Canalization of segmentation and its evolution in *Drosophila*

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Segmentation in *Drosophila* embryogenesis occurs through a hierarchical cascade of regulatory gene expression driven by the establishment of a diffusion-mediated morphogen gradient. Here, we investigate the response of this pattern formation process to genetic variation and evolution in egg size. Specifically, we ask whether spatial localization of gap genes *Kruppel* (*Kr*) and *giant* (*gt*) and the pair-rule gene *even-skipped* (*eve*) during cellularization is robust to genetic variation in embryo length in three *Drosophila melanogaster* isolines and two closely related species. We identified two wild-derived strains of *D. melanogaster* whose eggs differ by ≈25% in length when reared under identical conditions. These two lines, a *D. melanogaster* laboratory stock (*w1118*), and offspring from crosses between the lines all exhibit precise scaling in the placement of gap and pair-rule gene expression along the anterior–posterior axis in relation to embryo length. Genetic analysis indicates that this scaling is maternally controlled. Maternal regulation of scaling must be required for consistent localization of segmentation gene expression because embryo size, a genetically variable and adaptive trait, is maternally inherited. We also investigated spatial scaling between these *D. melanogaster* lines and single lines of *Drosophila sechellia* and *Drosophila simulans*, the latter two differing by ≈25% in egg length. In contrast to the robust scaling we observed within species, localization of gene expression relative to embryo length differs significantly between the three species. Thus, the developmental mechanism that assures robust scaling within a species does not prevent rapid evolution between species.

buffering | development | embryo size | genetic variation | scaling

Many developmental processes use mechanisms to assure stereotyped outcomes in the face of environmental noise and genetic variability acting in ontogeny. One such trait is the well studied pathway for establishing the anterior–posterior (A-P) axis in *Drosophila*, which has come into focus with renewed interest in the genetic basis of phenotypic robustness (1–5). In a seminal paper, Houchmandzedah, Wieschaus, and Leibler (1) measured spatiotemporal variability in the Bicoid (Bcd) diffusion gradient and its downstream target *hunchback* (*hb*). Spatial localization of Hb was shown to be remarkably precise and was resistant to both within-line variation in embryo length and experimentally produced shifts in the Bcd gradient profile, manipulated by raising embryos at different temperatures, thus changing development rate. That study reported greater variability in the morphogen gradient formed by Bcd, which activates *hb*, but challenges to this finding (2, 3) and carefully designed follow-up experiments (6) have shown that the Bcd gradient is as precise as Hb. Thus, spatial precision in A-P patterning system may be achieved simply by the biophysical process of Bcd morphogen diffusion and might not involve other factors or feedback loops.

Scaling, the expression of segmentation genes at the same relative position along the length of the embryo independent of embryo length, is a second reported property of the A-P patterning system (1, 4). The molecular or genetic basis for this feature of the patterning system is not known. A study of gene expression through the developmental stages during which Bcd activates *hb* expression (4, 5) indicates that the Bcd gradient is established along an absolute coordinate system, independent of (intrastrain) egg length, whereas Hb and even-skipped (Eve) patterns scale with embryo length. The establishment of spatial precision, as reflected in Hb and Eve expression, coincides with this transformation of scale (4). On the other hand, the Bcd gradient and Hb expression have also been investigated in three distantly related Dipteran species with egg sizes differing by a factor of five; Bcd gradients were found to scale with embryo size (5). Thus, the between-species and within-species observations conflict with respect to the dynamics of the Bcd gradient in relation to size variation. These conflicting observations, however, may represent differences in developmental time points when the gradient measurements were taken (3, 6).

Several models have been proposed to account for the precision and/or scaling of Hb (and other gap and pair-rule proteins), including a hypothetical reverse gradient of an unidentified factor (7) and presteady-state expression of Bcd targets (8). The latter model cannot easily account for size scaling, however, and the reverse gradient model lacks a viable candidate for the hypothetical factor. Another model proposes a mechanism involving the transport of *hb* RNA away from the anterior and posterior poles by the RNA-binding protein Staufen (9). Modeling of gap gene circuits also points to the existence of unknown factors contributing to the establishment of developmental precision (10). Further complicating this matter, the recent reappraisal of Bcd gradient precision (6) raises the possibility that precision and scaling may be established independently. Nevertheless, a full understanding of precision likely awaits a corresponding understanding of scaling.

