The embryonic expression pattern of a second, hitherto unrecognized, paralog of the pair-rule gene sloppy-paired in the beetle Tribolium castaneum

Ralf Janssen

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Abstract

In the fly Drosophila melanogaster, a hierarchic segmentation gene cascade patterns the anterior-posterior body axis of the developing embryo. Within this cascade, the pair-rule genes (PRGs) transform the more uniform patterning of the higher-level genes into a metameric pattern that first represents double-segmental units, and then, in a second step, represents a true segmental pattern. Within the PRG network, primary PRGs regulate secondary PRGs that are directly involved in the regulation of the next lower level, the segment-polarity genes (SPGs). While the complement of primary PRGs is different in Drosophila and the beetle Tribolium, another arthropod model organism, both paired (prd) and sloppy-paired (slp), acts as secondary PRGs. In earlier studies, the interaction of PRGs and the role of the single slp ortholog in Tribolium have been investigated in some detail revealing conserved and diverged aspects of PRG function. In this study, I present the identification and the analysis of embryonic expression patterns of a second slp gene (called slp2) in Tribolium. While the previously identified gene, slp, is expressed in a typical PRG pattern, expression of slp2 is more similar to that of the downstream-acting SPGs, and shows expression similarities to slp2 in Drosophila. The previously reported differences between the function of slp in Drosophila and Tribolium may partially account for the function of the newly identified second slp paralog in Tribolium, and it may therefore be advised to conduct further studies on PRG function in the beetle.

Keywords

Sloppy-paired · Slp · FoxG · Segmentation · Pair-rule · Segmental patterning · Forkhead

Introduction

In the dipteran fly Drosophila melanogaster, a hierarchic segmentation gene cascade almost synchronously controls the patterning of the early embryo along the anterior-posterior (AP) body axis into a defined number of single segments (reviewed in e.g. Akam 1987, Davis and Patel 2003). First, maternally provided factors form anterior to posterior and posterior to anterior morphogen gradients, that then regulate the zygotically expressed gap genes (GGs) (e.g. Fröhnhofner and Nüsslein-Volhard 1986). The GGs, which are expressed in broad overlapping domains along the AP axis of the developing embryo, regulate another group of zygotically expressed genes, the so-called pair-rule genes (PRGs). PRGs are typically expressed in the form of seven transversal stripes corresponding to alternating segment primordia (e.g. Jäckle et al. 1988, Cadigan et al. 1994a, recently reviewed in Clark et al. 2019). This mode of segment formation, in which all segments are patterned synchronously at the blastoderm stage, is derived, and most other insects, as well as all other arthropods, only
form their most anterior segments in a similar way (reviewed in Peel 2004). Posterior segments, however, are added from a posterior segment addition zone (SAZ). In most cases, single segmental units are added from the SAZ (e.g. Hughes and Kaufman 2002; Schoppmeier and Damen 2005; Janssen et al. 2011), but in some arthropods, all, or a number of segments, are added (or patterned) with a double-segment periodicity (e.g. Binner and Sander 1997; Dearden et al. 2002; Chipman et al. 2004; Erzyilmaz et al. 2009; Janssen et al. 2012; El-Sherif et al. 2012; Sarrazin et al. 2012). This latter mechanism is reminiscent of the initial expression of the PRGs in Drosophila and their pair-rule function. While it is still unclear if pair-rule patterning (and function) is a conserved ancestral trait of arthropod segmentation, it appears likely that the PRGs in general are involved in segmentation, as evident from functional studies (e.g. Liu and Kaufman 2005; Mito et al. 2007; Rosenberg et al. 2015; Xiang et al. 2017; Auman and Chipman 2018), and the analysis of gene expression patterns (e.g. Damen et al. 2000, 2005; Dearden et al. 2002; Hughes and Kaufman 2002; Chipman and Akam 2008; Janssen et al. 2011; Green and Akam 2013; Schönauer et al. 2016).

