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Different combinations of gap repressors for common stripes in Anopheles and Drosophila embryos

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Abstract

Drosophila segmentation is governed by a well-defined gene regulation network. The evolution of this network was investigated by examining the expression profiles of a complete set of segmentation genes in the early embryos of the mosquito, Anopheles gambiae. There are numerous differences in the expression profiles as compared with Drosophila. The germline determinant Oskar is expressed in both the anterior and posterior poles of Anopheles embryos but is strictly localized within the posterior plasm of Drosophila. The gap genes hunchback and giant display inverted patterns of expression in posterior regions of Anopheles embryos, while tailless exhibits an expanded pattern as compared with Drosophila. These observations suggest that the segmentation network has undergone considerable evolutionary change in the dipterans and that similar patterns of pair-rule gene expression can be obtained with different combinations of gap repressors. We discuss the evolution of separate stripe enhancers in the eve loci of different dipterans.

Keywords: Diptera; Eve; Gap genes; Oskar; Segmentation; Stripe enhancers

Introduction

Most studies on the evolution of insect segmentation have focused on comparing the expression profiles of just a few gap and pair-rule genes in a broad spectrum of short and long germband insects, including grasshoppers, flour beetles, lower dipterans (e.g., mothmidges), and higher dipterans (e.g., Drosophila) (reviewed by Davis and Patel, 2002; Tautz and Sommer, 1995). Questions have been raised about the extent to which the Drosophila segmentation gene cascade is conserved in other insects, particularly distantly related short germband insects. It has been argued that pair-rule stripes of even-skipped (eve) and hairy expression represent a highly derived feature of the Drosophila embryo. However, a recent study has shown that at least some of the hairy stripes are regulated by separate 5' regulatory DNAs in the short germband flour beetle, Tribolium, as seen in Drosophila (Eckert et al., 2004). Thus, there is a sense that the basic mechanisms of segmentation are highly conserved in diverse insects, but it is currently unclear whether detailed features of the Drosophila segmentation gene network rapidly fluctuate during insect evolution. We investigate this question by analyzing the expression of segmentation genes in a divergent dipteran: the malaria-bearing mosquito, Anopheles gambiae.

The recent elucidation of the complete Anopheles genome sequence permits the identification of mosquito orthologues of fly segmentation genes by simple sequence comparisons (Holt et al., 2002). Specific hybridization
‘probes’ were prepared for Anopheles segmentation genes via PCR. The Anopheles embryo posed a technical hurdle for in situ hybridization assays, since it is encased within a nontransparent double-layered chorion that is impermeable to hybridization probes. As a result, standard in situ hybridization protocols do not work (e.g., Wolff et al., 1995). We have developed a new method of fixation and dechorionation of Anopheles embryos that permits the visualization of gene expression by in situ hybridization. We have used this method to compare the expression profiles of nine different segmentation genes in early Anopheles and Drosophila embryos.

The Anopheles eve orthologue exhibits a dynamic pattern of expression that is similar to that seen in Drosophila (Frasch et al., 1987; Macdonald et al., 1986) with the exception that anterior eve stripes are repressed in dorsal regions of Anopheles embryos corresponding to the presumptive serosa. In situ hybridization probes were also prepared for two maternal determinants, oskar and nanos (reviewed by Rongo and Lehmann, 1996), as well as the five gap genes, hunchback, Kruppel, knirps, giant, and tailless (reviewed by Jackle et al., 1992). Despite the apparent conservation of the eve expression pattern, there are numerous differences in the expression profiles of the maternal determinants and gap genes. First, oskar and nanos transcripts are not restricted exclusively to the posterior plasm of Anopheles embryos, but instead, an additional domain of transient staining is also detected at the anterior pole. Second, there is an inversion of the posterior staining patterns of hunchback and giant, as compared with the patterns seen in Drosophila embryos (Jackle et al., 1992). And third, there is a marked expansion in the posterior expression pattern of the Anopheles tailless gene as compared with its Drosophila counterpart (Pignoni et al., 1990). These observations raise the possibility that the posterior borders of eve stripes 5, 6, and 7 are specified by different gap repressors in flies and mosquitoes. The borders are formed by Giant (stripe 5) and Hunchback (stripes 6 and 7) in Drosophila (Clyde et al., 2003; Fujioka et al., 1999) but might be specified by Hunchback (stripe 5), Taillless, and/or Giant (stripes 6 and 7) in Anopheles. We propose that different strategies are used to generate homologous segmentation stripes in flies and mosquitoes.

