Two distinct mechanisms for differential positioning of gene expression borders involving the Drosophila gap protein giant

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SUMMARY

We have combined genetic experiments and a targeted misexpression approach to examine the role of the gap gene giant (gt) in patterning anterior regions of the Drosophila embryo. Our results suggest that gt functions in the repression of three target genes, the gap genes Krüppel (Kr) and hunchback (hb), and the pair-rule gene even-skipped (eve). The anterior border of Kr, which lies 4-5 nucleus diameters posterior to nuclei that express gt mRNA, is set by a threshold repression mechanism involving very low levels of gt protein. In contrast, gt activity is required, but not sufficient for formation of the anterior border of eve stripe 2, which lies adjacent to nuclei that express gt mRNA. We propose that gt’s role in forming this border is to potentiate repressive interaction(s) mediated by other factor(s) that are also localized to anterior regions of the early embryo. Finally, gt is required for repression of zygotic hb expression in more anterior regions of the embryo. The differential responses of these target genes to gt repression are critical for the correct positioning and maintenance of segmentation stripes, and normal anterior development.

Key words: Drosophila melanogaster, Segmentation, Patterning, Repression, giant

INTRODUCTION

Gradients of maternal factors are important for the earliest patterning events in Drosophila embryogenesis (reviewed in St. Johnston and Nüsslein-Volhard, 1992). Along the anterior posterior axis, transcription factors encoded by bicoid (bcd) and the maternal component of hunchback (hbMAT) are distributed as gradients with high levels in anterior regions (Driever and Nüsslein-Volhard, 1988a; Tautz, 1988). These gradients initiate a hierarchy of genetic interactions that will eventually subdivide the embryo into fourteen segmental primordia by specifying positions of expression of target genes (reviewed by Pankratz and Jäckle, 1993; Small, 1997). For example, bcd has been shown to be a concentration-dependent activator of zygotic hb (hbZYG) expression in the anterior half of the embryo (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989, but see also Simpson-Brose et al., 1994). It has also been shown that the hbMAT gradient sets specific boundaries of target gene expression in posterior regions (Hülskamp et al., 1990; Struhl et al., 1992). In both cases, positions of gene expression boundaries are thought to be set by thresholds of protein concentration. Nuclei at specific positions within the gradients receive different concentrations of these factors, and respond by activating and repressing specific sets of genes.

Among the first targets of the maternal gradients are the gap genes, which are expressed in patterns of one or two broad bands at specific positions along the anterior posterior axis. The exact positioning of each gap gene expression domain involves initial cues from the maternal gradients as well as patterning events mediated by the gap genes themselves (Gaul and Jäckle, 1990; Kraut and Levine, 1991b). Since the gap genes also encode transcription factors, these events include autoregulation and repressive interactions between neighboring gap expression domains that refine borders of gene expression. The mature gap expression domains overlap at their edges, creating a situation where nuclei at each position along the axis contain unique concentrations and combinations of gap proteins (Fig. 1A). This system of overlapping gap and maternal proteins directs the expression of the pair-rule genes, each of which is expressed in a pattern of seven evenly spaced stripes that encircle the embryo (Hafen et al., 1984; Macdonald et al., 1985; Frasch et al., 1987). Despite intensive study, the exact mechanisms involved in pair-rule patterning are not well understood.

One possibility is that each gap gene expression domain acts as a source for a diffusion gradient that sets the position of target gene expression borders based on protein concentration. An example is the gap gene giant (gt), which is expressed in two broad domains located in anterior and posterior regions of the embryo respectively (Mohler et al., 1989; Fig. 1). Previous studies suggest that gt activity is required at two levels in the segmentation hierarchy, the gap level and the pair-rule level. At the gap level, the best candidate for a target of gt repression is Krüppel (Kr), which is expressed in a broad domain in the center of the embryo (Knipple et al., 1985). Heat shock misexpression experiments indicate that gt can act as a potent repressor of Kr (Kraut and Levine, 1991b; Eldon and Pirrotta, 1991), and several
high affinity gt-binding sites exist in the Kr regulatory region, suggesting that this repression may be direct (Capovilla et al., 1992). However, it is not clear whether gt repression of Kr is important for normal development. The anterior Kr border lies 3-4 nucleus diameters posterior to nuclei that express gt mRNA (Fig. 1A), and when Kr expression was examined in embryos lacking gt, no significant change was detected in the position of this border (Kraut and Levine, 1991b; Eldon and Pirrotta, 1991).

At the pair-rule level, a series of genetic and cotransfection experiments suggests that gt acts as a repressor to form the anterior border of stripe #2 of the even-skipped (eve) gene (Small et al., 1991, 1992; Stanojevic et al., 1991). This border lies adjacent to nuclei that express gt mRNA, and is therefore formed in a region that contains relatively high levels of gt protein. Three low affinity gt-binding sites have been mapped to a 480 bp eve enhancer sufficient for directing reporter gene expression at the stripe 2 position, suggesting that this interaction may be direct (Small et al., 1991; Capovilla et al., 1992). However, it is not clear whether gt activity is sufficient for repression of stripe 2 activation in vivo.