Compared with Drosophila, the beetle Tribolium castaneum displays a more conservative mode of development: The anterior segments are formed from the blastoderm, but posterior segments are added sequentially. In both the blastoderm that give rise to the anterior segments, and the posteriorly added segments, a clock-like mechanism including the function of PRGs appears to be involved. This mechanism generates/patterns segments with a double-segment periodicity (Choe et al. 2006; El-Sherif et al. 2012; Sarrazin et al. 2012), although the last-formed posterior segments indeed may be patterned one by one, at least on the level of SPGs (Janssen 2014). Generally, PRG orthologs have been in the focus of unravelling the segmentation mechanisms in Tribolium (e.g. Sommer and Tautz 1993, Brown et al. 1994, 1997, Maderspacher et al. 1998, Schröder et al. 2000, Eckert et al. 2004, Aranda et al. 2008, Bolognesi et al. 2009, Choe and Brown 2007, 2009, Peel et al. 2013, El-Sherif et al. 2014), and one of these genes of interest is the sloppy-paired (slp) gene (Choe et al. 2006; Choe and Brown 2007).

The PRG ortholog sloppy-paired (slp) (alternative name FoxG) encodes a forkhead-box containing transcription factor, that acts as a secondary PRG in Drosophila, where it exists in the form of two paralogs (slp1 and slp2) (Häcker et al. 1992; Grossniklaus et al. 1992). Both paralogs function directly on the SPGs and also act as SPGs. In this function, they are involved in the maintenance of parasegmental boundaries and segment polarity (Cadigan et al. 1994a). In accordance with this dual role, slp paralogs are first expressed in the form of seven transverse segmental stripes (PRG pattern), and later in the form of 14 stripes (SPG pattern) (Grossniklaus et al. 1992).

Similar to the two Drosophila paralogs, the single described Tribolium slp gene appears to function downstream of a regime of primary PRGs (Choe et al. 2006), and is involved in the regulation of SPGs, and thus in segmentation, albeit in a slightly different way than in Drosophila (Choe and Brown 2007, 2009).

In this paper, I present the discovery of a second, hitherto unrecognized, paralog of sloppy-paired in Tribolium. Its embryonic expression pattern suggests a potential role in segmentation, either downstream and/or in parallel with the other Tribolium PRG orthologs, including the earlier-described Tribolium slp gene (Choe et al. 2006). The presence of a second slp gene, designated as slp2, may have implications for the interpretation of earlier research conducted on PRG patterning and the gene regulatory network that governs segmentation in Tribolium.

Methods

Tribolium husbandry and preparing embryos

The used specimens of Tribolium castaneum stem from the culture in Göttingen/Germany. A colony of this strain was established in Uppsala, following the suggestions made in “The Beetle book” (link: http://wwwuser.gwdg.de/~gbucher1/tribolium-castaneum-beetle-book1.pdf). Embryos were collected and prepared for subsequent in situ hybridization experiments as per Schinko et al. (2009).

Extraction of total RNA, cDNA synthesis, gene cloning and whole mount in situ hybridization

Total RNA was isolated from complete embryos of mixed developmental stages using TRIZOL (Invitrogen). Total RNA was reverse transcribed into cDNA using the SuperScript First Strand kit (Invitrogen). Gene fragments were isolated by RT-PCR with gene-specific primers. Primer sequences are slp_forward: GGTGAAAAGGGAAGAAACGA, slp_backward: AGATCACCCGTCACTGGTTTA, slp2_forward: GGCAGAGAGCAAAGAAACCG and slp2_backward: ACAGTGACAGGTTTAAACCG (Grossniklaus et al. 1992). Fragment sequences were ligated into the PCRII vector (Invitrogen) and sequenced on an ABI3730XL automatic sequencer (Macrogen, Seoul, South Korea). Unique gene identifiers are listed in Supplementary File 1.