Materials and methods

Fly and mosquito stocks

Anopheles gambiae Kisumu strain was reared at 26°C, 75% humidity, with a 12-h light/dark cycle. Adults were maintained on a 10% sucrose solution, and females were blood-fed on anesthetized hamsters. Drosophila melanogaster strain yw67 was used for in situ hybridizations, as described previously (e.g., Statopoulos et al., 2002).

Mosquito embryo fixation

Embryo collection

A cup of deionized water was placed inside the mosquito population cage on the fourth night after the blood meal. The cups were kept inside the cage for 3 h, and then the embryos were aged outside the cage at 26°C for the period of time necessary to achieve the developmental stage of interest (usually an additional 4 h—until the onset of gastrulation).

Fixation

To remove the exochorion, the embryos were incubated in 25% household bleach for 75 s and then washed thoroughly in deionized water. The embryos were then placed in scintillation vials with a 1:1 mixture of heptane and 9% formaldehyde adjusted to pH 7 with NaOH, and then shaken on a rotary platform for 25 min. Afterwards, the formaldehyde phase was removed and replaced with deionized water. The water phase was then replaced once more, and the embryos were shaken an additional 30 min on the rotary platform. The water phase was then removed, and scintillation vials were filled to the top with boiling deionized water (without removing the heptane phase) and incubated for 30 s. The hot water phase was quickly removed and replaced with fresh deionized water prechilled on ice. Vials were then placed on ice for an additional 15 min. The water phase was then completely removed, and the heptane phase was exchanged. To crack the endochorion, an equal volume of methanol was added, and the vials were strongly swirled once to break the clumps of embryos. Vials containing the heptane–methanol mixture were allowed to stand for 10–15 min, and then the heptane and methanol phases were removed and the embryos were washed several times with methanol. The embryos can be stored in methanol at −20°C for several months. The endochorions were manually peeled from the embryos using needles from 1-ml insulin syringes and double-stick tape.

Whole-mount in situ hybridization

Embryos were hybridized with digoxigenin-labeled antisense RNA probes as described by Jiang et al. (1991). Double hybridization assays were done as described by Kosman and Small (1997). Hybridization probes were prepared against specific Anopheles segmentation genes identified by reciprocal BLAST analyses. The hybridization probes were generated by RT–PCR amplification from embryonic RNA. A 26-bp tail encoding the T7 RNA polymerase promoter (aagTAATACGACTCACTATAGGGAGA) was included on the reverse primer. PCR products were purified with the Qiagen™ PCR purification kit and used directly as templates for in vitro transcription reactions. Between 500 bp and 3 kb of coding sequence was used as a template for each probe. The following primer pairs were used to amplify each of the indicated Anopheles segmenta-
tion genes. (Note that the T7 promoter sequence is always included in the reverse primer):

eve:  gtaggtcagacaagcgcacggtagacagc, aagTAATACGACTCACTATAGGGAGACactctgacggtgctgaagtgctgaacgatt;

Kruppel:  cactgttctgcagcaaggcattcga, aagTAATACGACTCACTATAGGGAGAgtcctctgacggtgcaggtcgtgctcagcaga;

knirps:  cgcggctacatgcaacgctaatccttgat, aagTAATACGACTCACTATAGGGAGAgtcctctgacggtgcaggtcgtgctcagcaga;

oskar:  tcatgacaacggagtcaggtgcgcaaa, aagTAATACGACTCACTATAGGGAGAgtcctctgacggtgcaggtcgtgctcagcaga;

nanos:  aacgggactacttcggcagctgccctggag, aagTAATACGACTCACTATAGGGAGAgtcctctgacggtgcaggtcgtgctcagcaga;

hunchback:  ggctcggactgtgaggatggctcgtacgat, aagTAATACGACTCACTATAGGGAGAgtcctctgacggtgcaggtcgtgctcagcaga;

giant:  cgtctacgacgaggtgctgcggcttt, aagTAATACGACTCACTATAGGGAGAgtcctctgacggtgcaggtcgtgctcagcaga;

tailless:  gtcctagactgcagtctcggcagtt, aagTAATACGACTCACTATAGGGAGAgtcctctgacggtgcaggtcgtgctcagcaga;

orthodenticle:  tgtagcccgttcctcagatcgcggctctg, aagTAATACGACTCACTATAGGGAGAgtcctctgacggtgcaggtcgtgctcagcaga;

