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Mutually repressive interactions between the gap genes *giant* and *Krüppel* define middle body regions of the *Drosophila* embryo

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Summary

The gap genes play a key role in establishing pair-rule and homeotic stripes of gene expression in the *Drosophila* embryo. There is mounting evidence that overlapping gradients of gap gene expression are crucial for this process. Here we present evidence that the segmentation gene *giant* is a *bona fide* gap gene that is likely to act in concert with *hunchback*, *Krüppel* and *knirps* to initiate stripes of gene expression. We show that *Krüppel* and *giant* are expressed in complementary, non-overlapping sets of cells in the early embryo. These complementary patterns depend on mutually repressive interactions between the two genes. Ectopic expression of *giant* in early embryos results in the selective repression of *Krüppel*, and advanced-stage embryos show cuticular defects similar to those observed in *Krüppel*$^-$ mutants. This result and others suggest that the strongest regulatory interactions occur among those gap genes expressed in nonadjacent domains. We propose that the precisely balanced overlapping gradients of gap gene expression depend on these strong regulatory interactions, coupled with weak interactions between neighboring genes.

Key words: gap genes, cross-regulation, gradients, *giant*, segmentation, *Drosophila*.

Introduction

A central problem in the segmentation field is how relatively few, crudely distributed gap proteins generate organized sets of pair-rule and homeotic stripes in the early *Drosophila* embryo. The cascade of gene interactions that constitute the segmentation hierarchy result in a progressive refinement in the patterns of gene expression (reviewed by Ingham, 1988). This process transduces the broad gradients of maternal morphogens (reviewed by Nüsslein-Volhard *et al.* 1987) into the highly refined patterns of the segment polarity genes, which are expressed within the limits of just single cells (reviewed by DiNardo and Heemskerk, 1990). Interactions between gap genes and pair-rule genes lead to the single most dramatic increase in the spatial complexity of gene expression that occurs during the course of early development.

Recent efforts to understand how gap genes specify stripes have centered on the pair-rule genes *even-skipped* (*eve*) (Harding *et al.* 1986; Macdonald *et al.* 1986; Frasch *et al.* 1987) and *hairy* (*h*) (Rushlow *et al.* 1989). Promoter fusion studies suggest that separate *cis* elements are responsible for the initiation of individual *eve* and *hairy* stripes (Goto *et al.* 1989; Harding *et al.* 1989; Hooper *et al.* 1989; Howard and Struhl, 1990; Pankratz *et al.* 1990). Proteins encoded by gap genes have been shown to bind specific sequences within the *eve* promoter (Stanojević *et al.* 1989) and there is evidence that the same is true for the *h* promoter as well (Pankratz *et al.* 1990).

Meinhardt (1986) first proposed that borders between neighboring domains of gap gene expression define the sites where pair-rule stripes are initiated. However, a potential limitation of this model is that there are not enough gap borders to account for 7-stripe patterns of *eve* and *h* expression that are out of register with one another. Despite the fact that the gap genes *hunchback* (*hb*) (Tautz, 1988) and *knirps* (*kni*) (Pankratz *et al.* 1989) are each expressed in two discrete domains, one in anterior regions and another in posterior regions, the three best characterized gap genes, *hb*, *Krüppel* (*Kr*) (Gaul *et al.* 1987) and *kni*, together define only 3 or 4 gap borders. The paradox of too few gap borders for so many stripes would be resolved by the demonstration that additional gap genes participate in this process. The segmentation gene *tailless* (*tll*) was recently shown to have the properties of a gap gene (Pignoni *et al.* 1990), and here we provide evidence that *giant* (*gt*) (Mohler *et al.* 1989) is also a *bona fide* gap gene which interacts with the others to make pair-rule stripes.

Another mechanism for how relatively few gap genes might specify many stripes is suggested by recent immunolocalization studies, which have shown that gap

proteins are expressed in broad, overlapping gradients (Stanojević *et al.* 1989; Gaul and Jäckle, 1989; K. Howard, personal communication). This situation is in sharp contrast to the abutting zones of uniform expression originally envisioned by Meinhardt (1986, 1988) and supported by earlier studies (Knipple *et al.* 1985; Jäckle *et al.* 1986; Gaul and Jäckle, 1987*a*; Tautz *et al.* 1987). Gradients of gap proteins raise the possibility of dose-dependent effects on pair-rule expression, while overlapping patterns suggest a combinatorial mode of regulation which could generate more stripes than the sum of the gap borders. Evidence that gap genes exert dose-dependent effects on *eve* and *h* expression stems from genetic studies (Hooper *et al.* 1989; Warrior and Levine, 1990). For example, it has been shown that the progressive loss of Kr^+ gene activity causes a gradual expansion in the limits of the *eve* stripe 2 border. Just a 2-fold reduction in the level of *Kr* activity (in Kr^+/Kr^- heterozygotes) causes a significant expansion in the limits of this stripe (Warrior and Levine, 1990). Various models have been proposed to account for the role of overlapping patterns of gap gene expression in the combinatorial regulation of pair-rule stripes (i.e. Carroll, 1990). Overlapping expression of the *hb* and *Kr* proteins have been implicated in the organization of *eve* stripes 2 and 3 (Stanojević *et al.* 1989), while the overlapping *Kr* and *kni* patterns are thought to play an important role in the organization of *h* stripes 5 and 6 (Pankratz *et al.* 1990). Although not rigorously proven, the preponderance of the evidence to date suggests that striped patterns of pair-rule and homeotic genes depend on both the exact combinations and concentrations of gap proteins (Carroll and Scott, 1986; Ingham *et al.* 1986; White and Lehmann, 1986; Frasch and Levine, 1987; Harding and Levine, 1988; Gaul and Jäckle, 1989; Irish *et al.* 1989*b*; Reinitz and Levine, 1990). It is, therefore, of considerable interest to understand how the gap genes come to be expressed in such overlapping domains.

Maternal factors have been shown to be responsible for the initiation of the gap genes (Driever *et al.* 1989; Struhl *et al.* 1989; Hülskamp *et al.* 1990), while the refinement of these patterns depends on cross-regulatory interactions among the gap genes (Jäckle *et al.* 1986; Pankratz *et al.* 1989). The maternal morphogen *bicoid* (*bcd*) is critical for the activation of gap genes in anterior regions of the early embryo, while localized expression in posterior regions depends on *nanos*, which modulates the activity of maternal *hb* products (Hülskamp *et al.* 1989; Irish *et al.* 1989*a*; Struhl, 1989). The restriction of gap gene expression to central regions involves repression by *torso*, a maternal gene that is active at both poles (Gaul and Jäckle, 1987*b*; Tautz, 1988).

The initial gap patterns generated by these maternal factors are considerably broader than their limits of expression at the time when they are responsible for effecting pair-rule and homeotic stripes. For example, *Kr* transcripts are initially detectable in a region encompassing approx. 27% of the total length of the egg in nuclear cycle 12 embryos (Gaul and Jäckle, 1989), but by cycle 14 these transcripts are restricted to just 20–22% of the total egg length (Gaul *et al.* 1987; R. Kraut, unpublished observations). *gt* is expressed in two discrete domains along the A–P axis. The posterior pattern initially extends all the way to the posterior pole, from 30% to 0% egg length. However, expression is rapidly repressed in the posterior-most regions and comes to be restricted to a narrower band, between 30% to 17% egg length (Mohler *et al.* 1989; see accompanying report by Kraut and Levine, 1991). There is evidence that the refinement of the *Kr* and *gt* patterns involves regulatory interactions with other gap genes (Jäckle *et al.* 1986; Mohler *et al.* 1989). However, in general, there is sporadic and even contradictory information regarding the role of cross-regulation in the segmentation process.