Monochromatic in situ hybridizations were performed using the standard protocol provided in Janssen et al. (2018). Dichromatic in situ was performed as described in Janssen et al. (2008). Monochromatic in situ were performed with digoxigenin (DIG)-labelled probes; for the second staining in dichromatic in situ, a fluorescein-labelled second probe was used. First, the digoxigenin-labelled probes were detected...
with BMPurple (Roche), giving rise to a blue signal. After that, alkaline phosphatase on the DIG-labelled antibodies was inactivated with 0.1 M glycine, pH = 2.0 (2-min incubation at room temperature), and the second probe was detected with SIGMAFAST Fast Red TR/Naphthol AS-Mx (Sigma), giving rise to a red signal.

**Identification of potential paralogs of sloppy-paired and phylogenetic analysis**

Paralogs of Tribolium sloppy-paired (slp) were identified performing reciprocal BLAST searches (BlastX and BlastP) against the published genomic sequences of the beetle Tribolium using the Drosophila paralogs slp1 and slp2, and the previously identified Tribolium slp gene (Choe et al. 2006) as baits.

Amino acid sequences of the forkhead domains were aligned using T-Coffee (Notredame et al. 2000) using default parameters in MacVector v12.6.0 (MacVector, Inc., Cary, NC). A Bayesian phylogenetic analysis was performed using MrBayes (Huelsenbeck and Ronquist 2001) with a fixed WAG amino acid substitution model with gamma-distributed rate variation across sites (with four rate categories), unconstrained exponential prior probability distribution on branch lengths, and exponential prior for the gamma shape parameters for among-site rate variation. Tree topology was calculated applying 200,000 cycles for the Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) analysis (four chains; chain-heating temperature of 0.2). Markov chains were sampled every 200 cycles. Default settings were used, defining 25% of the samples as burn-in information. Clade support was calculated with posterior probabilities in MrBayes. Unique identifiers of all used sequences are summarized in Supplementary File 1.

**Data documentation**

Pictures of in situ–stained embryos were taken with a Leica DC490 digital camera mounted onto a MZ-FLIII Leica dissection stereo-loupe. Linear adjustments were made on contrast and brightness using the image-processing software Adobe Photoshop CC 2018 for Apple Macintosh (Adobe Systems Inc.)

**Results**

**Identification of Tribolium sloppy-paired genes**

The performed reciprocal BLAST search identified two genes with high sequence similarity to both Drosophila melanogaster sloppy-paired genes (slp1 and slp2) (Häcker
et al. 1992; Grossniklaus et al. 1992), the single previously
described Tribolium sloppy-paired gene (slp) (Choe et al.
2006), and confirmed sloppy-paired orthologs from other ar-
thropods (e.g. Damen et al. 2005, Liu and Patel 2010, Janssen
et al. 2011, Green and Akam 2013, Auman and Chipman
2018) and an onychophoran (Janssen and Budd 2013).

Phylogenetic analysis shows that these two Tribolium
genes cluster with panarthropod sloppy-paired orthologs and
form a well-supported monophyletic group (Fig. 1). Tribolium
Slp clusters with high confidence with Drosophila Sloppy-
paired1 (Slp1), and it seems clear that those two form an
orthology-pair. The newly identified Tribolium Slp2 and
Drosophila Slp2 do not form a sister-gene relationship in the
phylogenetic analysis. The posterior probability values, how-
ever, that separate Tribolium and Drosophila Slp2 from each
another are very low. Therefore, it is possible that the two
designated slp2 genes are in fact the result of a duplication
at the lineage leading to Tribolium + Drosophila, and thus
could represent paralogs. Note that the distribution of Slp
proteins does not represent our current understanding of ar-
thropod relationships.
Expression of the previously described *Tribolium* sloppy-paired (*slp*) in comparison with the newly identified second paralog, *slp2*

The *Tribolium* *slp* gene has been identified in Choe et al. (2006), and its expression has been described in some detail in Choe and Brown (2007). Here, I present a more detailed description of *slp* in a series of consecutive developmental stages, revealing some additional aspects of its expression, and at the same time compare its expression with that of *slp2* (Fig. 2).