Results

The segmentation cascade is one of the best-defined gene regulation networks in development (e.g., Arnone and Davidson, 1997). A combination of genetic screens, microarray assays, and bioinformatics methods has identified most of the genes that initiate segmentation in the early Drosophila embryo (see Clyde et al., 2003 and references therein). The most comprehensive information has been obtained for the formation of pair-rule stripes, which foreshadow the subdivision of the embryo into a repeating series of segments. The seven eve stripes are regulated by approximately 10 maternal determinants and early zygotic gap genes (e.g., St Johnston and Nusslein-Volhard, 1992). In an effort to understand evolutionary changes in the segmentation network, we examined the expression of a comprehensive set of orthologous genes in the early embryos of Anopheles gambiae.

Dynamics of eve expression in early Anopheles and Drosophila embryos

The establishment of seven pair-rule stripes of eve expression is a culminating event in the segmentation of the early Drosophila embryo (Frasch et al., 1987; Macdonald et al., 1986; Figs. 1g–l). These stripes arise from broad domains of expression that are first detected during early phases of nuclear cleavage cycle 14 (Figs. 1g–l). Discrete stripes are fully formed just after the midpoint of cleavage cycle 14, before the completion of cellularization (Fig. 1j). These stripes persist during gastrulation and the rapid phase of germ band elongation (Fig. 1k) but disappear after the completion of this process (Fig. 1l). A new site of staining is detected within the presumptive proctodeum that does not correspond to any of the original stripes (Frasch et al., 1987; Fig. 1l). Most of these detailed features of the dynamic eve expression pattern are conserved in Anopheles. As in Drosophila, there is no maternal expression of eve (Fig. 1a, compare with g). Staining is first detected in a broad band in anterior regions (Fig. 1b, compare with h) but rapidly extends into posterior regions (Fig. 1c, compare with i). Crude stripes can be seen under a general haze of staining before the resolution of the final expression pattern (Fig. 1d, compare with j).

A major difference in the early eve expression patterns is the repression of the first three or four stripes in dorsal regions that correspond to the presumptive serosa in Anopheles (arrow, Figs. 1c, d). This repression is already evident before the refinement of the stripes (Fig. 1c). In Drosophila, all seven stripes exhibit uniform levels of staining in both ventral and dorsal regions (e.g., Figs. 1j). Another difference in the two patterns concerns the presumptive proctodeum. In Drosophila, staining does not appear at this site until the completion of germ band elongation, after the loss of the seven original eve stripes (Fig. 1l). In Anopheles, it is possible that this staining pattern initially appears as an eighth “stripe,” which arises from an initially broad seventh stripe (asterisk, Fig. 1d; Fig. 6e), and then persists in the presumptive proctodeum as seen in Drosophila (Fig. 1f).

Anterior localization of maternal oskar and nanos RNAs in Anopheles embryos

The similar eve staining patterns in flies and mosquitoes suggested that the underlying maternal determinants and gap genes would display conserved patterns of expression in the two systems. To our surprise, this was not found to be the case. In Drosophila, the oskar and nanos germ line RNAs are exclusively restricted to the posterior plasm of unfertilized eggs and early embryos (reviewed by Rongo and Lehmann, 1996; Figs. 2e–h). The oskar RNA quickly disappears just as cleavage nuclei enter posterior regions and form pole cells (Figs. 2e, f). nanos RNAs persist a bit longer and become incorporated in all of the pole cells (Figs. 2g, h). In Anopheles, the oskar RNA is primarily localized in the posterior plasm (Fig. 2a), but weaker staining is also detected at the anterior pole (arrow). The oskar RNA becomes incorporated into pole cells, while anterior staining is lost (Fig.
Maternal nanos RNAs are similarly localized in the posterior plasm (Fig. 2c), but some early embryos exhibit a very weak dot of staining at the anterior pole (arrow). As seen in *Drosophila*, the nanos RNA becomes incorporated into all of the pole cells (Fig. 2d). The weak localization of oskar and nanos RNAs in anterior regions is never seen in *Drosophila*. In fact, mislocalization of these RNAs results in the formation of supernumerary pole cells in anterior regions, along with the suppression of head structures (Ephrussi and Lehmann, 1992; Smith et al., 1992). We suggest that Oskar, along with associated polar granules, localizes an unknown “head determinant” in anterior regions of the early *Anopheles* embryo (see Discussion).

