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Pax3/7 genes reveal conservation and divergence in the arthropod segmentation hierarchy

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Abstract

Several features of Pax3/7 gene expression are shared among distantly related insects, including pair-rule, segment polarity, and neural patterns. Recent data from arachnids imply that roles in segmentation and neurogenesis are likely to be played by Pax3/7 genes in all arthropods. To further investigate Pax3/7 genes in non-insect arthropods, we isolated two monoclonal antibodies that recognize the products of Pax3/7 genes in a wide range of taxa, allowing us to quickly survey Pax3/7 expression in all four major arthropod groups. Epitope analysis reveals that these antibodies react to a small subset of Paired-class homeodomains, which includes the products of all known Pax3/7 genes. Using these antibodies, we find that Pax3/7 genes in crustaceans are expressed in an early broad and, in one case, dynamic domain followed by segmental stripes, while myriapods and chelicerates exhibit segmental stripes that form early in the posterior-most part of the germ band. This suggests that Pax3/7 genes acquired their role in segmentation deep within, or perhaps prior to, the arthropod lineage. However, we do not detect evidence of pair-rule patterning in either myriapods or chelicerates, suggesting that the early pair-rule expression pattern of Pax3/7 genes in insects may have been acquired within the crustacean–hexapod lineage.

Keywords: Pax; Arthropod; Crustacean; Centipede; Spider; Paired; Gooseberry; Segmentation; Pair-rule; Pattern formation; Evolution

Introduction

Although we know a great deal about the genetic hierarchy responsible for progressively subdividing the *Drosophila* embryo into its future body segments, we are only just beginning to understand its evolutionary origins. In particular, we know little about how and to what extent the hierarchy is used by other insects and non-insect arthropods. Such comparative data are not only a prerequisite to any meaningful speculation as to how, during its ontogeny, the ancestral arthropod might have formed segments, but are also interesting in light of the rather diverse embryology exhibited by extant arthropods.

In *Drosophila*, gradients of maternal information act at the top of a hierarchy involving the sequential activation of the zygotic gap, pair-rule, and segment polarity genes. While segment polarity genes are defined by their loss-of-function phenotypes in which pattern defects are repeated in adjacent segments of the embryonic cuticle, pair-rule genes are instead defined by deletions of the cuticle occurring with a two-segment periodicity (Nüsslein-Volhard and Wieschaus, 1980). In order to better understand how segmentation is controlled in other organisms, as well as gain insight into how the *Drosophila* segmentation hierarchy evolved, a number of studies have examined the expression of orthologs of *Drosophila* pair-rule and segment polarity genes in various arthropods.

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Consistent with their phenotypes, most segment polarity genes are expressed in *Drosophila* just before and throughout the morphologically segmented germ band stage in a segmentally reiterated pattern. The segment polarity genes *wingless* (*wg*) and *engrailed* (*en*) are each expressed as single ectodermal stripes within each individual segment such that every *wg* stripe lies adjacent and anterior to an *en* stripe. Each *wg* and *en* stripe demarcates the posterior and anterior limits, respectively, of adjacent units known as parasegments. Thus far, similar patterns of *wg* and *en* have been found in all four of the major arthropod groups: hexapods (including insects) (reviewed by Davis and Patel, 2002), crustaceans (Duman-Scheel et al., 2002; Manzanares et al., 1993; Nulsen and Nagy, 1999; Patel, 1994a; Patel et al., 1989b; Scholtz and Dohle, 1996), myriapods (millipedes and centipedes) (Chipman et al., 2004b; Hughes and Kaufman, 2002a; Janssen et al., 2004; Kettle et al., 2003), and chelicerates (spiders, mites, scorpions, and horseshoe crabs) (Damen, 2002; Telford and Thomas, 1998). In all cases, *wg* stripes lie adjacent and anterior to stripes of *en* and these observations, together with functional studies in the flour beetle (Oppenheimer et al., 1999), suggest that in all these groups the *wg*–*en* interaction, and hence the parasegment, is conserved.

Such extensive conservation has not yet been observed among pair-rule genes. Thus far, those that have been examined outside of *Drosophila* include *even-skipped* (*eve*), *fushi-tarazu* (*ftz*), *runt*, *hairy*, *odd-skipped* (*odd*), and *paired* (*prd*). In *Drosophila*, all six are expressed in the early blastoderm in stripes of a two-segment periodicity. Following gastrulation, *eve*, *runt*, *odd*, and *prd* are additionally expressed in stripes of a one-segment periodicity, coinciding temporally with the early expression of segment polarity genes.

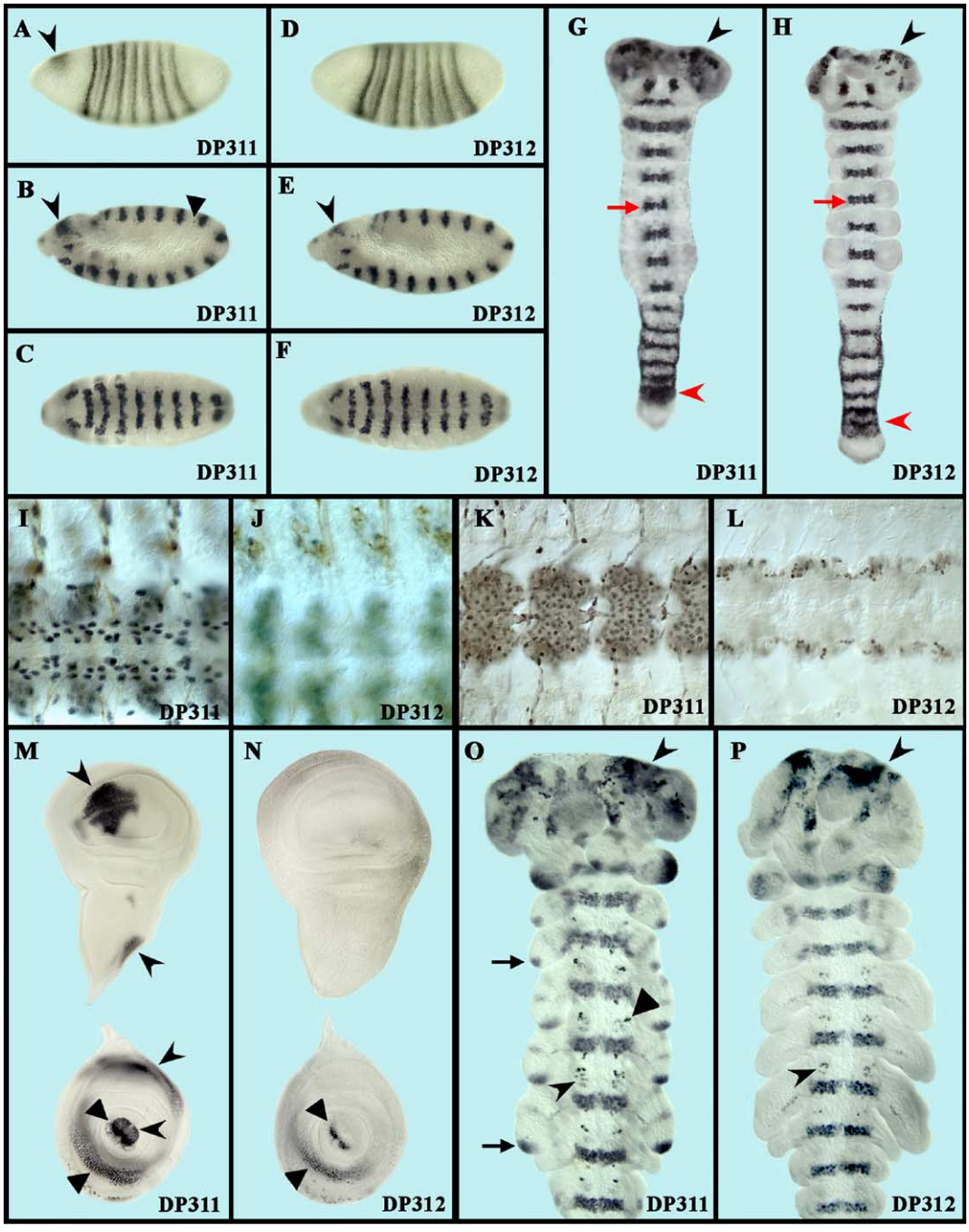
Among most holometabolous insects (including flies, moths, bees, and beetles), pair-rule genes are expressed in largely conserved patterns (reviewed by Davis and Patel, 2002). In the hemimetabolous grasshopper, however, presumed orthologs of *eve* and *ftz* are not expressed in periodic stripes in the embryo, but rather in broad posterior domains (Dawes et al., 1994; Patel et al., 1992). In general, however, it is non-insect arthropods that have furnished most of the more recent examples of divergent expression of pair-rule orthologs. For example, *ftz* expression has thus far been examined in the barnacle crustacean *Sacculina carcini* (Mouchel-Vielh et al., 2002), the centipede *Lithobius atkinsoni* (Hughes and Kaufman, 2002b), and the mite *Archegozetes longisetosus* (Telford, 2000). In contrast to insects, *ftz* in mites is expressed not in stripes, but in a Hox-like domain consistent with the position of this gene in the arthropod Hox cluster. In centipedes, *ftz* is expressed in a similar Hox-like pattern, as well as a posterior domain that gives rise to transient segmental stripes. Thus, with regard to *ftz*, there seems to have been an evolutionary transition from a Hox-like pattern to a striped pattern of expression (Hughes and Kaufman, 2002b). As yet, however, there is no convincing evidence of a pair-rule expression pattern for this gene outside of insects.