At the blastoderm stage, both paralogs of *slp* are expressed in identical patterns in the complete embryo, except for its anterior cap that will later give rise to the serosa (Fig. 2a/a’). In a slightly later blastoderm stage, expression of both paralogs disappears from the posterior of the embryo resulting in a broad central domain of expression (Fig. 2b/b’). In contrast to *slp* (Fig. 2b’), expression of *slp2* is weaker ventrally and finally fully disappears, resulting in two separate domains of expression in the following stage (Fig. 2e). The same ventral disappearance of expression happens with *slp*, but at a slightly later stage (Fig. 2c’/c’’). This is evident from the presence of an intermediate stage in which expression is visible in the form of a solid band (i.e. prior to ventral disappearance) (cf. Fig. 2c/c’/c’’). With the beginning of gastrulation (Fig. 2d/d’), two stripes of *slp* appear simultaneously and posterior to the anterior expression domain which is located in the head and likely contributes to the anlagen of the antennae and the eyes (see later developmental stages). The two new stripes correspond to the mandibular (md) and maxillary (mx) segment (Fig. 2d’). At the same time, only one stripe of *slp2* appears in the anlage of the mandibles (md) (Fig. 2d). The next stripe of *slp* expression appears in the first thoracic segment (T1) (Fig. 2e’), while *slp2* now appears in the mx (Fig. 2e). *slp* expression then appears in the labial (lb) segment and, in the form of a single broad domain, in the anlagen of the second and the third thoracic segments (T2 and T3) (Fig. 2f’). *slp2*, however, only appears newly in the lb. (Fig. 2f’). The broad domain of *slp* in T2/T3 then separates into two stripes (Fig. 2g’), while for *slp2*, the T1 stripe forms (Fig. 2g). Note the remnants of earlier expression (the broad domain) of *slp2* between the now-forming two stripes (Fig. 2g’, arrow; also see Fig. 2k’). Then another broad posterior domain of *slp* appears which is corresponding to the first two abdominal segments (A1/A2) (Fig. 2h’). At approximately the same time, expression of *slp2* appears in T2 (Fig. 2h). As the posterior expression of *slp* splits into separate stripes in A1 and A2, the T3 stripe of *slp2* appears (Fig. 2i’/l’). At the following stage, two new domains of expression appear for both genes. The first is the “delayed” appearance in the intercalary segment (ic) (Fig. 2j/j’). Additionally, another broad domain of *slp* appears representing the third and fourth abdominal segments (A3/A4) (Fig. 2j’), and, at this time, also the first abdominal stripe of *slp2* appears (Fig. 2j). This pattern of posterior stripe-addition and splitting (for *slp*) is maintained in the following developmental stages: addition of A3/A4 (*slp*) and A2 (A3 weakly) (*slp2*) (Fig. 2k’), addition of A5/A6 (*slp*) and A3 (A4 weakly) (*slp2*) (Fig. 2l’), addition of A7/A8 (*slp*) and A5 (A6 and A7 weakly) (*slp2*) (Fig. 2m’/m”), splitting of A7/A8 (*slp*) and A6 (A7 and A8 weakly) (*slp2*) (Fig. 2n’), addition of A9/A10 (*slp*) and A8 (*slp2*) (Fig. 2o/o”), splitting of A9 and A10 (*slp*) and addition of A9 (*slp2*) (Fig. 2p’/p”). Then, a single stripe of *slp* forms posterior to the A10-stripe (Fig. 2q’), and the A10 stripe of *slp2* forms (Fig. 2q). As this last added stripe of *slp* becomes stronger, the same stripe of *slp2* forms (Fig. 2r/r’ and s/s’).
Expression of slp in the labrum appears at the stage depicted in Fig. 2o. Expression of slp2 in the labrum, however, appears slightly later in the embryo shown in Fig. 2q.

In summary, segmental expression of slp appears in a double-segmental pattern and involves either the splitting of an initial broad domain into two distinct stripes in adjacent segments, or intercalation of a secondary stripe anterior to the last-formed stripe. Stripes of slp2, however, appear in an anterior to posterior order with a single-segment periodicity. The segmental stripes of slp appear earlier than those of slp2 (Fig. 2).