In this paper we use two complementary approaches to test the roles of gt in anterior patterning. The first involves a targeted misexpression approach to change the profile and shape of the gradient of gt protein emanating from the anterior domain. In the second, we reexamine gap and pair-rule expression patterns in gt mutant embryos. We find that gt activity diffusing from the anterior domain is critical for restricting Kr expression to central regions of the embryo during normal development, and that very low gt levels are sufficient for Kr repression. In contrast, the eve stripe 2 response is much less sensitive to gt repression compared with Kr, suggesting that the formation of the anterior border of this stripe involves gt and other unidentified factor(s). Thus, gt seems to be involved in two different repression mechanisms that set the anterior borders of eve stripe 2 and Kr respectively. Finally, we present evidence that gt-mediated repression is also transiently required for the normal refinement of the zygotic hb expression pattern. The implications of these results are discussed in light of current models for gap gene function.

MATERIALS AND METHODS

Targeted mis-expression of gt at the position of eve stripe 2

Previous experiments have identified a 480 bp enhancer that is sufficient for reporter gene expression at the position of eve stripe 2 (Small et al., 1992). This enhancer is activated by bcd and hb, and the anterior and posterior borders are formed by repression involving gt and Kr respectively (Stanojevic et al., 1991; Small et al., 1992). The st2-gt transgene used here contained a version of the 480 bp eve stripe 2 enhancer that was modified in two ways. First, since gt has been shown to be a repressor of this enhancer (Stanojevic et al., 1991; Small et al., 1991, 1992), all gt-binding sites in this sequence were deleted to avoid feedback repression. Second, the three low affinity bcd-binding sites (bcd-3, 4, and 5; Small et al., 1991) in the enhancer were changed to the consensus (GGGATTAGG/A) derived by Driever and coworkers (1989). Previous experiments showed that these changes increase the levels of expression driven by the enhancer (Arnori et al., 1996). Two copies of the modified enhancer (separated by 22 bp of polylinker sequence) were fused upstream of an eve fragment that contains the basal promoter and 80 bp of leader sequence to make a heterologous promoter fragment. Two versions of the gt cDNA were tested in these experiments. The wild-type version extends from an artificial Ndel site at the initiating codon (Small et al., 1991) to an EcoRI site located approx. 400 bp 3’ of the termination codon. The other version is identical to wild type except for the addition of the FLU epitope (YPYDVPDYA, Han and Manley, 1993) at the carboxy terminus of the coding sequence. This epitope was added by site-directed mutagenesis using the Mutagene kit (Bio-Rad Labs, Richmond, CA), and enabled us to distinguish ectopic gt expression from endogenous. The constructs also contained the eve 3’ UTR (Kosman and Small, 1997). To avoid problems with potential dominant embryonic lethality in making transgenic stocks, a transcriptional stop sequence flanked by two recombination targets for yeast FLPase was inserted between the heterologous promoter fragment and the gt coding sequence (Struhl et al., 1993).

Thirteen independent transgenic lines carrying st2-gt constructs (eleven with the FLU tag, and two without) were generated for these studies using standard microinjection techniques (Spradling, 1986). To activate misexpression, we generated males that contained both a st2-gt and a β2-tubulin-FLP transgene that is active during spermatogenesis (Struhl et al., 1993). These males were mated with w1118 females, and embryos were collected from these crosses for analysis of expression patterns. Ectopic expression was monitored by in situ hybridization using an antisense gt probe (Fig. 1), or by immunocytochemistry (data not shown) using an anti-FLU antibody (Berkeley Antibody Company, Berkeley, CA). Some variations in the level of ectopic gt were detected among these lines, probably due to different positions of genomic insertion. There were no qualitative differences between lines with respect to their effects on the expression profiles of other genes, and no discernible difference between lines carrying the FLU-tagged constructs and those with the wild-type gt sequence. Most of the data shown here was generated using a line (st2-gt5) that drives intermediate levels of misexpression when compared to other lines. Data from a second line (st2-gt7) that drives higher levels of misexpression are included in some cases. All embryos in this paper are oriented so that anterior is to the left and dorsal is on top.

In situ hybridization and cuticle analysis

Whole-mount in situ hybridization using anti-sense RNA probes was performed according to the method described by Kosman and Small (1997). Larval cuticles were prepared as follows (after Wiesnau and Nüsslein-Volhard, 1986). Embryos were collected for a 2-hour period, and aged for 20 hours before dechorionation. The vitelline membrane was removed by shaking in methanol/heptane (2:1 by volume). The embryos were fixed in a 1:4 ratio (V:V) of glycerol:acetic acid at 65°C overnight, mounted in a 1:1 ratio of Hoyer’s medium:lactic acid and baked in a 65°C oven overnight. The cuticle structures were analyzed and photographed using a 20× objective and phase contrast optics on a Zeiss Axioscope.