Additional non-insect data concern orthologs of the pair-rule genes *eve*, *runt*, and *hairy*. In amphipod crustaceans, all three genes are expressed in segmental stripes, but without any obvious pair-rule pattern (W. E. Brown, M. Gerberding, R. Parchem, N. H. Patel, unpublished data). In the brine shrimp, *Artemia franciscana*, *eve* stripes are present, but too transient to establish their periodicity with confidence (Copf et al., 2003). In the centipede *Lithobius*, *eve* is expressed in a broad posterior domain that subsequently resolves into stripes that persist transiently in newly formed segments (Hughes and Kaufman, 2002a). In the two-spotted spider mite, *Tetranychus urticae*, *runt* is expressed in segmental stripes (Dearden et al., 2002) and in the spider *Cupiennius salei*, *eve*, *runt*, and *hairy* are all expressed in stripes (Damen et al., 2000). In the case of the spider, *eve* and *runt* are both transiently expressed in stripes that arise in newly formed segments at the posterior, while *hairy* is expressed in a broad posterior domain that is periodically cleared, resulting in stripes. Importantly, it is not yet clear whether these stripes exhibit any sort of two-segment periodicity.

Fig. 1. DP311 and DP312 detect domains of Pax3/7 and non-Pax3/7 protein in *Drosophila* and *Schistocerca* embryos. Pax3/7 Patterns. DP311 and DP312 reveal Pax3/7 patterns in *Drosophila* (left half of figure: A–F, I–J, and M–N, anterior to left) and *Schistocerca* (right half of figure: G–H, anterior at top, K–L, anterior to left, and O–P, anterior at top). These include the pair-rule stripes found in *Drosophila* blastoderm embryos (due to Prd, A and D) and at the far posterior of *Schistocerca* germ bands (due to Pby1, red arrowheads in panels G–H), as well as the segment polarity stripes found in extended *Drosophila* germ bands (due to Gsb, B–C and E–F) and in the more mature segments of *Schistocerca* germ bands (due to Pby1 and Pby2, red arrows in panels G–H). Neural patterns due to Gsbn in *Drosophila* and Pby1 and Pby2 in *Schistocerca* are also detected by DP311 and DP312, but these patterns are largely out of focus in panels I–L, as these panels are intended to highlight the dorsal glial pattern recognized by DP311 but not DP312 (see below). DP311 and DP312 also detect a previously undescribed pattern that overlaps with Aristaless in *Drosophila* leg imaginal discs (black triangles in panels M–N) that is likely to be due *gsb* and *gsbn*, as both of these genes are expressed in leg imaginal discs (Carlos Estella and Richard Mann, personal communication). Non-Pax3/7 Patterns. Patterns detected by DP311 and not DP312 include: (1) An early anterior dorsal domain in *Drosophila* (black arrowhead in panel A, pattern absent in panel D) that is likely to be Homeobrain. (2) Stained glia in the CNS and PNS of *Drosophila* [black staining along dorsal surface of ventral nerve cord and along axon tracts (axon tracts stained brown) in panel I, pattern absent in panel J]. Note that panel J is in same focal plane as panel I. Out-of-focus staining (especially prominent in panel J) is due to Gsbn in more ventral neurons. This DP311-specific glial pattern is also seen in *Schistocerca* (dorsal glial pattern in panel K, absent in panel L), as well as younger embryos of both species as a pattern of glial precursors (black triangles in panels B and O, pattern absent in panels E and P). These glial patterns are likely to be Repo and/or CG2808. (3) A pattern in *Drosophila* 3rd instar wing and T3 leg imaginal discs (black arrowheads in panel M, pattern absent in panel N) and at the tips of gnathal palps and limb primordia in 35–37% *Schistocerca* embryos (black arrows in panel O, pattern absent in panel P). A complex pre-antennal pattern recognized by *both* DP311 and DP312 in *Drosophila* and *Schistocerca* likely includes Rx (black arrowheads in panels B, E, G–H, and O–P).

At present, clear molecular evidence for pair-rule patterning outside of holometabolous insects is limited to homologs of the gap gene *caudal* and the pair-rule genes *odd* and *prd*. In the centipede *Strigamia maritima*, an ortholog of *caudal* and a gene belonging to the *odd-skipped* family (*odr1*) are expressed in coincident, posterior stripes of a two-segment periodicity (Chipman et al., 2004a). *prd* belongs to a group known as the Pax3/7 genes (also known as Pax group III genes), which in *Drosophila* includes *prd*, as well as the segment polarity gene *gooseberry* (*gsb*) and *gooseberry-neuro* (*gsbn*), a gene that is expressed in the developing nervous system, but whose function has not yet been defined (reviewed by Noll, 1993). Together with their vertebrate homologs, *Pax-3* and *Pax-7*, the three genes belong to the Pax3/7 subgroup, one of four classically defined subgroups of the Pax family of transcription factors (Balczarek et al., 1997), whose members all possess both a paired domain (PD) and an extended S_{50} paired-type homeodomain (HD).

In the grasshopper *Schistocerca*, Pax3/7 genes are expressed in stripes of a two- and one-segment periodicity in grasshopper, suggesting that distantly related insects utilize these genes to pattern segments in both pair-rule and segment polarity fashion. In *Schistocerca*,



the pair-rule pattern is due solely to the Pax3/7 gene *pairberry1* (*Sa-pby1*), which is transiently expressed in stripes of a two-segment periodicity before resolving into a segmental pattern coincident with its paralog, *Sa-pby2* (Davis et al., 2001). In the spider *Cupiennius*, Pax3/7 genes are also expressed in coincident stripes of a one-segment periodicity, with stripes of *Cs-pby3* appearing early in the growth zone, prior to morphological segmentation (Schoppmeier and Damen, 2005). In contrast to the early Pax3/7 stripes in *Schistocerca*, however, these early Pax3/7 stripes in *Cupiennius* follow a one- rather than two-segment periodicity. In the spider mite *Tetranychus*, a Pax3/7 gene (*Tu-pax3/7*) is also expressed in stripes that appear in every segment. Intriguingly, however, *Tu-pax3/7* is expressed in prosomal stripes that exhibit a temporal pair-rule modulation: the appearance of stripes in segments of the 1st and 3rd walking legs are delayed relative to stripes in adjacent segments (Dearden et al., 2002).

How did the insect Pax3/7 expression pattern evolve? How far does the role that these genes apparently play in the segmentation of arachnids such as *Cupiennius* and *Tetranychus* extend to other chelicerates and other arthropods? When did Pax3/7 genes evolve a pair-rule mode of expression? To begin to answer these questions, we isolated monoclonal antibodies that recognize the products of Pax3/7 genes in a wide range of taxa, allowing us to investigate the expression of these genes in crustaceans, myriapods, and chelicerates. We report that, in these groups, Pax3/7 genes are expressed in patterns that are both similar to, and different from, the insect pattern. This suggests that while a role for Pax3/7 genes in segmentation is likely to be ancestral for arthropods, the details of this role have evolved within the arthropod lineage.

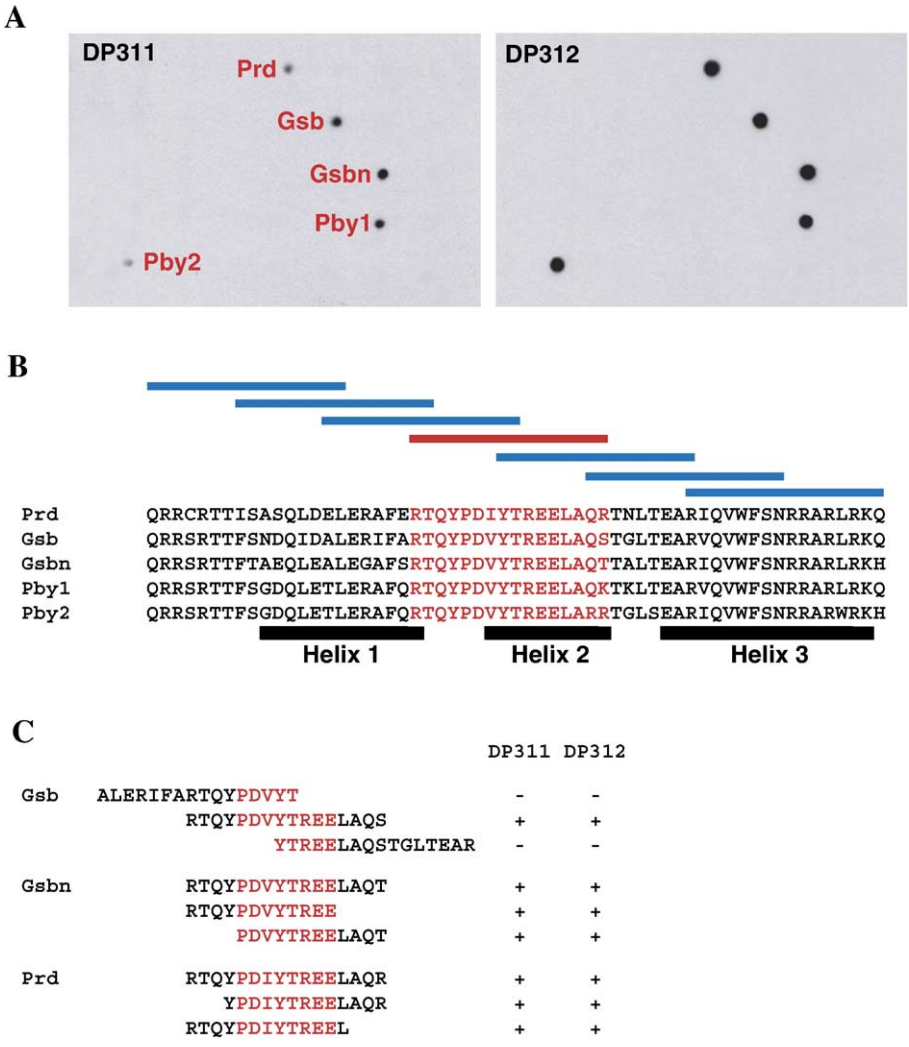


Fig. 2. Epitopes of DP311 and DP312 are represented by the same 8-amino acid stretch within the Pax3/7 homeodomain. (A) Exposed films of epitope-mapping array showing reactivity of DP311 and DP312 against overlapping 16-mer peptides derived from HDs of Prd, Gsb, and Gsbn from *Drosophila* and of Pby1 and Pby2 from *Schistocerca*. The MAbs did not show significant reactivity to other peptides on the array. (B) Schematic showing aligned HD sequences with screened 16-mer peptides indicated above. Data from panel A are shown as reactive peptides (red bars) and non-reactive peptides (blue bars). (C) More refined epitope mapping for DP311 and DP312 suggests that the core epitope for both MAbs lies within 8-amino acid stretch PD(V/I)YTREE (highlighted in red), which includes HD positions 26–33.