Investigations of precision and scaling in A-P axis formation to date have been restricted to a single isogenic laboratory stock, thus buffering of the segmentation phenotype with respect to embryo length has been demonstrated only for nongenetic sources of variability. In addition, although different Diptera species have been reported to exhibit scaling of Hb expression (5), subtle spatial shifts in gene expression cannot be excluded. This question might be better addressed by analyzing segmentation gene expression among more closely related species.

Egg length is genetically variable in *Drosophila melanogaster* (11) and related species. This variation is almost certainly adaptive because egg size exhibits latitudinal clines, with larger

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Abbreviations: A-P, anterior-posterior; Bcd, Bicoid; Eve, even-skipped; *gt*, *giant*; Hb, Hunchback; *Kr*, *Kruppel*.

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eggs being produced at higher latitudes, and egg length in laboratory populations evolves in response to temperature alone in the predicted directions (11). Phenotypic variability in egg size has been shown to have a polygenic mode of inheritance (12) and responds to artificial selection on egg length (13). As with many phenotypic characters, the adaptive value of egg size in the wild is not known. Egg size (and shape) differs between *Drosophila* species as well, although these differences have generally not been investigated.

Given this abundant source of genetic variation for egg size, we investigated whether the establishment of segmental boundaries, measured by the expression of the gap genes *giant* (*gt*) and *Kruppel* (*Kr*) and the pair-rule gene *eve*, is buffered developmentally against genetic variability for egg (embryo) length both within and between species. Embryo size may have consequences for the development of morphogen gradients in two ways: first, simply changing the physical space where these gradients are established could affect concentrations of transcription factors, and second, maternal contribution may differ with embryo size. We first surveyed isofemale lines of *D. melanogaster* and identified two strains producing substantially different-sized eggs representing the two tails of the distribution. Early-stage embryos collected from these two lines, along with a third laboratory stock, plus one stock each of *Drosophila simulans* and *Drosophila sechellia*, which also differ in egg size, were subjected to spatiotemporal analysis of *eve*, *gt*, and *Kr* expression. We analyzed gene expression by *in situ* hybridization along both absolute and relative egg length scales and could compare the localization of mRNA within and between species. We also carried out a genetic analysis between large- and small-egg *D. melanogaster* lines to investigate the mode of inheritance of pattern localization phenotypes. These data allowed us to address the extent to which expression patterns are buffered against genetic differences in egg length and whether patterning responds differently to within- or between-species sources of this variation.

Results

We first documented a substantial range of within- and between-species variation in egg size in 47 wild-derived and laboratory strains representing nine species [partial data shown in Fig. 1; also see supporting information (SI) *Text*]. There is genetic variation within species for egg size, and egg size can also evolve large differences over short evolutionary time, as shown by the newly evolved *Drosophila santomea* and *D. sechellia* having unusually large eggs compared with their parental species (*Drosophila yakuba* and *D. simulans*, respectively). For our investigation on the role of embryo length on spatial patterning, we focused on the *D. melanogaster* small- and large-egg lines *Fra* and *Ind* (as well as the laboratory strain *w1118*) and a small- and large-egg species pair, *D. simulans* (*FC* strain) and *D. sechellia* (*Robertson* strain). The magnitude of the egg size differences in the small- and large-egg pairs are similar within and between the species (Fig. 1), and egg size in these lines appears polygenic (SI Fig. 6), consistent with previous estimates (12).

For each line, we measured gene expression (i.e., stripe) boundary locations in ≈50 cellularization-stage embryos (cleavage cycle 14; see *Materials and Methods* and SI Fig. 7), recording both the absolute (in μm) and relative linear distance from the anterior pole of an embryo along a line bisecting the two poles. We used an ANOVA to investigate sources of variation in gene expression. Developmental stage has a significant effect on stripe boundary positions; however, the temporal dynamics of stripe formation does not differ significantly between lines or species (as measured by age × line interaction term in the ANOVA). Standard errors of within-line measurements, estimated from within-line residuals in the ANOVA, are similar across stripes and are on the order of a third of a cell width (see *Materials and Methods*), suggesting that the experimental design could detect shifts in stripe boundary localizations of as little as one cell.