RESULTS

Extending the anterior gt domain using targeted misexpression

In wild-type precellular embryos, gt mRNA is initially transcribed in a broad anterior band in response to the bcd morphogen gradient (Mohler et al., 1989; Eldon and Pirrotta, 1991; Kraut and Levine, 1991a; Fig. 1B). This band later resolves into two stripes, and a third stripe is activated very near the anterior tip of the embryo (Fig. 1C). Previous studies suggest that a gradient of gt protein diffusing posteriorly from nuclei expressing gt mRNA may be important for repressive interactions that set boundaries of expression of other segmentation genes. To change the diffusion profile of this gradient, we used a transgenic approach and the yeast FLP-FRT system to misexpress gt under the control of a modified version of the 480 bp eve stripe 2 enhancer (st2-gt; see Materials and Methods).

Whole mount in situ hybridization experiments were
performed to detect ectopic expression driven by the st2-gt transgene. Ectopic expression appeared as a wide band which overlapped most of the endogenous anterior gt domain, and extended 4-5 nucleus diameters toward the posterior (Fig. 1D,E). This expression persisted until the start of gastrulation. To test whether gt functions as a gradient of repressive activity in the region posterior to its normal expression domain, we analyzed the expression patterns of potential target genes in embryos containing the st2-gt transgene, and then compared them to patterns in wild-type and gt mutant embryos.

**gt repression is required for restricting Kr expression to central regions of the embryo**

The gap gene *Kr* is activated in a broad central region of precellular embryos (Knipple et al., 1985). Midway through cleavage cycle 14, this domain extends from 41-59% egg length. The initial positioning of the anterior border of this domain is thought to be controlled by repression involving a combination of *hb*<sup>MAT</sup> and *hb*<sup>ZYG</sup> (Hülskamp et al., 1990; Struhl et al., 1992; Schulz and Tautz, 1994). To test whether gt is also involved in setting or maintaining this border, we analyzed the *Kr* expression pattern in embryos containing the st2-gt transgene. These embryos showed no changes in the initial positioning of the *Kr* expression domain early in cleavage cycle 14, but slightly later there was a dramatic retraction of the anterior *Kr* border (Fig. 2C,D). The delay in the observed repressive effect on the *Kr* anterior border is probably due to the fact that the *Kr* domain is expressed earlier than the st2-gt transgene. Higher levels of ectopic gt resulted in a more severe retraction, suggesting that *Kr* transcription is very sensitive to repression by gt (compare Fig. 2D with 2C).

To test whether gt affects *Kr* expression during normal development, we examined *Kr* expression in embryos that carry a strong hypomorphic *gt* allele (*gt<sup>YAN2</sup>). In previous studies, no significant changes in *Kr* expression were detected in *gt* mutants (Kraut and Levine, 1991b; Eldon and Pirrotta, 1991). Consistent with these reports, we found that the initial *Kr* expression pattern was correctly established in *gt<sup>YAN2</sup>* embryos (data not shown). However, slightly later, we did detect a significant anterior expansion (from 59% to 65% egg length; Fig. 2B), suggesting that gt-mediated repression is essential for maintaining the position of the anterior border of the *Kr* domain.

**The role of gt in setting the position of eve stripe 2**

The anterior *gt* mRNA expression domain abuts the anterior border of *eve* stripe 2 in wild-type embryos. Several previous studies suggest that *gt* may act in vivo to set this border by repression. For example, there is a transient derepression in the interstripe region between *eve* stripes 1 and 2 in *gt* mutant embryos (Frasch and Levine, 1987), which suggests an anterior expansion of the stripe 2 response. Other studies suggest that *gt* acts as a direct repressor of stripe 2. For example, three *gt*-binding sites have been identified in the stripe 2 enhancer (Small et al., 1991), and deletions that remove these sites cause an anterior expansion of *lacZ* reporter gene expression (Stanojevic et al., 1991; Small et al., 1992). However, it is not clear whether *gt* is sufficient for repression of the in vivo *eve* stripe 2 response.

To test this, we examined *eve* expression in embryos containing the st2-gt transgene, which extends the *gt* domain so that it overlaps the position of *eve* stripe 2. Surprisingly, the ectopic *gt* caused only a weak transient reduction of the stripe early in cycle 14 (Fig. 3C). Later the stripe recovered to full strength, but expanded toward the posterior by about two nucleus diameters (Fig. 3F). Double in situ hybridization experiments

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**Fig. 1.** (A) Schematic representation of the overlapping gap gene expression patterns in blastoderm stage embryos. Note that *eve* stripe 2 occupies a position between the anterior *gt* domain and the central *Kr* domain. (B-E) *gt* mRNA expression patterns in wild-type embryos (B,C) and embryos that contain one copy of the st2-gt5 transgene (D,E). Staining patterns are shown early in cleavage cycle 14 (B,D) and midway through the cycle (C,E). In wild-type embryos, *gt* is expressed in broad bands located in anterior and posterior regions (B). The anterior band then resolves into two stripes, and a more anterior stripe is also activated (C). The position and extent of the ectopic *gt* domain driven by the st2-gt transgene is shown as a blue bar above D. This transgene directs a broad band of expression that overlaps the anterior *gt* domain at both stages, and extends four to five cells toward the posterior.

