Evolution of Developmental Control Mechanisms

Comprehensive analysis of Hox gene expression in the amphipod crustacean Parhyale hawaiensis

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A R T I C L E   I N F O

Article history:
Received 6 September 2015
Received in revised form 25 October 2015
Accepted 25 October 2015
Available online 10 November 2015

A B S T R A C T

Hox genes play crucial roles in establishing regional identity along the anterior–posterior axis in bilaterian animals, and have been implicated in generating morphological diversity throughout evolution. Here we report the identification, expression, and initial genomic characterization of the complete set of Hox genes from the amphipod crustacean Parhyale hawaiensis. Parhyale is an emerging model system that is amenable to experimental manipulations and evolutionary comparisons among the arthropods. Our analyses indicate that the Parhyale genome contains a single copy of each canonical Hox gene with the exception of fushi tarazu, and preliminary mapping suggests that at least some of these genes are clustered together in the genome. With few exceptions, Parhyale Hox genes exhibit both temporal and spatial colinearity, and expression boundaries correlate with morphological differences between segments and their associated appendages. This work represents the most comprehensive analysis of Hox gene expression in a crustacean to date, and provides a foundation for functional studies aimed at elucidating the role of Hox genes in arthropod development and evolution.

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1. Introduction

Few developmental gene families have been studied as extensively as the Hox genes, which encode homeodomain-containing transcription factors that determine regional identity along the anterior–posterior (AP) axis in bilaterian animals (Pourquié, 2009). Hox genes are typically organized within the genome in conserved clusters that display spatial colinearity—that is, their position along the chromosome correlates with the positions of their expression domains along the AP axis. Perturbation of Hox gene expression is associated with homeotic transformations, wherein body parts in one region of an animal are transformed to more closely resemble those of another region. Given the importance of Hox genes in establishing regional and segmental identity, it is not surprising that numerous lines of molecular, genetic, and developmental evidence have implicated Hox genes in generating morphological diversity during animal evolution.

Arthropods have proven to be especially well suited for studying the contribution of Hox genes to evolutionary changes in morphology (reviewed in Angelini and Kaufman, 2005; Hughes and Kaufman, 2002a). Arthropods have clearly segmented body plans, and each segment typically possesses a single, often unique, pair of appendages and other characteristics that provide useful markers for segmental identity. Furthermore, homologous segments between related arthropod species often display significant differences in appendage morphology. This allows one to ask questions about how differences in Hox gene expression, and the response of downstream target genes, generate segmental and appendage diversity, both within a single organism as well as between different species. Analyses of Hox gene expression in various arthropod lineages have led to a number of intriguing hypotheses linking observed shifts in Hox expression domains to specific morphological differences (Abzhanov and Kaufman, 1999a, 2000a; Averof and Patel, 1997; Hughes and Kaufman, 2002a; Hughes et al., 2004; Mahfooz et al., 2004; Peterson et al., 1999; Rogers et al., 1997). As a member of a major lineage, Malacostraca, within the Pancrustacean clade (Misof et al., 2014; Regier et al., 2010), which comprises all crustaceans and hexapods, Parhyale is well positioned as both an
outgroup of insects and as a reference crustacean for evolutionary
developmental studies of the diversity of arthropod bauplans (Rehm et al., 2009a).

The Parhyale body plan is fairly typical of the crustacean order
Amphipoda. From anterior to posterior, the head segments include
a pre-antennal segment (which lacks paired appendages), the first
antennal (An1), second antennal (An2), mandibular (Mn), first
maxillary (Mx1), and second maxillary (Mx2) segments. The
thorax is comprised of eight segments, T1 through T8. The T1
appendages are maxillipeds, which, along with the mandible and
the two maxillae, facilitate feeding. The gnathopods on T2 and T3
have a distinctive distal claw; the T3 claw grows especially large in
adult males. Segments T4 through T8 contain walking legs. The
walking legs on T4 and T5 are oriented anteriorly, while those on
T6 through T8 are larger, have thicker bristles, and are oriented
posteriorly (the name of the order, “Amphipoda”, derives from
having these two distinctly different walking legs. The abdomen
consists of six segments, A1 through A6, each bearing a pair of
biparous appendages. The pleopods (swimmerets) of A1 through
A3 are indistinguishable from one another, whereas the uropods
(anchor legs) of A4 through A6 significantly decrease in size from
anterior-to-posterior.

As a first step toward a comprehensive analysis of Hox gene
function and evolution in crustaceans, we have cloned and ex-
amined the expression patterns for the entire Parhyale Hox gene
suite. We have identified a single copy of each canonical Hox gene
with the exception of fushi tarazu (ftz). Our preliminary BAC data
indicate that many, if not all, Parhyale Hox genes are clustered in
the genome. Expression analyses demonstrate that, with a couple
of exceptions, they exhibit both temporal and spatial colinearity.
Many of the Hox expression boundaries coincide with obvious
morphological differences between appendage types and/or tag-
mata. The work presented here forms the foundation for future
studies aimed at examining the role that Hox genes play in crus-
tacean development and arthropod evolution.

