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Sequence and Expression of Grasshopper Antennapedia: Comparison to *Drosophila*

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We have cloned and characterized the *Antennapedia* (*Antp*) gene from the grasshopper *Schistocerca americana.* The Antennapedia protein contains seven blocks of sequence, including the homeodomain, that are conserved in the homologous proteins of other insects, interspersed with (usually repetitive) sequences unique to each species. There is no similarity between 1.8 kb of 3′ untranslated sequence in grasshopper and *Drosophila.* We examined Antennapedia protein expression in grasshopper using an antibody raised against a grasshopper fusion protein and reexamined its expression in *Drosophila* using several different antibodies. Early patterns of expression in the two insects are quite different, reflecting differing modes of early development. However, by the germband stage, expression patterns are quite similar, with relatively uniform epithelial expression throughout the thoracic and abdominal segments which later retracts to the thorax. Expression is observed in muscle pioneers, the peripheral nervous system, and the central nervous system (CNS). In the CNS expression is initially limited to a few neurons, but eventually becomes widespread. Both insects show strong expression in certain homologous identified neurons and similar temporal modulation of expression. © 1995 Academic Press, Inc.

INTRODUCTION

In *Drosophila,* maternal and segmentation gene products act in succession to control division of the embryo into progressively narrower zones, until the final number of segments has been achieved (Akam, 1987; Ingham, 1988). Concurrent with, and following, segmentation, the identity of the segments is determined by the homeotic genes. Progress in understanding the gene interactions underlying these developmental processes has been rapid, and there has been a tendency to generalize from *Drosophila* to other organisms. However, even among insects, *Drosophila* is unusual, for it is a ''long germ'' insect, in which the initial germ anlage divides simultaneously into the final number of segments to form the germband. In addition, more embryonic pattern formation takes place while the embryo is syncytial than in many other insects. While germ length alone is not a critical consideration, the Diptera are generally agreed to be among the most evolutionarily advanced insects (Kristensen, 1991).

The grasshopper, a ''short germ'' insect, lies near the other end of the continuum of germ lengths and shows a pattern of development commonly believed to be primitive (e.g., Sander, 1976), although other authors (e.g., Anderson, 1972a) have argued that the intermediate germ type is primitive. In short germ insects the initial germ anlage contains a defined head region, but the remaining body forms from a posterior proliferative zone. Various experimental manipulations suggest that this proliferative zone is not prepatterned and that segments form sequentially as the embryo grows (Anderson, 1972a,b; Sander, 1976; Akam and Dawes, 1992; Patel, 1993, 1994a). Thus, the grasshopper provides a particularly valuable comparison to all of the advanced holometabolous insects for which comparative gene expression data are now becoming available.

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The expression patterns of the grasshopper homologs of the pair-rule genes *even-skipped* (*eve*) (Patel *et al.,* 1992) and *fushi tarazu* (*Dax*) (Dawes *et al.,* 1994), the segment polarity gene *engrailed* (Patel *et al.,* 1989a, 1989b), and the homeotic genes *abdominal-A* (*abd-A*) (Tear *et al.,* 1990), *Abdominal-B* (*Abd-B*) (Kelsh *et al.,* 1993), and *Ultrabithorax* (*Ubx*) (Kelsh *et al.,* 1994) have been described and many of the genes have been partially or completely sequenced. All of these genes are expressed in both the ectodermal epithelium and the nervous system. With the exception of the two pair-rule genes, which both show neural expression that is similar to that of their *Drosophila* homologs but lack pair-rule expression, there is a fundamental similarity to the expression patterns of these genes between grasshopper and *Drosophila.* This similarity may seem surprising, given the quite different mechanisms of formation of abdominal segments in the two systems, but it is consistent with the suggestion of Sander (1988) that ''gene interactions guiding development up till the germband stage might differ more between various insect forms than the genes active in the germband itself.'' This suggestion is based on the concept of a conserved phylotypic stage, the germband stage, at which the embryo has become overtly metamerized and at which all embryonic arthropods are relatively similar (Sander, 1983).

The homeotic genes of *Drosophila* are organized into two complexes, the anterior-acting ANT-C and the posterior-acting BX-C. The BX-C contains the *Ubx, abd-A,* and *Abd-B* genes, all of whose homologs have been characterized in the grasshopper. No grasshopper homologs of members of the *Drosophila* ANT-C have yet been characterized in detail, although from partial sequence and *in situ* hybridization data it is clear that a homolog of *Sex combs reduced* is present (Akam *et al.,* 1988). Here we describe grasshopper *Antennapedia,* the first grasshopper representative of the ANT-C to be studied in detail. The *Antennapedia* gene, for which the complex is named, acts to determine the thoracic character of ectoderm, mesoderm, and nervous system in *Drosophila* (Kaufman *et al.,* 1990 and references therein).

Our results fall into three broad categories. First, since *Antp* homologs from several species have been sequenced, the predicted proteins can be compared with respect to conserved motifs and in relation to the experimental manipulations of the protein that have been done in *Drosophila.* The structure of the Antp protein in three species of *Drosophila* is so similar that little could be inferred about functionally important parts of the molecule (Hooper *et al.,* 1992), but addition of data from the grasshopper allows recognition of seven conserved regions interspersed with nonconserved, largely repetitive regions. Second, we characterize Antp expression during grasshopper development using *in situ* hybridization and polyclonal antibodies raised against a grasshopper Antennapedia fusion protein. For comparison, and to clarify some apparent differences, we reexamine Antp expression in *Drosophila.* Last, we compare Antp expression in the grasshopper central nervous system (CNS) and peripheral nervous system (PNS) with that in *Drosophila,* where possible at the level of identified neurons.

MATERIALS AND METHODS

Embryos

Schistocerca americana eggs were laid in damp sand and incubated at 28–30°C. Embryos were staged using the criteria of Bentley *et al.* (1979). *Drosophila* embryos were raised using standard techniques and fixed and processed in bulk. Staging of embryos was as described by Campos-Ortega and Hartenstein (1985).

Library, Probes, Hybridization Conditions, and Sequencing

Two clones were isolated from the Kai Zinn λ gt11 cDNA library prepared from 40–50% embryos of *Schistocerca americana* (Snow *et al.,* 1988) by their homology to a homeobox-containing fragment of a *Drosophila even-skipped* cDNA supplied by Tim Hoey and Michael Levine. In order to obtain clones which extended further 5′, three additional clones were isolated from the same library using fragments of one of the first isolates. For sequencing, cDNA inserts were subcloned into Bluescript KS(+) (Stratagene) or pEMBL8+. Randomly sheared fragments were sequenced using Sequenase (USB) or *Taq* polymerase (Promega). Sequence analysis was performed using the ANALYSEQ programs (Staden, 1984) and the MacVector package of programs (IBI).

Fusion Proteins

A 675-bp *Eco*RI fragment containing the *Schistocerca Antp* homeobox was subcloned into the glutathione S-transferase expression vector, pGEX-3X (Pharmacia). The plasmid was then transformed into RRI cells. A culture was grown from a single colony, induced, and lysed, and the protein was purified from the supernatant by addition of glutathione agarose beads (Sigma), from which it was eluted.

Antibodies and Immunocytochemistry

Polyclonal antibodies against the *Schistocerca* fusion protein were raised in rats using standard techniques (Harlow and Lane, 1988). Immunocytochemistry was as described in Patel (1994b) with additional techniques as noted below. Three antibodies to *Drosophila* Antp were available to us: the polyclonal antiserum of Carroll *et al.* (1986) and the 4C3 and 8C11 monoclonal antibodies of Condie *et al.* (1991). On initial staining the Carroll and 4C3 antibodies showed only subsets of the expression shown by MAb 8C11, but on optimization of staining conditions, all three antibodies showed similar patterns of expression. Because MAb 8C11 provides the cleanest and most sensitive result, our descriptions are

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based on this antibody. Primary antibodies used in addition to anti-Antp, and their specificities, are as follows: 4D9, engrailed/invected protein (Patel *et al.,* 1989b); 3B9, even-skipped protein (Patel *et al.,* 1994); I-5, an uncharacterized antigen specific to developing neurons and muscle pioneers in grasshopper (Chang *et al.,* 1983); 22C10, uncharacterized antigen specific to cytoplasm of developing neurons in *Drosophila* (Fujita *et al.,* 1982); and 8G7, uncharacterized antigen specific to CNS axons and some parts of the PNS in grasshoppers (M.J. Bastiani and C.S. Goodman, unpublished reagent). Embryos were photographed with and without DIC on Panatomic-X, T-Max 100, or Ektachrome 64. Some images were also captured directly from video.