Comparison of gap expression patterns in early *Anopheles* and *Drosophila* embryos

In *Drosophila*, different levels of the Hunchback and Knirps gap repressor gradients define the limits of *eve* stripes 3, 4, 6, and 7 (Clyde et al., 2003), while Giant and Kruppel establish the borders of stripes 2 and 5 (Fujioka et al., 1999; Small et al., 1991; summarized in Fig. 7). In situ hybridization probes were prepared for *Anopheles* orthologues of all four of these gap genes, as well as a fifth gap gene, *tailless* (Fig. 3). *hunchback* displays a broad band of expression in the anterior half of the *Anopheles* embryo, encompassing both the presumptive head and thorax (Fig. 3b). This pattern is similar to that
observed in *Drosophila* (Tautz, 1988; Fig. 3h), although there are a few notable deviations. First, there is no obvious maternal expression seen in early *Anopheles* embryos (Fig. 3a), whereas maternal *hunchback* mRNAs are strongly expressed throughout early *Drosophila* embryos (Fig. 3g). Second, there is a significant change in the posterior staining pattern. The *Drosophila* gene displays a strong posterior stripe of expression (Fig. 3h) that is comparable in intensity to the anterior staining pattern. In *Anopheles*, this staining is significantly weaker than that of the anterior domain, and the posterior pattern is shifted anteriorly into the presumptive abdomen (see below; Figs. 4b and 6a).

The *Kruppel* and *knirps* staining patterns are similar in *Anopheles* and *Drosophila* embryos (Figs. 3c, d; compare with i,j). In both cases, the principal sites of expression are seen in the presumptive thorax and abdomen, respectively. However, the remaining two gap genes, *giant* and *tailless*, exhibit distinctive staining patterns. In *Anopheles*, *giant* exhibits a continuous band of staining in anterior regions (Fig. 3e), whereas the *Drosophila* gene is excluded from the anterior pole (Fig. 3k). Moreover, there is a prominent band of staining in the presumptive abdomen of *Drosophila* embryos that is not seen in *Anopheles* (see below). Finally, *tailless* is expressed in a narrow stripe in the posterior pole of *Drosophila* embryos (Pignoni et al., 1990; Fig. 3l), whereas *Anopheles* embryos display a dynamic pattern (Figs. 5a–c) that (transiently) extends throughout the presumptive abdomen (Fig. 3f; see below).

**Changes in the hunchback and giant expression patterns**

The preceding observations document significant changes in the expression patterns of maternal determinants and gap genes in flies and mosquitoes, although the dynamic *eve* pattern is quite similar in the two systems. The most notable differences were seen for the gap genes *hunchback* and *giant*. Additional in situ hybridization assays were done in an effort to obtain a more comprehensive view of these changing patterns (Fig. 4); *hunchback* is initially expressed in the anterior half of *Anopheles* embryos, with no staining detected in posterior regions (Fig. 4a; same as Fig. 3b). Weak posterior staining is detected by the onset of gastrulation (Fig. 4b; arrow, Fig. 4c), but expression appears to be localized within the presumptive abdomen rather than the posterior pole as seen in *Drosophila* (Fig. 3h). This shift was confirmed by costaining with *eve* (see below). In *Drosophila*, the anterior *hunchback* pattern is lost except for a stripe of staining in the thorax, and this stripe persists along with the posterior pattern during gastrulation (e.g., Schroder et al., 1988). In *Anopheles*, the early *hunchback* expression pattern gives way to localized expression in the presumptive serosa (Figs. 4b, c). *Drosophila* lacks a comparable staining pattern,
although similar patterns have been documented in Tribolium (Wolff et al., 1995), grasshoppers (Patel et al., 2001), and mothmidges (Rohr et al., 1999). It is conceivable that the late hunchback pattern is responsible, directly or indirectly, for the repression of eve stripes in the presumptive serosa (see Figs. 1c, d).