2. Materials and methods

2.1. Cloning Parhyale Hox genes

Parhyale embryos of mixed ages were collected to make cDNA as
previously described (Price and Patel, 2008). We used a variety
of degenerate PCR primers targeting either the homeodomain or
hexapaptide regions of Hox genes. The forward primers we ini-
tially used were 5’-YTIGARYTNGARAAAGATT-3’, 5’-ACIT-
TAYACNCNGTAYCARAC-3’, 5’-ACICGTACRYCARACNTNGA-3’,
5’-CARACITYIGARYTNGARAA-3’, 5’-ATITAYCNCNTGATGMGN-3’,
5’-ATHTAYCNCNTGATGMGN-3’, 5’-ATITAYCNCNTGATGMGN-3’,
5’-CARATHTAYCNCNTGATGMGN-3’, and reverse primers were 5’-
CRRYTGYRAACCNATYTT-3’ and 5’-CATWCKWCRRTYTG-
GRAACCA-3’. We found the best way to be most useful for isolating
the broadest possible range of Hox genes involved creating a series
of less-degenerate primers targeting the highly conserved LELEKEF
and KIWFQNR motifs in the Hox homeodomain. Four LELEKEF
forward primers (5’-YTIGARYTNGARAAAGATT-3’, 5’-YTNGAR-
TYNGARAAAGATT-3’, 5’-YTNGARYTNGARAAAGATT-3’, 5’-YTNG-
ARYTNGARAAAGATT-3’) were used in every possible combina-
tion with four KIWFQNR reverse primers (5’-CRRYTGY-
GRAACCNATYTT-3’, 5’-CRRYTGYGRAACCNATYTT-3’, 5’-CRRYTGY-
GRAACCNATYTT-3’, 5’-CRRYTGYGRAACCNATYTT-3’). Because
the 3’-most nucleotides of each primer are not degenerate, PCR
amplifications tended to be specific for certain Hox genes but not
others—this circumvented the problem of repeatedly re-isolating
the same few (perhaps mostly high abundant) Hox genes. Each
primer was used at a final concentration of 5 μM in 50 μl PCR
reactions that included 2.5 units of Taq DNA Polymerase (New
England BioLabs), 1 x Thermopol buffer (New England BioLabs),
1.5 mM MgCl₂, 0.2 mM dNTPs, and embryonic cDNA. Forty cycles
of amplification were carried out at a melting temperature of
94 °C, a “step-down” annealing temperature starting at 63 °C and
decreasing 1 °C per cycle to 52 °C, and an extension temperature of
72 °C. PCR products were run on a 2% agarose gel, 120 nt fragments
were isolated via low melting point agarose and directly cloned
into pBluescript II (Stratagene). Miniprep DNA was directly se-
quenced. In some cases, colonies were pre-screened by PCR using
oligos specific for previously isolated Parhyale Hox genes in order
to identify those clones that would most likely represent novel
Hox genes.

5’ and 3’ flanking sequences for each identified Hox gene were
isolated using the GeneRacer (Invitrogen) and/or GenomeWalker
(Clontech Laboratories) kits. When possible, primers were de-
signed to the 5’- and 3’ most sequences of the untranslated regions
(UTRs) for each Hox gene and used to PCR-amplify full-length
cDNAs. Accession numbers for Parhyale Hox cDNA sequences are
as follows: labial, JQ952576; proboscipedia, JQ952587; Hox3,
JQ945948; Deformed, JQ952571; Sex comb reduced, JQ952579;
Antennapedia variant I, JQ952581; Antennapedia variant II, JQ952582;
Ultrabithorax isoform I, FJ628448; Ultrabithorax isoform II,
FJ628449; abdominal-A isoform I, Q952572; abdominal-A isoform
II, JQ952573; Abdominal-B isoform I, JQ952574; Abdominal-B iso-
form II, JQ952575.

2.2. BAC library screening

Parhyale BAC screening using radiolabelled Hox probes was
conducted according to (Parchem et al., 2010). Inverse PCR was
employed to generate sequence data for the 5’ and 3’ ends for
many of the Hox-positive BAC clones. In some cases, inverse PCR-
derived probes were used to re-screen the Parhyale BAC library.
Miniprep DNA from 96 unique Hox-positive BACs was spotted
onto “dot-blots,” and each blot was hybridized to probes derived
either from portions of Hox cDNAs and from the 5’ and 3’ ends
of specific BAC clones. This dot-blot data was used to construct
a rough map of the Parhyale Hox complex(es), and this map was
refined and verified using BAC sequencing data. Accession num-
bers for the Parhyale Hox BAC sequences are as follows: PA31-H15,
KR869963; PA24-C06, KR869964; PA93-L04, KR869965; PA264-
B19, KR869966; PA179-K23, KR869967; PA24-C06, KR869964; PA93-
L04, KR869965; PA264-B19, KR869966; PA179-K23, KR869967; PA24-
C06, KR869964; PA93-L04, KR869965; PA264-B19, KR869966; PA179-
K23, KR869967; PA24-C06, KR869964; PA93-L04, KR869965; PA264-
B19, KR869966; PA179-K23, KR869967; PA24-C06, KR869964; PA93-

2.3. In situ hybridization and imaging

Embryo dissection, fixation, and in situ hybridization were
conducted according to Rehm et al. (2009b,2009c) and Vargas-
Vila et al. (2010). Images were analyzed on a Zeiss Axioshot micro-
scope, captured in color with a Diagnostic Instruments Spot Camera,
and figures assembled using Adobe Photoshop. False-
color overlays of in situ hybridization images were carried out as
follows: The DAPI signal was photographed and shifted to cyan by
adjusting the hue to −35. A bright field image of the histochem-
ical staining (from the BCIP/NBT/alkaline phosphatase reaction;
Roche) was inverted, and the blue and green channels were
simultaneously.
2.5. CRISPR/Cas9-mediated homologous recombination

We synthesized a sgRNA targeting the codons 7–14 of Antp following the procedure of (Bassett and Liu, 2014). To generate a donor plasmid for homologous recombination around the cleavage site, we cloned a genomic DNA fragment of 2.1 kb approximately centered on plasmid for homologous recombination around the cleavage site, we cloned a genomic DNA fragment of 2.1 kb approximately centered on plasmid for homologous recombination around the cleavage site. We sequenced the homeobox sequences from all of the canonical Hox genes with the exception of ftz (see Supplemental Fig. 1). In subsequent cloning, we were able to isolate full-length cDNAs for la-bial (lab), Deformed (Dfd), Ultrabithorax (Ubx), abdominal-A (abd-A) and AbdB (Abd-B), and the entire open reading frame for Sex combs reduced (Scr). For proboscipedia (pb) and Antennapedia (Antp), we obtained cDNA sequence 5’ to 3’ to the homeobox, but not a full-length cDNA, and for Hox3 we only isolated sequence 3’ to the homeobox. Our analyses suggest that Parhyale has a single copy of each of these Hox genes. Multiple alternatively spliced variants for Ubx (Liubicich et al., 2009), abd-A, and Abd-B were identified; for each gene, these alternatively spliced versions are referred to as isoform-1 and isoform-2. For Antp, two classes of cDNA differing from one another at three distinct polymorphic regions within the coding region were identified. Since these polymorphic variants (alleles) encode slightly different proteins, we refer to them here as Antp variant I and Antp variant II. We also found that a subset of transcripts transcribed from the Ubx promoter(s) are spliced to exons from the Antp transcription unit, indicating that transcriptional read-through occurs between the two genes. Similar Ubx-Antp read-through transcripts have been reported for five other crustaceans, two myriapods, and an onychophoran (an outgroup to arthropods), suggesting that this may be a relatively ancient feature of arthropod Hox complexes (Brena et al., 2005; Jansen and Budd, 2010; Shiga et al., 2006).

