

## Interactions of the *Drosophila* gap gene *giant* with maternal and zygotic pattern-forming genes

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### Summary

The *Drosophila* gene *giant* (*gt*) is a segmentation gene that affects anterior head structures and abdominal segments A5–A7. Immunolocalization of the *gt* product shows that it is a nuclear protein whose expression is initially activated in an anterior and a posterior domain. Activation of the anterior domain is dependent on the maternal *bicoid* gradient while activation of the posterior domain requires maternal *nanos* gene product. Initial expression is not abolished by mutations in any of the zygotic gap genes. By cellular blastoderm, the initial pattern of expression has evolved into one posterior and three anterior stripes of expression. The evolution, position and width of these stripes are dependent on interactions between *gt* and the other gap genes. In turn, *gt* activity in these domains affects the expression of the

other gap genes. These interactions, typical of the cross-regulation previously observed among gap genes, confirm that *gt* is a member of the gap gene class whose function is necessary to establish the overall pattern of gap gene expression. After cellular blastoderm, *gt* protein continues to be expressed in the head region in parts of the maxillary and mandibular segments as well as in the labrum. Expression is never detected in the labial or thoracic segment primordia but persists in certain head structures, including the ring gland, until the end of embryonic development.

Key words: *Drosophila* segmentation/morphogenetic gradients/gap genes/head development.

### Introduction

The development of the anteroposterior pattern of the *Drosophila* embryo takes place in stages, progressing from simpler, block-like domains to units spanning double segments and then to individual segments. This strategy is revealed by the existence of zygotic genes whose localized expression is required for the differentiation of the corresponding pattern elements (Nüsslein-Volhard and Wieschaus, 1980). The first stage in the formation of the anteroposterior pattern of the embryo is controlled by the zygotic gap genes. These are expressed in broad, partially overlapping domains and, in combination, are responsible for activating the expression of the next genes in the segmentation hierarchy, the pair-rule genes. Lack of gap gene activity causes the loss of several contiguous segments from that part of the body plan that corresponds roughly to the domain of expression of the gene.

The initial domains of expression of the segmentation gap genes are specified by maternal cues laid down in the egg. The maternal cues are produced by three organizing systems responsible for the anterior, pos-

terior and terminal pattern elements, respectively (Nüsslein-Volhard *et al.* 1987). The anterior system, acting through a gradient of the *bicoid* (*bcd*) gene product, has been shown to activate at least one zygotic gap gene, *hunchback* (*hb*), in the part of the embryo in which *bcd* concentration is above a certain threshold (Driever *et al.* 1989). Genetic and molecular evidence (Hülskamp *et al.* 1989; Irish *et al.* 1989; Struhl, 1989) indicates that the posterior system, through the *nanos* (*nos*) product, acts negatively by preventing the accumulation of maternal *hb* product in a graded way from the posterior of the embryo. This activates posterior development because *hb* acts negatively on other gap genes. The terminal system, acting through the *torso* and *l(1)polehole* genes, activates the zygotic gap genes *tailless* (*tl*) and *huckebein* (*hkb*) in the terminal regions (Jürgens *et al.* 1984; Strecker *et al.* 1986; Pignoni *et al.* 1990; Weigel *et al.* 1990). By cellular blastoderm the initial domains of expression of the gap genes become sharper and their pattern is refined. Mutual interactions among the gap genes account for much of this elaboration of the pattern (Jäckle *et al.* 1986).

The *giant* (*gt*) gene is required for normal develop-

ment of the head and of the abdominal segments. Strong *gt* loss-of-function alleles or deficiencies of the gene were classified as segmentation gap mutations because they cause defects in the segmental pattern of two regions. One, in the posterior half of the larva, is a loss of contiguous abdominal segments A5, 6, 7 and sometimes 8, a type of pattern deletion characteristic of segmentation gap mutations. The other region is the head where defects have been interpreted as the loss of labral and labial structures resulting in failure to complete head involution and loss of parts of the cephalopharyngeal skeleton (Petschek *et al.* 1987; Mohler *et al.* 1989) and fusion of the labial and prothoracic segments (Petschek and Mahowald, 1990). In contrast to a classical gap mutation, lack of *gt* function causes less extensive gaps in the segmentation pattern and does not completely delete the abdominal segments that it affects but only their anterior parts, leaving a considerable expanse of naked cuticle (Petschek *et al.* 1987; Mohler *et al.* 1989; Petschek and Mahowald, 1990). Earlier studies also showed that *gt* mutations had only minor effects on the pattern of expression of the pair-rule gene *fushi tarazu* (Carroll and Scott, 1985). More recent reports show that *gt* mutations cause alterations in the expression of *even skipped* (Frasch and Levine, 1987), a drastic weakening of stripes 5 and 6 of *ftz* expression and the concomitant fusion of *engrailed* stripes 10–13 (Petschek and Mahowald, 1990). These results together with the pattern of defects, the early requirement for *gt* activity and the early onset of expression all indicate that *gt* functions very much like a gap gene. In this work we have studied the interactions of *gt* with the three maternal systems that organize the anteroposterior pattern and with the gap genes. We find that the initial expression of *gt* is dependent only on maternal factors but that *gt* affects the expression of other gap genes and is in turn influenced by them. Our results show that *gt* acts like a typical gap gene and is required for the establishment of the anteroposterior pattern of the embryo.

## Materials and methods

### Preparation of giant antibody

We inserted a 522 bp *SmaI*–*PvuII* fragment that encoded amino acids 263–436 of the cDNA open reading frame with no opa sequences (M. Capovilla, E. Eldon and V. Pirrotta, in preparation) into the pEX-1 expression vector of Stanley and Luzio (1984). High level expression of the *giant*– $\beta$ -galactosidase fusion protein was induced in NF-1 cells by growth at 42°C. Pelleted cells were lysed in 7 M guanidinium HCl and diluted with 6 volumes of Tris–EDTA, pH 8. Insoluble material was pelleted and resuspended in urea sample buffer (USB) containing 8 M urea, 100 mM Tris (pH 7.6), 2 % SDS and 5 % beta mercaptoethanol, sonicated and applied to 2 mm preparative 7.5 % polyacrylamide gels. The region of the gel containing the hybrid protein was excised, homogenized and injected into rabbits as described by Benson and Pirrotta (1987). Serum obtained from the rabbits was purified first through CM Affi-Gel Blue (BioRad), to isolate an enriched immunoglobulin fraction. This was then precipitated with ammonium sulfate, resuspended and applied to affinity

columns essentially as described by Pirrotta *et al.* (1988). The final product was greatly enriched for anti-*giant* activity and retained only very low levels of anti-beta galactosidase activity. Antibodies were used at a concentration of 1–2  $\mu\text{g ml}^{-1}$  when used with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cappel) or 0.1–0.6  $\mu\text{g ml}^{-1}$  when used with the Vectastain Elite kit (Vector Laboratories).

### Other antisera

Antisera directed against other segmentation gene products were kindly provided by other laboratories. In all cases they were preabsorbed by incubation with an excess of wild-type embryos prior to being used at the recommended dilutions. Rabbit anti-*ftz* antibodies (from H. Krause; Krause *et al.* 1988) were used at a 1/1000 dilution. Mouse anti-*hb* and rabbit anti-*Kr* antibodies (from R. Kraut and M. Levine) were used at a 1/300 and 1/100 dilution, respectively. Rabbit anti-*en* antibodies (from T. Kornberg; DiNardo *et al.* 1985) were used at a 1/400–1/1000 dilution. Rat anti-*kni* antibodies (obtained from K. Howard) were used at a 1/500–1/1000 dilution. Biotinylated secondary antibodies were obtained from Vector Laboratories (anti-mouse IgG and anti-rabbit IgG) or Jackson ImmunoResearch Laboratories (anti-rat IgG). These antibodies were also preabsorbed at a 1/10 dilution with an excess of Canton S embryos and used at a final dilution of 1/500.

### Embryo fixation and staining

Flies of the appropriate genotype were allowed to lay eggs on grape juice agar plates for specified periods of time. The embryos were aged, if necessary, at room temperature (approx. 22°C), then collected, dechorionated, fixed and devitellinized essentially as described by Mitchison and Sedat (1983) as modified by Karr and Alberts (1986) and DiNardo and O'Farrell (1987). Embryos to be stained with peroxidase-conjugated antibodies were pretreated with 0.3 %  $\text{H}_2\text{O}_2$  prior to rehydration in phosphate buffered saline (PBS, pH 7.4) containing 0.1 % Tween-20 and 0.1 % bovine serum albumin (BBT). Blocking was done in BBT containing 2 % serum (BBS). Primary antibody incubations were carried out in BBS at 4°C overnight. The embryos were washed extensively in BBT, reblocked in BBS and incubated with the appropriate preabsorbed secondary antibody for two hours at room temperature. They were then washed extensively in PBS containing 0.1 % Tween-20 (PBT) and incubated for one hour at room temperature with ABC reagent (Elite Kit, Vector Laboratories) at concentrations recommended by the manufacturer. Following extensive washes in PBT, the embryos were treated with the appropriate substrate. For horseradish peroxidase staining, we used diaminobenzidine (0.5 mg  $\text{ml}^{-1}$ ) in the presence of 0.001–0.003 % hydrogen peroxide. In the *exu* embryo shown in Fig. 4B, 2  $\mu\text{l}$  of a solution of 2 % nickel ammonium sulfate, 2 % cobalt chloride was added to 1 ml of substrate solution (DeBlas and Cherwinski, 1983). For alkaline phosphatase staining, we used 0.34 mg  $\text{ml}^{-1}$  nitroblue tetrazolium salt and 0.175 mg  $\text{ml}^{-1}$  of 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, in buffer containing 100 mM NaCl, 50 mM  $\text{MgCl}_2$ , 100 mM Tris, pH 9.5, 0.1 % Tween-20, 1 mM Levamisole. After staining, the embryos were washed in PBT, dehydrated, cleared briefly in methyl salicylate and mounted in GMM (Lawrence *et al.* 1986).