Materials and methods

Antibody production

Five balb-c male mice were each given intraperitoneal injections of ~100 μg of TrpE/full-length *Drosophila* Prd fusion protein, four times over a 6-week period, followed by 3 to 6 weeks of rest. During this time, test bleeds were screened for cross-reactivity by their ability to detect Gsb and Gsbn patterns in *Drosophila* embryos. One of three mice showing cross-reactivity was selected to receive a final boost consisting of 5–10 μg each of TrpE/*Drosophila* Prd, TrpE/*Drosophila* Gsb, and TrpE/*Drosophila* Gsbn fusion proteins. The portions of Gsb and Gsbn used in the boost included 164 amino acids of Gsb, containing both the octapeptide and the HD, and the C-terminal 291 amino acids of Gsbn, containing three amino acids of the octapeptide and all of the HD. We did not include the PD of Gsb and Gsbn since our previous attempts to produce cross-reactive antisera using the PD had failed. Hybridoma supernatants were screened for cross-reactivity on *Drosophila* embryos and positives re-screened on *Tribolium* and *Schistocerca* embryos (Davis et al., 2001). Two monoclonal antibodies (MAbs), DP311 and DP312, were successfully isolated.

Epitope mapping and characterization using peptide arrays

In order to map the epitopes of MAbs DP311 and DP312, peptide arrays were obtained from ResGen™ Invitrogen Corporation. Each array consisted of a polypropylene membrane on which discrete spots of peptides in 50 nM quantities were attached. Peptides were synthesized directly onto the membrane surface using standard Fmoc chemistry and covalently attached at their carboxy termini. Antibody binding analysis using DP11 and DP312 was performed on each array with either DP311 or DP312 at a dilution of 1:1000 in NGS-PTw, followed by detection with an HRP-linked goat anti-mouse IgG (Jackson ImmunoResearch Labs) at 1:40,000. Detection was by chemiluminescence with ECL (Amersham) and exposed film was quantified on a computing densitometer (Molecular Dynamics) using the Total Volume Integration feature in ImageQuant™ v. 3.2 (Molecular Dynamics). Arrays were stripped for re-use by immersion in 100 mM 2-mercaptoethanol/2% SDS/62.5 mM Tris–HCl pH 6.7 at 60°C for 30 min with occasional agitation. Stripping efficiency was confirmed by re-incubating with secondary antibody and reacting with ECL.

For the two epitope-characterization arrays (see Results), quantified signal intensities for each spot were averaged over multiple exposures for the two trials and these average values were expressed as a percentage or multiple of the least reactive Pax3/7 peptide on same array. Signal intensities >50% relative to the Pax3/7 reference peptide were judged not to differ significantly in light of observed variation in the amount of bound peptide and the differential effects of stripping. To further guard against the possibility of false negatives among peptides with single amino acid changes, changes that gave signal intensities <50% on Array #1 were re-analyzed on Array #2 and the lower value ignored. To confirm binding in the case of "representative peptides" (peptides based on naturally occurring paired class HDs from *Drosophila*, which contain multiple substitutions compared to Prd/Gsb/Gsbn), almost all that gave signal intensities >50% on Array #1 were re-analyzed on Array #2.

Reactivity of DP311 and DP312 to Single AA Changes

22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
R(K)	T	Q(H)	Y	P	D	V(I)	Y	T	R	E	E	L	A	Q(R/H)	T(S/R/K)
K G Q E A	A R N S	H R A K P	F		C	I A (22%, 11%) M (17%, 66%)	W N (25%, 40%) V (20%, 32%) S (16%, 28%) G (40%, 96%) F (266%, 12%)	F M V L A S C H			D R A M K Q L H	I V	S	R H M S V T L G I D A K	S R K W

Fig. 3. Single amino acid substitutions within the refined core epitope have both similar and differential effects on DP311 and DP312 reactivity. Chart shows the effects of single amino acid substitutions in the 16-mer epitope-containing stretch on the signal intensity produced by reacting with DP311 and DP312. The sequence from Gsbn is shown at the top of the chart along with HD position numbers; Pax3/7 variations on this Gsbn sequence are shown in parentheses. The refined epitope PD(V/I)YTREE is shown in larger font. Positions that are conserved among all paired class HDs are underlined. Tested substitutions are shown below the reference sequence with their resulting signal intensities indicated by highlights: green indicates functional substitutions that do not result in a significant decrease in signal intensity (>50%), red indicates nonfunctional substitutions that do result in a significant decrease in signal intensity (<50%), split green and red indicates substitutions that result in differential recognition by DP311 and DP312 (DP311 on left, DP312 on right). For nonfunctional and differential substitutions, the average signal intensity relative to the Pax3/7 reference peptide is shown in parentheses as (DP311, DP312). Note that positions 28 and 29 are critical to epitope function and are sensitive to variation found among paired class HDs. Note in particular the differential sensitivity of DP311 and DP312 to the change Y29F at position 29. For more details, see Supplementary Material 3.

Immunohistochemistry

Embryo staining was completed according to Patel et al. (1989b) and Patel (1994b), using DP311 and DP312 at a concentration of 1:20. In addition to staining with DP311 and DP312, staining was performed with MAbs 4D9 and 4F11 (anti-En, Patel et al., 1989b) and 22C10 (anti-Futsch, a protein expressed in the cell bodies and axons of all PNS cells and a subset of CNS cells in *Drosophila*, Fujita et al., 1982; Hummel et al., 2000). Further details of embryo procurement, fixation, and staining are available upon request.

Results

Two monoclonal antibodies cross-react to Pax3/7 proteins

Polyclonal antisera raised against conserved portions of the *Drosophila* Prd protein show reactivity not only to Prd, but also to the other *Drosophila* Pax3/7 gene products Gsb and Gsbn (Gutjahr et al., 1993a). When cross-reactive components of such antisera are purified and enriched, the resulting reagent also cross-reacts to Pax3/7 gene products in insects other than *Drosophila* (Davis et al., 2001). Because this enriched antisera did not work in non-insect arthropods, however, we attempted to raise similarly cross-reactive anti-Pax3/7 monoclonal antibodies (MAbs) in the hope that they would work reliably in a wider range of taxa, as well as provide a stable and permanent supply of cross-reactive reagent. First, we injected mice with *Drosophila* Prd and enriched for cross-reactivity by administering a final "cocktail" boost of Prd plus *Drosophila* Gsb and Gsbn. We then isolated two MAbs, DP311 and DP312, that cross-react to Prd, Gsb, and Gsbn in *Drosophila* (Figs. 1A–F), as well as the Pax3/7 proteins Pby1 and Pby2 in *Schistocerca* (Figs. 1G–H).

The MAb DP311 also reveals patterns in *Drosophila* that are not attributable to *prd*, *gsb*, or *gsbn*, representing additional cross-reactivity to non-Pax3/7 proteins. DP311, but not DP312, detects an early anterior dorsal domain (Figs. 1A, D, black arrowhead) that matches the expression pattern of the paired-like HD protein Homeobrain (Walldorf et al., 2000). In older embryos, DP311, but not DP312, stains the glia of the CNS and PNS (Figs. 1I–J). This glial pattern, which is also detected in *Schistocerca* by DP311 and not DP312 (Figs. 1K–L), is likely to be Repo and/or CG2808

Table 1
Reactivity of DP311 and DP312 to peptides representing Pax3/7 HDs and possible non-Pax3/7 paired class HD targets in *Drosophila*

Pax3/7 and possible non-Pax3/7 targets in *Drosophila*	Data from arrays (array #1/array #2)		Residue at position 29	Data from whole-mount immunostaining of embryos and imaginal discs (Fig. 1)
	DP311	DP312		
Pax3/7 (*Drosophila*, *Schistocerca*, chordate and cnidarian)	+/+	+/+	Y	Confirmed in *Drosophila* and *Schistocerca* (Figs. 1A–H). Also confirmed in zebrafish and *Ciona* (data not shown, Seo et al., 1998; Wada et al., 1996).
PHDP	+/+	+/+	Y	PHDP not expressed in embryos of *Drosophila melanogaster* (Lukacsovich et al., 1999).
Rx[a]	–/+	+/+	Y	Consistent with description (Eggert et al., 1998).
CG15782[b]	+/–	+/–	Y	Expression not yet described.
CG32532[b]	+/–	+/–	Y	Expression not yet described.
Aristaless	+/+	–/–	F	Confirmed (Figs. 1M–N, Schneitz et al., 1993).
Repo	+/+	–/–	F	Confirmed (Figs. 1B, E, I–L, and O–P, Halter et al., 1995).
CG31241	+/+	–/–	F	Expression not yet described.
Homeobrain	+/+	–/–	F	Confirmed (Figs. 1A, D, Walldorf et al., 2000)
OdsH[c]	+	–	F	OdsH not expressed in embryos of *Drosophila melanogaster* (Ting et al., 2004).
CG2808[b]	+/–	–/–	F	Consistent with description (Figs. 1I, K, Tomancak et al., 2002).
Pph13/Munster[b,d]	+/–	–/–	F	Not recognized (Goriely et al., 1999).
CG11294[b]	+/–	–/–	F	Expression not yet described.

+ and – under indicate >50% and <50% signal intensity, respectively, relative to signal intensity of the Pax3/7 peptide Gsbn. See Supplementary Material 4 for more details on array data. Bold designates strong support for being recognized by both DP311 and DP312 (upper half) or DP311 alone (lower half). Note that all peptides that are recognized by DP311 but not DP312 possess the substitution Y29F.