This study has made us keenly aware of the difficulties inherent in attempting to completely describe the expression pattern of a gene. In *Schistocerca,* our original (unpublished) observations based on fixation times of 20–30 min of expression were much more restricted than presented here. Once we discovered that our antiserum was extremely sensitive to fixation time, which needs to be kept to no more than 10 min, detectable expression was greatly increased. It is possible that better antibodies would reveal that the situation in the grasshopper is even more similar to that in *Drosophila* than described here, particularly in regard to expression in the PNS.

In Situ Hybridization

Nonradioactive *in situ* hybridizations were carried out according to the protocols of Tautz and Pfeifle (1989) and Patel and Goodman (1992). Template DNA consisted of a 1.8-kb fragment from the 3′ UTR of the *Antp* gene, cloned into the *Eco*RI site of Bluescript KS(+), which was then linearized with *Sma*I or *Eco*RV. Labeled anti-sense and sense DNA probes were then produced using T3 and T7 primers, respectively. We did not attempt to establish the first appearance of *Antp* message due to the technical difficulty of *in situ* hybridization to such early embryos.

RESULTS

Cloning and Sequence Analysis

Two initial clones were isolated by screening the Kai Zinn λ gt11 cDNA library prepared from 40–50% *Schistocerca* embryos. The longer insert, 2.35 kb, was sequenced in full and contains a homeobox sequence that codes for a homeodomain identical to that of *Drosophila melanogaster* Antp. A portion of the 5′ end of this clone was used to rescreen the same library at higher stringency and three more overlapping clones were obtained. The clone with the furthest 5′ extension was sequenced in turn. A long open reading frame extends to the 5′ end of the cDNA, but the clone includes the presumed translation start site, the sequence MSSYF, as identified by comparison to other homeotic genes which frequently begin with this sequence (Scott *et al.,* 1989). Figure 1A shows the deduced amino acid sequence of this protein. The arrowed methionine in Figure 1A marks the presumed translation start site. The coding region is rich in repeats, with stretches of glycine (GGC), alanine (GCG, GCC), and glutamine (CAG) residues upstream of the homeodomain, which lies near the C-terminal end of the protein. There is a long untranslated 3′ trailer of 1.8 kb which is probably incomplete, as neither a polyadenylation signal nor a poly(A) tail is present.

Expression of *Antp* mRNA in Grasshopper Embryos

The *Antp* mRNA distribution in grasshopper embryos at approximately 32, 40, and 42% of development (Fig. 2) is basically consistent with the distribution of *Antp* mRNA and protein in *Drosophila,* with *Antp* mRNA in young embryos absent from the head and present in the trunk epithelium from the posterior labial segment (S3) down the length of the body (Fig. 2A). It is, however, absent from the appendages in S3. In older embryos, mRNA is abundant in the thoracic epithelium and nervous system (Figs. 2B and 2C) and appears to tail off in abundance down the length of the abdominal nervous system.

Expression of Antp Protein in Drosophila Embryos

The actions and interactions of the segmentation and segment identity genes are best understood in the *Drosophila* embryo, which serves as a baseline to which the expression patterns of homologous genes in other insects can be compared. Our findings on Antp expression in the grasshopper, especially in the PNS, led us to reexamine expression in *Drosophila* using similar methods and reagents (except for the primary antibody) to facilitate direct comparison. Figure 3 shows Antp expression in developing *Drosophila* (A–G) and *Schistocerca* (H–M). In *Drosophila,* the *Antp* gene is transcribed from two promoters (Kaufman *et al.,* 1990) whose contributions to the overall expression pattern have been characterized by Bermingham *et al.* (1990) using *in situ* hybridization. Their paper thus provides an interesting comparison to the antibody results presented here, since it separates the contributions of the two promoters and examines the distribution of message rather than protein.

Based on the work of many previous authors, the single stripe of Antp protein seen in the gastrulating embryo in Fig. 3A is in parasegment (PS) 4. By stage 7 (Fig. 3B) there are two relatively strong stripes in the future neural region. Weaker expression associated with the anterior stripe forms a complete ring around the embryo. There is also lateral expression in the posterior abdomen (arrowhead), centered on PS14, which presumably corresponds to mRNA in PS14 at an earlier stage (Fig. 3A of Bermingham *et al.,* 1990). Expression then becomes more nearly uniform throughout the epithelium at stages 10 and 11, except that it is most intense in PS 4–6 (Fig. 3E) and extends anteriorly to the anterior border of PS 3 in the neural epithelium (Fig. 3D, left-pointing arrowhead) as demonstrated by double staining with anti-engrailed (not shown). Within

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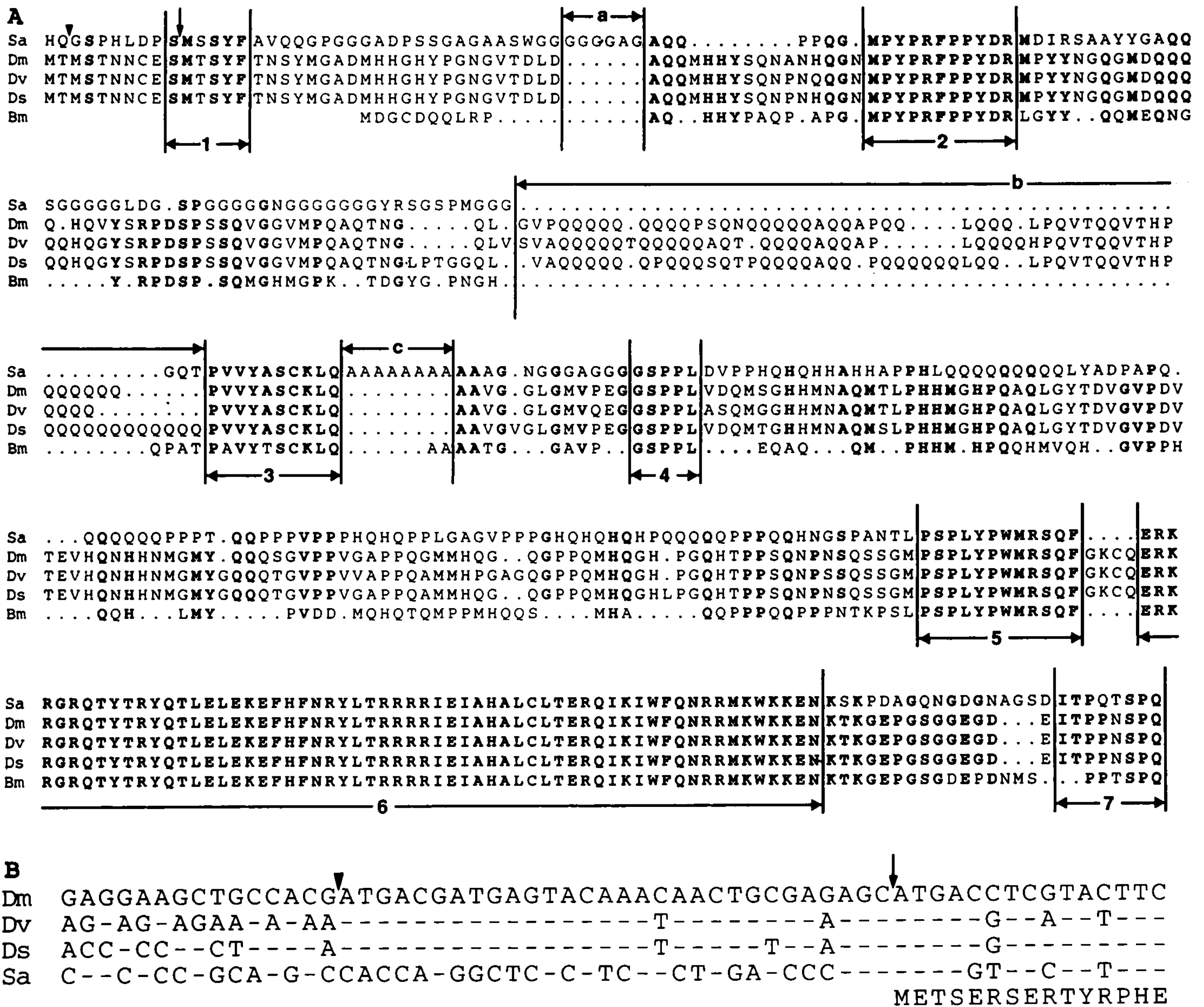