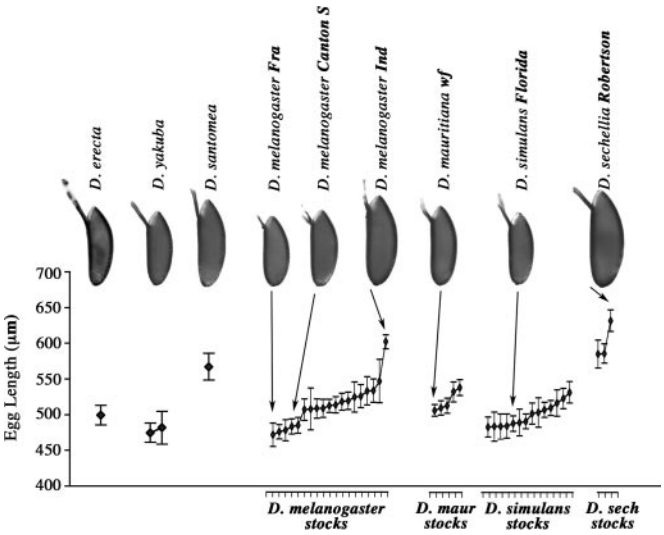

Fig. 1. Egg lengths within and among *Drosophila* species. Average egg lengths (in μm; $\pm$1 SD) for representatives of the *D. melanogaster* species subgroup, with multiple stocks from *D. melanogaster*, *D. mauritiana* (*D. maur*), *D. simulans*, and *D. sechellia* (*D. sech*). Images are representative eggs photographed at the same magnification and can be directly compared with one another. Measurement error is small compared with the length differences between sampled eggs. All strains were grown under the same conditions, and eggs were collected with the same protocol.

***eve, gt,* and *Kr* mRNA Localization Scales with Embryo Length Within *D. melanogaster*.** The analysis revealed statistically significant differences in absolute stripe boundary locations for all seven *eve* stripes between the three *D. melanogaster* lines (Fig. 2*A*), where predictably the stripes formed more posteriorly in the large-egg line (*Ind*) than the small-egg lines (*Fra* and *w1118*). Scaled to embryo length, however (Fig. 2*B*), none of the *eve* stripe boundaries differed significantly across *D. melanogaster* lines for all seven *eve* stripes (Fig. 2*D*, mel lines). In the same way, the locations of *gt* and *Kr* stripes also scaled with embryo length (SI Figs. 8 and 9, respectively).

Scaling of gene expression patterns for both gap and pair-rule genes echoes results on Bcd and Hb expression (1, 4, 7) and suggests that the absolute shift in gene expression between the large- and small-embryo lines is established early in embryogenesis. To test whether scaling is a maternal or zygotic property, we analyzed stripe locations in F_1, F_2, and F_3 embryos from reciprocal crosses between the *Ind* and *Fra* lines (Fig. 3*A*), following the same experimental design as in the previous analysis. Consistent with the previous results, stripe position in absolute measurements segregated with embryo size (Fig. 3*A*) in such a way that when scaled to embryo length all lines in this *D. melanogaster* cross had stripes placed in the same relative positions. F_1 embryo lengths resembled the maternal distributions of both length (*Ind* or *Fra*) and gene expression (SI Fig. 10 and Fig. 3*A*, respectively). An ANOVA was used to examine the variance in absolute units between the *Fra* and *Ind* parents and their reciprocal F_1 offspring, again using developmental age as a covariate. The effect of the mother ($P < 0.0001$ averaged across *eve* or across all patterns), but not line ($P = 0.13$ averaged over *eve*; $P = 0.22$ over all patterns) is statistically significant. The same scaling was also evident in the F_2 and F_3 embryos, although these generations produced embryos with an intermediate average length. As absolute stripe positioning is a maternally

