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Concentration-dependent patterning by an ectopic expression domain of the *Drosophila* gap gene *knirps*

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SUMMARY

The asymmetric distribution of the gap gene *knirps* (*kni*) in discrete expression domains is critical for striped patterns of pair-rule gene expression in the *Drosophila* embryo. To test whether these domains function as sources of morphogenetic activity, the stripe 2 enhancer of the pair-rule gene *even-skipped* (*eve*) was used to express *kni* in an ectopic position. Manipulating the stripe 2-*kni* expression constructs and examining transgenic lines with different insertion sites led to the establishment of a series of independent lines that displayed consistently different levels and developmental profiles of expression. Individual lines showed specific disruptions in pair-rule patterning that were correlated with the level and timing of ectopic expression. These results suggest that the ectopic domain acts as a source for morphogenetic activity that specifies regions in the embryo where pair-rule genes can be activated or repressed. Evidence is presented that the level and timing of expression, as well as protein diffusion, are important for determining the specific responses of target genes.

Key words: *Drosophila*, *knirps*, pair-rule gene, *even-skipped*, ectopic expression

INTRODUCTION

In *Drosophila*, genetic screens have identified more than 30 genes that act in a regulatory cascade to subdivide the embryo into 14 contiguous segments (Nüsslein-Volhard and Wieshaus, 1980; Nüsslein-Volhard et al., 1985). The process of segmentation is initiated by gradients of maternal gene products such as bicoid (bcd) and nanos (nos), which establish anterior-posterior polarity and specify positional information based on their concentration (reviewed in Driever, 1993; St. Johnston, 1993). These maternal factors control a cascade of zygotic gene expression, which results in increasingly refined striped patterns along the anterior-posterior axis (reviewed in Akam, 1987; Ingham, 1988). The zygotic segmentation genes have been classified according to their mutant phenotypes and expression patterns. These classifications include the 'gap' genes, each of which is expressed in one or two broad overlapping domains, the 'pair-rule' genes, which are expressed in patterns of seven transverse stripes in the precellular blastoderm, and the 'segment polarity' genes, which are expressed in patterns of fourteen stripes about one-cell wide. The segment polarity stripes demarcate the boundaries of the presumptive segments.

A critical step in this process is the initial establishment of the reiterated expression patterns of the pair-rule genes. Genetic experiments suggest that these patterns are set up by the combined activities of maternal morphogens and the products of the gap genes, which activate expression of individual stripes, or repress expression to create borders between stripes and interstripes (reviewed in Ingham, 1988; Carroll, 1990). Furthermore, the detailed analysis of the regulatory regions of the pair-rule genes *even-skipped* (*eve*) and *hairy* (*h*) has led to the identification of separate enhancers that control the initial expression of individual pair-rule stripes (Howard et al., 1988; Goto et al., 1989; Harding et al., 1989; Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991).

In the *eve* promoter, a 480 bp enhancer regulates the expression of stripe 2 of the seven-stripe pattern (stripe 1 is the most anterior stripe), and a separate 500 bp enhancer regulates stripes 3 and 7 (Small et al., 1992, 1996). Both enhancers contain multiple binding sites for maternal and gap proteins, suggesting that direct protein-DNA interactions are important for regulating these stripes (Stanojevic et al., 1989; Small et al., 1991, 1996). The stripe 2 enhancer is activated by the maternal morphogen bcd and the gap protein hunchback (hb), while its anterior and posterior borders are set by repression mediated by the gap proteins giant (gt) and kruppel (Kr), respectively. The stripe 3+7 enhancer is activated by a ubiquitous factor, D-stat/marelle, and the anterior and posterior borders of stripe 3 are set by the gap proteins hb and kni, respectively (Hou et al., 1996; Yan et al., 1996; Small et al., 1996). These two enhancers are positioned more than 1.5 kb away from each other in the *eve* regulatory region and function independently because of short-range repressive interactions that form the stripe borders (Small et al., 1993).

The *eve* stripes directed by these two enhancers are each about 4-5 cells wide and make up part of the initial seven-stripe pattern. This pattern is then refined via cross-regulatory inter-

actions between the pair-rule genes until each stripe is 2-3 cells wide and exhibits anterior posterior polarity. The refinement process involves a separate regulatory element that contains binding sites for pair-rule proteins including eve itself and paired (prd) (Jiang et al., 1991a; Fujioka et al., 1996).

In contrast, similar studies of the regulatory region of another pair-rule gene *fushi-tarazu* (*ftz*) have only identified promoter elements that direct all seven stripes of expression (Hiromi and Gehring, 1987; Pick et al., 1990; Schier and Gehring, 1993). This has given support to the classification of *ftz* as a secondary pair-rule gene, whose periodic expression pattern is regulated by other pair-rule genes (Ingham and Martinez-Arias, 1986; Carroll, 1990). Specifically, the pair-rule gene *runt* (*run*) has been implicated in *ftz* activation, while *hairy* (*h*) may be involved in repression events that form the posterior borders of the *ftz* stripes (Tsai and Gergen, 1995, and references therein). *run* and *h* stripes overlap the anterior and posterior borders of each *ftz* stripe respectively, consistent with this hypothesis (Ingham and Gergen, 1988; Kania et al., 1990). However, the initial pattern of *ftz* stripes is correctly established in *h* and *run* mutant embryos (Ingham and Gergen, 1988; Yu and Pick, 1995), suggesting that these genes may only be important for maintaining and refining pattern. Thus, the initial *ftz* stripes may be regulated by aperiodic cues, which may include the gap genes (Yu and Pick, 1995).

Most of the experiments described above focus on *cis*-acting sequences that control pair-rule pattern formation. However, less is known about the mechanisms whereby the *trans*-acting factors (maternal and gap proteins) set up the pattern. There is good evidence that the maternal factors bcd and hb (hb^{MAT}) act as gradient morphogens to establish the positions of target gene expression in anterior regions of the embryo (Driever and Nüsslein-Volhard, 1988, 1989; Struhl et al., 1989, 1992; Hülskamp et al., 1990; Simpson-Brose et al., 1994). The best-characterized target gene is *hb* itself, which is activated zygotically in response to the bcd and hb^{MAT} gradients. The posterior limit of zygotic *hb* expression can be shifted by genetic manipulations that change the shape of the maternal bcd and hb gradients, consistent with the hypothesis that the exact concentrations of these proteins controls the on/off state of *hb* transcription.

It has been suggested that the zygotic expression domains of the gap genes act as graded morphogens to establish sharp on/off patterns of pair-rule gene expression (Struhl, 1989; Gaul and Jäckle, 1990; Warrior and Levine, 1990; Pankratz et al., 1990; Kraut and Levine, 1991). Pair-rule stripes are severely disrupted in gap mutants, but these mutations also change the patterns of other gap genes, making the changes in pair-rule gene expression difficult to interpret. Similarly, heat-shock expression experiments have been used to overexpress gap proteins, causing severe effects on pair-rule expression patterns (Struhl, 1989; Kraut and Levine, 1991; Eldon and Pirotta, 1991; Hoch et al., 1992). However, these effects are also difficult to interpret since heat-shock induction results in ubiquitous expression that affects both gap and pair-rule expression domains.

In this paper, we use the *eve* stripe 2 enhancer and the yeast FLP-FRT system to create an ectopic expression domain of the gap gene *kni* in the blastoderm. We show that the strength and temporal profile of this ectopic domain can be manipulated by changing the enhancer copy number as well as the 3′ untranslated region of the misexpression construct. Relatively small changes in misexpression levels differentially affect individual *eve*, *h*, *run* and *ftz* stripes adjacent to the ectopic *kni* domain, suggesting unique mechanisms for the formation of each stripe. Increasing the levels of ectopic *kni* affects stripes positioned farther from the source of misexpression, suggesting that very low protein levels can significantly affect transcription. These results suggest that the ectopic *kni* domain acts as a source for a gradient of activity which represses or permits activation of individual pair-rule stripes.

MATERIALS AND METHODS

kni misexpression constructs

The constructs shown in Fig. 1B contain one or two tandem copies of the 480 bp *eve* stripe 2 enhancer that contains five high-affinity bcd-binding sites (Arnosti et al., 1996). When two copies were used, they were separated by 22 bp of polylinker sequence. The *Bss*HII site at the 3′ end of the stripe 2 enhancer(s) was fused to the *Pst*I site at –42 with respect to the *eve* initiation codon. All constructs contained the *eve* basal promoter, as well as the *eve* leader sequence up to the *Hin*fI site at +80 fused to a 2.3 kb fragment containing the 3′ termination signal from the *hsp70* gene flanked by two FLP recombination targets (FRTs). This FRT cassette was fused at the 3′ end to the *Nru*I site that lies 54 bp upstream of the *kni* translation initiation codon (Nauber et al., 1988). Therefore, when these constructs were activated, the 5′ UTR contained 80 bp of the *eve* leader, ~100 bp of FRT sequence and 54 bp of the *kni* leader. At the 3′ end of the *kni* cDNA, two different 3′ UTRs were fused to the *Bam*HI site located 240 bp downstream of the stop codon. The *eve* UTR consisted of a 1.5 kb *Bst*UI-*Eco*RI genomic fragment containing the *eve* transcription stop. This *Bst*UI site is located 5 bp downstream of the *eve* stop codon. The α-*tubulin* UTR consisted of an 800 bp fragment beginning at the *Sca*I site at position 1960 (Theurkauf et al., 1986). All constructs were generated by standard cloning procedures in the CaSpeR vector, which uses a mini-*white* gene as a selectable marker (Thummel et al., 1988). For each construct, between six and ten independent transgenic lines were generated in a yw^{67} background using standard P-element transformation procedures (Spradling, 1986).

