The *Drosophila* Gap Gene *giant* Has an Anterior Segment Identity Function Mediated Through *disconnected* and *teashirt*

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ABSTRACT

The C2H2 zinc-finger-containing transcription factors encoded by the *disconnected* (*disco*) and *teashirt* (*tsh*) genes contribute to the regionalization of the *Drosophila* embryo by establishing fields in which specific Homeotic complex (Hom-C) proteins can function. In *Drosophila* embryos, *disco* and the paralogous *disco-related* (*disco-r*) are expressed throughout most of the epidermis of the head segments, but only in small patches in the trunk segments. Conversely, *tsh* is expressed extensively in the trunk segments, with little or no accumulation in the head segments. Little is known about the regulation of these genes; for example, what limits their expression to these domains? Here, we report the regulatory effects of gap genes on the spatial expression of *disco*, *disco-r*, and *tsh* during *Drosophila* embryogenesis. The data shed new light on how mutations in *giant* (*gt*) affect patterning within the anterior *gt* domain, demonstrating homeotic function in this domain. However, the homeosis does not occur through altered expression of the Hom-C genes but through changes in the regulation of *disco* and *tsh*.

THE redundant, paralogous genes *disconnected* (*disco*) and *disco-related* (*disco-r*), referred to together as the *disco* genes below, and *teashirt* (*tsh*) are differentially expressed in the embryonic head and trunk segments and are therefore markers for head and trunk segment types. In the head segments the *disco* genes are required for the proper development of the larval mouthpart structures (MAHAFFEY *et al.* 2001; ROBERTSON *et al.* 2004), while in the trunk segments, these genes are necessary for development of the Keilin's organs, small thoracic sensory structures and some peripheral neurons (ROBERTSON *et al.* 2004; PATEL *et al.* 2007). By contrast, *tsh* is necessary for proper development of most of the ventral trunk epidermis (FASANO *et al.* 1991; RÖDER *et al.* 1992; DE ZULUETA *et al.* 1994). Both the *disco* genes and *tsh* are also members of the proximal-distal patterning network (ERKNER *et al.* 1999; WU and COHEN 2000; AZPIAZU and MORATA 2002; PATEL *et al.* 2007). The *disco* and *tsh* genes encode C2H2 zinc-finger transcription factors that are expressed early in embryonic development with precise, nearly reciprocal expression patterns in the trunk and head segments, but not much is known as to how these patterns are established. What is known is that ectopically expressing *tsh* in the head segments converts the expression of the

disco genes to a trunk-like pattern. The Spalt major (Salm) protein represses *tsh* expression in the posterior labial segment (KUHNLEIN *et al.* 1997), but otherwise little is known regarding the regulation of *disco* and *tsh*—in particular, what factors distinguish the head and trunk modes of expression. The gap genes are logical candidates for this role.

Patterning the *Drosophila* embryo involves initial establishment of the axes, regionalization of the embryo, definition of the segments and their polarity, and the specification of unique identities to each segment. The early acting components of this genetic cascade include both maternally and zygotically expressed genes that set in motion the segmentation and segment identity processes. The gap genes are among the earliest zygotic factors involved in these processes. Regulated by maternal morphogens in the blastoderm, and by one another, these genes act via overlapping gradients to divide the embryo into broad regions and to regulate the expression of downstream segmentation genes (see reviews in ST. JOHNSTON and NUSSLEIN-VOLHARD 1992; PANKRATZ and JACKLE 1993; RIVERA-POMAR and JACKLE 1996; NIESSING *et al.* 1997; SANSON 2001).

Comparative studies in other insects have revealed significant conservation in the function of many segmentation genes (see reviews in FRENCH 2001; DAVIS and PATEL 2002; HUGHES and KAUFMAN 2002), but less clear is the functional conservation of the gap genes between insect species. Two studies—one in *Tribolium castaneum*, examining a *giant* (*gt*) homolog (*Tc'giant*) (BUCHER and KLINGLER 2004), and one in *Oncopeltus fasciatus*, examining a *hunchback* (*hb*) homolog (*Of'hb*)

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(LIU and KAUFMAN 2004)—conclude that the function of these gap genes is one of segmentation and segment identity, differing somewhat from the segmentation function characterized in *Drosophila*. The authors further state that this difference is likely due to the differential patterning of long *vs.* short germ-band insect embryos. The results presented here indicate that *Drosophila gt*, in fact, has an embryonic segment identity role similar to that observed in *Tc'giant*. Surprisingly, this identity function arises not only through changes in homeotic (*Hom-C*) gene expression, but also from the regulation of *disco* and *tsh*. This, in conjunction with other gap genes, defines the position of the embryonic head and trunk segment types.

MATERIALS AND METHODS

Drosophila stocks and culture: Flies were reared on standard cornmeal-agar-molasses medium. The *giant*^{Q242} (*gt*^{Q242}), *giant*^{X11} (*gt*^{X11}), *hunchback*¹² (*hb*¹²), *Krüppel*² (*Kr*²), *caudal*³ (*cd*³), *tailless*¹ (*tl*¹), *huckebein*² (*hkb*²), *salm*¹, and *Antennapedia*²⁵ (*Antp*²⁵) lines were obtained from the Bloomington, Indiana, *Drosophila* Stock Center, and the *tsh*⁸ line from S. Kerridge (CNRS Marseille, France). The double-mutant embryos examined were obtained by crossing *gt*^{Q242}/*FM7c* or *gt*^{X11}/*FM7c* females to *Antp*²⁵/*TM3*, *salm*¹/*CyO*, or *tsh*⁸/*CyO* males. Virgin female progeny heterozygous for the *gt* allele and *Antp*²⁵, *salm*¹, or *tsh*⁸ were then crossed to *Antp*²⁵/*TM3*, *salm*¹/*CyO*, or *tsh*⁸/*CyO* males, respectively, to yield the genotypes of interest—*gt/y*; *Antp*²⁵/*Antp*²⁵, *gt/y*; *salm*¹/*salm*¹, or *gt/y*; *tsh*⁸/*tsh*⁸.

Cuticle analysis: Embryos were collected at 25° and prepared for cuticle examination as previously described (PEDERSON *et al.* 1996). Females were allowed to lay eggs for up to 24 hr, and embryos were aged for at least 24 hr at 25° before the mounting of unhatched terminal larvae.