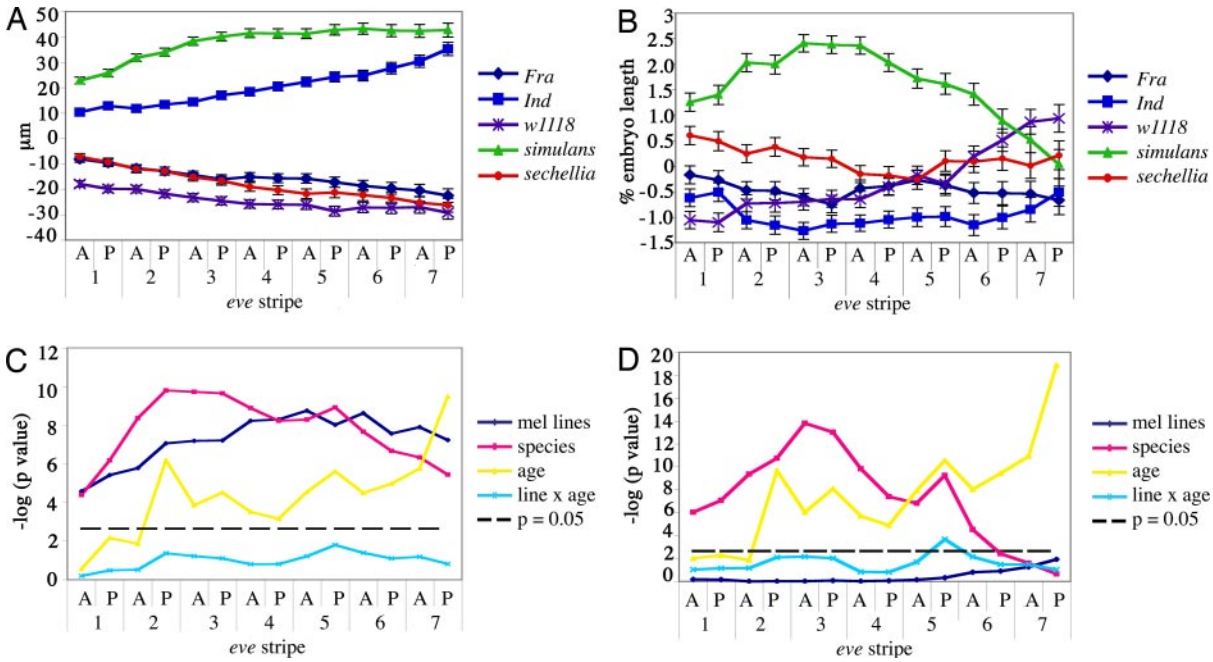

Fig. 2. *eve* stripe position varies between species, not within *D. melanogaster*. (*A* and *B*) Anterior (A) and posterior (P) *eve* stripe boundaries (least-square means ± 1 SE) in stage 14a embryos in three *D. melanogaster* isofemale lines (*Ind*, *Fra*, and *w1118*) and the closely related species *D. simulans* and *D. sechellia*. Stripe boundaries were measured as distances from the anterior pole of the embryo. Means are represented as deviations by line from the mean of all of the lines, with a shift toward positive numbers indicating more posterior localization compared with the grand mean. (*A*) Means in absolute units (μm), uncorrected for embryo length. Stripes are located further from the anterior pole in the two large-egg strains (*Ind* and *sechellia*). (*B*) Relative measurement means, represented as a percentage of the embryo length. *D. sechellia eve* stripes remain posterior-shifted when scaled to embryo length, whereas the *D. melanogaster Ind eve* stripes exhibit the same scaling with length as the other two *D. melanogaster* lines. (*C* and *D*) Statistical significance (negative logarithm of *P* value) of factors influencing stripe boundary positions based on an ANOVA. Factors are age, line within *D. melanogaster* (mel lines), species, and the line by age interaction. The dotted line is the Bonferroni-corrected $P = 0.05$ cutoff. These graphs are a confluence of different ANOVAs (see *Materials and Methods*), as the effects of line within *D. melanogaster* and species were modeled separately. The species term has a significant effect on both absolute (*C*) and relative (*D*) stripe position, whereas the significance of the within *D. melanogaster* term (mel lines) disappears when the measurements are corrected for embryo size.

inherited phenotype, we examined F_2 and F_3 generations to test for dominance effects: stripe patterning shows no statistically significant departure from complete additivity ($P = 0.75$). The results clearly indicate, therefore, that scaling of segmentation gene expression occurs through the expression of maternally derived determinants and does not depend on the zygotic genotype of the embryo.