Targeted misexpression and in situ hybridization

A transgenic line homozygous for a P(*ry*$^+$), *β2-tubulin*-FLP insertion was a generous gift from Gary Struhl. This line was crossed with individual lines containing various misexpression constructs to obtain males that contained both constructs. Embryos were then collected from crosses between these males and *yw* females, and stained by in situ hybridization using antisense RNA probes as previously described (Jiang et al., 1991b). In experiments where levels of expression were compared, embryos were stained in parallel and photographed under identical conditions. All experiments were repeated at least three times. All embryos in this paper are oriented so that anterior is to the left and dorsal is up.

The major modifications used for the double-label experiments are as follows. Embryos were hybridized simultaneously with antisense probes for two different genes, one containing digoxigenin-UTP and the other fluorescein-UTP (labelled nucleotides, antibodies and alkaline phosphatase (AP) substrates were obtained from Boehringer Mannheim Corp., Indianapolis, IN). After washing off the unbound probes, embryos were incubated with an AP-conjugated anti-fluorescein antibody for 1-2 hours at room temperature, washed extensively in PBT (1× PBS, 0.1% Tween 80), then washed twice in AP staining buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 80). The first colorimetric reaction was then performed at 25°C using Fast Red as a substrate in AP staining buffer. After staining, the embryos were washed several times in PBT to stop the

reaction and were then incubated for 10 minutes in 0.1 M glycine pH 2.2, 0.1% Tween 80 to remove the first antibody. After several more washes in PBT, the embryos were incubated overnight at 4°C with an AP-conjugated anti-digoxigenin antibody. After washing as before, the second colorimetric reaction was performed using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as previously described (Jiang et al., 1991b). Staining patterns using NBT and BCIP generally appear purple or blue, but after the red reaction, this stain appears black. (Figs 8, 9). To preserve the morphology of the embryos, after washing several times with PBT, we performed an extra postfixation (25 minutes in PBT, 5% formaldehyde). After several more washes in PBT, the embryos were mounted on microscope slides using Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA). Embryos were photographed using DIC optics on a Zeiss Axioscope.

RESULTS

Optimizing *kni* misexpression at the position of *eve* stripe 2

In precellular embryos, *kni* is normally expressed as a complex domain in the presumptive head region, and a broad posterior domain that encompasses parasegments 7 to 10 (Rothe et al., 1989). Since *eve* stripe 2 is positioned at parasegment 3, ectopic *kni* expression driven by the stripe 2 enhancer would create a third domain positioned between the two endogenous domains. Because this ectopic domain is likely to cause dominant embryonic lethality, we constructed silent transformation vectors in which the *kni*-coding region was separated from the stripe 2 enhancer by a transcriptional stop sequence flanked by two FLP recombination targets (FRTs; Fig. 1A). This stop sequence was removed by FLP-mediated recombination in the germ line of adult males that also carried a *β2-tubulin*-FLP construct (Struhl et al., 1993). The offspring from these males therefore contained a single copy of the activated *kni* transgene.

The *eve* stripe 2 enhancer is a 480 bp fragment that directs a broad domain of reporter gene expression in anterior regions early in nuclear cleavage cycle 14 (Small et al., 1992). By mid-cycle 14, this broad domain is refined to a stripe 4-5 nuclei wide, which persists until the beginning of gastrulation. The enhancer contains five DNA-binding sites for bcd protein, which are critical for enhancer activation (Stanojevic et al., 1991, Small et al., 1992). By increasing or decreasing the bcd-binding affinity of these sites, the level of reporter gene expression driven by this enhancer can be dramatically altered (Arnosti et al., 1996). To optimize expression of ectopic *kni* in these experiments, we have used a mutant version of the enhancer that contains high-affinity bcd sites at all five positions. We have augmented expression levels further by using two tandem copies of this enhancer (Fig. 1B). Moreover, we used the 3′ untranslated regions (UTRs) from α-1 *tubulin* (Theurkauf et al., 1986) and *eve* itself to direct significantly different developmental profiles of expression.

Embryos containing activated stripe 2-*kni* constructs were assayed for ectopic expression by in situ hybridization using an anti-sense *kni* RNA probe. Each construct shown in Fig. 1B was capable of directing a third *kni* expression domain between the two endogenous domains (Figs 2, 3). Double staining experiments using antisense *kni* and *eve* probes determined that the ectopic domain was centered over the position of *eve* stripe 2 (data not shown).

Generating different levels and temporal profiles of ectopic expression

We examined the *kni* staining pattern in embryos from several independently transformed lines for each construct. Ectopic expression levels varied minimally among individual embryos from a single transgenic line. However, we observed a range of levels among lines carrying the same construct, probably due to position effects at different genomic insertion sites (Fig. 2). For example, among seven independent lines that carried the 22FKE construct, we could discern at least four distinct levels of ectopic expression by comparing photographs of more than 20 embryos per line, and using the endogenous *kni* domain as an internal control for staining intensity. The highest levels of ectopic expression were very similar to the levels of endogenous *kni* RNA (Fig. 2G,H), and the ectopic stripe in these lines was wider than in the weaker lines, spanning an area about 6-7 cells wide even after refinement (Fig. 2H). The strongest of the six lines carrying the single copy enhancer construct (2FKE) displayed expression levels similar to the weak 22FKE lines (Fig. 2C,D).

The differences in expression levels caused by changing the enhancer copy number and the genomic location of the construct were apparent throughout the genesis of the ectopic domain (Fig. 2). However, changing the 3′ untranslated region (UTR) altered the temporal profile of ectopic expression. In cleavage cycles 12, 13, and early 14, the 22FKE construct directed high expression levels in a broad anterior domain (Fig. 3B) whereas in the 22FKT lines, we could detect only weak, scattered expression at this time (Fig. 3A). However, by mid-cycle 14, the intensity of the *kni* domain driven by the two constructs appeared very similar (Fig. 3C,D). Later in cycle 14, *kni* RNA possessing the *eve* 3′ UTR diminished more rapidly (compare Fig. 3F with 3E). Furthermore, the quality of the ectopic domain was also affected by changing the 3′ UTR. For example, the 2FKT construct, which contains the *α-tubulin* 3′ UTR, expressed at a low level in patches of nuclei in the *eve* stripe 2 area (data not shown), in contrast with the uniform stripe generated with the 2FKE construct (Fig. 2C). These results suggest that the *eve* 3′ UTR allows for early and uniform activation of the transgene and confers a rapid turnover rate in cycle 14.

In summary, analysis of the *kni* staining patterns suggests that we have created different types of localized gradients that vary in their shape and amplitude. By correlating these gradients with consistent perturbations in gap and pair-rule gene expression, we have attempted to determine whether ectopic kni has the properties of a morphogen.

Effects of stripe 2-*kni* on gap gene expression patterns

In situ hybridization experiments using RNA probes for the gap genes *gt*, *Kr* and *hb* were performed on stripe 2-*kni* embryos. We observed no effect on the expression patterns of *gt* and *Kr* at any level of *kni* misexpression (data not shown). However, the ectopic *kni* expression did significantly alter the *hb* pattern. Normally, zygotic *hb* is activated throughout the anterior half of the embryo (Fig. 4A) in response to bcd and hb^{MAT}, and, starting in mid-cycle 14, is refined into a broad stripe at parasegment 4 (Fig. 4B). It has been shown that a regulatory region upstream of the bcd response element forms this stripe (Margolis et al., 1995) and that its expression is essential for the correct establishment of neighboring parasegment 5

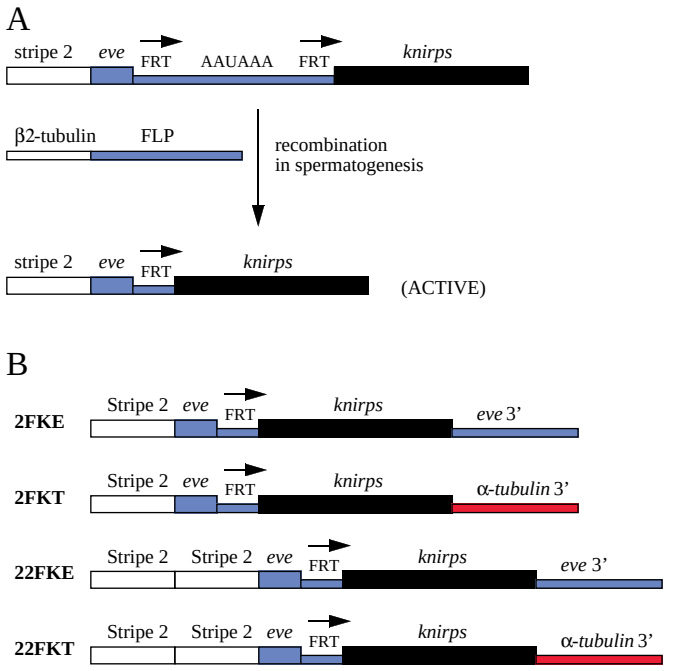

Fig. 1. (A) Strategy for *eve* stripe 2-*kni* misexpression in blastoderm stage embryos. Transgenic lines were generated that contain both the enhancer and the *kni*-coding region (top). This construct is inactive because of an intervening transcriptional stop (AAUAAA) flanked by two FLP recombinase targets (FRT), but is activated during spermatogenesis in males that also carry a β2-*tubulin*-FLP transgene (middle; Struhl et al., 1993). The active construct contains a single FRT positioned between the enhancer and the *kni* coding region. (B) Schematic representations of the activated forms of the constructs used in this study. One or two copies of the *eve* stripe 2 enhancer containing five high-affinity bcd-binding sites were fused upstream of the *eve* TATA box (see Materials and Methods). Constructs contained either the *eve* or the *α-tubulin* 3′ UTR.

