford and Thomas, 1998). These designations allow us to directly compare patterns of *Tu-pax3/7* and *Tu-run* expression with those of their *Drosophila* homologues. For these purposes we will adopt the same system used to number engrailed stripes (and thus parasegment boundaries) in *Drosophila* (DiNardo and O'Farrell, 1987) (Fig. 7). Thus stripe 1 (mandibular in *Drosophila*), refers to the first walking leg segment. In the prosoma, odd numbered segments are thus the chelicera, first walking leg and third walking leg segments. Even numbered segments bear the pedipalps, second walking leg and fourth walking leg (Fig. 7).

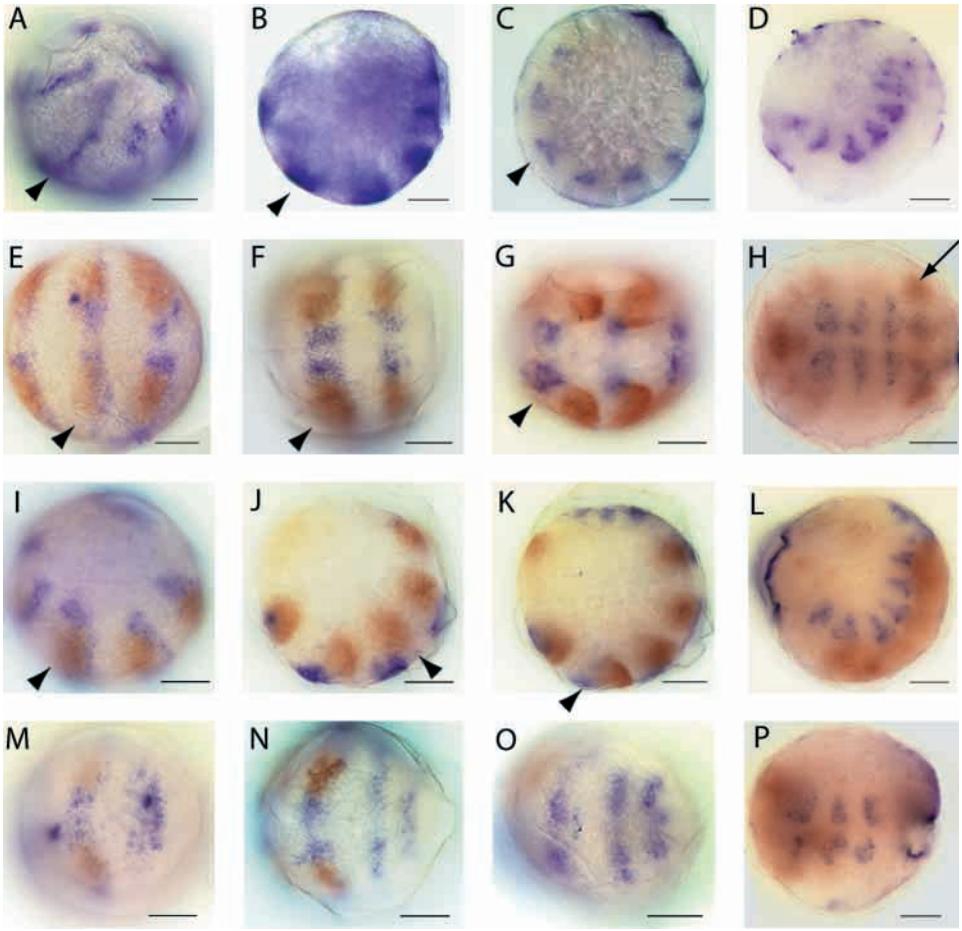

Fig. 6. Expression of *Tu-pax3/7* and Distal-less. (A-D) Lateral view of embryos hybridised for *Tu-pax3/7* RNA (blue). Anterior to the left, dorsal is up. (A) Surface view of an embryo showing the full pattern of *Tu-pax3/7* RNA stripes in the prosoma. (B) Deeper focal plane of an embryo showing one stripe of cells expressing *Tu-pax3/7* RNA in the opisthosoma, as well as the prosoma pattern. (C) Later epidermal expression of *Tu-pax3/7*. Cells expressing *Tu-pax3/7* RNA are visible in all the prosoma segments and in three segments in the opisthosoma. (D) Late expression of *Tu-pax3/7* in the nervous system. (E-H) Ventral view of embryos hybridised for *Tu-pax3/7* RNA (blue) and stained for Dll protein (brown). Anterior to the left. (E) Cells within the anterior region of the *Tu-pax3/7* RNA-expressing stripes in the prosoma begin to express Dll protein. In cells expressing Dll protein, expression of *Tu-pax3/7* RNA rapidly becomes undetectable. (F) Dll expression spreads across the entire *Tu-pax3/7* expression domain. *Tu-pax3/7* becomes restricted to a square group of cells just ventral to the Dll-expressing limb bud. (G) Dll expression retreats from the proximal regions of the limb bud. (H) Expression of *Tu-pax3/7* in the nervous system. The arrow indicates the primordia of the fourth walking leg. (I-L) Lateral view of embryos hybridised for *Tu-pax3/7* RNA (blue) and stained for Dll protein (brown). Anterior to the left, dorsal up. (I) Expression of Dll protein appears in *Tu-pax3/7*-expressing cells in each stripe. These cells rapidly lose expression of *Tu-pax3/7*. (J) Expression of Dll and loss of *Tu-pax3/7* expression spreads across the stripe. (K) Dll expression retreats from proximal regions of the