Class 3 Hox genes in insects and the origin of *zen*

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ABSTRACT We have cloned, from a beetle and a locust, genes that are homologous to the class 3 Hox genes of vertebrates. Outside the homeobox they share sequence motifs with the *Drosophila zerknullt* (*zen*) and *z2* genes, and like *zen*, are expressed only in extraembryonic membranes. We conclude that the *zen* genes of *Drosophila* derive from a Hox class 3 sequence that formed part of the common ancestral Hox cluster, but that in insects this (Hox) gene has lost its role in patterning the antero-posterior axis of the embryo, and acquired a new function. In the lineage leading to *Drosophila*, the *zen* genes have diverged particularly rapidly.

The Hox genes encode homeodomain transcription factors that specify the fate of cells during development (1, 2). In insects, vertebrates, and probably most other metazoans they are used to specify the position of cells along the antero-posterior axis of the embryo (3). Different Hox genes are expressed in each region of the body axis, from a precise anterior limit. The genes are clustered, and for reasons that are not yet understood, the order of the genes along the chromosomes parallels their spatial sequence of expression along the body.

The conservation of gene sequence, chromosomal order, and colinear expression suggests that many of the distinct subclasses of Hox genes arose before the insect and vertebrate lineages split. The Hox class 3 genes are an exception. Vertebrate and cephalochordate class 3 genes show characteristics of structure and expression typical of other Hox genes (4), but *Drosophila* contains no recognizable class 3 gene. Any such gene would be expected to lie between proboscipedia (*pb*) (a class 2 homolog) and Deformed (*Dfd*) (class 4) in the *Drosophila* Antennapedia complex (5). This position is occupied by three homeobox genes that have no close homologs in the vertebrate Hox clusters. These genes do not show colinear expression, and do not specify axial position during embryogenesis. Two of them, *zen* and its paralog *z2*, are expressed specifically in extraembryonic membranes (*zen* at least is necessary for the formation of these membranes) (6). The third, *bicoid*, is expressed only during oogenesis, and encodes a maternal RNA that is localized to the anterior pole of the egg (7). The origin of these three genes has not been clear. They encode homeodomains that are divergent with respect to all other sequences (8), and outside the homeodomains there is no strong similarity to other proteins in the homeotic complexes.

We report here the cloning from other insects of genes that specifically link the class 3 Hox genes and *zen*. One of these, *Sgzen*, derives from an Orthopteran, the grasshopper *Schistocerca*, a distant relative of *Drosophila*. The other, *Tc zen*, derives from the beetle *Tribolium*, a closer relative, but still 250 million years distant. The sequences of the encoded homeodomains place these genes in the Hox 3 subfamily. However, they share sequence motifs outside the homeodomains with the *Drosophila zen* genes, and like *zen*, are expressed in

extraembryonic tissues. In addition, the *Tribolium zen* homolog is located in the homeotic complex in the expected position of a Hox 3 class gene.

MATERIALS AND METHODS

Isolation and Analysis of cDNAs. The grasshopper cDNA was isolated from a library constructed as described (9) using poly(A)⁺ RNA extracted from whole eggs at 35% stage of development. Subpools of this library were screened using a set of degenerate primers designed to amplify Antennapedia class homeoboxes until a single positive clone was obtained (10). The portion of the clone surrounding the homeobox was sequenced on both strands using a transposon insertion strategy (United States Biochemical). The sequence of the *Sgzen* open reading frame is available in the EMBL database (accession no. X92654). A genomic fragment of *Tc zen* was amplified using degenerated primers designed to recognize empty spiracles class homeoboxes. The resulting fragment was cloned, sequenced, and used to screen a λ ZAP (Stratagene) cDNA library prepared from poly(A)⁺ RNA of 0–96-hr-old *Tribolium* embryos. A single recombinant, identified in a screen of 1.5×10^5 plaques from an amplified aliquot of the library, was recovered by mobilizing the plasmid from the vector as described by the manufacturer and the insert sequenced by a shotgun procedure. The sequence of the *Tc zen* open reading frame is available in the EMBL database (accession no. X97819).