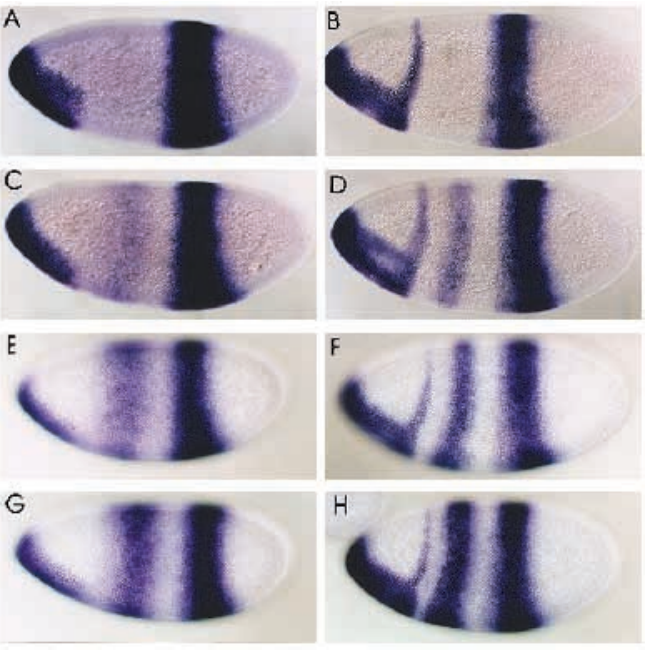

Fig. 2. Individual transgenic lines direct different levels of ectopic *kni*. Wild-type embryos (A,B) and embryos from lines containing *eve* stripe 2-*kni* misexpression constructs (C-H) were stained to detect *kni* RNA expression. Expression patterns are shown for embryos early (left) and late (right) in cycle 14. Low levels of ectopic *kni* are directed by construct 2FKE (C,D), which contains a single copy of the stripe 2 enhancer (Fig. 1B). Lines containing the 22FKE construct showed significant differences in levels of ectopic expression that probably reflect different genomic insertion sites (see Fig. 10A). Of the seven 22FKE lines analyzed, two (K12 and K15) expressed low levels that were similar to those directed by the 2FKE construct (C,D). Three 22FKE lines (K13, K14 and K16) expressed intermediate levels (e.g., E,F), although there was some variation in this group. Finally, two 22FKE lines (K10, K11) showed very high levels of ectopic *kni* (e.g., G,H).

(Hülskamp et al., 1994). Stripe 2-*kni*, centered on PS3, completely prevents the expression of the PS4 *hb* stripe (Fig. 4C,D). This repression occurs even in embryos that contain the lowest levels of ectopic *kni*.

Effects of stripe 2-*kni* on *eve* expression

During the first 20 minutes of cycle 14, a seven-stripe pattern of *eve* expression is formed (MacDonald et al., 1986; Frasch et al., 1987; Fig. 5A,B). These stripes appear in a particular temporal order, suggesting that the initiation of each stripe depends on a different mechanism. As outlined above, several lines of evidence suggest that gap proteins set the borders of *eve* stripes 2 and 3 by binding to discrete *cis*-elements and repressing transcription. It is likely that kni functions as a repressor to set the posterior border of stripe 3: in *kni* mutants, there is a posterior expansion of reporter gene expression driven by the stripe 3 enhancer (Small et al., 1996). Thus we expected that ectopic *kni* produced by the stripe 2 enhancer,

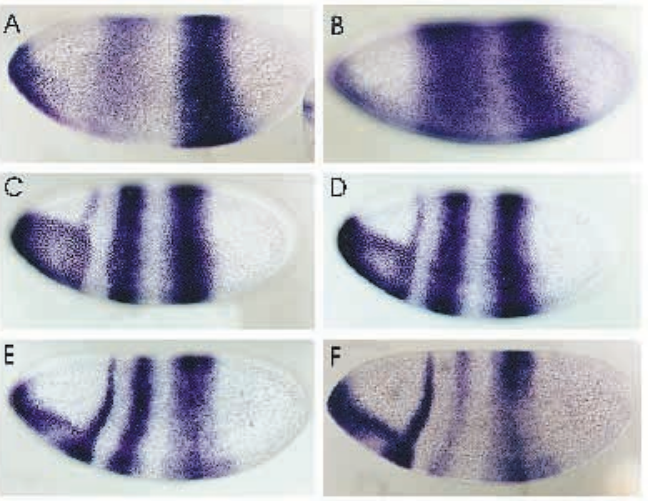

Fig. 3. Different 3′ UTRs change the temporal profile of ectopic *kni* expression. *kni* RNA expression patterns at early (A,B), mid (C,D), and late cycle 14 (E,F) were assayed in embryos transformed with construct 22FKT (*α-tubulin* 3′ trailer; A,C,E) or 22FKE (*eve* 3′ trailer; B,D,F). At mid cycle 14, the expression levels directed by the two constructs are nearly identical (compare C with D). However, the construct containing the *eve* 3′ trailer is expressed at much higher levels early (compare B with A), but is significantly weaker late in cycle 14 (compare F with E).

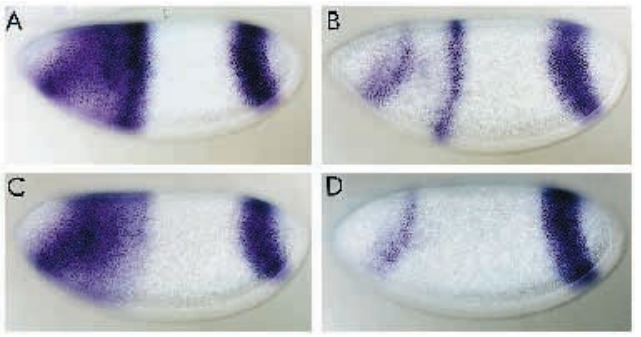

Fig. 4. stripe 2-*kni* expression abolishes expression of the *hb* stripe at parasegment 4. Wild-type embryos (A,B) and embryos carrying the 2FKE construct (C,D) were stained to detect *hb* RNA expression. Wild-type embryos express a strong stripe of *hb* RNA near the posterior limit of the anterior domain early in cycle 14 (A) that persists until midway through cycle 14 (B). This stripe is not expressed in stripe 2-*kni* embryos (C,D).

two parasegments distant, might cause a repression of the endogenous stripe 3.

In wild-type embryos, *eve* stripe 3 is initiated after stripes 1 and 2, but before the resolution of stripes 4, 5 and 6. Embryos containing low levels of ectopic *kni* did not form stripe 3 at the correct time (Fig. 5D), and this delay was also apparent in slightly older embryos, whose stripe 3 transcription level was reduced relative to other stripes (Fig. 5E). Later in cycle 14, stripe 3 appeared nearly normal. Intermediate levels exacerbated the delay in stripe 3 initiation (Fig. 5G), but a stripe finally did appear at the correct position (Fig. 5H). In embryos with the highest levels, *eve* stripe 3 was completely abolished early in cycle 14 (Fig. 5J), but reappeared later in cycle 14 in a more posterior position (Fig. 5K).

To test whether the early repression of stripe 3 was mediated through the previously characterized stripe 3 enhancer, stripe 2-*kni* constructs were crossed with a line carrying a *lacZ* reporter gene under the control of both the *eve* stripe 2 and stripe 3 enhancers. This reporter gene contains a 300 bp spacer sequence between the two enhancers (Small et al., 1993) and directs similar levels of both stripes (Fig. 5C). Ectopic *kni* specifically represses stripe 3 *lacZ* expression driven by this construct in a dose-dependent manner (Fig. 5F,I,L). These results support the hypothesis that kni may be a direct repressor of the *eve* stripe 3 enhancer.

Compared with stripe 3, other *eve* stripes were not as severely affected by ectopic *kni*. No effect was detected on stripe 1 at any level. Also, low and intermediate levels had no effect on the expression of *eve* stripe 2 (Fig. 5D-F,G-I). These results confirm that individual *eve* stripes are controlled by distinct mechanisms and that the enhancers that control the initiation of these stripes function independently (Small et al., 1993). However, the highest levels of ectopic *kni* caused a mild but consistent reduction in the levels of stripe 2 in some embryos (Fig. 5J,L). It is unlikely that this repression is direct, since there are no high affinity kni-binding sites in the stripe 2 enhancer and the high levels of *kni* do not interfere with the expression of the stripe 2-*kni* transgene. Interestingly, although there are strong repressive effects on stripes 2 and 3 early, these stripes recover by the time gastrulation starts, although not always in the correct position (Fig. 5K). We suggest that this recovery is due to the activities of other pair-rule genes that normally refine and stabilize the *eve* striped pattern, and that ectopic *kni* does not significantly affect these later patterning mechanisms (see Discussion).

