Gene expression suggests conserved aspects of \textit{Hox} gene regulation in arthropods and provides additional support for monophyletic Myriapoda

Ralf Janssen* and Graham E Budd

Abstract
Antisense transcripts of Ultrabithorax (aUbx) in the millipede Glomeris and the centipede Lithobius are expressed in patterns complementary to that of the Ubx sense transcripts. A similar complementary expression pattern has been described for non-coding RNAs (ncRNAs) of the bithoraxoid (bxd) locus in Drosophila, in which the transcription of bxd ncRNAs represses Ubx via transcriptional interference. We discuss our findings in the context of possibly conserved mechanisms of Ubx regulation in myriapods and the fly.

Bicistronic transcription of Ubx and Antennapedia (Antp) has been reported previously for a myriapod and a number of crustaceans. In this paper, we show that Ubx/Antp bicistronic transcripts also occur in Glomeris and an onychophoran, suggesting further conserved mechanisms of Hox gene regulation in arthropods.

Myriapod monophyly is supported by the expression of aUbx in all investigated myriapods, whereas in other arthropod classes, including the Onychophora, aUbx is not expressed. Of the two splice variants of Ubx/Antp only one could be isolated from myriapods, representing a possible further synapomorphy of the Myriapoda.

Background
The Hox genes are expressed in broad overlapping domains along the anterior-posterior axis of developing arthropods, and specify the segment identity under the control of upstream acting segmentation genes [1,2]. In \textit{Drosophila}, the initially established expression patterns of the Hox genes are maintained by the trithorax (trxG) and Polycomb group (PcG) factors [3]. These factors act through sets of response or maintenance elements (MEs), the best investigated of which are involved in the regulation of the \textit{Ultrabithorax} (\textit{Ubx}) gene [4,5].

A number of non-coding RNAs (ncRNAs) have been reported for \textit{Drosophila}, which are transcribed through MEs in the bithoraxoid (bxd) region located between \textit{Ubx} and \textit{abd-A}. The ncRNAs including \textit{bxd} are expressed in similar patterns to those of the neighbouring Hox genes [6,7]. Although it was initially thought that \textit{bxd} would activate \textit{Ubx}, a recent study suggests that transcription of ncRNAs promoted by Trithorax represses \textit{Ubx} in cis by means of transcriptional interference [4].

Elongated transcription of \textit{bxd}-ncRNAs through the \textit{Ubx} locus prevents the transcription of the latter in the same cells. However, in cells that do not express \textit{bxd} \textit{Ubx} is expressed [4]. The expression patterns of \textit{bxd} ncRNAs and \textit{Ubx} are therefore complementary in \textit{Drosophila}.

In organisms other than \textit{Drosophila}, the mechanisms that regulate \textit{Ubx} transcription are less well known. It is unclear whether MEs or \textit{bxd} are conserved or if transcription of \textit{bxd} interferes with the transcription of \textit{Ubx} in a similar way to that in \textit{Drosophila}. However, some evidence has recently accumulated suggesting that a similar mechanism could be involved in the regulation of \textit{Ubx} outside \textit{Drosophila}. Data from the beetle \textit{Tribolium} show that ncRNAs of the \textit{Ubx} region are expressed in patterns similar to those of the neighbouring Hox genes, resembling the observations in \textit{Drosophila} [8]. In the centipede \textit{Strigamia}, the non-coding antisense transcript of \textit{Ubx} is expressed in a pattern complementary to that of the coding \textit{Ubx} sense transcript, suggesting that bidirectional transcription of a non-coding RNA, antisense \textit{Ubx}, is also involved in the regulation of \textit{Ubx} in this myriapod [9].

In this paper, we present data from two distant myriapod relatives - the millipede \textit{Glomeris marginata} and the
centipede *Lithobius forficatus* - which show conserved expression of antisense *Ubx* (**aUbx**) in a pattern complementary to that of *Ubx* in Myriapoda. Data from species of other arthropod groups and the onychophoran *Euperipatoides kanangrensis* reveal that **aUbx** expression does not represent an ancestral feature but a synapomorphy of the Myriapoda. The latter provides support for the still controversially discussed idea that the Myriapoda form a monophyletic group [10].

An mRNA that encodes a single protein, which describes the typical case for eukaryotic genes, is termed monocistronic, whereas mRNAs encoding two or several proteins are termed bicistronic and polycistronic respectively. We show here that bicistronic transcripts of *Ubx* and *Antp* (**Ubx/Antp**), as described for a number of crustaceans and the centipede *Strigamia* [9,11], also exist in *Glomeris* and *Euperipatoides*. This finding suggests that bicistronic transcription is an ancestral feature that is likely to be involved also in arthropod Hox gene regulation by means of transcriptional interference and the blockade of *Antp* translation.

**Materials and methods**

**Species husbandry and embryo treatment**

The general handling of *G. marginata* is described in Janssen et al. [12]. The embryos were allowed to develop at room temperature (22 to 25°C). The developmental stage of the embryos was determined by 4’-6-diamidino-2-phenylindole (DAP) staining. Staging was performed as described previously [12,13].

Specimens of *L. forficatus* were collected from a leaf litter stack in the backyard of the Evolutionary Biology Centre (EBC) in Uppsala/Sweden in spring (May/June). Around 50 centipedes were held at room temperature in a spacious plastic box filled with washed leaf litter (washing away small particles makes the later finding of the eggs easier). The adults were fed with pieces of common earthworms (*Lumbricus*) every few days. The often detritus-covered eggs were collected by hand and incubated in plastic dishes on damp paper tissues until they reached the desired developmental stage. Staging was performed as described previously [14]. Generally, the handling was carried out similarly to the method described for *Lithobius atkinsoni* [15].

Embryos of the spider *Cupiennius salei*, the onychophoran *E. kanangrensis* and the red flour beetle *Tribolium castaneum* were obtained and treated as described previously ([16-18], respectively).