limb. (L) Expression of *Tu-pax3/7* in the nervous system. Dark staining in the anterior of the embryo is caused by damage during preparation. (M-P) Embryos oriented to examine expression of *Tu-pax3/7* RNA (blue) and Dll protein (brown) in the opisthosoma. Anterior parts of the germ band curl away from the focal plane to the left; embryos are viewed from the ventral surface. (M) One broad stripe of *Tu-pax3/7*-expressing cells is visible posterior to the fourth walking leg stripe (marked by expression of Dll). This is joined by a second stripe (N), and a third (O). Later, expression of *Tu-pax3/7* RNA is visible in three stripes in the central nervous system (P). Arrowheads indicate the first walking leg segment. Scale bars: 50 μm.

Tu-pax3/7 has the characteristics of an ancestral arthropod Pax III gene

The sequence and expression pattern of *Tu-pax3/7* have the characteristics of an ancestral arthropod PgIII gene. Phylogenetic analysis of PgIII proteins using the paired domain and a small part of the extended homeobox domain (boxed in Fig. 3) implies that *Tu-pax3/7* forms a clade with *pax3* and *pax7* from mouse, to the exclusion of insect PgIII genes. *Tu-pax3/7* may thus be derived from an ancestral PgIII group gene from before the separation of the *prd* and *gsb* genes in *Drosophila* and *pby1* and *pby2* from *Schistocerca*.

Consistent with the ancestral character of the *Tu-pax3/7* sequence, the expression pattern of *Tu-pax3/7* appears to combine those of *Drosophila prd* and *gsb*. In *Drosophila* the PgIII genes *prd*, *gsb* and *gsb-n* are vital components of both the pair-rule, and segment polarity cascades. *Prd* activates the segment-polarity gene *engrailed* in odd numbered parasegments and *gsb* in both odd and even numbered parasegments (DiNardo and O'Farrell, 1987). In *Drosophila*, *prd* is first expressed around the 13th nuclear division, in an anterior domain. After cellularisation, this domain splits and is joined by more posterior stripes, forming a pair-rule type pattern of eight stripes of *prd*-expressing cells. These eventually split to form segmentally iterated stripes (Gutjahr et al., 1993a). Initially *gsb* is expressed only in odd numbered parasegments, but expression rapidly appears in all parasegments (Gutjahr et al., 1993b). *gsb* is also expressed in the nervous system where it activates expression of *gsb-n* (Gutjahr et al., 1993b).