The first evidence for cross-regulation involved pairs of gap genes expressed in neighboring domains, such as *hb–Kr* and *Kr–kni* (Jäckle *et al.* 1986). For example, mutations in *hb* cause a slight expansion in the limits of the *Kr* pattern and *vice versa*. It is unclear whether these relatively weak interactions account for the refinement in the patterns of gap gene expression that occur during the course of normal development. Here we re-examine the question of cross-regulation by analyzing the effects of ectopically expressed gap products on the expression of other gap genes. We show that ectopic expression of the segmentation gene *gt* results in the rapid and selective repression of *Kr*. This result lends strong support to the view that *gt* is a *bona fide* gap gene which interacts with *hb*, *Kr*, and *kni* to specify stripes. Moreover, interactions between *gt* and *Kr* suggest that the strongest regulatory interactions occur among gap genes that are expressed in nonadjacent domains. Further support for this proposal is provided by the analysis of gap gene expression in embryos that ectopically express *hb* and *tll* proteins.

Materials and methods

Antibody staining

Embryo collections, fixations and staining procedures were done exactly as described by Frasch *et al.* (1987). The polyclonal anti-*gt*, anti-*Kr*, anti-*kni*, and anti-*eve* antibodies are all the same as those used in the accompanying report (Kraut and Levine, 1991). The histochemical stainings shown in Figs 7 and 8 were done using biotinylated secondary antibodies and DAB staining with horseradish peroxidase, as described in the 'Elite' ABC kit purchased from Vector laboratories (Burlingame, CA).

P-transformations

P-mediated germline transformation was done as originally described by Rubin and Spradling (1982). A full-length *gt* cDNA (see accompanying report for the details of its isolation) was placed downstream of a 315 bp DNA fragment spanning the hsp70 promoter (Kuziora and McGinnis, 1988). The cDNA includes 50 bp from the *Xenopus* beta-globin gene leader sequence (derived from the pNB40 cDNA library; see Brown and Kafatos, 1988) and 40 bp of the *gt* untranslated leader. The hsp70–*gt* fusion gene was cloned into the P-transformation vector, C20-X, which has a wild-type copy

of the *rosy* gene as a selectable marker. The C20-X vector is a derivative of the Carnegie 20 vector described by Rubin and Spradling (1982). Injections were done with approximately 400 ng of P-transposon DNA along with approx. 100 ng of the pΠ25.7 helper P-element μl^{-1} of injection buffer. Five independent transformed lines were obtained, and two of these were homozygosed.

Heat shock

For cuticle preparations, embryos were collected at room temperature for 1½ h, aged for another 1½ h and examined under Voltalef oil. Gastrulating or older embryos were discarded, while others were heat shocked on apple juice–agar collection plates on a pre-heated metal block in a 36°–37°C water incubator. Embryos were incubated at this temperature for 20 to 45 min, and then aged for at least 24 h at room temperature. We found that heat shocks of a total time of 30 min or longer, with a slow increase in temperature were the most effective in inducing severe phenotypes. Cuticle preparations were done as described by Wieschaus *et al.* (1984*a*,*b*). Embryos were rinsed with heptane to remove the Voltalef oil prior to dechorionating.

Embryos used for antibody staining were treated as described above, except that they were not monitored with Voltalef oil prior to heat shocking. The embryos were heat shocked for 30 min and allowed to recover for 30 to 45 min at room temperature prior to fixation.

Fly stocks

The following fly stocks were used for these studies. *rosy*$^-$ for P–transformation: *rosy*^{506}; *spliced* (dominant gain-of-function *torso* mutation): *torso*^{RL3} (Klingler *et al.* 1988; Strecker *et al.* 1989); *Kr*$^-$: *Kr*^{9}, a null point mutation (Wieschaus *et al.* 1984*b*). Embryos from hsp70–*hb* P-transformants, provided by Dr Gary Struhl, were collected from a homozygous line on the third chromosome (details described in Struhl, 1989). hb$^-$, *gt*$^-$ double mutants: *hb*^{14F21}, a null mutation (Lehmann and Nüsslein-Volhard, 1987) and *gt*^{YA82}, a strong ems-induced mutation (Wieschaus *et al.* 1984*a*); *hb*$^-$, *gt*$^-$ double mutants were identified among mixed progeny by staining with a mixture of anti-*gt* and anti-*Kr* antibodies. Hemizygotes for the *gt*^{YA82} allele can be identified since they express much lower levels of the *gt* protein than wild-type. *hb*$^-$/*hb*$^-$ embryos can be identified by the anterior shift in *Kr* expression (not seen in *gt*$^-$ alone). Double mutants were identified by both the lack of strong *gt* staining and the shift in the *Kr* pattern. Fluorescent–stained embryos were grouped into three classes: *hb*$^-$ homozygotes; *hb*$^-$, *gt*$^-$ double mutants; and *trans* heterozygotes or single *hb*$^-$ heterozygotes (which are phenotypically wild-type). These were photographed, the numbers in each class were counted and the extent of *Kr* expression was measured as percentage egg length for each embryo. Figures for each group were averaged and the differences in *Kr* expression between groups was found to be significant according to the calculated standard deviation.

Results

Kr *represses* gt *expression*

Polyclonal antibodies raised against a full-length *gt* protein made in *E. coli* were used for immunolocalization studies (for details, see accompanying report by Kraut and Levine, 1991). The *gt* protein is expressed in both anterior and posterior regions of early embryos (Fig. 1A, red stain). These sites of expression are consistent with previous RNA localization studies (Mohler *et al.* 1989) and correspond to the regions of the embryonic fate map that are disrupted in *gt*$^-$ embryos. Such mutants lack pattern elements in the fifth through seventh abdominal segments and show deletions of head structures (Gergen and Wieschaus, 1986; Petschek *et al.* 1987; Mohler *et al.* 1989). Double immunolocalization studies with mixtures of anti-*gt* and anti-*Kr* antibodies show that the two proteins are expressed in complementary sets of cells, such that the two *gt* domains bracket the central *Kr* pattern (Fig. 1A). Although the *gt* and *Kr* expression patterns appear to directly straddle one another, no cells contain detectable levels of both proteins.

Genetic studies have shown that the initiation of *gt* expression depends on maternal factors, particularly *bcd* and maternal *hb* proteins (see accompanying report by Kraut and Levine). However, the maintenance of the normal limits of *gt* expression depends on cross-regulatory interactions with other gap genes. For example, there is premature loss of *gt* expression in *kni*$^-$ embryos. The most dramatic cross-regulatory interaction involves *Kr*. Both the anterior and posterior domains of *gt* expression expand into central regions of *Kr*$^-$ embryos (Fig. 1B), indicating that *Kr* exerts a negative effect on *gt* expression. This repression is important for inhibiting *gt* expression in central regions and restricting the two *gt* domains near the poles.

gt *represses* Kr *expression*

The *Kr* pattern is not strongly disrupted in *gt*$^-$ embryos (Gaul and Jäckle, 1987*b*, and data not shown). At first glance this result suggests that *gt* does not participate in the regulation of *Kr* expression. However, *Kr* is subject to very complex and redundant control (Gaul and Jäckle, 1987*b*; Jäckle *et al.* 1988; Lehmann and Frohnhöfer, 1989; see Discussion). Evidence that *gt* does indeed regulate *Kr* expression was obtained by creating a 'dominant' *gt* mutation using the hsp70 heat shock promoter to drive ectopic expression of the *gt* protein.

An hsp70–*gt* fusion gene was introduced into the germ line by P-transformation (see Materials and methods for details). The fusion gene that was used is shown in Fig. 2. It includes the entire *gt* coding sequence, as well as untranslated leader sequences from *gt* and the *Xenopus* beta-globin gene (Brown and Kafatos, 1988). Several independent transformed lines containing the hsp70–*gt* fusion gene were obtained, and all displayed the same mutant phenotype, but with varying degrees of severity, upon transient misexpression of *gt* following heat shock. Immunolocalization studies with anti-*gt* antibodies showed that heat shock caused ubiquitous expression of the *gt* protein at low levels in all embryonic nuclei (data not shown). After heat shock, P-transformed embryos were allowed to grow at room temperature until the secretion of the cuticle. Typical cuticular phenotypes are presented in Fig. 3.