**Gene cloning**

Fragments of *Ubx* and *Antp* transcripts of *G. marginata* were obtained via 5’ and 3’ rapid amplification of cDNA ends (RACE)-PCR (Gene Racer RACE Kit; Invitrogen, Carlsbad, CA, USA). A fragment (383 bp) of *Tribolium Ubx* corresponding to the C-terminal end of the open reading frame (ORF) (94 bp) and the beginning of the 3’ untranslated region (UTR) was isolated with gene-specific primers (Table 1). General Hox primers, as described previously [19], were used to isolate a small fragment of *Ubx* from *Euperipatoides* cDNA. An extended fragment was subsequently obtained by 3’-RACE.

A fragment of *Lithobius forficatus Ubx* was isolated with gene-specific primers based on the published sequence of *Lithobius atkinsoni Ubx* [15]. The isolated *L. forficatus* fragment is only 221 bp long, but works well in hybridization experiments.

Part of the bicistronic transcripts containing *Ultradistal* and *Antennapedia* (**Ubx/Antp**) were isolated from the brine shrimp *Artemia* (first PCR), the onychophoran *Euperipatoides* and the millipede *Glomeris*. The gene-specific primers used were directed against the homeodomains of *Ubx* (forward primer) and *Antp* (backward primer). Gene-specific primers to amplify a possible *Tribolium Ubx/Antp* transcript failed, even though we used the primers (Table 1) in all possible combinations including nested PCRs.

Sequences of the fragments were determined from both strands by sequencing (Big Dye Terminator Cycle Sequencing Kit; Perkin-Elmer Applied Biosystems, Foster City, CA, USA) chemistry on an automatic analyser (ABI3730XL; Perkin-Elmer Applied Biosystems) by a commercial sequencing service (Macrogen, Seoul, Korea). Sequences are available in GenBank under the accession numbers FN687748 (**Gm-Ubx**), FN687749 (**Gm-Antp**), FN687750 (**Gm-Antp_variant II**), FN687751 (**Ek-Ubx**), FN687752 (**Ek-Ubx/Antp_variant I**), FN687753 (**Ek-Ubx/Antp_variant II**), FN687754 (**Lf-Ubx**), and FN687755 (**Af-Ubx/Antp_variant II**).

**In situ hybridization and nuclear staining**

Whole-mount in situ hybridization for all species was performed as described previously for *Glomeris* [20]. Double whole-mount in situ hybridization and cell nuclei detection using DAPI was performed as described by Janssen et al. [21]. Embryos were analyzed under a dissection microscope (Leica, Heerbrugg, Switzerland) equipped with a digital camera (Axiocam; Zeiss, Jena, Germany) or a DC100 (Leica) digital camera. Brightness, contrast and colour values were corrected in all images using image processing software (Adobe Photoshop CS2, V.0.1 for Apple Macintosh; Adobe Systems Inc. San Jose, CA, USA).

**Results**

**Ultrabithorax and Antennapedia transcripts**

Partial sequences of the transcripts of all ten Hox genes of *G. marginata* were published previously [19]. In all cases
except *fushi-tarazu*, only part of the homeodomain and 3' UTR sequence was obtained. The published *Ubx* fragment neither ends in a poly-A tail nor has one of the typical polyadenylation sites and is therefore likely to be incomplete. Recent 3'-RACE experiments demonstrated the presence of additional 3' UTR transcript. The extended fragment ends in a poly-A tail, but lacks an obvious polyadenylation site close to this. The 3' UTR region contains nine possible polyadenylation sites more distant from the poly-A tail, allowing for the presence of transcripts with different 3' UTR length. Whether the recovered '3' UTR' sequence is a typical UTR that occurs in the monocistronic transcript of *Ubx* or if is merely the result of the bicistronic transcript of *Ubx* and *Antp* (see following section) is unclear.

We recovered 5'-RACE fragments of *Ubx* and *Antp*. The *Ubx* fragment represents the complete N-terminal region of the ORF and 5' UTR sequence. The 5'-Antp fragment is incomplete and does not include the N-terminal region of the protein coding sequence and the 5' UTR. The fragments encode conserved motifs that are characteristic for *Ubx* and *Antp* orthologs in arthropods.

### Table 1: Primers used for PCR.

| Gene                  | Direction | Primer sequence 5' T 3'                      |
|-----------------------|-----------|---------------------------------------------|
| *Tribolium Ubx*       | Forward   | CCCAATTACGTATATAGTTG                        |
|                       | Reverse   | GATCAAGAACTCAACGAC                          |
| *Lithobius forficatus Ubx* | Forward   | GAGAGAGGGCGGATAGAGATG                       |
|                       | Reverse   | TTATTTGTTTGGGTAGGGG                        |
| *Artemia Ubx/Antp*    | Forward (1) | TACAGCGAGACGAAGG                           |
|                       | Reverse (1) | CTCTCTTTCATTTCCATTGG                      |
|                       | Forward (2) | CAGATCGATATGCTTCC                         |
|                       | Reverse (2) | GCGAACATAAGAGCATAGG                      |
| *Euperipatoides Ubx/Antp* | Forward   | GCCGAGGATAGAAATTGGCGTACAGC                 |
|                       | Reverse   | CGGAGTCTACGTGCGCTTTCCGTGCG                 |
| *Glomeris Ubx/Antp*   | Forward   | GGGGAGGAGCCGAGATAAGAATGG                   |
|                       | Reverse   | TTTATACGTGCGGTTCGTACAGG                    |
| *Tribolium Ubx/Antp*  | Forward (1) | GAGAGAGAGAGATCCACAAA                      |
|                       | Reverse (1) | CCCTATTCGTCCCATGTCC                      |
|                       | Forward (2) | GATCAACAGACTCAACGAGC                      |
|                       | Reverse (2) | GAGATGCTCCTCCGGTATAC                      |
|                       | Forward (3) | CAGGGTAAAAAGCGGCGG                     |
|                       | Reverse (3) | Against N-terminal part of ANTP             |
(Figure 1A). Note that the *Glomeris* ANTP protein lacks the characteristic SQFE motif between the hexapeptide and the homeodomain. Instead, this short peptide is replaced by a single lysine (K) in *Glomeris* (Figure 1A). The expression pattern of all newly recovered fragments is identical to those described previously [19] (not shown).