To determine whether Parhyale Hox genes are organized into clusters, we screened a Parhyale BAC library (Parchem et al., 2010) using probes specific for each Hox gene. Sequences from the ends of each positive BAC clone were also used as templates for probes to both re-screen the BAC library and to map individual BACs in relation to one another. Once sequenced, we were able to confirm our BAC map and precisely define the overlap between BACs (Fig. 1). As shown in Fig. 1, one set of BACs together span four linked Hox genes (5’-Ubx, Antp, Scr, Dfd-3’) and a second spans two linked Hox genes (5’-pb, lab-3’). In both cases, the Hox genes are organized in a linear, 5’-to-3’ orientation consistent with what has been reported for vertebrate and other Hox clusters. BAC clones corresponding to the three remaining Hox genes (Abd-B, Abd-A and Hox3) were also isolated, but we were unable to determine whether they are located near or adjacent to each other and/or the aforementioned Hox mini-clusters. Given that Parhyale has a relatively large genome size (Parchem et al., 2010), additional genomic data will be required to determine whether all nine Hox genes.

3. Results

3.1. Isolation and genomic mapping of Parhyale Hox genes

We used a comprehensive degenerate PCR strategy to identify the entire set of Parhyale Hox genes. This approach resulted in the isolation of the homeobox sequences from all of the canonical Hox genes with the exception of ftz (see Supplemental Fig. 1). In subsequent cloning, we were able to isolate full-length cDNAs for labial (lab), Deformed (Dfd), Ultrabithorax (Ubx), abdominal-A (abd-A) and AbdB (Abd-B), and the entire open reading frame for Sex combs reduced (Scr). For proboscipedia (pb) and Antennapedia (Antp), we obtained cDNA sequence 5’ to 3’ to the homeobox, but not a full-length cDNA, and for Hox3 we only isolated sequence 3’ to the homeobox. Our analyses suggest that Parhyale has a single copy of each of these Hox genes. Multiple alternatively spliced variants for Ubx (Liubicich et al., 2009), abd-A, and Abd-B were identified; for each gene, these alternatively spliced versions are referred to as isoform-1 and isoform-2. For Antp, two classes of cDNA differing from one another at three distinct polymorphic regions within the coding region were identified. Since these polymorphic variants (alleles) encode slightly different proteins, we refer to them here as Antp variant I and Antp variant II. We also found that a subset of transcripts transcribed from the Ubx promoter(s) are spliced to exons from the Antp transcription unit, indicating that transcriptional read-through occurs between the two genes. Similar Ubx-Antp read-through transcripts have been reported for five other crustaceans, two myriapods, and an onychophoran (an outgroup to arthropods), suggesting that this may be a relatively ancient feature of arthropod Hox complexes (Brena et al., 2005; Jansen and Budd, 2010; Shiga et al., 2006).

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genes are organized within a single, large genomic cluster.

3.2. Hox gene expression during Parhyale embryogenesis

To gain insight into the potential function of Parhyale Hox genes in patterning and segmental identity, we examined their embryonic expression patterns via in situ hybridization. We started by examining the expression of all nine Hox genes at stage 22–24, a time when all segments are clearly visible and the different appendage types have taken on their unique morphologies (Fig. 2). Each Hox gene is expressed in a unique, but often overlapping, domain along the anterior–posterior (AP) axis. Hox genes generally display spatial colinearity, with the relative domains of expression along the embryonic AP axis aligned to the relative genomic position of the individual genes within the complex. This spatial colinearity appears conserved in Parhyale, at least within the limits of our preliminary data on clustering: lab, pb, Hox3 and Dfd expression is restricted to head segments, Scr and Antp span the head and thoracic segments, Ubx is expressed predominantly in the thorax, abd-A spans the thoracic and abdominal segments, and Abd-B is restricted to the abdomen (Fig. 2).

For each Hox gene, we then analyzed its expression through all stages of development (see below). The stages of Parhyale embryogenesis discussed here have been previously described (Browne et al., 2005). The germ band forms shortly after gastrulation, when most of the cells in the embryo migrate to the anterior end and condense to form the early germ disc. Over time, ectodermal cells organize themselves into a germ band composed of an orderly grid beginning in the posterior part of the head. We refer to the first row that forms as “row 1,” and the row that eventually forms just anterior to it as “row 0.” Rows 0 and 1 together appear to comprise the future parasegment 1, which ultimately gives rise to the posterior of the Mn and the anterior of Mx1. Each transverse cell row that forms posterior to row 1 will give rise to a single parasegment, and is thus termed a parasegmental precursor row (PSPR). In succession, PSPR 2 (the cell row immediately posterior to row 1) through PSPR 16 (which gives rise to the posterior-most parasegment) undergo two rounds of mitotic division that proceed in waves from medial to lateral and from anterior to posterior. The first mitotic wave (FMW) results in two rows of progeny cells: a/b and c/d. These cells divide again during the second mitotic wave (SMW) to yield four rows of cells (a, b, c, d).