### Mutant strains

Wild type embryos were obtained from Canton S parents. The *giant* alleles *gt*<sup>x11</sup>, *gt*<sup>YA82</sup> and *Df(1)62g18* were balanced over an FM7 chromosome carrying a P-element insertion express-

ing  $\beta$ -galactosidase under the control of the *ftz* promoter (Kania *et al.* 1990). We used embryos from stocks carrying the following mutations: *Krüppel*, *Kr<sup>1</sup>* (Gloor, 1950); *knirps*, *kni<sup>IE72</sup>* (Tearle and Nüsslein-Volhard, 1987); *hunchback*, *hb<sup>6N47</sup>* (Lehmann and Nüsslein-Volhard, 1987); *bicoid*, *bcd<sup>E1</sup>* (Frohnhofer and Nüsslein-Volhard, 1986); *exuperantia*, *exu<sup>QR</sup>* (Schüpbach and Wieschaus, 1986); *nanos*, *nos<sup>L7</sup>* (Nüsslein-Volhard *et al.* 1987); *oskar*, *osk<sup>166</sup>* (Lehmann and Nüsslein-Volhard, 1986); *torso*, *tor<sup>4021</sup>* (Klingler *et al.* 1988) and *tor<sup>177111</sup>* (Schüpbach and Wieschaus, 1986); *tailless*, *tl<sup>8</sup>* (Pignoni *et al.* 1990); *empty spiracles*, *Df(3R)red3L* (Dalton *et al.* 1989); *buttonhead*, *btd<sup>XG</sup>* (Jürgens *et al.* 1984); *orthodenticle*, *Df(1)KA14* (Wieschaus *et al.* 1984); *tailless-huckebein*, *tl<sup>8</sup> hkb<sup>2</sup>* (Weigel *et al.* 1990).

### Data analysis

Embryos were examined on a Zeiss Axiophot with differential interference contrast illumination. Measurements of the domains of protein expression were made midlaterally using a Zeiss E7 eyepiece micrometer and converted to egg length percentages. Ages of blastoderm embryos were estimated by measuring the length of the nuclei and invaginating cell membranes at the cell periphery as a percent of egg diameter at the broadest part of the embryo (at approximately 50 % EL). After the onset of gastrulation, morphogenic criteria were used to stage embryos according to Campos-Ortega and Hartenstein (1985). Because the *gt* pattern of expression changes quite rapidly, care was taken to compare only those embryos at the same developmental stage. For the mutant studies, this was the brief period between completion of cellularization (when the cellular layer was at least 10 % of the diameter of the embryo) and the earliest signs of gastrulation. At least 10 embryos of the appropriate stage and orientation were measured for embryos from maternal effect collections. At least 25 embryos of the appropriate stage and orientation were measured from zygotic lethal collections. Mean and standard deviations were calculated for the position of each stripe boundary. In most cases, determining which embryos were mutant was straightforward: the pattern of protein expression was obviously altered in the appropriate percentage of embryos. In a few cases of zygotic mutants, however, changes in the pattern were subtle. In these cases generally a standard deviation at one or more domain boundaries was greater than 2 %, suggesting a large degree of scatter in the data points. The values were then plotted to see if two peaks could be resolved, consistent with a reproducible shift in approximately 25 % of the embryos. As a wild-type standard, we took the pattern obtained with Canton-S embryos but, in measuring the zygotic mutants, the *giant* domains of wild-type and heterozygous embryos were measured as well and compared to the domains in the 25 % of embryos that were mutant. Shifts of less than 2 % EL were considered insignificant. To confirm the identification of mutant embryos, whenever possible we used double-stained embryos with a second antibody against *ftz* or *en* protein to reveal changes in the embryonic fate map corresponding to the mutant phenotype.

### Results

The *gt* RNA is first detected by *in situ* hybridization during nuclear cycle (NC) 12 (Mohler *et al.* 1989). Our antiserum begins to detect *gt* protein at the end of NC 12, localized in nuclei in two distinct domains that parallel the pattern observed by *in situ* hybridization: a

broad stripe at 62–80 % EL and a posterior domain at 2–33 % EL, excluding the pole cells (Fig. 1A). Expression is weak initially but gradually increases in strength through NC 13 and the pattern begins to sharpen. Embryos carrying strong *giant* mutations such as *gt<sup>X11</sup>* or *gt<sup>YA82</sup>* still express the protein and show a very similar evolution of the pattern as the wild-type but the intensity of the antibody staining does not increase, suggesting that functional *gt* protein may stimulate its own expression. Embryos hemizygous for *Df(1)62gl8*, a deletion that removes the *gt* gene entirely, show no staining at all under our conditions.

By the middle of NC 13, the posterior domain has retracted and sharpened forming a stripe whose posterior boundary is at 15–20 % EL (Fig. 1B). During NC 14 the pattern changes rapidly. As membranes progress inward from the cortex to form cells, the anterior domain begins to resolve into two stripes, a stronger one at 63–73 % and a somewhat weaker one at 73–84 % EL (Fig. 1C). By the time cellularization is complete, a new band of expression appears anteriorly at position 88–95 % EL (Fig. 1D). This domain, stripe 1, extends only laterally and dorsolaterally. Meanwhile, the separation of stripes 2 and 3 has progressed so that they now occupy positions 74–81 % and 62–70 % EL, respectively. The posterior stripe gradually shifts forward, reaching a final position at 24–35 % by the end of cellularization. The complete pattern of expression at cellular blastoderm consists then of four stripes.

The pattern of expression in post-blastoderm development is complex, with some regions becoming strong and others fading throughout germ band extension. We cannot determine at this point whether all the cells expressing *giant* at later stages are descendants of the cells that constitute stripes 1–3 at blastoderm. When we speak of elongation or changes in the three head stripes of expression, we refer only to patterns that show some local continuity and apparent evolution through time. The rapid disappearance of *giant* expression from certain regions indicates that the protein as well as the mRNA must be rapidly turning over. In fact the pattern is continuously evolving throughout the process of cellularization. The posterior band, stripe 4, begins to fade towards the end of NC14. The ventral part of stripe 2 also fades at this time and is replaced by a distinct domain of expression that develops as a short ventral stripe slightly more anterior and more intense than the part of stripe 2 that faded away. As gastrulation begins, the anterior end of the ventral furrow reaches the ventral stripe and the *giant*-expressing cells line the interior and the edges of the resulting anterior midgut invagination (Fig. 1E,F). These cells become rapidly internalized forming a mass that stains most intensely bilaterally (Fig. 2A). These cells are not part of the anterior midgut primordia, which also differentiate at this time in the same region and are displaced posteriorly by the stomodeal invagination. Instead, the *gt*-expressing cells appear to move laterally and dorsally during germ band extension, forming an arch that meets anteriorly and medially with a group of strongly expressing cells inside the clypeolabrum (Fig. 2B,C).

Initially, stripe 3 straddles the cephalic furrow, staining cells on both sides of it and with its posterior border overlapping with the thin maxillary stripe of *engrailed*-expressing cells (Fig. 2A). At no time from now on is there appreciable *giant* expression more posterior than this *engrailed* stripe, hence no expression in the primordia of the labial lobe. As the germ band begins to extend, stripe 3 decreases in intensity dorsally and laterally while it expands ventrally as cells enter the deepening cephalic furrow. Expression is seen internally anterior of the furrow in a region between the maxillary stripe of *engrailed* and the anterior midgut invagination (Fig. 2A). On the ventral side, the cells of stripe 3 fold into the ventral furrow and give rise to a thin layer of strongly expressing cells that extends to the posterior border of the maxillary segment, directly under the ectodermal layer (Fig. 2D). Most of these cells will gradually move forward as the stomodeal invagination deepens and will cease expressing *gt* as they fold into the stomodeum (Fig. 2D,E,F). Intriguing, but difficult to explain, is the appearance of staining in the germ cell precursors that are found in the amnio-proctodeal invagination (arrowhead in Fig. 2B). This staining, observed in many embryos during germ band elongation and again during germ band retraction (Fig. 3A), is not detectable in the pole cells at earlier stages. Whether it is a transient stage of expression or an odd artefact, we cannot tell.