Effects of stripe 2-kni on expression of run and h

In wild-type embryos, *run* stripes are formed that overlap the posterior half of each *eve* stripe. In early cleavage cycle 14, individual *run* stripes also appear in a stereotypic sequence. A

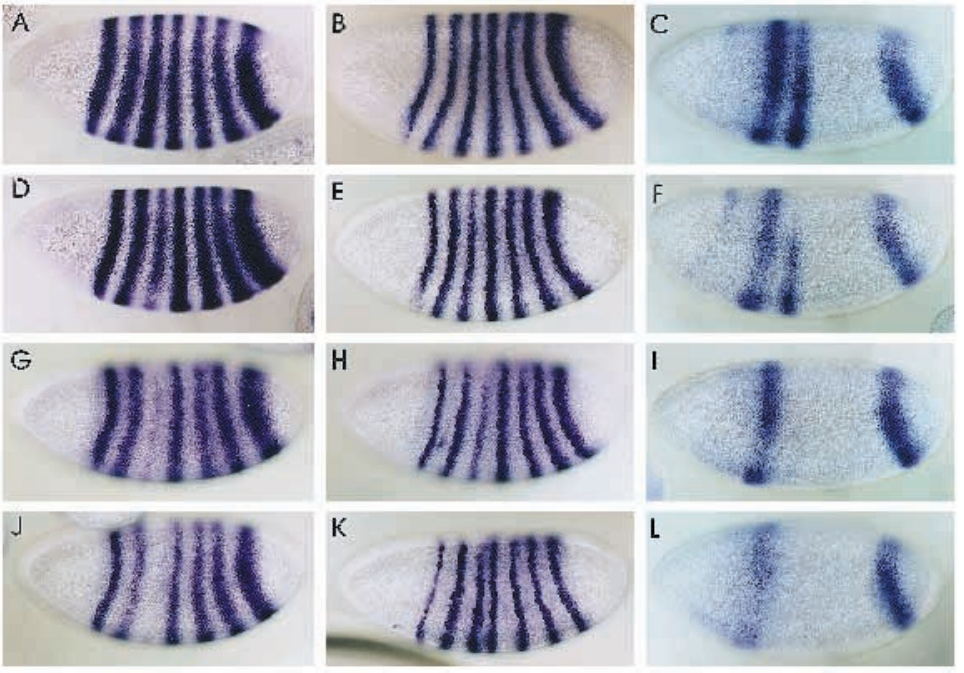

Fig. 5. Effects of stripe 2-*kni* on *eve* expression. Wild-type embryos (A-C) and embryos containing low (construct 2FKE; D-F), intermediate (construct 22FKE; G-I) or high levels of ectopic *kni* (construct 22FKE; J-L) were stained to detect *eve* (left, middle columns) or *lacZ* RNA (right). *eve* expression patterns are shown for embryos early (left) and late (middle) in cycle 14. In wild-type embryos, the initial *eve* stripes are each 4-5 cells wide (A), but are subsequently refined to narrower stripes two to three cells wide that exhibit anterior posterior polarity (B). Low levels of ectopic *kni* cause a mild reduction of *eve* stripe 3 (D,E). This repressive effect is more severe in embryos containing intermediate (G,H) and high levels of ectopic *kni* (J,K). In embryos containing the highest levels, stripe 3 is shifted toward the posterior (H). The embryos in the right column contain a *lacZ* reporter gene driven by the *eve* stripe 3 and stripe 2 enhancers separated by a 300 bp spacer sequence (Small et al., 1993). This reporter drives expression of *lacZ* in stripes 2, 3 and 7 in wild-type embryos (C). Increasing levels of ectopic *kni* causes a dose-dependent repression of stripe 3 (F,I,L).

transient pattern is detected at this time that is composed of stripes 1, 2, 3 and 6, which evolves into a mature seven-stripe pattern (Kania et al., 1990; Fig. 6A,B). Previous genetic experiments suggest that *run* is a direct target of gap gene regulation (Klingler and Gergen, 1993), although stripe-specific enhancers such as those in the *eve* and *h* promoters have not yet been identified.

Stripe 2-*kni* constructs caused disruptions of *run* stripes 2 and 3, but had no effect on stripe 1. Early in cycle 14, low levels of ectopic *kni* repressed *run* stripe 2 quite strongly, but stripe 3 only mildly (Fig. 6C). At the same age, higher levels increased repression of both stripes: stripe 2 is absent and stripe 3 is severely reduced (Fig. 6E,G). These results suggest that a similar threshold of kni-mediated repression may exist for both stripes. Nuclei in more posterior positions cross this threshold in embryos with higher levels of ectopic *kni*. However, a simple concentration-dependent repression mechanism cannot explain the late cycle 14 *run* patterns in these embryos. In the presence of low levels of ectopic *kni*, the early repression of *run* stripe 2 is alleviated, and the mature stripes 2 and 3 appear nearly normal (Fig. 6D). In contrast, the repression caused by intermediate levels persists longer, resulting in reduced levels of both *run* stripes 2 and 3 (Fig. 6F). In embryos containing the highest levels, *run* stripe 3 is more severely reduced, while expression near the position of stripe 2 recovered to near wild-type levels (Fig. 6H).

run stripes 2 and 3 respond differently to changing the levels of *kni* at the position of *eve* stripe 2. The repression of stripe 3 increases in proportion to the level of ectopic *kni*, a response similar to that seen for *eve* stripe 3. For stripe 2, all levels cause repression early in cycle 14, but there is a restoration of expression in this region that increases with the level of ectopic *kni*. Since these nuclei are adjacent to the source of misexpression, they must contain higher levels of kni protein than stripe 3 nuclei. Thus, it is possible that low concentrations of kni mediate repression, while higher levels permit activation. Alternatively, the activation of *run* in this region may be caused by the combination of higher concentrations of kni with other regulatory factors (see Discussion).

In contrast to the strong disruptions observed in the *eve* and *run* expression patterns, we could not detect any disruption of *h* expression in lines that expressed low or moderate levels of ectopic *kni*. However, the highest levels caused a subtle effect on the *h* pattern late in cycle 14. These embryos showed a significant delay in the separation of *h* stripes 3 and 4, which is one of the last events in the evolution of the *h* pattern (data not shown). It has been proposed that the separation of these stripes is regulated by *run*-mediated repression (Hartmann et al., 1994). As shown above, the highest levels of ectopic *kni* also cause a reduction of *run* stripe 3, consistent with this hypothesis.

Effects of stripe 2-*kni* on *ftz* expression

Although *ftz* has been classified as a secondary pair-rule gene, the sequence of initiation of individual stripes is unique among the pair-rule genes (Yu and Pick, 1995). In early cycle 14, the first stripes formed are 1, 2 and 5, followed by stripes 3, 6 and 7, and finally stripe 4 (Fig. 7A,B). The mature seven-stripe pattern is exactly reciprocal to *eve*; thus, the ectopic *kni* domain directed by the *eve* stripe 2 enhancer is positioned between *ftz* stripes 1 and 2.

Different levels of ectopic *kni* caused disruptions of *ftz* stripes 2 and 3, but had no effect on the expression of *ftz* stripe 1. Early in cycle 14, even low levels caused a dramatic repression of *ftz* stripe 2 (Fig. 7C). This repression of *ftz* stripe 2 was also observed in embryos containing intermediate and high levels (Fig. 7E,G). Stripe 3 expression was unaffected at low levels, mildly delayed at intermediate levels, and strongly repressed by the highest levels (Fig. 7C,E,G). The repression of *ftz* stripe 3 represents the posterior-most effect that we could detect resulting from the highest levels of ectopic *kni* driven by the *eve* stripe 2 enhancer.

The early repression of *ftz* stripe 2 mediated by low levels of ectopic *kni* was still evident later in cycle 14 (Fig. 7D). These embryos showed only a very narrow stripe of *ftz* RNA expression about 1-2 cells wide. Similarly, embryos containing high levels continued to show a significant reduction of *ftz* stripe 3 (Fig. 7H), while expression was strongly activated near the normal position of *ftz* stripe 2. The late *ftz* expression pattern detected in embryos containing intermediate levels showed significant reductions in the levels and widths of both stripes 2 and 3 (Fig. 7F).

In summary, in a situation similar to that observed for *run*, changing the levels of ectopic *kni* creates a continuum of effects on the formation of *ftz* stripes 2 and 3. There is a strong correlation between high levels of ectopic *kni* at *eve* stripe 2 and the repression of *ftz* stripe 3. In contrast, after the initial repression of *ftz* stripe 2, expression is activated near the original position of stripe 2. This expression increases with the levels of ectopic *kni*.

The stripe 2-*kni* disruptions of *ftz* are not mediated by other pair-rule genes

The preceding experiments suggest that the observed changes in *ftz* expression are due to direct effects of ectopic kni protein. Alternatively, it is possible that these effects are indirect and may be mediated through other segmentation genes. It has previously been shown that *run* and *h* are important for maintenance and refinement of the *ftz* pattern (Kania et al., 1990; Tsai and Gergen, 1995), and thus are good candidates for intermediate factors involved in disrupting the *ftz* pattern. If this is so, their altered expression patterns should parallel those observed for *ftz*. By this criterion, it is unlikely that *h* plays any role in the *ftz* disruptions, since the early *h* expression pattern is unperturbed in embryos containing ectopic *kni*. However, the disruptions of the *run* expression pattern (Fig. 6) seem very similar to those observed for *ftz* (Fig. 7). To test whether the effects on these two genes coincide, we performed double-label experiments to simultaneously visualize both expression patterns. From these experiments, it is clear that the effects on *run* can be uncoupled from those on *ftz* (Fig. 8). For example, a relatively low level of *kni* that was sufficient to repress *ftz* stripe 2 had very little effect on the expression of *run* stripe 2 (Fig. 8B). Conversely, a higher level caused a severe reduction of *run* stripe 3, while *ftz* stripe 3 was not significantly affected (Fig. 8C).

The uncoupling of the effects on individual *run* stripes from those on *ftz* stripes, and the fact that *h* expression is virtually unaffected in stripe 2-*kni* embryos suggests that the observed effects on *ftz* are not mediated through these other pair-rule genes. Rather, it seems more likely that the *ftz* effects are caused by direct activity of the kni protein driven by the *eve* stripe 2 enhancer. These results support the hypothesis that *kni*,

and possibly other gap genes, are important aperiodic cues that establish the *ftz* pair-rule stripe pattern (Yu and Pick, 1995).

Effects of changing the temporal profile of the stripe 2-*kni* domain

The highest levels of ectopic *kni* caused a significant repression of *ftz* stripe 3, which is located eight to ten nuclei away from the position of *eve* stripe 2. This long-range effect could be the result of diffusion of kni protein from the position of *eve* stripe 2 to the position of *ftz* stripe 3. Alternatively, since the ectopic expression domain driven by the *eve* stripe 2 enhancer is very broad before being refined to a stripe, it is possible that effects on *ftz* stripe 3 reflect activity of kni protein that is produced before refinement. The early patterns generated by the strongest lines extend quite far into the middle regions of the embryo (e.g. Fig. 3B), consistent with the latter model.