DNA Isolation and Southern Blot Analysis. Nuclei were isolated from 0.84 g of Lab-S *Tribolium* pupae (11) embedded in 10 ml of 0.75% InCert agarose (FMC) and lysed with Proteinase K and SDS. For enzyme digestion, 60 μ l of InCert agarose was melted and the included DNA was digested with *Bss*HII or *Nhe*I (Promega). Each digest was divided between three lanes and large DNA fragments were resolved in a 1% pulsed-field certified-agarose (Bio-Rad) gel at 10°C in a contour clamped homogeneous electrophoretic field system (CBS Scientific, Del Mar, CA) using a switching time of 7–45 sec, ramped over 29 hr. The gel was blotted for >24 hr to Micron Separations magnacharge nylon (following the manufacturer's instructions), UV crosslinked, and hybridized at 65°C (6 $\times$ SSC/10 $\times$ Denhardt's solution/25 mM phosphate buffer, pH 7.0/0.5% SDS/10% PEG/200 μ g/ml denatured, sonicated herring testes DNA). Radiolabeled probes were generated by random priming (Promega).

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from *Schistocerca* eggs, dissected embryos, and extraembryonic membranes using RNazol (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. The entire amount of resulting RNA was separated by denaturing gel electrophoresis, blotted to a nylon membrane, and hybridized with the 4.5 kb

Sgzen cDNA. Hybridization was estimated by densitometric analysis of the digitized autoradiogram (NIH Image).

In Situ Hybridization and Immunocytochemistry. The *Tc zen* cDNA was linearized at the 5' end and used as template to synthesize digoxigenin-labeled anti-sense riboprobe (BRL). Hybridization to 0–12-hr embryos, detection, and documentation were performed as described (12). The anti-Sgzen antibody was generated by ligating a 647-bp *XmnI*/*PstI* fragment from the Sgzen gene encoding part of the homeodomain and the C terminus of the protein, into pQE31 (Quiagen, Chatsworth, CA). The fusion protein, containing a 6-histidine tag in frame with the Sgzen peptide, was overexpressed in *Escherichia coli* cells, purified on a nickel column, and used to immunize rabbits. The serum was affinity purified using the same fusion protein. The purified antibody was used to immunolocalize the Sgzen protein in whole mount *Schistocerca* embryos, dissected embryos, and extraembryonic membranes as described (13).

RESULTS

The grasshopper and beetle cDNAs were identified using degenerate homeobox primers. The 4.5-kb *Schistocerca* and

0.8-kb *Tribolium* cDNAs encode homeodomains that are most similar to the vertebrate and cephalochordate Hox 3 genes (12–14 mismatches) with which they share most of the class 3 diagnostic residues (Fig. 1A). With other homeodomains they show at least 19 differences (19–22 mismatches with Hox 2, *pb*, or Antennapedia (*Antp*) and 20–22 with *zen* and *z2*). To assess the relationship between these genes more rigorously we analyzed phylogenetically a matrix containing class 2, class 3, Sgzen, *Tc zen*, and the *Drosophila zen* homeodomains. A heuristic algorithm produced a single most parsimonious tree (Fig. 1B) that grouped Sgzen and *Tc zen* with the vertebrate and cephalochordate class 3 sequences, and placed in separate branches the class 2/*pb* and the *Drosophila zen* genes. When this tree is rooted with other classes of Hox genes the *zen* branch is associated with either class 2 or class 3 genes, reflecting the high divergence of the *Drosophila zen* sequences.

Although the homeodomains of these new insect genes are not highly similar to *zen*, they share sequence motifs outside the homeodomain, which indicate that they are *zen* homologs. Three motifs in the beetle and grasshopper proteins, one near the amino terminus and the others downstream of the homeodomain, are conserved to varying extents with *zen* and *z2* (overlined in Fig. 1C and D, respectively). Unlike most other

FIG. 1. (A) Comparison of the homeodomains encoded by Sgzen and *Tc zen* with the most closely related homeodomains, encoded by class 2 and class 3 genes, and with the *Drosophila zen* genes (14, 15). Dashes indicate identity with *Antp*. Asterisks mark sequences reported as the consensus of a paralogy group. The highlighted residues are well conserved within classes, but differ between classes and thereby distinguish class 3 from class 2 homeodomains. Sgzen and *Tc zen* are relatively divergent in the helix 2 region (residues 30–40), but retain most of the diagnostic class 3 residues at the N and C termini of the homeodomain. Short PCR fragments isolated from the horseshoe crab (*Limulus*) suggest that better conserved class 3 genes are retained in other arthropod groups. (B) Maximum parsimony tree generated by PAUP 3.0Q (16) for a matrix of homeodomain peptide sequences from mouse (mHoxB3), human (mHoxB3 and mHoxA2), Amphioxus (AHoxB3), grasshopper (Sgzen), beetle (*Tc zen*), and fruitfly (*zen*, *z2*, and *pb*). The consensus tree from 500 bootstrap replicates using the heuristic search function with all default options was identical to the most parsimonious tree. Bootstrap values greater than 50% are indicated to the left of each node. The tree is unrooted. (C) Alignment of N-terminal regions of the insect *zen* proteins. Sequences were aligned using Clustal W (17). A region near the N terminus (overline) is conserved to various extents in each protein. Amino acids shared by two or more proteins are shown in boldface type. (D) Alignment of two conserved motifs downstream of the homeodomain. Each sequence is shown from the residue immediately following the homeodomain. Conserved sequence blocks (overlines) in the insect *zen* genes were identified by Blockmaker (18) using the blossom 62 substitution matrix. Dots indicate gaps required to align the blocks and additional residues at the C termini. Amino acids shared by two or more proteins are shown in boldface type.