The staining region that corresponds to stripe 2 continues fading on the sides of the embryo but remains strong on the dorsal side where it moves posteriorly towards and then into the cephalic furrow (Fig. 2B,C). Strong expression persists in a group of dorsolateral cells while the rest fade away as the germ band reaches its maximal extension. The anteriormost stripe in the labral region, stripe 1, broadens laterally and advances anteriorly and then ventrally as the clypeolabrum begins to form and fold ventrally into the stomodeal invagination. A group of cells at the tip and inside the infolding clypeum express strongly and continue to do so until the beginning of germ band retraction. The intensity and extent of *gt* expression in the head peaks shortly before the germ band reaches its maximum extent. The number of cells expressing *gt* continues to decrease during germ band retraction (Fig. 3A) but some staining continues to be detectable even after the cuticle is secreted, nearly up to the end of embryonic development. It is interesting to note that among the last cells in which expression persists is a cluster located dorsally just behind the dorsal sac and the pharyngeal musculature but above the brain and corresponding in shape and position to the ring gland (Fig. 3B). Petschek *et al.* (1987) have observed that in strong *gt* mutants the ring gland appears to be missing or defective and have speculated that very mild defects in the ring gland of the viable *gt*<sup>1</sup> allele might be responsible for the insufficiency of ecdysteroid that is responsible for the eponymous *giant* larva phenotype in this mutant.

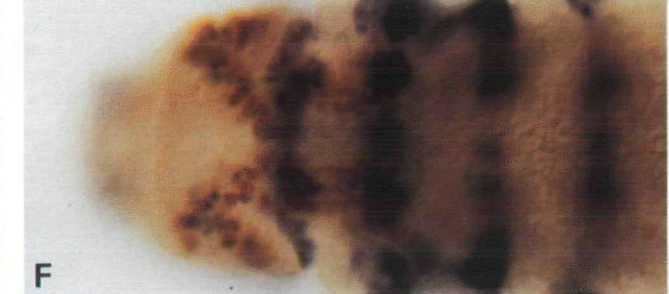
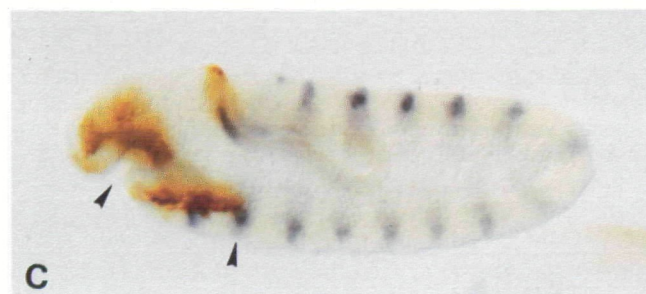
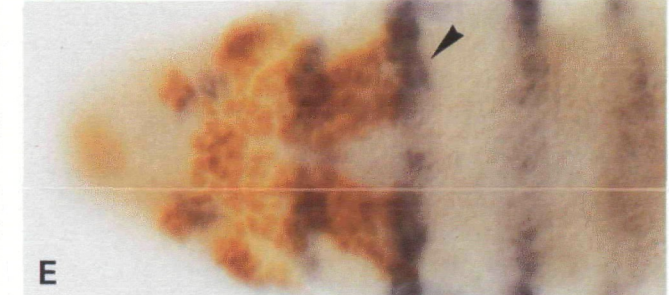
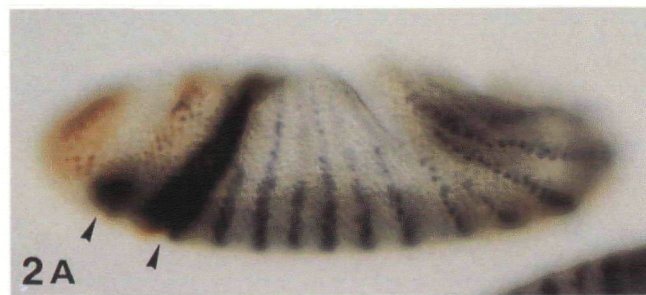
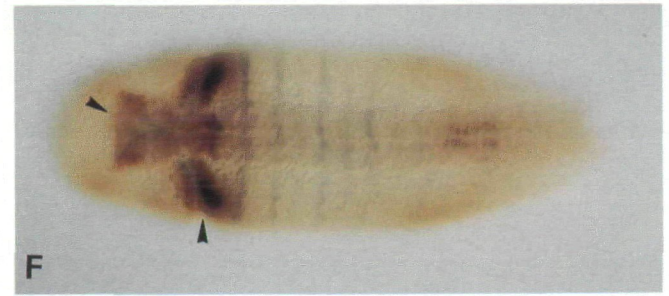
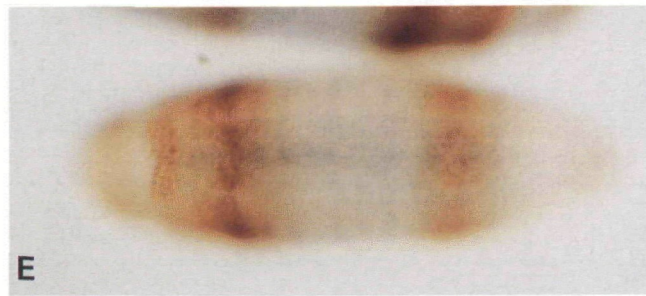
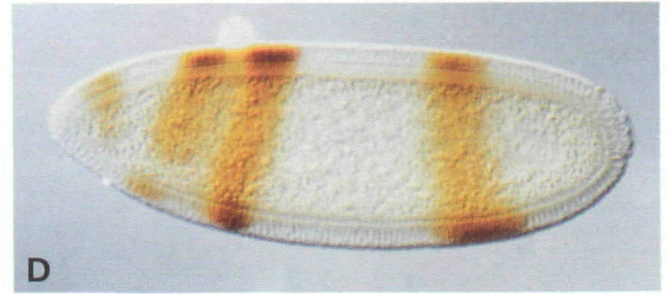
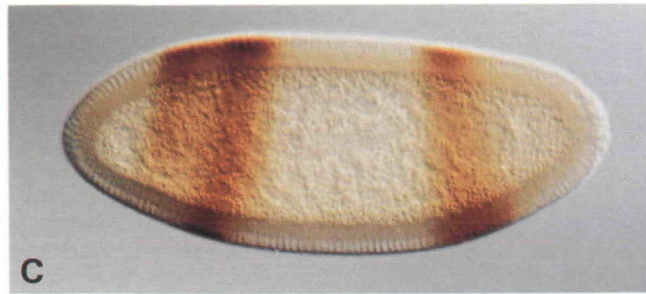
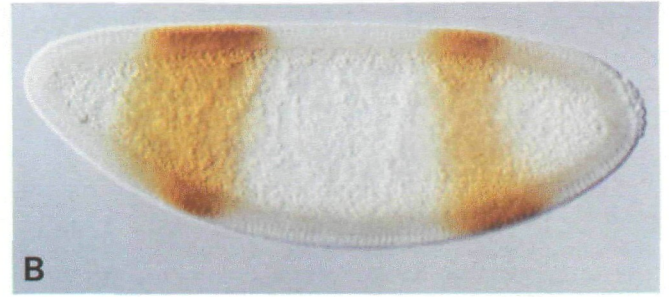
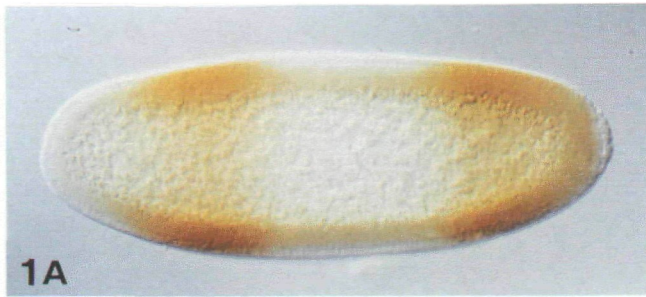
#### Maternal effects

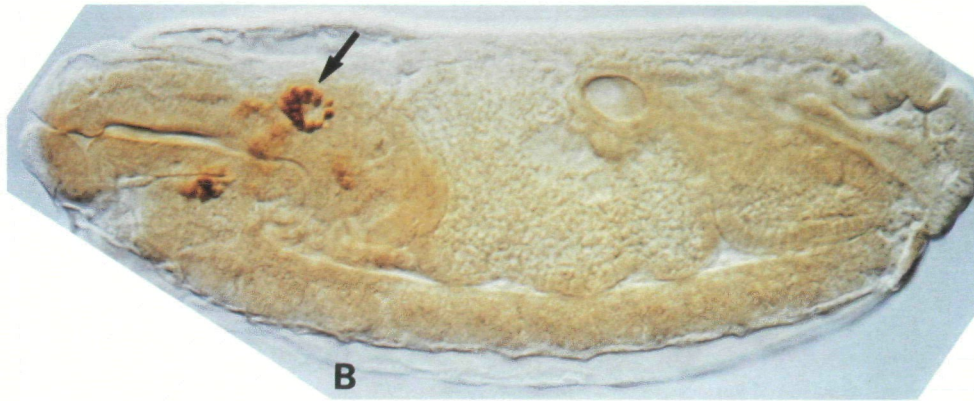
Three classes of maternally acting genes have been