The intra-segmental position of slp has been shown to be anteriorly adjacent to the expression of the SPG engrailed (en) (Choe and Brown 2007). Double expression of slp with the downstream target of En, hedgehog (hh), corroborates this finding (Fig. 3a/b). The intra-segmental position of slp2 is almost identical with that of slp as revealed by co-expression with hh (Fig. 3c/d). Double in situ with slp and slp2, however, reveal that the expression of slp extends somewhat more towards anterior than that of slp2 (Fig. 3e, f, g, h).

With respect to their temporal appearance during segment addition, the appearance of the segmental expression of slp predates the onset of slp2-expression in stripes (Figs. 2 and 3e, f, g, h).
Discussion

The expression profiles of the two Tribolium slp genes share common features with their Drosophila orthologs

In Drosophila, the Sloppy-paired locus contains two copies of the gene, slp1 and slp2 (Häcker et al. 1992; Grossniklaus et al. 1992). It is therefore tempting to assume that the presence of two paralogs in Tribolium may be correlated with the function(s) of the two Drosophila slp genes, i.e. that in both species, slp1 and slp2, respectively, share the same function(s). In Drosophila, slp1 is expressed slightly earlier than its paralog, slp2 (Grossniklaus et al. 1992; Häcker et al. 1992). Therefore, and although the overall function of the two paralogs is largely redundant, it has been assumed that slp1 may be responsible for the early function of the Slp locus, i.e. that of a PRG, and that the later expressed slp2 may be (in concert with slp1) involved in the later function, i.e. that of a SPG (Cadigan et al. 1994b).

Interestingly, this situation appears to be conserved in Tribolium, where slp (the likely ortholog of Drosophila slp1) is expressed earlier than its paralog slp2. In addition, slp is expressed in a PRG-like pattern in the form of initial broad domains that later split and then correspond to expression in two adjacent segments, or indeed by intercalation (discussed below) (Fig. 2). In contrast, Tribolium slp2 expression is that of a typical SPG (cf. expression of Tribolium SPGs; e.g. Farzana and Brown 2008) with a clear single-segment periodicity (Fig. 2). The expression profiles of Tribolium slp and slp2 are thus remarkably similar to those of the two Drosophila slp genes, suggesting at least partially conserved regulation and interaction of these genes in the fly and the beetle.

Pair-rule patterning: Intercalation or splitting?

In Drosophila, the secondary PRG-stripes of slp1 and slp2 form by intercalation, i.e. de novo formation of stripes in between the first-formed primary PRG-stripes (Grossniklaus et al. 1992). In Tribolium, the situation is not that clear. It is obvious that the segmental expression of slp2 appears with a single-segment periodicity (this study). Posterior stripes of slp, however, appear in pairs, or do they not? The mandibular (md) and maxillary (mx) stripes seem to appear as a pair (Fig. 2d) (see also Choe and Brown 2007). In the following labial (lb) segment, however, appearance of slp is delayed; this stripe intercalates (like the secondary stripes of slp1 and slp2 in Drosophila). For the next two pairs of stripes, the situation is unclear. It appears that these stripes form by splitting of an...
initially broad single domain (Fig. 2f’, g’, h’, i’) (see also Choe and Brown 2007, their Fig. 2g), rather than by intercalation of the secondary (anterior) stripes. A remnant of the initial broad domain (Fig. 2f’) that may document the splitting is present (Fig. 2g’, arrow). Such remnant of expression has been interpreted as evidence of splitting of a PRG’s initial expression in the grasshopper Schistocerca (Davis et al. 2001). The formation of stripes in the third and fourth abdominal segments (A3/A4), however, may imply intercalation (note the spacing (black bar) between the last-formed stripe in the embryo shown in Fig. 2j’). However, again, in the following stage, possible remnants of this initial domain are present between the two stripes (Fig. 2k’, arrow). The situation in A7 and A8 is similar, displaying a large distance between a first-formed stripe and the stripe in A6 (Fig. 2m’). It is therefore unclear if the secondary (anterior of each pair) stripes of slp appear by intercalation or splitting. Possibly, there are also differences in the regulation of the patterning of anterior abdominal versus posterior abdominal segments as shown for the regulation of the SPG H15 in this species (Janssen 2014).