[a] Rx failed to show significant reactivity to DP311 on array #1 but was clearly recognized by both MAbs on array #2. In *Drosophila*, *Rx* is expressed in two dorsolateral spots of the procephalic region beginning at stage 9 and in cells of the presumptive clypeolabrum and developing CNS by germ band extension (Eggert et al., 1998). While this pattern is consistent with DP311 and DP312 staining, due to overlapping Pax3/7 patterns, we were unable to confirm that these MAbs recognize Rx in whole-mount tissue.

[b] These peptides gave significant signal intensities on array #1 (either for both DP311 and DP312 or for DP311 alone), but not on array #2. It is unclear whether these are due to false positives (array #1) or false negatives (array #2), except in the case of PpH13/Munster (see footnote d).

[c] OdsH was included only on array #1 and thus has not been confirmed. *OdsH* transcript has been detected in *D. melanogaster* testes (Ting et al., 2004); however, we did not observe staining in whole-mount testes using either DP311 or DP312 (data not shown).

[d] *Pph13/Munster* is expressed in the developing Bolwig organs, or larval eyes (Goriely et al., 1999). No such expression has been detected by immunostaining *Drosophila* embryos with DP311, suggesting that the observed recognition of Pph13/Munster by DP311 on array #1 either does not extend to whole-mount tissue or was a false positive.

(Halter et al., 1995; Tomancak et al., 2002). Consistent with this suggestion, glial precursors, which express *repo*, are detected by DP311 and not DP312 (Figs. 1B, O, black triangles). A pattern detected by DP311 in *Drosophila* wing and leg imaginal discs (Fig. 1M, black arrowheads) and at the tips of gnathal palps and limb primordia in *Schistocerca* embryos (Fig. 1O, black arrows), but not by DP312 (Figs. 1N, P), is likely to be Aristaless (Schneitz et al., 1993). Possible non-Pax3/7 patterns that are detected by *both* DP311 and DP312 include a complex pre-antennal pattern in both *Drosophila* and *Schistocerca* that likely includes Rx (Eggert et al., 1998). Because these non-Pax3/7 proteins are found in non-stripe patterns–mostly in the head or later in development–this additional cross-reactivity did not interfere with our analysis of the role of Pax3/7 genes in segmentation. It did, however, motivate us to delimit the range of proteins recognized by DP311 and DP312.

The core epitope of DP311 and DP312 is PD(V/I)YTREE

In order to delimit the proteins recognized by DP311 and DP312, an effort was made to define the epitopes to which these MAbs bind. Because the HD was the only conserved region from Gsb and Gsbn used in the final boost, it seemed likely that the HD would contain the epitopes of these cross-reactive MAbs. Because DP311 and DP312 reacted positively to Pax3/7 HDs on Western blots under denaturing conditions (data not shown), the epitopes were likely to be contiguous and independent of tertiary or complex secondary structure. We thus attempted to map and characterize the epitopes of DP311 and DP312 using peptide arrays.

We first tested DP311 and DP312 against an "epitope-mapping" array, which consisted of sets of overlapping 16-mer peptides that covered the extended HDs of *Drosophila* and *Schistocerca* Pax3/7 proteins (Supplementary Material 1). Reacting DP311 and DP312 against this array revealed a single reactive peptide for each Pax3/7 HD (Figs. 2A–B). This reactive peptide was the same for both DP311 and DP312, suggesting that the epitopes of the two MAbs were likely to overlap and might be identically positioned. In order to further delimit the epitope within this 16-amino acid stretch, two additional "epitope-characterization" arrays were designed (Supplementary Material 2). Using the epitope-containing 16-amino acid stretch of either Gsbn or Prd as a reference peptide, these arrays included 12-mer peptides with overlaps of a single residue and peptides truncated at either the N- or C-termini (Fig. 2C). These data show that the core epitope for both DP311 and DP312 lies within the 8-amino acid stretch PD(V/I)YTREE, which overlaps a large portion of helix 2.

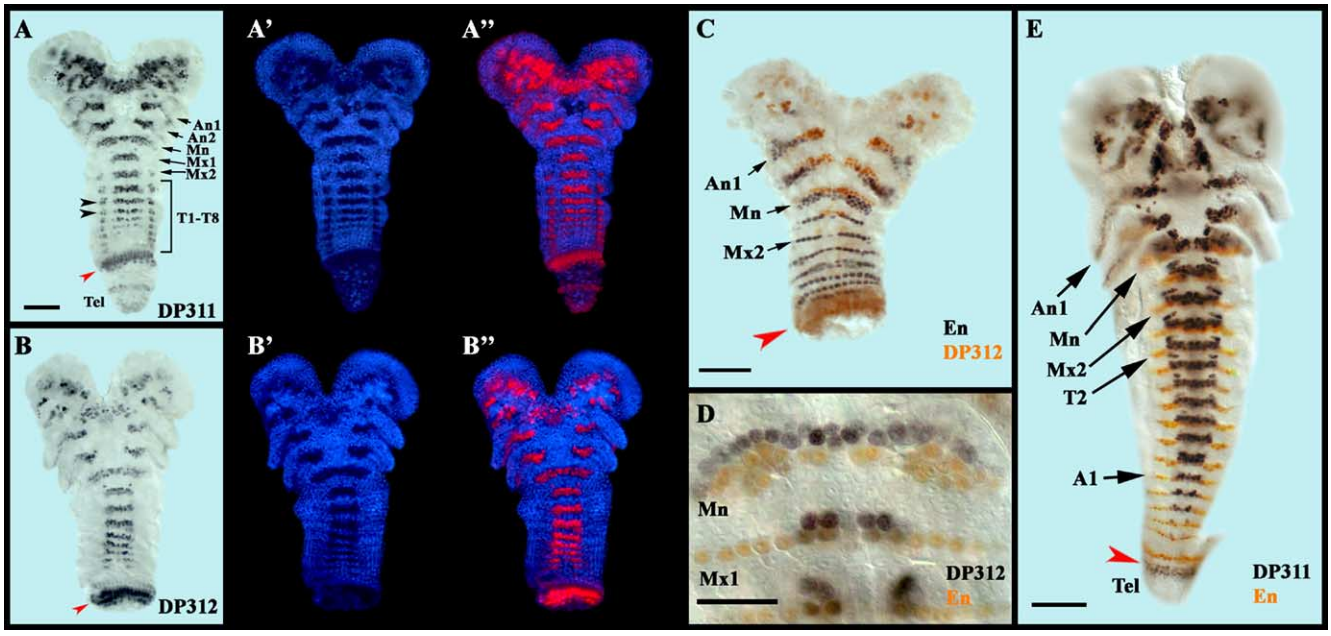


Fig. 4. Pax3/7 expression in *M. columbiae*. (A–B) Two mysid embryos stained with DP311 and DP312 are shown along with Dapi counterstains that reveal unlabeled nuclei (A′ and B′) and merged images in which the black stain is false-colored red (A″ and B″). Note that post-mandibular Pax3/7 stripes are laterally restricted to the neuroectoderm from the point of their initial appearance. Also note Pax3/7 expression in 3–4 cell rows of the posterior region, which includes the ectoteloblasts (red arrowheads, see also panels C and E). (C–E) Embryos double-labeled with either DP311 or DP312 and anti-En (MAb 4D9) reveal that, although Pax3/7 expression occurs earlier in the posterior region, the appearance of laterally restricted Pax3/7 stripes lags behind the appearance of En stripes (C). Beginning with the 1st maxillary segment (Mx1), Pax3/7 stripes appear sequentially and are restricted to two cell rows in the neuroectoderm (C and D), the more posterior of which overlaps with the adjacent En stripe (D). Non-Pax3/7 Patterns. Domains that may not be Pax3/7 protein are also detected, including pre-antennal domains of protein (A, C, and E) that are detected by both DP311 and DP312, as well as lateral ectodermal spots detected by DP311, but not DP312 (black arrowheads in panel A, pattern absent in panel B). Anterior is up and all views are ventral. Scale bars: panels A–B, C, and E, 100 μm; panel D, 50 μm.

DP311 and DP312 react differentially to amino acid substitutions within the core epitope

Additional portions of the epitope characterization arrays were designed to determine which naturally occurring variants among paired class HDs, including the HDs of all Pax transcription factors, constitute viable epitopes for DP311 and DP312. This was done by testing 16-mer peptides with single amino acid substitutions that represent almost all known natural variation among paired class HDs. In particular, substitutions were based on variation found among all paired class HD proteins known from *Drosophila* and all Pax3/7 proteins known in general (Supplementary Material 2).

Importantly, all the single amino acid variants found among known Pax3/7 proteins represent functional epitopes for both DP311 and DP312 (Fig. 3). This is consistent with the observation that both MAbs also bind to "representative peptides" (i.e., peptides based on naturally occurring paired class HDs, which contain multiple substitutions compared to Prd/Gsb/Gsbn) for all known Pax3/7 proteins, including those of chordates and the cnidarian Pax3/7 protein, PaxD (Table 1). That DP311 and DP312 indeed recognize chordate Pax3/7 proteins is confirmed by staining patterns in embryos of zebrafish and the urochordate *Ciona* (data not shown, Seo et al., 1998; Wada et al., 1996).

