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Maternal expression and early zygotic regulation of the *Hox3/zen* gene in the grasshopper *Schistocerca gregaria*

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SUMMARY In insects, a key step in the early patterning of the egg is to distinguish the primordium of the embryo proper from those regions that will form extra-embryonic membranes. In *Drosophila*, where these processes are well understood, the structure of the extra-embryonic membranes is highly derived. The distinct amnion and serosa typical of lower insects is replaced by a single, fused, and much reduced membrane, the amnioserosa, which never secretes an embryonic cuticle.

We have used the *Zen* gene as a marker to study the formation of the extra-embryonic membranes, and other aspects of early embryonic patterning, in the grasshopper *Schistocerca gregaria* (African Plague Locust). Zen genes are derived from Hox genes, but in *Drosophila* they appear to have lost any role in patterning the A/P axis of the embryo; instead, they are involved in D/V patterning and the specification of the extra-embryonic membranes.

We show that the *Schistocerca zen* gene is expressed during embryogenesis in three distinct phases. The first of these is during cleavage, when *Sgzen* is transiently expressed in all energids that reach the cell surface. The second phase of expression initiates in a ring of "necklace cells" that surround the forming embryo, and demarcate the boundary between the amnion and serosa. This leads to expression throughout the serosa. The final phase of expression is in the amnion, after this has separated from the serosa. This complex pattern implies that the role of *Sgzen* in *Schistocerca* is not limited solely to the specification of cell identity in the extra-embryonic membranes.

We also report that the *Schistocerca zen* gene is expressed maternally, unlike its *Drosophila* and *Tribolium* counterparts. A distinct maternal transcript, and maternal Zen protein, accumulate in the developing oocyte from early post-meiotic stages. They remain uniformly distributed in the oocyte cytoplasm until late vitellogenic stages, when the protein and RNA become somewhat concentrated at the egg cortex and in the posterior polar cap of the oocyte, probably by passive exclusion from the yolk. The cytoplasmic localization of Sgzen protein in the oocyte, and at some stages during embryogenesis, implies that nuclear exclusion of this transcription factor is specifically controlled.

INTRODUCTION

Drosophila is the one arthropod in which early development is well understood. In this species, the pattern of the embryo is generated during syncytial cleavage stages. Localized maternal determinants provide signals that result in the activation of different zygotic genes in nuclei at different positions within the egg. By the time cell membranes have closed around these nuclei, each cell is already transcribing a characteristic set of genes that define the antero–posterior pattern of segments, and the dorso–ventral pattern of tissue types.

In grasshoppers, the initial steps of embryogenesis appear to be very different from those in *Drosophila*. Early cleavage is syncytial, but cells form while there are still few nuclei in the egg (Ho et al. 1997). An embryonic primordium is formed by the aggregation of cleavage cells at the posterior pole of the egg. These cells continue to divide, forming a dense blastoderm disc within which the pattern of the embryo becomes visible (Roonwal 1936; Ho et al. 1997). Elsewhere, cells differentiate rapidly to form an extra-embryonic membrane, the serosa, which secretes an embryonic cuticle. A similar pattern of development is found in many of the more basal insect orders, and in other arthropod classes (Dohle 1972; Dohle 1974), though in few cases do we know exactly when closed cells form around the cleavage nuclei.

Several key questions need to be answered before we can understand the process of early development in this and other lower insects. What specifies the site where the embryonic primordium will form? What genes define the difference between the embryonic and extra-embryonic territories? To what extent is pattern established in the syncytium, and carried to the blastoderm by the aggregation of cleavage cells, and to what extent is pattern generated de novo as these cells interact with one another and their surroundings.

In this paper, we use the *zen* gene of the African Plague Locust, *Schistocerca gregaria* (*Sgzen*), as a marker to ad-

dress one of these questions: the establishment of differences that distinguish the embryonic and extra-embryonic regions of the blastoderm. In *Drosophila*, the two *zen* genes are among the earliest markers of the future extra-embryonic territory of the embryo (Rushlow et al. 1987a; Rushlow et al. 1987b). They are not transcribed maternally, but are activated in nuclei throughout the dorsal half of the syncytial embryo, under the control of the maternal dorso/ventral patterning cascade. Their expression rapidly becomes restricted to the dorsal 10% of the egg's circumference, in cells fated to form the amnio-serosa. Expression persists in the amnio-serosa.

Homologs of *zen* have been cloned from the beetle *Tribolium* as well as from *Schistocerca* (Falciani et al. 1996). Like the *Drosophila zen* genes, these are persistently expressed in extra-embryonic tissues, implying that they may serve a role in defining this tissue. Thus, we reasoned that the early expression of *Sgzen* might provide a useful marker to monitor the establishment of embryonic versus extra-embryonic territories in *Schistocerca*.

In this paper, we analyze the expression of the *Sgzen* gene during early embryogenesis. We show that it is expressed in several distinct phases during embryogenesis, implying that it plays roles additional to those defined in *Drosophila*. At gastrulation, when extra-embryonic membranes are forming, it is expressed in cells of the presumptive serosa, and serves to define a precise boundary between cells fated to form this outer membrane, and the primordium that will detach from it to form the embryo with its encircling amnion. We also report that, exceptionally among genes of the Hox clusters, the *Schistocerca zen* gene is expressed during oogenesis, though at this stage Sgzen protein is localized to the cytoplasm, implying that the nuclear localization of this transcription factor is regulated.

MATERIALS AND METHODS

Cloning

A Lambda Zap cDNA library (Stratagene) was prepared from adult ovary RNA following the manufacturer's instructions (Frenk Mora 1993). Two *Sgzen* cDNA clones were isolated from this library, by screening with PCR-amplified fragments from the zygotic *Sgzen* cDNAs described previously (Falciani et al. 1996). Sequencing of these clones showed that the ovary transcripts are similar to the previously described zygotic cDNAs, except that they lack sequences present at the 5′ end of the zygotic cDNA. The ovary clones differ from one another in the number of copies of a 220 base repeat present in the 3′ untranslated region. Three tandem copies of this sequence are present in the longer (2.6 kb) clone, matching the zygotic cDNA. Only two copies are present in the shorter (2.4 kb) clone. However, we have no evidence that this shorter variant exists in vivo. Amplification of ovary RNA by RT-PCR across the repeats consistently gives a product corresponding to the longer 3 kb transcript. The shorter clone may have arisen from a deletion event during cloning. The sequence of the longer clone is available under the accession No. X92654.