In situ localization of mRNA and protein: Colorimetric localization of mRNA and protein followed the protocols essentially as described in PEDERSON *et al.* (1996). The antisense digoxigenin-labeled RNA probes for *disco* and *tsh* were prepared as previously described (MAHAFFEY *et al.* 2001; ROBERTSON *et al.* 2004). Fluorescent *in situ*s were prepared as described in PATEL *et al.* (2007), and images were acquired using a Zeiss Pascal laser-scanning microscope. For the *pb* mRNA localization, the probe template was amplified from *Drosophila* genomic DNA using PCR with the primers ATCG TGTC AAGGCTGCGAA (forward) and TAAGCCCGACATT CATTGGC (reverse).

Identification of mutant embryos and cuticles: Whole embryos lacking a functional gap gene allele were identified either by the alteration of anti-Invected/Engrailed staining or by aberrant segment morphology. The loss of *gt*, *hb*, or *Kr* in cuticle preparations was evident by denticle belt alterations. Homozygous *Antp*²⁵ cuticles were identified by the transformation of the T2 and T3 denticle belts toward T1. For the experiments presented in Figure 6, double-mutant embryos were identified by the *gt* abdominal phenotype and detection of either *tsh* mRNA or Antp protein as described in the Figure 6 legend.

RESULTS

***disco* mRNA distribution is altered in gap mutants:** To explore the regulation of the *disco* gene during embryogenesis, we examined *disco* mRNA accumulation

in homozygous gap mutant embryos. *disco* is normally expressed in the clypeolabrum, the optic lobe region, the antennal segment, the gnathal segments (mandibular, maxillary, and labial), the embryonic leg primordia, and transiently in similar positions in the abdominal segments and the proctodeum (Figure 1, A and B; see LEE *et al.* 1991; MAHAFFEY *et al.* 2001). Five homozygous gap gene mutants exhibited altered *disco* mRNA distribution—*hunchback* (*hb*), *Krüppel* (*Kr*), *giant* (*gt*), *tailless* (*tl*), and *caudal* (*cad*); the results are summarized in Table 1. Of these, *hb*, *Kr*, and *gt* affected the gnathal/thoracic *disco* expression domains. As noted above, a redundant paralog of *disco*, *disco-r*, is present in the *Drosophila* genome. *disco-r* mRNA accumulation was examined in *hb*¹², *Kr*², *gt*^{Q292}, and *gt*^{X11} mutant embryos. Alterations in *disco-r* expression mirrored those of *disco* (data not shown). Because the effects on *disco* and *disco-r* mRNA accumulation appeared to be identical, our remaining studies focused on the regulation of *disco*. We noted that *gt* mutations had a particularly interesting effect, indicating a central role for *gt* in *disco* regulation and possibly head-trunk boundary formation. Therefore, we will concentrate on *gt* for the rest of our description. Indeed, the effects of *hb* and *Kr* could be interpreted through their known cross-regulation of *gt* (HULSKAMP *et al.* 1990; ELTON and PIRROTTA 1991; KRAUT and LEVINE 1991a,b; WU *et al.* 2001).

We examined *disco* expression in two *gt* alleles, *gt*^{X11} and *gt*^{Q292}. Both alleles are described as amorphic (PETSCHKE *et al.* 1987); however, as we discuss below, we suspect that *gt*^{Q292} is a strong hypomorphic allele, an important distinction with respect to this study. *disco* mRNA accumulation was significantly reduced in the labial segments of *gt*^{Q292} embryos. In the labial segments of early embryos, *disco* expression was reduced from encompassing the majority of the epidermis to a small patch in the dorsomedial portion of the lobe (Figure 1, C and D). At later stages, little or no *disco* mRNA was detected in the labial region (Figure 2, C and D). What remained resembled the thoracic appendage primordia. *disco* mRNA was also detected in portions of the maxillary segments. Similar effects on *disco* mRNA accumulation were noted in hemizygous *gt*^{X11} embryos (Figure 1, E and F; Figure 2B).

***tsh* mRNA distribution is altered in gap mutants:** Earlier work demonstrated that Tsh represses *disco* expression in the trunk and that ectopic Tsh in the gnathal segments represses and remodels the expression of the *disco* genes (ROBERTSON *et al.* 2004). Therefore, we suspected that the alterations in *disco* and *disco-r* expression in *gt* mutants might be mediated through altered *tsh* expression. To test this, we examined *tsh* expression in *gt* embryos and observed that *tsh* mRNA accumulation was altered in a nearly a reciprocal manner to the changes in *disco* mRNA distribution.

In mid-stage 11, wild-type embryos, when the gnathal segments are evident, *tsh* mRNA accumulates in the first

FIGURE 1.—*disco* and *tsh* mRNA localization is affected by *gt* mutations. (A, C, E, G, I, and K) Stage 11 to early stage 12 embryos (CAMPOS-ORTEGA and HARTENSTEIN 1997). (B, D, F, H, J, and L) Late stage 12 or stage 13 embryos. (A and B) In wild-type embryos, *disco* expression is normally present in the gnathal (mandibular, maxillary, and labial) segments, as well as in the embryonic leg primordia, optic lobe, clypeolabrum, antennal segment, and the proctodeum. (C and D) In hemizygous *gt*^{Q292} embryos, the labial domain of *disco* expression is significantly reduced (white arrows). As the germ band retracts (D), labial expression of *disco* continues in the dorsomedial portion of the labial lobe in these mutant embryos. (E and F) Hemizygous *gt*^{X11} embryos exhibit alterations in *disco* expression similar to those observed in hemizygous *gt*^{Q292} individuals; however, the loss *disco* expression in the labial segment is more extensive. (G and H) In wild-type embryos, *tsh* mRNA accumulates in the epidermis of the first thoracic through eighth abdominal segments. (I and J) In *gt*^{Q292} mutant embryos, *tsh* expression expands through the labial segment (white arrows) and often occurs in a stripe at the mandibular/maxillary border (white arrowhead). (K and L) Hemizygous *gt*^{X11} embryos exhibit altered *tsh* mRNA accumulation very similar to that observed in *gt*^{Q292} individuals, although the appearance of *tsh* expression in the anterior maxillary segment occurs more frequently.

thoracic through the eighth abdominal segments (FASANO *et al.* 1991; RÖDER *et al.* 1992), and this is maintained throughout remaining embryogenesis (Figure 1, G and H). The change in *disco* mRNA accumulation in the labial segment of *gt* mutant embryos was reminiscent of the change in *disco* caused by the ectopic *tsh* expression (ROBERTSON *et al.* 2004). Indeed, we found that *tsh* mRNA accumulates throughout the labial segment of *gt* mutants (Figure 1, I–L). Ectopic maxillary expression was observed in some individuals, primarily localized to the anterior edge of the segment. We also noted an affect on the parasegmental modulation of *tsh* in the trunk.