The most consistent defect is the deletion of pattern elements in the thorax and abdomen, including the

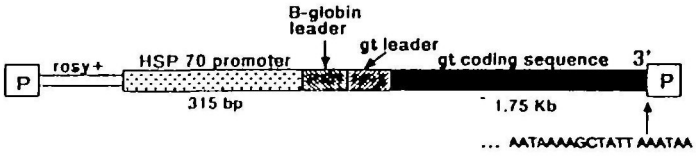

Fig. 2. P-transformation expression plasmid used to misexpress *gt*. The P-element transformation vector contains the *rosy* gene as a selectable marker. The cDNA lies downstream of a 315 bp DNA fragment from the hsp70 promoter (stippled rectangle) which is activated upon heat shock. The composite 5′ untranslated leader sequence includes 40 bp from the *Xenopus* beta-globin leader as well as 40 bp from the *gt* leader. The nucleotide sequence corresponds to a putative polyadenylation signal located approx. 1.6 kb downstream from the initiating ATG.

second (T2) and third (T3) thoracic segments as well as the second (A2) and fourth (A4) abdominal segments (Fig. 3A and B). The most severe defects were obtained in homozygous lines containing two copies of the hsp70–*gt* fusion gene. In these cases most of the pattern elements in the thorax and anterior abdomen were deleted, resulting in embryos containing normal denticles for A6 through A8 but lacking most of the segments between the posterior head and A6 (Fig. 3C). This phenotype is very similar to that observed in Kr^- embryos (Wieschaus *et al.* 1984*b*) (compare Fig. 3C with D), although even the most severely affected hsp70–*gt* transformants retain some pattern elements in the anterior abdomen (Fig. 3C). Further evidence for a link between the hsp70–*gt* and *Kr* phenotypes is that less severely affected hsp70–*gt* embryos resemble weaker (i.e. hypomorphic) *Kr* mutants. For example, the first disruptions seen in the least severely affected hsp70–*gt* embryos center around T2–T3 and A4, similar to the weakest *Kr* mutants, which lose T2, T3, A2 and A4 (Wieschaus *et al.* 1984*b*).

The striking similarity of the cuticular defects resulting from ectopic expression of *gt* and those seen in Kr^- embryos suggest that *gt* selectively represses *Kr* and does not cause a general disruption of segmentation gene activities. For example, the normal head skeleton and tail structures observed in even the most severely affected hsp70–*gt* transformants suggest that the activities of the gap genes *hb* (Lehmann and Nüsslein-Volhard, 1987) and *tailless* (*tll*) (Strecker *et al.* 1986) are not altered significantly by ubiquitous expression of the *gt* protein. The specificity of the *gt–Kr* interaction was confirmed by immunolocalization studies, using antibodies against the gap proteins *hb* and *kni*, and the pair-rule protein *even-skipped* (*eve*) to stain hsp70–*gt* transformants after heat induction.

There is a rapid loss of *Kr* protein following heat shock (compare Fig. 4A and B), while the *hb* and *kni* patterns appear essentially normal (data not shown). Specific repression of *Kr* is also indicated by the altered

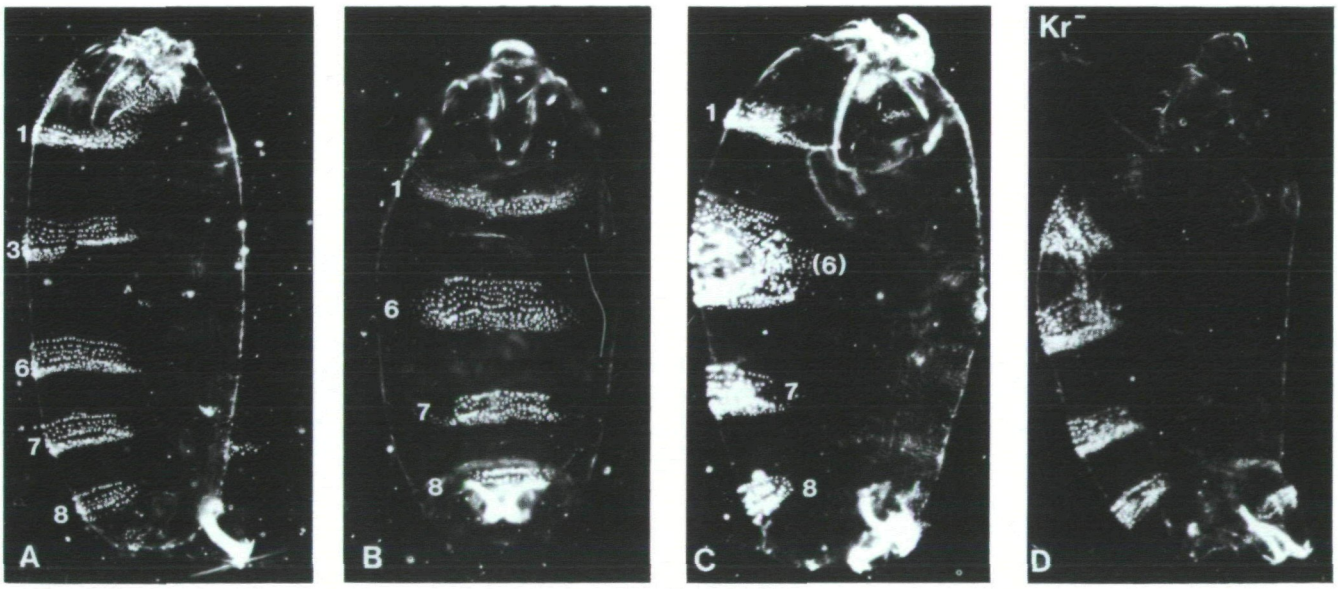

Fig. 3. Misexpression of *gt* disrupts the embryonic cuticle. Cuticle preparations are oriented so that anterior is up. A, B and C show cuticles of P-transformants containing the hsp70–*gt* expression plasmid. A typical series of cuticular disruptions are presented, with increasing severity from left to right. (A) The A6, A7 and A8 denticles appear normal but the region between T1 and A5 is severely disrupted, and contains only two abdominal denticle belts. This pattern is similar to the defects seen in weak (hypomorphic) *Kr* mutations, in which T2, T3, A2 and A4 are deleted (Wieschaus *et al.* 1984*b*). (B) The A7 and A8 denticles appear normal, while the A6 denticle is abnormally broad and might contain additional abdominal denticles due to fusions of the cuticle. The region between T1 and the abnormal A6 segment contains just one abdominal denticle belt, which appears to correspond to A1. In wild-type embryos, the *Kr* protein is found at peak levels in the region that gives rise to A1. After heat shock, the *Kr* protein is largely repressed, but a remnant persists in the A1 primordium. (C) A lateral view of an embryo similar to that shown in B; (D) Cuticle preparation from a Kr^9/Kr^9 null mutant. Kr^- embryos show extensive deletions in the region between T1 and A5, similar to the disruptions observed after heat-induced misexpression of *gt*.