FIG. 1. The *Schistocerca Antennapedia* nucleotide and deduced amino acid sequences compared to homologous sequences from three species of *Drosophila* (Hooper *et al.,* 1992) and the moth *Bombyx mori* (Genbank Accession No. D16684). Abbreviations: Sa, *Schistocerca americana,* Dm, *Drosophila melanogaster,* Dv, *Drosophila virilis,* Ds, *Drosophila subobscura,* Bm, *Bombyx mori.* (A) Comparison of the derived amino acid sequences of the Antp protein from *Schistocerca,* from three species of *Drosophila,* and from *Bombyx mori.* Alignments were established by fitting the *Schistocerca* and *Bombyx* data to the alignments determined for the three *Drosophila* species by Hooper *et al.* (1992) in order to maximize agreement. Identities extending beyond the *Drosophila* species are shown in bold. Dots indicate spaces put in to achieve a best fit. The presumed translation start site in grasshopper and possibly in the *Drosophila* species is indicated by the vertical arrow. Sequence upstream of the arrow is included due to uncertainty about the actual start site. The arrowhead marks the *Drosophila* translation start site suggested by Hooper *et al.* (1992). Letters located above the sequence indicate areas where the grasshopper and fly sequences differ, apparently mainly due to the insertion of repetitive nucleotide sequences. Numbers below the sequence indicate areas of high sequence conservation which are discussed in more detail in the text. (B) Comparative nucleotide sequence data do little to clarify the true site of translation initiation. The sudden shift from nonconserved to conserved sequence at the first ATG (arrowhead) in all of the *Drosophila* species might indicate it as the translation start site (Hooper *et al.,* 1992). However, the only ATG in this region of the grasshopper sequence (arrow) corresponds to the second ATG in all of the *Drosophila* species and this ATG is associated with the amino acid sequence MXSYF, which is commonly found near the translation start site of HOM/Hox genes (Hooper *et al.,* 1992).

these parasegments, expression is modulated by the activities of several other genes (Figs. 3D–3G). By stages 11–12 (Figs. 3E and 3F), abdominal epithelial expression fades while expression in the CNS becomes more intense (Fig. 3F). As the general epithelial expression fades, the nuclei of neurons and support cells in the PNS become apparent (Fig. 3F). In stages 13–16, neural expression continues to rise and expression in the epithelium outside the thorax

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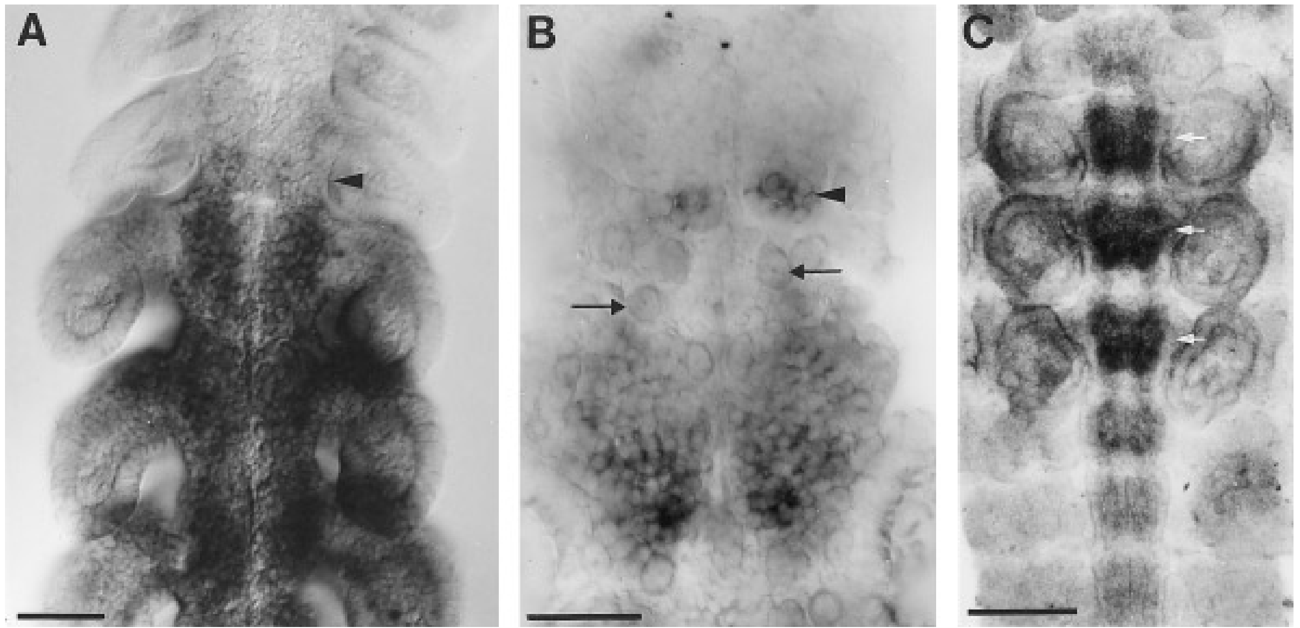

FIG. 2. Nonradioactive *in situ* hybridization reveals the distribution of *Antp* mRNA in the grasshopper. (A) At 32% of embryonic development, hybridization is found in the posterior part of the labial segment (arrowhead) as well as in the thoracic limbs and throughout the thorax. (B) The anterior thorax of a 40% embryo shows that message is present in some neuroblasts (arrows), as well as in their neural progeny, and that message extends into the posterior portion of the labial segment (arrowhead). (C) A 42% embryo shows high concentrations of message in the three thoracic segments (arrows), a lower concentration in the central nervous system of the posterior third of the labial segment, and a falling but still significant concentration down the length of the anterior abdominal central nervous system. Scale bar, A, 200 μm; B, 100 μm; C, 1 mm.

continues to fall (Figs. 3G and 5D). By stage 17, the CNS is retracted and Antp expression in all tissues has fallen considerably (not shown).

Early Expression of Antp Protein in Grasshopper Embryos

The earliest Antp expression is at 19–20% of embryonic development (Fig. 3H). At this stage, embryo morphology is changing rapidly and the future thoracic segments are recognizable only as bulges in the body wall and by engrailed expression, which is present as well-formed stripes in the thorax and subesophageal segments and is just about to appear in the developing abdomen (Patel *et al.,* 1989a). Antp expression at this stage is not sharply delimited, but is clearly present in the metathorax (T3), the widest part of the body (Fig. 3H, arrowhead). The abdomen has begun to form posterior to this point. The thoracic segment in which expression begins seems to vary: in some cases expression comes on in all thoracic segments simultaneously, while in others a particular thoracic segment appears to lead. During the next 1–2% of development the abdomen grows into a paddle-shaped, unsegmented structure and the three thoracic segments become clearly demarcated (Fig. 3I). At this stage expression is usually present in all three thoracic segments, with a narrow, darker-staining border one or two cells wide at the anterior edge of each (shown at a slightly later stage in Fig. 3J). Expression fades out gradually down the length of the abdomen (Fig. 3I). The anterior limit of early epithelial expression appears to be segmental (Figs. 3I and 3J), although relatively soon the anterior border of expression in T1 becomes somewhat ragged (Fig. 3J).