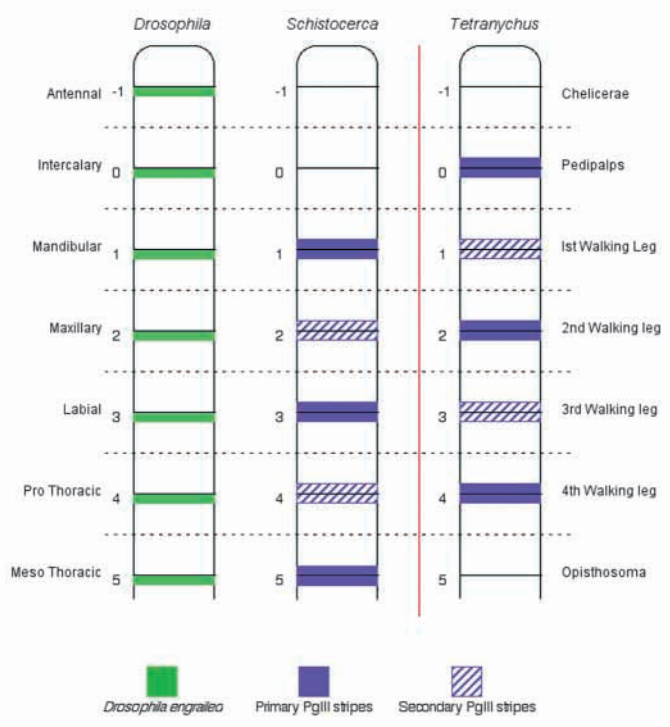

Fig. 7. Schematic diagram of PgIII expression and segment identity in the anterior segmented germ band in *Drosophila*, *Schistocerca* and *Tetranychus*. Dotted horizontal lines define the boundaries of homologous segments in insects and chelicerates as described by Damen et al. (Damen et al., 1998) and Telford and Thomas (Telford and Thomas, 1998). Solid horizontal lines represent parasegment boundaries in *Drosophila*, and possible parasegment boundaries in *Schistocerca* and *Tetranychus*. Solid green bars represent *engrailed* expression in *Drosophila*; solid blue bars represent primary stripes of PgIII genes; hatched blue bars represent secondary stripes. Pair-rule modulated stripes of PgIII genes in insects and *Tetranychus* form with a different register.

The grasshopper (*Schistocerca*) contains two PgIII group genes, *pby1* and *pby2* (Davis et al., 2001). Phylogenetic studies have shown these two genes to be derived from an ancestral PgIII gene before the duplication that formed *prd* and *gsb*. *pby1* is first expressed in the embryonic primordium in a faint posterior domain, which splits into a thoracic domain and a gnathal arc. These arcs resolve into pair-rule stripes of cells in odd numbered segments (i.e. mandibular, labial and second thoracic). Shortly after the formation of the odd numbered stripes, stripes of cells in even numbered segments begin expressing *pby1* de novo (i.e. the maxillary and first thoracic segment). In both *Drosophila* and *Schistocerca*, PgIII genes are first expressed in broad regions of the germ band, and then come to designate, initially, odd numbered parasegment (or putative parasegment) boundaries.

In *T. urticae*, by contrast, *Tu-pax3/7* is not initially expressed in broad domains, and the stripes that form have the opposite phasing to those in *Drosophila* and *Schistocerca* (Fig. 7). In *T. urticae*, *Tu-pax3/7* is initially expressed at the center (as defined by the placement of the limb bud) of even numbered segments (pedipalp, second and fourth walking leg). We suggest that these domains are the equivalent of the initial domains of *prd* in *Drosophila* and thus lie on a potential parasegment boundary. The odd numbered stripes (first and third walking leg) appear later, from lateral foci, in anterior to posterior progression, expanding in width in a similar manner to that described for *gsb* stripes in *Drosophila* (Gutjahr et al., 1993b).