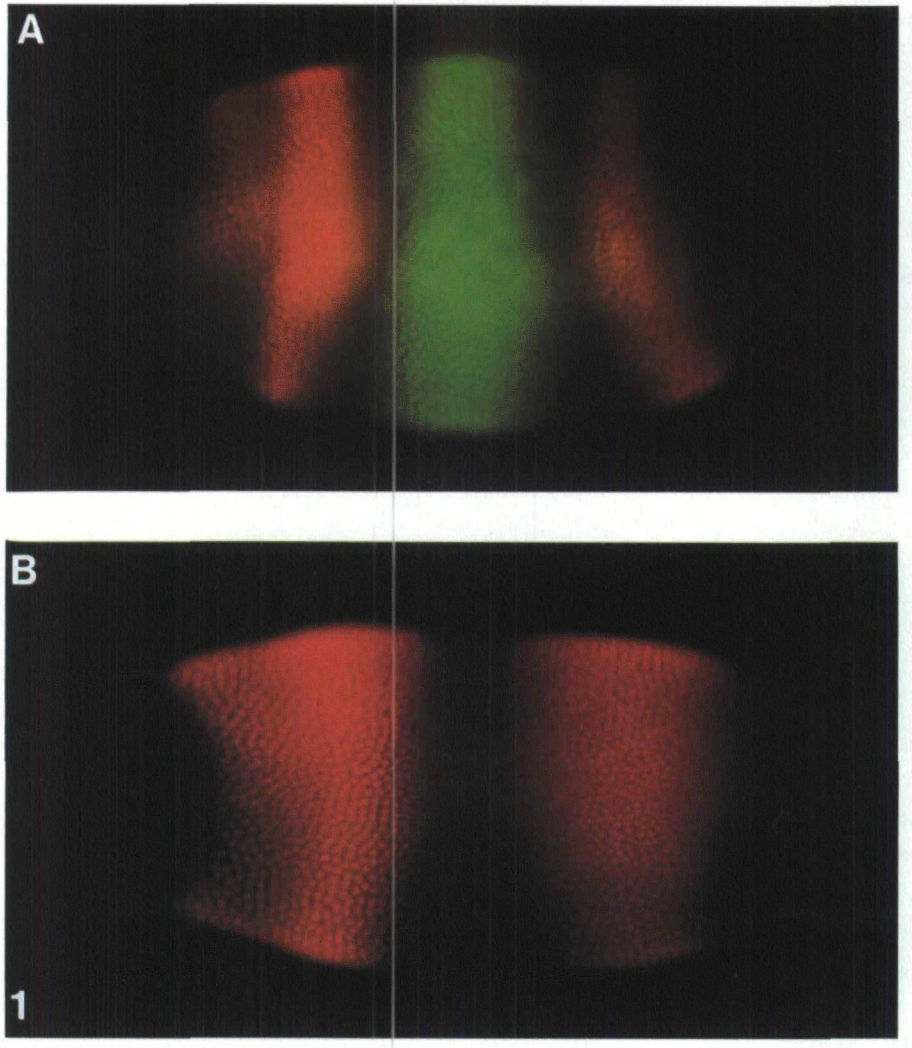

Fig. 1. Localization of *giant* and *Kruppel* proteins in wild-type and *Kruppel*$^-$ embryos. (A) Wild-type embryo at cell-cycle 14 stained with a mixture of anti-*gt* and anti-*Kr* antibodies. The *gt* protein stains red and the *Kr* protein stains green. The two *gt* domains bracket the central *Kr* pattern. (B) *Kr*$^-$ embryo stained with anti-*gt* antibody. Both the anterior and posterior *gt* domains expand towards the center of the embryo, which normally contains high levels of the *Kr* repressor.

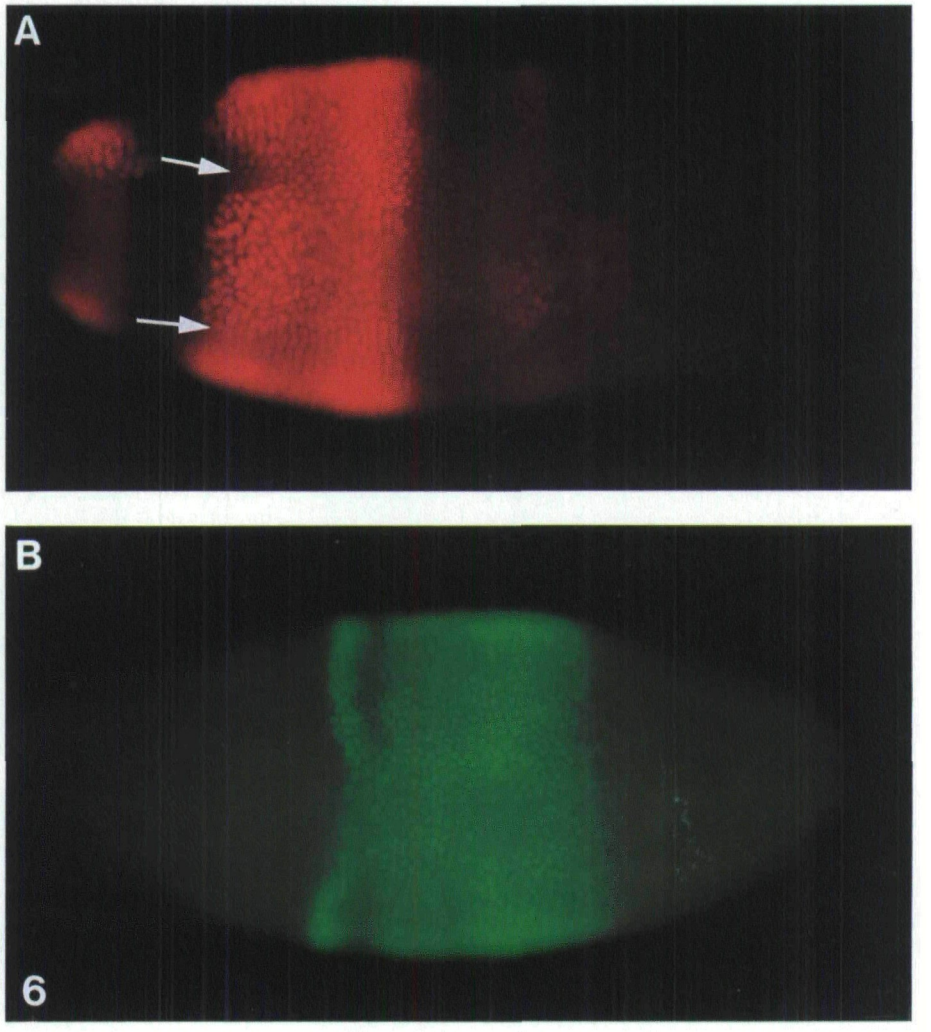

Fig. 6. *giant* and *eve* expression patterns in a *tor* gain-of-function embryo. (A) a tor^{RL3} mutant embryo during cellularization stained with anti-*giant* antibodies. The pattern is severely disrupted in both anterior and posterior domains. The posterior pattern is noticeably reduced, while anterior expression remains strong but is shifted posteriorly. (B) Same embryo stained with *eve* antibody. Stripe 1 appears nearly normal, but stripes 2 through 7 are fused into a single broad band.