As the embryo grows, the limb buds extend and express Antp throughout their epithelium as they do so (Fig. 3K). As abdominal segmentation occurs, Antp expression spreads gradually posteriorly, starting at the anterior edge of each forming segment. It is never strong in unsegmented regions. The dimensions of a segment are usually clear before Antp expression is apparent. The pattern of a narrow, segmentally repeated anterior stripe of more intensely expressing cells is repeated down the length of the abdomen. At 25–30% of development, neuroblasts appear to be weakly expressing. Expression in neuroblasts and ganglion mother cells is less obvious than in neurons, but *Antp* RNA is clearly present in neuroblasts as revealed by *in situ* hybridization (Fig. 2B, arrows). By ~30% of development, small clumps of cells in the ventral midline at the anterior border of each segment begin to stain more darkly (not shown). By 30–35%, neural expression is apparent in the thoracic segments. In S3, there is a U-shaped pattern of expressing neurons (not shown) extending anteriorly beyond the overlying ectodermal parasegment boundary,

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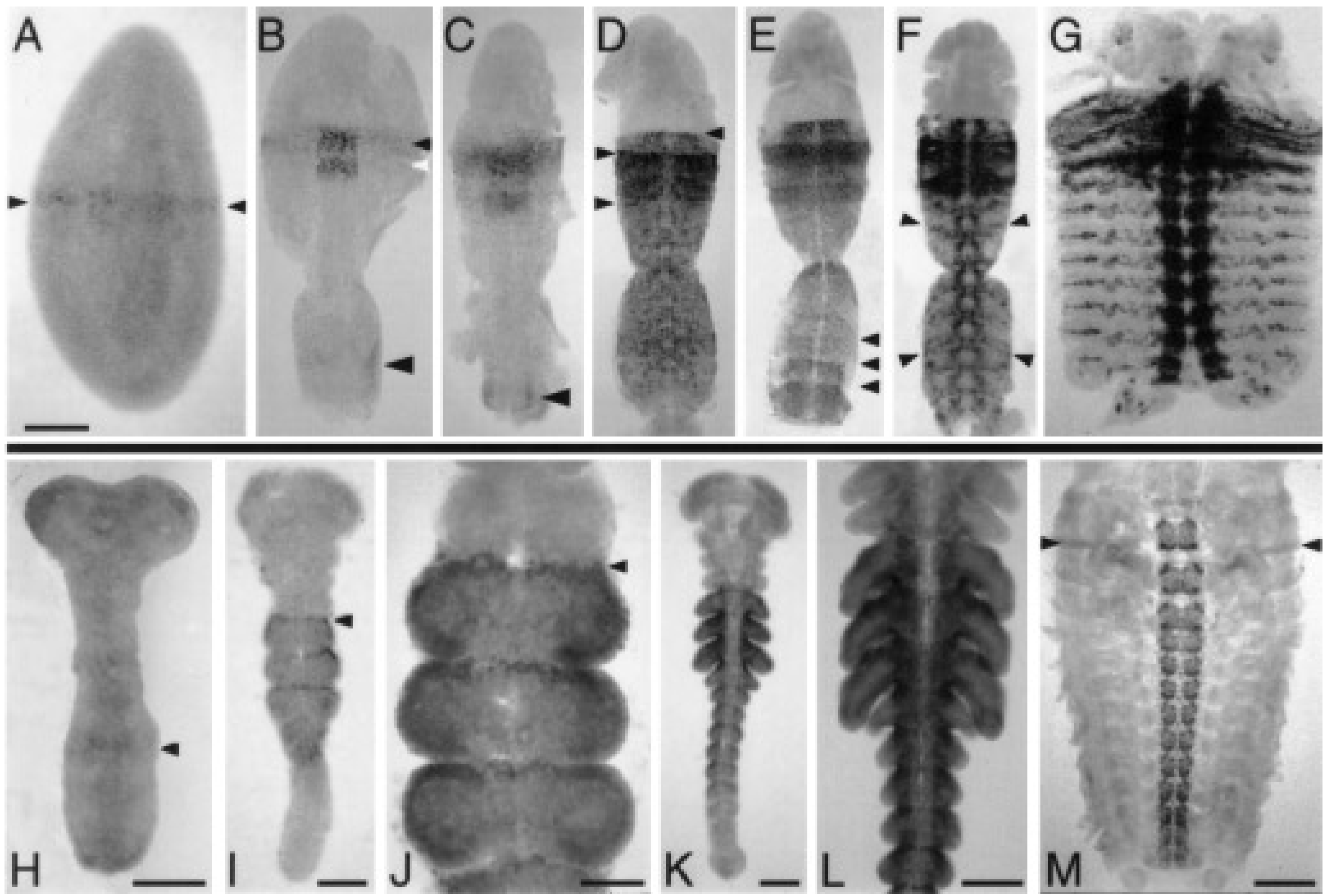

FIG. 3. Comparative distribution of Antp protein during embryonic development in *Drosophila* (A–G; A intact, B–G dissected) and grasshopper (H–M) embryos. (A) at stage 6 in *Drosophila,* there is a single band of protein in parasegment (PS) 4 (arrowheads). (B) At stage 7 there is a broad band of expression in PS 4 (small black arrowhead), a narrower band, with most expression in the ventral midline, in PS 6 (small white arrowhead), and lateral expression, on the sides of the abdomen, in PS 14 (large black arrowhead). (C) At stage 8 the bands of ventral expression have broadened and are less sharply defined and the expression in PS 14 is stronger (arrowhead). (D) By stage 10 there is Antp expression throughout the epithelium posterior to the anterior margin of PS 3 (arrowhead pointing to left), with particularly intense, modulated, expression in PS4 and PS5 (delimited by arrowheads pointing to the right). (E) At stage 11 epithelial expression in the abdomen begins to fade. Segment boundaries are visible at this stage (arrowheads). (F) At stage 11–12 expression continues to fade in the epithelium outside of PS 4–6 at the same time as it is increasing in the CNS. The nuclei of peripheral neurons and some associated support cells appear (arrowheads), as general epithelial expression fades. (G) At stage 13 the CNS and the segmentally repeated cells of the PNS are clearly apparent, while epithelial staining has retreated to the thorax. (H) In grasshopper at 19–20% of embryonic development, before a clearly defined abdomen is visible, the first expression is apparent in the area that will become the metathorax (T3) (arrowhead). (I) At 22% an abdomen is clearly visible. Expression appears uniform across the thoracic segments, ending abruptly at the segment boundary anteriorly (arrowhead) and fading away gradually posteriorly. (J) Slightly later epithelial expression at the anterior boundary of T1 becomes ragged (arrowhead), as shown here for a 24% embryo. A more intensely expressing band of cells at the anterior border of each segment is also clearly visible in this embryo. (K) At 29% all of the thoracic segments are expressing strongly and expression is spreading down the abdomen along with segmentation. (L) At 35% expression remains strong in the same pattern. (M) At 42% CNS expression is strong, but epithelial expression has become limited to the thorax, especially the T1–T2 boundary (arrowheads). Scale bar, A and G, 125 μm; B–F, 100 μm; H–L, 200 μm; M, 500 μm.

which will ultimately mark the anterior border of neural Antp expression. In a few intensely stained older preparations (e.g., Fig. 4C, arrow), weakly staining neurons can still be found in this area. Abdominal epithelial expression continues strongly until 37–38%, when it gradually fades. Some peripheral neurons have already become apparent before it starts to fade (Fig. 5A). From ~40%, CNS expression is strong, but epithelial expression has faded away except in the thoracic segments, especially T2–T3 (Fig. 3M, arrowheads). At no time, until expression starts to fade, have we seen modulation of the level of epithelial Antp expression in the grasshopper, either in the trunk, as occurs in *Drosophila* (Fig. 3, especially D–G), or in the limbs, as occurs with grasshopper UBX (Kelsh *et al.,* 1994).