Both splitting of initially formed broad double-segment wide stripes and intercalation of secondary stripes as seen in Drosophila appear to be a characteristic of PRG expression in general, as it is seen in a wide range of drosophilids, non-drosophilid insects with a long-germ developmental mode and in short-germ arthropods such as Tribolium, myriapods and even a mite (e.g. Binner and Sander 1997; Dearden et al. 2002; Chipman et al. 2004; Choe and Brown 2007; Wilson et al. 2010; Janssen et al. 2012). If these double-segmental patterns in the various arthropods are conserved, or have evolved independently, again, remains unclear.

The intra-segmental position of slp genes in arthropods is conserved

In both the beetle and the fly, both slp genes are expressed in stripes anterior adjacent to engrailed (en) and hedgehog (hh), and thus, at least partly, co-expressed with wingless (wg) (Grossniklaus et al. 1992, Choe and Brown 2007, this study). The relative expression of Drosophila slp1 and slp2 in comparison with each other has not been investigated in detail, but the two slp genes are described as fully co-expressed (Grossniklaus et al. 1992, Erik Clark (personal communication)). The broader expression of slp in Tribolium compared with slp2 thus represents a difference between these two species. Data on slp expression from other insects are scarce. In another beetle, Dermentes maculatus, a single slp gene has been identified, that is expressed in a comparable pattern with that of the Tribolium slp genes, and plays a classic function as PRG (complementary to that of the other secondary PRG, paired (prd)) (Xiang et al. 2017). Its intra-segmental position is not clear. In the true bug Oncopeltus fasciatus, the single identified slp gene is expressed as stripes with a single-segment periodicity in the anterior SAZ and newly forming segments, is under control of the bona fide gap gene giant (Liu and Patel 2010) and appears to have a conserved function as segment-polarity gene (Auman and Chipman 2018; Reding et al. 2019). Data on crustacean slp expression are not available. In the myriapods Glomeris marginata and Strigamia maritima, slp is expressed with a single-segment periodicity in posteriorly added segments and anterior to en (Janssen et al. 2011; Green and Akam 2013). The intra-segmental position is thus conserved between insects and myriapods. Finally, in the spider Cupiennius salei, slp is expressed in stripes in newly forming segments, but its intra-segmental position is not clear (Damen et al. 2005).

Possible implications for earlier studies

The finding that Tribolium, like Drosophila, possesses two slp genes with shared and comparable (to Drosophila) expression profiles could explain some of the differences in the regulation and function of Tribolium slp reported in earlier studies, and suggests the need for a re-investigation of the function(s) of slp and slp2 in Tribolium (Choe and Brown 2007).

In Drosophila slp null mutants, expression of wg disappears from alternating segments, while in the same segments, expression of en extends towards anterior into the natural domain of wg (summarized in Choe and Brown 2007). In Tribolium, however, downregulation of slp leads inter alia to defects in all anterior segments, but in posterior segments, alternating segments are affected (Choe and Brown 2007). Interestingly, however, the register in which slp affects segmentation is shifted (the alternating pattern is reversed) in these two species (Choe and Brown 2007). Another difference is that in Drosophila and the true bug Oncopeltus, but not in Tribolium, en expands towards anterior in the absence/downregulation of Slp function (Choe and Brown 2007; Auman and Chipman 2018; Reding et al. 2019). While the former cannot easily be explained by the presence of a second paralog in Tribolium (i.e. slp2), it could be that en remains restrained to its natural domain in the absence of slp in Tribolium because slp2 acts as a potential repressor of en-expression. Alternatively, the remaining low activity of slp after RNAi may be responsible for this difference.

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