Further characterization of the zygotic clone described by Falciani et al. (1996) showed that 1.9 kb of sequences at its 5′ end are probably not derived from the *Sgzen* gene, but represent a cloning artifact. They have strong homology to bacterial rDNA and probably represent the spurious ligation of sequences derived from bacterial contaminants to the 5′ end of an incomplete *Sgzen* cDNA. These bacterial sequences are not detectable in embryo RNA by RT PCR or by Northern blotting, but they do hybridize to RNA prepared from whole eggs (which contain symbiotic bacteria and/or soil-borne bacteria on the eggshell). The Northern blots shown in Falciani et al. (1996) were probed with a clone of *Sgzen* containing this bacterial DNA, and almost certainly contain spurious bands in the lanes prepared from whole-egg RNA.

The genuine *Sgzen* sequences in our zygotic clone account for only 2.6 kb of the approximately 4.5 kb zygotic transcript and are entirely contained within the span of the 2.6 kb ovary transcript. Attempts to isolate sequences unique to the zygotic transcript have so far proved unsuccessful. We have performed 5′ RACE PCR on cDNA from zygotic stages using three different methods (Chen 1996; Hug and Klobeck 1996; Lung and Chan 1996), and on two separate zygotic cDNA libraries using nested primers in the vector and in the *Sgzen* coding sequence. Thus at present we cannot determine the precise point at which the ovary and zygotic transcripts diverge.

Northern blotting

RNA was extracted from timed egg collections using the Poly-A pure kit (Ambion, Austin, TX, USA). Northern blotting was performed as described by Kim, Febbraio et al. (1995).

Embryo staining

Tissues for antibody staining and in situ hybridization were prepared as follows: Ovaries were prepared by the methods of Dearden and Akam (2000). *Schistocerca* eggs were collected from adults in sand-filled pots and washed with water and PBS. For antibody staining of whole eggs (up to 70 h after egg laying (AEL), individual eggs were removed from egg pods and placed in 4% formaldehyde. The eggs were then pricked 20–30 times with a fine needle and left in fixative for 2 h. Eggs were then washed in PBS and peeled free of chorion before staining.

To analyze antigen expression in the developing embryos, amnion, and serosa, locust eggs were dechorionated in 50% bleach solution. (Eggs younger than 70 h AEL with incomplete serosal cuticle, cannot be dechorionated in this way). Embryos and extra embryonic membranes were dissected in locust saline and fixed for 30 min as described above.

Fluorescent antibody stainings were conducted according to the protocol described in Grbic et al. (1996) with *Sgzen* polyclonal antibody (Falciani et al. 1996) and mAb 7c11 (anti grasshopper Hunchback, Patel, personal communication). Histochemically stained specimens were prepared using the methods of Dawes et al. (1994), counter-stained with Hoescht 33,258 (1 h) and destained for 30 min. Embryos and ovaries stained fluorescently were counter-stained with FITC conjugated phalloidin to visualize cell and embryo outlines. Fluorescently stained tissues were examined on a 1024 BioRad Laser Scanning Confocal Microscope.

In situ hybridization to ovaries was performed as described by Dearden and Akam (2000).

RESULTS

Maternal and zygotic products of *Schistocerca zen*

The *Sgzen* homeobox hybridizes to transcripts of different sizes in ovary and embryonic RNA (Fig. 1A). Ovary RNA contains a transcript of approximately 2.6 kb; zygotic RNA contains a transcript of approximately 3.5 kb. Zygotic transcripts have replaced the ovary transcript by 1 day after egg laying (AEL).

To determine the difference between these transcripts, we isolated *Sgzen* cDNA clones from ovary and embryonic cDNA libraries (see methods). Representative clones from both libraries were sequenced. Near full-length clones (2681 basepairs) of the ovary transcript encode a protein of 379 amino acids. We do not have full-length clones of the zygotic isoform, but the available clone matches the ovary transcript throughout its length and terminates at the same polyadenylation site. We suggest that the difference in size between maternal and zygotic RNA may be due to an elongated 5′UTR present in the zygotic transcript but not represented in our zygotic cDNA clone. However, we cannot rule out the possibility that this difference could be due to differential polyadenylation of the zygotic transcript, though this would entail the addition of just less than 1kb of polyA.

In the course of this work we also discovered that the zygotic cDNA clone previously characterized (Falciani et al. 1996) was not representative of the major transcript species, and almost certainly contains a cloning artifact at the 5′ end. (See methods for details.)

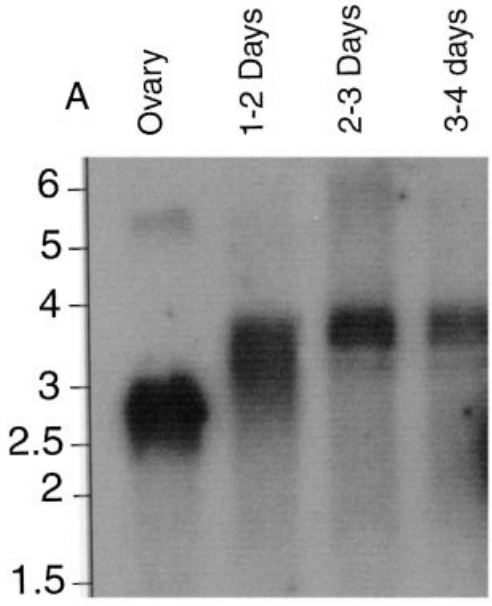


Fig. 1. Northern blot showing expression of *Sgzen* from ovary to 3–4 days AEL (after egg laying). Note ovary transcripts run at 2.6 kb and zygotic transcripts run at 3.5 kb. The smearing in the 1–2 day lane is probably due to degradation of RNA. Very little RNA is present in the egg at these early stages, making extraction of sufficient amounts for Northern blots difficult. The distribution of signal in that lane strongly implies that a transcript similar in size to the zygotic transcript is present, and the maternal transcript absent. (5 μg of poly A+ RNA loaded on each lane corresponding to RNA from 1, 0.2, 0.18 grams of eggs at 1–2, 2–3, 3–4 day AEL, respectively.)