The origin of the difference in register between stripes of PgIII genes in insects and *T. urticae* is unclear. It is possible that this difference reflects the evolutionary distance between insects and chelicerates, with PgIII stripes simply having moved during the passage of time to pattern a different set of segments in one taxon. The difference in register between the stripes of *pby1* in the *Schistocerca* abdomen and *Drosophila* is perhaps another example of this kind of shift (Davis et al., 2001). It is also possible that the difference in register may have come about because a PgIII gene has become involved in, or regulated by, segmentation twice, once in the lineage leading to mites, and once in the lineage leading to insects. If PgIII genes ancestrally had a role in segmental patterning, they might easily become modulated by pair-rule genes, and may eventually take up that function. Indeed the expression pattern of *Tu-pax3/7* is more reminiscent of a segment-polarity gene being regulated by a pair-rule gene, than as a pair-rule gene itself.

In later development, *Tu-pax3/7* stripes are expressed in domains shared with grasshopper *pby1* and *pby2*, including rings in spider mite limbs and in the nervous system (also shared with *Drosophila*). The segmental expression in the CNS is consistent with the proposal that an ancestral PgIII gene should combine functions in segmentation and neurogenesis (Gutjahr et al., 1993b). Even though we cannot completely exclude the possibility that *T. urticae* contains another PgIII homologue, collectively, the expression pattern

of *Tu-pax3/7* may represent an ancestral pattern of PgIII genes in arthropods.

Runt domain genes in chelicerates

The *Tu-run* cDNA was the only *run*-like sequence obtained either in our EST screen or using degenerate PCR on genomic DNA. This implies that it may be the only *runt* homologue in the spider mite genome. Despite this, *Tu-run* is significantly different in its sequence and expression from other *run*-like genes. The most obvious sequence difference is in the VWRPY motif at the carboxyl terminus of the protein. This motif is conserved in all arthropod and vertebrate *runt* homologues examined except spider mites, where the sequence is modified to LWRPF, and *C. elegans*. In *Drosophila*, this motif mediates interaction between *run*-like proteins and Groucho, a transcriptional co-repressor (Aronson et al., 1997). While the changes in sequence of this motif in *Tu-run* are conservative, they may affect the interaction of *Tu-run* with Groucho (a spider mite homologue of the *groucho* gene has been identified in our EST screen). The lack of apparent pair-rule expression of this protein in *T. urticae* is also significantly different from *run* expression in *Drosophila* (Kania et al., 1990) and *Manduca sexta* (Kraft and Jackle, 1994). The later expression of this gene in spider mites is, however, consistent with the role of *run* in segmentation, cell fate specification in the nervous system in *Drosophila*, and with the expression of a *run* homologue in the opisthosoma of the spider *C. salei* (Damen et al., 2000).

Early expression of *Tu-run* may be involved in limb specification

The earliest expression of *Tu-run* is in oval domains in each prosoma segment (excluding the chelicera segment). This expression precedes the morphological differentiation of limbs. Dll expression, however, appears before *Tu-run* rings, implying that *Tu-run* does not play a role in the specification of the initial limb primordia but rather in delimiting their outer perimeters. Cells expressing *Tu-run* do not initially express Dll, implying that they are not initially included in the limb primordia. As the limb extends posteriorwards, however, cells posterior to the initial primordia that once expressed *Tu-run*, lose this expression, and express Dll instead. These cells are incorporated into the limb bud. Expression of *Tu-run* thus does not preclude limb bud cell fate.