We also employed two additional markers to assist in assigning the precise boundaries of Hox gene expression relative to segmentation and appendage development: engrailed (en) and Distal-less (Dll). Engrailed expression can be detected in the “a” cell of each parasegment (Browne et al., 2005; Patel et al., 1989). As in other arthropods, engrailed expression marks the anterior boundary of each parasegment and the posterior boundary of each morphological segment. Cells anterior to parasegment 1 (e.g., those that primarily give rise to the An1, An2, and the anterior majority of Mn) do not seem to organize into precise rows, nor do they exhibit regular division patterns as far as we have observed. Individual segments begin to express Distal-less-early (Dll-e) shortly after the onset of engrailed expression and, for most segments, Dll-e expression is initially restricted to a subset of the daughters of the “d” row, but then expands to include more anterior row progeny that contribute to the developing appendages (Browne et al., 2005; Liubicich et al., 2009). By stage 15, developing limb buds are evident in An1, An2 and Mn, and by stages 23–24 (just prior to cuticle secretion and, as such, the latest stages we can assay by in situ hybridization) a full complement of limbs are present and readily distinguished from one another (Fig. 2).

3.3. Head-specific Hox genes (lab, pb, Hox3, and Dfd)

The first Hox genes to be expressed during Parhyale embryogenesis are lab and Dfd. Beginning at stage 8, they are co-expressed in a single, imperfectly aligned row of cells (row 1) prior to grid formation (Fig. 3A–A’). After the onset of lab and Dfd expression, the ectodermal cells that will form PSPR 2 begin to organize just posterior to row 1 (see progression in Fig. 3A’–D’). Around this same time, cells that will become PSPR 0, just anterior to row 1, begin to weakly express lab (Fig. 3B and B’). As lab expression levels increase in these cells, they decrease within row 1 (Fig. 3C and C’). By the time that the FMW is initiated in PSPR 2, row 1 typically lacks any detectable lab expression—the sole exception to this is the midline cell of row 1, which sometimes remains lab-positive during the initial progression of the FMW (Fig. 3D and D’).

When the lab and Dfd expression domains first become distinct from one another, Dfd is strongly expressed in row 1 and lab is strongly expressed in two anterior patches on either side of the midline (Fig. 3C and E). There is a one- to two-cell wide (in the A–P direction) region that is composed of cells that express both lab
and Dfd. This co-expression region persists as the exclusively lab and Dfd expression domains on either side of it begin to expand (Fig. 3D and E). We observe very few cell divisions in this region during this period (based on fixed DAPI preparations and preliminary time-lapse movies), suggesting that the expansion might not be simply due to proliferation of the initial lab and Dfd expressing cells. This conjecture could be tested in the future with live imaging of GFP tagged versions of these genes.

With the onset of en1 expression (when it becomes possible to clearly demarcate parasegmental boundaries), it is clear that Dfd expression occurs throughout parasegments 0 and 1 (Fig. 4A and B). Segmentally, this corresponds to the very posterior of the An2 segment (in the ventral body region, but not the appendages), the entire Mn segment, and most of M × 1 (with the exception of the en1-expressing cells at the very posterior of this segment). By stage 19, as the head appendages are extending, Dfd expression in the Mn segment is strongest in the ventral-most region of the segment (where the paragnaths will form) and in the distal tip of the extending mandible, whereas Dfd expression throughout the anterior of M × 1 is more uniform (Fig. 4C). At stage 21, this pattern remains relatively unchanged, but there is additional expression in the abdomen, specifically, in the distal regions of the A1-3 appendages (Fig. 4D). By stage 24, Dfd expression is localized to the two bimodal branches of each developing pleopod (swimmeret) (Fig. 4E). For lab, during most of germ band elongation, expression levels in An2 are stronger in the lateral regions of the segment, where limb buds will eventually form, than in the ventral region (Fig. 4F). Expression of lab can be observed throughout the An2 appendages as they grow, and is also detected at the posterior base of the labrum (Fig. 4G).

The third head-specific Hox gene to be expressed during Palaemonidae embryogenesis is Hox3. Its transcripts are first detected around stage 12 in two patches in the head lobes just anterior to the Dfd expression domain (Fig. 4H). These Hox3 domains appear to be predominantly in the posterior half of the future An2 segment, although we also observe Hox3-positive cells throughout and immediately posterior to the An2 engrailed stripe (Fig. 4I). Around stage 17, there is a significant decrease in Hox3 expression anterior to the An2 engrailed stripe (Fig. 4K), such that, by stage 20, Hox3 is only found in posterior and medial (with regards to the proximal-distal axis) regions of the developing mandibles (Fig. 4J). By stage 24, Hox3 expression becomes localized to a small patch of mesodermal cells in the mandible (Fig. 2).

Compared to the other three head Hox genes, pb comes on relatively late in development. Transcripts are first observed around stage 17 at the anterior base of the second antennae (not shown). This expression domain then extends posteriorly as embryogenesis progresses, such that, by stage 19, pb transcripts can be found at the ventral-most base of antenna 2 (Fig. 4L). This staining is still observed through stages 22–24, at which point additional pb staining can be detected at the ventral, anterior-most region of the paragnaths of the Mn segment (Figs. 2 and 4M).