**Fig. 2.** The gap gene *Kr* is sensitive to changes in the anterior *gt* expression domain. (A,B) *Kr* mRNA expression is shown in wild-type (A) and *gt<sup>YAN2</sup>* (B) embryos midway through cleavage cycle 14. Note that the *Kr* expression domain expands anteriorly in *gt* mutants at this time. (C,D) *gt* and *Kr* mRNA expression in embryos from two different lines carrying the st2-gt transgene. This transgene extends the expression domain of *gt* toward the posterior, resulting in a retraction of the anterior border of *Kr*. Higher levels of ectopic *gt* cause a more severe retraction (compare C with D).
showed that the timing and the extent of the expansion correlated well with the retraction of the 
Kr domain (Fig. 3I), suggesting that the expansion of 
eveto stripe 2 is indirectly caused by relief 
from Kr repression. Doubling the ectopic gt expression levels still 
neither reported constructs (Fig. 4E,F; data not 
shown), suggesting a posterior 
expansion of the 
eveto stripe 2 caused by the st2-gt transgene is mediated through the early 
acting enhancer.

The recalciitrance of the 
eveto stripe 2 response to ectopic gt 
expression led us to reexamine the expression pattern in 
gt mutant embryos. Early in cycle 14, these mutants showed a 
derepression in the interstripe region between stripes 1 and 2 
(Fig. 3B), consistent with previous reports (Frasch and Levine, 
1987). However, later in cycle 14, gt mutants showed a dramatic 
reduction in stripe 2 expression levels (Fig. 3E), suggesting a 
role for gt in maintaining the stripe. Since Kr has been 
previously implicated as the repressor that forms the stripe 2 
posterior border, it is possible that the stripe 2 reduction in 
gt mutants is indirectly caused by Kr, which expands anteriorly to 
completely overlap the diminishing stripe (Fig. 3I).

To test this possibility, we generated gt mutant embryos that 
also contained one copy of the Kr\(^{null}\) mutation (Preiss et al., 
1985). Early in cycle 14, gt; Kr\(+\) embryos showed a slight 
posterior shift of gt stripe 2 (Fig. 5C). Both the anterior and 
posterior borders of this stripe appeared fuzzy at this time, 
suggesting that activities important for setting both borders are 
compromised in these embryos. Later, stripe 2 diminished 
somewhat in intensity (Fig. 5D), but was visibly stronger than 
the comparable stripe in gt mutants (Fig. 5B). Therefore, the 
repression of 
eveto stripe 2 observed in gt mutants can be relieved 
by reducing Kr levels. These results suggest that a major 
function of the anterior gt domain is to prevent Kr from 
expanding anteriorly, thus permitting the expression of 
eveto stripe 2. Furthermore, since gt repression maintains the position 
of the anterior Kr border in wild-type embryos, it indirectly 
defines the position of the posterior border of 
eveto stripe 2.

![Fig. 3. gt has both repression and maintenance functions in 
eveto stripe 2 regulation. 
eveto (blue and 
black) and Kr (red) mRNA expression patterns are shown for wild-type (A,D,G), 
gt\(^{DAS}\) (B,E,H) and st2-gt (C,F,I) embryos early in cycle 14 (A-C) or midway through the cycle (D-I). 
gt mutants can be unambiguously identified by the fusion of 
eveto stripes 5 and 6 in posterior 
regions. In gt mutant embryos, there is a transient fusion of 
eveto stripes 1 and 2 early in cycle 14 
(B), but later, stripe 2 is nearly abolished (E). A double in situ hybridisation experiment shows that 
the expanded Kr expression domain completely overlaps the weak 
eveto stripe 2 in these 
embryos (H). Embryos containing the st2-gt5 transgene show a very weak repression of 
eveto stripe 2 early in cycle 14 (C), but later the stripe recovers, expanding 1-2 cells toward the 
posterior (F). A retraction of the Kr expression domain coincides with the expanding stripe 2 (I).](image-url)
A gt concentration gradient is not required for setting the anterior border of eve stripe 2

In principle, the preceding experiments support the hypothesis that gt acts as a concentration-dependent repressor to set the anterior borders of the Kr and eve stripe 2 expression domains in different positions. Ectopic gt is an effective repressor of Kr, but has little effect on the activation of eve stripe 2. In situ hybridization experiments indicate that endogenous gt levels are significantly higher than the ectopic gt driven by even the strongest st2-gt transgenic lines (Figs 1, 2; data not shown). Perhaps these higher endogenous levels are required for effectively setting the anterior border of eve stripe 2. If this is the case, the early expansion of eve stripe 2 toward stripe 1 detected in gt mutants (Fig. 6B) should not be affected in embryos in which the endogenous gt gene is replaced by the st2-gt transgene. To test this, we examined eve expression in gt mutants that contained the st2-gt5 transgene. Surprisingly, a sharp anterior eve stripe 2 border was formed in these embryos, with a clear interstripe between eve stripes 1 and 2 (Fig. 6C). Furthermore, the st2-gt domain rescued eve stripe 2 to full strength with a posterior expansion which is probably due to repression of the anterior Kr border.