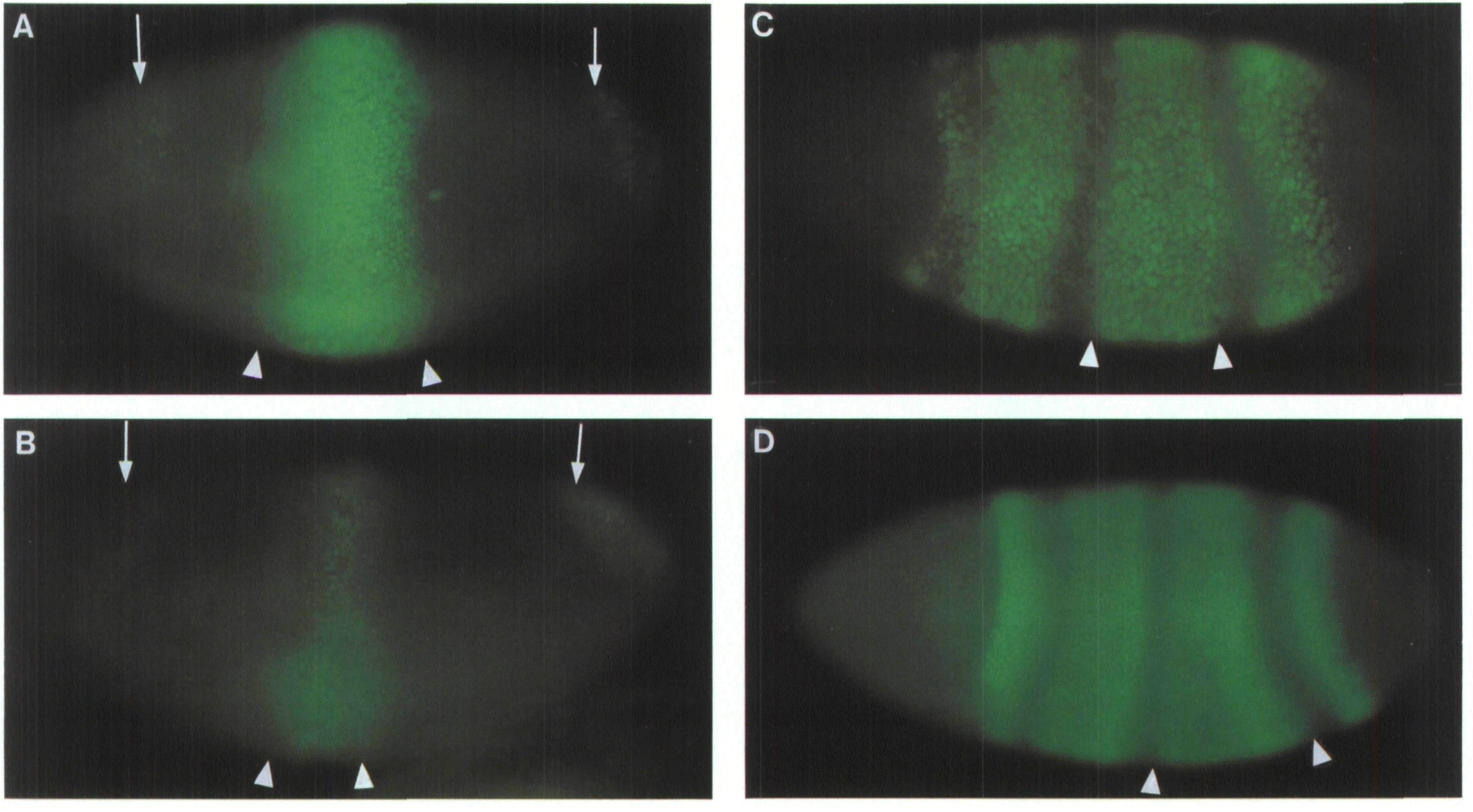

Fig. 4. *Kr* and *eve* expression patterns in hsp70–*gt* transformants. Embryos were collected from hsp70–*gt* P-transformed lines (A–C) and Kr^- balanced stocks (D). Embryos were stained with anti-*Kr* (A, B) or anti-*eve* (C, D) antibodies. (A) A heat shocked embryo showing an almost normal *Kr* staining pattern. The range of effects on *Kr* expression in any given experiment was variable from embryo to embryo, due to variation in timing and levels of misexpressed *gt* protein. The arrowheads show the limits of the central *Kr* domain and the arrows indicate the weak patches of expression at the anterior and posterior poles. (B) The *Kr* staining pattern in a transformed embryo that was heat shocked during cleavage cycle 14. In this embryo the *gt* protein is ubiquitously expressed in all embryonic nuclei at low levels (data not shown). Both the limits and intensity of *Kr* expression are drastically reduced in central regions (arrowheads). The arrows indicate that the polar expression is essentially normal (compare with A), which serves as an internal control for staining. (C) The *eve* staining pattern in a transformed embryo that was heat shocked during cleavage cycle 14. There is a severe disruption of the normal *eve* staining pattern, such that stripes 2 through 6 are fused into two broad bands. The arrowheads indicate the composite band encompassing stripes 4 through 6. This altered *eve* staining pattern is similar to that seen in Kr^- mutants; (D) A Kr^9 homozygous embryo stained with anti-*eve* antibodies. The pattern is similar to that seen in C.

eve staining patterns that are observed (Fig. 4C). Instead of 7 periodic stripes, the *eve* pattern consists of only two stripes (1 and 7) and two broad bands in place of stripes 2 through 6. This pattern is very similar to the altered *eve* pattern observed in Kr^- mutants (Frasch and Levine, 1987) (Fig. 4D). Control experiments indicate that the altered *Kr* and *eve* patterns are a specific consequence of ectopically expressed *gt* protein. Untransformed parental stocks ($rosy^{506}$) were subjected to the same conditions of heat shock, but consistently showed normal patterns of *Kr* and *eve* expression (data not shown).

gt *expression in* torso *gain-of-function mutants*

The above results indicate that complementary patterns of *Kr* and *gt* expression involve mutually repressive gene interactions. The two genes are expressed in alternating, not adjacent, domains (summarized in Fig. 7). *hb* intervenes between *gt* and *Kr* in anterior regions, while *kni* is expressed between *Kr* and *gt* in posterior regions. In order to examine the possibility that strong regulatory interactions occur among gap genes expressed in nonadjacent domains, we examined interactions between *gt* and *tll* as well as *hb* and *kni*.

Previous studies suggest that *tll* does indeed exert a negative effect on *gt* expression (Mohler *et al.* 1989; see accompanying report by Kraut and Levine). In tll^- embryos the posterior *gt* domain expands towards the posterior pole. The negative effect that *tll* exerts on *gt* could account for the normal refinement of the *gt* pattern seen in wild-type embryos. Upon its initiation the posterior *gt* domain normally extends to the posterior pole, but soon thereafter *gt* expression is rapidly lost in the posterior-most regions. *tll* has been cloned and characterized and recently shown to be expressed from 0 % to 15 % egg length (where 0 % = the posterior pole and 100 % = the anterior pole) (Pignoni *et al.* 1990). The posterior *tll* domain closely coincides with the region where *gt* expression is lost during the course of normal development.

Additional support for a strong regulatory interaction between *gt* and *tll* stems from studies on the pattern of *gt* expression in embryos where *tll* is active in ectopic regions. As discussed above, *tll* expression is normally restricted to the poles where it is activated by the maternal *torso* (*tor*) gene (Klingler, 1989; Strecker *et al.* 1989). *tor* encodes a transmembrane tyrosine kinase that is ubiquitously distributed throughout the embryo, but is activated only at the poles where it somehow directs the initiation of *tll* expression (Klingler *et al.* 1988; Casanova and Struhl, 1989). Several different dominant gain-of-function *tor* mutations have been identified, including a temperature-sensitive mutation, tor^{RL3}, also called *spliced* (Schüpbach and Wieschaus, 1989). These are thought to cause constitutive acti-