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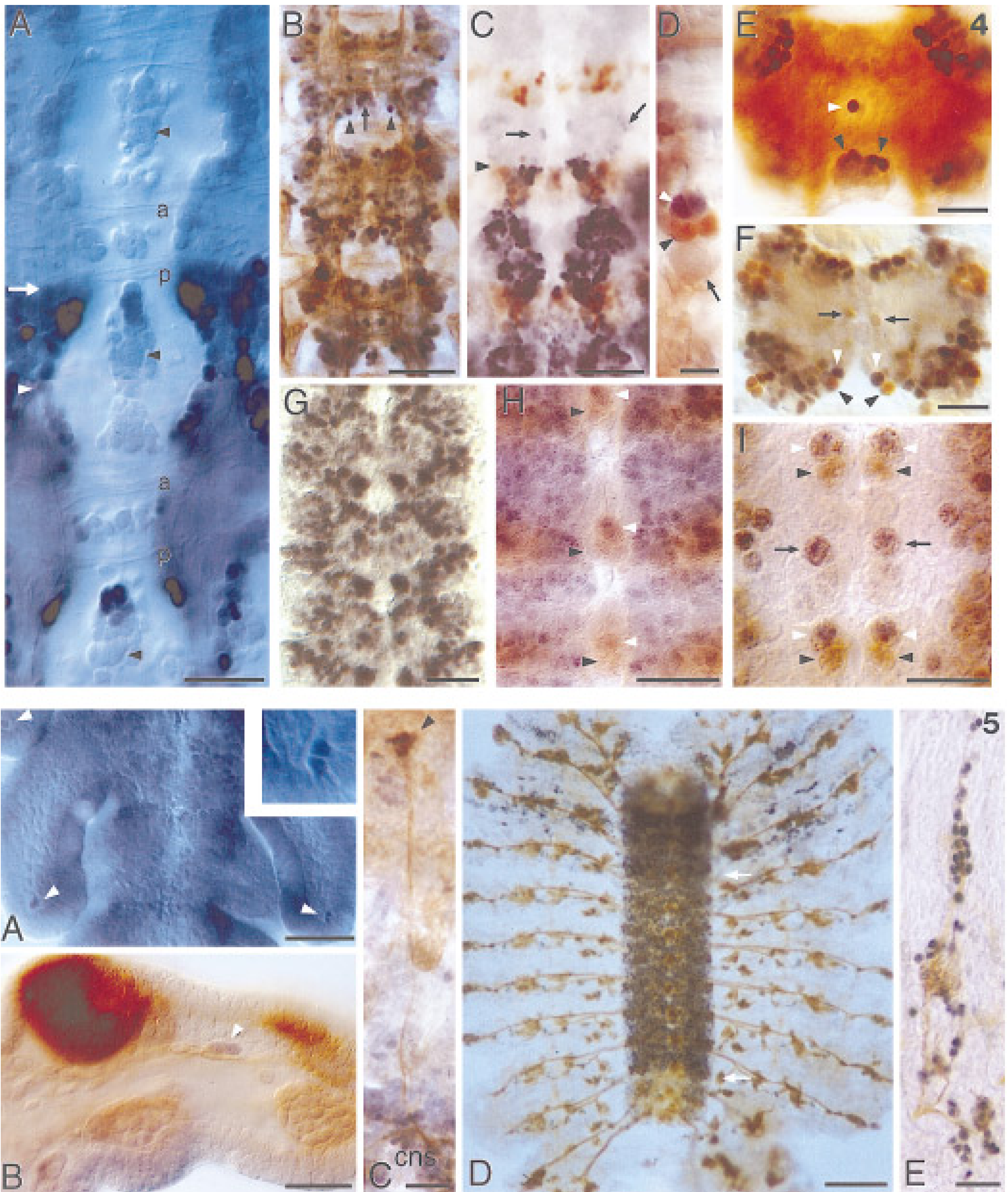

FIG. 4. Antp expression in the central nervous system of grasshopper (A–F) and *Drosophila* (G–I). (A) In the anterior thorax at 39% in the grasshopper embryo, homologous neurons on the two sides of the parasegment boundary in the labial segment (white arrow) are quite different with respect to Antp expression. Thus, none of the progeny of the median neuroblast (the serially homologous median neuroblasts are marked with a black arrowhead in each segment) express Antp in the anterior-most PS shown, nor do any of the lateral neurons,

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The antibody also fails to show any clear posterior inhibition of epithelial Antp expression such as is seen for abd-A in grasshopper (Tear *et al.,* 1990).

Comparison of *Drosophila* (Figs. 3A–3G) and *Schistocerca* (Figs. 3H–3M) Antp expression would seem to lend considerable support to the idea that the germband represents a conserved stage and that genes expressed at this stage show considerable similarities in their expression patterns (Sander, 1983). Thus, a stage 11 *Drosophila* embryo, in the interval shown in Figs. 3D–3E, would be comparable to a fully segmented grasshopper embryo of ~30% (i.e., slightly older than the one shown in Fig. 3K). For some time beyond this point in development, the similarities in Antp expression appear great, with CNS and PNS expression increasing as epithelial expression gradually becomes limited to the thorax.

Expression of Antp Protein in the Grasshopper CNS

As described above, from 30–40% of embryonic development, a segmentally repeating pattern of intensely expressing neurons begins to appear, associated with neuroblasts and ganglion mother cells in the ventral area that will be occupied by the CNS. Because the neural staining arises against a background of ectodermal staining, it is difficult to give an order in which the progeny of various neuroblasts begin to stain. However, the pattern is nonrandom within a ganglion, and serially homologous neurons show a sequential turning on of expression of the Antp protein, with expression appearing first in thoracic segments T2 and T3 and then in T1 and sequentially down the length of the abdomen. At ~40%, neural expression is just appearing in the terminal ganglion (not shown).

The sharp suppression of Antp expression, even in homologous neurons, anterior to the posterior commissure in the labial segment (Fig. 4A, white arrow), is the most striking feature of Antp expression in the CNS (Figs. 3M and 4A). This cutoff is also coincident with the parasegment boundary in the ectoderm, as determined by double staining with anti-Antp and anti-engrailed (Fig. 4C, arrowhead). However, suppression of Antp expression is not total, for a few weakly staining neurons may sometimes be seen anterior to the boundary (Fig. 4C, arrows). Initially, Antp expression is limited to specific neurons (Figs. 4A, 4C, 4E, and 4F), but by 45–50% it is widespread and there is some degree of expression in most, if not all, neurons posterior to the parasegment boundary in S3 (Fig. 4B).

Early in embryonic development, the situation at the midline is particularly clear because the ganglia are still relatively two dimensional. At 39%, there is no expression in midline cells in S2 (Fig. 4A), but from S3 posteriorly the anterior-most cells in the group associated with the median neuroblast (MNB, arrowheads) express Antp strongly. The

although their serial homologs in posterior S3 and T1 do. The white arrowhead marks the location of the S3–T1 segment boundary. a, anterior commissure; p, posterior commissure. (B) anterior abdominal ganglia at 47% stained with anti-Antp (black) and I-5 (brown). By this age most neurons are expressing Antp, although at highly varying levels. aCC (arrowheads) and the anterior progeny of the MNB (arrow) can be readily recognized by their intense staining. (C) Anterior CNS of an embryo double stained for the presence of engrailed (brown) and Antp (black). The anterior border of Antp staining clearly corresponds to the parasegment boundary in the overlying epithelium, as indicated by the coincident cutoff of engrailed and Antp in the labial ganglion (black arrowhead). Usually the cutoff of Antp staining at this point appears total. However, in this intensely stained preparation, a few staining cells are apparent anterior to the cutoff (arrows). Note also the high percentage of Antp-expressing cells posterior to the boundary. (D) Median neuroblast and associated cells. The anterior-most cells are stained with anti-Antp (white arrowhead), then there is a zone of cells staining with anti-engrailed (black arrowhead), and posteriorly the weakly stained MNB (arrow). (E) An abdominal ganglion stained with anti-Antp (black) and 8G7 (brown, labeling neural cytoplasm) showing a single, intensely expressing VUM cell (white arrowhead). Antp-expressing progeny of the MNB are apparent in the posterior midline of the ganglion (black arrowheads). (F) An abdominal ganglion stained with anti-Antp (black) and anti-even-skipped (brown). Identified neurons include aCC, clearly staining with anti-Antp (white arrowheads), pCC (black arrowheads), and RP2 (black arrows). Both of the latter neurons do express Antp weakly, although only anti-eve staining is clearly apparent here. (G) The great majority of *Drosophila* neurons stain to different degrees with anti-Antp at stage 13. (H) Double staining of the *Drosophila* CNS at stage 14 with anti-Antp and anti-en reveals a staining pattern in the group of cells associated with the MNB similar to that seen in the grasshopper (cf. D), with the anterior-most cells in the group expressing Antp (white arrowheads) and a more posterior zone of cells expressing en (black arrowheads). This experiment was done using an en–lacZ line. Thus, some brown staining is also seen in Antp-positive cells since the lacZ protein persists longer than en protein. (I) Stage 16 *Drosophila* embryo double labeled for Antp (purple) and even-skipped (brown) showing that aCC (white arrowheads) expresses Antp in *Drosophila* just as its homolog does in grasshopper (F). pCC (black arrowheads) shows obvious staining only for anti-even-skipped, as is true of grasshopper (F). In this *Drosophila* embryo Antp expression in RP2 (black arrows) is more apparent than it is in *Schistocerca* (cf. F). Scale bars: A, E, and F, 50 μm; B and C, 100 μm; D, G, H, and I, 20 μm.