As seen for hunchback, there is no detectable expression of giant in posterior regions of early Anopheles embryos (Figs. 4d, e). Weak staining appears in the posterior pole by the onset of gastrulation (arrow, Fig. 4f). This staining is clearly posterior of the hunchback pattern in the presumptive abdomen (compare panels f and c). Thus, the posterior hunchback and giant patterns are reversed in Anopheles as compared with Drosophila (see Figs. 3h, k). The anterior giant pattern encompasses the entire anterior half of Anopheles embryos and extends into the anterior pole (Fig. 4d). The staining pattern is refined at gastrulation, including the loss of expression in the presumptive serosa (Fig. 4e) and the formation of discrete bands (Fig. 4f). Nonetheless, unlike the situation in Drosophila (Fig. 3k), expression persists in the anterior pole (Fig. 4f), thereby raising the possibility that different mechanisms are used to

Fig. 3. Comparison of gap gene expression patterns in Anopheles and Drosophila. Drosophila embryos (g–l) were hybridized with antisense RNA probes for: (g,h) hunchback, (i) Kruppel, (j) knirps, (k) giant, (l) tailless. Anopheles embryos (a–f) were hybridized with probes directed against the orthologous genes: hunchback (a,b), Kruppel (c), knirps (d), giant (e), and tailless (f). Early Anopheles embryos appear to lack maternal hunchback mRNAs (a), while comparable Drosophila embryos display strong, uniform staining (g). At the stages shortly preceding cellularization, hunchback is expressed in the anterior half of Anopheles embryos (b) but is expressed in both anterior and posterior regions of comparable Drosophila embryos (h). Kruppel and knirps are expressed in central and abdominal regions of Anopheles embryos, respectively (c,d). Similar patterns are seen for comparable Drosophila embryos (i,j). There is an anterior domain of knirps staining in both Anopheles (d) and Drosophila (j). The giant gene displays distinct patterns of expression in Anopheles (e) and Drosophila (k). In particular, giant staining extends to the anterior pole in Anopheles and is absent in posterior regions (e). In contrast, Drosophila embryos exhibit a strong band of staining in both anterior and posterior regions, although anterior staining does not extend to the anterior pole (k). Before the completion of cellularization, the Anopheles tailless gene exhibits broad staining in posterior regions (f) but exhibits a tight posterior stripe of expression in Drosophila, in addition to a spot of staining in the head (l).
establish the anterior border of eve stripe 2 in flies and mosquitoes (see Discussion).

Changes in the tailless expression patterns

The altered patterns of hunchback and giant expression in posterior regions raise the possibility that different combinations of gap repressors are used to establish eve stripes 5, 6, and 7 in Anopheles and Drosophila. It is unlikely that Giant establishes the posterior border of eve stripe 5 and that Hunchback delimits the posterior border of stripe 7, as seen in Drosophila (Clyde et al., 2003; Fujioka et al., 1999). The expression profiles of additional gap genes were analyzed in an effort to identify potential repressors for these stripe borders. The most obvious candidates are huckebein and tailless, since both are expressed in the posterior pole of Drosophila embryos (Bronner and Jackle, 1996; Pignoni et al., 1990). We did not detect expression of huckebein in early embryos, although strong staining appears after germband elongation (data not shown).

The gap gene tailless is initially detected at the anterior and posterior poles, with roughly equivalent levels of staining at the two sites (Fig. 5a). At slightly later stages, the anterior domain is lost, and the posterior pattern expands throughout the presumptive abdomen (Figs. 3f and 5b). The tailless transcripts detected in posterior regions exhibit a graded distribution, with peak levels at the posterior pole and progressively lower levels in more anterior regions.
During cellularization, staining is reduced in posterior regions and reappears near the anterior pole (Fig. 5c). This broad and dynamic staining pattern is consistent with the possibility that the Tailless repressor specifies the posterior borders of one or more posterior eve stripes (see below).

Torso signaling was examined in the Anopheles embryo in an effort to understand the basis for the expanded tailless expression pattern. In Drosophila, tailless is activated by the Torso signaling pathway (e.g., Cleghon et al., 1996), which can be visualized with an antibody against diphospho (dp)-ERK (Gabay et al., 1997; Schroder et al., 2000). The antibody detects localized staining in the terminal regions of early Drosophila embryos. A similar staining pattern is detected in Anopheles, although staining may be somewhat broader in Anopheles than Drosophila (Figs. 5d–f). It is therefore conceivable that the expansion of the posterior tailless expression pattern seen in Anopheles might be due to an expanded activation of the Torso signaling pathway.