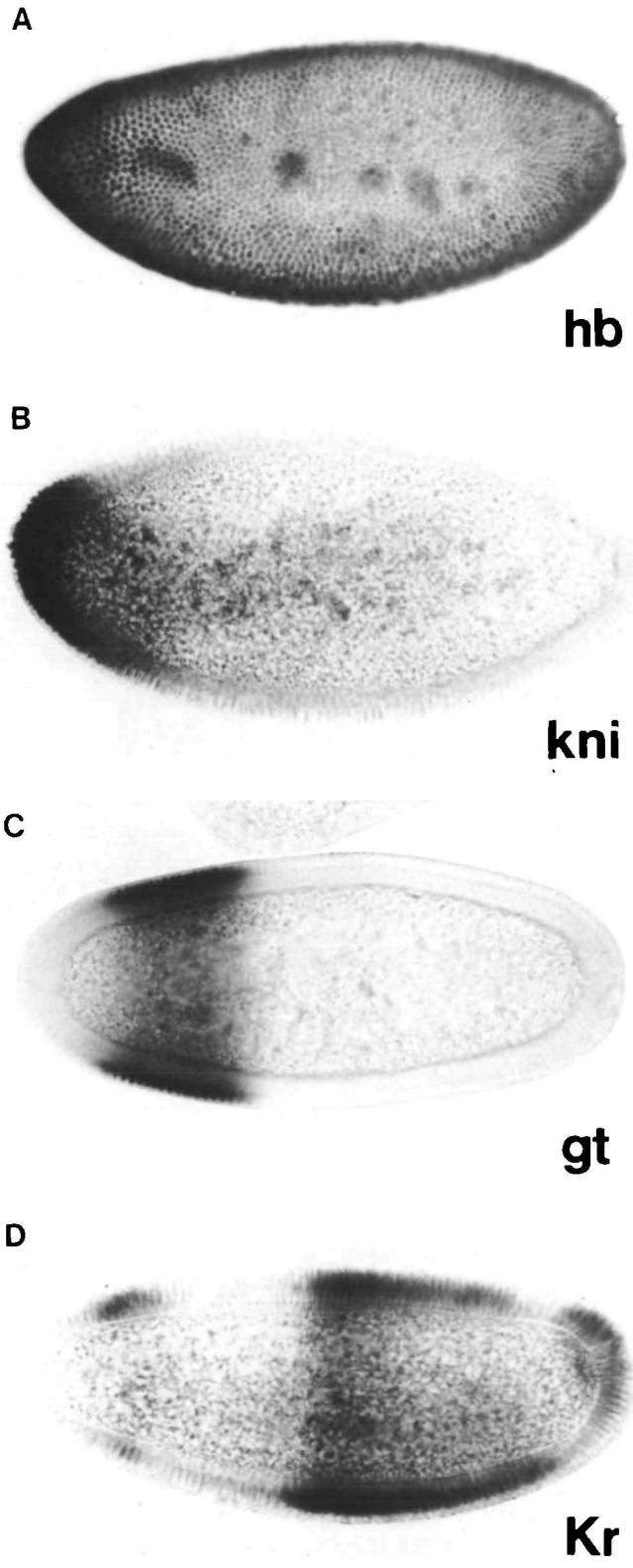

Fig. 5. Gap gene expression in hsp70–*hb* embryos. Embryos in A–D carry two copies of the hsp70–*hb* transposon. All are at nuclear cycle 14 and were fixed approximately 45 min after heat shocking. Antibodies were detected with horseradish peroxidase–DAB.
(A) Misexpression of *hb* protein throughout the embryo. (B) *kni* expression. The posterior domain is repressed, while the anterior domain appears normal. (C) *gt* expression. Again, expression is repressed only in the posterior domain. (D) *Kr* expression. There is a severe posterior expansion in the limits of the *Kr* pattern, but no detectable reduction in the level of protein.

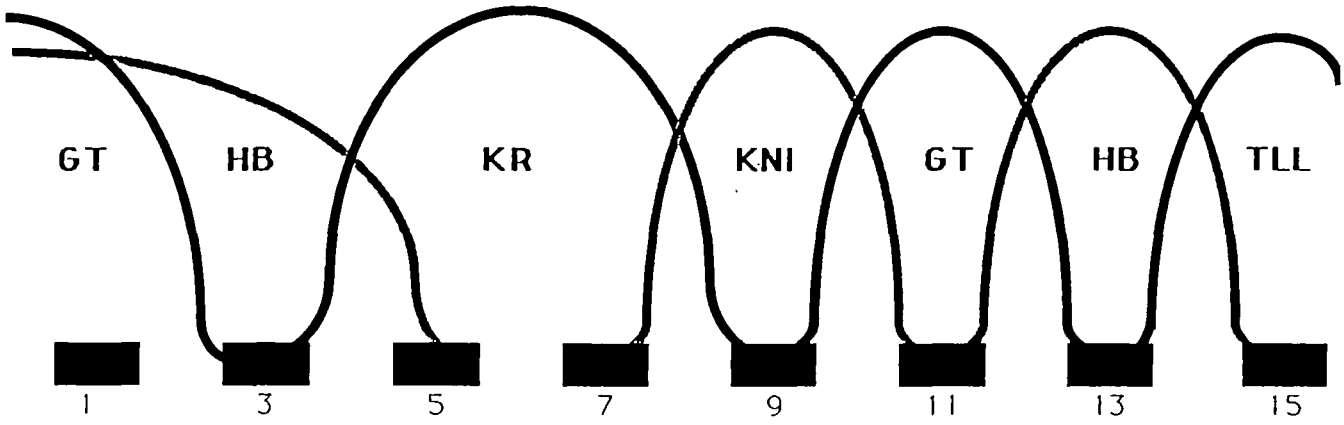

Fig. 7. Summary of the *hb*, *Kr*, *kni*, *gt* and *tll* expression patterns. Relative protein distribution patterns based on the experiments reported here, and on previously published reports (Stanojević *et al.* 1989; Gaul and Jäckle, 1989; Gaul *et al.* 1987; Tautz, 1988; Pignoni *et al.* 1990). The limits of the *kni* protein distribution pattern are based on unpublished studies done by K. Howard (personal communication) and were independently confirmed by one of us (R.K.). The bars at the bottom of the figure represent the limits of the indicated odd-numbered parasegments. Note that the *Kr* and *gt* patterns tightly abut one another but do not overlap. Similarly, the *hb* and *kni*, and the *gt* and *tll* patterns do not overlap. The very mild alteration of the *Kr* pattern observed in gt^- embryos might be due to redundant repression by *hb* and *kni*. This diagram is not quantitative, but simply summarizes the extent to which gap proteins are expressed in broad, overlapping gradients.

vation of the *tor* tyrosine kinase, resulting in ubiquitous expression of *tll* (Klingler, 1989; Strecker *et al.* 1989). The consequences of such misexpression are quite severe, resulting in embryos lacking virtually all middle body segments. Previous studies have shown that this mutant phenotype results from the repression of *Kr* in middle body regions (Klingler, 1989) coupled with an expansion in the limits of *hb* expression into central regions (Warrior and Levine, 1990). Ectopic *tll* activity also causes a severe reduction in the posterior *gt* pattern, as shown in Fig. 6. This repression is either due to ectopically expressed *tll* products, or is an indirect consequence resulting from the expanded *hb* pattern (see below). Either way, this result establishes a strong regulatory interaction between *gt* and a second gap gene, *tll* that is normally expressed in a nonadjacent region.

hb *is a strong repressor of* gt *and* kni

Previous studies have shown that ectopic expression of the *hb* protein in early embryos results in a phenotype similar to that observed in *nanos* mutants (Hülskamp *et al.* 1989; Irish *et al.* 1989*a*; Struhl, 1989). This observation was taken as evidence that *nanos* organizes the posterior pattern by repressing the activity of maternal *hb* products, thereby restricting *hb* function to anterior regions of early embryos. Both the *kni* and *gt* posterior patterns are severely reduced or absent in $nanos^-$ embryos (see accompanying report by Kraut and Levine), raising the possibility that *hb* is a strong repressor of these genes. To test this we examined the expression of *kni* and *gt* in P-transformed lines carrying an hsp70–*hb* fusion gene (Struhl, 1989).

Heat shock results in strong ubiquitous expression of the *hb* protein (Fig. 5A), as was shown previously (Strûhl, 1989). Both the posterior *kni* and *gt* patterns are virtually abolished in these embryos (Fig. 5B and C). This result provides additional evidence that the strongest cross-regulatory interactions occur among gap genes expressed in nonadjacent domains (see Fig. 7 summary). In marked contrast to the effect that ectopic *hb* expression has on *kni* and *gt*, *Kr* expression remains quite strong (Fig. 5D). Thus, *hb* has little or no repressive effect on *Kr* expression, indicating that regulatory interactions between these neighboring genes are quite weak. The severe posterior expansion of the *Kr* pattern seen in hsp70–*hb* embryos (Fig. 5D) is probably due to the loss of both *kni* and *gt* products, which normally repress *Kr* expression (Jäckle *et al.* 1986; Fig. 4B).

gt *acts in concert with other gap genes to repress* Kr

The ectopic expression studies and mutant analysis described above indicate that *gt* is a strong repressor of *Kr*. However, the results of these studies are paradoxical in that the *Kr* expression pattern is not disrupted in gt^- embryos until after cellularization (Gaul and Jäckle, 1987*b*; Reinitz and Levine, unpublished observation). Such mutants show only a very mild anterior and posterior expansion of the *Kr* pattern during gastrulation (data not shown). One possible explanation for this apparent inconsistency is that *gt* functions either redundantly or cooperatively with *hb* and *kni* to define the anterior and posterior limits of the *Kr* pattern, respectively (see Fig. 7 summary). If repression of *Kr* is achieved through cooperative interactions between *hb* and *gt* and/or *gt* and *kni*, then the expansion of the *Kr* pattern should be greater when both products are removed in double mutants than in single mutants. To test this possibility we examined the *Kr* staining pattern in hb^-, gt^- double mutants (Fig. 8). Previous studies have shown that there is an anterior expansion of the *Kr*