**Bicistronic transcript of *Ultrabithorax* and *Antennapedia***

For *Glomeris*, we identified an *Ubx/Antp* bicistronic transcript that encodes the *Ubx* homeodomain C-terminal to the upstream primer position and 38 bp of the *Ubx* 3’ UTR, which is directly adjacent to the complete N-terminal part of the *Antp* homeodomain up to the downstream primer position (splice variant II; see below) (Figure 1B,B’). Whether the sequence C-terminal to this sequence is part of the fusion transcript is unclear; however, the sequence N-terminal to the described short fusion transcript has been independently recovered by 5’ RACE using gene specific primers (GSPs) against the *Antp* homeodomain that amplified the *Ubx/Antp* fusion transcript instead of the *Antp* 5’ transcript. This sequence is part of the *Ubx* transcript as proven by 5’-RACE PCR for *Ubx*.

We also successfully isolated a splice version (splice variant I) of *Ubx/Antp* bicistronic transcripts from an onychophoran (*Euperipatoides*). This splice variant I is also described for a number of crustaceans including the brine shrimp *Artemia* [11] (Figure 1B). For *Euperipatoides* and *Artemia*, we also isolated the shorter splice variant II of the bicistronic transcript described for *Strigamia* [9] (Figure 1B,B’). A splice variant I is not described for *Strigamia* and we could not isolate it from *Glomeris* either. We failed to detect any *Ubx/Antp* bicistronic transcripts in the beetle *Tribolium* (Insecta).

**Extension and nature of the *Ubx* antisense (a*Ubx*) transcript**

The information on *aUbx* transcription is based on probes detecting the *Ubx* antisense strand during in situ hybridization experiments (Figure 1C). It was thus necessary to unravel the true extension of the *aUbx* transcript by in situ hybridization experiments with minimum size probes (around 300 bp for *Glomeris*) detecting *aUbx* complementary to the ends of the available *Ubx* fragments (Figure 1C). In all cases these sense probes detected the *aUbx* expression pattern (described below) suggesting their complete transcription. Whether the *aUbx* transcript extends the *Ubx* transcript is unclear; however, it does not extend into the transcripts of *abdominal-A* (abad-A) or *Antennapedia* (Antp), because in situ hybridization experiments with anti-abad-A and anti-Antp probes did not detect any transcription. The longest possible ORF of the *aUbx* transcript is 113aa long and encodes a repetitive sequence of the type (LLLLR/cSE) (Figure 1D).

**Expression of *aUbx***

Transcripts of *aUbx* can already be detected at the blastoderm stage in a broad posterior domain (Figure 2A); at stage 0.2, this expression intensifies (Figure 2B). At the next stage (0.3) the centre of the initial broad domain is cleared from the transcripts (Figure 2C). At stage 0.4, the domain splits into an anterior stripe and a broad posterior domain (Figure 2D). The broad domain lies anterior to the future proctodaeum; the anterior stripe covers the intersegmental indentation between trunk segment two (T2) and T3, and is thus located in the posterior part of T2. At stage 1, the posterior domain has broadened and its anterior and posterior margins show enhanced expression (Figure 2E). At stage 1.2, the complete T2 segment expresses *aUbx*, although the expression in its anterior part is weak (Figure 3A). The anterior margin of the former broad domain (Figure 2E) has now transformed into an independent stripe in the posterior of T3 (Figure 3A). The posterior-most expression is in the anal valves (AV). Ventrally, the expression of *aUbx* is weaker than in its corresponding lateral and dorsal tissue (Figure 3A). At the subsequent stage (stage 2) three stripes of *aUbx* expression are detectable: in the posterior areas of T1, T2 and T3 (Figure 3B). This expression is restricted to the ventral tissue only for the stripes in T1 and the T3, whereas the stripe in T2 extends into the dorsal tissue. All stripes are discontinuous at the ventral midline. At around stage 3, an additional stripe forms in the posterior of T4 (Figure 3C). In subsequent stages (4 to 6), additional discontinuous stripes of *aUbx* appear in the ventral germ band with the formation of additional segments. Expression in dorsal tissue, legs and anal valves remains unchanged. Expression of the anterior-most *aUbx* stripe (the posterior stripe in T1 (T1p)), is enhanced at these stages (Figure 3D and data not shown). Note that although the legs posterior to T3 are forming, *aUbx* is not expressed in their tips (Figure 3D). The posterior-most part of the developing early embryo, which will later give rise to the hindgut and the proctodaeum, remains free from *aUbx* expression (Figure 2).