3.4. Hox genes spanning the head and thorax (Scr and Antp)

Two Hox genes span head and thoracic segments: Scr and Antp. Scr is first expressed around stage 12 in the PSPR 2 cells as they undergo their first round of division to produce the a/b and c/d
Fig. 4. Dfd, lab, Hox3 and pb in situ hybridization of stage 14–24 embryos. Anterior is at top. A, C–H, and L–M are DAPI stained with in situ patterns shown in red or yellow; B and I–K are brightfield images with in situ hybridization patterns in purple and red. In panels A, B, F and I–J, en1 is used as a marker for the posterior boundary of each segment. (A) Dfd (red) and en1 (yellow) expression in a stage 14 embryo. Arrow marks Mx1 en1 stripe. (B) Dfd (purple) and en1 (red) expression in a stage 17 embryo; arrow marks Mx1 en1 stripe. In this embryo, Dfd transcripts are observed throughout parasegments 0 and 1, which corresponds to the posterior of An2 through the anterior of M + 1. (C) Dfd (red) expression in a stage 19 embryo. Arrowhead, Mn segment; arrow, M + 1 segment. (D–E) Dfd (red) expression in the developing pleopods during stages 21 and 24, respectively. Arrowheads mark the developing A3 pleopod. (F) lab (red) and en1 (yellow) expression in a stage 16 embryo; arrowhead marks Mn en1 stripe. (G) lab (red) expression in An2 (arrow) of a stage 19 embryo. (H) Hox3 (red) and Dfd (yellow) expression in a stage 12 embryo. (I) Close up of Hox3 (purple) and en1 (red) expression in a stage 13 embryo; arrowhead marks Mn en1 stripe. Hox3 expression is observed in the posterior half of the An2 segment and the anterior-most cells of the Mn segment. (J) Hox3 expression (purple) is restricted to the developing mandibles in a stage 17 embryo. Expression of en1 shown in red; arrowhead marks Mn en1 stripe. (K) Hox3 (purple) and en1 (red) expression in a stage 20 embryo; arrowhead marks Mn en1 stripe. (L) pb (red) and Dfd (yellow) expression in a stage 19 embryo. Pb is restricted to the base of the An2 appendages (arrow). (M) pb expression (red) at the base of the An2 appendages (arrow) and in the paragnaths of a stage 22 embryo. Scale bars 100 μm in all panels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
daughter rows (Fig. 5A). By stage 15, Scr expression expands anteriorly into parasegment 1 and posteriorly into the a and b cells of parasegment 3 (Fig. 5B and C). Together, these cells will give rise to Mx1 through the anterior region of the first thoracic segment (T1). It should be noted that Scr expression levels tend to be weaker in Mx1 than in Mx2 throughout development (Figs. 5C, D and 2). During stage 19, as the limb buds begin elongating, Scr expression appears more intense in the most distal and proximal regions of the maxillipeds, with only weak or no expression in the medial region, whereas expression throughout the maxillary appendages appear more uniform (Fig. 5D). By stage 23, Scr expression is present in both maxillary segments and their appendages, as well as in the T1 appendages (maxillipeds), but not the T1 ventral body wall (Fig. 2).

Antp is initially expressed during stage 12 in PSPR 3 cells as they undergo their first division to give rise to a/b and c/d daughter cells (Fig. 6A). The expression boundaries of Antp then expand in both the anterior and posterior directions. Anteriorly, in the second parasegment, the c/d cells begin to express Antp at stage 13; these cells will eventually be a part of the Mx2 segment (Fig. 6B). At stage 15, the Antp expression domain extends from the 2c and d rows through at least parasegment 7 (Fig. 6C). By stage 17–18, Antp expression can be found in the very posterior, ventral region of Mx1, throughout all of Mx2 and the developing thoracic segments, and at lower levels in the most ventral (neurogenic) region of the abdominal ectoderm (Fig. 6D). Antp expression in Mx2 appears weaker in general than Antp expression in the thoracic segments. This expression pattern persists through at least stage 24, the latest time at which we can carry out in situ hybridization of whole mount embryos, and the late abdominal Antp expression is restricted to the nervous system (Figs. 2 and 6E).

The description provided above for Antp transcript distribution in Parhyale is based on in situ hybridization data, but relating this to where Antp functions presents an unusual problem in crustaceans. In at least five previously studies crustacean species, a Ubx-Antp bi-cistronic transcript is produced, presumably the result of transcriptional read-through from the Ubx promoter (Shiga et al., 2006). In the case of Daphnia and Artemia, these bi-cistronic transcripts are incapable of generating functional Antp protein (Shiga et al., 2006). Thus, in situ probes for Antp actually detect the combined expression domains of Antp plus Ubx. In the case of Daphnia and Artemia, it was shown that Antp protein was not produced by the bi-cistronic transcripts, and indeed Antp protein was produced in a domain that did not overlap with Ubx.

Parhyale also produces Ubx-Antp bi-cistronic transcripts. Ubx transcript and protein in Parhyale are expressed at moderate levels in T2 and T3, and higher levels from T4-T8 (Liubicich et al., 2009; also summarized below). Therefore, it seemed possible that Antp transcripts produced from the Antp promoter—i.e., those that make functional Antp protein—are not expressed in the more posterior thoracic segments and appendages, and that the staining observed in this region is solely due to our Antp probe hybridizing to Ubx-Antp hybrid transcripts. To test this possibility, we used two approaches to detect the distribution of Antp protein. First, we used a previously characterized pair of monoclonal antibodies (MAb 8C11 and MAb 4C3) that have been shown to detect Antp protein across various arthropods (Condie et al., 1991; Hayward et al., 1995; Saenko et al., 2011). Immunostaining reveals that Antp protein is initially broadly expressed in the neurogenic region from Mx2 on posterior, but within the developing appendages, expression is not detected posterior to T3 (Fig. 6F and G). At later stages, the discrepancy between the in situ results and antibody staining is even more marked (compare Fig. 6E and H). Antp protein is detected...
throughout the entirety of the T1–T3 limbs, but restricted to just a small subset of what appear to be neurons and muscle within the developing limbs in T4–T8 (Fig. 6H). Within the nervous system, expression is detected throughout the entire CNS starting at M×2 (Fig. 6H). Thus, there is overlap of Ubx and Antp protein expression in the CNS, but within the limbs, the two overlap in T2 and T3, but in the remaining thoracic segments, Ubx is expressed throughout the appendages, but Antp is restricted to a small subset of cells (Fig. 6H’ and H”).

