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In vivo zygotic- and maternal-effect characterization of the *Drosophila melanogaster* gene, *tango*

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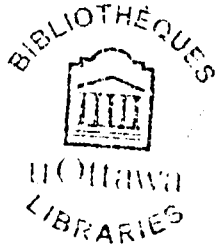

This work is dedicated to my parents.

Abstract

This study involved the *in vivo* phenotypic characterization of the *Drosophila melanogaster* gene *tango* (*tgo*) through classification of both its zygotic-effect during CNS development and maternal-effect during early embryonic patterning. Particularly, this study involved characterization of the *prd-repeat* domain of the Tango protein, or its allelic equivalent, tgo^3. *tgo* has been previously isolated as a bHLH-PAS nuclear transcription factor required in the regulation of CNS development through its dimerization of the CNS master transcriptional regulatory gene, *single-minded*. Having performed an inter-allelic analysis, I grouped the CNS zygotic effects of *tgo* into various phenotypic classifications. The phenotypes exhibited a pleiotropy of CNS mutant effects and were classified as fused commissure, neurogenic, stalled or ambiguous nervous system defects. These phenotypes may possibly be attributed to different but partially overlapping functions of the various Tgo domains. Additionally, I analyzed the maternal-effect of *tgo* during precellular blastoderm development. Germline clonal analysis of the tgo^3 allele revealed that *tgo* has an important role during early embryogenesis. Moreover, maternal tgo^3 phenotypes resemble those of the more well-characterized maternal coordinate and gap segmentation genes. tgo^3 germline clones were embryonic lethal and were classified as severe or intermediate, depending on the extent of deleterious effects on the resultant cuticular phenotype. Germline clones that showed a deletion of the entire anterior end, abdominal segments A1 to A5 and missing the Filzkorper and spiracular opening in the posterior end were classified as severe and are comparable to the cuticular phenotypes of amorphic *bicoid* mutants and *Krüppel* (*Kr*) phenotypes. Less severe deleterious effects, such as improper development of various anterior and posterior end structures or aberrant denticle band formation, were classified as intermediate tgo^3 germline clones, comparable to weaker bicoid alleles. Subsequent stainings to observe the effect on embryonic patterning through gap and pair-rule protein localization revealed alterations in spatial expression patterns. Alterations in both Krüppel and Even-skipped localization patterns in embryos mutant for maternal tgo^3 suggest a role for *tgo* during early embryonic patterning, perhaps through the activity of its *prd-repeat* domain. Ectopic expression studies, employing the GAL4-UAS system, involved heat shock trials eliciting the ubiquitous expression of a truncated C-terminal Tgo protein (UAS-$tgo^{\Delta C}$)

and both the ubiquitous and targeted expression of UAS-*tgo*$^{\Delta c}$. This targeted misexpression analysis revealed *tgo* as both a possible activator and repressor of *Kr* gap gene expression, in addition to affecting the protein distribution pattern of the later-acting segmentation gene, *engrailed*. Taken together, these germline clonal and ectopic expression studies suggest that the role of Tgo during early embryonic patterning may function through combinatorial interactions with maternal-effect and/or early-acting segmentation gene products. Moreover, this role may depend on the function(s) of the C-terminal sequences encoded by Tgo, including the *prd repeat* domain. Ultimately, this early embryonic role of *tgo* seems to be distinct from its role as a nuclear transcription factor.

ACKNOWLEDGEMENTS

In these acknowledgements, I feel that the weight of my gratitude to the people below cannot be known simply from the jotting of a few words on paper, but here goes.....