Because of this maternal inheritance of both embryo size and spatial scaling of gene expression, the F_3 embryos are the first generation in which segregation of size and stripe-positioning determinants can be investigated. As expected for a quantitative character, F_3 embryos exhibited a wide range of lengths (≈20%), but retained very nearly perfect linear scaling of all stripe positions across the entire distribution of embryo lengths (Fig. 3*B*). Thus, we find no evidence for genetic differences between the lines in the regulation of stripe positioning. The linear scaling of gene expression in these F_3 embryos, whose lengths include a genetic contribution, implies that this buffering mechanism is effective across the entire spectrum of embryo lengths present in *D. melanogaster*. Holloway *et al.* (4) presented evidence for scaling of *eve* stripes over 20% embryo size differences induced within a single (presumably isogenic) laboratory strain. We can now extend this finding to heritable embryo size differences of the same magnitude within this species: pattern formation in segmentation exhibits robustness within this species against both environmental and genetic sources of variation, consistent with a general theory of evolutionary canalization (14, 15).

Interestingly, we did observe a slight anterior shift of scaled gene expression with increasing embryo length (average slope ± standard deviation: $-3.28e\text{-}5 \pm 1.54e\text{-}5$), which translates to an average stripe localization difference of 1% embryo length between the smallest and largest embryos in our sample. Although the differences show statistical significance at only some of the stripe positions, this finding suggests that buffering may be incomplete. We note, however, that because in nature egg size is strongly correlated with latitude, and therefore temperature, and development time increases with decreasing temperature, a posterior shift in Bcd is expected, which may compensate for larger egg size in a natural environment. Thus, scaling may have both genetic and environmental underpinnings.

We also investigated the variation in stripe positions within individual embryos. In particular, we could ask whether within-individual deviations are correlated and, if so, whether the strength of the correlations depends on the physical distance separating stripes. The results are clear: variation in stripe locations shows strong linear dependence on the distance between stripes ($P < 0.0001$, intercept 0.93, slope of -1.99, and $R^2 = 0.83$) (Fig. 4). Remarkably, this relationship holds for all genes and is not restricted to expression boundaries of the same mRNA. Correlations between stripe boundary variations are slightly stronger if the mRNA is produced by the same locus (data not shown and Fig. 4). Although technical biases introduced by the preparation of specimens for photography (the embryos are slightly compressed under a coverslip) cannot be excluded, the uniformity of the correlation structure across the length of the embryo indicates that it is probably not an artifact.

Spatially restricted correlations in stripe localization within individual embryos are consistent with the establishment of subtle errors in the spatial patterning of individual gap genes that then propagate to pair-rule gene expression. Interestingly, we did not observe coordinate shifts of all stripes within individual embryos, as one might predict would have occurred if these

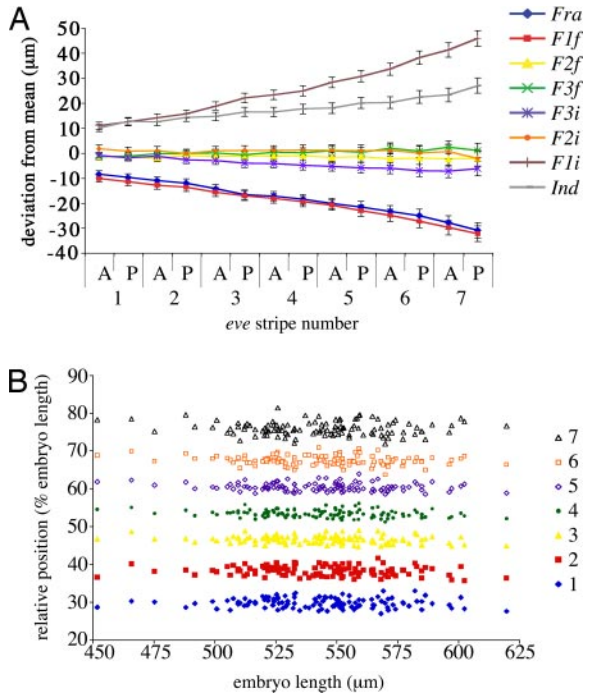

Fig. 3. Maternal inheritance of *eve* stripe boundaries. Genetic analysis of *D. melanogaster Fra* and *Ind* reciprocal crosses to F_3s. (*A*) Inheritance of absolute stripe position is consistent with an additive, maternally inherited trait. Line means for absolute measures of stripe positions (μm from anterior pole), shown as the deviation from the grand mean, for each stripe. The designation *f* (*Fra*) or *i* (*Ind*) after a cross (F1*f*, F1*i*, etc.) indicates the maternal lineage. The stripe locations in *F1f* and *F1i* lines resemble those of their mothers, *Fra* and *Ind*; stripes in F_2 and F_3 lines are localized midway between the parental lines. (*B*) F_3 embryos exhibit a wide range of embryo lengths (as expected for a segregating trait) but retain nearly perfect linear scaling of *eve* stripe boundary locations across this length distribution. Relative stripe positions in individual F_3 embryos for the anterior boundaries of each *eve* stripe are plotted against each embryo's length.

deviations propagated from embryo-to-embryo differences in the Bcd concentration gradient. Thus, the fact that correlations are spatially restricted is consistent with Bcd gradient precision.