FIG. 5. Antp expression in the peripheral nervous system (PNS) of grasshopper (A–C) and *Drosophila* (D–E). (A) The first Antp expression in the PNS is in the paired Ti1 pioneer neurons as they emerge from the ectoderm at the tip of each leg (white arrowheads). (Inset) These cells at higher magnification in another preparation. (B) Ti1 pioneer neurons (arrowhead), after their proximal migration, express Antp in a prothoracic leg at 40%; anti-Antp (purple) and I-5 (brown) double stain. (C) Dorsal body wall sense organs in the wall of the abdomen expressing Antp at 39% (arrowhead), anti-Antp/I5 double stain; cns, central nervous system. (D) Stage 16 *Drosophila* embryo double stained with anti-Antp (black) and 22C10 (brown). Two steps are clearly apparent in the intensity of Antp expression in the CNS (arrows). (E) PNS of *Drosophila* abdominal segment double stained with anti-Antp (black nuclei) and 22C10 (brown, to reveal the associated neural tissue). All of the sensory neurons stain as do the accessory cells of the ES organs. Scale bars: A, 100 μm; B, C, and D, 50 μm; E, 20 μm.

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more posterior cells in this group also express, but they do so at a much lower level. At this stage, there are six to seven intensely Antp-expressing cells in this group in each of the thoracic and anterior abdominal ganglia, with the number then falling progressively in the posterior abdominal ganglia, reflecting their more recent formation. The number of intensely Antp-expressing cells in this group continues to rise as the group grows in number and becomes assimilated into the ganglion (Fig. 4E, black arrowheads).

Examination of the CNS of a 45% embryo reveals a pattern of neurons in the ventral midline similar to that described for the H cell and its sibling (Bate *et al.,* 1981) in that some segments contain two strongly expressing neurons, others a strong and a weakly expressing neuron, a single expressing neuron (Fig. 4E), or none at all. The aCC neuron expresses from the time it is clearly identifiable and shows strong and consistent expression up until at least 45% (Figs. 4B and 4F). The sibling pCC neuron never achieves the levels of Antp protein seen in aCC, but it does express the protein later in development. At least three of the four clumped cells Q1, Q2, G, and C stain early and strongly with Antp (not shown), and RP2 also expresses the protein, but never strongly.

Expression of Antp Protein in the Drosophila CNS

Antp expression is widespread in the CNS of *Drosophila,* although it varies greatly over time (Figs. 3F, 3G, 4G, and 5D). As in the epithelium, regulation by other genes is clearly apparent (Fig. 5D), to a greater extent than in the grasshopper CNS. As in the grasshopper, there is a sharp cutoff of Antp staining in the CNS that corresponds to the ectodermal parasegment boundary in S3. Staining neurons are abundant from PS3 posteriorly at stage 13 (Fig. 4G).

Of the easily recognized identified cells, it is only a few in the central part of the group of cells associated with the MNB that we have never observed staining. At the midline, the anterior-most cells in the MNB bundle express Antp strongly (Fig. 4H, white arrowheads), just as they do in *Schistocerca* (Fig. 4D, white arrowhead), while posterior to them lies a set of cells which express engrailed (black arrowhead). The aCC, pCC, and RP2 neurons all stain at some stage during their development (Fig. 4I).

Expression of Antp Protein in the Grasshopper PNS

The first peripheral neurons to express Antp at a detectable level are the paired Ti1 pioneer neurons, which are the first neurons to differentiate and appear at the tips of the limb buds at ~31% of embryonic development (Fig. 5A). These neurons migrate proximally to the mid-tibia region of each leg later in embryonic development (Fig. 5B). Presumably the epithelial progenitors of other early neurons are also staining at this time, but it is only these cells at the tip of the limb that are recognizable as neurons. Later, as other neurons move from the ectoderm into the lumen of the leg, they also express Antp. Antp expression in the PNS shows clear temporal modulation, since previously expressing cells no longer stain and new cells appear as development proceeds. In addition, there are staining nuclei associated with the developing subgenual organ (sgo) and femoral chordotonal organ (fco) (Fig. 6A). Some of these nuclei belong to neurons, but others may be the nuclei of support cells.

Sensory neurons of the body wall also express Antp from the time they appear. Figure 5C shows the dorsal body wall (dbw) cells (Meier *et al.,* 1991) in the wall of an abdominal segment at 40%, while Fig. 6E shows the dbw cells plus the wing chordotonal organ (sr) in the T3 body wall at 45%.

Expression of Antp Protein in the Drosophila PNS

Scattered strong nuclear expression becomes apparent in the periphery at stage 11 as epithelial expression is fading (shown at stage 12 in Fig. 3F) and well before there is any sign of neural staining with either the anti-HRP or 22C10 antibodies (which stain developing axons as they appear in the periphery). By stages 14 and 15, when PNS axons have developed, it is apparent that the nuclei belong to peripheral neurons and their associated support cells (Figs. 5D and 5E). In the abdomen we find all of the sensory neurons previously described by Bodmer *et al.* (1989) and Merritt and Whitington (1995) as well as a few additional staining nuclei, which belong to the accessory cells of ES organs (Fig. 5E). Although all neuronal nuclei stain, the ch nuclei appear to stain less strongly than the others.

Expression of Antp Protein in Muscle Pioneers and other Mesoderm Cells in Grasshopper Embryos

Antp expression in mesoderm nuclei associated with the muscle pioneers (mps) (Ho *et al.,* 1983) of the legs begins not long after their appearance (Ball *et al.,* 1985) and the number of Antp-expressing nuclei continues to grow along with the muscle pioneers (Fig. 6A). We cannot yet explain the pattern of this expression, as there are mp nuclei present before Antp expression begins, and with double staining it appears that some of the nuclei belong to the mps (Figs. 6B, 6C, and 6D), while others belong to the surrounding mesoderm cells, which will later fuse with the mps (not shown).

Antp expression in the body wall mesoderm shows a simple pattern. The first nuclei to express form a row down the length of the body on either side of the midline, with a single nucleus at the anterior edge of each segment (arrowheads, Figs. 6C and 6D). These are the nuclei of the TM1 mps (Xie *et al.,* 1992). Next to stain is a group of nuclei on the dorsal body wall (Fig. 6E, black arrowheads), associated with the DM1 mps (Xie *et al.,* 1992). Some of these nuclei appear to belong to the mps and others to adjacent mesoderm cells which will fuse with the mps.

DISCUSSION

As cDNA sequences for homologous homeobox-containing genes from a variety of species have become avail-

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able, conserved regions of the encoded proteins outside the homeodomains have been revealed (Bürglin, 1994). These conserved regions are typically at the N-terminal end of the protein or just upstream or downstream of the homeodomain (Wright *et al.,* 1989; Acampora *et al.,* 1989; Bürglin, 1994). The sequencing of *Antp* from three distantly related species of *Drosophila* has provided a comparison of homologous homeodomain-containing proteins from within a single insect genus (Hooper *et al.,* 1992). However, this analysis revealed such a high degree of sequence conservation that the comparison did not reveal conserved domains that are likely to be important for Antp protein function.