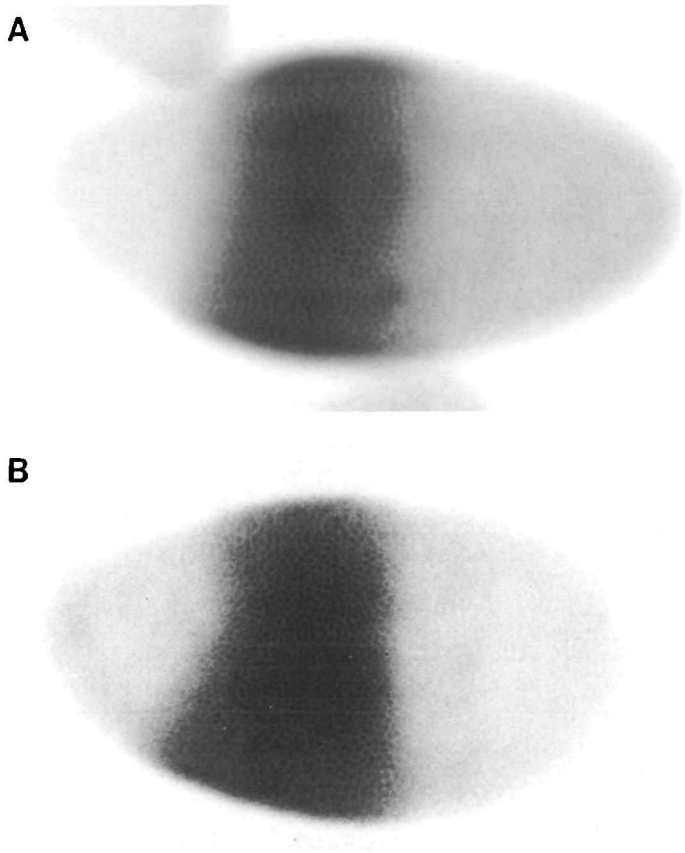

Fig. 8. Expansion of the *Kr* pattern in hb^-, gt^- double mutants and in bcd^- embryos. Embryos were stained with anti-*Kr* antibodies and are oriented so that anterior is to the left. (A) hb^-, gt^- embryo. The *Kr* pattern is substantially broader than that seen in wild-type (compare with Fig. 1A) and hb^- (Gaul and Jäckle, 1989). The *Kr* protein can be unambiguously detected in a band of 16 to 18 nuclei in ventral regions of wild-type embryos. The protein is found in additional anterior nuclei in both hb^- and hb^-, gt^- double mutants. The *Kr* protein is expanded an additional 3 % to 4 % egg length in double mutants as compared with hb^-. (B) bcd^- embryo. The *Kr* pattern is greatly expanded into anterior regions (Gaul and Jäckle, 1989). The extent of expansion is similar to that observed in hb^-, gt^- double mutants.

pattern in hb^- embryos (Jäckle *et al.* 1986; Harding and Levine, 1988). As indicated above, *gt* mutants have little or no effect on the *Kr* pattern prior to cellularization. In hb^-, gt^- double mutants there is a dramatic anterior expansion of the *Kr* pattern in precellular embryos, which is significantly greater than the broadening observed in hb^- or gt^- single mutants (Fig. 8A).

The expanded *Kr* pattern in hb^-, gt^- double mutants is similar to that observed in bcd^- embryos (Fig. 8B, compare with A). This expansion of *Kr* observed in *bcd* mutants prompted the proposal that high concentrations of the *bcd* morphogen repress *Kr*, thereby restricting it to central regions (Gaul and Jäckle, 1987*b*; Hülskamp *et al.* 1990). However, our results suggest that *bcd* may not directly repress *Kr*, but instead might exert a negative effect on *Kr* expression *via* the *hb* and *gt* repressors. The anterior *hb* and *gt* patterns are activated by *bcd*, and both are absent in bcd^- embryos (Kraut and Levine, 1991), resulting in a situation similar to that seen in hb^-, gt^- double mutants.

Discussion

We have shown that the complementary patterns of *gt* and *Kr* expression depend on mutually repressive gene interactions. The demonstration that *gt* selectively represses *Kr* provides strong evidence that *gt* is a *bona fide* gap gene which participates with *hb*, *Kr*, and *kni* to initiate striped patterns of gene expression in the early embryo. Previous studies on the regulation of gap gene expression have focused primarily on interactions between gap genes expressed in neighboring domains (Jäckle *et al.* 1986; Pankratz *et al.* 1989). An important implication of the current study is that the strongest regulatory interactions occur among gap genes expressed in nonadjacent domains. In addition to interactions between *Kr* and *gt*, we have shown that *hb* strongly represses the expression of *kni* and *gt*. Finally, studies on double mutants suggest that *gt* functions cooperatively with *hb* to restrict *Kr* expression to central regions of the early embryo.

gt *is a bona fide gap gene*

There has been considerable debate regarding the role of *gt* in the segmentation process. Hesitation to include it among the ranks of the gap genes stems from the comparatively mild cuticular disruptions seen in gt^- mutants (Wieschaus *et al.* 1984*a*; Petschek *et al.* 1987; Mohler *et al.* 1989). The head defects and partial fusions of abdominal segments A5–A7 observed for *gt* are not nearly as dramatic as the altered segmentation patterns seen in hb^-, Kr^-, or kni^- embryos (Nüsslein-Volhard and Wieschaus, 1980; Wieschaus *et al.* 1984*b*; Lehmann and Nüsslein-Volhard, 1987; Nauber *et al.* 1988). In the case of *Kr*, there is a deletion of the entire thorax and first five abdominal segments along with a mirror-image duplication of A6 (Wieschaus *et al.* 1984*b*). Furthermore, *gt* mutants exert a relatively weak effect on the initiation of pair-rule stripes (Carroll and Scott, 1986;

Frasch and Levine, 1987; K. Howard, personal communication). *hb*, *Kr* and *kni* mutations result in severe disruptions in the initiation of the 7–stripe *eve* pattern, with complete fusions or deletions of at least three of the stripes. In contrast, gt^- embryos show partial fusions of *eve* stripes 1 and 2, as well as 5 and 6 (Frasch and Levine, 1987).

The demonstration that ectopic expression of *gt* causes the rapid and specific repression of *Kr* provides strong evidence that *gt* is a *bona fide* gap gene, which participates with *hb*, *Kr*, and *kni* to generate pair-rule and homeotic stripes. The hsp70 promoter has been used quite extensively to drive ectopic expression of a variety of segmentation genes, including the gap gene *hb* (Struhl, 1989), the pair-rule genes *ftz* (Struhl, 1985), *eve* (G. Struhl and M. Levine, unpublished results) and *hairy* (Ish-Horowicz and Pinchin, 1987) and the segment polarity genes *engrailed* (Poole and Kornberg, 1988) and *wingless* (P. Lawrence, personal communication). In no case does the misexpression of a member of one segmentation gene class lead to a mutant phenotype characteristic of another class. For example, misexpression of *hb* causes a gap mutant phenotype, while misexpression of *ftz* or *hairy* cause pair-rule phenotypes. Thus, an important criterion for the classification of *gt* as a gap gene is that it influences the expression of other gap genes. This is fulfilled by the demonstration that ectopic expression of *gt* leads to a Kr^- phenotype.

The comparatively mild *gt* phenotype may be due to the existence of one or more '*gt*-like' genes that partially duplicate *gt* function. This possibility is suggested by the nature of the *gt* protein. It contains a b type leucine zipper (E. Eldon and V. Pirrotta, personal communication) that mediates a sequence-specific DNA binding activity (T. Hoey and R. Kraut, unpublished results). Leucine zipper proteins have been shown to bind DNA as obligate dimers (reviewed by Vinson *et al.* 1989). In many instances such proteins function as heterodimers, as has been shown in detail for the mammalian proto-oncogenes *fos* and *jun* (Nakabeppu and Nathans, 1989; Gentz *et al.* 1989; Turner and Tjian, 1989). Perhaps a second leucine zipper protein active in the head partly duplicates *gt* activity. The removal of both *gt* and this putative *gt*-like gene might be expected to cause a far more severe phenotype than that observed in gt^- mutants. Support for the existence of a second *giant*-like gene stems from recent aneuploidy studies, which have permitted the systematic search for novel gap gene activities (Vavra and Carroll, 1989). These studies revealed a potentially novel gap gene on the left arm of the second chromosome that is similar to *gt* with respect to the regulatory effect it exerts on the initiation of the 7-stripe *eve* pattern.