**Fig. 1.** Early embryonic expression of *giant* protein in Canton S embryos. Embryos B to D are oriented with anterior to the left and dorsal side up. (A) Horizontal view of the initial pattern of *gt* expression in a precellular NC 13 embryo showing pale staining in two broad domains. (B) Refinement and intensification of the expression pattern in a slightly older embryo. (C) Anteriorly, stripes 2 and 3 begin to resolve and stripe 4 continues to be refined as cellularization begins during NC 14. (D) As cellularization is completed, during NC14, stripe 1 is detected anteriorly, stripe 2 ventral is seen ventrally and slightly anteriorly to stripe 2, and stripe 4 achieves its final position. Embryos E and F are seen from the ventral surface and oriented with the anterior to the left; *giant* expression is seen in brown, *engrailed* expression in blue. (E) During gastrulation, the ventral furrow extends to stripe 2 ventral, seen here as the anterior-most *giant* stripe in the plane of focus. (F) The anterior midgut begins to invaginate carrying the cells of stripe 2 ventral with it (arrowhead on the left). Stripe 3 flanks the cephalic furrow (arrowhead on the right) obscuring *engrailed* stripe 2.

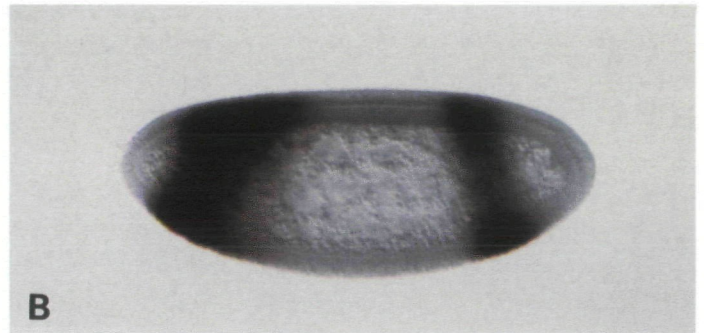
**Fig. 2.** Post-blastoderm expression of *giant* protein. In all embryos, *giant* protein is stained brown and *engrailed* protein in blue. Embryos A to C are oriented with anterior to the left and dorsal up. (A) Early stage 7 embryo just beginning germ band extension. Posterior expression of *giant* is barely detectable. From the anterior tip, arrowheads indicate the site of the anterior midgut invagination and *engrailed* stripe 2, respectively. (B) Stage 9 embryo nearing the end of germ band extension. The arrow indicates the pole cells which are transiently stained. Anteriorly, *gt* stripe 1 is at the anterior tip of the embryo, the cells from *gt* stripe 2 ventral that invaginated with the anterior midgut primordia form a cluster midlaterally, cells from *gt* stripe 2 form the patch seen dorsally and cells from *gt* stripe 3 are found ventrally anterior to *engrailed* stripe 2. (C) Stage 10 embryo, fully germ band extended. Arrowhead indicates the stomodeal invagination. Pole cells no longer stain, and *gt* expression is restricted to regions of the embryo anterior to *engrailed* stripe 2, which marks the posterior border of the maxillary segment. Cells formerly associated with stripe 3 are now internalized. Internalized cells from stripe 2 ventral and stripe 1 surround the region of the invaginating stomodeum, and a few cells from stripe 2 are still seen dorsally. D to F are ventral views of the anterior end of progressively older embryos, beginning at stage 10 (fully germ band extended) and progressing to stage 12 (midway through germ band retraction). The arrowheads indicate *engrailed* stripe 2. During this time the process of head involution begins and the ventrolateral lobes of the gnathal segments (mandibular, maxillary and labial) begin to be drawn ventrally and anteriorly into the stomodeum. This movement is reflected in the pattern of *gt* staining. As head involution proceeds, cells that move into the region of the stomodeum extinguish *gt* expression.

identified, whose function during oogenesis is to lay down the cues that result in the establishment of the anteroposterior axis of the embryo. Their domains of activity are independently established, largely non-overlapping and affect a) the anterior segments, b) the posterior segments and c) the terminal structures at both ends of the embryo (Nüsslein-Volhard *et al.* 1987). *giant* expression is found in all three of these domains. It is not surprising therefore, to find that loss of activity





**Fig. 3.** Later embryonic expression of *giant* in Canton S embryos. Both embryos are oriented with anterior to the left and dorsal up. (A) Late stage 12 embryo (germ band retraction) in which transient staining is again observed in the germ cells which are accumulating in the developing gonad. Anteriorly the number of cells expressing *gt* is much reduced and restricted primarily to a group of cells surrounding the pharynx and a smaller group of cells dorsally. (B) Stage 16 embryo (germ band fully retracted, prior to the secretion of cuticle), showing high levels of *gt* expression in very few cells. The arrow indicates site of the ring gland. A second cluster of cells still stains ventral to the pharynx.



**Fig. 4.** Expression of *giant* in maternal mutants. In all cases embryos are at cellular blastoderm (late stage 5), anterior end to the left and dorsal side up. (A) *bicoid* embryos. Anterior *gt* expression is entirely absent while the posterior domain is broadened and shifted anteriorly. (B) *exuperantia* embryos. The anterior domain consists of a single broad band and stripe 4 is shifted posteriorly. (C) *oskar* embryos. Anterior expression is normal but posterior expression is lacking entirely. (D) *torso* embryos. Stripe 1 is absent leaving only stripe 2 and 3 anteriorly. Posterior expression persists to the posterior tip but not including the pole cells.

of any of these three pattern-forming systems has profound effects on the distribution of *giant* protein in the early embryo.

#### *The anterior domain*

The key maternal anterior gene, *bicoid* (*bcd*), is distributed in the early embryo in a concentration gradient with a high point near the anterior tip (Driever and Nüsslein-Volhard, 1988a,b). The *bcd* gradient can activate the *hb* gene only in the anterior part of the embryo, where *bcd* concentration is high enough to bind to its response elements in the *hb* regulatory region (Driever *et al.* 1989; Struhl, 1989). By manipulating the number and affinity of the *bicoid* binding sites, the domain of activation of the *hb* gene can be shifted more anteriorly or more posteriorly, demonstrating how this maternal morphogen can generate a pattern of zygotic gene expression. The activation of *hb* by *bcd* is insufficient to account for the complexity of the anterior *gt* pattern since the specification of head structures requires *bcd* levels higher than those required to activate *hb* expression and mutations in *hb* do not account for the totality of the phenotypes produced by lack of maternal *bcd*. Driever *et al.* (1989) hypothesized a gene X that would be activated by higher concentrations of *bicoid* and might therefore serve to specify the more anterior head segments. Our results show that *giant* is such a gene X, whose anterior domain of expression is most likely directly controlled by *bcd*.

In embryos produced by *bcd* mothers, the anterior pattern of *giant* expression is never observed. Stripes 1–3 fail to appear and stripe 4 develops as a broader domain shifted to a more anterior position (28–47% EL instead of 24–35% EL; Fig. 4A). This indicates that the anterior expression of *giant* requires *bcd* activity either directly or indirectly but that stripe 4 is established independently of *bcd*. The anterior activation of *giant* might be mediated by some other zygotic gene dependent on *bicoid*, either *hb* or some other *bcd*-dependent gene, but several lines of reasoning suggest that the *bcd* controls *gt* directly: (1) *gt* anterior expression begins very early, before appreciable expression of other zygotic genes; (2) it is independent of *hb*, the major known mediator of *bcd*; (3) it is not abolished by mutations in the other maternal factors or in the other known zygotic genes.

Furthermore, the anterior pattern of *gt* expression depends on the gradient of *bcd* concentration. This is shown by the distribution of *gt* expression in embryos derived from mothers mutant for *exuperantia* (*exu*), a gene required for the proper localization of *bcd* mRNA in the oocyte. Phenotypically such mutant embryos have greatly reduced anterior structures and expanded gnathal and thoracic structures (Schüpbach and Wieschaus, 1986). In *exu* eggs, the *bcd* RNA fails to be preferentially localized anteriorly and as Driever and Nüsslein-Volhard (1988b) have shown, the *bcd* protein gradient in these embryos is much shallower than normal, with the highest concentration at the anterior end corresponding to that normally found at about 65%. In *exu* embryos, *gt* expression at cellular

blastoderm is found in a broad domain from 70–92% EL anteriorly and 15–30% EL posteriorly (Fig. 4B). We interpret this as a broadened and anteriorly shifted stripe 3, in agreement with the general anterior shift of thoracic and gnathal structures. We suppose that *bcd* concentrations are insufficient to specify stripes 1 and 2 but that levels adequate to activate stripe 3 are now found over a good part of the head region. The fact that the domain of *giant* expression does not extend to the anterior tip of the embryo suggests that factors that prevent *giant* expression are present in the terminal region. The posterior shift of stripe 4 is probably an indirect effect mediated through other gap genes (see below). These results argue in favor of a direct dependence on the *bcd* concentration at least for the initial *gt* anterior domain. The separation of stripe 2 from stripe 3 and the appearance of stripe 1 are probably due to interactions with zygotic gene products.