***eve*, *gt*, and *Kr* mRNA Localization Differs Between Closely Related Species.** Unlike the within-species contrasts, relative stripe boundary locations are significantly different between the three

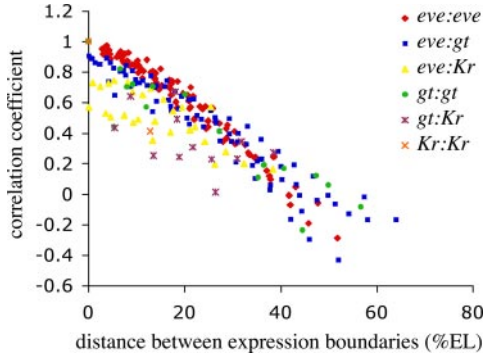

Fig. 4. Correlations of expression pattern boundary positions within embryos. The Pearson's correlation coefficient for all pairs of expression domain boundaries (all lines and ages, for *eve*, *gt*, and *Kr*) are plotted as a function of average relative distance (%EL) between pairs. Deviations of neighboring stripe boundaries within an individual embryo relative to the mean are highly correlated. The correlation falls off with increasing distance between stripes, suggesting regional influences of stripe positioning.

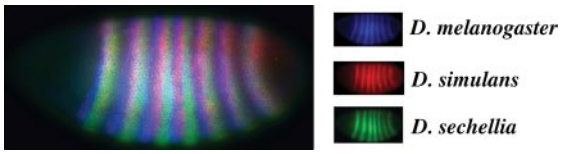

Fig. 5. Species differences in *eve* localization. The relative positioning of *eve* stripes differs between the three species studied (*D. melanogaster*, *D. simulans*, and *D. sechellia*). Embryos with stripe positions closest to the mean for each species were chosen, and images were scaled to the same length and overlaid.

closely related species, especially evident in the comparison of *D. sechellia* with either *D. simulans* or *D. melanogaster* lines (Fig. 5). Under the mixed-model ANOVA, the effect of species is significant for all but the posterior-most stripe (Fig. 2*D*, species). Also notable are the two large-egg lines, the *D. sechellia* line and *D. melanogaster* (*Ind*), exhibiting stripes shifted in opposite directions relative to mean stripe position, posterior and anterior of the mean, respectively. Thus, egg size, *per se*, is not itself a causal factor in the relative stripe positioning shifts observed between the species. Rather, the evidence indicates that relative positioning of *eve* stripes has evolved among these closely related species.

Discussion

Here, we report results regarding developmental stability and its evolution. First, scaling in *D. melanogaster* embryogenesis is effective against both environmental and genetic sources of length variation. In fact, because the two lines we investigated span most of the known range of egg length variation in the species, our results show that the scaling mechanism, whatever its molecular basis, is likely effective across the natural range of this phenotype. Second, scaling is established maternally and not zygotically. This observation is evolutionarily inevitable because egg size, a genetically variable trait, is also maternally inherited. Maternal regulation of scaling implies that robustness of pattern formation in relation to embryo length is not a property of segmentation network complexity or feedback loops, two often-invoked theories of developmental robustness. Third, subtle noise in the placement of gap and pair-rule stripes is strongly correlated over short (but not long) distances along the A-P axis within individual embryos. We hypothesize that this noise can be traced back to stochastic fluctuations in the spatial activation of gap genes. Although this is an example of localization differences in scaling caused by nongenetic variation, it reveals the biological possibility for genetic variation for stripe scaling. Finally, spatial patterns of segmentation gene expression differ between closely related species and therefore can evolve rapidly.

The term "canalization" was coined by Waddington (16) to describe a development process that produces stereotypical or discrete outputs. Precision and scaling of segmentation gene expression qualifies, we believe, as a canalized trait because in the absence of a mechanism to assure scaling, the dynamics of the Bcd gradient formation (the morphogen required for establishing A-P patterning) cannot produce stereotypical outputs in response to embryo length variation.