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Abbreviations

AC = anterior commissure

AEL = after egg laying

Ahr = aryl hydrocarbon receptor

AIF = aryl hydrocarbon receptor interaction factor

Arnt = aryl hydrocarbon receptor nuclear translocator

βgal = beta-galactosidase

bHLH-PAS = basic-helix-loop-helic-Per-Arnt-Sim

bcd = *bicoid*

cad = *caudal*

cDNA = complementary deoxyribonucleic acid

CME = central nervous system midline enhancer element

CNS = central nervous system

DA = dorsal arm

DAB = diaminobenzidine tetrahydrochloride

DB = dorsal bridge

DER = *Drosophila* epidermal growth factor receptor

DFS = dominant female sterile mutation

DNA = deoxyribonucleic acid

EGFR = epidermal growth factor receptor

EMS = ethane methyl sulfonate

en = *engrailed*

eve = *even-skipped*

exu = *exuperentia*

FITC = fluoresceine iso-thocyanate

FLP = flippase

FRT = flippase recombinase target

Gal4 = galactosidase

glc = germline clone

gt = *giant*

hb = *hunchback*

HRP = horseraddish peroxidase

hs = heat shock

hsp70 = heat shock promoter 70

IgG = immunoglobulin G

lacZ = *lactose Z*

LG = lateralgraten

Lr = labrum

K_o = Keilin organ

Kr = Krüpple

lacZ = lactose Z

mAb = monoclonal antibody

MG = midline glia

MGC = mother ganglion cell

Mh = mouth hook

neo = *neomycin*

neu = *neuralized*

NS = nervous system

NGS = normal goat serum

nos = *nanos*

odd = *odd-skipped*

prd = *paired*

P = P-element

PBT = phosphate buffer + Triton

PC = posterior commissure

per = *period*

ry = *rosy* (eye colour)

Sb = Stubble (bristle length)

sim = *single-minded*

ss = *spineless*

swa = *swallow*

stau = *staufen*

tgo = *tango*

tgo^{3glc} = *tango* allele 3 germline clone

$tgo^{\Delta b}$ = *tango* transgene, basic domain deleted

$tgo^{\Delta C}$ = *tango* transgene, C-terminal end deletion

tgo^{full} = *tango* transgene full

TM3 = third multiple 3 (third chromosome)

tor = *torso*

tsl = *torsolike*

tub = tubby

ubx = *ultrabithorax*

w^{+} = wild-type (eye colour)

w^{118} = wild-type (fly line)

XRE = xenobiotic response element

yw = *yellow* (eye colour)

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I. INTRODUCTION

I-1 *tango*: A Background and its Role During Early Central Nervous System Development

I-1a bHLH-PAS Transcription Factors

Many transcription factors bind DNA as dimers. With the formation of dimeric complexes, there is an extension of the DNA-binding site and an increase in combinatorial possibilities of distinct binding sties (Lamb and McKnight, 1991). An example of this lies in a prominent class of transcriptional regulators known as the family of basic-helix-loop-helix - Period (Period) - Aryl hydrocarbon Receptor Nuclear Translocator (ARNT) - Single-minded (Sim) transcription factors. Members of this family are responsible for the regulation of a wide variety of developmental and physiological events in vertebrates, invertebrates, plants and prokaryotes, including neurogenesis, segmentation, normoxic and hypoxic responses, circadian rhythms, tracheal and salivary function (Murre et al., 1994; Crews, 1998; Hahn, 1998). Defined by the presence of the basic helix-loop-helix (bHLH) and Per-Arnt-Sim (PAS) homology domains, bHLH-PAS proteins share a number of similarities with other bHLH-PAS subfamilies (see Crews, 1998).

One striking similarity lies in the highly conserved sequence organization (Figure 1.1). Located in the N-terminal end of the bHLH-PAS family members, the bHLH motif is characteristic of proteins involved in modulation of cell proliferation and differentiation (Hahn, 1998) and is required for DNA-binding and dimerization, respectively (Mitchell and Tjian, 1989). First described by Murre et al., (1989), the basic region, approximately 15 basic amino acids long, is just N-terminal to the helix and the motif as a whole consists of two amphipathic alpha-helices separated by a non-helical loop. Proteins containing the bHLH motif are capable of forming homo- or hetero- dimers acting as sequence-specific, DNA-binding proteins (Hahn, 1998).

The PAS domain is a 200-300 amino acid region comprised of two 51 amino acid degenerate subregions PAS-A and PAS-B separated by an unconserved spacer region (Crews et al., 1988). Within the A and B subregions are 44 amino acid-repeats (PAS