**Complementary expression patterns of *Ubx* and *aUbx***

The *Gm-aUbx* transcript is regulated in a similar, but complementary, specific pattern to that of *Gm-Ubx* (Figure 2, Figure 3, Figure 4). Expression of *aUbx* starts earlier (stage 0) than expression of *Ubx* (stage 0.2 or 0.3), but in a comparable posterior area. Double in situ hybridization to detect possible overlap of early *Ubx* and *aUbx* expression is not possible because the signal of *Ubx* is too weak in the early stages (Figure 2G-I). At stage 1, *Ubx* expression is still restricted to the posterior growth zone
Figure 1 Sequence information on Ubx, Antp and Ubx/Antp. (A) Conserved N-terminal region of UBX and hexapeptide sequence of UBX and ANTP in arthropods. Dashes indicate conserved positions, dots represent gaps, question marks stand for unknown sequence. Amino acids contributing to the homeodomain are underlined. (B) Overview of bicistronic transcripts of Ubx/Antp and their splice variants in arthropods. Ubx sequence is in light grey; Antp sequence is in dark grey; positions of primers for the detection of Ubx/Antp are indicated by arrows. In splice variant I, Antp is almost exactly abutting the open reading frame of Ubx with only few base pairs of Ubx 3' UTR in between. In splice variant II, all sequence of Antp 5' to the homeodomain (HD) is missing. (B) Sequences of the Ubx/Antp splice variant II from Glomeris, Euperipatoides and Artemia. The homeodomain is underlined, primers are shaded, and the 3' UTR of Ubx is in bold. (C) Extension of isolated Ubx mRNA and inferred extension of the aUbx transcript. Probes (P1 to P5) detecting Ubx and aUbx are indicated (not to scale). Whether 5'- and 3' UTR transcripts extend beyond the detected area is unclear (question marks). Positions of start codon (ATG), hexapeptide (hex), homeodomain (HD), stop codon (TAA) and poly-A tail (dot_AAAAAA) are indicated. (D) Twelve conserved 21bp-repeats situated in the 3' UTR of Glomeris Ubx. The sequences are abutting each other without bases in between. Consensus sequence is on top. Differences from the consensus are marked by shaded bases, changed amino acids are in bold.
and is not present in the nascent segment T3 (Figure 2I), unlike the previously reported expression in T3 in stage 1.2 embryos [19]. At this stage, the anterior margin of \textit{aUbx} is clearly more anterior (T2) than that of \textit{Ubx} (T3).

At stage 2, it becomes obvious that the expression patterns of \textit{Ubx} and \textit{aUbx} are indeed broadly complementary (Figure 4A-C). The stripe of \textit{aUbx} expression extending into the dorsal tissue lies in the posterior of T2, and is thus anteriorly abutting the expression of \textit{Ubx} (Figure 4A-C). The ventral \textit{aUbx} stripe in T3 coincides with a lack of \textit{Ubx} expression in this area (Figure 4A-D). Very faint expression of \textit{Ubx} extends minimally into T2 ventrally (Figure 4B), and \textit{aUbx} is weakly expressed anterior to this (Figure 4A-C). Whereas the ventral expression of \textit{Ubx} at stage 4-6 becomes more complex, the expression of \textit{aUbx} remains as stripes (Figure 4D), which are complementary to the expression of \textit{Ubx}. (Figure 4D). The anterior shift of \textit{aUbx} into the posterior of T1 (Figure 3A,B) coincides with a shift of \textit{Ubx} expression into the complete ventral part of T2 (Figure 4F) [19]. The anterior border of dorsal \textit{Ubx} is shifted towards the posterior compared with its anterior border in ventral tissue (Figure 4F) [19]. In dorsal tissue, the expression of \textit{aUbx} still abuts the expression of \textit{Ubx} and is hence also shifted towards posterior (Figure 3C, Figure 4E; also seen in Figure 4F for a stage 4 embryo).

**Transcript and expression of Lithobius \textit{Ubx} and \textit{aUbx}**

The isolated fragment of \textit{L. forficatus} (Uppsala/Sweden) \textit{Ubx} is 93% (206 of 221 bp) identical with the orthologous sequence of \textit{L. atkinsoni} [15] and 98% (64 of 65 bp) identical with the sequence of \textit{L. forficatus} (UK) [22]. The expression pattern of \textit{Lf-Ubx} is identical to that described for \textit{L. atkinsoni} [15]. As expected from the data for \textit{Glomeris} and \textit{Strigamia}, the antisense transcript (\textit{Lf-\textit{aUbx}}) is also transcribed. The expression pattern of Lf-
aUbx is complementary to that of Ubx and very similar to that of Strigamia antisense Ubx in embryos with 30 leg-bearing segments (LBS) (Figure 5) [9]. A broad central domain in the first walking leg segment (L1) abuts the anterior-most expression of Ubx which extends into the very posterior of L1. Dorsal to that, in the region of the developing legs, aUbx is expressed as a thin stripe at the border of the maxillipedal segment (mxpd) and L1 (Figure 5). We expect that the expression pattern of Lf-aUbx is more complex in older developmental stages [9].

Detection of aUbx in arthropods other than myriapods

We investigated the possible expression of aUbx in members of other arthropod classes and an onychophoran. Sense probes of the same length as the antisense probes were used for the detection of Ubx in Triboliom (Insecta), the two known Ubx paralogs in Cupiennius (Chelicera) [16], and Ubx in Euperipatoides (Onychophora) failed to detect any transcripts. In all cases, positive controls detecting the Ubx signal were successfully probed with antisense probes in parallel experiments (data not shown).

Discussion

Conserved transcription and complementary expression of Ubx and aUbx supports myriapod monophony

Sequence and expression data of Ultrabithorax are presently known from four myriapod species: the geophilomorph Strigamia maritima (Chilopoda) [9]; the lithobiomorph species L. atkinsoni and L. forficatus ([15] and this study); and the pill millipede G. marginata (Progoneata) [19]. In all cases, the antisense DNA strand complementary to Ubx is transcribed and the expression pattern of the antisense transcripts (aUbx) is complementary to that of the sense transcript (coding transcript; Ubx) ([9] and this study). This finding suggests that complementary expression of sense and antisense transcripts generated from the Ubx locus is conserved between all myriapods.

Because aUbx expression has not yet been detected outside the Myriapoda, but has been detected in Chilopoda and Progoneata, it probably represents a synapomorphy for the Myriapoda, although this conclusion is dependent on the phylogenetic position of symphylans and pauropods [23-25]. This finding further supports myriapod monophony, which is to date mainly based on nucleotide sequence data ([26,27] morphological data are still controversial in this context [10,25,28,29]).

Similarities of Ubx regulation in Drosophila and myriapods: evidence for a conserved mechanism?