Sgzen protein is expressed in the oocyte cytoplasm.

In the ovary, expression of *Sgzen* is restricted to the germline cells. The *Schistocerca* ovary is panoistic; it contains no nurse cells. Surrounding each of the older oocytes is a layer of follicle cells that secrete the chorion of the egg and die before egg laying. The ovary comprises three sections (Nelsen 1934) (Fig. 2A): the terminal filament, which is made up of somatic cells; the germarium, where the oocytes and follicle cells are generated from stem cell populations; and the vittelarium, where the oocytes mature, becoming large and yolky. The mature oocytes are approximately 8 mm long and filled with yolk. In-situ hybridization demonstrates that *Sgzen* mRNA is restricted to the oocytes themselves and is absent both from the terminal filament and the follicle cells (Figs. 2 F, 2G, and 2H). The gene is first transcribed in the germline cells of the germarium and transcripts are present in all older oocytes. A similar pattern is observed with an antibody raised against the *Sgzen* protein (Figs. 2B, 2C, 2D, and 2E).

In *Drosophila*, Bicoid and some other maternal proteins are transported to and localized at the poles of the egg during pre-vitellogenic stages (St. Johnston and Nusslein-Volhard 1992). We see no evidence for such localization of the *Sgzen* protein or RNA in *Schistocerca*. In all pre-vitellogenic oocytes, Sgzen protein appears to be uniformly distributed in the oocyte cytoplasm. *Sgzen* RNA has a similar distribution, except that it is relatively concentrated around the germinal vesicle. Sgzen protein is totally excluded from the oocyte nucleus. This contrasts with the distribution of another maternally expressed transcription factor, Hunchback, which is localized within the large germinal vesicle (Fig. 2C).

During vitellogenesis, in the oldest oocytes, Sgzen protein and mRNA become enriched near the posterior pole of the egg, around the germinal vesicle. *Sgzen* mRNA also becomes enriched at the cortex of the oocyte (Fig. 2H). Oocytes stained for total RNA with Methylene Blue show a similar pattern of localization (Fig. 2J), implying that it reflects the passive exclusion of many cytoplasmic components by the packed yolk granules in the middle of the egg.

Sgzen protein accumulates in the nuclei of all cleavage energids

Immediately after fertilization, the Sgzen antibody stains a thin layer of cytoplasm at the surface of the egg (Fig. 3A). As cleav-