Double-labeling assays suggest distinctive strategies of stripe formation

The combinations of gap repressors that define the borders of eve stripes 2 to 7 are known in Drosophila (summarized in Fig. 6f). Stripes 2 and 5 are formed by the combination of Giant and Kruppel repressors, while distinctive borders for stripes 3, 4, 6, and 7 are established by the differential repression of the stripe 3/7 and stripe 4/6 enhancers in response to distinct concentrations of the Hunchback and Knirps repressor gradients (Clyde et al., 2003). Double-staining assays provide immediate insights into the likely combination of gap repressors that are used for any given stripe. For example, the giant and Kruppel expression patterns abut the borders of eve stripes 2 and 5 (Fig. 6f). Double-staining assays were done to determine the potential regulators of the Anopheles eve stripes (Fig. 6). These experiments involved the use of digoxigenin-labeled hunchback, Kruppel, knirps, and giant hybridization probes along with an FITC-labeled eve probe. Different histochemical substrates were used to separately visualize the two patterns (Kosman and Small, 1997).

The anterior hunchback pattern extends through eve stripe 2 and approaches the anterior border of stripe 3, while the posterior pattern extends through stripes 6 and 7 (Figs. 6a, e). As discussed earlier (Fig. 4), this pattern is quite distinct from the posterior hunchback pattern seen in Drosophila, which abuts the posterior border of eve stripe 7. The anterior giant pattern extends from the anterior pole to eve stripe 2 (Fig. 6b), while the posterior pattern abuts the posterior border of eve stripe 7. In Drosophila, the posterior

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**Fig. 6. Colocalization assays.** Cellularized Anopheles embryos are oriented with anterior to the left and dorsal up. They were stained with mixtures of an FITC-labeled antisense RNA probe directed against eve (red) and a digoxigenin-labeled antisense RNA probe (blue) directed against hunchback (a,e), giant (b), Kruppel (c), or knirps (d). The bracket in a indicates the limits of the posterior hunchback staining pattern, which encompasses eve stripes 6 and 7. The posterior giant pattern (b) is restricted to the posterior pole rather than the presumptive abdomen as seen in Drosophila (e.g., Fig. 3k). (f) Summary of the gap gene expression patterns in Drosophila (top) and Anopheles (bottom) based on double-staining assays. eve stripes 2 to 7 are represented by the cross-hatched vertical bars, while the limits of the gap genes are indicated by the solid horizontal lines. Uncertainty in the exact limits of the gap patterns is indicated by breaks in these lines. The anterior hunchback (hb) and giant (gt) expression patterns are similar in flies and mosquitoes, as are the limits of the knirps (kni) pattern. The Kruppel (Kr) pattern may be somewhat narrower in mosquitoes than flies, while the posterior hunchback (hb) and giant (gt) patterns are quite distinct in the two systems. The posterior hunchback (hb) pattern encompasses eve stripes 6 and 7 in Anopheles but abuts the posterior border of eve stripe 7 in Drosophila. The posterior giant (gt) pattern abuts the posterior border of stripe 7 in Anopheles but encompasses stripe 6 in Drosophila.
The maternal promoter leads to the same limits of expression in *Anopheles as Drosophila*. In both cases, the staining pattern extends from *eve* stripes 4 to 6 (Fig. 6d). In *Anopheles*, the anterior *knirps* pattern straddles the anterior border of *eve* stripe 1. Some of the *eve* stripes are associated with the same combinations of gap repressors in flies and mosquitoes (e.g., stripes 2, 3, and possibly 4), whereas others show distinctive combinations of gap repressors (e.g., stripes 5, 6, and 7; Fig. 6f).

**Discussion**

The systematic comparison of segmentation regulatory genes in *Anopheles* and *Drosophila* suggests that the segmentation gene network has undergone considerable evolutionary change among dipterans despite highly conserved patterns of *eve* expression. We discuss three particular changes in the network: the localization of maternal determinants, the formation of the anterior border of *eve* stripe 2, and the formation of the posterior borders of *eve* stripes 5, 6, and 7.

**Maternal determinants**

In *Drosophila*, *hunchback* contains two promoters (Schroder et al., 1988), and the maternal promoter leads to the ubiquitous distribution of *hunchback* mRNAs throughout early embryos (e.g., Fig. 3g). Although a negative result, we do not detect *hunchback* mRNAs in early *Anopheles* embryos (Fig. 3a). This apparent absence of maternal transcripts raises the possibility that localized Nanos products are not required for inhibiting the synthesis of Hunchback proteins in posterior regions of *Anopheles* embryos. In *Drosophila*, the embryonic lethality caused by *nanos* mutants can be suppressed by the removal of maternal Hunchback products (Irish et al., 1989; Struhl, 1989). This *nanos–hunchback* interaction is ancient and probably operating in basal insects, and possibly basal arthropods (e.g., Lall et al., 2003). However, the potential absence of this interaction in *Anopheles* is consistent with the idea that *nanos* has an additional essential function. Indeed, a recent study suggests that Nanos is required for maintaining stem cell populations of germ cells in *Drosophila* (Wang and Lin, 2004).