The fact that Ubx and aUbx are expressed in conserved and complex complementary patterns strongly suggests that one (or its transcription) is involved in the regulation of the other. Striking similarities to the situation in myriapods can be found in Drosophila, in which transcription of bxd non-coding RNAs (ncRNAs) upstream of Ubx prevents transcription of the latter. This repression is probably caused by transcriptional interference as the bxd transcript(s) elongate into the region of Ubx promoters and prevent the binding of the transcription machinery [4,30]. As a result, bxd ncRNAs are expressed in a complementary pattern to that of Ubx, causing a mosaic-type expression pattern of Ubx within its overall expression domain [4,6].

A similar situation is found in myriapods, in which a putative ncRNA, aUbx, is expressed in a complementary pattern to that of Ubx. Like the bxd ncRNAs in Drosophila, aUbx also precedes expression of Ubx, and also as in Drosophila, expression of Ubx in myriapods occurs in the anterior of each segment and expression of bxd and aUbx occur in the posterior of each segment (this study, [9,31]).

The most obvious difference between the expression of bxd ncRNAs in Drosophila and aUbx in myriapods is that aUbx (or its promoter) is located on the complementary DNA strand in myriapods and not oriented in a tandem position to Ubx on the same strand. How can this disparity be explained if we assume that aUbx expression in myriapods is homologous to bxd expression in Drosophila?

The simplest explanation of this pattern would be to postulate an inversion event in the Ubx locus back in the stem lineage leading to the myriapods, placing the aUbx (bxd) promoter on the complementary strand (Figure 6A). Subsequent transcription through the promoter site(s) of Ubx in myriapods would then cause expression of aUbx in a complementary pattern. However, this would require a stage at which Antp and Ubx were on different strands, and as we show in this paper, bicistronic transcripts of Ubx/Antp and their splice versions (vari-
ants I and II) are conserved and thus are most probably of strong developmental importance, thus they are unlikely to have been separated in this way. A single inversion event putting \textit{Ubx} alone on the complementary strand can also be excluded because of the presence of \textit{Ubx}/\textit{Antp} bicistronic transcripts that are very unlikely to be a result of a \textit{trans}-splicing event (discussed below) [9,32]. An alternative to these unlikely possibilities is that a new \textit{bxd}/\textit{aUbx} promoter site evolved on the complementary strand located between \textit{Antp} and \textit{Ubx} (Figure 6B). Functional studies or a fully sequenced genome, which could possibly help shed light on the role of \textit{aUbx} transcription in myriapods and answer the question of whether the mechanisms suggested for \textit{Ubx} regulation in myriapods are related to those in \textit{Drosophila}, are currently not available.

**Alternative functions of \textit{aUbx} expression**

A number of theories have been suggested over the past few years to explain how noncoding antisense transcripts or bidirectional transcription may regulate the expression of the coding unit ([33] and references therein). A case of possible transcriptional interference displaying much similarity between \textit{Drosophila} and myriapods has been discussed in the previous section. However, although this possibility appears to be likely, \textit{aUbx} or its transcription could nevertheless also act differently. We therefore summarize and discuss some of those mechanisms in the light of our data.

First, transcription of the antisense strand can cause epigenetic modifications, methylation of sense-strand promoters, and conversion of the chromosome structure, causing repression of gene transcription on the sense strand [34]. Epigenetic modification could explain or cause the complementary pattern of \textit{Ubx} and \textit{aUbx} if \textit{aUbx} represses the transcription of \textit{Ubx} in tissues or cells that are generally \textit{Ubx}-competent.

Second, transcriptional interference can also occur via promoter collision, when RNA polymerases meet on opposite strands and cannot pass each other. This can cause the premature termination of one or both transcripts [30,35].

Third, sense and antisense transcripts could form double-stranded (ds)RNA, a source for small interfering RNAs that would mediate RNA interference (RNAi) [36]. The complementary expression pattern of \textit{Ubx} and \textit{aUbx} would be explainable by the rapid degeneration of \textit{Ubx} due to perfectly matching miRNAs descendent from the possible \textit{Ubx-aUbx} dsRNA [37].

The fact that \textit{aUbx} is expressed significantly earlier than \textit{Ubx} may also have important implications on the regulatory mechanisms discussed. It would guarantee the immediate binding of incorrectly expressed \textit{Ubx} to pre-existing \textit{aUbx} in an RNAi-based mechanism, or provide a head start for transcription of \textit{aUbx} in cases of transcriptional interference. In the case of epigenetic modification, it would prevent the later transcription of \textit{Ubx} by silencing its promoter(s).

**A 21 bp repeat in the \textit{Ultrabithorax} 3’ UTR of \textit{Glomeris}**

We discovered a repetitive sequence of exactly 21 bp (Figure 1D) in the 3’ UTR of \textit{Ubx}. This sequence most probably represents a minisatellite (or short sequence repeat; SSR) common in bacterial and metazoan genomes [38]. It may represent multiple recognition sites for micro (mi)RNAs [39]. Alternatively, it could represent an ORF encoding a small 113 amino acid protein, possibly
involved in the regulation of \textit{Ubx}. The finding of an SSR could generally also be of interest for investigating population genetics in 	extit{Glomeris} [40].

**Presence of \textit{Ubx/Antp} bicistronic transcripts in myriapods, crustaceans and onychophorans, but not in insects?**
The finding that bicistronic transcripts of \textit{Ubx} and \textit{Antp} (\textit{Ubx/Antp}) are present in myriapods and crustaceans suggests that this represents a conserved state of at least the Mandibulata or potentially the Arthropoda. Despite this, we failed to isolate \textit{Ubx/Antp} fusion transcripts from the beetle \textit{T. castaneum}. The latter may merely represent a loss in higher insects that finally allowed the Hox complex to split between \textit{Ubx} and \textit{Antp}, as is the case in \textit{Drosophila melanogaster} [41]; however, in \textit{Tribolium}, the Hox cluster is still intact [8]. Alternatively, it may represent the early loss of \textit{Ubx/Antp} in the stem lineage of the insects or hexapods. If the hexapods have evolved from a crustacean ancestor (as in the Pancrustacea theory), a loss of \textit{Ubx/Antp} may be present in the suggested recent sister-group crustacean orders Remipedia and/or Cephalocarida [42]. The presence of \textit{Ubx/Antp} fusion transcripts in an onychophoran shows that the evolutionary origin of bicistronic transcription of \textit{Ubx} and \textit{Antp} dates back to the common ancestor of onychophorans and euarthropods, suggesting that \textit{Ubx/Antp} is also likely to occur in chelicerates.