Expression domains surrounding the limb primordia have not been observed in *Drosophila*. *Drosophila* limbs form from imaginal discs, a derived mode of limb specification peculiar to holometabolous insects. In most other arthropods, the appendages develop as an outgrowth of the body wall. *run* homologues have not been isolated from non-holometabolous insects, nor has the expression of the *run* homologues discovered in the spider (*C. salei*) been examined during limb primordium specification. It is thus not possible to determine if the limb bud-associated expression of *Tu-run* represents a conserved pathway found in other arthropods, but missing from holometabolous insects, or a derived gene expression pattern specific to mites or chelicerates.

The relative timing of the expression of *Drosophila run* and *prd* is also not conserved in spider mites. In *Drosophila*, *run* is expressed earlier than *prd* and modulates *prd* expression (Gutjahr et al., 1993a). Such changes are not surprising given the differences in early *run* expression and 520 million years of independent evolution.

Different mechanisms pattern the prosoma relative to the opisthosoma

As mentioned previously, spider mite embryogenesis resembles that of intermediate germ band insects. Both the morphological development and the expression patterns of *Tu-run* and *Tu-pax3/7* imply that the prosoma is patterned by a mechanism that differs from that of the opisthosoma. While the pattern of *Tu-pax3/7* is pair-rule modulated in the prosoma of the spider mite, no evidence for pair-rule patterning of the opisthosoma exists. In this tissue, stripes of both *Tu-pax3/7* and *Tu-run* appear to form one by one, in an anterior to posterior progression. In the absence of segmental markers we presume that the opisthosomal stripes represent segmental repeats. This assumption is based on the fact that the stripes display the same width and inter-stripe spacing as those in the prosoma.

This pattern of segmentation gene expression is consistent with the expression of the spider homologues of the segment polarity genes *engrailed*, *wingless* and *cubitus interruptus* (Damen, 2002). In this species expression of these genes first appears as stripes simultaneously formed in all the prosomal segments, followed by the appearance of individual stripes, in anterior to posterior sequence, in the opisthosoma. This observation supports the notion that two mechanisms exist to segment the chelicerate germ band. In the prosoma, a mechanism exists that deploys *pax3/7* expression in a pair-rule-like manner and leads to all segments expressing segment polarity genes simultaneously. In the opisthosoma, *pax3/7* is not regulated in a pair-rule like manner but, like segment polarity gene expression, appears in anterior to posterior sequence.

Differences in the patterning of different body domains have also been shown in grasshoppers, where several genes are expressed differently during segmentation of the gnathum and thorax, as compared to the abdomen (Davis et al., 2001; Dearden and Akam, 2001; French, 2001). The gnathum and thorax of the grasshopper are first demarcated by the expression of the *hunchback* gene (Patel et al., 2001). Within this domain *pby1* stripes appear in a pattern that reflects pair-rule modulation, with secondary stripes forming de novo (Davis et al., 2001). Early stripes of *wingless* (*wg*) also appear in this region with the mandibular *wg* stripe appearing first, followed by the simultaneous formation of all the thoracic stripes (Dearden and Akam, 2001). *Wingless* stripes in the maxillary and labial segments appear de novo between the mandibular and first thoracic stripes. All of these stripes form before the expression of Engrailed protein. In the *Schistocerca* abdomen, by contrast, pair-rule *pby1* domains form segmental stripes, but by splitting of initially broad stripes, rather than de novo appearance of inter-stripes. *Wingless* stripes form with anterior to posterior progression, with Engrailed protein being expressed soon after each *wg* stripe forms.

Conservation of limb positioning between chelicerates and insects

The relationship between *Tu-pax3/7*-expressing cells and the initial domains of Dll-expressing cells provides some evidence that the mechanism specifying placement of the limb primordia in *Drosophila* may be conserved in spider mites. In *Drosophila*,

prd is expressed in a stripe of cells that spans the parasegment boundary (Gutjahr et al., 1993a). This expression is required to activate both *wg* and *engrailed* expression in their respective domains on either side of the parasegment boundary (reviewed by Nasiadka and Krause, 1999). The initial expression of Dll in *Drosophila* is also regulated by the parasegment boundary. The leg imaginal discs derive from *wg*-expressing cells just anterior to the parasegment boundary (Cohen et al., 1993).