Mechanism of the Kr–gt *interaction*

It is quite possible that regulatory interactions between *gt* and *Kr* are direct and occur at the level of transcription. Both the *gt* and *Kr* proteins possess sequence-specific DNA binding activities (T. Hoey and R. Kraut, unpublished results; Stanojević *et al.* 1989; Treisman and Desplans, 1989). As mentioned above, *gt* encodes a leucine zipper protein, while *Kr* contains multiple copies of the zinc finger motif (Rosenberg *et al.* 1986). In Kr^- embryos the two *gt* domains expand towards central regions of the embryo, which normally contain high levels of the *Kr* repressor. The most straight-forward interpretation of this result is that the *Kr* protein binds to specific sites within the *gt* promoter to repress its expression in central regions of wild-type embryos. Consistent with this possibility is the recent demonstration that the *Kr* protein functions as a transcriptional repressor in transient cotransfection assays (Licht *et al.* 1990).

A potential problem with this model of direct repression is the finding that the expansion of the *gt* pattern in Kr^- is relatively mild (see Fig. 1B) and does not include central regions containing peak levels of the *Kr* protein in wild-type embryos. Why is there not a more dramatic expansion in the limits of *gt* expression in Kr^- embryos? This could be explained by spatial constraints on the distribution of the *gt* activators. The accompanying report by Kraut and Levine (1991) suggests that the maternal morphogen *bcd* activates *gt* expression in anterior regions. The *bcd* protein is distributed in a broad gradient along the anterior–posterior axis, with peak levels at the anterior pole (Driever and Nüsslein-Volhard, 1988). This gradient is not altered in Kr^- embryos, and the limited posterior expansion of the *gt* pattern could reflect the need for a certain minimal threshold level of *bcd* protein to trigger *gt* expression. In central regions, there might not be a sufficient level of *bcd* to activate *gt* even in the absence of the *Kr* repressor. Similar arguments suggest that the posterior *gt* pattern depends on a localized activator emanating from the posterior pole, but its identity is currently unknown.

Redundant repressors of Kr *expression*

The restriction of *Kr* products to central regions of the early embryo involves multiple tiers of repression. One set of repressors emanate from the anterior and posterior poles, under the control of maternal *tor* activity (Gaul and Jäckle, 1987*b*). Dominant gain-of-function *tor* mutations abolish the central *Kr* pattern, and severely disrupt segmentation (Klingler, 1989). This effect is probably due to ectopic activation of *tll* throughout the embryo, since the removal of *tll* in a *tor* gain-of-function mutant background (i.e. $spliced^-$,tll^- double mutants) rescues segmentation (Klingler, 1989; Strecker *et al.* 1989). While not directly tested, it is likely that the central *Kr* expression pattern is fully restored in such double mutants.

An implication of the current study is that *Kr* is subject to a second level of repression which is completely independent of *tor*. The second set of repressors are controlled by the maternal factors *bcd* and *nanos*. *bcd* activates *gt* and *hb* in anterior regions of the embryo (Driever *et al.* 1989; Struhl *et al.* 1989), where they help define the anterior margin of the central *Kr* pattern. *nanos* modulates the activity of maternal *hb* (Hülskamp *et al.* 1989; Irish *et al.* 1989*a*;

Struhl, 1989), which participates in the localization of *kni* and *gt* expression within the presumptive abdomen (Hülskamp *et al.* 1990; Kraut and Levine, accompanying report). *kni* and *gt* set the posterior limit of the central *Kr* pattern (see summary of gap patterns in Fig. 7). *Kr* expression is not significantly expanded in tor^- embryos due to the independent action of the *bcd*, *nanos* system (Jäckle *et al.* 1988). In bcd^- or nos^- embryos there are only partial expansions of *Kr* towards the poles since *tor* still functions to activate the *tll* repressor (Gaul and Jäckle, 1987*b*; Lehmann and Frohnhöfer, 1989). Redundant repressors are likely to ensure the accurate and reproducible localization of *Kr* to central regions of the embryo.

Cooperative action of Kr *repressors*

It is possible that *gt* functions in a cooperative manner with *hb* and *kni* to repress *Kr* expression. For example, the dramatic anterior expansion of the *Kr* pattern seen in hb^-, gt^- double mutants (Fig. 8A) raises the possibility that optimal repression of *Kr* involves *hb–gt* protein-protein interactions. Consistent with this possibility is the observation that even the most severely affected hsp70–*gt* transformants retain a remnant of the *Kr* pattern, which corresponds to the peak of the normal *Kr* domain in parasegment (PS6) (Stanojević *et al.* 1989) (see Fig. 4B). Previous immunolocalization studies have shown that the anterior *hb* domain extends through PS5, but does not include PS6 (Tautz, 1988; Stanojević *et al.* 1989; see summary in Fig. 7). Thus, it would appear that ectopic *gt* proteins repress *Kr* only in those nuclei that also contain at least low levels of *hb*. Similarly, the *kni* expression pattern extends anteriorly through PS7 but does not include PS6 (K. Howard, and own observations), suggesting that *gt* might act in concert with *kni* to repress the posterior portion of the central *Kr* domain and leave PS6 expression intact. This idea is supported by the dramatic posterior expansion of *Kr* that we observe in hsp70–*hb* embryos, concomitant with the loss of both *gt* and *kni* (see Fig. 5).

Such a combinatorial mechanism for *Kr* repression suggests that the bell-shaped distribution profile of the *Kr* protein (see Fig. 7) is a direct consequence of the action of the maternal organizing centers. For example, *hb* and *gt* appear to be activated by different thresholds of the *bcd* gradient (Kraut and Levine, 1991). Since *hb* is triggered by a lower threshold than *gt*, the *hb* pattern extends at least 8 to 10 cells posterior to the limit of *gt*. Since *hb* is only a weak repressor it reduces, but does not abolish, *Kr* expression in those cells containing little or no *gt*. A precipitous reduction in *Kr* occurs in cells containing at least low levels of both repressors, culminating with a sharp border formed by cells containing high levels of both proteins.

Strong interactions among nonadajcent genes

The specification of cell fate in the early embryo appears to depend on both the exact concentrations and combinations of gap proteins (Gaul and Jäckle, 1989; Lehmann and Frohnhöfer, 1989; Stanojević *et al.* 1989; Warrior and Levine, 1990). Here we have presented evidence that strong regulatory interactions between gap genes expressed in nonadjacent domains are critical for establishing the precise patterns of their expression. The original studies on cross-regulatory interactions involved *hb* and *Kr* as well as *Kr* and *kni* (Jäckle *et al.* 1986), which are expressed in adjacent domains (see Fig. 7 summary). These interactions are relatively weak in that only partial expansions of the *Kr* pattern are observed in either hb^- or kni^- embryos and misexpression of *hb* does not reduce *Kr* levels. In contrast, interactions between *hb* & *kni* and *gt* & *Kr*, which are expressed in alternating domains, are quite strong. Ectopic expression of *hb* in early embryos causes a failure to initiate *kni* and *gt* expression, but has virtually no effect on the neighboring *Kr* pattern. Similarly, misexpression of *gt* results in the strong repression of *Kr*, but has no discernible effects on the neighboring *hb* and *kni* patterns. These results suggest that the limits of gap gene expression depend most critically on regulatory interactions between genes expressed in nonadjacent domains. Weak interactions between neighboring gap genes coupled with strong interactions between nonadjacent genes could account for the establishment of precisely balanced, overlapping gradients of gap gene expression.

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