To further observe the association between *disco* and *tsh* expression in *gt* mutants, we fluorescently labeled probes to detect both mRNAs simultaneously (Figure 2). In the wild-type embryos before (data not shown) and after (Figure 2A) germ-band retraction, there was no colocalization of *disco* and *tsh* mRNAs at the labial/first thoracic border. Coexpression was observed in the appendage primordial of the trunk. By contrast, in

embryos hemizygous for *gt* mutations, there was mixed and overlapping *disco* and *tsh* expression in the head. This was noted in the labial segment and somewhat in the maxillary (Figure 2, B–D). Overlapping expression in the maxillary was transient, while in *gt*^{Q292} embryos coexpression persisted in the aberrant labial segment. This labial expression appeared similar to that in the thoracic appendage primordial (Figure 2D). After germ-band retraction, there was no colocalization in the maxillary region (Figure 2, B and D). As in wild-type embryos, colocalization was observed in the trunk appendage primordial of all *gt* embryos.

Cuticle phenotype of *gt* mutants: *disco*, together with Sex combs reduced (Scr), directs a labial identity (ROBERTSON *et al.* 2004), while *tsh* acts with Scr to properly pattern the first thoracic segment (FASANO *et al.* 1991; RÖDER *et al.* 1992; ANDREW *et al.* 1994; DE ZULUETA *et al.* 1994; TAGHLI-LAMALLEM *et al.* 2007). It seemed possible that, in the labial segment of the *gt*^{Q292} mutants, loss of *disco* with concomitant ectopic expression of *tsh*

TABLE 1
The effects of examined genes on *disco* mRNA accumulation

Gene	Effect on <i>disco</i> expression						
	Mandibular	Maxillary	Labial	First thoracic	Second thoracic	Third thoracic	Posterior domain
<i>giant</i> (<i>gt</i>)	0/— ^a	0/— ^b	—/— ^c	0	0	0	0
<i>hunchback</i> (<i>hb</i>)	0	0	—	—/— ^d	—/—	—/—	0
<i>Krüppel</i> (<i>Kr</i>)	0	0	0	—/+ ^e	—	—	0
<i>Knirps</i> (<i>Kni</i>)	0	0	0	0	—	—	0
<i>tailless</i> (<i>tll</i>)	0	0	0	0	0	0	—
<i>caudal</i> (<i>cad</i>)	0	0	0	0	0	0	—
<i>huckabein</i> (<i>hkb</i>)	0	0	0	0	0	0	0
<i>buttonhead</i> (<i>btd</i>)	0	0	0	0	0	0	0

—, complete loss of *disco* expression; —, decrease of *disco* expression; 0, no effect on *disco* expression; +, increase in *disco* expression.

^aThe effect on *disco* expression is allele dependent. *gt*^{Q292} homozygotes exhibit no effect on mandibular expression. *gt*^{X11} homozygotes occasionally exhibit some loss of mandibular expression.

^bThe effect on *disco* expression is allele dependent. *gt*^{Q292} homozygotes exhibit no effect on maxillary expression. *gt*^{X11} homozygotes exhibit some loss of maxillary expression.

^cThe effect on *disco* expression is allele dependent. *gt*^{Q292} homozygotes exhibit less reduction of *disco* expression than that observed in *gt*^{X11} homozygotes.

^dThe effect on thoracic *disco* expression is variable—from a decrease in expression to the complete loss of expression.

^eThe effect on the first thoracic *disco* expression combines both a gain and a loss. The normal expression in the embryonic leg primordia is lost, and a dorsal thoracic domain of *disco* expression (reminiscent of the labial segment) is gained.

might cause a transformation of the labial segment to a first thoracic identity. This would imply that at the gnathal/trunk border *gt* has a segment identity role in addition to a segmentation role. Further supporting this hypothesis was the remodeling of *disco* expression in the labial segment of *gt*^{Q292} mutants to resemble normal thoracic *disco* expression (Figure 1, C and D, and Figure 2D). Therefore, we next closely reexamined the *gt* mutant cuticles with particular attention to the gnathal and anterior trunk segments.

Overall, the gross morphology of both *gt* genotypes was quite similar, and mainly as previously reported (PETSCHKE

et al. 1987; PETSCHKE and MAHOWALD 1990). Terminal larval cuticles displayed defects in head involution, giving the embryo a “buttonhead”-type appearance (Figure 3, B and C). However, careful examination of the dorsal cuticle in both genotypes revealed evidence of a homeotic transformation. Ectopic thoracic-like hairs were present anterior to the normal rows of first thoracic dorsal hairs (Figure 3, H and I), which likely originate from the dorsal ridge. The appearance of dorsal hairs in the gnathal region has previously been interpreted to indicate a transformation of these segments toward a thoracic identity (JÜRGENS 1988; DE ZULUETA *et al.* 1994).

FIGURE 2.—Overlap in *disco* and *tsh* mRNA accumulation in the labial segment of *gt* mutant embryos. Embryos were double labeled to detect *disco* (green) and *tsh* (red) mRNAs. (A) A wild-type embryo after germ-band contraction (early stage 13). No overlap of *disco* and *tsh* was noted in the head region, although there was overlapping expression in the limb primordium of the thorax (arrows). (B) In similar staged *gt*^{X11} individuals, little overlap was detected, although at earlier stages there was significant overlap in the maxillary region (data not shown). Note the greater width of the first thoracic segment, T1, in the *gt* mutant. An asterisk

marks a remaining bit of the labial segment in this embryo that has not fused with first thoracic. (C) Overlapping expression of *disco* and *tsh* in the labial region of an early stage 12 *gt*^{Q292} embryo. Note that in later stage *gt*^{Q292} embryos (D), *tsh* mRNA was still present in the transformed labial lobe (T1*), which resembles the limb primordium of the thoracic segments. The arrowhead marks where *disco* mRNA accumulation resembles that of the normal thoracic segments. Mn, mandibular; Mx, maxillary; Lb, labial; T#, first through third thoracic segments; T1*, transformed labial segment.