As noted above, the relatively low levels of ectopic gt driven by the st2-gt construct overlap the endogenous gt domain and extend 4-5 nucleus diameters posteriorly. The fact that a sharp anterior eve stripe 2 border is formed in embryos containing only this domain argues against a simple concentration-dependent mechanism for setting this border. Rather, we propose that other factor(s) are involved along with gt in defining the anterior border of eve stripe 2 in vivo (see Discussion).
Fig. 6. The loss of eve stripe 2 expression and the anterior cuticle defects in gt mutant embryos can be rescued by the st2-gt transgene. eve mRNA expression patterns (A-C), and corresponding larval cuticles (D-F) are shown for wild-type embryos (A,D), gt mutants (B,E), and gt mutants carrying one copy of the st2-gt5 transgene (C,F). The cuticles of gt mutants can be unambiguously identified by the loss of ventral denticle bands in abdominal segments A5-A8. The position of the A5 denticle band is marked with an asterisk.

Ectopic expression driven by the st2-gt transgene rescues the loss of eve stripe 2 expression detected in gt mutants and shows a posterior expansion of the stripe (compare C with B). The larval cuticles of strong gt mutant embryos exhibit a typical ‘buttonhead’ phenotype and the loss of A5-A8 dentical bands (E). The st2-gt domain can rescue the anterior defects found in gt mutants (F). Some of these rescued cuticles are indistinguishable from wild type (D).

analyzed for rescue of the cuticular defects. More than 70% of the gt mutants carrying a single copy of the activated st2-gt5 transgene showed some phenotypic rescue of the anterior cuticular defects, while posterior defects were unaffected (Table 1). Of these, more than 40% showed a cuticular head morphology that was indistinguishable from wild-type (Fig. 6F).

A role for gt in regulating zygotic hb expression

Zygotic expression of the gap gene hb is initially activated by the bcd and hbMAT gradients in a broad domain that spans the anterior half of the embryo (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989). This expression is then rapidly refined during nuclear division cycle 14, leaving a secondary pattern that includes a variable head domain, a stripe at the position of parasegment 4 (PS4), and a posterior stripe (Hülskamp et al., 1994; Margolis et al., 1995; Fig. 7A). The PS4 stripe overlapped the anterior border of the Kr domain (Fig. 7E). By examining hb expression in gt mutants, we detected significant changes in the anterior expansion of the Kr domain in gt mutants (Fig. 7F). To test this possibility, we examined hb expression in gt mutants that also contained reduced Kr activity. In both gt;Kr/+ (Fig. 7C) and gt;Kr/Kr (Fig. 7D) embryos, there was a partial recovery of the stripe as well as a posterior shift. This is consistent with the hypothesis that Kr acts as a repressor to form the posterior border of this stripe (Hülskamp et al., 1994). However, the expression levels of the shifted stripe were significantly lower than the wild-type stripe. The cause of this reduction is unknown, but a similar reduction was also detected in Kr single mutants (data not shown), suggesting that Kr activity is required for wild-type expression levels of the hb PS4 stripe.

We also noted that high levels of hb expression persisted in more anterior regions of gt mutant embryos. The persistent hb expression domain appeared very similar in shape to the normal gt domain (compare to Fig. 1B), suggesting that gt may act as a repressor to clear hb expression from this part of the embryo during wild-type development. To test whether endogenous gt levels were required for this repression, we examined hb expression in gt mutants that also contained the st2-gt transgene. hb expression was repressed normally by a single copy of the st2-gt5 transgene (data not shown), suggesting that relatively low levels of ectopic gt can replace this function of the endogenous gene.

Since gt seems to be involved in repression of hb in anterior regions, it is possible that this repression is important for setting the anterior border of the hb PS4 stripe during wild-type development. To test this, we examined hb expression in embryos containing the st2-gt transgene (Fig. 7G,H). The position of the anterior border of the hb PS4 stripe appeared unchanged in these embryos, suggesting that the levels of ectopic gt tested here are not sufficient to repress hb PS4 expression. However, a slight posterior expansion of this stripe could be detected in embryos with high levels of misexpression (Fig. 7H), which is probably caused by the retraction of the Kr domain. This supports the hypothesis that Kr activity is important for setting the posterior PS4 stripe border, and

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In this experiment, 219 gt mutant cuticles were identified by missing denticle bands in posterior regions, and examined for rescue of defects in head structures. Based on the genetic cross used to generate these embryos (see text), only half (110) should have contained the st2-gt transgene. The number of st2-gt embryos that showed no rescue* was estimated by subtracting the number of embryos showing partial or full rescue from 110. This experiment was repeated twice with similar results.
further demonstrates the importance of gt-mediated restriction of Kr expression to central regions of the embryo.