To test whether the early *kni* prepattern might be responsible for the distant effects on pair-rule patterning, we compared a strong misexpression line containing the *eve* 3′ UTR (22FKE) with one containing the *α-tubulin* 3′ UTR (22FKT). These two lines generate indistinguishable domains of RNA expression midway through cycle 14 (Fig. 3C,D), from which similar amounts of kni protein might diffuse. Earlier, however, the 22FKE construct directs a much stronger prepattern of expression (compare Fig. 3B with 3A). Conversely, the domain produced by the 22FKT construct persists significantly longer (compare Fig. 3F with 3E). If these different expression profiles change the effective gradient of ectopic kni protein, the resulting pattern disruptions of pair-rule genes should be significantly different.

We simultaneously visualized *eve* and *ftz* mRNAs in embryos from these two lines. While both constructs caused a repression of *eve* stripe 3 early in cycle 14 (Fig. 9A,C), only the 22FKE construct caused a repressive effect on *ftz* stripe 3 (Fig. 9A), which lies farther from the misexpression source. This suggests that the early prepattern generated by 22FKE is very important for repression of *ftz* stripe 3. However, the repression of *eve* stripe 3 caused by the 22FKT construct persisted significantly longer compared to the 22FKE construct (compare Fig. 9B with 9D). Since the 22FKT domain lasts longer (Fig. 3), this later repression may reflect the activity of kni protein diffusing from the nuclei at the position of the mature *eve* stripe 2. Together these results suggest that both the early prepattern of expression and protein diffusion contribute to the effective gradient of ectopic kni protein.

DISCUSSION

Localized ectopic expression in blastoderm stage embryos

A regulatory relationship between the gap and pair-rule classes of segmentation genes was originally inferred by examining pair-rule pattern disruptions in gap mutants. Ubiquitous expression of gap proteins also caused severe disruptions of pair-rule patterns, suggesting that the localized expression of gap genes was required for their patterning activity (Struhl, 1989; Eldon and Pirrotta, 1991; Kraut and Levine, 1991; Hoch et al., 1992). However, it was not clear that gap proteins function as gradient morphogens in this process because it was not possible to spatially control the distribution of ectopic protein in these experiments. In this paper, we have created an ectopic domain of *kni* mRNA using the *eve* stripe 2 enhancer.

Since the ectopic activation of *kni* might cause dominant lethality, the yeast FLP-FRT system was used in our experiments. In this system, silent constructs containing a FRT-Stop-FRT cassette were activated by FLP recombinase in the male germ line (Struhl et al., 1993). This activation before fertilization permits the generation of high expression levels during early blastoderm development. Thus, this approach may be significantly more effective than other indirect strategies for enhancer driven ectopic expression. One such strategy is the Gal4-UAS system (Brand and Perrimon, 1993), in which the enhancer drives expression of the yeast transcriptional activator Gal4, which then activates expression of a target gene through Gal4-binding sites. This strategy has worked well in several studies later in development, but may not be effective in the early blastoderm because of the delay between activating the enhancer and expressing the target gene.

We have also shown that the developmental profile of ectopic expression varies significantly depending on the 3′ UTR used in the misexpression construct. Early in cycle 14, constructs containing the *eve* 3′ UTR directed higher levels of expression than those containing the *α-tubulin* 3′ UTR. The accumulated levels of RNA driven by the two types of constructs were very similar in mid cycle 14, but transcripts containing the *eve* 3′ UTR disappeared before those containing the *α-tubulin* 3′ UTR. It has been previously shown that the UTRs of several patterning genes, including *eve* itself, control RNA localization to a particular region of the embryo, or to a particular subcellular region (Davis and Ish-Horowicz, 1991). Our results suggest that sequences in the UTR may be important for RNA stability and possibly for early activation of transcription as well. Also, we found that the levels of transcription directed by the stripe 2-*kni* constructs vary to a significant degree according to the position of insertion in the genome. These factors have permitted us to generate a series of lines that contain qualitatively different ectopic *kni* domains at the position of *eve* stripe 2.

Ectopic *kni* causes specific disruptions of gap gene expression patterns

The PS4 stripe of *hb* expression was abolished by stripe 2-*kni* (Fig. 4), even in embryos containing very low levels of ectopic expression. This suggests a mechanism of activation for this stripe that is very sensitive to repression by kni. In contrast, no changes were observed in the expression patterns of *gt* or *Kr* even in embryos containing high levels of ectopic *kni*, suggesting that the observed repressive effects are specific for *hb* among the gap genes.

The absence of an effect on the *Kr* pattern apparently contradicts previous experiments that suggested a direct role for endogenous kni in setting the posterior border of the *Kr* domain (Hoch et al., 1992). These experiments focused on a 16 bp *Kr* regulatory sequence that contains overlapping bcd- and kni-binding sites. In P-element transformation experiments, six copies of this sequence activated *lacZ* expression in the anterior part of the embryo. This activation was repressed by inducing ubiquitous *kni* expression using a heat-shock approach. There are several explanations for these conflicting results. First, the levels of ectopic expression driven by the stripe 2 enhancer

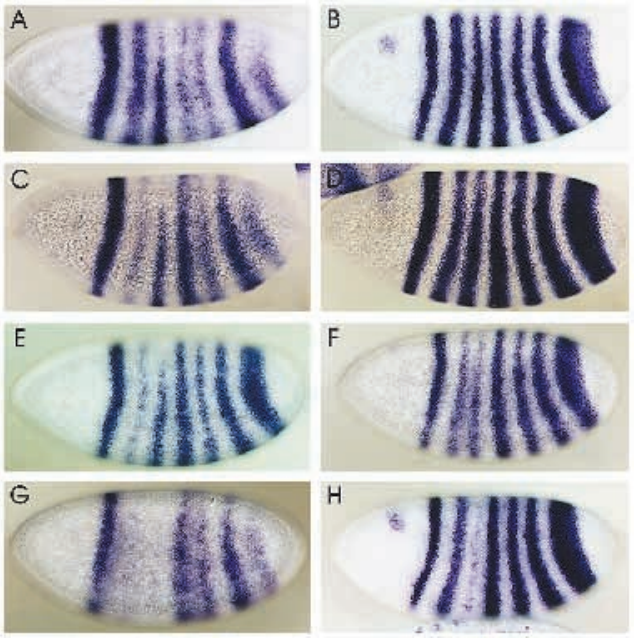

Fig. 6. Effects of stripe 2-*kni* on expression of *run* stripes. Wild-type embryos (A,B), and embryos containing low (construct 2FKE; C,D), intermediate (construct 22FKE; E,F), or high levels of ectopic *kni* (construct 22FKE; G,H) were stained to detect *run* RNA expression. Expression patterns are shown for embryos early (left) and late (right) in cycle 14. Wild-type embryos show strong expression of stripes 1, 2, 3, and 6 early in cycle 14 (A). This early pattern evolves into a pattern of seven evenly spaced stripes later in cycle 14 (B). Low levels of ectopic *kni* cause a delay of the expression of *run* stripe 2 early (C); however this stripe appears to be only mildly affected later in cycle 14 (D). Intermediate levels cause a delay in the initiation of *run* stripes 2 and 3 early in cycle 14 (E). Both of these stripes are significantly repressed later (F). The highest levels cause the complete repression of *run* stripes 2 and 3 early (G). Later, *run* stripe 3 is still nearly abolished, while expression reappears between stripes 1 and 3 (H). This stripe seems to be shifted one or two cells toward the posterior.

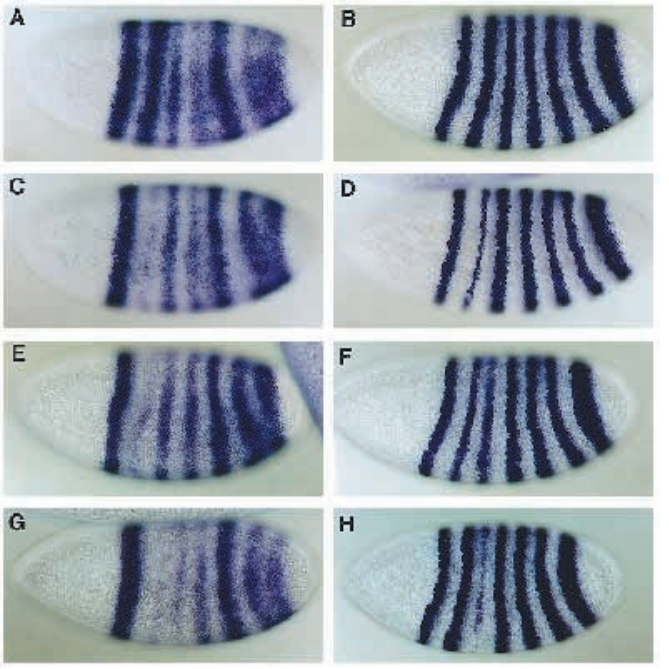

Fig. 7. Effects of stripe 2-*kni* on expression of *ftz* stripes. Wild-type embryos (A,B) and embryos containing low (construct 2FKE; C,D), intermediate (construct 22FKE; E,F), or high levels of ectopic *kni* (construct 22FKE; G,H) were stained to detect *ftz* RNA expression. Patterns are shown for embryos early (left) and late (right) in cycle 14. Early in cycle 14, the *ftz* pattern is composed of strong bands of expression at stripes 1, 2, and 5, and weaker expression at stripes 3, 4, 6 and 7 (A). Later, this pattern evolves into seven stripes about 4 cells wide (B). Low levels of ectopic *kni* cause a dramatic reduction of *ftz* stripe 2 expression early (C) and late (D) in cycle 14. Intermediate levels cause a reduction of *ftz* stripes 2 and 3 early in cycle 14 (E), which is still visible later (F). The highest levels of ectopic *kni* cause repression of *ftz* stripes 2 and 3 early in cycle 14 (G). Later, *ftz* stripe 3 is still reduced, while a strong stripe appears in the region between stripes 1 and 3 (H). This stripe appears to be shifted posteriorly from the normal position of *ftz* stripe 2.

may be insufficient for effective repression of *Kr*. This seems unlikely because the strongest stripe 2-*kni* lines, which direct levels similar to the endogenous *kni* gene (Fig. 2H), do not show any detectable *Kr* repression. Alternatively, perhaps the activities mediated by the 16 bp sequence represent only a small part of the in vivo mechanism of *Kr* regulation. Much larger fragments (~700 bp) are required to drive *lacZ* expression in the central *Kr* domain (Hoch et al., 1990). In summary, it seems that kni probably plays only a minor role in setting the posterior *Kr* border in vivo. This view is supported by the observation that the endogenous *Kr* and *kni* domains overlap to a significant extent (Pankratz et al., 1989; D. K., unpublished data). This overlap would not be possible if a strong negative interaction existed between these two genes (Kraut and Levine, 1991).