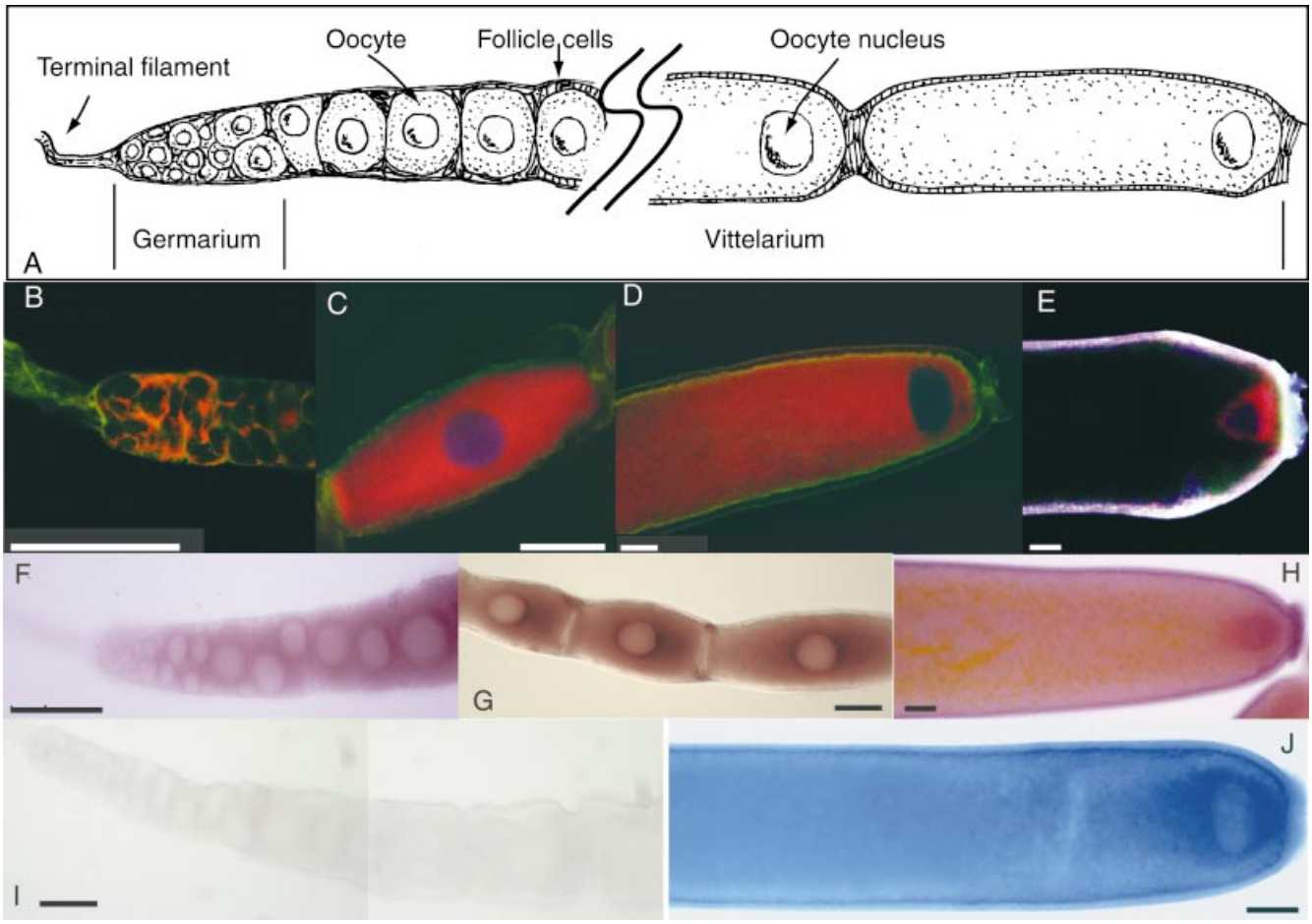


Fig. 2. Sgzen protein and RNA distribution in the ovary; scale bars represent 50 μM. (A) Cartoon of a panooistic ovary. Oocytes form in the germarium. Oocytes grow larger, and become enclosed in a single layer of follicle cells, in the vittelarium. As the oocytes mature, their cytoplasm fills with yolk granules. In *Schistocerca,* the oocyte nucleus migrates to the posterior pole of the egg during the later stages of oogenesis, before the germinal vesicle breaks down. The somatic follicle cells arise as interstitial cells between the maturing oocytes in the germarium. They divide to cover the oocytes in the germarium, and die after secreting the chorion and before egg laying.

(B-E) Expression of Sgzen protein (red) and *Schistocerca* Hunchback (blue) counter stained with phalloidin (green). In early stages Sgzen is found in the cytoplasm of newly formed oocytes (B), Sgzen is absent from the nucleus or follicle cells. As the eggs mature (C and D) Sgzen accumulates uniformly in the oocyte cytoplasm. At this stage Hunchback is restricted to the nucleus (C). In eggs just prior to deposition (E), Sgzen protein can be seen around the nucleus at the posterior pole. (F–I) Expression of *Sgzen* mRNA in the ovary. mRNA appears first in the earliest oocytes (F) and is continually expressed in maturing oocytes (G). In these oocytes, *Sgzen* mRNA appears concentrated around the germinal vesicle. In mature eggs, the mRNA is concentrated at the posterior end, and in the cortex (H). (I) Control ovary hybridized with sense *Sgzen* probe. (J) Mature oocyte stained with Methylene blue to reveal total RNA. RNA is localized to the cortex and around the germinal vesicle.

age energids emerge at the egg surface, this staining concentrates, first into a network of strands around each energid, and then into the energid nucleus. This sequence of events occurs about 12–16 h AEL near the posterior pole of the egg, where the first cleavage energids emerge, and then successively more anteriorly. This timing parallels the formation of closed cells, which also takes place shortly after cleavage energids reach the cortex (Ho et al. 1997). By 12 h, the earliest energids to reach the surface of the egg have Sgzen protein in the nucleus (Fig. 3b).

We cannot distinguish what contribution maternal and zygotic RNA make to early embryonic protein. Zygotic message is easily detected by 24 h AEL (Fig. 1A), at a time when there are still relatively few zygotic cells. It is thus likely that cleavage energids begin to transcribe the *Sgzen* gene when they emerge at the surface, or very soon thereafter.

By 24 h, cleavage cells are distributed throughout the posterior half of the egg, but they have only just begun to aggregate at its posterior pole, where the blastoderm disc will form (Figs. 3C, 4A, and 4B). At this time, the subcellular localiza-

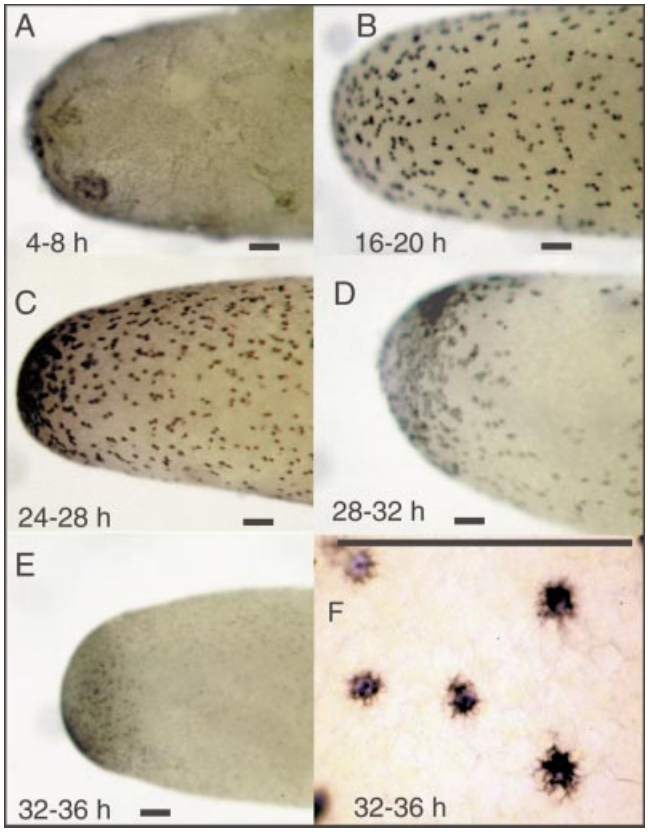


Fig. 3. Sgzen protein distribution in early eggs; scales represent 100 μM. Posterior pole of the egg to the left. (A) 4–8 h egg, Sgzen protein appears as a thin layer on the surface of the entire egg (somewhat disturbed by removal of eggshell). (B) 16–20h Sgzen is present in the nucleus of all energids as they migrate to the pole. (C) 24–28h Sgzen expression is high as cells aggregate at the posterior pole to form the blastoderm disc. At this stage, down-regulation of Sgzen expression begins at the posterior pole (Figs. 4C and 4D). (D) Down regulation of Sgzen expression spreads from the pole across the aggregating cells forming the blastoderm. (E) 32–36 h, only stellate nuclei in a layer underlying the embryo still stain strongly. (F) 32–36 h. Egg with overlying embryo peeled away showing stellate nuclei underlying embryo. All nuclei strongly stain for nuclear Sgzen.