Interestingly, only the short splice variant II (Figure 1B, B') has been isolated from myriapods. We therefore believe that variant I may be lacking in myriapods exclusively, again supporting myriapod monophyly. However, we are aware that negative results are less reliable arguments than positive results, and therefore we can only see a loss in higher insects that finally allowed the Hox complex to split between \textit{Ubx} and \textit{Antp}, as is the case in \textit{Drosophila melanogaster} [41]; however, in \textit{Tribolium}, the Hox cluster is still intact [8]. Alternatively, it may represent the early loss of \textit{Ubx/Antp} in the stem lineage of the insects or hexapods. If the hexapods have evolved from a crustacean ancestor (as in the Pancrustacea theory), a loss of \textit{Ubx/Antp} may be present in the suggested recent sister-group crustacean orders Remipedia and/or Cephalocarida [42]. The presence of \textit{Ubx/Antp} fusion transcripts in an onychophoran shows that the evolutionary origin of bicistronic transcription of \textit{Ubx} and \textit{Antp} dates back to the common ancestor of onychophorans and euarthropods, suggesting that \textit{Ubx/Antp} is also likely to occur in chelicerates.

**Conserved regulatory aspects of \textit{Ubx/Antp} expression**
In crustaceans, bicistronic transcripts of \textit{Ubx/Antp} are not (\textit{Daphnia}) or only partially (only \textit{Ubx} in \textit{Artemia}) translated. Expression of the translated monocistronic transcripts, and therefore the protein, differs significantly from expression of \textit{Ubx/Antp} [11]. It is tempting to speculate that transcription of \textit{Ubx/Antp} under control of the \textit{Ubx} promoter interferes with the proper transcription of monocistronic \textit{Antp} in these crustaceans.

The conserved appearance of \textit{Ubx/Antp} in arthropods and onychophorans suggests their involvement in the regulation of \textit{Ubx}, \textit{Antp} or both \textit{Hox} genes. In particular, repression of \textit{Antp} via \textit{Ubx/Antp} transcription appears likely, not least because the transcript is apparently spliced in such a way that it lacks most of its coding capacity (variant II).

For \textit{Glomeris} and \textit{Euperipatoides}, it is unclear whether the detected expression patterns of \textit{Ubx} and \textit{Antp} are a result of mono- or bicistronic transcription. However, in both, as in crustaceans [11], the \textit{Ubx/Antp} transcript is probably under control of the \textit{Ubx} promoter, as the expression pattern of \textit{Ubx/Antp} is identical with that of \textit{Ubx} (not shown). Thus, it is possible \textit{Ubx/Antp} contributes to or even replaces monocistronic \textit{Ubx} expression in \textit{Glomeris} and \textit{Euperipatoides} as it does in \textit{Artemia} [11]. If part of the detected mRNA expression patterns of \textit{Ubx} and \textit{Antp} [19] is a result of \textit{Ubx/Antp}, it might not correlate with the protein pattern. Specific antibodies to detect UBX and ANTP protein are not available, and the cross-reacting antibody FP6.87 [43] does not detect UBX in \textit{Glomeris} (data not shown). Further investigation is thus needed to unravel the role of \textit{Ubx/Antp} transcription in arthropods.

**Regulation of limb development in \textit{Glomeris}**
\textit{Ubx} expression is likely to be involved in the delayed outgrowth of the walking legs posterior to T3 in \textit{Glomeris} by repressing \textit{Distal-less (Dll)} as shown for other arthropods [44-46]. The finding that \textit{aUbx}, a possible repressor of \textit{Ubx} (as discussed above), is strongly expressed in the tips of the legs in T2 and T3 further supports this view, suggesting that the absence of \textit{Ubx} is indeed crucial for the accelerated development of walking legs in T1 to T3 in \textit{Glomeris} [19]. The exclusion of \textit{Ubx} from the distal part of the legs possibly caused or supported by \textit{aUbx} could represent a developmental novelty in the 'battle' of appendage growth in \textit{Ubx}-expressing segments. In \textit{Strigamia} and \textit{Lithobius}, \textit{Ubx} seems not to repress \textit{Dll}, possibly because of a number of phosphorylation sites in the C-terminal end of the protein that interfere with the assumed repressor function of \textit{Ubx} on \textit{Dll} [19,45]. Consequently, there is no need to keep the tips of the legs free from \textit{Ubx} or, in other words, to express \textit{aUbx}.

**Conclusions**
A number of conserved aspects of \textit{Ubx} and \textit{Antp} regulation are found across the Arthropoda. Repression of \textit{Ubx} transcription, and thus formation of a complex segmental pattern of \textit{Ubx} expression, may depend on transcriptional interference as shown for \textit{Drosophila}, and suggested and visualized by \textit{aUbx} expression in \textit{Glomeris}. Furthermore, bicistronic transcription of \textit{Ubx} and \textit{Antp} and subsequent splicing of these transcripts as shown for \textit{Crustacea}, \textit{Myriapoda} and \textit{Onychophora}, but possibly not \textit{Insecta}, suggests that \textit{Ubx/Antp} transcription is an important ancestral feature of \textit{Hox} gene regulation as well. As shown for \textit{Crustacea}, runthrough transcription and sub-
sequent nontranslucence of *Ubx/Antp* may compete with the proper transcription of the (translated) monospecific *Ubx* and *An* transcripts [11], and thus transcriptional interference via *Ubx/Antp* transcription might contribute to a defined protein expression pattern within areas of ubiquitously expressed Hox gene mRNA. Presence of all*Ubx* transcription and the possible lack of *Ubx/Antp* splice variant I in myriapods represent possible synapomorphies for the Myriapoda.