To further confirm the restriction of Antp to the anterior part of the thorax, we used a CRISPR/Cas9 homology mediated knock-in strategy to create embryos in which GFP is inserted in frame with the Parhyale Antp coding sequence (see Section 2 and Fig. 7A). This approach yielded GFP-expressing G0 embryos that confirmed the results seen with the 8C11 and 4C3 monoclonal antibodies. Especially striking were late stage embryos (stage 24 and beyond that are stages in which we cannot carry out routine in situ or immunohistochemistry due to the cuticle) where we see GFP thoracic appendage expression restricted T1–T3 (Fig. 7B and C). Thus, we are able to conclude that, as in Daphnia and Artemia, Antp protein does not appear to be expressed from the

![Fig. 6. Antp in situ hybridization and immunolocalization. (A) Close up of a stage 12 embryo with Antp transcripts accumulating in rows a/b and c/d. (B) Antp expression expands anteriorly into 2 c/d by stage 13. (C) Stage 15 embryo displaying Antp expression from parasegments 2 through 8. (D) Stage 19 embryo. The most anterior domain of expression is in the CNS in the posterior portion of the Mx1 segment. Expression is rather uniform throughout M×2 and the entire thorax (thoracic segments posterior to T5 are not visible in this view due to the flexure of the embryo at this stage). Within the abdomen, Antp transcripts are expressed in primarily in the neurogenic region. (E) Stage 24 embryo with Scr in red and Antp in purple. At this stage, Antp expression in the head is largely confined to the CNS, and it is detectable in the T1 appendage where it overlaps with Scr expression. (F–G) Immunodetection of Antp proteins with the 8C11 monoclonal antibody suggests post-transcriptional repression in the T4–T8 appendages. (F) Extended focus view of a stage 20 embryos shows expression throughout the thorax in the nervous system and body wall, but (G) optical sections through the developing limbs reveals that Antp protein is not detected in the T4–T8 appendages. (H–H”) At stage 24, neuronal expression of Antp (red) is seen from the posterior of the head through to the end of the abdomen. In the appendages and body wall, however, widespread expression is seen in T2 and T3, but in the remaining thoracic appendages, expression is restricted to a small subset of neurons and mesodermal cells. Ubx protein (green) is detected from T2 through T8. Scale bar 20 μm in panel A, 100 μm in panels B–H”.

![Fig. 7. GFP reporter of Antp via CRISPR/Cas9 genome editing. (A) Diagram of the strategy for somatic fluorescent tagging of Antp. A CRISPR gRNA targets the 5′ region of the Antp coding sequence (yellow) and was co-injected with a Cas9 recombinant protein at the one-cell stage. A donor plasmid was provided as a repair template for homologous recombination, generating alleles that incorporate eGFP and the T2A ribosome-skipping site to the N-terminus of the Antp protein. Secondary cleavage of repaired alleles is avoided by a degenerate protospacer-adjacent motif (PAM) sequence (blue). (B, C) Live fluorescent imaging of an eGFP-positive G0 CRISPR-induced somatic transgenic animal at stage 27. Note the strong signal in T2-T3 limbs, while little signal is seen in the remaining more posterior thoracic limbs. Scale bar 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)]
bi-cistronic transcripts in Parhyale, and Antp and Ubx overlap less than expected from the in situ data.

3.5. Thoracic Hox gene (Ubx)

We previously published a detailed account of Ubx transcript and protein expression during Parhyale embryogenesis (Liubicich et al., 2009). By stage 23, when Ubx expression reaches its full fruition, it is expressed at lower levels in the gnathopods of T2 and T3, and more robustly in the walking legs of T4 through T8 (Figs. 2, 6H, and 8D). There is transient expression of Ubx transcripts in the neurogenic region of the abdomen at stages 17–18, which then resolves to a small subset of neuronal cells before ceasing expression in the abdomen.

3.6. Abdominal Hox genes (abd-A and Abd-B)

The Hox genes that are expressed in the Parhyale abdomen are abd-A and Abd-B. In Parhyale, we first detected abd-A transcripts at stage 17 in the a/b and c/d cells of parasegments 12 and 13—these cells will eventually give rise to the posterior of A1 through the anterior of A3 (Fig. 8A). At stage 18, we also observe weaker levels of abd-A in parasegments 8 through 11, which will go on to form the very posterior of T5 through the anterior of A1. As the limb buds start protruding during stage 19, Abd-A is expressed strongly in A1-3, more weakly in T6-8, and it is just starting to come on in A4 (Fig. 8B). During stages 21-22, abd-A expression appears in the T6-8 appendages, but is absent from the distal most tips, whereas the strong A1-3 and very weak A4 expression appears more homogeneously throughout the limbs (Fig. 8C). By stage 24, we consistently observe abd-A expression in the ventral ectoderm from the posterior of T5 through the anterior of A3, and in the appendages of T6 through A3, with very weak staining in the limbs of A4, and no staining in A5, A6 or the telson (Figs. 2 and 8D).

Abd-B is first expressed at stage 17–18 in the b, c, and d cells of parasegment 11, and in the a/b and c/d cells of parasegment 12 (Fig. 8E). By stage 19, Abd-B expression remains absent from the T8 en1 stripe, but is present in all of the other cells of parasegment 11, plus the developing parasegments 12, 13 and 14, which together give rise to the anterior of A1 through the anterior of A4 (Fig. 8F). As the germ band continues to expand, Abd-B is expressed in all of the developing abdominal segments and appendages (Fig. 8G). Finally, at stage 24, Abd-B is rather homogeneously expressed throughout the ventral ectoderm from the very posterior of T8 through A6, and in the developing limbs of A1 through A6, but is excluded from the developing telson (Fig. 2).

4. Discussion

Previous studies have examined the expression of subsets of crustacean Hox genes, most notably in the branchiopods Artemia and Daphnia, the maxillopod Sacculina, and in the malacostracans Porcellio, Procambarus and Asellus (Abzhanov and Kaufman, 1999a, 1999b, 2000a, 2000b; Averof and Akam, 1995; Blin et al., 2003; Brenner et al., 2005; Copf et al., 2003; Mouche-Vielh et al., 2002; Papillon and Telford, 2007; Shiga et al., 2002; Vick and Blum, 2010). The work reported here represents the most thorough cloning and expression analysis of Hox genes in a crustacean to date. We have isolated sequences (in most cases, full-length cDNAs) for nine Parhyale Hox genes and have shown that at least six of them are linked to other Hox genes in the genome. Further experiments are necessary to determine whether all of these genes lie together in a single cluster, or whether the Parhyale Hox complex has been split, as is the case for other arthropod species (Negre and Ruiz, 2007; Yasukochi et al., 2004).