#### *The posterior domain*

Embryos derived from mothers mutant for genes of the posterior group fail to differentiate abdominal structures (Nüsslein-Volhard *et al.* 1987). Unlike the anterior and terminal systems, the posterior system controls the embryonic pattern by negative regulation. It has been clearly established that *nanos* (*nos*), the key gene of the posterior class, acts solely by preventing the expression of the maternal *hb* protein in the posterior half of the embryo and is not required in embryos lacking maternal *hb* product (Hülkamp *et al.* 1989; Irish *et al.* 1989; Struhl, 1989). The maternal *hb* protein is uniformly distributed in a *nos* mutant but forms a decreasing gradient in the posterior half of *nos*<sup>+</sup> embryos. Similarly, the distribution of maternal *hb* remains uniformly high along the entire anteroposterior axis of embryos derived from mothers mutant for *oskar* (*osk*) (Tautz, 1988), another member of the posterior group of genes which is required for correct positioning of *nos* product in the embryo. These results show conclusively that it is the abnormal persistence of the maternal *hb* product that suppresses abdominal development in these mutants.

In embryos derived from mothers mutant for *nos* or *osk*, the posterior stripe of *gt* fails to appear while the anterior pattern appears to be normal (Fig. 4C). This suggests that maternal *hb* product inhibits the expression of *gt* in the posterior domain but has no effect on the anterior expression. However, we have not generated *hb*<sup>−</sup> germ line clones to demonstrate this directly. This distinction between the anterior *gt* expression, which is *bcd* dependent and not repressed by maternal *hb*, and posterior expression, which is independent of *bcd* and repressed by *hb*, strongly suggests that they are controlled by two separate regulatory elements in the *gt* gene that act independently and respond to different regulatory proteins.

#### *The terminal domains*

The nonsegmental termini, anteriorly the acron and posteriorly the telson, require the action of the

maternal genes of the terminal class. These are thought to act through a signalling pathway that includes genes such as *torsolike*, *trunk* etc. and results in the activation of a membrane-bound tyrosine kinase, the product of the *torso* gene, and of the *D-raf l(1)polehole* ser/thr kinase (Sprenger *et al.* 1989; Ambrosio *et al.* 1989; Stevens *et al.* 1990). In contrast to *bicoid*, these gene products are not localized in the embryo but are instead activated only at the poles by a localized signal and in turn activate zygotic genes such as *tailless* and *huckebein* in the terminal domains (Klingler *et al.* 1988; Strecker *et al.* 1989a; Weigel *et al.* 1990). We studied *gt* expression in embryos produced by mothers mutant for two members of the terminal class, *torso* (*tor*) and *torsolike* (*tsl*). As expected from the signal transduction model, their effects on *gt* expression are virtually identical. The posterior domain of expression appears normally at early syncytial blastoderm but fails to mature and to withdraw from the posterior region. At cellular blastoderm it still extends up to, but not including, the pole cells. The anterior domain of expression is also affected. Stripe 1 never forms. Stripe 2 is shifted anteriorly by 4–7 % EL but stripe 3 remains in its normal position (Fig. 4D). This indicates that the terminal genes have contrasting effects on *giant*: a repressive effect posteriorly and an inductive effect anteriorly. The fact that both of these effects take place after the initial stages of *giant* expression suggests that they are mediated by one or more zygotic effectors.

A confirmation of these conclusions was given by the pattern of *gt* expression in embryos produced by mothers carrying a dominant allele of *torso*, *tor*<sup>4021</sup> (not shown). This mutation is thought to result in a constitutively active form of the *torso* product which would therefore activate terminal-specific zygotic gene functions throughout the embryo (Klingler *et al.* 1988; Strecker *et al.* 1989a). The phenotypic result is the expansion of the terminal, nonsegmental domains of the embryo at the expense of the segmental primordia. The distribution of *giant* product in these embryos is revealing: the posterior domain is completely absent and only two stripes are seen anteriorly. We interpret these results as showing that stripes 3 and 4, which normally correspond to segmented regions of the blastoderm fate map, are suppressed by the ectopic expression of terminal genes. Two anterior stripes remain. We suppose that these are stripes 1 and 2, which normally correspond to the anterior nonsegmented portion of the map and are shifted more posteriorly than normal by the expansion of the terminal domains. In approximately 10% of the embryos, the second stripe (63–73 % EL) succeeds in resolving into two stripes (69–77 % and 59–64 % EL). This incomplete suppression may reflect the fact that segmental primordia, though greatly reduced, are not altogether absent in these embryos.

The results with the three maternal classes of pattern-forming genes do not tell us conclusively whether *giant* is directly affected by maternal cues. However, the fact that the anterior (*bcd*) and posterior (*nos*) systems affect the earliest expression of *gt* argues for a direct

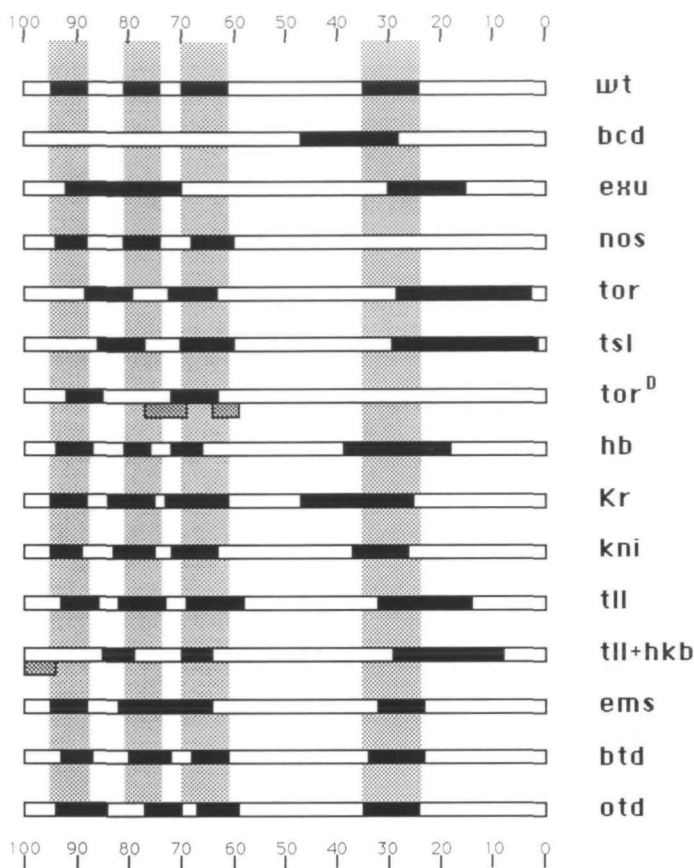
activating effect of *bcd* on stripe 3 and of a repressing activity of maternal *hb* on stripe 4 expression. The effects of the terminal system on the later-appearing stripe 1 and on the retraction of stripe 4 are instead most likely mediated by zygotic gene products.

#### Gap gene interactions with giant

The earliest zygotic genes that affect the antero-posterior pattern belong to the segmentation gap class. The gap genes begin to be expressed very early at the syncytial blastoderm stage and are thought to respond directly to the maternal cues. It has been shown in addition that the gap genes interact both positively and negatively with one another so that the final pattern of expression is a complex resultant of the maternal and zygotic influences (reviewed by Gaul and Jäckle, 1990). To determine how other gap genes influence *gt* expression, we examined the *gt* protein distribution in embryos homozygous for *Krüppel* (*Kr*), *knirps* (*kni*), *hb* or *ill* mutations. In addition we looked for the effects of *gt* mutations on the expression pattern of *hb*, *Kr* and *kni*. To aid in identifying mutant embryos, we frequently double-stained using antibodies to *ftz* or *en*. As a rule, however, we measured the positions of the staining domains in a large number of unselected embryos and plotted each in a histogram. A statistically significant separate peak accounting for approximately 25 % of the embryos was taken to represent a domain of expression affected by the mutation (see the methods section). The results obtained with each mutation are summarized diagrammatically in Fig. 5.