homeotic proteins, the homeodomain is located near the amino terminus in all four insect proteins. They all also lack the hexapeptide motif, the four central residues (YPWM) of which are conserved in most Hox proteins (8).

The *Tribolium zen* Homolog Is Located in the HOMC. Long-range Southern mapping was performed with the *Tc zen* cDNA and genomic fragments including either the *Tribolium pb* homolog, maxillopedia (*mxp*) (unpublished data), or *Tc Dfd* (19). The *mxp* and *Tc zen* (but not *Tc Dfd*) probes hybridize with a 70-kb *Bss*HII fragment (Fig. 2A), whereas *Tc zen* and *Tc Dfd* (but not *mxp*) probes identify a 100-kb *Nhe*I fragment (Fig. 2B). Thus, the beetle and fly *zen* homologs lie between *pb/mxp* and the *Dfd* homologs. Since *pb/mxp* and *Dfd* are orthologous to the Hox 2 and 4 subfamilies, respectively, these *zen* genes occupy the position expected for an insect Hox 3 gene.

Expression of *zen* Homologs in Beetle and Grasshopper Embryos. In both insects and vertebrates, most Hox genes are first expressed after formation of the body axis (20), in the developing trunk. The *zen* homologs in flies (21), beetles, and grasshoppers are expressed much earlier, and in a fashion that is not colinear with their homeotic neighbors. In *Drosophila* the *zen* genes are among the first zygotic genes to be transcribed in the syncytial blastoderm. They are expressed in a broad domain that covers the dorsal-most 40% of the egg surface and includes the anterior and posterior poles. Later, just before the onset of gastrulation, this pattern is refined to a strip 5–6 cells wide at the dorsal midline. These cells will differentiate into the *Drosophila* extraembryonic membrane, the amnioserosa. *zen* is also expressed in two groups of cells just anterior to the cephalic furrow in an area fated to become the optic lobe.

FIG. 2. Long-range southern mapping of *Tc zen*. *Tribolium* DNA was restricted with *Bss*HII (lanes B) or *Nhe*I (lanes N), electrophoretically separated, and blotted to generate identical panels. *Tc zen*, *mxp*, and *Tc Dfd* probes were used individually or combined in the same hybridization reaction to maximize the resolution of similarly sized fragments. *Tc zen* and *mxp* probes recognize a 70-kb *Bss*HII fragment (A), whereas *Tc zen* and *Tc Dfd* recognize a 100-kb *Nhe*I fragment (B). Note that the *Tc zen* and *mxp* probes hybridize to markedly different length *Nhe*I fragments (A). The *Bss*HII fragments recognized by *Tc zen* and *Tc Dfd* probes are similar in size (B), but careful alignment indicates that the fragment hybridizing to *Tc Dfd* is slightly larger. These data indicate that *Tc zen* is positioned between the homologs of *pb* and *Dfd* as predicted from the location of *Drosophila zen*.

The Hox 3/*zen* homologs of *Schistocerca* and *Tribolium* are also expressed very early, in nuclei fated to become extraembryonic membranes (Fig. 3 B and D). However, the organization of the extraembryonic membranes is rather different in these embryos (23). The first membrane to appear is the serosa, which forms from cells of the blastoderm that lie outside the embryonic primordium. In *Schistocerca*, the embryonic primordium is small, and the serosa covers most of the egg surface (Fig. 4). It differentiates by 48 hr, the cells becoming polyploid and flattened, and secreting a cuticle around the entire egg surface. In these embryos, Sgzen transcripts are already detectable at 18 hr, when cleavage nuclei are just reaching the surface of the egg and before the embryonic primordium is distinct (Fig. 3 A and B). They are most abundant in the first 36 hr, but persist until at least the sixth day of development. If the embryo is dissected free of the serosa, transcripts are detectable only in the serosa. An antibody raised against the Sgzen protein stains the large nuclei of the serosa very strongly, from gastrulation onwards (Fig. 3 C–E). In *Tribolium*, the embryonic primordium is more extensive (Fig. 4), and the serosa forms from cells at the anterior pole and along the dorsal anterior midline. These cells become polyploid and flattened as they cover the entire surface of the egg. *Tc zen* transcripts are detected very early in a dorsally shifted cap at the anterior pole of the blastoderm (Fig. 3 F and G). Prior to gastrulation, cells along the dorsal anterior midline, in the developing serosa, also express *Tc zen* (Fig. 3 H and I). The squamous serosal cells continue to express *Tc zen* during gastrulation but the staining is very faint (not shown).