Parhyale has at least eleven distinct appendage types, and each type develops in the presence of a unique set of Hox genes (Fig. 9).

![Fig. 8. abd-A and Abd-B in situ hybridization in stage 17–22 embryos. Anterior is at top. A, B, E and F are DAPI stained with in situ patterns in red and yellow; C, D and G are brightfield images with in situ hybridization patterns in purple and red. (A) Close up of a stage 17 embryo with abd-A expression in developing parasegments 12 and 13; en1 expression is shown in yellow. (B) abd-A (red) and en1 (yellow) expression in a stage 19 embryo. (C) Stage 21 embryo with abd-A expression (purple) at moderate levels in the T6-T8 appendages, at higher levels in the A1–A3 appendages, and at lower levels in the A4 appendage; en1 staining shown in red. (D) Stage 24 embryo with Ubx in red and abdA in purple. The anterior boundary of Ubx is at T2, while abdA is expressed thought T6-A3, with low levels in A4. (E) Stage 17 embryo with Abd-B transcripts first detectable in rows 11b, c and d, and in 12 a/b and c/d; en1 expression is shown in yellow. (F) Abd-B (red) and en1 (yellow) expression in a stage 19 embryo. Expression is seen throughout the entire abdomen. (G) Stage 22 embryo with Abd-B expression (purple) throughout segments A1-6; en1 staining shown in red. Scale bars 100 μm in all panels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
For example, as the appendages are developing in the head, the first antenna does not express any Hox genes. The second antenna expresses lab and pb. For the mandibular (Mn) segment, Dfd is expressed throughout the segment, with additional expression of pb in the paragnaths and Hox3 in the mandibles. The first maxillae express both Dfd and Scr; the second maxillae express Scr strongly and Antp weakly. Within the anterior thorax Antp is the only Hox gene expressed in the T1 body wall, although both Scr and Antp are strongly expressed in the T1 appendages (maxillipeds), with Scr appearing significantly later in embryogenesis; the gnathopods of T2 and T3 are unique in that they express Antp plus moderate levels of Ubx. High levels of Ubx are found in the walking legs of T4–T8, with the posteriorly oriented T6–T8 walking legs additionally expressing abd-A. Analysis of Antp protein expression by immunohistochemistry and GFP knock-in reveals that there is initial Antp expression throughout the thorax in the neurogenic region, but expression in the developing appendages is largely restricted to T1–T3, with T4–T8 expression in the limbs restricted to a small subset of cells, most likely neurons and muscle cells. Within the abdomen, the pleopods (swimmerets) of A1–3 express both abd-A and Abd-B; and Abd-B is the only Hox gene expressed at high levels in the uropods of A4–6. Antp protein is expressed in the abdomen, but is restricted to the nervous system. All these patterns are summarized schematically in Fig. 9. These patterns suggest that a complex “Hox code” may be responsible for establishing the morphological differences that exist between these appendage types (Fig. 9). We also note that while the initial expression patterns of the Hox genes follow parasegmental boundaries, and such parasegmental boundaries are maintained for expression in the central nervous system, the expression boundaries in the developing and mature limbs are instead segmental. We previously showed for Ubx that the initial parasegmental expression boundary retracted back to a segmental boundary in the ectoderm before limb growth began (Liubicich et al., 2009), and this behavior is also seen here for several of the other Hox genes.

As in many other animals, Parhyale Hox genes also exhibit temporal colinearity—that is, those genes located at the proximal or 3′ end of the Hox cluster (e.g., lab) are transcribed prior to more distal, 5′ end genes (e.g., Abd-B). The only exceptions to this temporal colinearity in Parhyale are pb, which is located on the proximal end of the Hox cluster but is one of the last Hox genes to be expressed, and Hox3, which is first expressed after Dfd, but before (or perhaps coincident with) Scr.

The only Hox ortholog that we were unable to isolate from Parhyale is ftz. Typically, ftz protein sequence, function and expression profiles are highly divergent in arthropods, where they appear to play a role in neurogenesis, segmentation, and/or segment identity depending on the species (Damen, 2002; Heffer...
et al., 2010, 2013). While we cannot rule out the existence of a ftz ortholog in Parhyale, our degenerate PCR primers targeted the LELEKF and KIWFNQR motifs that are conserved in all pancrustaceans (insects and crustaceans) where ftz has been isolated (Heffer et al., 2013). Furthermore, genomic sequences obtained from contiguous Parhyale BAC clones show that there is no ftz between Scr and Antp, its canonical position in arthropods (Chipman et al., 2014). It is worth mentioning that while ftz genes have been isolated from brachiopod and maxilipod crustaceans, no ftz ortholog has been reported for any malacostracan crustacean—this includes both Porcellio and Procambarus, for which numerous Hox genes (including Scr and Antp) have already been isolated (Abzhanov and Kaufman, 1999a, 1999b, 2000a, 2000b). Whether ftz was lost in the entire malacostracan lineage poses an interesting question that awaits further analysis.

The other Hox gene that has diverged significantly during arthropod evolution is Hox3. While Hox3 appears to play a segment identity role in chelicerates and myriapods, in insects (where it is often called zen) it typically serves a function in extra-embryonic tissues (Abzhanov and Kaufman, 1999b; Falciani et al., 1996; Hughes and Kaufman, 2002b; Janssen and Damen, 2006; Panfilio et al., 2006; Telford and Thomas, 1998). Prior to this report, only one Hox3 ortholog had been identified in a crustacean: Daphnia Hox3. Its expression pattern, initially expressed in An2 and Mn before ultimately becoming restricted to the mesoderm of the developing mandible (Papillon and Telford, 2007), is strikingly similar to what we observe for Parhyale Hox3. Interestingly, Hox3 is also expressed in the mandibular mesoderm in the centipede Lithobius and in Thermobia, a member of the early diverging insect lineage Thyasura (Hughes and Kaufman, 2002a; Hughes et al., 2004). It has been suggested that this conserved pattern may have arisen early within Mandibulata, and perhaps may have even played a role in the origin of mandibles (Papillon and Telford, 2007). Our results demonstrate that Hox3 expression in the mandibular mesoderm also occurs in at least one malacostracan, lending further credence to this hypothesis.