#### hb-gt interactions

As expected from the analysis of *nos* mutations, zygotic *hb*, like maternal *hb*, has a negative effect on *gt* expression in the posterior domain. In *hb* mutant embryos, stripe 4 expands posteriorly into the region normally occupied by a late-appearing band of zygotic *hb* expression (10–20 % EL; Fig. 6A). In addition, the anterior border is shifted 4 % EL anteriorly. This confirms the negative effect of *hb* on the expression of stripe 4 and indicates that the withdrawal of this domain from the posterior end of the embryo is at least in part caused by the synthesis of zygotic *hb* in this region. However, in *hb* mutants *giant* expression does not continue all the way to the posterior pole, indicating that other negative interactions are involved, most likely with the zygotic interpreters of maternal terminal information. The shift of the anterior border is harder to account for. Loss of zygotic *hb* function is necessarily accompanied by the reduction of maternal *hb* to only one dose of the gene. This might lower the maternal *hb* concentration in the posterior half of the embryo and permit the activation of stripe 4 up to a more anterior position. Hülkamp *et al.* (1990) invoke a similar explanation for the anterior expansion of the abdominal band of *knirps* expression. This broadening of the *kni* band in response to a reduction in maternal and zygotic *hb* may have additional direct or indirect effects on the domain of *gt* expression. We do not know what causes stripe 4 to fade at the end of cellular blastoderm but it



**Fig. 5.** Summary of *giant* expression patterns in wild-type and mutant backgrounds. The diagrams display the averaged positions of the *giant* domains of expression, converted to percent egg length (EL). Only changes of greater than 2% EL from Canton S and internal wild-type (where possible) values were judged significant (see Materials and methods). The stippled boxes shown underneath the *tor<sup>D</sup>* and *tll+hkb* diagrams indicate domains of expression seen in a minority of the embryos.

cannot be simply inhibition by increasing levels of *hunchback* posterior expression since the fading occurs also in *hb<sup>-</sup>* embryos.

The only significant effect of the absence of zygotic *hb* on the anterior pattern of *gt* is a slight anterior shift of stripe 3. This indicates that *hb* does not mediate the activating effect of *bicoid* on *gt* anterior expression. Given the complete overlap between the anterior domains of *gt* and of *hb*, this minor effect is not likely to be directly caused by *hb*. A more plausible explanation is that it is mediated through *Kr*. It has been shown that high levels of *hb* repress *Kr* and set the anterior edge of the *Kr* central domain. In the absence of zygotic *hb*, the *Kr* domain expands anteriorly by 10% EL (Hülskamp *et al.* 1989) and could affect *gt* anterior expression.

There are no obvious changes in the domains of zygotic *hb* expression in embryos deficient for *gt*. A very subtle effect may be observed in the anterior of the embryo where, at cellular blastoderm, the previously continuous expression of *hb* becomes modulated into three stripes that appear to be complementary to the

three anterior *gt* stripes. If this is the case, it would suggest a negative effect of *gt* on the anterior expression of *hb*. However, the anterior modulation of *hb* is difficult to ascertain under our staining conditions and we cannot be certain that it fails in *gt*-deficient embryos.

#### *Kr-gt interactions*

*Kr* has a distinct negative effect on *gt* expression. In *Kr* mutant embryos stripe 4 is greatly expanded anteriorly, reaching 48% EL and invading therefore both the *kni* and *Kr* domains of expression (Fig. 6B). This effect could be direct or mediated through *kni*. Pankratz *et al.* (1989) have shown that the full expression of *kni* requires *Kr* activity. In *Kr* mutants, the posterior expression of *kni* never reaches its normal intensity, though its position is not shifted. The effect of *Kr* on *gt* stripe 4 could therefore be a direct negative effect of *Kr* on *gt* or an indirect effect caused by *Kr* enhancement of *knirps*-dependent repression of *gt*. We conclude that it is most likely a direct effect because *kni* mutations have no broadening effect on *gt* stripe 4 (see below) while, in *Kr* mutants, stripe 4 expands nearly to 50% EL. What prevents this *gt* domain from expanding further is probably the steeply rising concentration of *hb* whose anterior domain extends to 48% EL. In the anterior region, *Kr* mutations have only slight effects on *gt*, consisting of a slight broadening of stripe 3 and stripe 2. This could also be explained as a repressive effect caused by *Kr* expression in the central domain and in the *Kr* anterior domain (a stripe around 82% EL).

The effects of *gt* mutations on *Kr* expression are very mild. In agreement with Gaul and Jäckle (1987), we could not observe a significant broadening of the *Kr* central domain. However, several lines of evidence suggest that *gt* has a negative effect on *Kr*: (1) *bcd* embryos, in which *gt* stripe 4 expands forward, show a contraction of the *Kr* posterior border; (2) conversely, in cases in which *gt* stripe 4 is suppressed, *Kr* posterior border expands posteriorly, e.g. in *nos* embryos; (3) more directly, we have shown that ectopic expression of *gt* under control of the heat shock promoter causes suppression of *Kr* expression in the central domain and generates cuticular phenotypes similar to those of *Kr* mutant embryos while *gt* protein binds *in vitro* to specific sites in the *Kr* gene in the regulatory region responsible for expression in the central domain (M. Capovilla, E. Eldon and V. Pirrotta, unpublished data). Similar observations with ectopic expression of *gt* have been made by Kraut and Levine (1991).

#### *kni-gt interactions*

Mutations in the *knirps* gene affect the development of the first seven abdominal segments, thus including most of the abdominal region affected by *giant*. In *kni* mutants, stripe 4 of *gt* expression is affected only very slightly, if at all (Fig. 6F). It is possible that the posterior border becomes less well defined and that the intensity of posterior expression, which normally begins to fade at the end of cellular blastoderm, fades somewhat more rapidly in *kni* embryos. However, it is clearly still detectable at the beginning of germ band

extension and we hesitate to attach much significance to these slight effects. The lack of significant changes in the *gt* stripe 4 domain in these mutants indicates that neither the *hb* nor the *Kr* effect on *gt* are to be explained by a mediation through *knirps* and are therefore best interpreted as direct interactions. In the head region, it is interesting to note that the *knirps* anterior domain of expression is roughly complementary with that of *giant* (Fig. 6E). At syncytial blastoderm, *knirps* forms a cap at the anterior pole that extends ventrally until about 75% EL. At cellular blastoderm, a thin ring of expression appears at this position that corresponds to the interval between stripe 2 and 3. In spite of this pleasing complementarity, *knirps* mutants show the normal separation between *gt* stripe 2 and stripe 3 which cannot therefore be accounted for by a repressive action of *kni* on *gt* anterior expression.

Of the gap genes, *knirps* is the one whose pattern of expression is most strongly altered in embryos deficient for *gt*. In these embryos, the posterior domain of *kni*, which is normally at 34–47% EL, expands posteriorly to 28% EL. The strong negative effect of *gt* on *kni* that is implied by this result may in fact help to explain the previously reported interactions of *kni* with *Kr*. Pankratz *et al.* (1989) found that in *Kr* mutant embryos the abdominal *kni* stripe is present at the normal position but its intensity is greatly decreased. They concluded from this that *Kr* enhances *kni* expression. This apparent enhancement may be better explained as an indirect effect: in *Kr* mutants, *gt* stripe 4 expands anteriorly up to the middle of the embryo, fully overlapping and inhibiting expression of *kni* in its posterior domain.