DISCUSSION

We have used a combination of genetics and a targeted misexpression approach to examine the in vivo patterning functions of the anterior domain of the gap gene gt. The results are summarized in Fig. 8. We find that gt functions as a repressor of the gap genes Kr and hb, and the pair-rule gene eve. Furthermore, we show that gt is involved in distinct mechanisms that set the borders of two expression domains. These borders are the anterior border of eve stripe 2, which lies adjacent to nuclei that produce gt mRNA, and the anterior border of Kr, which lies 4-5 nucleus diameters further posterior (Fig. 8A). Our results suggest that the anterior Kr border is set by a threshold mechanism involving very low levels of gt protein that diffuse toward the posterior. However, higher gt levels are not sufficient for setting the anterior border of eve stripe 2. Rather, a combination of gt activity and at least one other factor is required to set this border.

gt-mediated repression of Kr

Expression patterns of the gap genes are initially established by maternal morphogens, and then refined by autoregulatory mechanisms and repressive interactions between neighboring domains. In the case of Kr, previous experiments suggested that the anterior border is set by a combination of maternal and zygotic hb, with a minor role for gt. Genetic removal of zygotic hb resulted in an anterior Kr expansion (Hülskamp et al., 1990; Struhl et al., 1992), but only subtle changes were detected in embryos lacking gt function. A more severe anterior Kr expansion was detected in gt; hb double mutants (Kraut and Levine, 1991b). These experiments led to the hypothesis that hb and gt repression activities are redundant, or part of a cooperative mechanism. The results reported here confirm that gt is critical for maintaining the anterior Kr boundary. Contrary to previous reports, we detect a significant anterior expansion of the Kr border in gt mutants, and a retraction in embryos that contain the expanded gt domain directed by the st2-gt transgene. Since the highest levels of ectopic gt repress Kr to a position just beyond the posterior border of hb expression (Fig. 7H), low gt levels appear to be sufficient for this repressive

![Fig. 7](image.png)

**Fig. 7.** The gap genes gt and Kr are both involved in refining the mature hbZYG expression pattern. (A-D) hb mRNA expression patterns are shown for wild-type mid-cycle 14 embryos and several mutant combinations as indicated. The wild-type pattern at this stage includes a variable head domain, the PS4 stripe, and the posterior stripe (A). In gt mutant embryos, hb expression persists in regions anterior to the normal position of the PS4 stripe, which is nearly abolished (B). Genetically reducing Kr function results in a posterior shifting of the reduced PS4 stripe, suggesting that Kr may be important for setting the posterior border. (E-H) Double-stained mid-cycle embryos showing hb (red) and Kr (black) mRNA expression patterns in wild-type embryos (E), gt mutants (F), and embryos containing extended gt expression domains (G,H). In wild-type embryos, the hb PS4 stripe partially overlaps the anterior border of the central Kr domain (E). In gt mutants, the anterior expansion of the Kr domain overlaps with the derepressed hb in more anterior regions. Ectopic gt (G,H) causes a posterior retraction of the Kr domain, but has no effect on the anterior border of the PS4 hb stripe. However, a slight posterior expansion of the hb PS4 stripe is detected in embryos containing high levels of ectopic gt (H).

![Fig. 8](image.png)

**Fig. 8.** A summary of the changes in gene expression patterns caused by manipulations of the anterior gt expression domain. Initial expression patterns of the gap genes (A) are refined by mid cycle 14 (B). Adding ectopic gt with the st2-gt transgene (C) causes a strong retraction of the anterior Kr border, and a concomitant posterior expansion of the eve stripe 2 response and the hb PS4 stripe. In gt mutants (D), Kr expands anteriorly, resulting in the severe reduction of both eve stripe 2 and the hb PS4 stripe. In gt mutants that contain the st2-gt construct (E), the levels and positions of the PS4 hb stripe and eve stripe 2 are rescued.
activity, arguing against a cooperative mechanism involving hb. This leaves open the possibility that the repressive effects of hb and gt on Kr are redundant, but we propose that each repression activity is required for setting the border at different times in development. Our experiments suggest that the expansion in gt mutants (Fig. 2B) occurs later than that observed in hb mutants (data not shown). Thus hb activity sets the initial border, but gt activity is required later to maintain its position.

In wild-type embryos and those containing the st2-gt transgene (Fig. 2 C,D), there is an apparent space (3-4 nucleus diameters) between the posterior-most row of nuclei that express detectable levels of gt mRNA and those that express Kr. This space defines the distance that gt protein levels, sufficient for repression, can diffuse away from nuclei that produce gt mRNA. This space suggests that Kr is very sensitive to gt-mediated repression. Since gt has been shown to bind in vitro to several high-affinity sites in the Kr regulatory region (Capovilla et al., 1992), this repression is probably direct, and the high affinity sites allow Kr to respond to very low levels of gt protein.