Prior to this work, the isopod Porcellio was the only crustacean for which lab, pb and Dfd expression patterns were known (Abzhanov and Kaufman, 1999b). Given that Porcellio are isopods (closely related to amphipods), it is not surprising that such expression patterns are similar to, albeit slightly different from, those we observe in Parhyale. In both species, lab is expressed exclusively in An2 and its appendages, while pb is expressed at the base of the second antennae as well as in the paragnaths, a pair of lobes that act as the lower lips of the mandibular apparatus (Wolff and Scholtz, 2006). Both species also exhibit Dfd expression in Mn (including the paragnaths and mandibles) and the anterior portion of the Mx1 segment. However, they differ in that Parhyale Dfd is additionally expressed in the first maxillae and in the developing swimmerets of A1–A3, while Porcellio Dfd is not. To the best of our knowledge, Parhyale is the only arthropod for which Dfd expression has been observed in abdominal appendages. It will be interesting to examine whether Dfd plays a significant role in pleopod development.

Compared to the head-specific Hox genes, Scr, Antp and Ubx have all received considerably more attention in crustaceans. Ubx expression is typically restricted to the thorax, and its anterior boundary marks the transition between feeding appendages (e.g., maxillae or maxillipeds) and locomotory appendages (Averof and Patel, 1997). Our previously published expression and functional data on Parhyale Ubx confirms that this gene plays a role in specifying this transition (Liubicich et al., 2009; Pavlopooulos et al., 2009).

The expression patterns associated with Scr have been previously examined in two malacostracan crustaceans: Porcellio (which, like Parhyale, has a single pair of maxillipeds on T1) and the crayfish Procambarus (which have three pairs of maxillipeds located on segments T1–T3). In Porcellio, Scr transcripts are expressed in Mx1 and Mx2 segments and their appendages, and in the T1 maxilliped (Abzhanov and Kaufman, 1999a); these expression boundaries coincide with what we see in Parhyale. The Procambarus Scr pattern is similar to this except for the absence of Scr transcripts in Mx1 (Abzhanov and Kaufman, 2000a). In both Porcellio and Procambarus, Scr protein is detected early in second maxillae, but comes on much later in the T1 maxilliped, presumably as a result of post-transcriptional regulation (Abzhanov and Kaufman, 1999a). Whether a similar post-transcriptional regulation of Scr protein occurs in Parhyale awaits future experiments.

Our in situ hybridization experiments using probes for Parhyale Antp reveal a pattern similar to that reported for Porcellio Antp in that both are observed in Mx2 through most of the thorax, with additional staining in the abdominal neuroectoderm and CNS. A similar pattern of Antp mRNA expression (i.e., from Mx2 through most or all of the thorax) has been observed in the branchiopods Artemia and Daphnia, and during the early stages of Procambarus development (Abzhanov and Kaufman, 2000a; Averof and Akam, 1995; Shiga et al., 2002). However, it has been shown that all of these species produce Ubx-Antp read-through transcripts, and in at least two of these species (Artemia and Daphnia), Antp protein is not translated from these chimeric transcripts (Shiga et al., 2002, 2006); we find the same appears to be true in Parhyale. This suggests that the previously described posterior boundaries for Antp expression (i.e., at the border between the thorax and abdomen) may merely reflect transcription of the Ubx-Antp read-through transcripts from the Ubx promoter(s) rather than bona fide Antp expression. Consistent with this, Antp protein expression has been found to be more anteriorly restricted in several of these species than the initial in situ hybridization analyses suggested. For example, Antp protein is only expressed in Mx1 in Artemia, and in Mx1 and T1 of Daphnia (Shiga et al., 2002, 2006). Furthermore, during later stages in Procambarus, Antp transcripts are restricted to Mx1 through T3, suggesting that no Ubx-Antp read-through transcripts are being produced at this late stage (Abzhanov and Kaufman, 2000a).

Intriguingly, in all three of these cases, Antp-specific transcripts and/or Antp protein are only found in Mx2 and in those thoracic appendages that do not express Ubx protein, or, in the case of Parhyale, express Ubx at lower levels than the rest of the thorax. This raises the possibility that Ubx may negatively regulate Antp in these species, perhaps by acting as a transcriptional repressor of the Antp promoter as it does in Drosophila (Beachy et al., 1988). Alternatively, the production of Ubx-Antp readthrough transcripts might somehow physically interfere with the transcription of Antp-specific transcripts from the Antp promoter in these species.

We found that Parhyale abd-A is expressed in T6–8 and A1–A3, and is detected at very low levels in A4. This general pattern of being expressed in the posterior-most thoracic segments and the anterior-most abdominal segments has also been observed in the three other malacostracans for which abd-A expression has been examined: Asellus, Porcellio and Procambarus. In situ hybridizations for Asellus abd-A revealed staining in T6-T8, in A1-A2, but not in A3-A6 (Vick and Blum, 2010). Porcellio abd-A has three splicing variants: abd-A1 and abd-A2 are expressed in a graded manner (stronger in anterior, weaker toward the posterior) in A1-5, but not A6. abd-A3 also shows a graded expression pattern in A1-A5, with additional expression in T6–T8 (Abzhanov and Kaufman, 2000b). Procambarus abd-A is similarly expressed in A1-A5, and shows additional expression in the appendages of T7 and T8 (Abzhanov and Kaufman, 2000a). The authors suggested that abd-A may play two roles in Procambarus, namely, specifying abdominal identity and differentiating posterior thoracic appendages from more anterior ones. Consistent with this idea, in Parhyale, the
**References**