In both *Schistocerca* and *Tribolium*, a second extraembryonic membrane, the amnion, forms and covers the ventral side of the embryo. In *Schistocerca*, this membrane forms from cells at the boundary between the embryo and serosa (Fig. 4). It folds over the ventral surface of the embryo, and detaches from the serosa as the embryo sinks into the yolk. The cells of the amnion also become polyploid and attenuated, although they never reach the size or ploidy of the serosal cells. Once the amnion has formed, it expresses low levels of Sgzen protein. In *Tribolium*, the amnion forms somewhat differently (Fig. 4) and does not express *Tc zen*.

DISCUSSION

The early and persistent expression of *Schistocerca* Sgzen and *Tribolium* *Tc zen* in extraembryonic membranes, but not in the embryo, directly parallels the expression of the *Drosophila zen* genes in the amnioserosa. In *Drosophila*, the amnioserosa is a highly derived structure, representing an evolutionary vestige of the amnion and serosa, which never separates into two layers or covers the ventral side of the embryo. Some lower Dipterans (*Culex*) form a separate amnion and serosa, directly analogous to those of lower pterygotes, but the folding of the membranes over the ventral surface of the embryo is delayed until later in development. At gastrulation, the presumptive extraembryonic membrane lies in the same relation to the embryo as the amnioserosa in *Drosophila*, closing the dorsal surface (24). In these insects the membranes start to fold but never merge on the ventral side of the embryo. In all of these extraembryonic membranes the cells rapidly cease division and become polyploid. Thus, although the morphogenesis of these insects is very different, *zen*, *Tc zen*, and Sgzen are expressed in homologous structures that share certain important aspects of subsequent differentiation. In other respects it is more difficult to compare the expression of these genes. In *Drosophila* the early expression of *zen* is controlled by some of the components of the dorsal/ventral and terminal patterning systems (21). In *Schistocerca* at least, this is not obvious. Finally, no expression of the Hox 3/*zen* genes has been detected in the optic lobe anlagen of *Schistocerca* or *Tribo-*

FIG. 3. Expression of *Tc zen* and *Sgzen* during embryogenesis. (A) Relative abundance of *Sgzen* RNA in early cleavage stage *Schistocerca* eggs. Single pods of approximately 50 eggs were used for each time point. (Eggs within a pod are synchronous to within ≈ 3 hr). Four eggs from each pod were fixed, stained, and observed under UV fluorescence (13) to determine the stage [estimated from the distribution of nuclei according to Ho (22)]. Each lane (time point) contains the total RNA extracted from the remaining eggs of a pod. Approximately 5 g of RNA were recovered from each pod, indicating that the total egg RNA content does not change dramatically during this period. -/+ indicates the relative hybridization of *Sgzen* probe to total RNA extracted from eggs at the stages illustrated. Dots indicate the distribution of superficial nuclei. In the youngest pod, no nuclei had yet migrated to the surface of the egg, and no *Sgzen* RNA is detected. A and P indicate the antero-posterior axis of the egg. The arrowhead on the 35-hr egg indicates the approximate boundary between embryonic and extraembryonic tissues. (B) Hybridization to RNA isolated from ovaries, whole eggs, serosa, and embryos. For each time point several pods were pooled. Total RNA was extracted from one-half the eggs in each pool. The remainder were dissected, and RNA prepared separately from yolk/serosa and embryos. 'Serosa' samples were prepared by removing the embryo and the enveloping amnion. The samples contain the major extraembryonic membrane and traces of any RNA present in the yolk mass. (C-E) Distribution of *Sgzen* protein in *Schistocerca*. A dissected egg at about 45% development (C). The serosa is still around the embryo but the yolk has been removed. The serosa nuclei stain strongly with the *Sgzen* antisera (C and D, at higher magnification). The small nuclei of the amnion stain only at the level of the embryonic head (C-E, at higher magnification). (F-I) Distribution of *Tc zen* in *Tribolium* embryos. Digoxigenin-labeled, antisense RNA probes (12) initially detect *Tc zen* transcripts at the anterior pole of wild-type blastoderm embryos (F). Hoechst staining of the same embryo (G) shows that condensation of the embryonic rudiment has not yet begun. Slightly later, but still before formation of the embryonic rudiment (H), expression extends further posteriorly on the dorsal surface, in the presumptive serosa. As the embryo forms and gastrulation begins (I), expression diminishes and is restricted to the serosa; *Tc zen* transcripts are not detected in the embryo or amnion.

lium, suggesting that this aspect of *zen* expression may be restricted to higher insects.