Ectopic *kni* causes specific changes in pair-rule gene expression patterns

For the pair-rule genes, different levels of ectopic *kni* caused disruptions of specific *eve*, *run* and *ftz* stripes, but there was no detectable effect on the initial *h* pattern. The absence of an effect on *h* was unexpected since individual *h* stripes are thought to be directly regulated by gap proteins (Pankratz et al., 1990; Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991). In particular, the *hb* PS4 stripe has been previously shown to be required for the expression of *h* stripe 3 (Hülskamp et al., 1994), and this stripe is missing in stripe 2-*kni* embryos. The results reported here suggest that, even in the absence of the PS4 *hb* stripe, *h* stripes are formed that are indistinguishable from the endogenous pattern. The exact mechanism for how this occurs is not clear at present, but one possibility is that the ectopic kni may be capable of replacing some activities normally mediated by PS4 *hb* (see below).

The effects of stripe 2-*kni* on *eve*, *run* and *ftz* patterning can be summarized as follows. First, there are significant effects on individual stripes that lie posterior to *eve* stripe 2, but no effects on anterior stripes. This suggests that the regulatory mechanisms governing the expression of stripes anterior and posterior to *eve* stripe 2 must be significantly different. Second, the level and profile of the ectopic *kni* domain determines which pair-rule stripes will be affected. Low levels cause disruptions of pair-rule

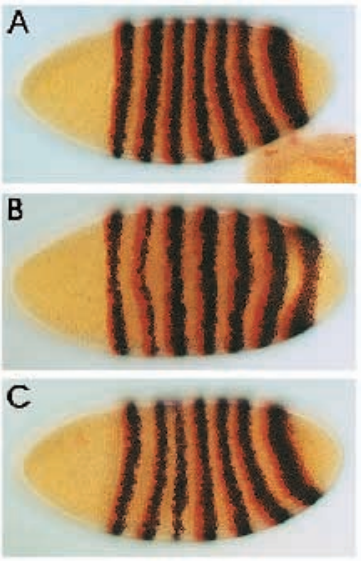

Fig. 8. The stripe 2-*kni* disruptions of *ftz* and *run* are qualitatively different. Expression patterns of *run* RNA (red) and *ftz* RNA (black) are shown for a late cycle 14 wild-type embryo (A), and embryos containing low (B) and intermediate levels of ectopic *kni* (C) respectively. In wild-type embryos at this stage, each *run* stripe overlaps the anterior half of a *ftz* stripe (A). Low levels of *kni* misexpression (line K12, 15) cause a significant reduction of *ftz* stripe 2, but have little effect on *run* stripe 2 (B). In contrast, higher levels (line K14, K16) dramatically reduce *run* stripe 3, but have little effect on *ftz* stripe 3 (C).

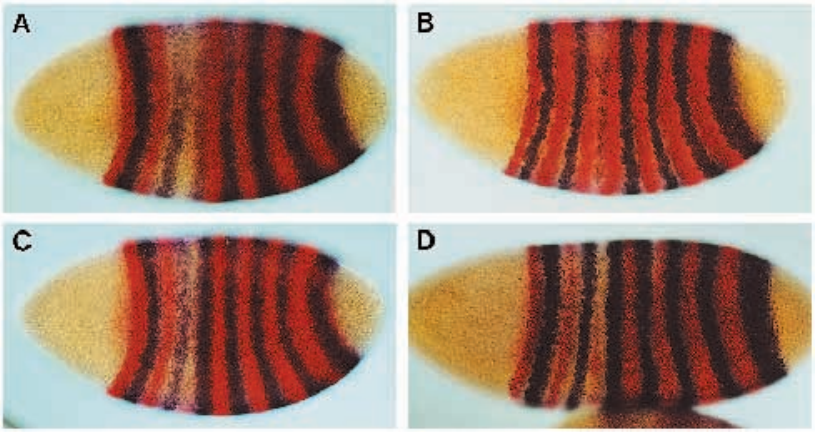

Fig. 9. The temporal profile of expression determines the effective activity of the gradient. Embryos containing the 22FKE (A,B) and the 22FKT construct (C,D) were stained to simultaneously detect *eve* (red) and *ftz* RNA (black). Patterns are shown for embryos early (left) and later (right) in cycle 14. The accumulated levels of ectopic *kni* midway through cycle 14 were indistinguishable in these lines. The ectopic *kni* domain generated by the 22FKE construct mediates a strong repression of *ftz* stripe 3 (A,B). In contrast, the domain generated by the 22FKT construct has little effect on *ftz* stripe 3, but represses *eve* stripe 3 for a longer period.

stripes near the source, while higher levels cause disruptions further away (Fig. 10A). Third, changing the strength and profile of the ectopic domain causes qualitatively different effects on individual pair-rule stripes. For example, embryos with very high levels of ectopic *kni* exhibit repression of *ftz* stripe 3 and *run* stripe 3, but these genes are both activated near their normal stripe 2 positions, which lie closer to the source of misexpression.

Ectopic kni is a direct repressor of *eve* stripe 3

Previous studies indicated that kni acts as a repressor to set the posterior border of *eve* stripe 3 (Small et al., 1996). The identification of five kni-binding sites in the stripe 3+7 enhancer suggested that this interaction may be direct. This hypothesis is supported by the finding here that ectopic *kni* at *eve* stripe 2 caused a significant repression of *eve* stripe 3 early in cycle 14. The repression mediated by the ectopic *kni* was transient, however, suggesting that the complete abolishment of this stripe might require relatively high kni concentrations for a longer period of time. This is supported by the observation that the 22FKT construct, which directs ectopic expression longer than the 22FKE construct, also repressed *eve* stripe 3 for a longer period (Fig. 9).

Later in cycle 14, stripe 3 expression was restored, even in embryos carrying the 22FKT construct. This recovery is probably due to the activities of other pair-rule genes that are normally involved in maintenance and refinement of the early stripes. It has been previously suggested that *h*, *prd* and *eve*

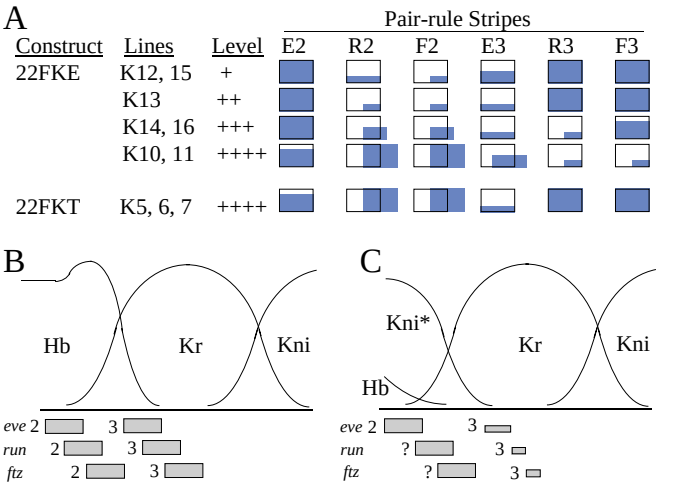

Fig. 10. (A) Summary of the effects of different levels of ectopic *kni* on the formation of individual pair-rule stripes midway through cycle 14. Individual transgenic lines and their relative levels of misexpression are shown on the left. The wild-type positions of individual stripes are schematically depicted by the rectangles on the right. The expression levels and positions of the stripes in embryos that contain ectopic *kni* are shown as shaded areas. These diagrams are not meant to be quantitative, but merely represent relative changes observed when compared to the wild-type patterns. (B) Schematic representation of the overlapping expression domains of the relevant gap genes in wild-type embryos midway through cycle 14, and the positions of stripes 2 and 3 of the *eve*, *run* and *ftz* patterns. The parasegment 4 stripe of *hb* expression is depicted as an increase in *hb* levels that is centered over the position of *ftz* stripe 2. (C) In embryos containing high levels of ectopic *kni* (*), the PS4 stripe of *hb* expression is abolished. Furthermore, there is a significant reduction in the stripe 3 response for all three pair-rule genes. In the case of both *ftz* and *run*, a strong stripe of expression (?) is detected in a position just posterior to that of the normal stripe 2.

itself are involved in positive regulation to maintain *eve* expression, while *run* represses from the posterior to refine the stripes (Jiang et al., 1991a; Fujioka et al., 1996). Embryos containing the highest levels of ectopic *kni* exhibit a posterior shift of the recovered stripe. In these embryos, there is also a severe reduction of *run* stripe 3 (Fig. 6H), and a posterior expansion of *prd* stripe 3 (D. K., unpublished results), which could account for the observed shift.

Several previous experiments suggest that *eve* function is normally required for the late striped pattern. For example, in *eve* mutant embryos, all seven stripes fail to refine, and are prematurely lost (Frasch et al., 1988; Lawrence and Johnston, 1989). Furthermore, there are two eve-binding sites in a minimal *eve* autoregulatory element that directs reporter gene expression in seven late stripes (Jiang et al., 1991a). Mutating these sites causes a reduction in reporter gene expression, suggesting a direct role for eve in autoregulation. In the studies presented here, embryos containing intermediate and high levels of ectopic *kni* exhibit a recovery of *eve* stripe 3 expression in the absence of the early stripe 3 response (Fig. 5H,K), apparently contradicting this earlier work. However, since the ectopic kni-mediated repression of stripe 3 is transient, it is possible that low levels of eve protein are produced slightly later. Perhaps these low concentrations of eve, together with positive inputs from prd and h, may then activate the stripe maintenance response.