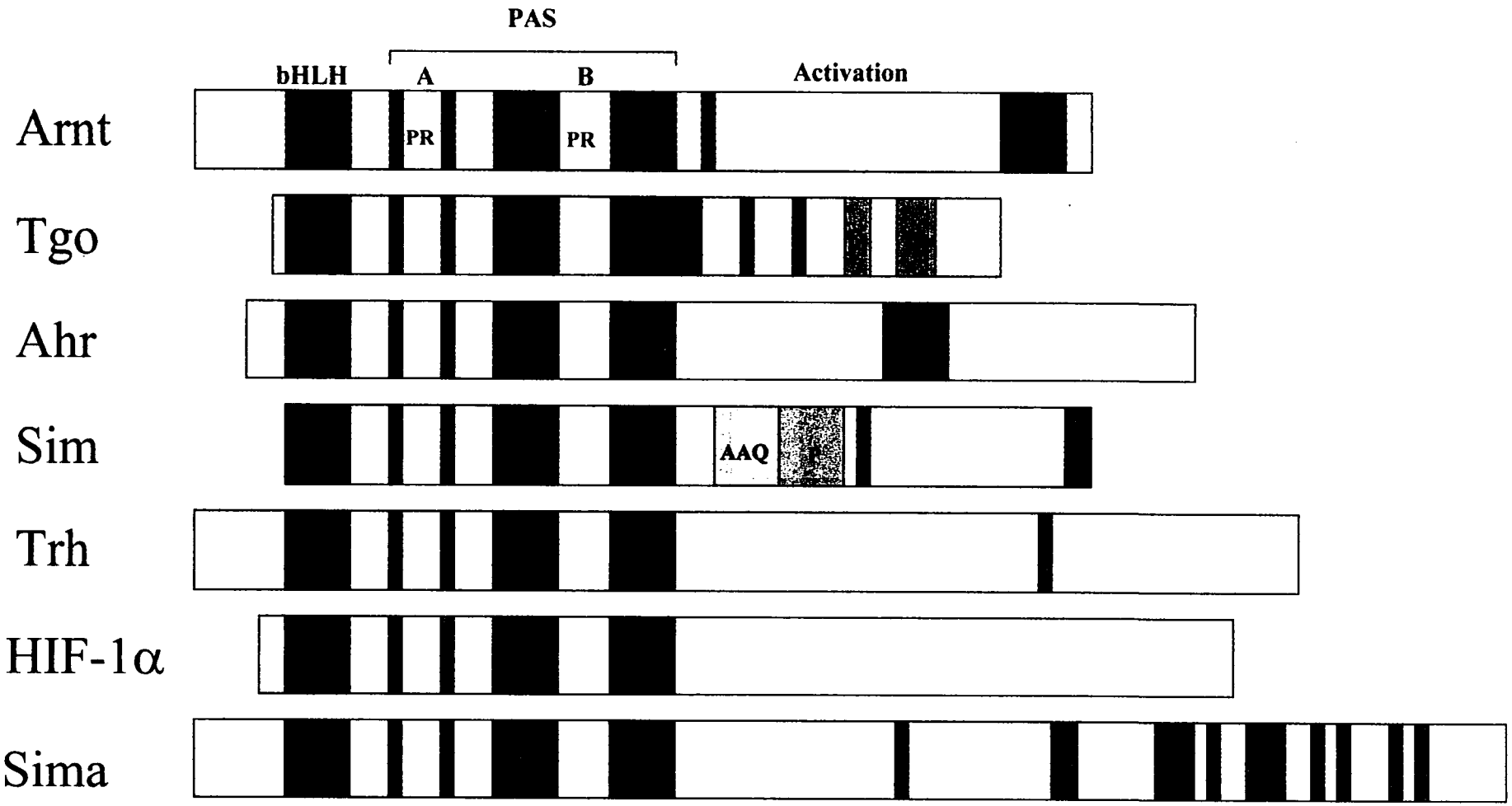


Figure 1.1 Structural conservation among bHLH-PAS proteins is high. Shown are human Arnt, *Drosophila* Tango, murine Ahr, *Drosophila* Sim, human HIF-1α and *Drosophila* Sima proteins. The N-terminus is to the left. The bHLH domain (red) is housed in the N-terminus, followed by the PAS domain. The PAS domain consists of two well-conserved regions, Pas A (blue) and Pas B (green), separated by a poorly conserved spacer. Within each PAS region is a 44 amino acid PAS repeat (PR). The carboxyl termini of these bHLH-PAS family members function as transcriptional activation domains. Poly[glutamine] repeats, shown as unlabelled purple blocks, are associated with activation function. Unique to Tango is a histidine-proline-rich (HP) *paired repeat* (orange). Also shown is an Alanine-Alanine-Glutamine (AAQ) repeat and proline-rich (P) in Sim. After Crews (1998).