*Anopheles* lacks *bicoid* and contains a lone *Hox3* gene that is more closely related to *zen* and specifically expressed in the serosa (Y. Goltsev, unpublished observations). How is *hunchback* activated in the presumptive head and thorax in *Anopheles*? The homeobox gene *orthodenticle* can substitute for *bicoid* in *Tribolium* (SCHRÖDER, 2003). However, *orthodenticle* does not appear to be maternally expressed in *Anopheles*, but instead, staining is strictly zygotic and restricted to anterior regions, similar to the pattern seen in *Drosophila* (Figs. 4g–i). Sequential patterns of *orthodenticle*, *giant*, and *hunchback* expression are established by differential threshold readouts of the Bicoid gradient in *Drosophila*. It is possible that an unknown maternal regulatory gradient emanating from the anterior pole is responsible for producing similar patterns of expression in *Anopheles*. We propose that this unknown regulatory factor may be localized to the anterior pole by Oskar (see Fig. 2a). Oskar coordinates the assembly of polar granules and is essential for the localization of Nanos in the posterior plasm (e.g., Rongo and Lehmann, 1996). It might also localize one or more unknown determinants in anterior regions of *Anopheles* embryos.

**eve Stripe 2**

The *eve* stripe 2 enhancer is the most thoroughly characterized enhancer in the segmentation gene network. It can be activated throughout the anterior half of the embryo by Bicoid and Hunchback, but the Giant and Kruppel repressors delimit the pattern and establish the anterior and posterior stripe borders, respectively. Removal of the Giant repressor sites within the stripe 2 enhancer in *cis* or removal of the repressor in *trans* causes an anterior expansion of the stripe 2 pattern (Small et al., 1992). However, ectopic expression does not extend to the anterior pole, suggesting that an additional anterior repressor regulates the stripe 2 enhancer. Recent studies identified Sloppy-paired as the likely anterior repressor (Andrioli et al., 2002). The limits of the *giant* and *Kruppel* expression patterns seen in *Anopheles* suggest that they might define the *eve* stripe 2 borders, just as in *Drosophila*. However, at the critical time when *eve* stripe 2 is formed in *Anopheles*, the *giant* staining pattern extends to the anterior pole, while the corresponding *Drosophila* gene is repressed in these regions (see Figs. 3e, k). It is therefore possible that Giant is sufficient to form the anterior border in *Anopheles* and that repression by Sloppy-paired represents an innovation in *Drosophila*.

**Posterior borders of eve stripes 5, 6, and 7**

There are numerous differences in the patterns of gap gene expression in *Drosophila* and *Anopheles*. In *Drosophila*, the posterior stripe of *hunchback* expression is the source of a repressor gradient that specifies the posterior borders of *eve* stripes 6 and 7 (Clyde et al., 2003; Fujioka et al., 1999; summarized in Fig. 6a). *Anopheles* exhibits a distinct posterior staining pattern, with expression extending through stripes 6 and 7 (see Fig. 6). It is therefore unlikely that Hunchback regulates these stripes as seen in *Drosophila*. Instead, the location of the posterior *hunchback* pattern suggests that it regulates the posterior border of *eve* stripe 5 in *Anopheles* (Fig. 6). In *Drosophila*, this border is formed by Giant, but in *Anopheles*, the posterior giant
expression pattern is restricted to the posterior pole where it abuts stripe 7. Thus, a combination of Kruppel and Giant defines the eve stripe 5 borders in Drosophila, whereas Kruppel and Hunchback might be used in Anopheles (summarized in Fig. 7).