**Multiple roles for gt in the regulation of eve stripe 2**

Previous experiments suggest that gt acts as a repressor to form the anterior border of eve stripe 2 (Frasch and Levine, 1987). A 480 bp minimal enhancer that drives lacZ reporter expression at the position of stripe 2 contains three gt-binding sites, and deleting these sites causes an anterior expansion of the lacZ stripe (Stanojevic et al., 1991; Small et al., 1992; Arnosti et al., 1996). Furthermore, cotransfecting gt into cultured Drosophila cells causes a dose-dependent repression of a CAT reporter gene containing sequences from the stripe 2 enhancer (Small et al., 1991). Here we have shown, however, that ectopic expression driven by the st2-gt transgene is not sufficient for effective repression of the endogenous stripe. One possible explanation is that the expression levels driven by the st2-gt transgene are not high enough for efficient repression. Two results argue against this. First, doubling the levels of ectopic expression does not increase the repression of the in vivo stripe. Second, the anterior border of eve stripe 2 can be set by the ectopic st2-gt expression domain in the absence of endogenous gt function. Since the ectopic domain completely overlaps the stripe from the anterior, a steep concentration gradient of gt is not required for setting the anterior border.

These results indicate that other factor(s) are required along with gt for setting the anterior border of stripe 2. At least one of these factor(s) must also be localized to anterior regions of the embryo, because the stripe 2 anterior border is correctly set in gt; st2-gt embryos. Thus, gt may act as a potentiatior of repression mediated by these localized factors. Previous experiments support the possibility that other factors are involved in setting the anterior border of eve stripe 2. For example, when a stripe 2-lacZ transgene is crossed into a gt mutant, there is a moderate anterior expansion of the stripe response (Small et al., 1992). However, deleting the gt-binding sites in the enhancer results in a more severe expansion, suggesting that these deletions also interrupt the function of another repressive activity. Since gt encodes a putative leucine zipper (b-ZIP) protein (Vinson et al., 1989), one possibility is that this activity is also a b-ZIP protein that can heterodimerize with gt as part of an effective repressor complex. Repressive function in the absence of gt would be provided by a homodimer of this protein. Alternatively, since the gt site deletions tested in previous experiments removed relatively long sequences (14-43 bp), it is possible that these deletions may have removed or interrupted binding sites for other protein(s).

Genetic studies using segmental aneuploids have localized an activity that may be involved in repression of the anterior eve stripe 2 border to the left arm of the chromosome II (Vavra and Carroll, 1989). Embryos lacking this region exhibit an anterior expansion of stripe 2 similar to that detected in gt mutants.

The later reduction of eve stripe 2 in gt mutants suggests a role in maintaining the stripe response. Several lines of evidence suggest that this maintenance occurs by restricting Kr to central regions of the embryo. First, Kr has been previously proposed as a repressor that forms the posterior border of stripe 2, and binds in vitro to several sites in the stripe 2 enhancer (Stanojevic et al., 1989). Second, the derepressed Kr domain in gt mutants overlaps virtually all of eve stripe 2 (Fig. 3H), consistent with its role as a repressor. Third, the stripe 2 reduction in gt mutants can be rescued by reducing Kr activity, or by repressing the anterior border of the Kr domain using the st2-gt transgene. Thus, gt plays several important roles in eve stripe 2 regulation. It functions in combination with other factor(s) to set the anterior border, and it indirectly controls maintenance of the stripe and the position of the posterior border by setting the anterior Kr border.

**gt represses zygotic hb in anterior regions of the embryo**

Zygotic hb is initially activated throughout the anterior half of the embryo, and then refined to a pattern that includes a variable head stripe and the PS4 stripe (Figure 7A). It has been previously shown that phosphorylation of bcd by the terminal system leads to the down-regulation of hb expression anterior to the head stripe (Ronchi et al., 1993). Other experiments suggest that the PS4 stripe is activated via autoregulation, while its posterior border is formed by high levels of Kr (Hülskamp et al., 1994). However, the mechanism for forming the anterior border of this stripe is not known. One possibility is that hb expression activated by bcd is repressed by other factors in this region, leaving a stripe at the most posterior part of the domain, which then initiates autoregulation. The anterior gt domain lies within this region; removing gt function causes a transient derepression of hb (Reinitz and Levine, 1990; Fig. 7B), suggesting that gt may be part of such a mechanism. This activity can be replaced by the st2-gt transgene, suggesting that lower levels are sufficient for this repressive activity. However, while gt may be important for repressing hb anteriorly, it is clear that gt repression is not the primary activity that sets the anterior border of the PS4 stripe.
First, the anterior border of the PS4 stripe in Kr mutant embryos does not expand in Kr; gt double mutants (Fig. 7C). Second, extending the gt domain with the st2-gt transgene has no effect on the position of the anterior PS4 stripe border. Thus, we suggest that the anterior border of this stripe is formed by other factors, but gt may play a role in repressing hb in more anterior regions.

