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Evolution of Developmental Control Mechanisms

Evolution of the pair rule gene network: Insights from a centipede ☆

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ABSTRACT

Comparative studies have examined the expression and function of homologues of the *Drosophila melanogaster* pair rule and segment polarity genes in a range of arthropods. The segment polarity gene homologues have a conserved role in the specification of the parasegment boundary, but the degree of conservation of the upstream patterning genes has proved more variable. Using genomic resources we identify a complete set of pair rule gene homologues from the centipede *Strigamia maritima*, and document a detailed time series of expression during trunk segmentation. We find supportive evidence for a conserved hierarchical organisation of the pair rule genes, with a division into early- and late-activated genes which parallels the functional division into primary and secondary pair rule genes described in insects. We confirm that the relative expression of *sloppy-paired* and *paired* with respect to *wingless* and *engrailed* at the parasegment boundary is conserved between myriapods and insects; suggesting that functional interactions between these genes might be an ancient feature of arthropod segment patterning. However, we find that the relative expression of a number of the primary pair rule genes is divergent between myriapods and insects. This corroborates suggestions that the evolution of upper tiers in the segmentation gene network is more flexible. Finally, we find that the expression of the *Strigamia* pair rule genes in periodic patterns is restricted to the ectoderm. This suggests that any direct role of these genes in segmentation is restricted to this germ layer, and that mesoderm segmentation is either dependent on the ectoderm, or occurs through an independent mechanism.

Introduction

The genetic dissection of the mechanism of segmentation in the fruit fly, *Drosophila melanogaster*, has laid the foundation for a growing body of comparative research on other arthropods (Nusslein-Volhard and Wieschaus, 1980; Peel et al., 2005). The *Drosophila* work identified a number of genes involved in segment pattern formation, and divided them into four functional categories based on their mutant phenotypes. These categories are maternal factors, gap genes, pair rule genes and segment polarity genes. Work over the years has pieced together the hierarchy of interactions between these genes, and shown how this hierarchy is capable of reproducibly generating precise patterns (Akam, 1987; Jaeger et al., 2004; Pankratz and Jaeckle, 1993).

Homologues for most of the *Drosophila* segmentation genes can be identified throughout the arthropods. The key segment polarity genes *wingless, engrailed, hedgehog* and *Cubitus interruptus* show highly conserved expression at the parasegment boundary in a range of arthropod species, including chelicerates and myriapods, as well as insects (Damen, 2007; Farzana and Brown, 2008). Thus, the parasegment is likely to be an ancient feature of arthropod segmentation. However, the extent of conservation in the gene regulatory network that acts upstream of the segment polarity genes is not yet clear (Peel et al., 2005).

Besides *Drosophila*, there are two other insects for which extensive functional data are available on the role of pair rule gene homologues: the red flour beetle *Tribolium castaneum* (Choe and Brown, 2007, 2009; Choe et al., 2006) and the honeybee *Apis mellifera* (Wilson and Dearden, 2012). In the honeybee, transcripts for a number of the pair rule gene homologues are deposited into the oocyte and have acquired novel roles in early developmental patterning, which obscure any later roles in segmentation in RNAi experiments (Wilson and Dearden, 2012). Therefore, the main work able to address the role of these genes in segment pattern formation is in *Tribolium*. There are a number of differences in the roles of pair rule genes between *Tribolium* and *Drosophila*. For example, the beetle homologues of the pair rule genes *hairy*, *odd-paired* (*opa*) and *fushi-tarazu* (*ftz*) appear to have no essential role in *Tribolium* trunk segmentation, or at least give no detectable RNAi phenotype (Aranda et al., 2008; Choe et al., 2006).

One aspect of segment patterning that is largely conserved between *Tribolium* and *Drosophila* is the division of the pair rule

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genes into primary and secondary tiers, based on their functional position in the hierarchical cascade. Primary pair rule genes are upstream in the cascade and are regulated by other factors (maternal coordinate factors and gap genes), whereas the secondary pair rule genes are downstream in the cascade and regulated by the primary pair rule genes. In *Drosophila*, *even-skipped* (*eve*), *runt* and *hairy* are considered the primary pair rule genes; *sloppy-paired* (*slp*) and *paired* (*prd*) are considered the secondary pair rule genes. *ftz* and *odd-skipped* (*odd*) were originally considered to be secondary, but a more recent and thorough analysis of their cis-regulatory architecture has shown that in some respects they qualify as primary pair rule genes (Schroeder et al., 2011). In *Tribolium*, *eve*, *runt,* and *odd* are found to be primary pair rule genes, and *slp* and *prd* to be secondary pair rule genes. Thus, not only do many of the *Tribolium* pair rule gene homologues play a role in segmentation, they are also found to occupy similar levels in the gene regulatory hierarchy. (Choe et al., 2006).