Comparisons between the Hox genes of protostomes and deuterostomes have often been used to infer the structure of an ancestral Hox complex (20), but the relationship between possible ancestral genes, the vertebrate Hox 2 and Hox 3 subfamilies, and putative insect orthologs has been uncertain. Recently, the amplification of homeodomain fragments resembling Hox 3 genes (although not themselves mapped to a complex) has provided some evidence that the ancestral complex contained a specific Hox 3 representative (25, 26). Our analysis of *Tc zen* and *Sgzen* reveals that insect *zen* genes are specific homologs of the vertebrate Hox 3 subfamily genes, and demonstrates the inclusion of a Hox 3/*zen* gene in the Hox complex of the last ancestor common to arthropods and vertebrates.

Based on distance matrix analysis, it has been suggested that two genes, *Gsh-1* and *Gsh-2* (27, 28) may be vertebrate homologs of *Drosophila zen* (14). The mouse genes are not located in a Hox cluster and are not assignable to any Hox class, including Hox 3. Their homeodomain sequences show 17–19 differences with that of *zen* (Fig. 1A). They are equally similar to *Tc zen* and *Sgzen* (16–19 differences), but lack most of the Hox 3 diagnostic residues. Because they display no additional sequence identity outside the homeodomain, it is most likely that *Gsh-1* and *Gsh-2* show convergent similarities with the insect *zen* homeodomains.

The insect *zen* genes comprise a relatively rapidly diverging group among the Hox genes. The homeodomains of the insect *zen* genes differ from one another considerably more than do the sequences of mouse and amphioxus Hox 3, or

FIG. 4. Comparison of extraembryonic membranes in different insect orders. Diagrams in top row illustrate the relative size of the embryonic primordium and the extraembryonic membranes at the onset of gastrulation. The center row shows the amnionic folds (dotted lines) forming over the ventral surface of the beetle and grasshopper germ rudiments. The bottom row consists of diagrammatic cross sections of the eggs or germ rudiments at the level indicated by the vertical lines in the panels above. In the higher Diptera (e.g., *Drosophila*), the large embryo forms around the yolk (top row, left panel). The only extraembryonic membrane is the amnioserosa (As), which lies over the yolk, joining the dorso-lateral edges of the embryo. This situation is derived from a more primitive condition, retained in *Schistocerca* and *Tribolium*, where the embryo is smaller in relation to the egg (top row, center and right panels). Formation of extraembryonic membranes—the serosa, which surrounds the entire egg, and the amnion, which folds over the ventral surface of the embryo, is very similar in these embryos. In *Schistocerca* the amnion is formed from cells in a narrow region surrounding the embryo (top row, right panel). As the embryo sinks caudal end first into the yolk, the amnion folds over the posterior and lateral edges of the embryo. *Tribolium* is in some respects intermediate. The embryo is larger and the caudal end only sinks transiently into the yolk. The amnion is formed from cells in a large region on the dorsal side of the egg (top row, center panel). During elongation and retraction the germ band remains near the surface of the egg, similar to *Drosophila*.

mouse and insect genes within either class 2 or 4. Comparing insect and vertebrate Hox 3 sequences suggests divergence has been particularly fast in the lineage leading to the *zen* genes of *Drosophila*, which may account for the difficulty in recognizing *zen* sequences as members of Hox class 3 (29). In this respect, the *zen* genes resemble another nonhomeotic ANT-C gene, *fushi tarazu* (12, 13, 30), which also shows a more rapid rate of sequence divergence than its homeotic neighbors.

It appears that the critical feature of the ancestral Hox complex was the existence of a group of transcription factor-encoding genes that were regulated in a colinear fashion. This regulatory paradigm has been adapted to many different developmental processes, including assignment of developmental fate along the anterior-posterior axis (10), and in the limbs of vertebrates (31). We therefore expect that colinear expression is the ancestral state for the Hox 3 genes. Further phylogenetic comparisons will be necessary to determine when *zen* lost its colinear expression and acquired a role in defining the distinction between embryonic and extraembryonic tissues.

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