#### *tll-gt interactions*

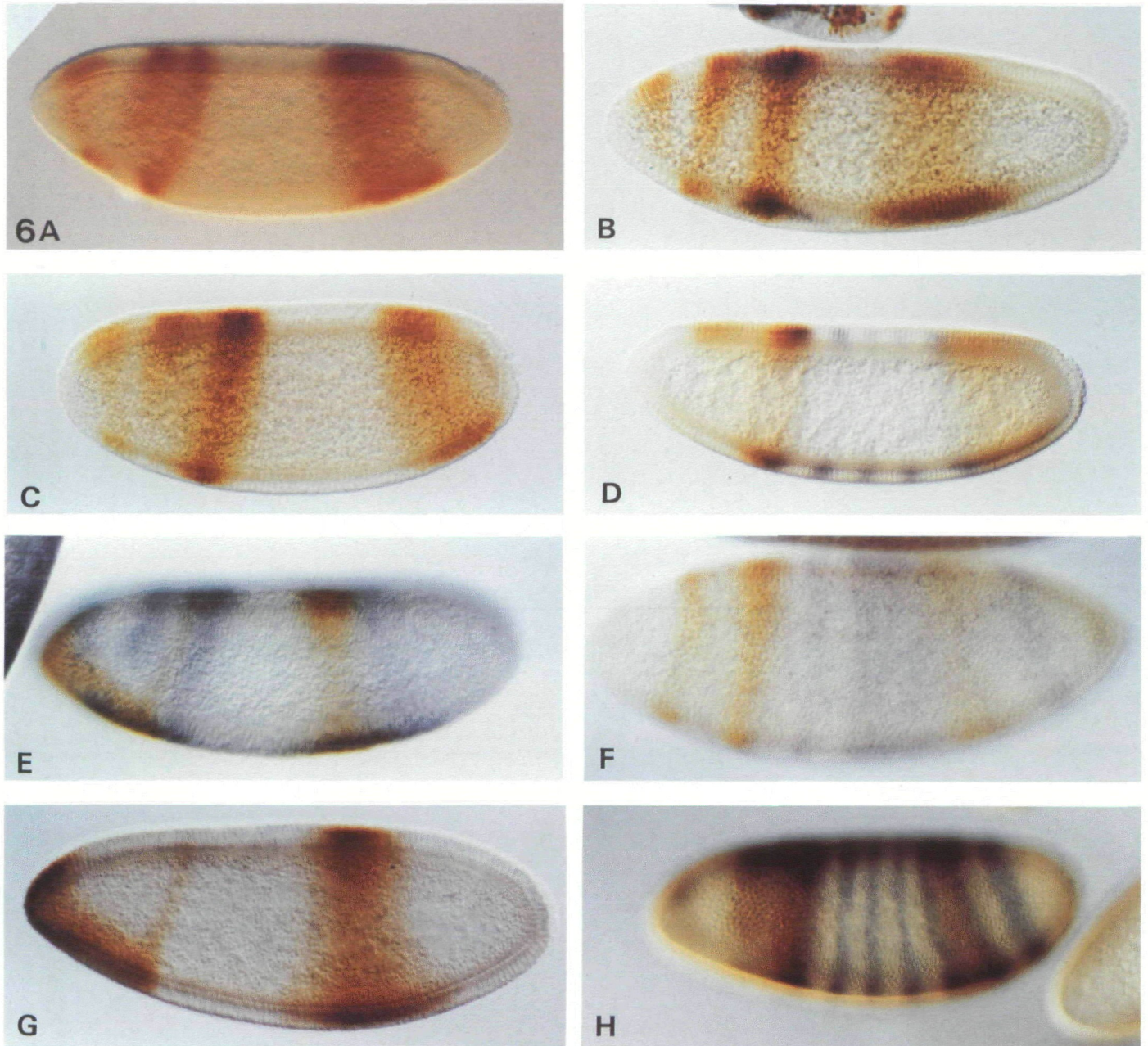
Lack of *tll* function affects the structures derived from both anterior and posterior terminal domains. In *tll* mutants abdominal segments 8–10 are missing and a decrease in the extent of the procephalon is accompanied by an expansion of the remaining body segments (Strecker *et al.* 1986). While *tll* is not the only zygotic target of maternal terminal class genes, it is clearly one of the mediators of these maternal signals (Klingler *et al.* 1988; Strecker *et al.* 1989b). In the absence of *tll* activity, the posterior domain of *gt* expression never matures properly. At the end of cellularization, it does not complete its withdrawal from the posterior terminal region and forms an expanded band whose posterior border is shifted 10% EL posteriorly (Fig. 6C). This position is in good agreement with the shift in the fate map seen in *tll* embryos by Mahoney and Lengyel (1987) and with the distribution of *tll* mRNA (0–15% EL at cellular blastoderm, Pignoni *et al.* 1990). Surprisingly, lack of *tll* has little effect on the anterior domains of *gt* expression. The apparent posterior shift of stripe three was not considered significant because the internal wild-type controls in this set of embryos showed a similar range of positions for this stripe.

More significant shifts are seen with *tailless-huckebein* double mutant embryos (Fig. 6D). Accord-

ing to Weigel *et al.* (1990), the zygotic gene *huckebein* (*hkb*) is required to specify parts of the fate map corresponding to the posterior midgut and to anterior terminal structures. They suggest that *hkb* acts like a gap gene and, together with *tll*, mediates maternal terminal cues from the *tor* gene product. In *tll-hkb* double mutants, the posterior border of *gt* stripe 4 retracts even less than in *tll* mutants but at cellular blastoderm it does not extend completely to the tip of the embryo as in *tor* or *tsl* embryos. This might suggest the existence of additional components that mediate the effect of *tor* but a more likely explanation is that the *hkb* mutation used does not cause complete loss of function (Weigel *et al.* 1990). While *tll* alone has little effect on *gt* in the head region, the sum of the two mutations results in a *gt* pattern approaching the greatly shifted pattern seen in the absence of *tor* function. Stripe 1 is shifted to the anterior pole and is present at barely detectable levels while stripes 2 and 3 are displaced forward as in *tor* embryos. This indicates that, at the anterior end, *torso* effects on *gt* are mediated primarily through *hkb* and not through *tll*.

#### *gt expression in the head primordia*

Three genes have been recently described as having a gap-like role in mediating the development of head segments. These are *orthodenticle* (*otd*), *empty spiracles* (*ems*) and *buttonhead* (*btd*) (Cohen and Jürgens, 1990). The genes for *otd* and *ems* have been cloned by Finkelstein and Perrimon (1990) and Dalton *et al.* (1989), respectively, who found that they both encode homeodomain proteins. Since, after blastoderm, *gt* protein is found almost exclusively in the head primordia, we looked at the *gt* expression pattern in embryos mutant for each of these three genes. Both *ems* and *otd* affect *gt* expression in the anterior domain. In *ems* embryos, stripe 1 is normal but stripes 2 and 3 fail to sharpen, and separate at cellular blastoderm (Fig. 6H). In *otd* mutants, the posterior border of stripe 1 and all of stripe 2 are shifted posteriorly (not shown). In both cases, the part of the *gt* pattern most affected lies in the region of expression of the corresponding gene and involves the part of the fate map affected by the corresponding mutations. Both *otd* and *ems* are positively regulated by *bcd* and it has been proposed that they are direct targets of the *bcd* protein (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Dalton *et al.* 1989). However, they cannot be the mediators of *bcd* effect on *gt* since their effect on *gt* anterior expression is negative rather than positive. Instead, the shifts in the *gt* stripes of expression that result from their loss of function are similar to those caused by other gap gene mutations. This is consistent with the interpretation that *otd*, *ems* and *btd* act as segmentation gap genes in the head region, where a greater complexity may require a greater number of components to specify the pattern. However, not all of these genes interact with *giant*. In *btd* embryos the changes in the *gt* anterior pattern are so slight as to be most likely insignificant. Although molecular probes for *btd* product are not available, the structures affected by



**Fig. 6.** Expression of *giant* in gap mutants. In all cases, embryos are at cellular blastoderm (late stage 5), anterior end to the left and dorsal side up. (A) *hunchback*. Stripe 3 is shifted anteriorly and stripe 4 expands posteriorly. (B) *Krüppel*. Anterior expression is nearly normal but stripe 4 expands anteriorly to mid-embryo. (C) *tailless*. Anterior expression is normal but stripe 4 retracts only partially from the posterior tip. (D) *tailless-huckebein* double mutants. Stripe 1 fails to appear and stripe 4 extends closer to the posterior tip. Blue staining indicates the stripes of *fushi tarazu* expression. (E) Wild type embryo showing *knirps* expression in brown and *gt* expression in blue. Compare with patterns seen in F and G. (F) *knirps* mutant. Note the altered pattern of *fushi tarazu* expression seen in blue. *gt* expression (brown) is nearly normal, though faint in this batch of embryos. Expression of stripe 4 continues to be detectable up to early germ band extension. (G) *giant* mutant embryo showing altered *knirps* expression. The posterior domain is expanded posteriorly, although anterior expression is normal. (H) *empty spiracles* embryo. *giant* is stained brown and *fushi tarazu* blue. *gt* stripes 2 and 3 fail to separate but posterior expression is normal.

*btd* mutations imply that its domain of expression should include the mandibular, intercalary and antennal segments. Of these, at least the mandibular region should overlap with sites of *gt* expression at cellular blastoderm.

## Discussion

Our results show that the *giant* gene behaves like a typical segmentation gap gene. Like other gap genes, *gt* produces a nuclear factor very likely involved in regulating the expression of other genes. The defects exhibited by *giant* loss-of-function mutants fall into two regions. One, in the posterior half of the larva, is a loss of contiguous abdominal segments A5, 6, 7 and sometimes 8, a type of pattern deletion characteristic of segmentation gap mutations. The posterior domain of *giant* expression corresponds well to this phenotype and the alterations of the *gt* pattern in various mutant backgrounds are indicative of interactions typical of segmentation gap genes. While the posterior domain of *gt* expression fades off soon after cellular blastoderm, the *in situ* hybridization results (Mohler *et al.* 1989) and the localization of the protein show that the head is the site of most intense, complex and prolonged *gt* expression. This later expression in the head region represents a second phase of *giant* activity that takes place after its function as a gap gene is completed.

The anterior defects caused by *gt* mutations are more difficult to characterize because of the complex events and the structural rearrangements that normally result from head involution but that are perturbed or fail to take place in *gt* mutants. The reported lack of labral structures, epi- and hypostomal sclerites, H-piece and dorsal bridge (Mohler *et al.* 1989) are difficult to interpret as segmentation gaps. Rather, the defects within the head appear restricted to individual structures or elements rather than whole segments. The segmental attribution of these defects does not always correspond well with the pattern of expression that we observe in the head region. The anteriormost stripe and the later strong expression in the clypeolabrum fit well with the labral defects and the failure of head involution but there is a discrepancy between the position of stripes 2 and 3 and the sites of the other head defects. Petschek *et al.* (1987) and Petschek and Mahowald (1990) noted the disappearance of the labial lobe and the transient fusion of T1 and T2 in *gt* embryos at the germ band retraction stage, accompanied by defects in the expression of *Antennapedia* and *Scr* genes. Carroll and Scott (1985) and Petschek and Mahowald (1990) have also shown that stripes 1 and 2 of *fz* are significantly broadened in *gt* mutants. Our results show that the posterior boundary of *gt* expression is within the posterior compartment of the maxillary segment and coincides with stripe 2 (maxillary) of *engrailed*, just in front of the labial lobe. This discrepancy can be accounted for in part by supposing that some of the mature head defects might have been incorrectly assigned to labial primordia and in part by assuming

that the effects on the labial and thoracic segments are indirect, not primarily due to the lack of *gt* product but to the effect that it produces on other gap genes, most likely *Kr*. The spread of pattern defects to regions broader than the domains of strong expression is a phenomenon common to the other gap genes (*hb*, *Kr*, *kni*, *ill*) and is most likely explained through combinatorial and indirect effects produced by the cross-interactions between these genes. The fact that in *gt* mutants no specific defects have been attributed to maxillary and mandibular derivatives may also be due in part to the early internalization of most of the cells that express *gt* during the complex infolding of the initial blastoderm sheet of cells in the cephalic furrow. It is possible that, as a result, the *gt*-expressing cells contribute principally to internal structures, most of which are not preserved in cuticle preparations and whose ontogeny is more difficult to follow.