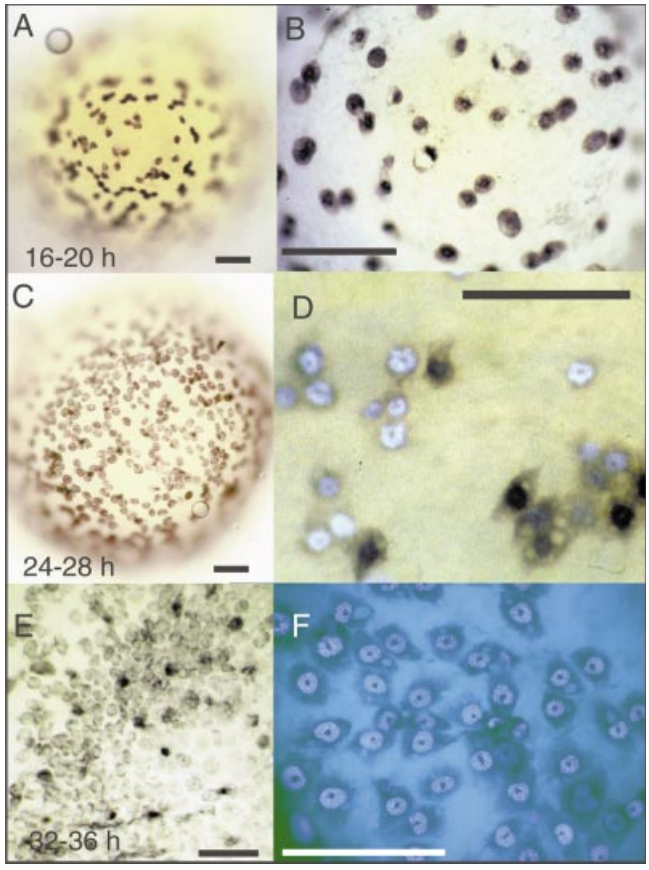


Fig. 4. Sgzen protein in the posterior pole; scale bars represent 100 μM. Looking down onto the posterior pole. (A and B) 16–20 h, all energids at the post pole contain nuclear Sgzen protein. (C and D) 20–24 h: Sgzen protein clears from the nuclei of cells at the posterior pole. Note adjacent cells can have very different levels of Sgzen protein. (D) is counterstained with Hoescht 33258 to visualize unstained nuclei. (E and F) Part of a 32–36h-blastodisc embryo. All superficial cells have lost Sgzen protein from all but one spot in the nucleus, though some cytoplasmic staining remains. Nuclei underlying the embryo still contain Sgzen protein. (F) Close up showing embryonic cells, stained with Hoescht 33258 with nuclei free of Sgzen protein.

tion of Sgzen protein changes within a small group of cleavage cells located at the posterior pole of the egg. Staining is lost from most of the cell nucleus and expression of the gene is greatly reduced (Figs. 4C and 4D). Over the course of the next 12 h, this altered pattern of expression spreads to surrounding cells, until by 36 h virtually all cells in the egg have lost nuclear Sgzen staining (Figs. 3C, 3D, 4E, and 4F).

The exception to this pattern is a population of nuclei that lie slightly below the surface of the embryo, and retain the stellate appearance characteristic of cleavage energids (Figs. 4E and 4F). These energids retain high levels of Sgzen protein even at the posterior pole of the egg. We interpret them as the population of energids that remain in contact with the yolk syncytium (Ho et al. 1997). Sgzen protein can be detected in these cells until they are obscured from view by the consolidation and thickening of the embryonic blastodisc.

Sgzen expression demarcates the boundary between the amnion and the serosa

By 36 h the embryo is visible as a circular disc of cells at the posterior pole of the egg, generated partly by the posterior directed migration of blastoderm cells over the yolk, and partly by the rapid proliferation of these posterior cells. Although the fate map of the egg is not well defined at this stage, we can infer from subsequent development that cells

Fig. 5. Sgzen expression in extra embryonic membranes, scale bars represent 100 μM (anterior of embryos at top). (A) 44–48 h "heart stage" embryo. Sgzen marks a double line of cells (termed "necklace cells") surrounding the embryo. (B) Close-up of part of the same embryo (boxed in A) counterstained with Hoescht 33258 to visualize unstained nuclei. Necklace cells demarcate a sharp boundary around the embryo. Staining of embryos is cytoplasmic and may be due to background in this thick tissue. (C) At 48–52 h, the necklace cells move over the embryo, dragging both the serosa and the amnion over the posterior pole. (D) Right side of a 50–52 h embryo. Necklace cells are now moving over the head-lobes. (E) Posterior end of the embryo from D (boxed) focused on the amnion covering the embryo. Counterstained with Hoescht 33258 to visualize unstained nuclei. Note the amnion does not contain Sgzen protein at this stage. Nuclei of the amnion (arrowhead) are intermediate in size between the large necklace cell nuclei and the smaller nuclei of the embryonic primordium (arrow).

of the embryo proper are surrounded by a halo of extra-embryonic cells that will form the amnion, outside of which lie cells that will form the serosa. (The amnion is a membrane that remains in contact with the embryo, but detaches from the egg surface to form a closure over the ventral surface of the embryo. The serosa seals along the line where the amnion has detached to form a continuous membrane around the surface of the egg, freeing the embryo to sink within the yolk [see Figs. 5 and 6]).

At 44 h, Sgzen expression demarcates a sharp boundary around the embryo, which coincides with a change in cell shape at the crest of the infolding extra-embryonic membranes (Figs. 5A and 5B). We believe that this expression marks the future boundary between the amnion and the serosa, the line at which these membranes will detach and reseal to release the embryo from the serosa. Cells of the serosa abutting the amnion express the highest levels of Sgzen protein. We call these the necklace cells. The necklace cells move over the embryo (Figs. 5C and 5D), dragging both the amnion and the serosa behind them to encapsulate the embryo in its double membrane.

We have found it difficult to determine the fate of the necklace cells once the amnion has closed. Most likely, they form part of the serosa. By removing the posterior cap of the egg and fixing and staining for Sgzen, we were able to confirm that the serosa forms at the posterior pole of the egg at the same time as closure of the amnion. At 40 h, staining the posterior pole yields only embryonic cells that do not express Sgzen. By 52 h, a group of Sgzen-staining nuclei forms a continuous layer at the posterior pole. These are similar in size and number (approximately 120) to the necklace cells (Figs. 6A and 6B).