Canalization poses difficulties for evolution, as canalized traits show no phenotypic variation upon which selection can act. Given the fact that spatial patterning is a strongly buffered trait, we were surprised to find evidence for its rapid evolution. *D. sechellia* is a genetically invariant island endemic that is likely to have evolved as a small population for most, if not all, of its history (perhaps as long as 500,000 years) (17). Consistent with a small population size, it exhibits a number of characteristic patterns of sequence evolution indicating the accumulation of deleterious mutations (by genetic drift) (17). It also exhibits

obvious adaptations, such as oviposition and larval survival on Morinda fruit, which is toxic to *D. simulans* (18). *D. sechellia* produces many fewer ovarioles than *D. simulans* (19); here, we show that it also produces an uncharacteristically large egg. As both of these traits are tightly tied to fecundity, it seems likely that these are both adaptive trait differences, although the fixation of deleterious mutations for these traits cannot be excluded. The same arguments can be applied to the evolved difference in segmentation gene patterning. The segmentation pattern difference between this species and *D. simulans* can be genetically mapped, in principle, as hybrids and backcross generations are fertile. If genes responsible for the difference can be identified, it should be possible to experimentally distinguish between the adaptive vs. deleterious models for the evolution of this trait.

Although the three *D. melanogaster* strains investigated did not exhibit genetic variation for the specification of stripe locations, the fact that differences have evolved between closely related species is an indication that such genetic variation can exist. The ability to suppress environmental or genetic noise is the hallmark of genetic canalization, as illustrated by the classical experiments of Waddington (16, 20–23), Waddington and Robertson (24), Rendel (25, 26), and others (27–30). One possible explanation of our results, therefore, is that the expression of within-species variation is suppressed or is too small to be detected by our experiments. Another possibility is simply that stripe localization is under strong stabilizing selection and is not genetically variable. The experiments presented here were not designed to investigate these alternatives; genetic variation might be revealed by surveying other natural isolates of this species or manipulating environmental or genetic conditions.

The evolved changes in spatial patterning between species are likely to involve differences affecting the "set points" of gene expression (i.e., the specific location, timing, and abundance of gene product), rather than the process controlling precision and scaling. The main argument supporting this hypothesis is that the shift in segmentation gene patterning between species is not associated with the evolution of egg size *per se* because the *D. melanogaster Ind* egg and the *D. sechellia* egg are similar in length but produce divergent segmentation patterns.

One possible way to alter a gene expression set point is evolution of the cis-regulatory elements controlling its spatial expression. Transgenic experiments with the *eve* stripe 2 enhancer indicate, however, that spatial localization of stripe expression (but not activation or expression levels) is highly conserved in *Drosophila* (31–33). Furthermore, parallel evolution of many such cis-regulatory modules would be required to produce a coordinate shift in segmentation gene patterning. Thus, it seems unlikely that the patterning differences between the species are driven by changes in the cis-regulatory architecture of the surveyed genes. The scenario might be different for traits like thorax bristles and wing pigmentation, where recent evidence illustrates the role of cis-regulatory evolution (34–36).

In theory, heterochronic differences in the segmentation process could also underlie differences in segmentation patterning between the species, which might include the timing of cell division, for example, allowing more or less time for diffusion of morphogens. Arguing against this possibility, we find no evidence for strain by stage interactions in the statistical analysis of the data. We investigated, however, only a narrow window of developmental time, which may have been insufficient for detecting heterochronic shifts.

A third possibility involves the evolution of egg or blastoderm architecture, including the density of nuclei along the A-P axis in precellular embryos, or the morphology of the cortex space in which cellularization occurs. Traits such as these could readily evolve in response to natural selection for optimal egg shape or downstream morphology. These traits would have to influence A-P axis formation, the possibilities of which have yet to be explored.