The antibodies are also able to recognize paired class HDs other than those of Pax3/7 proteins. While some non-Pax3/7 paired class HDs are recognized by both DP311 and DP312, others are recognized by DP311 and not DP312 (Table 1). A look at the effects of single amino acid substitutions suggests that the residues at positions 28 and 29 are critical for both the DP311 and DP312 epitopes (Fig. 3). Position 29 is not only critical for epitope function, but is the source of most of the differential cross-reactivity of DP311 and DP312 to paired class HDs other than Pax3/7. Most importantly, DP312 is not able to tolerate a substitution of Y with F at position 29. Although the change does not significantly decrease signal intensity for DP311 (instead it resulted in a 266% *increase* in signal relative to the control Pax3/7 peptide), for DP312 this change results in a >88% decrease in signal, representing more than a 22-fold

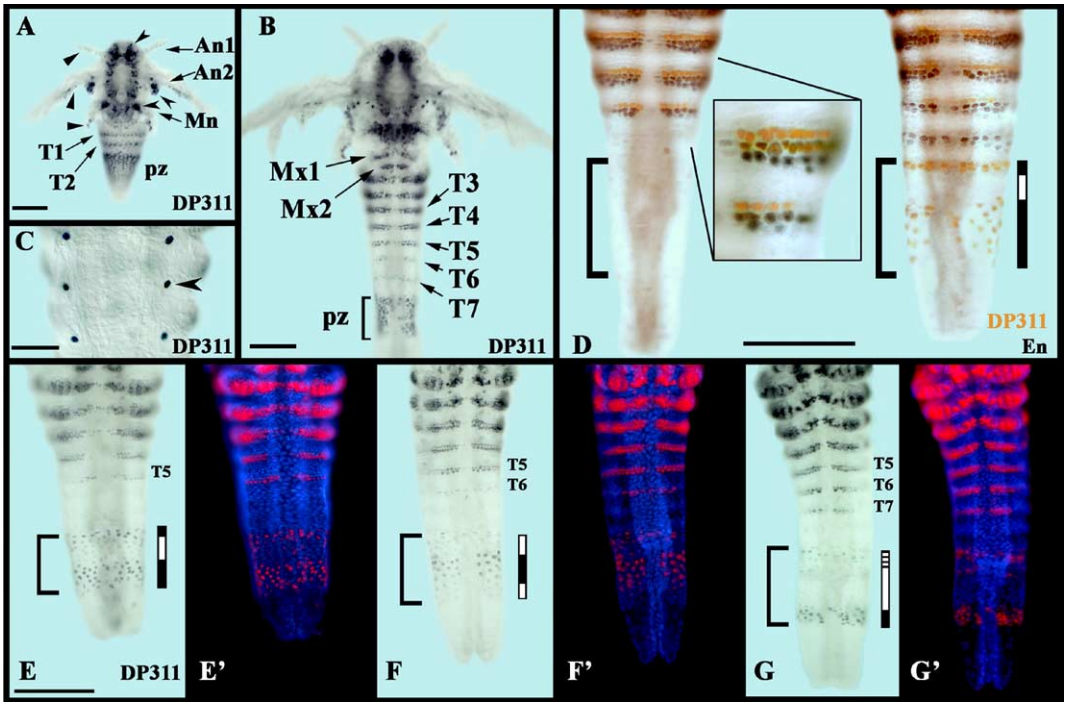


Fig. 5. Pax3/7 expression in *A. fransciscana*. (A–B) In young L1 larvae, Pax3/7 expression is found in the An1, An2, Mn, T1, and T2 segments, with clearly distinguished stripes in T1 and T2, as well as a gradient of expression in a posterior zone (A, pz). An older larva (L4) possesses Pax3/7 stripes of Mx1, Mx2, T1–5 and the first hints of staining in T6 and T7 (B). (D) Double labels of L4 larvae with DP311 and anti-En (4F11) reveal that Pax3/7 stripes are positioned just anterior to En stripes, initially as a single row of cells, but then expanding posteriorly into the En domain with an overlap of one cell row (high magnification inset). Stained larvae also reveal that expression in the posterior zone is very dynamic [compare posterior zones (brackets) of double-labeled larvae in which expression is absent in one case and bimodal in the other]. (E–G) A time series of L4 larvae stained with DP311 (with Dapi counterstains merged with black stain false-colored red) further illustrates the dynamic pattern: at times the posterior zone exhibits an anterior and posterior domain of expression in which expression is down-regulated in the center (E and E′), while a slightly older larva shows expression concentrated in the center (F and F′) and an even older larva shows a strong posterior and weak anterior domain (G and G′). Non-Pax3/7 Patterns. Possible non-Pax3/7 protein detected by DP311 (and DP312, not shown) include domains in the pre-antennal brain, the endites of the 2nd antennae and the base of the mandibules (black arrowheads in panel A), as well as isolated nuclei scattered along the length of the 1st antennae, the exopods of the 2nd antennae and the mandibles (black triangles in panel A). (C) Dorsal view of thorax shows unidentified DP311-labeled cells at T1–3 (black arrowhead points to T2). These cells are also labeled by DP312 (not shown) and the antigen is not known. Anterior is up and all views except for panel C are ventral. Scale bars: panels A, B, D, and E–G, 100 μm; panel C, 50 μm.

difference between the two MAbs. Together with its differential recognition by DP311 and DP312, the high frequency of this variant among paired class HDs accounts for most, if not all, of the observed differential cross-reactivity of the MAbs against the peptides representing non-Pax3/7 paired class HDs from *Drosophila* (Table 1).

Pax3/7 genes are expressed in a posterior domain and segmental stripes in crustaceans

In most malacostracan crustaceans, such as *Mysidium columbiae*, thoracic and abdominal segments form sequentially from a posterior row of ectodermal stem cells during embryogenesis. These ectoteloblasts are arranged in a crescent shape and undergo a series of longitudinal asymmetric divisions to generate a reiterated series of cell rows known as Roman numeral rows (Dohle, 1970; Dohle, 1976; Dohle and Scholtz, 1988). Each Roman numeral row (abcd) will then undergo two rounds of equal longitudinal divisions to eventually form four rows (first to two rows called a/b and c/d, then to a, b, c, and d). It was suggested that these Roman numeral row descendent groups might be the equivalents of insect parasegments (Dohle and Scholtz, 1988; Martinez-Arias and Lawrence, 1985), a claim that was verified by the discovery that *en* is expressed in the a row (Patel et al., 1989a; Scholtz and Dohle, 1996).

In *Mysidium* embryos, DP311 and DP312 reveal Pax3/7 expression both in a posterior domain and in segmentally iterated ectodermal stripes (Fig. 4). Prior (i.e., more posterior) to the expression of En, DP311 and DP312 detect a posterior domain of expression (Figs. 4A–C, E, red arrowheads). The domain consists of 3–4 cell rows and includes the ectoteloblast row, at least one nascent Roman numeral row, and a row posterior to the ectoteloblasts. Segmental stripes of Pax3/7, in contrast, appear only *after* (i.e., more anterior) to En stripes (Fig. 4C). Like insects, each Pax3/7 stripe lies anterior and adjacent to stripes of En, with some degree of overlap. The anterior two antennal "stripes" each consists of separate bilateral domains (Figs. 4A–C). The antennal and mandibular stripes appear at the same time, while the maxillary (2), thoracic (8), and abdominal (6) stripes appear sequentially. The thoracic and abdominal stripes initiate in row d cells and then expand posteriorly to occupy cells of rows d and a. The resulting overlap with adjacent En stripes in the a row indicates that Pax3/7 stripes span the parasegmental boundary, as in insects (Fig. 4D). Unlike insects, however, these more posterior stripes appear to be laterally restricted to the neuroectoderm from the point of their initial appearance (Figs. 4A–B, E).

In the branchiopod crustacean *Artemia fransciscana* (brine shrimp), the majority of segments are formed post-embryonically in the free-swimming nauplius larva. Upon hatching, the nauplius possesses only a few anterior segments, typically the 1st and 2nd antennal and mandibular segments. As the nauplius develops, the thoracic, genital, and post-genital segments are formed sequentially (the maxillary segments are formed only after the formation of more posterior thoracic segments) during a phase of extension from a reportedly cone-shaped posterior "growth zone" that does not contain teloblasts and lacks the highly ordered division patterns observed in malacostracans (Anderson, 1967). In *Artemia*, this post-embryonic segmentation process progressively generates the maxillary (2), thoracic (11), genital (2), and post-genital (6) segments over a period of about 2 weeks (Manzanares et al., 1993).

Like *Mysidium*, DP311 and DP312 in *Artemia* reveal Pax3/7 expression in both a posterior domain as well as segmentally iterated ectodermal stripes. In *Artemia*, however, this posterior domain is particularly dynamic. Newly hatched larvae show a gradient of expression in the posterior zone (Fig. 5A, pz), while older larvae exhibit both phases of very little expression in the posterior zone, as well as phases

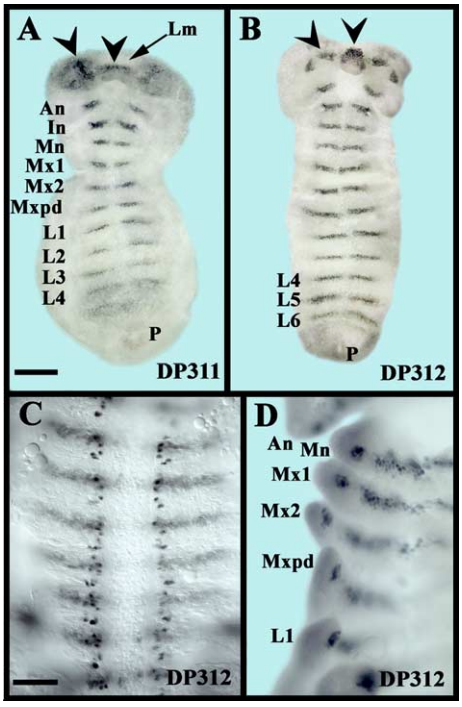


Fig. 6. Pax3/7 expression in the centipede, *L. atkinsoni*. (A–B) Incompletely segmented embryos stained with DP311 and DP312, showing ectodermal Pax3/7 stripes corresponding to the head and trunk segments. The stripes corresponding to leg-bearing segments 4 and 6 (L4 and L6) are just beginning to appear in panels A and B, respectively. Embryo in panel B still needs to form leg-bearing segments 7 and 8 before it hatches. Both MAbs detect pre-antennal, possibly non-Pax3/7, staining in the head, including the labrum (black arrowheads). Proctodeum is visible in the posterior. (C) Close-up dorsal view of fully segmented DP312-stained embryo focused on possible non-Pax3/7 pattern in the CNS with Pax3/7 stripes in a more ventral focal plane. (D) Close-up of fully segmented DP312-stained embryo showing Pax3/7 stripes, as well as domains detected at the ventral tip of appendage primordia, including the antennae, mandibles, maxillae, maxillopeds and legs. Lm = Labrum, An = antennal, In = intercalary, Mn = mandibular, Mx1 = maxillary1, Mx2 = maxillary2, Mxpd = maxilliped, L1–6 = leg-bearing segments 1–6, P = proctodeum. Anterior is up and all views are ventral except for panel C. Scale bars: panels A–B, 250 μm; panels C–D, 500 μm.

in which expression is limited to either the center or the anterior and posterior portions of this posterior region (Figs. 5D–G). In newly hatched larvae, DP311 and DP312 also detect staining associated with the 1st and 2nd antennal and mandibular segments, as well as two Pax3/7 stripes corresponding to the 1st and 2nd thoracic segments (Fig. 5A). Only after the appearance of these stripes are the more anterior maxillary stripes added, as is the case for *en* (Manzanares et al., 1993). The remaining posterior thoracic, genital, and abdominal stripes are progressively added as the larva grows (Figs. 5B, E–G). As in *Mysidium*, the stripes initially span a single cell row, but then expand to two cell rows, overlapping En in the more posterior row (Fig. 5D, high magnification inset). Except for the midline, the *Artemia* Pax3/7 stripes extend across the whole ventral surface, rather than being restricted to the neuroectoderm, as in *Mysidium*. Interestingly, *Artemia* stripes also differ from those of *Mysidium* in that they are less delayed relative to the onset of En stripes (Fig. 5D).