External to the boundary formed by the necklace cells, and further from the embryo, the levels of Sgzen protein are initially low. From 50 h onwards, Sgzen expression spreads throughout the serosa from posterior to anterior. By four days, high levels of Sgzen protein have accumulated in all cells of the serosa (Fig. 6C). Cells flatten and nuclear size increases—a visible sign of the dramatic polyploidy that accompanies differentiation of the serosal cells. However, serosal cells at the posterior pole remain smaller, and more closely packed, than the rest of the serosal cells (Fig. 6C)

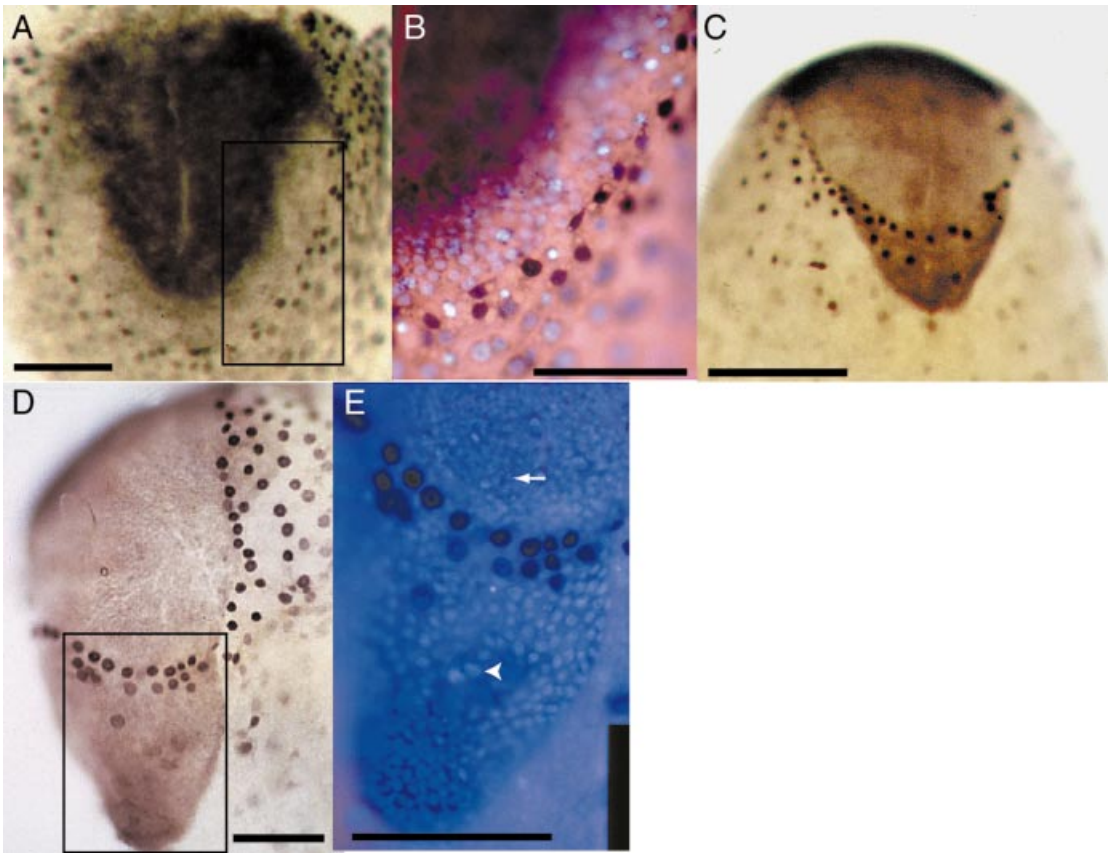
Cells of the amnion initially remain cuboidal, and are not sharply demarcated from those of the embryo. They show no staining with the Sgzen antibody. By four days, however, the amnion also becomes polyploid, the cells flatten (Fig. 5E) and express Sgzen (Falciani et al. 1996).

Neither *Sgzen* RNA nor protein can ever be detected in the embryo itself.

DISCUSSION

The *Schistocerca zen* gene is expressed in the germ line throughout oogenesis, and then in three distinct zygotic phases. The first of these is during cleavage, when Sgzen is transiently expressed in all energids that reach the cell surface. The second phase of expression initiates in the necklace cells demarcating the boundary between the amnion and serosa, and leads on to expression throughout the serosa. The final phase of expression is in the amnion, after it has separated from the serosa. This complex pattern implies that the role of *Sgzen* in *Schistocerca* is not limited solely to the specification of cell identity in the extra-embryonic membranes.