### Competing interests

The authors declare that they have no competing interests.

### Authors’ contributions

RU carried out the experiments, wrote the first draft of the manuscript and was mainly responsible for the experimental outline. GEB was involved in drafting the final version of the manuscript and discussed the experimental outline. GEB also initiated work on *Apomorphia for the Myriapoda*. (Correspondence to Janssen and Budd; licensee BioMed Central Ltd. 2010. This article is available from: http://www.evodevojournal.com/content/1/1/4© 2010 Janssen and Budd; licensee BioMed Central Ltd. 2010. This article is available from: http://www.evodevojournal.com/content/1/1/4

### Acknowledgements

This work was mainly supported by the European Union via the Marie Curie Research and Training Network ZOONET (MRTN-CT-2004-050624). The work was also supported by the Swedish Research Council (VR) and the Swedish Royal Academy of Sciences (KVA). We thank WGM Damen for the Ubx1 and Ubx2 clones and embryos of the *C. salei*. Adults of the beetle *T. castaneum* to establish our own lab culture were provided by G. Bucher and N-M. Prpic-Schäper. Genitalia specimens of *K. kanangensis* were collected with the most appreciated help of Noel Tait (Sydney). We would like to thank the three anonymous reviewers for their helpful comments on the manuscript.

### Author Details

Department of Earth Sciences, Palaeobiology, Villavagen 16, SE-75236 Uppsala, Sweden