In both *Mysidium* and *Artemia*, likely non-Pax3/7 domains detected by DP311 and DP312 include large numbers of pre-antennal nuclei (Figs. 4A–B, C, E and 5A–B). This anterior pattern represents expressing cells of the developing brain and is likely due to the same antigen(s) responsible for a similar non-Pax3/7 pattern in insects (Figs. 1B, E, G–H, O–P). In *Mysidium*, DP311 detects lateral ectodermal spots beginning with the 2nd maxillary segment that are not detected by DP312 (Figs. 4A–B, black arrowheads). These spots appear to be associated with the development of limbs and it is possible that they are related to lateral ectodermal spots detected by DP311 (and not DP312) in the grasshopper abdomen (not shown). In *Artemia* larvae, possible non-Pax3/7 protein detected by DP311 (and DP312, not shown) include domains found in the endites of the 2nd antennae and at the base of the mandibles (Fig. 5A, black arrowheads), as well as isolated scattered nuclei along the length of the 1st antennae, the exopods of the 2nd antennae and the mandibles (Fig. 5A, black triangles) and these continue to express as the larva develops (Fig. 5B). Finally, in *Artemia*, an unidentified antigen is detected in one nucleus per hemisegment on the dorsal side of older larvae by DP311 (Fig. 5C) and DP312 (not shown).

Pax3/7 genes are expressed in segmental stripes in centipedes

Centipedes and millipedes make up the bulk of the class Myriapoda. During development, centipede embryos form their trunk (leg-bearing) segments successively (Chipman et al., 2004b; Hughes and Kaufman, 2002a; Kettle et al., 2003). In embryos of the lithobiomorph centipede *Lithobius atkinsoni*, DP311 and DP312 detect a pattern of segmentally iterated ectodermal stripes (Figs. 6A–B). These Pax3/7

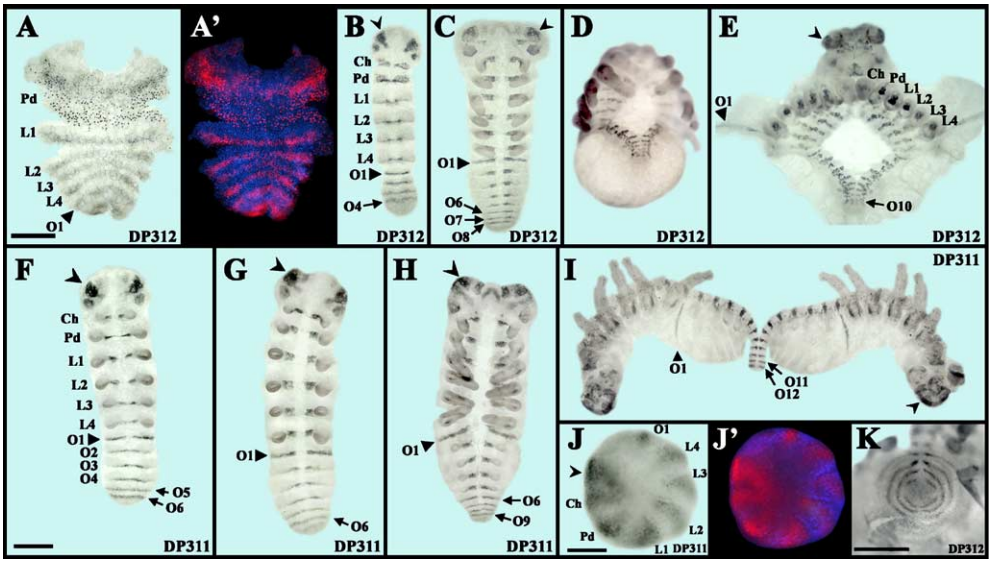


Fig. 7. Pax3/7 striped expression in chelicerates. (A–I) Increasingly older embryos of the spider *S. ocreata*, stained with DP312 (A–E) and the spider *C. salei*, stained with DP311 (F–I). Note the continuing dorsal extent of Pax3/7 stripe O1 (black triangles in panels B–C, E, and F–I). Possible non-Pax3/7 expression in bilateral clusters of nuclei in the far anterior of the head is detected by both DP311 and DP312 (black arrowheads in panels B–C, E, and F–I). Embryos in panels E and I have fully elongated and are in the process of "inversion", whereby a longitudinal furrow splits the germ band along the midline while the head and posterior remain joined. Embryo in panel D is the same as that of panel E, prior to being dissected off the yolk. In the case of panel I, the head has been manually split in order to mount the embryo. (J) Optical cross-section of an embryo of the two-spotted spider mite *T. urticae* stained with DP311, along with Dapi counterstain merged with black stain false-colored red. Pax3/7 stripes corresponding to Ch, Pd, L1–4, and O1 are apparent. The O2 Pax3/7 stripe is present, but in a different focal plane. (K) Embryo of the horseshoe crab *L. polyphemus* stained with DP312. Pax3/7 stripes originate as circumferential rings from the posterior. In addition to the stripe pattern, both DP311 and DP312 detect domains of expression in the pre-cheliceral CNS of the two spiders and the spider mite (black arrowheads in panels B–C and E–J). Ch = Cheliceral, Pd = pedipalpal, L1–L4 = leg-bearing segments, O1–O12 = opisthosomal/abdominal. For panels A–I, anterior is up; all views are ventral except for panel J, which is a cross-section, anterior to the left, ventral down, and panel K, which is a posterior view, ventral up. Scale bars: panels A–E and F–I, 500 μm; panel J, 100 μm; panel K, 250 μm.

stripes appear de novo in or close to the "growth zone" as the embryo elongates. Like insects and crustaceans, the stripes restrict ventrally somewhat relative to their initial, more circumferential, appearance, possibly representing restriction to the neuroectoderm. Although posterior stripes of two-segment periodicity have been reported for a gene belonging to the *odd-skipped* family, as well as a presumed ortholog of *caudal*, in the geophilomorph centipede, *Strigamia maritime* (Chipman et al., 2004a), we do not detect such a pair-rule pattern for Pax3/7 genes in *Lithobius*. In particular, we do not detect any obvious splitting or intercalation of secondary stripes that would be expected of a Pax3/7 pair-rule pattern; nor did we detect anything similar to the dynamic patterns that we observe in *Artemia*. The fact that our sample of embryos was limited, however, compels us to regard this result as provisional.

Possible non-Pax3/7 domains of expression were detected by both DP311 and DP312 in the pre-antennal region of the head, including the labrum (Figs. 6A–B, black arrowheads), as well as the CNS and ventral tips of appendage primordia in older embryos (Figs. 6C–D). The appendage domains are detected by both MAbs (DP311 data not shown) and hence are likely not to be Aristaless (which in *Drosophila* is detected only by DP311), and may even be Pax3/7 protein.

Pax3/7 genes are expressed in segmental stripes in chelicerates

The class Chelicerata includes horseshoe crabs and arachnids (spiders, scorpions, mites, etc.), and among these, spider development has received the most attention (for review, see Foelix, 1996). In the early germ band of spiders, segmental furrows delimit the five segments that bear the pedipalps and legs. These segments appear synchronously, just prior to formation of the cheliceral segment, which is derived from the cephalic lobe. The caudal lobe in turn splits to form the first opisthosomal (abdominal) segment and the germ band continues to add opisthosomal segments sequentially as it elongates.

In embryos of the spiders *Schizocosa ocreata* and *Cupiennius salei*, DP311 and DP312 detect a pattern of segmentally iterated ectodermal stripes (Figs. 7A–I), their order of appearance presaging the order of segmental furrows: the earliest Pax3/7 expression detectable in the *Schizocosa* germ band already exhibits Pax3/7 stripes of the pedipalpal and leg segments, with only the beginnings of the 1st opisthosomal stripe (Fig. 7A); as the spider embryo elongates, Pax3/7 stripes appear sequentially in, or close to, the posterior "growth zone" (Figs. 7B–I and 8). The stripes are initially broad and diffuse at the far posterior tip of the germ band, but soon narrow and strengthen as expression clears from the posterior. Like insects, the stripes are initially circumferential, but quickly restrict ventrally to the neuroectoderm (Figs. 7B–I and 8G–H). The only exception in this regard is the stripe of the 1st opisthosomal segment, which does not restrict, retaining its dorsal length late into embryogenesis (Figs. 7C, E, G–I and 9A–B, black triangles). The maintenance of a circumferential stripe in the 1st opisthosomal segment may indicate a role for this stripe in establishing the distinct border between the tagmata of the opisthosoma (abdominal segments) and the prosoma (head and leg segments).