Fig. 6. Sgzen expression in the forming serosa; scale bars represent 100 μM. (A) Posterior cap from 36–40 h egg, viewed from the inside after removal of the yolk. The central portion of an embryonic disc remains flattened against the egg shell. It shows few staining cells. (B) Posterior cap from 48–52 h egg. A complete serosal cell layer lines the inner surface of the egg shell. (C) Dissected serosal membrane from 56–60 hour egg showing tightly packed smaller cells at the posterior pole. (D) Cartoon of morphogenetic movements associated with the formation and separation of the amnion and serosa, viewed as a longitudinal section down the egg. (1) Egg at 36–40 h. The embryo (blue) lies at the posterior pole of the egg, anterior to the left. Between the embryo and the Sgzen positive necklace cells (black circles), lies the presumptive amnion (pink). Outside the ring of necklace cells is the presumptive serosa (light green). (2) The necklace cells move over the embryo, dragging the amnion (red) and the serosa (green) behind them. (3) The ring of necklace cells contracts over the embryo to give a complete serosal membrane (green). The amnion remains attached to the serosa by the necklace cells. (4) Egg at 56–60 h. The amnion seals and detaches from the serosa, wrapping the ventral surface of the embryo. The serosa now begins to secrete cuticle, first at the posterior of the egg and then progressively anteriorly.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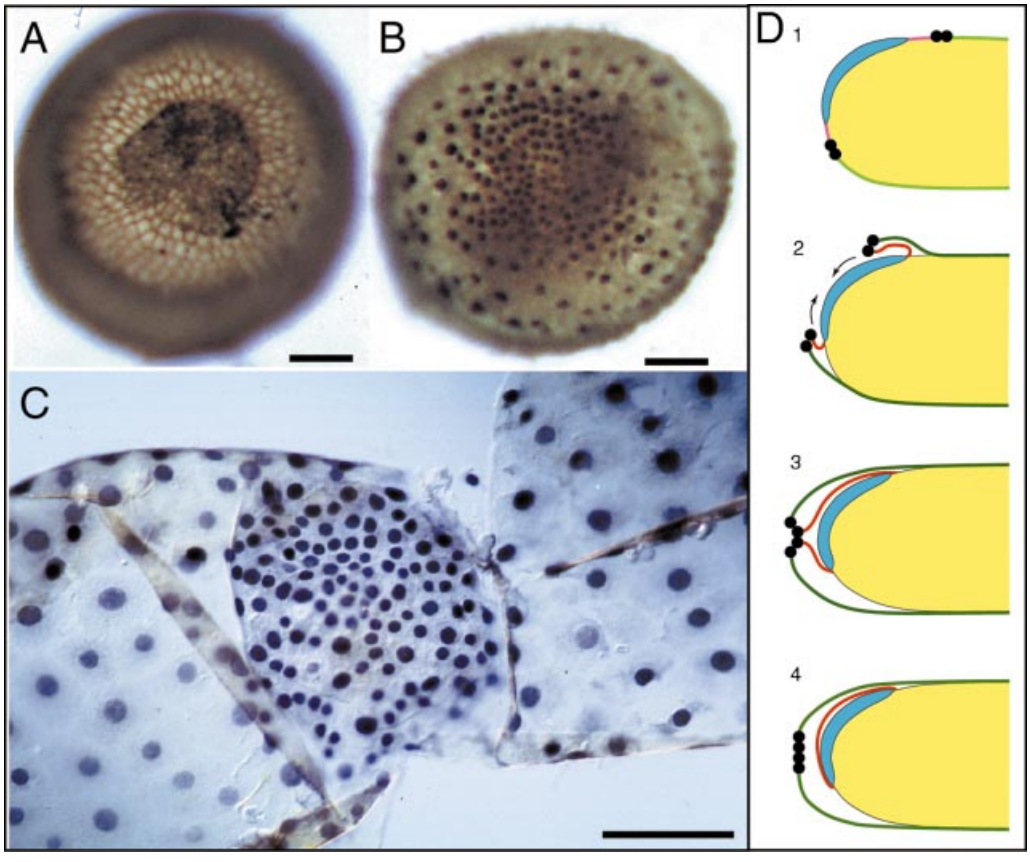
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Maternal expression

The maternal expression of *Sgzen* in *Schistocerca* has no counterpart in *Drosophila* (Rushlow et al. 1987b) or in *Tribolium* (Dearden and Falciani, unpublished results). *Sgzen* RNA and protein are initially distributed uniformly in the cytoplasm of the oocyte, but become concentrated at the cortex, and near the posterior pole, during late stages of oogenesis. We suggest that this localization reflects simply the exclusion of free cytoplasm from the bulk of the egg by the accumulation of packed yolk granules.

The cytoplasmic localization of Sgzen protein in the oocyte is noteworthy. Sgzen is a homeodomain protein, predicted to act as a transcription factor. It contains a putative nuclear localization signal, RPRR at residue 88 (determined by the methods of Horton and Nakai 1996), and in later embryonic stages, is specifically concentrated in the nucleus. However, it appears to be excluded from the germinal vesicle of the growing oocyte. This may simply be a way of blocking the function of Sgzen protein during oogenesis, while allowing the maternal provision of a stockpile for the embryo. However, it is also possible that the differential subcellular localization of different nuclear proteins represents a method of controlling their distribution in the mature egg. A different maternally expressed transcription factor, Hunchback, is restricted to the nucleus in these same oocytes and carried with it to the posterior pole of the egg (Fig. 2C, N. Patel, personal communication).

Our Sgzen antibody also stains a maternal protein in the early oocytes of a distantly related Orthopteran species, the cricket *Gryllus bimaculatus*. However, in this species, the protein is present in the nucleus of early oocytes, and redistributes to the cytoplasm in later oocytes, before vittelogenesis (Cartwright-Finch and Dearden, unpublished results). This strongly implies that nuclear exclusion of Sgzen is tightly controlled. There are precedents for similar controls operating in *Drosophila*. The nuclear import of the nuclear lamina protein FsYa is controlled by phosphorylation during oogenesis (Yu et al. 1999).

Zygotic expression and embryonic patterning

Cleavage energids accumulate maternal Sgzen protein as they reach the cortical cytoplasm, from 16 h onwards. At least some nuclei also activate zygotic transcription of the gene within the first 24 h. These cleavage nuclei have an extensively branched, almost stellate appearance but acquire a more conventional rounded shape around the time of cellularization. (The same changes in nuclear morphology are visible after staining for nuclear lamin; data not shown.) This early phase of *Sgzen* expression persists while energids and cells are moving toward the posterior of the egg, but is quite distinct from later expression in the serosa. Sgzen protein has been eliminated from the nuclei of virtually all surface cells by 36 h.

The elimination of Sgzen protein first from nuclei at the posterior pole of the egg reveals a sharp focus in the egg from which patterning appears to spread. This focus seems much sharper than can be accounted for by any difference in developmental age resulting from the time at which nuclei reach the surface. Nuclei do surface at the posterior of the egg long before they reach more anterior regions, but no difference in timing is obvious within the posterior cap of the egg, which is almost hemispherical. Nuclei reach the surface at about the same time throughout this cap, but the initial loss of Sgzen protein occurs in only one small region of the cap, at the posterior pole.

We suggest that cells at the posterior pole may receive a signal that initiates down regulation of the *Sgzen* gene, and perhaps other aspects of embryonic patterning. The nature of this signal is not known, but a possible source is the follicle cells at the posterior pole. A maternal signal, set up in the follicle cells, could be transmitted zygotically to the embryo to initiate *Sgzen* down regulation. A series of three distinct chorion types are concentrically arranged around the posterior pole of the egg, suggesting that these terminal follicle cells are distinct from those surrounding the rest of the oocyte (Dawes 1996). Obvious parallels can be drawn with the terminal patterning system of *Drosophila*, which patterns both the follicle, and the underlying embryo through the activation of the Torso receptor (Stevens et al. 1990).