The biological significance of this repression is evident if the pair-rule patterns and cuticular structures of gt; Kr/+ embryos are compared with gt mutant embryos that also contain the st2-gt transgene (gt; st2-gt). The pair-rule patterning defects associated with gt mutants are rescued to some degree in both genetic combinations (Figs 5C,D, 6C) but the larval cuticles resulting from these genotypes are distinctly different. As noted above, the st2-gt transgene can fully or partially rescue the anterior cuticular defects in gt mutants (Table 1; Fig. 6F). In contrast, the head cuticle defects of gt; Kr/+ larvae are identical to those observed in gt mutants (data not shown). Our results suggest that a major patterning difference between these two genotypes is the derepression of hb expression in gt; Kr/+ embryos. Perhaps the persistence of hb in this region causes some of the head defects observed in gt mutants.

**Opposing gradients of gt and Kr and anterior body patterning**

Previous studies suggested a minor role for gt in maintaining the anterior border of the Kr expression domain. The results presented here suggest that this may be one of gt’s most important functions. gt mutants exhibit a significant anterior Kr expansion that may cause the repression of the hb PS4 stripe and eve stripe 2. These stripes are positioned in adjacent parasegments, with the hb PS4 stripe overlapping the anterior border of the Kr domain (Fig. 8B). Since Kr has been implicated as a repressor of both stripes, their respective posterior borders may be set by a gradient of Kr protein where low levels are sufficient for repression of eve stripe 2, while high levels are required to repress the PS4 stripe. Future experiments will be required to test this model.

The importance of gt-mediated repression of Kr is underscored by the fact that the anterior defects of strong gt mutants can be rescued by a single copy of the st2-gt transgene, which represses Kr and restores the expression of the hb PS4 stripe and the pair-rule stripes in this region. It is worth noting that the st2-gt transgene drives low levels of expression in a broad domain that overlaps the region normally occupied by the endogenous gt domain (Fig. 8E). In contrast to the endogenous gt domain, which refines into two stripes and persists through germ band elongation, the st2-gt domain does not refine significantly, and persists only until the beginning of gastrulation. The rescue activity mediated by this transgene suggests that some flexibility is permitted in gt concentration levels and in the shape of the anterior domain. One possibility is that gt’s major roles in anterior embryonic patterning are restricted to early developmental stages before its further refinement into three stripes, or that there is some redundancy in its later functions.

**Differential repression: a mechanism for multi-level pattern integration**

Repressive interactions among the gap genes are critical for establishing their precise overlapping expression patterns in the blastoderm. Kraut and Levine (1991b) have previously proposed that strong interactions between non-adjacent gap genes, coupled with weak interactions between adjacent genes could generate the observed overlapping pattern. Since gt and Kr represent non-adjacent gap genes, the demonstration here that Kr is very sensitive to gt repression is consistent with this model. A consequence of this interaction is the creation of a space (3-4 nucleus diameters) between nuclei that express gt mRNA and those that express Kr. This is precisely the space where eve stripe 2 is activated because its enhancer is much less sensitive to gt repression compared with Kr. We have shown that the correct positioning of the anterior border of eve stripe 2 requires gt and other factors(s), but in principle, this border could have been formed by higher levels of gt alone. Perhaps combining the activities of more than one localized repressor is a more effective mechanism for establishing a sharp border of gene expression than a simple concentration threshold involving one protein.

Mechanisms similar to the one described here for eve stripe 2 may also be involved in the positioning of other pair-rule stripes. An example is eve stripe 3 (Fig. 9). Previous experiments suggest that hb, and its non-adjacent neighbor, kni, act as repressors that form the anterior and posterior borders of this stripe (Small et al., 1996; Fig.1A). However, hb is also involved in setting the anterior border of kni (Hülskamp et al., 1990; Struhl et al., 1992). Perhaps different sensitivities to hb-mediated repression are involved in setting these borders in different positions. Both the eve stripe 3+4+ enhancer (Small et al., 1996) and the kni regulatory region (Pankratz et al., 1992) contain multiple hb-binding sites, suggesting that they could respond to different levels of hb protein. Thus differential sensitivities between gap and pair-rule targets to gradients of gap proteins may be a general mechanism involved in pair-rule patterning.

A key aspect of this type of mechanism is that it integrates positional information between different levels of the segmentation hierarchy. Such multi-level integration may provide a mechanism for stabilizing relative positions of gene expression patterns. It has been previously shown that significant flexibility can be tolerated in the exact positions of segmentation patterns along the anterior posterior axis. This is most dramatically seen in embryos containing multiple copies of the maternal effect gene bcd (Driever and Nüsslein-Volhard, 1988b; Struhl et al., 1989). The extra copies of bcd cause the expression patterns of the segmentation genes to be compressed into posterior regions of the embryo. Nevertheless, many embryos containing up to six bcd genes hatch and appear wild-type, suggesting that the spatial order of expression patterns required for normal development is not disturbed. We propose that multi-level integration mechanisms such as the one described here are important for coordinating the positional shifts of individual genes in this extreme case, and for maintaining the spatial order of gene expression patterns during the normal segmentation process.

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