The relative positions of the expression domains of Dll and *paired* in *Drosophila* appears conserved in spider mites. Dll expression domains appear in ovals centered on top of the anterior parts of the stripes of *Tu-pax3/7*-expressing cells. If *Tu-pax3/7* is expressed across a putative parasegment boundary, then the limb bud is placed just anterior to the parasegment boundary, as in *Drosophila*. As the limb bud grows, apparently recruiting cells in the posterior of the *Tu-pax3/7* stripe, and beyond it, *Tu-pax3/7* expression is repressed in the majority of the stripe, but remains active in a block of cells, ventral to the limb bud. These cells form a domain that lies just anterior to and extends posterior of, the edge of the cells of the limb bud. It is possible that the juxtaposition of cells expressing these two genes represents conservation of the pathways specifying the anterior-posterior positioning of the limbs in spider mites. These data imply that the parasegment boundary may be conserved in spider mites, and is possibly an ancestral feature of arthropod development. Similar observations in spiders (Damen, 2002) also imply that the placement of the limb and the parasegment boundary are conserved in arthropods.

Pair-rule patterning in chelicerates?

The expression pattern of *Tu-pax3/7* in the prosoma is consistent with that expected for a gene regulated by a pair-rule like process. The appearance of the stripes with an alternate-segment periodicity is similar to the expression patterns of some segment polarity genes in *Drosophila* that are modulated by pair-rule genes. This may provide indirect evidence for a pair-rule mechanism acting in spider mite segmentation. Similar expression patterns of PgIII genes in the gnathal and thoracic regions of the grasshopper have been interpreted as evidence for a pair-rule mechanism acting during segmentation of these areas of the grasshopper embryo (Davis et al., 2001). In the spider mite opisthosoma however, *Tu-pax3/7* is not expressed in a pair-rule like pattern, supporting recent findings (Damen, 2002) that the prosoma and the opisthosoma are patterned differently in the spider *C. salei*.

A recent model (Wilkins, 2001) proposes a scenario for the evolution of the complex set of pair-rule genes seen in *Drosophila*. According to this model, co-option of a new set of gap genes is necessary for the simultaneous patterning of the entire embryo. Incorporation of these new gap genes in turn requires modulation and refinement by recruiting new pair-rule genes so that in *Drosophila* they form a complex regulatory hierarchy formed to correct regulatory imbalances. Ultimately, in *Drosophila*, these interactions modulate the expression of the segment-polarity cascade. This model predicts that only a few proto-pair-rule genes would be present in primitive arthropods, and that homologues of *Drosophila* pair-rule genes might have alternative functions in basal arthropods.

The expression of *Tu-pax3/7* provides the first suggestion that a pair-rule patterning mechanism may exist outside insects. If pair-rule patterning does act in the development of chelicerates then the evolutionary distance between spider mites and *Drosophila* would suggest that pair-rule patterning is an ancient pathway, and is probably deployed in segmentation, of at least some of the body, in all arthropods. However, expression of two other homologues of the *Drosophila* pair-rule cascade in mites, *Tu-run* (described in this paper) and *ftz* (Telford, 2000) suggests that they are not involved in pair-rule patterning in chelicerates. This implies that the upstream gene(s) that regulate pair-rule modulation of *Tu-pax3/7* could be different from 'traditional' pair-rule genes isolated in *Drosophila*, though they may illustrate utilization of a 'pair-rule logic'.

Confirmation that a pair-rule pathway exists will require cloning of upstream modulator(s) of *Tu-pax3/7* and functional studies of genes involved in segmentation. Examining the expression patterns of pair-rule genes in other arthropod groups (such as myriapods and crustaceans), as well as close relatives of arthropods (such as onychophorans and tardigrades) will provide a better understanding of the origins of pair-rule patterning in arthropods.

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