FIGURE 3.—Cuticle analysis of hemizygous *gt* mutant embryos reveals homeosis of the labial segment. Cuticles of wild-type (A, D, and G), *gt*^{Q292} (B, E, and H), and *gt*^{X11} (C, F, and I) terminal larvae. Hemizygous *gt* mutant embryos (B and C) display defects in the ventral denticle patterning. The fifth-through-seventh abdominal denticle belts are missing or fused. In some *gt*^{Q292} embryos (B), the labial segment is transformed to a first thoracic identity. In D, E, and F, the solid arrows point to the normal anterior first thoracic denticle belt. The solid arrowheads point to the normal posterior first thoracic denticle belt, or “beard.” At high magnification, a duplication of the first thoracic denticle belt is evident in occasional *gt*^{Q292} individuals (E). Open arrows point to the ectopic anterior first thoracic denticle belt. The open arrowheads point to the ectopic “beard.” More commonly, the anterior first thoracic denticle belt is split at the ventral midline as in *gt*^{X11} (F). An alteration in the pattern of the dorsal hairs is also evident (H and I), with ectopic patches of hairs (open arrowheads) present anterior to the normal first thoracic segment hairs (solid arrows). mh, mouth hooks; ci, cirri; T#, thoracic segment; A#, abdominal segment; T1', segment resembling first thoracic anterior to the normal first thoracic segment; Lb/T1, fusion of the labial and first thoracic segments.

The denticle pattern of the ventral cuticle also provided indications of a transformation. Most *gt* mutant embryos (both alleles) appear to have a fusion of the labial and thoracic segments with labial tissues developing as first thoracic, yielding an enlarged first thoracic segment. In these, the first thoracic denticle belt appeared to form at the labial/maxillary border (Figure 3F), given its proximity to the maxillary-derived cuticular structures (the cirri and mouth hooks). In some instances, the anterior denticle row of the first thoracic segment was directly adjacent to the maxillary cirri. This denticle belt was frequently “split” at the ventral midline (Figure 3F), which could be a consequence of incomplete fusion between the labial and first thoracic segments (discussed further below). These observations

are compatible with the loss of the labial segment, which was previously reported (PETSCHER *et al.* 1987; PETSCHER and MAHOWALD 1990). An additional observation, however, strongly supported a labial/first thoracic fusion and concomitant segment identity change in the labial segment. Some *gt*^{Q292} individuals ($n = 30$ of 138) had obvious homeosis of the labial segment to yield two nearly complete first thoracic segments. In these individuals, the labial and thoracic segments did not fuse, and a complete duplication of the first thoracic denticle belt was present in the transformed labial segment (Figure 3, B and E). We did not observe this unequivocal transformation of the labial segment in any hemizygous *gt*^{X11} cuticles examined ($n = 115$); it was observed only in *gt*^{Q292} individuals.

FIGURE 4.—Varying degrees of labial/first thoracic segment fusion as indicated by α -Engrailed/Invected staining of *gt* mutant embryos. In wild-type embryos (A, D, G, and J), En protein accumulates a striped pattern in the posterior compartment of each segment. In *gt*^{Q292} embryos (B, E, H, and K), En accumulation is reduced in the labial segment (Lb) and is severely altered in the fifth-through-seventh abdominal segments (not shown), while it appears somewhat expanded in the posterior maxillary segment (Mx). Labial En accumulation is variable in *gt*^{Q292} embryos (compare H and K). The amount of En accumulation remaining in the posterior labial segment appears to correspond to the location of and degree of segment border formation between the labial and first thoracic segment. Arrowheads point to the residual segmental grooves between these two segments. The dorsal ridge usually remains visible in these embryos, but is frequently fused, at least in part, to the dorsal labial segment. In *gt*^{X11} embryos (C, F, I, and L), the labial En stripe is more consistently and more completely lost. Correspondingly, the fusion between the labial and first thoracic segment is virtually complete. The dorsal ridge is quite reduced or absent in these individuals. DR, dorsal ridge; Mn, mandibular; Mx, maxillary; Lb, labial; T#, first through third thoracic segments.

Segment border formation in the two *gt* mutants:

The differences in the labial/first thoracic fusion between the two *gt* genotypes prompted us to closely examine Engrailed (En) protein distribution. In wild-type embryos, En accumulates in the posterior of each gnathal and trunk segment (DINARDO *et al.* 1985) and thus provides a good marker for segmentation and segment morphology. Further, LARSEN *et al.* (2003) demonstrated the integral role of *en*-expressing cells in the morphological changes accompanying segment groove formation. It was previously reported that, in homozygous *gt* mutants, the third (labial) En stripe is severely reduced, and this was interpreted to indicate that the posterior compartment of the labial segment was deleted (PETSCHKE and MAHOWALD 1990). We found that the loss of En staining was primarily in the lateral and ventral portions of the labial segment in both *gt* mutant genotypes, but there was significant variation as to the extent of the loss. While most *gt*^{Q292} embryos displayed nearly complete loss of the lateral and ventral En accumulation (Figure 4H), some embryos maintained significant En protein in the posterior labial segment (Figure 4, E and K). In these individuals, we noted that a portion of the dorsal ridge was fused with the labial segment to form a contiguous segment that

appeared thoracic-like. Any portion of this extended labial segment lacking En appeared to fuse with the normal first thoracic segment, but the regions accumulating En protein remained separated (see arrowheads in Figure 4 and compare Figure 4, H and K). *gt*^{X11} embryos displayed a consistent and nearly complete loss of En protein in the posterior labial segment (Figure 4, C, F, I, and L), and fusion between the labial and first thoracic segments generally appeared complete. In light of the results from LARSEN *et al.* (2003) demonstrating the integral role of En in segment border formation, we conclude that the persistent accumulation of En protein in some hemizygous *gt*^{Q292} embryos explains the retention of a labial/first thoracic segment border in these individuals. The presence of this segment border likely contributes to the formation of a separate ectopic first thoracic-like denticle belt in the transformed labial segment in some *gt*^{Q292} embryos. Thus, the variable loss of labial En expression provides an explanation for the difference in cuticle phenotype between the two *gt* genotypes that we examined.