In Drosophila, eve stripes 6 and 7 are regulated by different concentrations of Knirps and Hunchback (Clyde et al., 2003). Low levels of Knirps define the anterior border of stripe 7, while higher levels are needed to repress eve stripe 6. Conversely, low levels of Hunchback establish the posterior border of eve stripe 6, while higher levels regulate stripe 7 (summarized in Fig. 7). The position of the knirps expression pattern (Fig. 6) is consistent with the possibility that it defines the anterior limits of stripes 6 and 7, just as in Drosophila. However, as discussed above, the posterior borders of these stripes are probably not regulated by Hunchback. The expanded pattern of tailless expression seen in Anopheles (see Figs. 3f, i) might permit it to establish the posterior border of eve stripe 6 and possibly stripe 7 (see Figs. 5a–c). An alternative candidate for the posterior stripe 7 border is giant, which is expressed in a tight domain within the posterior pole. Consistent with this possibility is the observation that the posterior giant pattern comes on relatively late, and the posterior stripe 7 border is the last to form among the seven eve stripes (see Fig. 1). The reversal of the posterior hunchback and giant expression patterns, along with the expanded tailless pattern, strongly suggests that different combinations of gap repressors are used to define eve stripes 5, 6, and 7 in Drosophila and Anopheles (summarized in Fig. 7).

Organization of eve regulatory DNAs and opposing gap repressor gradients

An implication of the preceding arguments is that each of the seven eve stripes is regulated by a separate enhancer in Anopheles. Only five enhancers regulate eve in Drosophila since four of the seven stripes (3, 4, 6, and 7) are regulated by just two enhancers (3/7 and 4/6) that respond to different concentrations of the opposing Hunchback and Knirps repressor gradients (Clyde et al., 2003). The change in the posterior hunchback pattern virtually excludes the use of this strategy in Anopheles. Thus, stripes 3 and 7 are probably regulated by separate enhancers since different combinations of gap repressors appear to define the stripe borders (Hunchback/Knirps and Knirps/Giant, respectively). Similar arguments suggest that stripes 4 and 6 are also regulated by separate enhancers.

Why do some enhancers generate two stripes, while others direct just one? Consider the eve stripe 2 and stripe 3/7 enhancers in Drosophila. The stripe 3/7 enhancer is activated by ubiquitous activators, including dSTAT, and the two stripes are “carved out” by the localized Hunchback and Knirps repressors (Fujioaka et al., 1999; Small et al., 1996). Knirps establishes the posterior border of stripe 3 and anterior border of stripe 7, while Hunchback establishes the anterior border of stripe 3 and posterior border of stripe 7. The stripe 2 enhancer directs just a single stripe due to the localized distribution of the stripe 2 activators, particularly Bicoid. In principle, a ubiquitous activator would cause the stripe 2 enhancer to direct two stripes, stripes 2 and 5. Opposing Giant and Kruppel repressor gradients would carve out the borders of the two stripes, similar to the way in...
which Hunchback and Knirps regulate the stripe 3/7 and stripe 4/6 enhancers. Presumably, the eve stripe 5 enhancer directs a single stripe of expression because it is regulated by a localized activator, possibly Caudal (Fujikoka et al., 1999).

We suggest that ancestral dipterans contained an eve locus with separate enhancers for every stripe. Anopheles eve might represent an approximation of this ancestral locus. The consolidation of enhancers that generate multiple stripes was made possible by cross-repression of gap gene pairs. In Drosophila, there are mutually repressive interactions between Hunchback and Knirps, as well as between Giant and Kruppel (e.g., Kraut and Levine, 1991; Struhl et al., 1992). The use of these interacting gap pairs along with ubiquitous activators permits the formation of two stripes from a single enhancer. It is possible to envision two ways in which mutual cross-repression of these gap genes helps to establish the precise patterns of pair-rule gene expression. First, it ensures that there are zones free of repressor activity on both sides of Kruppel (for the Kruppel and Giant pair) and Knirps (for the Knirps and Hunchback pair) domains. Second, it protects the patterns of pair-rule gene expression from mutations that could potentially shift the domains of gap gene expression. For example, a mutation that could shift the expression of Kruppel would simultaneously shift the expression of Giant always leaving a repressor-free zone where Eve stripes would be established. Therefore, the evolution of the eve locus depends on the changes in the preceding tier of the segmentation network: refinement in gap gene cross-regulatory interactions.

Finally, it is easy to imagine that certain dipterans have a single enhancer for stripes 2 and 5, rather than the separate enhancers seen in Drosophila. Perhaps, the symmetric repression of Giant and Kruppel is a relatively recent occurrence, only now creating the opportunity for consolidated expression of stripes 2 and 5.

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