Received: 12 March 2010 Accepted: 5 July 2010

Published: 5 July 2010

### References

1. Irish VF, Martinez-Arias A, Akam M: Spatial regulation of the *Antennapedia* and *Ultrathorax* homeotic genes during *Drosophila* early development. *EMBO J* 1989, 8:1527-1537.
2. Carroll SB, DiNardo S, O'Farrell PH, White RA, Scott MP: Temporal and spatial relationships between segmentation and homeotic gene expression in *Drosophila* embryos: distributions of the *fushi tarazu*, *en*grailed, *Sex comb reduced*, *Antennapedia*, and *Ultrathorax* proteins. *Genes Dev* 1988, 2:350-360.
3. Grimaud C, Negre N, Cavalli G: From genetics to epigenetics: the tale of *Polycomb* and trithorax group homeobox genes. *Chromosome Res* 2006, 14:363-375.
4. Petruk S, Sedkov Y, Riley KM, Hodgeson J, Schweisguth F, Hirose S, Jaynes JB, Broch HW, Mazo A: Transcription of *bcd* noncoding RNAs promoted by trithorax represses *Ubx* in cis by transcriptional interference. *Cell* 2006, 127:1209-1221.
5. Hodgson JW, Arigropoulos B, Broch HW: Site-specific recognition of a 70-base-pair element containing a d(GA)n repeat mediates bithoraxoid polycomb group response element-dependent silencing. *Mol Cell Biol* 2001, 21:4528-4543.
6. Rank G, Prestel M, Paro R: Transcription through intergenic chromosomal memory elements of the *Drosophila* Bithorax complex correlates with an epigenetic switch. *Mol Cell Biol* 2002, 22:8026-8034.
7. Bae E, Calhoun VC, Levine M, Lewis EB, Drewell RA: Characterization of the intergenic RNA profile at *abdominal-A* and *Abdominal-B* in the *Drosophila* bithorax complex. *Proc Natl Acad Sci USA* 2003, 99:16467-16472.
8. Shippy TD, Ronshaugen M, Cande J, He J, Beeman RW, Levine M, Brown SJ, Denell RE: Analysis of the *Tribolium* homeotic complex: insight into mechanisms constraining insect Hox clusters. *Dev Genes Evol* 2008, 218:127-139.
9. Brena C, Chipman AD, Minelli A, Akam M: Expression of trunk Hox genes in the centipede *Strigamarpa* *maritima*: sense and anti-sense transcripts. *Evolution Dev* 2006, 8:252-265.
10. Koch M: Monophyly of the Myriapoda? Reliability of current arguments. *Proceedings of the 12th International Congress of Myriapodology (Afri Inverts 2003)*, 44:137-153.
11. Shiga Y, Sagawa K, Takai R, Sakaguchi H, Yamagata H, Hayashi S: Transcriptional readthrough of Hox genes *Ubx* and *Antp* and their divergent post-transcriptional control during crustacean evolution. *Evolution Dev* 2006, 8:407-414.
12. Janssen R, Prpic N-M, Damen WGM: Gene expression suggests decoupled dorsal and ventral segmentation in the millipede *Glomeris marginata* (*Myriapoda: Diplopoda*). *Dev Biol* 2004, 268:89-104.
13. Doehle W: Die Embryonalentwicklung von *Glomeris marginata* (Villers) im Vergleich zur Entwicklung anderer Diplopoden. *Zool Jb Anat* 1964, 81:241-310.
14. Kadner D, Stollewerk A: Neogenesism in the chilopod *Lithobius forficatus* suggests more similarities to chelicerates than to insects. *Dev Genes Evol* 2009, 219:249-264.
15. Hughes CL, Kaufman TC: Exploring the myriapod body plan: expression patterns of the ten Hox genes in a centipede. *Development* 2002, 129:1225-1238.
16. Damen WGM, Hausdorff M, Seyfarth EA, Tautz D: The expression pattern of Hox genes in the spider *Cupiennius salei* suggests a conserved mode of head segmentation in arthropods. *Proc Natl Acad Sci USA* 1998, 95:10665-10670.
17. Eriksson BJ, Tait NN, Budd GE, Akam M: The involvement of *engrailed* and *wingless* during segmentation in the onychophoran *Euperipatoides kanangensis* (*Peripatopodae*: Onychophora) (Reid 1996). *Dev Genes Evol* 2009, 219:249-264.
18. Wolff C, Sommer R, Schroder R, Glase G, Tautz D: Conserved and divergent expression aspects of the *Drosophila* segmentation gene *hunchback* in the short germ band embryo of the flour beetle *Tribolium*, *Development* 1995, 121:4227-4236.
19. Janssen R, Damen WGM: The ten Hox genes of the millipede *Glomeris marginata*. *Dev Genes Evol* 2006, 216:451-465.
20. Prpic N-M, Tait NN: The expression of the proximal/distal axis patterning genes Distal-less and dachshund in the appendages of *Glomeris marginata* (*Myriapoda: Diplopoda*) suggests a special role of these genes in patterning the head appendages *Dev Biol* 2003, 260:97-112.
21. Janssen R, Budd GE, Damen WG, Prpic N-M: Evidence for Wg-independent tergite boundary formation in the millipede *Glomeris marginata*. *Dev Genes Evol* 2008, 218:361-370.
22. Cook CE, Smythe ML, Telford MJ, Bartanello A, Akam M: Hox genes and the phylogeny of the arthropods. *Curr Biol* 2001, 11:59-763.
23. Doehle W: Progoneata. In *Spreizelle Zoologie Teil 1: Einzeller Und Wirbellosa* Tiere Edited by: Westheide W, Rieger R, Stuttgart: Jena; Gustav Fischer Verlag. 1996:592-600.
24. Edgecombe GD: Arthropod phylogeny: An overview from the perspective of morphology, molecular data and the fossil record. *Arth Struct Dev* 2010, 39:74-87.
25. Shear WA, Edgecombe GD: The geological record and phylogeny of the Myriapoda. *Arth Struct Dev* 2010, 39:174-190.
26. Regier JC, Wilson HM, Shultz JW: Phylogenetic analysis of Myriapoda using three nuclear protein-coding genes. *Mol Phylo Evol* 2005, 34:147-158.
27. Gai Y-H, Song D-X, Sun H-Y, Zhou K-Y: Myriapod monophyly and relationships among myriapod classes based on nearly complete 28S and 18S rDNA sequences*'. *Zool Sci* 2006, 23:101-110.118.
28. Loesel R, Strausfeld NJ: Common design in a unique midline neuropil in the brains of arthropods. *Arth Struct Dev* 2002, 31:77-91.
29. Strausfeld NJ, Strausfeld CM, Loesel R, Rowell D, Stowe S: Arthropod phylogeny: onychophoran brain organization suggests an archaic relationship with a chelicerate stem lineage. *Proc Roy Soc B* 2006, 273:1857-1866.
30. Mazo A, Hodgson JW, Petruk S, Sedkov Y, Broch HW: Transcriptional interference: an unexpected layer of complexity in gene regulation, *J Cell Sci* 2007, 120:2755-2761.
31. Petruk S, Sedkov Y, Brock HW, Mazo A: A model for initiation of mosaic Hox gene expression patterns by non-coding RNAs in early embryos. RNA Biol 2007, 4:1.
32. Douris V, Telford MJ, Averof M: Evidence for multiple independent origins of trans-splicing in Metazoa. Mol Biol Evol 2010, 27:684-693.
33. Osato N, Suzuki Y, Ikeo K, Gotoh T: Transcriptional interferences in cis natural antisense transcripts of humans and mice. Genetics 2007, 176:1299-306.
34. Tusie-Luna C, Stanley JA, Garrick D, Sharpe JA, Assyub H, Wood WG, Higgs DR: Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. Nat Genet 2003, 34:157-165.
35. Crampton N, Bonass WA, Kirkham J, Rivetti C, Thomson NH: Collision events between RNA polymerases in convergent transcription studied by atomic force microscopy. Nucleic Acids Res 2006, 34:5416-5425.
36. Okamura K, Balla S, Martin R, Liu N, Lai EC: Two distinct mechanisms generate endogenous siRNAs from bidirectional transcription in Drosophila melanogaster. Nat Struct Mol Biol 2008, 15:998.
37. Wienholds E, Plasterk RHA: MicroRNA function in animal development. FEBS Lett 2009, 579:5911-5922.
38. Mouton L, Nong G, Preston JF, Ebert D: Variable-number tandem repeats as molecular markers for biotypes of Pasteuria ramose in Daphnia spp. App Environ Microbiol 2007, 73:3715-3718.
39. Lai EC: Micro RNAs are complementary to 3’ UTR sequence motifs that mediate negative post-transcriptional regulation. Nat Genet 2002, 30:363-364.
40. Ellis JR, Burke JM: EST-SSRs as a resource for population genetic analyses. Heredity 2007, 99:125-132.
41. Kaufman TC, Lewis R, Wakimoto B: Cytogenetic analysis of chromosome 3 in Drosophila melanogaster: The homeotic gene complex in polytene chromosome interval 84A-B. Genetics 1980, 94:115-133.
42. Regier JC, Shultz JW, Zwick A, Hussey A, Ball B, Wetzer R, Martin JW, Cunningham CW: Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. Nature 2010, 463:1079-1083.
43. Kelsh R, Weinzierl RO, White RA, Akam M: Homeotic gene expression in the locust Schistocerca: an antibody that detects conserved epitopes in Ultrabithorax and abdominal-B. Dev Genet 1994, 15:19-31.
44. Mann RS, Hogness DS: Functional dissection of Ultrabithorax protein in D. melanogaster. Cell 1990, 60:597-610.
45. Ronshaugen M, McGinnis N, McGinnis W: Hox protein mutation and macroevolution of the insect body plan. Nature 2002, 415:914-917.
46. Galant R, Carroll SB: Evolution of a transcriptional repression domain in an insect Hox protein. Nature 2002, 415:910-913.

doi: 10.1186/2041-9139-1-4
Cite this article as: Janssen and Budd, Gene expression suggests conserved aspects of Hox gene regulation in arthropods and provides additional support for monophyletic Myriapoda. EvoDevo 2010, 1:4