Homeotic gene expression in *gt* mutant embryos:

The cuticle results and the alterations in *disco* and *tsh* expression observed in *gt* mutant embryos indicated that the labial segment was homeotically transformed

FIGURE 5.—Alterations in *Hom-C* gene. Expression in *gt^{Q292}* mutant embryos indicates a homeotic change in labial identity (A). *Dfd* protein is detected in the mandibular lobe, the maxillary lobe, and the anterior dorsal ridge in wild-type embryos and is essentially unchanged in *gt^{Q292}* embryos (B). No *Dfd* protein is detectable in the portion of the dorsal ridge that fuses to the labial segment. When compared to wild type (C), *pb* mRNA accumulation is significantly reduced in the labial segment of *gt^{Q292}* embryos (E). *Scr* protein normally accumulates in the labial lobe and the first thoracic segment in wild-type embryos. In *gt^{Q292}* embryos (F), *Scr* protein accumulates throughout the fused labial/dorsal ridge, such that the pattern of accumulation mimics that observed in the first thoracic segment. The anterior limit of *Antp* in the wild-type embryo is the posterior half of the first thoracic segment (G), while *Antp* protein accumulates through much of the labial epidermis in *gt^{Q292}* embryos (H). DR, dorsal ridge; Mn, mandibular; Mx, maxillary; Lb, labial; T1, first thoracic segment; T3, third thoracic segment.

toward a first thoracic identity. The differential expression of the *Hom-C* genes guides unique and specific segment identities in the developing *Drosophila* embryo (reviewed in ROBERTSON and MAHAFFEY 2005). To further assess the identity of the labial segment in *gt^{Q292}* mutant embryos, we examined the protein or mRNA accumulation from four *Hom-C* genes normally expressed in the gnathal or anterior trunk segments—*Dfd*, *proboscipedia* (*pb*), *Scr*, and *Antennapedia* (*Antp*). We chose to examine only *gt^{Q292}* mutant individuals because this allele exhibited a recognizable transformation (on the basis of cuticle phenotype) and suffered less fusion between the labial and first thoracic segments (on the basis of *Inv/En* staining). Further, the expression of these *Hom-C* genes was previously reported in *gt^{X11}* mutant embryos, but not in *gt^{Q292}* (JACK *et al.* 1988; PETSCHKE and MAHOWALD 1990; REINITZ and LEVINE 1990; RUSCH and KAUFMAN 2000).

The *Dfd* protein normally accumulates in the mandibular and maxillary segments and in the anterior dorsal ridge (Figure 5A) and is necessary for the proper embryonic development of the cephalopharyngeal structures derived from these regions (MERRILL *et al.* 1987; REGULSKI *et al.* 1987). As with previous studies of *gt^{X11}* (JACK and MCGINNIS 1990; REINITZ and LEVINE 1990), we observed no change in *Dfd* staining in *gt^{Q292}* (Figure 5B). The retention of *Dfd* protein accumulation suggests that there was no homeotic transformation of the mandibular segment, maxillary segment, or in the anterior portion of the dorsal ridge.

pb mRNA normally accumulates in the maxillary and labial segments (Figure 5C) (PULTZ *et al.* 1988). Because *tsh* was previously described as a negative regulator of *pb* in the first thoracic segment (RUSCH and KAUFMAN 2000), we expected a reduction in *pb* mRNA accumulation in *gt* mutants in response to the anterior expansion of *tsh* in these individuals. Indeed, in *gt^{Q292}* mutant embryos, *pb* accumulation was slightly reduced in the maxillary segment and significantly reduced in the labial (Figure 5D). The affected region coincided with ectopic *tsh* expression. *pb* transcripts were present only in the dorsal posterior portion of the labial/dorsal ridge. Loss of *pb* from the labial lobe supports a transformation of this segment from a gnathal toward a trunk-like identity.

In wild-type embryos, *Scr* protein accumulates first throughout the labial segment and then in the anterior first thoracic segment (Figure 5E). Early *Scr* protein accumulation was reported to be altered in *gt* mutant embryos (HIROMI *et al.* 1985; REINITZ and LEVINE 1990). We, however, found no evidence of significantly altered *Scr* accumulation in *gt^{Q292}* embryos. As the germ band extended and the gnathal lobes began to form, *Scr* protein accumulated throughout the developing labial segment. The only detectable change was a slight decrease in staining intensity in the ventral portion of the segment (data not shown). Yet, as the germ band contracted and the labial segment and dorsal ridge fused to take on a thoracic-like morphology, *Scr* protein accumulation was concentrated in the anterior portion of the fused labial/dorsal ridge, mimicking the pattern

in the adjacent first thoracic segment (Figure 5F). The expansion of Scr protein into the portion of the dorsal ridge that fuses with the labial segment in *gt^{Q292}* embryos is consistent with transformation to a first thoracic identity. Occasionally, a persistent lobe-like protrusion was observed in the anterior labial segment of germ-band-retracted embryos, which continued to retain a strong labial-like Scr pattern (data not shown).

The initial accumulation of the Antp protein in wild-type embryos is parasegmental and it quickly resolves to include the posterior first thoracic segment and all of the second and third thoracic ectoderm (CARROLL *et al.* 1986). When we examined the localization of the Antp in *gt^{Q292}* embryos, we observed ectopic Antp accumulation in the labial segment epidermis, as previously described for *gt^{X11}* and *gt^{VA82}* embryos (PETSCHKE and MAHOWALD 1990; REINITZ and LEVINE 1990; RILEY *et al.* 1991).

The presence of Antp in the labial segment of *gt* mutants could be significant for two reasons. First, Antp was previously shown to activate *tsh* expression in the gnathal segments when ubiquitously expressed (RÖDER *et al.* 1992). Thus, this ectopic Antp protein might be responsible for the presence of *tsh* mRNA in the labial segments of *gt* mutant embryos (discussed further below). Second, the presence of Antp in the labial segment, along with *tsh* and Scr, supports a transformation of the labial segment toward a first thoracic identity, as this combination of factors is also present in the wild-type first thoracic segment.