A common regulatory mechanism for *eve* stripe 3, *run* stripe 3 and *ftz* stripe 3?

Previous genetic experiments suggested that hb and kni act as repressors to set the anterior and posterior borders of *eve* stripe 3, as well as *run* stripe 3, and *ftz* stripe 3. There are expansions of the anterior borders of *eve* 3 and *run* 3 in mutants that lack zygotic *hb* (Klingler and Gergen, 1993; Small et al., 1996). Furthermore, the *hb* PS4 stripe has been shown to be important for establishing the interstripe between *ftz* stripes 2 and 3, suggesting a role for *hb* in forming the anterior border of *ftz* stripe 3 (Hülskamp et al., 1994). In mutants that lack *kni* function, the posterior borders of all three of these stripes fail to form (Carroll and Scott, 1986; Klingler and Gergen, 1993; Small et al., 1996). However, the positions of these three stripes are offset by about two cells along the anterior posterior axis in wild-type embryos (Fig. 10A). We suggest that the different positions of the three stripes reflect their different sensitivities to the repressive activities of *hb* anteriorly and *kni* posteriorly. In this model, *eve* stripe 3 would be most sensitive to repression by *kni*, followed by *run* stripe 3 and then *ftz* stripe 3. The repression of all three of these stripes by high levels of ectopic *kni* is consistent with a model that kni-mediated repression normally forms the posterior border of these stripes. Intermediate levels repress *eve* stripe 3, whereas higher levels are required to repress *run* stripe 3 and *ftz* stripe 3.

Even though the PS4 *hb* stripe may be involved in setting the anterior border of *ftz* stripe 3, repressing the *hb* stripe with ectopic *kni* does not significantly derepress *ftz* transcription. One explanation for this result is that the ectopic *kni* domain may be able to substitute for some *hb* functions in the absence of the PS4 stripe. If this is so, perhaps both the anterior and posterior borders of *ftz* stripe 3 are set by kni in embryos that contain the ectopic domain. Consistent with this view, a one nucleus anterior expansion of *ftz* stripe 3 can be detected in some embryos containing low levels of ectopic *kni* (data not shown). Increasing the levels of ectopic *kni* first turns off the expression in the one cell expansion, and then represses the stripe itself.

Does ectopic kni protein function as a gradient morphogen?

A morphogen was originally defined as a 'form-producing' substance that creates pattern by diffusion within an embryonic field (Turing, 1952). A localized source of such a morphogen would establish a concentration gradient that might specify different cell fates at different positions (reviewed in Slack, 1987), possibly by specifically affecting the transcriptional state of individual target genes. Based on these criteria, the ectopic *kni* domain may generate a gradient of morphogenetic activity. The *hb* PS4 domain is sensitive to repression by very low levels of ectopic *kni*, while the expression of *Kr* is unaffected. Also, different levels are required to repress the expression of individual pair-rule genes (see above).

It is especially interesting that embryos containing the highest levels of ectopic *kni* show severe reductions of *run* stripe 3 and *ftz* stripe 3, but activate these genes in regions closer to the source of misexpression (summarized in Fig. 10C). This suggests that low concentrations of ectopic *kni* repress transcription of these genes, while higher concentrations activate, or at least permit activation. Several mechanisms could account for the observed concentration-dependent change in kni activity. One possibility is that kni protein might exist in different forms (e.g. monomers versus dimers) depending on concentration and that these forms have different activities. In transient cotransfection assays, it has been shown that the gap proteins hb and Kr can change their transcriptional activities depending on concentration (Zuo et al., 1991; Sauer and Jäckle, 1991). However, the in vivo relevance of these studies is not clear. Alternatively, interactions with other proteins may be involved in changing the transcriptional activity of kni in different positions along the anterior posterior axis. Since increasing *kni* levels at the *eve* stripe 2 position causes more overlap with the endogenous *Kr* domain, one possibility is that some interaction between kni and Kr may change the transcriptional state of *ftz*. The simplest model might involve a Kr-kni heterodimer that could activate *ftz* transcription. This possibility is supported by the demonstration that Kr and kni can form a heteromeric complex in vitro (Sauer and Jäckle, 1995).

Prepattern and diffusion contribute to the effective kni gradient

Mutations in the gap genes cause disruptions in embryonic pattern outside the regions where their protein products are detectable by histochemical analysis. In the case of *kni*, the posterior domain of RNA expression encompasses about four presumptive parasegments at the beginning of cycle 14 (Rothe et al., 1989), but strong *kni* mutants show pattern disruptions of at least seven contiguous segments (A1 to A7; Nauber et al., 1988). The discrepancy between expression pattern and mutant phenotype suggests that sufficient kni protein for pattern formation exists a significant distance away from the region where its mRNA is detectable. There are at least two major factors that contribute to the effective kni gradient. Since *kni* and the other gap genes are initially expressed in very wide domains that are refined by interactions between the gap genes, the initial prepatterns could be the source of activity that is

undetectable later. The second factor is continuous protein diffusion from the refining domain.

The expression domain directed by the *eve* stripe 2 enhancer is initially very broad and then refined to form a discrete stripe, and thus serves as a good model for an endogenous gap expression domain. Furthermore, since changing the 3′ UTR of the stripe 2-*kni* constructs changes the developmental profile of expression, it is possible to separate the effects of the early prepattern from the effects of protein diffusion. The 22FKE construct, which directs a strong prepattern, represses *eve* stripe 3 early, as well as *run* stripe 3 and *ftz* stripe 3. In contrast, the 22FKT construct, with a very weak prepattern, represses *eve* stripe 3, but not *ftz* stripe 3 (Fig. 9). However, the RNA generated by this construct lasts longer, resulting in a longer delay in the activation of *eve* stripe 3. Since the ectopic *kni* domain is well-refined at this time, and is four to five cells anterior to the position of *eve* stripe 3, the most likely source of kni protein for the extended repression is protein diffusion. For repression of *ftz* stripe 3, however, it seems clear that a strong early prepattern is required. Interestingly, once *ftz* stripe 3 has been repressed, the stripe cannot fully recover, as is observed for the *eve* stripes. This may reflect differences in the mechanisms of maintenance and refinement for these two genes.

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REFERENCES

Akam, M. (1987). The molecular basis for metameric pattern formation in the Drosophila embryo. *Development* **101**, 1-22.

Arnosti, D., Barolo, S., Levine, M. and Small, S. (1996). The *eve* stripe 2 enhancer employs multiple modes of transcriptional synergy. *Development* **122**, 205-214.

Brand, A. and Perrimon, **N.** (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Carroll, S. and Scott, M. (1986). Zygotically active genes that affect the spatial expression of the *fushi tarazu* segmentation gene during early Drosophila embryogenesis. *Cell* **45,** 113-126.

Carroll, S. B. (1990). Zebra stripes in fly embryos: activation of stripes or repression of interstripes? *Cell* **60**, 9-16.

Davis, I. and Ish-Horowicz, D. (1991). Apical localization of pair-rule transcripts requires 3′ sequences and limits protein diffusion in the Drosophila blastoderm embryo. *Cell* **67**, 927-940.

Driever, W. and Nüsslein-Volhard, C. (1988). A gradient of Bicoid protein in Drosophila embryos. *Cell* **43**, 59-69.

Driever, W. and Nüsslein-Volhard, C. (1989). The Bicoid protein is a positive regulator of *hunchback* transcription in the early *Drosophila* embryo. *Nature* **337**, 138-143.

Driever, W. (1993). Maternal control of anterior development in the *Drosophila* embryo. In *The Development of Drosophila melanogaster*. (ed. M. Bate and A. Martinez Arias). pp. 301-324. Cold Spring Harbor Laboratory Press.

Eldon, E. and Pirrotta, V. (1991). Interactions of the *Drosophila* gap gene *giant* with maternal and zygotic patterning genes. *Development* **111**, 367-378.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the *even-skipped* protein of Drosophila. *EMBO J.* **6**, 749-759.

Frasch, M., Warrior, R., Tugwood, J. and Levine, M. (1988). Molecular analysis of *even-skipped* mutants in Drosophila development. *Genes Dev*. **2**, 1824-1938.

Fujioka, M., Miskiewic, P., Raj, L., Gulledge, A. A., Weir, M. and Goto, T. (1996). *Drosophila* paired regulates late *even-skipped* expression through a composite binding site for paired domain and the homeodomain. *Development* **122**, 2697-2707.

Gaul, U. and Jäckle, H. (1990). Role of gap genes in early Drosophila development. *Advances in Genetics* **27**, 239-275.

Goto, T., MacDonald, P. and Maniatis, T. (1989). Early and late periodic patterns of *even-skipped* expression are controlled by distinct regulatory elements that respond to different spatial cues. *Cell* **57**, 413-422.

Harding, K., Hoey, T., Warrior, R. and Levine, M. 1989. Autoregulatory and gap response elements of the *even-skipped* promoter of Drosophila. *EMBO J.* **8**, 1205-1212.

Hartmann, C., Taubert, H., Jackel, H. and Pankratz, M. (1994). A two-step mode of stripe formation in the Drosophila blastoderm requires interactions among primary pair-rule genes. *Mech. Dev.* **45,** 3-13.

Hiromi, Y. and Gehring, W. J. (1987). Regulation and function of the Drosophila segmentation gene *fushi tarazu*. *Cell* **50**, 963-974.

Hoch, M., Schroder, C., Seifert, E. and Jäckle, H. (1990). Cis-acting control elements for *Kruppel* expression in the Drosophila embryo. *EMBO J.* **9**, 2587-2595.