In non-insect arthropods there is indirect evidence that the functional division of the pair rule network into primary and secondary levels is conserved. Importantly, the timing of expression of the pair rule genes in segment patterning reflects the functional division of the hierarchy. That is to say, the alternate expression of the primary pair rule genes is established first in the process, and the periodic patterning of the secondary pair rule genes appears afterwards. Therefore in arthropods where functional tools are not available, indirect evidence for the functional division can still be obtained from the temporal order in which the genes are expressed during segment pattern formation. This has been done for spider (Damen et al., 2005) and millipede segmentation (Janssen et al., 2011). A consistent finding across studies is the early expression, and where tested, upstream function of *eve*, *runt* and *odd* homologues during segment patterning; and the late expression, and where tested, downstream function of *prd* and *slp* homologues. This suggests that the functional division between primary and secondary pair rule genes, and at least some of the genes that occupy these categories, may be a conserved feature of arthropod segmentation.

A second aspect of segmental patterning conserved between *Tribolium* and *Drosophila* concerns the role of the secondary pair rule genes, *prd* and *slp*, in the regulation of the key segment polarity genes, *wingless* (*wg*) and *engrailed* (*en*) (Choe and Brown, 2009). In both insects, the *prd* homologue is expressed in both *wg*- and *en*-positive cells, and thus overlaps the parasegment boundary, whereas the *slp* homologue is restricted to *wg*-positive cells. The same relationship is observed between these four genes in the pill millipede *Glomeris marginata* (Janssen et al., 2011), and the available data suggests that it holds for a *pax3/7* homologue and *en* in the grasshopper *Schistocerca americana* (Davis et al., 2001). This striking conservation of relative expression suggests an ancient origin for the regulatory module in which these four genes act, even accepting that conserved transcriptional output cannot be taken to imply that the underlying transcriptional networks are also conserved (Ludwig et al., 2000; Romano and Wray, 2003).

We have tested whether these features of the pair rule gene network are also conserved in another ancient branch of the myriapods – the Chilopoda. Our study species is the geophilomorph centipede, *Strigamia maritima*. Previous work on *Strigamia* has characterised a number of the major features of segmentation (Brena and Akam, 2012; Chipman et al., 2004b; Kettle et al., 2003). At the time of germ band formation, a large population of progenitor cells forms a disc of unsegmented tissue at the posterior of the embryo. As the germ band elongates, this posterior disc narrows and segments emerge from it sequentially in anteroposterior order. Because segments are added in a temporal sequence, the progression of segment patterning can be visualised in each embryo as a sequence from posterior to anterior.

Expression of some of the pair rule gene homologues has already been examined in *Strigamia* – two *even-skipped* homologues (*Sm-eve1* and *Sm-eve2*); one member of the *odd-skipped* gene family (*Sm-odr1*); and two members of the *hairy*/*deadpan* family (originally named *Sm-hes1* and *Sm-hes4*) (Chipman and Akam, 2008; Chipman et al., 2004a). This work has shown that there are two phases of patterning during the major stage of trunk segmentation. In the first phase, dynamic patterns of gene expression resolve to a series of concentric rings in the posterior disc, defining a double segment repeat. In the second phase, the double segment pre-pattern resolves to a single segment repeat by the splitting and/or intercalation of expression domains. The region where this segmental resolution occurs, in between the posterior disc and the segmented germ band, is called the transition zone. The domain of dynamic expression is restricted to a population of cells surrounding, but largely anterior to, the proctodeum and lying within the first resolved ring of expression. We refer to this territory as the peri-proctodeal region.

We set out to test four hypotheses about the degree of conservation of the pair rule gene homologues in segment pattern formation. First, are the pair rule genes in *Strigamia* hierarchically organised into primary and secondary tiers, as has been described in other arthropods? Second, are the relative expression domains of key genes conserved during the specification of the parasegment boundary? In particular, is the spatial registration of *slp* and *pax3/7* homologues conserved in relation to the abutting *wg*- and *en*-expressing cells? Third, is there evidence from the relative expression of *eve*, *runt*, *odd* and *hairy* homologues that the upstream tiers of segment patterning are more divergent? Fourth, is the expression of the *Strigamia* pair rule genes restricted to the ectoderm germ layer? And if not, are any of these genes expressed in patterns that suggest an early role in mesoderm segmentation? We have addressed these hypotheses by examining the spatial and temporal dynamics of expression of a complete set of *Strigamia* pair rule homologues during segment patterning.

Materials and methods

Embryo collection, fixation and staging

Embryos were collected in the field from a population near Brora, Scotland and fixed as described previously (Chipman et al., 2004b). Embryos were staged by counting the total number of morphologically visible leg-bearing segments, either in whole embryos or in flat-mount preparations.

Gene identification and cloning

A genome and adult and embryonic transcriptomes have recently been assembled for *Strigamia maritima* (genome release Smar_1.0 http://www.ncbi.nlm.nih.gov/assembly/322118/). Pair rule gene homologues were identified by similarity searches against these resources, and orthology of the genes was confirmed by reciprocal similarity searches. To clone genes, gene-specific primers were designed against the genomic or transcriptomic sequence, and products amplified by standard PCR from embryonic cDNA. Genes were cloned into a pGEM-T Easy vector (Promega). The clones of *ftz* and *twist* were a gift of C. Brena, the clones of *slp* and *opa1* were a gift of V. Hunnekuhl and the clone of *wg* was a gift of L. Hayden. An annotated gene set for *Strigamia* is provided at EnsemblMetazoa (http://metazoa.ensembl.org/Strigamia_maritima/Info/Index). The Ensembl IDs of the pair rule and segment polarity gene homologues examined in this paper are provided in Supplementary Table 1.