***tsh* is required for the gnathal-to-trunk transformation in *gt* mutants:** Because *tsh* is required for proper trunk specification (FASANO *et al.* 1991; RÖDER *et al.* 1992; DE ZULUETA *et al.* 1994), and ectopic Tsh can transform the gnathal segments toward a trunk identity (JÜRGENS 1988; DE ZULUETA *et al.* 1994; ROBERTSON *et al.* 2004), we suspected that embryos doubly mutant for *gt* and *tsh* might reduce or eliminate the labial-to-first-thoracic transformation. Therefore, we examined cuticles from embryos lacking both *tsh* and *gt* and indeed observed no evidence of dorsal or ventral labial-to-thoracic transformation (data not shown). We detected no ectopic thoracic-like hairs in the dorsal cuticle anterior to the first thoracic segment, and no cuticles were found to have fused or partially fused labial/first thoracic denticles or ectopic first thoracic denticles in the ventral cuticle. The interpretation of these results, however, was confounded by the effects of the loss of *tsh*. Head involution was severely disrupted and there was frequently a hole in the dorsal cuticle anterior to the first thoracic segment, making a definitive assessment of dorsal transformation on the basis of dorsal cuticle patterning difficult. Further, the ventral denticle belts were quite reduced, particularly in the thoracic segments, making clear identification of a labial-to-first-thoracic transformation problematic.

To clarify the effect of removing both *tsh* and *gt*, we examined the mRNA accumulation of two gnathal

factors that are altered in *gt* mutants. As described above, *disco* and *pb* transcript abundance was quite reduced in the labial segment of *gt^{Q292}* mutant embryos (Figure 1D and Figure 5D, respectively), while *tsh* mRNA spread anteriorly to include the labial segment (Figure 1I; Figure 2, B and D). If ectopic *tsh* were responsible for the homeotic transformation of the labial segment in *gt^{Q292}* mutant embryos, then we might expect some recovery of normal *disco* and *pb* expression in *gt*; *tsh* double mutants. Indeed, we observed significant recovery of the normal *disco* expression (Figure 6B). *disco* transcripts were detected throughout most of the labial segment. The morphology of the labial segment appeared more lobe-like, and the dorsal ridge was fully separated from the labial lobe. *disco* expression did not fully return to normal, however. The loss of *tsh* alone results in ectopic *disco* transcript accumulation in the ventrolateral regions of the thoracic and abdominal segments (ROBERTSON *et al.* 2004), and this was present in the *gt^{Q292}*; *tsh⁸* double mutants. Further, ectopic *disco* transcripts accumulated strongly at the anterior edge of the first thoracic segment, which is not normally observed in *tsh* mutant embryos. *pb* mRNA accumulation also recovered much of its normal expression in the labial segment in embryos lacking both *gt* and *tsh* (data not shown).

Antp is not required for the ectopic expression of *tsh* in the gnathal segments of *gt^{Q292}* embryos: Antp is required for high-level expression of *tsh* in the thoracic epidermis of the *Drosophila* embryo, and ectopic expression of Antp activates a *lacZ* reporter element derived from the upstream regulatory region of the *tsh* gene (RÖDER *et al.* 1992; MCCORMICK *et al.* 1995). It seemed possible, therefore, that the ectopic activation of Antp in the labial segment of hemizygous *gt^{Q292}* mutant embryos was responsible for the ectopic accumulation of *tsh* and the corresponding loss of *disco*. However, it was also possible that *gt* was affecting *tsh* directly or through another pathway. To address this, we examined *tsh* mRNA accumulation in embryos hemizygous for the *gt^{Q292}* and homozygous for *Antp²⁵* (Figure 6D). *tsh* expression was significantly reduced in the thoracic segments, as would be expected, but the ectopic expression of *tsh* in the gnathal segments persisted (arrows in Figure 6D). Thus, the anterior expansion of Antp into the gnathal segments was not required for ectopic activation of *tsh* and the change in morphology of the labial segment. We note that the reciprocal was also true. Antp protein accumulated in the labial segment of *gt^{Q292}/y*; *tsh⁸/tsh⁸* mutant embryos (Figure 6F) as with embryos lacking only *gt* (Figure 6E). The continued presence of Antp in the labial segment of *gt^{Q292}*; *tsh⁸* embryos supports the conclusion that *tsh* is required for the labial-to-first-thoracic transformation in *gt* mutants, as the remaining ectopic Antp was unable to cause the labial-to-thoracic transformation.

FIGURE 6.—*tsh* is required for homeosis of the labial segment in *gt*^{Q292} mutants. Embryos lacking both a functional *gt* allele and a functional *tsh* allele recover significant *disco* expression in the labial segment (B) when compared to embryos lacking only *gt* (A). Additionally, the labial segment recovers its normal lobe-like morphology and the dorsal ridge, although enlarged, is well separated from the labial segment (arrow) in the late stage double mutants (also see F). [In B, both *disco* and *tsh* mRNAs were detected. Embryos lacking both *gt* and *tsh* were identified by the abdominal segment phenotype (*gt*) and lack of *tsh* mRNA.] (C) The anterior expansion of *tsh* mRNA into the gnathal segments of *gt* mutants does not require *Antp*. Embryos doubly mutant for *gt* and *Antp* retain *tsh* transcript accumulation in the gnathal segments (D).

(Embryos doubly mutant for *gt* and *Antp* had the *gt* abdominal segmentation defects and were not positive for *Antp* protein.) The reciprocal is also true. *Antp* protein accumulates in the labial segment of embryos lacking both *gt* and *tsh* (F), as it does in embryos lacking only *gt* (E). (Embryos doubly mutant for *gt* and *tsh* had the *gt* abdominal segmentation defects and were not positive for *tsh* mRNA protein.) DR, dorsal ridge; Mx, maxillary; Lb, labial; T1, first thoracic segment.

DISCUSSION

We draw two significant conclusions from this study: (1) In its anterior expression domain, *gt* acts in both segment identity and segmentation roles, and these two roles are functionally separable; and (2) the distinction between the gnathal and trunk segment types is determined by the gap genes and is reflected by the head and trunk expression patterns of *disco* and *tsh*, which appear to be regulated by a series of repressive interactions (described below).