Hoch, M., Gerwin, N., Taubert, H. and Jäckle, H. (1992). Competition for overlapping sites in the regulatory region of the Drosophila gene *Kruppel*. *Nature* **256**, 94-97.

Hou, X., Melnick, M. and Perrimon, N. (1996). *marelle* acts downstream of the Drosophila HOP/JAK kinase and encodes a protein similar to the mammalian STATS. *Cell* **84**, 411-420.

Howard, K., Ingham, P. and Rushlow, C. (1988). Region-specific alleles of the Drosophila segmentation gene *hairy*. *Genes Dev.* **2**, 1037-1046.

Howard K. and Struhl, G. (1990). Decoding positional information: regulation of the pair-rule gene *hairy*. *Development* **110**, 1123-1232.

Hülskamp, M., Pfeifle, C. and Jäckle, H. (1990). A morphogenetic gradient of *hunchback* protein organizes the expression of the gap genes *Kruppel* and *knirps* in the early Drosophila embryo. *Nature* **346**, 577-579.

Hülskamp, M., Lukowitz, W., Beermann, A., Glaser, G. and Tautz, D. (1994). Differential regulation of target genes by different alleles of the segmentation gene *hunchback* in Drosophila. *Genetics* **138**, 125-134.

Ingham, P. (1988). The molecular genetics of embryonic pattern formation in Drosophila. *Nature* **335**, 25-34.

Ingham, P. and Martinez-Arias, A. (1986). The correct activation of Antennapedia and Bithorax complex genes requires the *fushi tarazu* gene. *Nature* **324**, 592-597.

Ingham, P. and Gergen, J. P. (1988). Interactions between the pair-rule genes *runt*, *hairy*, *even-skipped*, and *fushi tarazu* and the establishment of periodic pattern in the *Drosophila* embryo. *Development* **104 Supplement**, 51-60.

Jiang, J., Hoey, T. and Levine, M. (1991a). Autoregulation of a segmentation gene in Drosophila: combinatorial interaction of the *even-skipped* homeobox protein with a distal enhancer element. *Genes Dev*. **5**, 265-277.

Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991b). The dorsal morphogen gradient regulates the mesoderm determinant twist in early Drosophila embryos. *Genes Dev.* **5**, 1881-1891.

Kania, M. A., Bonner, A. S., Duffy, J. B. and Gergen, J. P. (1990). The Drosophila segmentation gene *runt* encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. *Genes Dev*. **4**, 1701-1712.

Klinger, M. and Gergen, P. (1993). Regulation of runt transcription by Drosophila segmentation genes. *Mech. Dev.* **43**, 3-19.

Kraut, R. and Levine, M. (1991). Mutually repressive interactions between the gap genes *giant* and *Kruppel* define middle body regions of the *Drosophila* embryo. *Development* **111**, 611-621.

Lawrence, P. and Johnston, P. (1989). Pattern formation in the *Drosophila* embryo: Allocation of cells to parasegments by *even-skipped* and *fushi-tarazu*. *Development* **105**, 761-767.

MacDonald, P., Ingham, P. and Struhl, G. (1986). Isolation, structure, and expression of *even-skipped*: a second pair-rule gene of Drosophila containing a homeobox. *Cell* **47**, 721-734.

Margolis, J., Borowsky, M., Steingrimsson, E., Shim, C., Lengyel, J. and Posakony, J. (1995). Posterior stripe expression of *hunchback* is driven from two promoters by a common enhancer element. *Development* **121**, 3067-3077.

Nauber, U., Pankratz, M., Kienlin, A., Seifert, E. Klemm, U. and Jäckle, H. (1988). Abdominal segmentation of the *Drosophila* embryo requires a

hormone receptor like protein encoded by the gap gene *knirps*. *Nature* **336**, 489-492.

Nüsslein-Volhard, C. and Wieshaus, E. (1980). Mutations affecting segment number and identity in *Drosophila*. *Nature* **287**, 795-801.

Nüsslein-Volhard, C., Kluding, H. and Jurgens, G. (1985). Genes affecting the segmental subdivision of the Drosophila embryo. *Cold Spring Har. Symp. Quant. Biol*. **50**, 145-154.

Pankratz, M., Hoch, M., Seifert E. and Jäckle, H. (1989). *Kruppel* requirement for *knirps* enhancement reflects overlapping gap gene activities in the *Drosophila* embryo. *Nature* **341**, 337-339.

Pankratz, M., Seifert, E., Gerwin, N., Billi, B., Nauber, U. and Jäckle, H. (1990). Gradients of *Kruppel* and *knirps* gene products direct pair-rule gene stripe patterning in the posterior region of the Drosophila embryo. *Cell* **61**, 309-317.

Pick, L., Schier, A., Affolter, M., Schmidt-Glenewinkel, T. and Gehring, W. (1990). Analysis of the *ftz* upstream element: germ layer-specific enhancers are independently regulated. *Genes Dev*. **4**, 1224-1239.

Riddihough, G. and Ish-Horowicz, D. (1991). Individual stripe regulatory elements in the Drosophila *hairy* promoter respond to maternal, gap, and pair-rule genes. *Genes Dev.* **5**, 840-854.

Rothe, M., Nauber, U. and Jäckle, H. (1989). Three hormone receptor-like Drosophila genes encode an identical DNA-binding finger. *EMBO J*. **8**, 3087-3094.

Sauer, F. and Jäckle, H. (1991). Concentration-dependent transcriptional activation or repression by Kruppel from a single binding site. *Nature* **353**, 563-566.

Sauer, F. and Jäckle, H. (1995). Heterodimeric Drosophila gap gene protein complexes acting as transcriptional repressors. *EMBO J*. **14**, 4773-4780.

Schier, A. and Gehring, W. (1993). Analysis of a *fushi tarazu* autoregulatory element: multiple sequence elements contribute to enhancer activity. *EMBO J*. **12**, 1111-1119.

Simpson-Brose, M., Treisman, J. and Desplan, C. (1994). Synergy between the Hunchback and Bicoid morphogens is required for anterior patterning in Drosophila. *Cell* **78**, 855-865.

Slack, J. M. W. (1987). Morphogenetic gradients – past and present. *TIBS* **12**, 200-204.

Small, S., Kraut, R., Hoey, T.,Warrior, R. and Levine, M. (1991). Transcriptional regulation of a pair-rule stripe in Drosophila. *Genes Dev*. **5**, 827-839.

Small, S., Blair, A. and Levine, M. (1992). Regulation of *even-skipped* stripe 2 in the Drosophila embryo. *EMBO J*. **11**, 4047-4057.

Small, S., Arnosti, D. and Levine, M. (1993). Spacing ensures autonomous expression of different stripe enhancers in the *even-skipped* promoter. *Development* **119**, 767-772.

Small, S., Blair, A. and Levine, M. (1996). Regulation of two pair-rule stripes by a single enhancer in the Drosophila embryo. *Dev. Biol*. **175**, 314-324.

Spradling, A. (1986). P-element mediated transformation. In *Drosophila: a Practical Approach* (ed. D. B. Roberts). Oxford: IRC Press Limited.

Stanojevic, D., Hoey, T. and Levine, M. (1989). Sequence-specific DNA binding activities of gap proteins encoded by *hunchback* and *Kruppel* in *Drosophila*. *Nature* **341**, 331-335.

Stanojevic, D., Small, S. and Levine, M. (1991). Regulation of a segmentation stripe by overlapping activators and repressors in Drosophila. *Science* **246**, 1385-1387.

Struhl, G. (1989). Morphogen gradients and the control of body pattern in insect embryos. *Ciba Foundation Symp*. **144**, 65-98.

Struhl, G., Struhl, K. and MacDonald, P. (1989). The gradient morphogen Bicoid is a concentration-dependent transcriptional activator. *Cell* **57**, 1259-1273.

Struhl, G., Johnston, P. and Lawrence, P. (1992). Control of Drosophila body pattern by the *hunchback* morphogen gradient. *Cell* **69**, 237-248.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74**, 331-345.

St. Johnston, D. (1993). Pole plasm and the posterior group genes. In *The Development of Drosophila melanogaster*. (ed. M. Bate and A. Martinez Arias). pp. 325-364. Cold Spring Harbor Laboratory Press.

Theurkauf, W., Baum, H., Bo, J. and Wensink, P. (1986). Tissue-specific and constitutive α-*tubulin* genes of Drosophila melanogaster code for structurally distinct proteins. *Proc. Nat. Acad. Sci. USA* **83**, 8477-8481.

Thummel, C., Boulet, A. and Lipshitz, H. (1988). Vectors for Drosophila P-element mediated transformation and tissue culture transformation. *Gene* **74**, 445-446.

Tsai, C. and Gergen, P. (1995). Pair-rule expression of the *Drosophila fushi tarazu* gene: a `nuclear receptor response element mediates the opposing regulatory effects of *runt* and *hairy*. *Development* **121**, 453-462.

Turing, A. (1952). The chemical basis of morphogenesis. *Philos. Trans. R. Soc. London B.* **237**, 37-72.

Warrior, R. and Levine, M. (1990). Dose dependent regulation of pair-rule stripes by gap genes and the initiation of segment polarity. *Development* **110**, 759-768.

Yan, R., Small, S., Desplan, C., Dearolf, C. and Darnell, J. (1996). Identification of a Stat gene that functions in Drosophila development. *Cell* **84**, 421-430.

Yu, Y. and Pick, L. (1995). Non-periodic cues generate seven *ftz* stripes in the Drosophila embryo. *Mech. Dev*. **50**, 163-175.

Zuo, P., Stanojevic, D., Colgan, J., Han, K., Levine, M. and Manley, J. L. (1991). Activation and repression of transcription by the Drosophila gap proteins hunchback and Kruppel. *Genes Dev*. **5**, 254-264.

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