Patterning of extra embryonic membranes

By 40 h, the embryo has formed as a disc of cells at the posterior pole. Around the embryo lie cells, some of which may still be migrating, whose eventual fate is unknown. Many of these cells will form the extra embryonic membranes; some will form yolk nuclei and some may still join the embryo.

The expression of Sgzen in the necklace cells between 44–48 hours shows that Sgzen is regulated by a process that establishes an abrupt boundary between two distinct extra-embryonic cell types, the presumptive amnion and serosa. Sgzen may also control some of the subsequent morphogenetic behavior of these necklace cells. They appear to act like a drawstring, migrating over the embryo and pulling the amnion behind them until they meet. If our interpretation is correct, they then fuse, detach from the embryo, and, later, form the first patch of the serosa. Extra-embryonic cells in this membrane secrete the serosal cuticle over the whole egg surface, first in the posterior part of the egg, and then more anteriorly. The embryo, in its amnion, is at this stage devoid of Sgzen expression. Only somewhat later does the amnion also express Sgzen protein (Falciani et al. 1996).

Closure of the amnion over the embryo has been described in the beetle *Tribolium* using SEM (Handel et al. 2000). In this species, it is thought that the migration of the membranes over the embryo is led by cells of the amnion, which do not express *Tribolium zen*, rather than the serosa.

Parallels with Drosophila

In *Drosophila*, *zen* is activated in the blastoderm by ubiquitous transcription factors, and initially repressed in the ventral half of the embryo by the dorsal/ventral patterning system, and at the poles by the terminal system (Ray et al. 1991). Its expression is further restricted to the amnioserosa by *dpp* mediated signaling (Rusch and Levine 1997). *Zen* expression persists in the amnioserosa at least until germ band shortening, but this tissue never secretes an embryonic cuticle. In this respect, it resembles the amnion rather than the serosa of *Schistocerca*.

It seems premature to equate any one phase of *Sgzen* expression in *Schistocerca* with specific events in *Drosophila*. It is not clear whether the segregation of embryonic and extra-embryonic tissues in *Schistocerca* bears any specific relation to the specification of the amnioserosa in *Drosophila*. An alternative scenario might draw parallels between the formation of the amnioserosa and the relatively late differentiation of the amnion from the dorsal embryonic epidermis in *Schistocerca*. Neither is there any parallel in *Drosophila* for the early patterning process that divides the extra-embryonic tissues into two distinct cell populations, and regulates *Sgzen* so strikingly. This patterning process, which is probably ancestral to the pterygote insects, appears to have been suppressed completely in the higher Diptera.

Thus, although we can point to the general similarity that both *Schistocerca* and *Drosophila zen* genes are expressed in extra-embryonic tissues, a more detailed examination implies that Sgzen is subject to multiple and quite distinct phases of gene regulation in *Schistocerca*, that are without direct parallel in *Drosophila*. Experimental manipulation will be required to establish what mechanisms are involved, and what gene interactions may have been conserved between these two species.

Zen and *bicoid*

The *zen* genes of insects are the orthologues of class 3 Hox genes, but in insects, these genes appear to have diverged rapidly, to have lost the presumed ancestral role in patterning the A/P axis of the embryo, and to have acquired new functions (Falciani et al. 1996). The same is not true of all arthropod *Hox3* genes. The *Hox3* genes of two chelicerates contain homeoboxes very similar to their vertebrate homologs, and like them, show a pattern of expression restricted to particular body segments (Damen and Tautz 1998; Telford and Thomas 1998). None is known to be involved in the specification of extra-embryonic membranes. Presumably, the extra-embryonic role of the *Hox3/zen* genes evolved within the arthropod lineage leading to the insects.

Adjacent to *zen* in the *Drosophila* Hox cluster is another homeobox gene, *bicoid*, with an even more divergent homeobox sequence. *Bicoid* encodes the key anterior determinant of the *Drosophila* embryo (Driever and Nüsslein-Volhard 1988a; Struhl et al. 1989). It is transcribed maternally, and the maternal RNA becomes localized to the anterior pole of the *Drosophila* egg. This RNA is not translated until after fertilization, when Bicoid protein forms a graded distribution throughout the anterior half of the *Drosophila* egg (Driever and Nüsslein-Volhard 1988b). Levels of Bicoid protein, acting together with other maternal gene products, determine the boundaries of expression of the first zygotically expressed segmentation genes (Driever and Nüsslein-Volhard 1988b; Driever and Nusslein-Volhard 1989; Struhl et al. 1989).

No *bicoid* gene had been identified in any species outside the cyclorraphan Diptera. Within the Diptera, the sequence of *bicoid* is diverging rapidly (Seeger and Kaufman 1990). We have previously suggested that *bicoid*, like *zen*, may be a highly derived Hox class 3 gene (Falciani et al. 1996; Dearden and Akam 1999). This hypothesis has recently derived support from a phylogenetic analysis of the *bicoid* and *zen* genes from a dipteran with less derived sequences, the Phorid *Megaselia abdita* (Stauber et al. 1999). Thus, the ancestral *Hox3* gene of insects may have given rise to two early embryonic patterning genes in insects, which in the higher Diptera have quite distinct roles in development.

In this context, we were intrigued to discover that the *zen* gene of *Schistocerca* is expressed maternally as well as zygotically. This raises the possibility that the *zen* genes of insects may have acquired a maternally active promoter before the gene duplication event that generated distinct *bicoid* and *zen* derivatives. The origin of these two genes would then be seen as separating already established maternal and zygotic functions of the ancestral gene (Force et al. 1999). Alternatively, the maternal expression of *zen* in *Schistocerca* may represent a convergence with *Drosophila bicoid*. Further species sampling is needed to distinguish these hypotheses: to resolve when distinct *zen* and *bicoid* genes first arose, and whether maternal expression of Hox3-derived genes is observed in branches of the lineage that predate the origin of *bicoid*.

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