In addition to segmentation, the anterior *gt* domain functions in segment identity: Our assertion that *gt* acts in both segment identity and segmentation is based upon the following observations:

1. The dorsal cuticle of *gt* embryos displayed ectopic dorsal hair development anterior to the first thoracic segment, and the ventral cuticle of some *gt*^{Q292} individuals displayed first thoracic-like denticle belts in the labial segment. This ventral transformation was masked in some *gt* mutant alleles (*i.e.*, *gt*^{X11}).
2. In many *gt* mutant embryos, the reduction or loss of En protein from the posterior labial segment likely prevented the formation of the labial/first thoracic segmental groove, resulting in the fusion of the two segments and, consequently, the presence of only one organizer for denticle development in the fused segment.
3. In the labial segment of *gt* mutant embryos, the expression of the homeotic genes *Scr*, *Antp*, *disco*, and *tsh* recapitulated their expression in the first thoracic segment, indicating a transformation to a first thoracic identity.
4. In *gt* mutant embryos, expression of the gnathal-specific *Hom-C* gene *pb* is significantly reduced in the

labial segment, indicating a loss of gnathal identity. Such a separation of segmentation and segment identity functions as we describe for the anterior domain of different *gt* alleles was previously observed in the posterior *gt* domain (PETSCHKE *et al.* 1987).

Initial characterizations of the *gt* mutant phenotype, based on SEM studies, described a fusion between the labial, first thoracic, and second thoracic segment, which, later in development, resolved such that the first and second thoracic segments separated, but the labial segment remained fused with the thoracic segment (PETSCHKE *et al.* 1987). PETSCHKE and MAHOWALD (1990) describe the loss of the third (labial) En stripe as indicating the deletion of the labial posterior compartment, and they suggest that this may be the extent of the “gap” phenotype in the anterior *gt* domain. Our examination of *gt*^{Q292} embryos revealed clear indications of a homeotic transformation of the labial segment to a first thoracic identity. MOHLER *et al.* (1989) noted the presence of ectopic hairs in the dorsal cuticle of *gt*^{X11} mutants, but did not relate this observation to a change in segment identity.

LARSEN *et al.* (2003) determined that En-expressing cells were the first to regress during the formation of the segment groove. They describe the absolute requirement for En expression in cells adjacent to the developing groove. Thus, in *gt* mutant embryos, the amount of En accumulation retained in the posterior labial compartment likely determines the extent of segmentation that will occur between the labial and first thoracic segments. Embryos hemizygous for *gt*^{X11} almost completely lose the third En stripe, coinciding with the virtually complete fusion between the labial and first thoracic segments in these individuals. Consequently,

we never observed a duplication of the first thoracic segment in embryos of this genotype. This lack of ventral denticle duplication in *gt^{X11}* embryos follows when considering the loss of En in the posterior labial segment. Since more of the labial En stripe remains in *gt^{Q292}* embryos, a segment border can form. Interestingly, both *gt^{X11}* and *gt^{Q292}* develop ectopic dorsal hairs anterior to the first thoracic segment. Our En staining revealed that at least a portion of the dorsal ridge fuses (or never properly separates) from the dorsal labial segment, creating a segment that resembles the first thoracic segment. It is likely that the ectopic dorsal hairs arise from the dorsal ridge, which has been transformed toward dorsal first thoracic identity.

The case for a *gt* segment identity function is strengthened by the alterations in the homeotic genes expressed in the gnathal and thoracic regions. In all *gt^{Q292}* mutants examined, the labial segment expressed *Scr*, *Antp*, and *tsh*. This combination of segment identity factors is normally found in the first thoracic segment. Further, the labial segment shows significant reduction or alteration in *pb* and *disco* expression, both markers of gnathal identity.

There are two potential explanations for the differential effects on En accumulation and the ventral cuticle phenotype in the *gt* alleles that we examined. First, the available *gt^{Q292}* stock may, over time, have acquired second site suppressors responsible for the occasional persistence of En accumulation in the posterior labial segment. However, when we crossed this allele into a different genetic background, we continued to observe the presence of En accumulation and ventral cuticle transformation. If it is a second site suppressor of the *gt* mutation, then it must lie on the X chromosome carrying the *gt^{Q292}* allele. A second possibility is that the *gt^{Q292}* allele is a strong hypomorph, rather than an amorphic allele, and the residual Gt function is sufficient in some individuals to allow the labial En segmentation process to proceed, although the segment identity process remains faulty. Regardless of which explanation proves to be true, it appears that the anterior *gt* domain regulates embryonic patterning at two different levels—segmentation and segment identity—and that these two processes are functionally separable from one another.

This conclusion is not without precedent. Early reports suggested a possible homeotic function in addition to the segmentation function of Gt in its posterior expression domain (HARDING and LEVINE 1988; MOHLER *et al.* 1989). Homeotic transformations and segmentation defects are observed in the mutant phenotypes of other gap genes. For example, in *hb* mutants, the loss of mid-abdominal segmentation is accompanied by mirror image duplications (LEHMANN and NUSSLEIN-VOLHARD 1987; JÜRGENS 1988). Our results are significant, as they are the first to definitively demonstrate a segment identity role of the anterior *gt* domain.

The function of the anterior *gt* domain in *Drosophila* is similar to *Tribolium*: A recent study characterized the expression and function of a *gt* homolog in *Tribolium* (*Tc'gt*) (BUCHER and KLINGLER 2004). As in *Drosophila*, *Tc'gt* mRNA is expressed in two primary domains—one in the anterior of the embryo, overlying the gnathal region, and a second in the region of the third thoracic segment to the second abdominal segment. Although the anterior *Tc'gt* domain is similarly placed as compared to *Drosophila*, the posterior domain is shifted forward approximately five segments. The authors used RNA interference and morpholinos to knock down the expression of *Tc'gt* to explore its function. Interestingly, they found that *Tc'gt* has a role in the identity specification of the maxillary and labial segments, but did not have a role in segmentation. The maxillary and labial segments were transformed to a first and second thoracic identity, respectively, while all three thoracic segments exhibit a third thoracic identity. There was no loss of the gnathal *Tc'en* (*Tc'En*) stripes, although thoracic and abdominal *Tc'En* accumulation was affected to varying degrees in different embryos. Although the region affected by the loss of *Tc'gt* function is broader than the transformed region we observed in *Drosophila* *gt^{Q292}* mutants, the nature of the homeotic change is quite similar. A gnathal segment(s) is transformed to a thoracic identity, and this identity change is separate from the segmentation process. The segment identity function for *gt* may have been present in the last common ancestor of the holometabolous insects, and the segmentation role of the anterior *gt* might have been acquired separately to accommodate the long germ-band mode of development.