It is now possible to add data from two more distantly related insects, the moth *Bombyx* (Genbank Accession No. D16684) and the grasshopper *Schistocerca* (this paper), to this comparison, with the result that highly conserved regions emerge (Fig. 1). From the N-terminal end, the first conserved region is M(S/T)SYF (region 1), a motif that was noted by Hooper *et al.* (1992) as commonly occurring at or near the N-terminus of homeotic gene products. The sequence data from three *Drosophila* species led Hooper *et al.* (1992) to suggest that translation probably started at the next ATG upstream of this motif (arrowhead, Figs. 1A and 1B) because of the sharp change from nucleotide divergence to conservation at this point (Fig. 1B). *Schistocerca,* however, has only a single ATG in this area (at the point indicated by the arrow), and 3′ from this point nucleotide conservation between *Drosophila* and *Schistocerca* is good. The *Drosophila* translation initiation consensus sequence, (C/A)AA(A/C)AUG (Cavener, 1987), is of little help in choosing between the two potential initiation sites, since the *Drosophila* species agree with it equally poorly at both sites. Thus, in the absence of protein sequence data we are left with several alternatives: (1) all four species start translation at the M in MXSYF (arrow, Figs. 1A and 1B); (2) all three *Drosophila* species start translation at the ATG (arrowhead, Figs. 1A and 1B) a short distance 5′ to the putative grasshopper start site; or (3) the grasshopper protein might be significantly larger, since the reading frame remains open for another 258 nucleotides (as far as we have data) 5′ to the putative translation start site (arrowed in Fig. 1B) without any further ATGs.

No functions are known for conserved domains 2, 3, and 4. The conserved peptide YPWM in region 5 is commonly found just upstream of the homeodomain (Scott *et al.,* 1989) and is present in all of the Antp sequences shown in Fig. 1. However, alteration of the equivalent peptide in three different ways in HOXB7, which shares 59 of 60 amino acids with the homeodomain of *Drosophila* Antp, did not prevent binding of the altered protein to DNA, nor did similar alterations to the *Drosophila* protein prevent activation of the *Ultrabithorax* promoter (Baier *et al.,* 1991). The homeodomain itself (domain 6) is highly conserved, being identical in all of the insect species shown, and differing by only two or three amino acids in presumably homologous genes in the honeybee, *Apis* (Walldorf *et al.,* 1989), and the brine shrimp, *Artemia* (Averoff and Akam, 1993). At the C-terminal end of the protein (domain 7), the insects also show sequence conservation, and this part of the molecule does have demonstrated functional importance (see below).

If we assume a common ancestral sequence, a substantial portion of the difference between the insect sequences is due to highly repetitive insertions of glycine (region a) and alanine (region c) residues in the grasshopper and mainly of glutamine residues (region b) in *Drosophila.*

Hooper *et al.* (1992) observed "dramatic sequence conservation of much of the 3′ nontranslated part" of mRNAs in three *Drosophila* species. However, we find no significant conservation in the 3′ untranslated region between grasshopper and *Drosophila.*

Relation of Conserved Antp Protein Sequences to Deletion and Substitution Experiments

An attractive hypothesis would be that regions of amino acid sequences have been conserved where they are important for protein function. In the case of *Drosophila,* experiments by Gibson *et al.* (1990) have provided a direct test of this hypothesis by ubiquitously expressing various *Antp* gene constructs in transformed flies and observing the effect on segmental identity. The results of these experiments support the hypothesis that nonconserved portions of the protein are dispensable, in that the glutamine-rich portion (area b), which is the one extended area of difference between the *Drosophila* and grasshopper proteins, can be deleted without apparent effect.

The N-terminal region of the protein has a general potentiating effect, thus determining overall levels of Antp activity, with the area around the conserved MXSY essential for activity in all cells. The agreement between the *Drosophila* and grasshopper proteins at their C-terminal ends is particularly gratifying, as domain switching experiments between Antp and Scr proteins indicate that this region is important for functional specificity (Gibson *et al.,* 1990). Functional studies by Zeng *et al.* (1993) and Chan and Mann (1993) have confirmed and refined these results, establishing that it is the N-terminal end of the homeodomain and the short sequence of amino acids just C-terminal to the homeodomain that are critical to the segment identity functions of *Drosophila* Antp. However, the mouse gene *Hoxb-6* (as *Hox-2.2*), which has little similarity to *Drosophila Antp* outside of the homeobox, can functionally substitute for it in *Drosophila* overexpression assays (Malicki *et al.,* 1990). Our present assays may still be inadequate to determine the function of all of the conserved regions of the Antp protein.

Antp Protein Expression in Drosophila and Grasshopper

Although the overall pattern of Antp expression and its tissue specificity are broadly conserved between *Drosophila* and grasshopper there are some interesting differences. In order to examine these differences in detail, and to attempt to better understand the different staining patterns initially produced by different antibodies to *Drosophila* Antp, we

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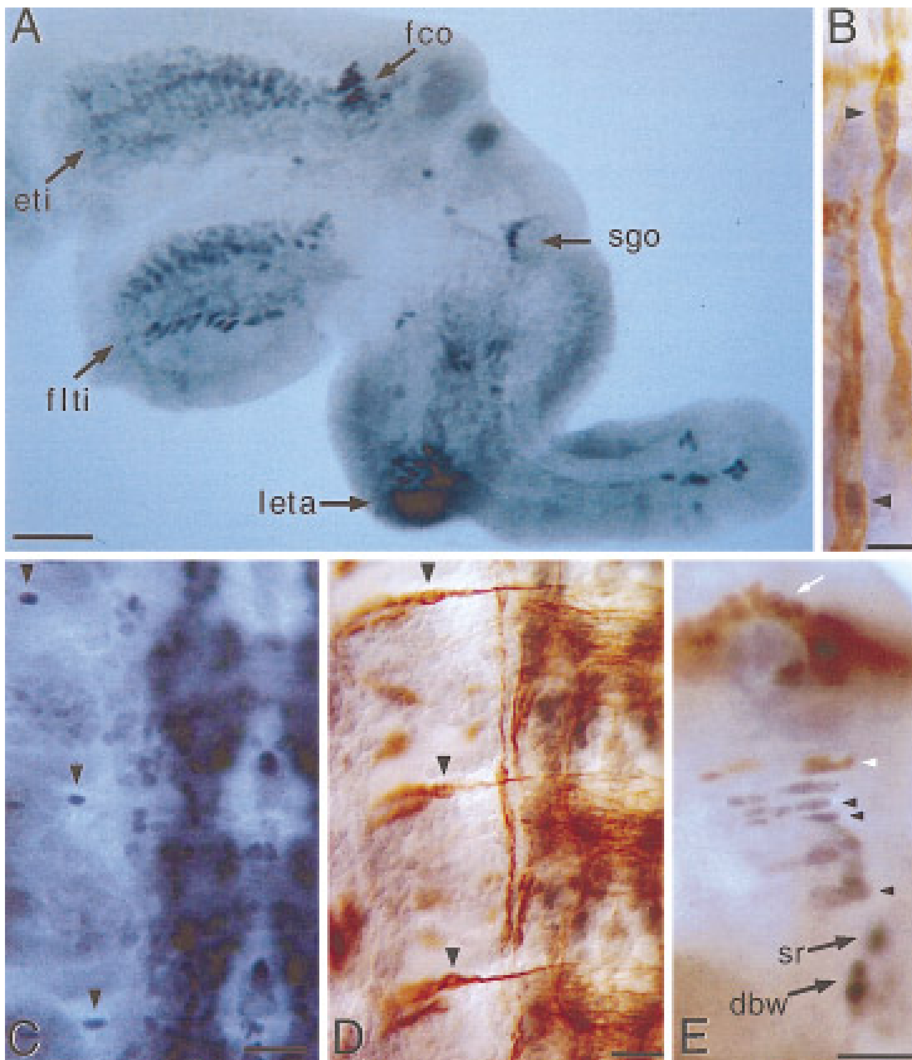

FIG. 6. Antp expression in muscle pioneers of grasshopper. (A) Muscle pioneers, pioneer neurons, and sense organs expressing Antp in a metathoracic leg at 45%. Muscle pioneers include those of the extensor tibiae (eti), flexor tibiae (flti), and levator tarsus (leta). Obvious sense organs include the subgenual organ (sgo) and the femoral chordotonal organ (fco). The other expressing cells are pioneer neurons or sensory neurons. (B) Muscle pioneers in the thoracic body wall double stained with anti-I5 (reddish-brown) and anti-Antp. Grey, Antp-expressing nuclei (arrowheads) are clearly apparent within the cells. (C) CNS and adjacent body wall in a 40% embryo stained with anti-Antp. The three large nuclei in the body wall (black arrowheads) belong to TM1 muscle pioneers. This is clearly shown in the embryo in (D) which is double-stained with anti-Antp (grey nuclei) and I-5 (brown cytoplasm) to show the full extent of the cells associated with these nuclei. (E) Dorsal part of the thoracic body wall. Anti-Antp (grey) is expressed in dorsal body wall sense organs (dbw), in the stretch receptor (sr), and in pioneers of the dorsal longitudinal muscles (black arrowheads). Anti-eve (brown) stains other dorsal longitudinal muscles (white arrowhead) and tissue associated with the heart (white arrow). Scale bars: A, 100 μm; B, 25 μm; C, D, and E, 50 μm.

reexamined Antp expression in *Drosophila* using techniques and reagents as similar as possible to those used for the grasshopper.