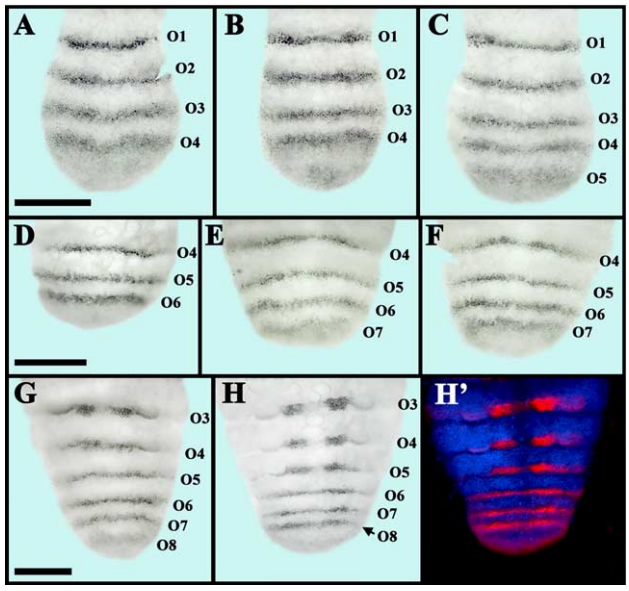


Fig. 8. Examples of the formation of individual Pax3/7 stripes at the posterior tip of elongating spider embryos. Shown are the development of the O4 and O5 stripes in *Schizocosa* (A–C), the O7 stripe in *Cupiennius* (D–F) and the O8 stripe in *Schizocosa* (G–H). Note that stripes first appear as broad diffuse domains at the posterior tip (O5 in panels B and C, O7 in panel E, and O8 in panel G), but narrow and strengthen as they mature (O7 in panel F, and O8 in panel H). The faintest appearance of O9 at the posterior tip can be detected in the Dapi counterstain merged with the black stain in panel H false-colored red (H′). Note the ventral restriction of more mature (i.e., more anterior) stripes (for example, O5 in panels G–H). As noted above, this restriction does not occur in O1. Scale bars: panels A–C, L–N, and O–P, 1 mm.

This description of Pax3/7 protein is consistent with the description of *pax3/7* mRNA in *Cupiennius*: dynamic expression of the Pax3/7 gene, *Cs-pby-3*, in the far posterior of the germ band is subsequently joined by the overlapping striped expression of the Pax3/7 genes, *Cs-pby-1* and *Cs-pby-2*, with the latter restricted to the developing neuroectoderm (Schoppmeier and Damen, 2005). Both *Cs-pby-1* and *Cs-pby-3* are responsible for the circumferential stripe of Pax3/7 protein observed in the 1st opisthosomal segment (Schoppmeier and Damen, 2005). By closely examining the formation of individual stripes in closely timed embryos, we concur with the description of *pax3/7* mRNA in *Cupiennius* by concluding that there is no difference in the way odd- versus even-numbered stripes of Pax3/7 protein form in either *Schizocosa* or *Cupiennius* (Fig. 8) and, like *Lithobius*, the formation of stripes does not appear to be associated with any sort of two-segment periodicity.

In addition to spiders, DP311 and DP312 were able to detect a segmentally repeated pattern of Pax3/7 stripes in

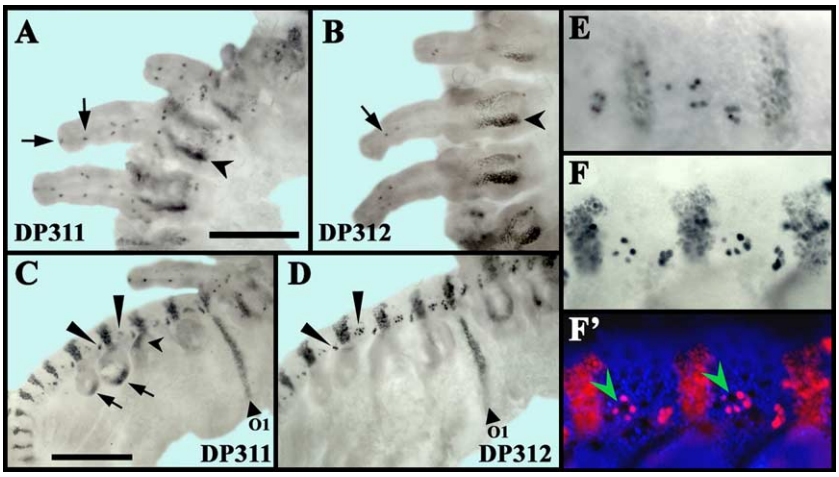


Fig. 9. Non-stripe staining with DP311 and DP312 in chelicerates. (A–B) The pedipalps and legs (L1–L2) of *Cupiennius* stained with DP311 and DP312. Note Pax3/7 protein at base of pedipalps and legs detected by both DP311 and DP312 (black arrowheads). Possible non-Pax3/7 staining is found in nuclei scattered along the length of developing appendages with both DP311 and DP312 (black arrows). Note that we detect more cells more distally with DP311 than with DP312. (C–D) One half of the developing opisthosoma of *Cupiennius* stained with DP311 and DP312. As in Fig. 7, note dorsal extent of Pax3/7 stripe O1 (black triangles). Possible non-Pax3/7 patterns detected by both DP311 and DP312 include two clusters of labeled nuclei of the CNS (long black triangles, see also panel F). Non-Pax3/7, possibly Aristaless, staining in the dorsal portions of spinneret primordia in O4 and O5 (black arrows) and tubular tracheae in O3 (black arrowhead) is detected by DP311 but not DP312. (E–F) High-magnification images of CNS staining in *Limulus* (E) and *Cupiennius* (F) with DP311 showing a similar pattern of two neural cell clusters per segment. Dapi counterstain in *Cupiennius* merged with black stain false-colored red reveals that the more posterior cluster surrounds an invagination from which neural cells arise (F′, green arrowheads). For panels A–D, anterior is towards the upper right; for panels E–F, anterior is to the right. All views are ventral. Scale bars: panels A–B and C–D, 1 mm.

embryos of the two-spotted spider mite *Tetranychus urticae* and the horseshoe crab *Limulus polyphemus*. We have not examined a sufficient number of embryos in either case to describe the process of stripe formation in detail. Our limited observations in *Tetranychus* (Fig. 7J), however, are consistent with the described expression pattern of the Pax3/7 gene, *Tu-pax3/7*, in embryos of this mite (Dearden et al., 2002). In the case of *Limulus*, our observations suggest that Pax3/7 stripes first appear as rings of expression at the far posterior, with a subsequent maintenance of expression in the ventral portion of each ring (Fig. 7K).

In addition to the pattern of ectodermal stripes, a domain of mesodermal Pax3/7 expression at the base of the pedipalps and legs in both species of spider–due to the *Cupiennius* Pax3/7 gene, *Cu-pby-2*–is detected by both DP311 and DP312 (Figs. 9A–B, black arrowheads) (Schoppmeier and Damen, 2005). Although Distal-less is expressed in all prosomal appendages, we have not detected Aristaless-like domains at the tips of developing limbs with DP311, as we have in insects. However, we do detect likely non-Pax3/7 protein in nuclei (possibly peripheral glia) scattered along the length of developing spider appendages with both DP311 and DP312 (Figs. 9A–B, black arrows), though some of these cells are likely to possess a DP311-specific antigen since we detect more cells more distally with DP311 than with DP312. Other–possibly non-Pax3/7–domains detected by both DP311 and DP312 in spiders include two bilateral clusters of labeled nuclei in the far anterior of the head (Figs. 7B–C, E, F–I, black arrowheads), as well as two clusters of nuclei in the developing CNS located between ectodermal Pax3/7 stripes (Figs. 9C–D, long black triangles). Aspects of the latter pattern are shared between *Limulus* (Fig. 9E) and *Cupiennius* (Fig. 9F). These clusters of cells within the CNS surround specific invaginations from which neural cells arise (Stollewerk et al., 2001), suggesting that these individual invaginations give rise to unique sets of neural cells, as is the case for insect neuroblasts. This association with one of the specific invaginations is best seen for the more posterior clusters (Fig. 9F, green arrowheads). Non-Pax3/7 staining detected by DP311 and not DP312 includes domains on the dorsal portion of the spinneret primordia (Figs. 9C–D, black arrows), as well as the ventral portion of the tubular tracheae (Figs. 9C–D, black arrowhead). While Aristaless is a candidate for these appendage domains, we have so far not detected DP311 staining in the book lung primordia, though such domains may appear at later stages.

Discussion

Cross-reactive antibodies, such as those against Engrailed/Invected (Patel et al., 1989b), the Distalless family (Panganiban et al., 1994), and Ubx/AbdA (Kelsh et al., 1994), have proved to be powerful tools in comparative molecular embryology. They allow rapid assessment of developmental patterning programs and help identify key organisms and genes for subsequent detailed molecular and functional studies. Here, we document the isolation of two monoclonal antibodies (MAbs) that cross-react to Pax3/7 gene products across a wide range of taxa.

Because the epitopes bound by cross-reactive antibodies are necessarily shared among orthologous proteins, it is likely

that such epitopes will also be found in paralogous or even more distantly related proteins. In order to define the extent of their cross-reactivity beyond their intended targets, we mapped and characterized the epitopes of our two anti-Pax3/7 MAbs and compared the *Drosophila* expression patterns that they detect to the known expression patterns of all potential targets. This allowed us to conclude that at early stages, the reiterated patterns seen in the trunk of the embryo are due to staining of known Pax3/7 family members (Prd, Gsb, and Gsbn). The MAbs were then used to investigate Pax3/7 expression in crustaceans, myriapods, and chelicerates in an effort to resolve when Pax3/7 genes evolved their multiple roles in arthropod segmentation.