***gt* establishes the difference between gnathal and trunk segments via differential expression of *tsh* and *disco*:** In the head, *disco* is expressed in most cells of the segmental epidermis, while there is little or no expression of *tsh*. By contrast, *tsh* is expressed throughout most of the trunk segmental epidermis, while *disco* is limited to the limb primordia. The genetic studies presented here demonstrate that the difference between head and trunk expression patterns, and therefore segment types, is dependent upon the gap genes, and particularly, on *gt*. We summarize this regulation in Figure 7.

In *gt* mutant embryos, both *disco* and *tsh* expression are altered reciprocally. *disco* expression is severely reduced in the labial segment and in fact is altered such that the remaining *disco* mRNA resembled the embryonic limb primordia expression observed in the thoracic segments. There is a concomitant expansion of *tsh* expression into the labial segment. We previously demonstrated that UAS-driven ectopic *tsh* expression in the gnathal segments reduces and alters *disco* expression such that it mimics the expression pattern of the thoracic segments (ROBERTSON *et al.* 2004). Similarly, in *gt* mutants, it is the expansion of *tsh* expression into the labial segment that is responsible for the changes in

FIGURE 7.—The gap gene *giant* has both homeotic functions and segmentation functions. (A) The anterior *Giant* domain regulates segmentation through the regulation of the pair-rule and segment polarity genes and segment identity through regulation of the Hom-C genes, *tsh*, and *disco*. (B) A summary of the genetic interactions acting to position the gnathal/trunk boundary. Zygotic gap protein gradients are shown above a diagram of the expression domains of the homeotic genes involved in gnathal/trunk specification. Repressive interactions are shown with a barred line. *gt* is a key player in this cascade of repressive interactions, with the alterations that we observed in *hb* and *Kr* mutants likely due to their interactive regulatory relationship with *gt*. The anterior domain of *gt* is limited by its interactions with the zygotic gap proteins Hb and Kr, both of which act as repressors of *gt* expression (see text for references). Gt, in turn, limits the anterior expression of *tsh*. Tsh represses *disco*, such that *disco* is expressed throughout the gnathal segments, but is limited to only the embryonic leg primordia in the trunk. *tsh* is further limited by the expression of *salm* in the anlagen of the maxillary and labial segments. *Antp*, *Antennapedia*; *disco*, *disconnected*; Gt, Giant; Hb, Hunchback; Kr, Krüppel; Scr, *Sex combs reduced*; *salm*, *spalt major*; *tsh*, *teashirt*; PS, parasegment; Man, mandibular; Max, maxillary; Lab, labial; T, thoracic segments; A, abdominal segments.

disco expression. When both *gt* and *tsh* are absent, *disco* expression in the labial lobe recovers significantly, and the overall morphology of the labial segment and adjoining dorsal ridge is notably improved.

Our results may support a direct role for *gt* in the regulation of *tsh*. Although previous work demonstrated the requirement of Antp for appropriate *tsh* expression in the thoracic segments, and the loss of *gt* results in ectopic Antp protein in the labial segment (PETSCHKE and MAHOWALD 1990; REINITZ and LEVINE 1990; RILEY *et al.* 1991; RÖDER *et al.* 1992), Antp is not required for ectopic *tsh* activation in the labial and maxillary segments of *gt* mutants. It is likely that *gt* directly limits the

anterior expression of both *tsh* and *Antp*. Gt functions as a short-range repressor and has been shown to bind with high affinity to the CD1 sequence (TAT GAC GCA AGA) derived from the Kr regulatory region (CAPOVILLA *et al.* 1992; HEWITT *et al.* 1999). There is a sequence ~0.5 kb upstream of the transcription start site of *tsh* that is similar to the CD1 sequence (TAT GAA GGA AGG), differing by only three bases. Although it remains to be investigated as to whether the Gt protein can bind to this sequence, the similarity in sequence to a known *in vivo* Gt-binding site supports direct repression of *tsh* by Gt.

The results that we present in this study, taken together with previous work, outline a model for the

positioning of the gnathal/trunk boundary in the *Drosophila* embryo, involving a network of repressive factors. This model is presented in Figure 7, and *gt* is a key player in this model. The anterior domain of *gt* is limited by its interactions with the zygotic gap genes *hb* and *Kr*, both of which act as repressors of *gt* expression (HULSKAMP *et al.* 1990; ELDON and PIRROTTA 1991; KRAUT and LEVINE 1991a,b; WU *et al.* 2001). *Gt* in turn limits *tsh* expression, preventing expression in the labial segment. *tsh* expression is further limited by the expression of *salm* in the anlagen of the maxillary and labial segments. However, our results demonstrate that *salm* alone is insufficient for repressing *tsh* in the posterior labial segment in embryos lacking *gt* function. In the trunk segments, *Tsh* limits *disco* expression to only the embryonic appendage primordial, so that, lacking *Tsh*, *disco* expression is expanded through much of the gnathal segments.

Questions remain regarding the activation of *tsh* and *disco*. *disco* mRNA accumulates in the cellular blastoderm prior to gastrulation, implying the involvement of maternal factors or early acting gap genes. However, none of the gap mutants that we tested affected the initiation of the initial anterior *disco* domain. *disco* was significantly affected by the loss of maternal *bcd* (data not shown). This suggests that *bcd* and/or maternal *hb* may play a role in the initial activation of the anterior *disco* domain, after which *Tsh* acts to limit the *disco* to the gnathal region. *tsh* expression initiates prior to gastrulation, first with a central stripe that resolves to form a striped pattern reminiscent of the pair-rule genes. Again, none of the gap genes that we examined eliminated *tsh* expression. Although *Kr* is expressed in the central region of the embryo, where *tsh* is first transcribed, it is not the activator of *tsh* (data not shown). Early *tsh* may respond to maternal factors and/or a combination of gap gene products in a concentration-specific manner, which would account for our inability to detect a single activator in our gap mutant studies (data not shown). Finally, although we have found several instances where *Tsh* represses *disco* (ROBERTSON *et al.* 2004; LAUGIER *et al.* 2005; PATEL *et al.* 2007), we have no evidence that the reverse is true. What leads to the repression of *tsh* and concomitant maintenance of *disco* in the maxillary segment of *gt* mutant embryos is unclear at this time.

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