repeats) (Crews et al., 1988; Nambu et al., 1996). The PAS domain is named for the first three proteins identified with this motif: *Drosophila* Period, required for proper circadian rhythmic establishment, human ARNT, required for mammalian toxin metabolism and *Drosophila* Single-minded, essential for the initiation of central nervous system (CNS) midline formation (Nambu et al., 1991). Consistent with its size and variability in sequence, the PAS domain can regulate a number of biochemical functions, namely protein-protein interactions and therefore serve as a secondary dimerization surface (Hahn, 1998). It is used in heterodimeric activity between other PAS proteins (Huang et al., 1993), interactions between non-PAS proteins (Coumailleau et al., 1995), small molecules (Dolwick et al., 1993; Coumailleau et al., 1995) and can function as a homodimerization surface (Probst et al., 1997). Previous investigations have provided a characterization of *tango* as the *Drosophila* orthologue of mammalian ARNT and the dimerization partner of both *Drosophila* Sim and *Drosophila* Trachealess (Trh) via their respective PAS domains during the establishment of the CNS midline and tracheal development in early embryogenesis, respectively.

The carboxyl termini of bHLH-PAS proteins have been reported to function as either transcriptional activators (Franks and Crews, 1994; Jain et al., 1994; Li et al., 1994) or repression domains (Moffett et al., 1997). The C-termini of the bHLH-PAS proteins shown in Figure 1.1 function as transcriptional activation domains (Crews, 1998), however the variability in C-terminal structure among these transcription factors is noteworthy. The poly [glutamine] repeats (shown in unlabeled purple blocks) are associated with activation function. *Drosophila* Sim is the master transcriptional regulator of CNS development and is the dimerization partner of Tango (see below). An interesting feature of the *Drosophila* Tango C-terminus is the presence of a histidine-proline-rich domain. This region is known as the *paired* (*prd*) *repeat* domain and little consensus has been reached regarding its functional significance, but further light is shed upon the requirement of the *prd repeat* during early embryogenesis in this present body of work.

An important aspect of bHLH-PAS protein function is that some members act as receptors and regulate cell signaling pathways. An example is the mammalian aryl hydrocarbon receptor complex (AHRC, also known as the dioxin receptor) (Hankinson, 1995; Whitlock et al., 1996; Hahn, 1998). The *aryl hydrocarbon receptor* (AHR) is a ligand-activated transcription factor involved in the activation of genes for xenobiotic-metabolizing enzymes such as cytochrome P_{4501a1} and glutathione S-transferase (GST), required in toxin metabolism (Crews, 1998). These detoxifying enzymes have been found in the liver (Burbach et al., 1992; Zelzer et al., 1997). The functional DNA-binding complex consists of the Ahr ligand-binding bHLH-PAS protein (Ema et al., 1992) and another bHLH-PAS protein, ARNT (Hoffman et al., 1991) (Figure 1.2). In the unliganded state, the Ahr is complexed with two molecules of a 90kDa heat-shock protein (hsp90) and other proteins, such as Ahr interaction factor (AIF) (Ma and Whitlock, 1997). With the binding of a ligand to the AHR PAS domain, ligands (such as chlorinated dioxins and related halogenated aromatic hydrocarbons), hsp90 and associated proteins dissociate and association with ARNT ensues (Hoffman et al., 1991; Hahn, 1998). This interaction leads to the initiation of transcriptional regulation involving the binding of the aryl hydrocarbon receptor complex (AHRC) to the xenobiotic response element (XRE), comprised of a GCGTG core binding sequence. The AHRC has been classified as a paradigmatic member of the bHLH-PAS family. Its mechanism of transcriptional regulation through signal transduction by ligand binding, control of nuclear localization and protein-protein interactions has aided in the genetic characterization of how *Drosophila* Sim mediates CNS development and transcription as the dimeric partner of Tango.

In the developing spinal cord of vertebrates and ventral nerve cord of insects lie specialized cells that play key roles in CNS development (Klämbt et al., 1991). In both vertebrates and insects, most CNS neurons are interneurons that project axons across the midline in one of the commissures (anterior or posterior) and then extend rostrally or caudally along one of the contralateral longitudinal tracts or connectives (Klämbt et al., 1991). In *Drosophila*, the midline cells provide the cellular substructure required in the proper establishment of the axon scaffold during CNS formation (Figure 1.3; see A1 in