There have been numerous previous studies on the distribution of Antp protein in *Drosophila,* so our studies mainly allow us to fill in details in comparison to Antp expression in the grasshopper. There are three observations that appear particularly worthy of discussion. (1) At stages 10–11, Antp staining extends down the length of the abdomen in both the lateral and neurogenic epithelium. Thus, at the extended germband stage, Antp expression in *Drosophila* and grasshopper appears quite similar, extending throughout the thoracic and abdominal epithelium. (2) Antp expression in the posterior CNS is never fully suppressed, but remains at a low level, consistent with the distribution of *Antp* transcripts (Bermingham *et al.,* 1990). (3) All nuclei of peripheral neurons in the thorax and abdomen from parasegment 3 posteriorly express Antp, as do the nuclei of several of the

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accessory cells of the ES organs. These peripheral nuclei become apparent as epithelial expression fades.

This universality of expression in the PNS, as seen with MAb 8C11, contrasts with our own initial observations using other antibodies and with previous reports that only subsets of *Drosophila* peripheral neurons express Antp. Clearly, these subsets comprised those cells containing the most Antp protein. Based on these results, we may have a comparable situation in the grasshopper, with the PNS cells that we describe here being those that express the most Antp protein at a given time, while the remainder are expressing at levels below what we can detect. Similarly, our ability to detect earlier expression in the grasshopper may have been limited by the signal-to-noise ratio that we could achieve. With those caveats, there are still several differences between Antp expression in *Drosophila* and grasshopper. First, the early patterns of expression (Figs. 3A–3C and 3H–3J), which lead up to the relatively similar patterns at the germband stage (Figs. 3D, 3E, 3K, and 3L), are quite different. Second, once segments begin to be demarcated there also appears to be a difference in the anterior limit of expression in the two systems. The anterior limit of expression in the neurogenic ectoderm of *Drosophila* is the parasegment boundary, apparently from the time of its appearance [Carroll *et al.,* 1986 (for protein); and Bermingham *et al.,* 1990 (for mRNA)]. In the grasshopper, in contrast, the earliest well-defined expression appears to be segmental (Fig. 3I). Only later do Antp-expressing cells appear in S3, in a pattern quite different from that seen in *Drosophila.* A simple explanation for this result could be that our grasshopper antibody lacks sensitivity and that the two systems are similar. Under this hypothesis, a weakly Antp-expressing epithelium in S3 would give rise to the more strongly expressing neurons that we describe above. However, the anterior border of Antp expression appears sharp and strong, while posteriorly expression fades away gradually (Fig. 3H), thus indicating that there is at least a substantial step in expression levels at the anterior border of T1. A third difference is that while on a gross scale the patterns of epithelial expression are similar, with widespread expression (Figs. 3D, 3E, 3K, and 3L) gradually becoming limited to the thorax (Figs. 3F, 3G, and 3M), the grasshopper shows none of the modulated expression that is so prominent in the thoracic epithelium of *Drosophila* (Figs. 3D–3G).

Modulation of Antp expression in the nervous system is also much more apparent in *Drosophila* (e.g., Fig. 5D), although it does occur in the grasshopper as well. In the *Drosophila* CNS there are two distinctly stepped reductions in Antp expression (Fig. 5D, arrows). The anterior step disappears on removal of the BX-C (Hafen *et al.,* 1984; Wirz *et al.,* 1986), and from its position could be mediated by Ubx. Removal of the BX-C also derepresses transcription from the P2 promoter in PS 13-14 [probably by removing Abd-B repression (Boulet and Scott, 1988)], but transcription from the P1 promoter remains repressed in these parasegments (Bermingham *et al.,* 1990). The same patterns of inhibition are present in the grasshopper, but are less apparent because the ganglia are separate and much larger by the time posterior modulation appears. Thus, by 70%, expression has disappeared from formerly expressing cells in the last two abdominal ganglia (which represent primitive ganglia 7–11), presumably corresponding to the posterior modulation in *Drosophila,* and by hatching, Antp-expressing neurons are numerous only in T1 and T2 and greatly reduced in number elsewhere, corresponding to the situation in the last stages of *Drosophila* embryogenesis (e.g., Fig. 3D of Carroll *et al.,* 1986).

The pattern of Antp expression in the anterior-most neurons associated with the MNB is of particular interest in view of recent findings of Condron and Zinn (1994) concerning the origin of the cells within this common sheath. They find that the MNB produces neuronal ganglion mother cells for 3–4 divisions (28–31%), then glial precursors for 8–10 divisions from 31–39%, and then goes back to producing neuronal GMCs. All of the progeny of the MNB initially express engrailed, but this expression persists only in a subset of neural progeny. We have examined both Antp and engrailed expression, individually and in double-stained preparations. We confirm the results of Condron and Zinn with engrailed and find that, in embryos at 39–40%, Antp levels are very high in the anterior-most six to eight cells (Figs. 4A and 4D), and much lower in the more posterior cells, which Condron and Zinn have shown to be glia. We see an essentially identical pattern of expression of these two transcription factors in *Drosophila* (Fig. 4H), suggesting that the same mechanisms may be working to determine neuronal and glial cell fate in both systems.

Similarities and Differences of Grasshopper and Drosophila Development

The germband stage of embryonic development probably represents an evolutionarily constrained, or phylotypic, stage, for it is at, and for some time after, this stage that embryonic insects exhibit maximum morphological similarity. This similarity is reflected at the molecular level, with expression patterns of the homeotic genes, as well as the segment polarity genes, similar in all of the insects studied thus far (Patel, 1994a). *Schistocerca* and *Drosophila,* however, exhibit modes of early development apparently very different, both morphologically and mechanistically, and it seems likely that the way in which the expression of the homeotic and segment polarity genes is initiated differs between them.

Early development in *Drosophila* is controlled by asymmetrically distributed signals, provided maternally, and a cascade of spatially restricted, zygotically produced transcription factors, acting in a syncytium. The same mechanism cannot occur in *Schistocerca* development, where at least the patterning of the abdominal segments occurs after the initiation of gastrulation, in a cellular environment. There are also differences at the molecular level. In *Drosophila,* the zygotic pair-rule genes, *eve* and *ftz,* play a key role in establishing the segmental pattern of *engrailed* expression. The pair-rule genes and gap genes also act to refine the boundaries of homeotic gene expression. However, the grasshopper homologs of the *Drosophila eve* (Patel *et al.,*

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1992) and *ftz* genes (Dawes *et al.,* 1994) have no obvious pair-rule patterns of expression and therefore probably do not play the same roles that they do in *Drosophila.* One possibility is that other pair-rule genes serve their function in the grasshopper. Obvious candidates are the homologs of the *Drosophila* genes *hairy* and *runt.*

Looking at the expression of genes higher up the hierarchy may provide clues about the earliest stages of grasshopper development. The expression pattern of grasshopper *hunchback* is of particular interest for several reasons. First, *hunchback* plays a role in determining the expression patterns of *Antp* and other homeotic genes in *Drosophila* (Harding and Levine, 1988; Irish *et al.,* 1989), so if it plays a similar role in the grasshopper it must be appropriately distributed. Second, a gradient of hunchback protein plays a key role in initiating segmentation in the *Drosophila* embryo. A distribution inconsistent with the formation of such a gradient would seem to indicate an important difference in the control of development in the two systems. Considerably more comparative data, both for other genes and other insects, will be necessary to allow informed speculation on whether the apparent differences between the modes of development exhibited by these two insects are significant and, if they are, which is closer to the ancestral pattern.

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