Consistent with previous descriptions of *pax3/7* mRNA expression in arachnids (Dearden et al., 2002; Schoppmeier and Damen, 2005), the MAbs reveal segmental stripes of Pax3/7 protein that appear to straddle compartment boundaries in all arthropods examined, as well as reiterated neural patterns that are similar to the known patterns of *Drosophila* Gsb and Gsbn. Obvious pair-rule patterns, such as those observed in insects (Prd in *Drosophila* and Pby1 in *Schistocerca*, for example), were not observed. Based on these data, we suggest that the Pax3/7 role in segmentation predates the divergence of the four major arthropod lineages, but that pair-rule patterning for Pax3/7 within the trunk region appears to have evolved somewhere within the crustacean–hexapod lineage.

The monoclonal antibodies DP311 and DP312 recognize a subset of paired class homeodomain proteins, which includes the entire Pax3/7 subgroup

The staining patterns of the MAbs DP311 and DP312 in both *Drosophila* and *Schistocerca* embryos indicate that these MAbs cross-react to known Pax3/7 proteins in insects, as well as non-Pax3/7 gene products expressed in the head and later in the trunk nervous system and the appendages (Fig. 1). Peptide arrays confirm that both DP311 and DP312 react to the full range of known Pax3/7 proteins within the metazoa (Fig. 3 and Table 1).

The array data also identify particular non-Pax3/7 paired class HDs from *Drosophila* that are likely to be recognized by both DP311 and DP312, as well as those that are likely to be recognized by DP311 and not DP312 (Table 1). Although their epitopes appear to overlap almost entirely, if not perfectly (Fig. 2), differences between the two Mabs in non-Pax3/7 cross-reactivity are due mostly to the fact that DP311 is capable of binding to paired class HDs possessing a phenylalanine at position 29, while DP312 is not (Fig. 3). Many of the potential non-Pax3/7 targets were either confirmed or rejected based on our ability to detect them using whole-mount immunostaining, provided they have been reported to be expressed in either *Drosophila* embryos or imaginal discs in patterns that are not obscured by Pax3/7 staining (Fig. 1 and Table 1).

Taken together, the data suggest that both DP311 and DP312 allow visualization of the Pax3/7 patterns that are present in early embryos. Both MAbs also detect patterns in the head that include expression of genes such as Rx orthologs. DP311 will potentially detect additional patterns, such as the late patterns of glia in the nervous system (due to Repo and CG2808 orthologs) and the tips of appendages (Aristaless orthologs). As a cautionary note, however, it is worth noting that expression detected by a cross-reactive antibody is often best confirmed by the cloning of genes and use of species-specific probes, particularly when dealing with unexpected or divergent patterns.

The segment polarity role of Pax3/7 is conserved among arthropods

The Pax3/7 genes *prd* and *gsb* play pair-rule and segment polarity roles, respectively, during *Drosophila* embryogenesis. Prd protein is found in an early pair-rule pattern of seven primary stripes of a two-segment periodicity, which subsequently resolves into a segmental pattern of 14 secondary stripes (Gutjahr et al., 1993a). Gsb protein is found in 14 segmental stripes at the posterior of each parasegment, consistent with *gsb*'s role as a segment polarity gene (Gutjahr et al., 1993b). At the end of germ band extension, each Gsb stripe includes a *wg* stripe and extends across the parasegmental boundary 1–2 cell rows into the anterior portion of the En domain. Following germ band extension, most of the *gsb* stripes undergo restriction to the ventral neuroectoderm.

Consistent with its role as a pair-rule gene, *prd* is required for the activation of the odd-numbered stripes of *wg* and *en* (DiNardo and O'Farrell, 1987; Ingham and Hidalgo, 1993), as well as *gsb* (Baumgartner et al., 1987). *gsb* mutants show loss of naked cuticle and this phenotype appears to be mediated almost entirely by *wg*, which requires *gsb* for its maintenance after stage 11 (Li and Noll, 1993; Nüsslein-Volhard and Wieschaus, 1980). *gsb* is also required for the proper patterning of neuroblasts in rows 5 and 6, plus the most medial neuroblast of row 7 (Duman-Scheel et al., 1997), as well as the subsequent expression of *gsbn* in a subset of the ganglion mother cell and neuronal progeny of the *gsb*-expressing neuroblasts (Gutjahr et al., 1993b). Interestingly, the Prd, Gsb, and Gsbn proteins appear to be interchangeable with regard to patterning the embryonic cuticle and nervous system; differences in the roles played by *prd* and *gsb* in segmentation (as well as *gsbn* in the developing nervous system) appear to derive solely from their different cis-regulatory systems and their resulting differential expression (Li and Noll, 1994; Xue and Noll, 1996).

The non-insect arthropods examined here (two crustaceans, one centipede, and several chelicerates) all exhibit a conserved pattern of segmental Pax3/7 stripes throughout the head and trunk, which appears prior to morphological segmentation. In the crustaceans examined, each of these

stripes is positioned anterior and adjacent to a stripe of En, with a slight overlap (Figs. 4 and 5). This is likely to be the case for the centipede and spiders as well, based on descriptions of *en* expression in these organisms (Damen, 2002; Hughes and Kaufman, 2002a). It is also likely that, in all three groups, the anterior border of each Pax3/7 stripe coincides with the anterior border of a *wg/wnt* stripe, based on descriptions of *wg/wnt* expression in crustaceans (Duman-Scheel et al., 2002; Nulsen and Nagy, 1999), centipedes (Hughes and Kaufman, 2002a), and spiders (Damen, 2002).

Although functional inferences based on expression data must be made with caution, these data suggest that a Pax3/7 role in segmentation was acquired deep within, or prior to, the arthropod lineage. Beyond this basic description, however, some details of Pax3/7 segment polarity patterns vary, even among crustaceans. Although the staining in the ectoteloblasts of *Mysidium*, for example, might be regarded as a circumferential pattern that extends across the entire ectoderm, these embryos lack the distinct circumferential stripes observed in all other arthropods examined. This may suggest that mysids have lost the Pax3/7 role in segmentation most analogous to the early circumferential stripes of *gsb* in *Drosophila*, while retaining the earlier Pax3/7 role in segmentation analogous to *prd* plus the later Pax3/7 role in the developing nervous system analogous to late *gsb* and *gsbn*.

The pair-rule patterning role of Pax3/7 genes is derived within arthropods

In the extending germ bands of both flour beetle and grasshopper, broad Pax3/7 stripes of a two-segment periodicity split to form segmental stripes (Davis et al., 2001). This suggests that a Pax3/7 role in pair-rule patterning may be conserved among insects. In contrast, the myriapod and chelicerates we examined do not exhibit obvious hints of a two-segment periodicity such as alternating broad domains that split or the intercalation of secondary stripes. In spiders in particular, we find no differences in the Pax3/7 expression dynamics associated with the formation of both odd- and even-numbered segments (Fig. 8), consistent with previous descriptions of *pax3/7* mRNA in the spider *Cupiennius* (Schoppmeier and Damen, 2005). The Pax3/7 genes of both crustaceans examined, however, do display more complicated dynamic expression in broad posterior domains, well before the appearance of En stripes (Figs. 4 and 5). Although it is tempting to homologize these early patterns to the pair-rule stripes of insects, in neither *Mysidium* nor *Artemia* is the pattern obviously pair-rule. Based on these data, we venture that a pair-rule patterning role for Pax3/7 genes is likely to have evolved within the mandibulates (crustaceans + hexapods) and possibly within insects.

This is not to say that basal arthropods do not utilize pair-rule patterning at all; rather, several lines of evidence suggest they do. The Pax3/7 gene *Tu-pax3/7* from the mite *Tetranychus* is expressed in segmental stripes that display a temporal pair-rule modulation in the head (Dearden et al., 2002), and this may reflect an underlying pair-rule mechanism, as is the case with several segment polarity genes in *Drosophila* (Davis and Patel, 2003; Dearden et al., 2002). In embryos of the geophilomorph centipede *Stigmia*, the expression of an *odd*-related gene, *odr1*, provides more direct evidence of pair-rule patterning: *odr1* stripes appear transiently in alternating segments (Chipman et al., 2004a). This pattern is highly suggestive of a pair-rule mechanism of sorts and helps to explain the fact that centipedes always possess odd numbers of segments (Arthur and Farrow, 1999; Chipman et al., 2004a; Minelli and Bortoletto, 1988).

In addition to its pair-rule striped pattern, *odr1* in *Stigmia* also exhibits a dynamic broad domain at the far posterior of the germ band, where it appears to cycle with a two-segment periodicity (Chipman et al., 2004a). Rather than the static pair-rule patterning observed in *Drosophila*, the dynamic *odr1* pattern appears more reminiscent of the cycling of several genes in the presomitic mesoderm of vertebrates, raising the possibility that pair-rule patterning mechanisms have evolved independently in the centipede and insect lineages (Chipman et al., 2004a; Damen, 2004). Here, we detect a dynamic Pax3/7 pattern in the posterior zone of *Artemia* larvae (Figs. 5D–G). In contrast to *odr1* in *Stigmia*, however, there is no continuity of expression between the domains of the posterior zone and the subsequent formation of Pax3/7 stripes. Nevertheless, this and similar dynamic patterns detected for both *caudal* and *eve* in the posterior of *Artemia* (Copf et al., 2003) may play a role in the formation of segments. A more detailed examination of these expression dynamics, along with functional studies, ought to clarify the role these genes play in the formation of segments in *Artemia*.

Concluding remarks

A picture of the evolution of arthropod segmentation is emerging. It asserts the ancestry and stasis of the segment polarity level of the *Drosophila* segmentation hierarchy while hinting at an evolved diversity of the mechanisms used to establish it. The results presented here support this general view, as the segment polarity aspects of Pax3/7 expression appear to be more widely conserved than the pair-rule aspects.

While future comparative work should continue to refine our picture of the evolution of Pax3/7 genes, this task should be eased considerably by the cross-reactive MAbs DP311 and DP312, which possess several advantages over MAbs 4D9 and 4F11, which have been widely used in comparative analyses of *en* expression. These include the ability to simultaneously assay both the segment polarity and pair-rule levels of the segmentation hierarchy, more intense and robust staining (particularly

as a second label following in situ hybridization), epitopes that are better characterized, and cross-reactivity over a broader range of taxa.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.06.014.

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