### Two different mechanisms of *gt* activation

An account of the *gt* pattern of expression must begin with the realization that the anterior expression is controlled by a different mechanism from that which activates the posterior. Anterior expression is *bcd*-dependent, the posterior is not. Posterior expression is *hb*-sensitive, the anterior is not. Molecular studies of several other segmentation genes have shown that their regulatory regions are complex and constituted by multiple elements that can act independently of one another (Harding *et al.* 1989; Pick *et al.* 1990; Hoch *et al.* 1990). A preliminary analysis of the *gt* regulatory region shows in fact that the control elements responsible for expression in the posterior domain are distinct, independent and widely spaced from those that control anterior expression (P. Bagnaresi and V. Pirrotta, unpublished results). The results presented in this paper show that *bcd* is responsible, most likely directly, for activating the initial anterior expression of *gt*. We may suppose that this occurs through a mechanism similar to that demonstrated for *hb* and anticipate that molecular analysis will demonstrate the presence of *bcd* binding sites in the regulatory domain responsible for anterior expression of *gt*. The failure of this *bcd*-dependent activation to extend as far as the anterior tip even at the earliest stages suggests that maternal factors inhibit *gt* expression in the terminal region. If these were part of the terminal system, we would expect that in *tor* or *tsl* embryos this repression would be lifted and expression would extend fully to 100% EL. Since this is not the case, we suppose that the highest *bcd* concentrations have a repressive effect on *gt* expression. Although such repressive activities of *bcd* have not yet been demonstrated at the molecular level they have been postulated by Pignoni *et al.* (1990) to explain anterior inhibition of *ill* by *bcd* and by Hülkamp *et al.* (1990) to set the anterior border of *Kr* expression.

The posterior initial pattern, like much of the rest of abdominal development, appears to be negatively rather than positively controlled. Several groups have shown that the removal of maternal *hb* suffices for

normal abdominal development even in the absence of *nos* function (Hülskamp *et al.* 1989; Irish *et al.* 1989; Struhl, 1989). The activation of *gt* in the posterior domain must therefore be constitutive, dependent on ubiquitous transcription factors and severely inhibited by even relatively low levels of *hb*. Maternal *hb* RNA is initially uniformly distributed but, under the influence of *nos* product, the accumulation of *hb* protein tapers off in the posterior half of the embryo (Tautz, 1988). We can explain the activation of *gt* in the posterior third of the embryo as the derepression that occurs when *hb* concentrations drop below a threshold. Initial expression of *gt* would then take place from the position at which this threshold is reached to the posterior tip. The posterior expression of *kni* could be similarly derepressed but at a higher threshold of *hb* concentration. Maternal *hb* product, in this interpretation, acts as a graded morphogen, exerting negative control on genes that differ in their sensitivity to *hb* inhibition. Additional control may be exerted by *bcd* on the posterior expression of *gt*. This is likely because if maternal *hb* is removed entirely, normal development can still ensue (Lehmann and Nüsslein-Volhard, 1987). Though it has not been shown directly, this implies that *gt* expression in these embryos is normal and not ubiquitous. Some other maternal factor, in addition to maternal *hb*, must therefore inhibit *gt* expression in the anterior two thirds of the embryo. A likely candidate for this inhibitor is the *bcd* product which would then have a positive effect on the anterior regulatory element of *gt* and a negative effect on the posterior regulatory element. That this may be the case is also suggested by the fact that a consequence of the lack of maternal *bcd* is the broadening and anterior shift of the entire posterior stripe of *gt* expression (Fig. 4A) as well as of *kni* and *Kr* expression (Hülskamp *et al.* 1990). The result of this contrasting effect on anterior and posterior gap gene expression is to cause both the loss of anterior structures and the anterior shift of the rest of the pattern.

The gradual withdrawal of *gt* stripe 4 from the posterior, which occurs at the end of nuclear cycle 13, is clearly dependent on the terminal system: it is abolished in *tor* or *tsl* embryos. The effect of *tor* is mediated in part through the zygotic gap genes *tl* and *hkb*, as soon as their products begin to accumulate. In part, however, the withdrawal of stripe 4 is caused by the appearance of the posterior stripe of zygotic *hb*, which exerts a repressive effect on posterior *gt* expression.

#### Interactions with other gap genes

While maternal mutations can abolish *gt* expression in the anterior (*bcd*) or posterior (*nos*) domains, none of the known gap genes is required for initiating *gt* expression. This strongly suggests that *gt* is a direct interpreter of maternal cues and confirms the status of *gt* as a gap gene. In typical gap gene fashion, *giant* enters into a network of cross-regulatory interactions with other members of the gap gene class. To understand the interactions of *gt* with other gap genes,

it is once again important to consider the different stripes of *gt* expression as independently controlled. It is also important to bear in mind that, because of the complex cross-regulatory interactions between genes of this class, it has been difficult to distinguish direct effects of one gene product on the expression of another and indirect effects mediated by other members of the group. In many cases, molecular experiments will be necessary to establish the direct regulatory relationships. As already discussed, *hb*, both maternal and zygotic, plays a major role in inhibiting or sharpening the expression of stripe 4 but our results show that it has little effect on the anterior *gt* stripes. At the same time, *gt* has little effect on *hb* expression. *Kr* clearly has a strong negative effect on the *gt* posterior stripe and *gt* in turn has a negative effect on *Kr*. This negative effect of *gt* on *Kr* is not very visible under normal conditions, as found also by Gaul and Jäckle (1987), not because it is not strong but probably because *Kr* is also repressed by *kni* at its posterior border and by *hb* at its anterior border. Hence, in *gt* mutants, the expansion of the *Kr* domain is immediately checked by the presence of *kni* and *hb* immediately flanking it. However, the strong repressing effect of *gt* on *Kr* is evident when *gt* is expressed ectopically under the control of the heat shock promoter (Kraut and Levine, 1991; M. Capovilla, E. Eldon and V. Pirrotta, unpublished data). The anterior border of *Kr* is therefore set by *hb* repression and also by *gt* anterior expression, as hypothesized by Hülskamp *et al.* (1990). This is yet another case in which expression boundaries are multiply determined.

We found very little effect of *kni* on *gt* expression in the posterior domain but strong repressive effects of *gt* on the *kni* posterior stripe. The interactions of *gt* and *Kr* and of *gt* and *kni* may help to explain the apparent activating effect of *Kr* on *kni* expression as caused indirectly by *Kr* repression of *gt*. This negative role of *Kr* is more consistent with the demonstration that *Kr* protein acts as a transcriptional repressor (Licht *et al.* 1990). However, *Kr* may also interact directly with *kni* as suggested by Pankratz *et al.* (1989) who have identified a binding site for *Kr* protein in the *kni* regulatory region. The terminal gap genes *tl* and *hkb* appear to have negative effects on the other gap genes, both at the anterior and at the posterior end, with the exception of *hb* whose late-appearing posterior stripe actually requires terminal gene function (Tautz, 1988). Although no direct data are yet available, it is possible that *gt* in turn represses *tl* since the initial expression of *tl* over a broader posterior domain recedes to a posterior cap by NC14 (Pignoni *et al.* 1990). Failure to reduce *tl* posterior expression in a *gt* mutant might perhaps account for the appearance of secondary filzkörper in the abdominal region, noted by Petschek *et al.* (1987) and Mohler *et al.* (1989).

The domains of expression of the gap genes are set by a network of mutual interactions and their boundaries are often multiply determined by the maternal morphogens, the neighboring gap genes as well as the non-adjacent gap genes. These interactions are essential to account for the stability and robustness of the patterns

of expression and probably for the broad and graded morphogenetic effects that result from gap mutations. Accordingly we can interpret the extensive defects seen in *Kr* mutations as due not only to absence of *Kr* but also to the resulting expansion of *gt* stripe 4, which invades and overruns the *kni* domain and most of the *Kr* domain. In contrast, the more restricted effects of *gt* mutations in the abdomen could be attributed to the fact that the posterior expansion of *kni* is limited by the *hb* and *tl* domains.

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