Materials and Methods

Egg Length Measurement of Fly Strains. Wild-derived lines were mostly isofemale lines, whereas laboratory strains are largely of unknown origin, but all are relatively inbred and are assumed to be largely isogenic. Egg lengths were measured in samples of 8–10 eggs per strain, produced by multiple females, 2–4 days after eclosion, grown in uncrowded conditions. A study of the effect of female age and age of egg (postlaying) on egg size in these lines showed no differences across the range used. Zero to 12-h eggs (postlaying) were harvested and placed in 70% glycerol (volume: volume with 1× PBS) on glass slides, rolling them if necessary to ensure horizontal orientation on the slide. A coverslip was placed over the egg, supported on either side by additional coverslips to avoid any compression or deformation of the egg. Eggs were viewed at the same magnification, focusing on both the anterior and posterior egg poles (sagittal plane), and photographed. All measurements of egg length (in μm) were performed in OpenLab (version 3.1.7; Improvision, Emeryville, CA), tabulated, and analyzed in SAS version 8.2 (SAS Institute, Cary, NC). The least-square means and standard deviation were calculated for the strains and species, and their differences were estimated with the Tukey option in Proc GLM. The genetic analysis of the *Fra* and *Ind* lines were summarized similarly.

Genetic Analysis. Crosses were simultaneously established from 3- to 5-day posteclosion females between *Fra* × *Fra*, *Ind* × *Ind*, *Fra* × *Ind*, and *Ind* × *Fra* adults. Adult (sibs) from the reciprocal cross were allowed to mate to produce F_2 (and similarly F_3) embryos. After the establishment of each cross, 0- to 4-h embryos were collected for analysis.

Visualization and Measurement of *eve*, *gt*, and *Kr* Expression. Embryos were collected and *in situ* hybridization was carried out as described (37) with SDS used in place of Proteinase K (protocol available on request), DAPI-stained to visualize nuclei, and stored and mounted in 90% glycerol + *n*-Propyl galate to protect against fading. Embryos were prescreened under a dissecting microscope to preliminarily assess developmental stage and further selected by stage and orientation to identify ≈50 cleavage cycle 14a embryos for analysis for each line and cross. Each selected embryo was assigned to one of five age classes as described in SI Fig. 7 (substages 4–8 on the Fly-Ex web Site, http://flyex.ams.sunysb.edu/flyex) (38).

Optical Z-sectioning (0.8-μm per step) was carried out with an Axioplan2 microscope (Zeiss, Thornwood, NY). To separate and prevent overlap of the DAPI, Alexa 546, and Alexa 647 signals, we used the following filters from Chroma Technology (Rockingham, VT): 31000v2 for DAPI, 41002b for Alexa 546, and 31023 for Alexa 647. Photographs were taken with an Orca C4742–95 camera (Hamamatsu, Hamamatsu City, Japan) and Openlab software, version 3.1.7.

The measurements of stripe position were performed in Openlab by one investigator (S.E.L.). First, a midline was drawn, and points were laid down at the anterior and posterior boundaries of each stripe. Points were also laid down for the anterior and posterior of the embryo along this midline, on the sagittal section. Distances from the anterior of the embryo to each stripe boundary were calculated, tabulated, and imported into SAS for analysis.

Statistical Analysis. A linear model ANOVA (SAS, version 8.2) was fitted with Proc GLM with the main effect of line, and age (the inferred age of development) was used as a covariate. The significance was estimated with restricted maximum likelihood, and the denominator degrees of freedom were determined with

the Satterthaite method. Least-square means were calculated with the Lsmeans option:

$$Y = \mu + \text{line} + \text{age} + \text{line} \times \text{age} + \varepsilon.$$

A related model studying the differences between the species was fitted, with a species term substituting line. A similar model was created to test for maternal inheritance of stripe phenotypes in the *D. melanogaster Fra* and *Ind* crosses, with mother indicating the maternal contribution to the variance, cross indicating the effect of being a parental or F_1, with age as a covariate, and interaction terms between all three. Notably, the interaction term mother × cross is equivalent to the line term in the other models. The significance of model terms for each stripe is corrected for the total number of stripe boundaries tested in the experiment ($14\,eve + 6\,gt + 2\,Kr = 22$ tests). For *D. melanogaster Fra* and *Ind* crosses the tests for additivity were calculated according to standard theory (39).

The Pearson correlation coefficients between stripe boundaries were evaluated with the Proc CORR function in SAS. The correlations were calculated for the entire data set and also for subsets broken down by lines, species, and ages (data not shown) with similar results. We then proceeded to test with a simple regression model whether the strength of the correlations between stripes depends on physical distance between their boundaries. The results were evaluated with the Proc REG function.

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