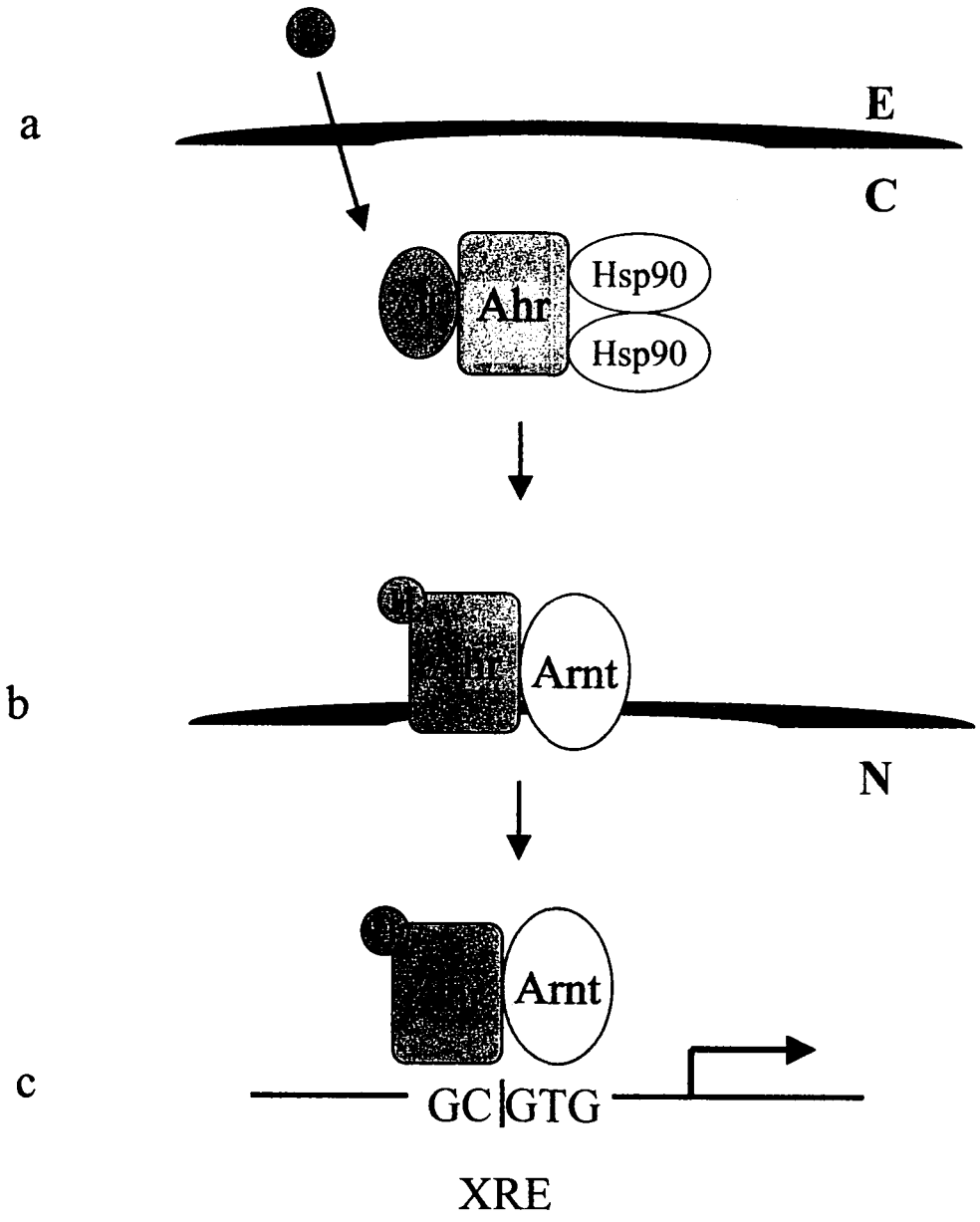


Figure 1.2 Ligand-dependent regulation of bHLH-PAS protein function by AHRC.

(a) The unliganded state. The aryl hydrocarbon receptor (Ahr) complexes with the aryl hydrocarbon interacting factor (AIF) and two molecules of Hsp90. All three components reside in the cytoplasm (C). Aryl hydrocarbons, such as dioxin (H) from the extracellular (E) side, will diffuse through the membrane will bind Ahr.

(b) The ligand-state. Upon dimerization with Arnt, the accessory proteins become dissociated from the Ahr and the Ahr::Arnt complex enters the nucleus. Dimerization occurs via their respective PAS domains.

(c) The Ahr::Arnt complex. The complex enters the nucleus (N), binds the xenobiotic response element (XRE) and activates target gene transcription regulating toxin metabolism. On the XRE, Ahr binds the GC (guanine/cystosine) half-site and Arnt will bind the GTG (guanine/tyrosine/guanine) half-site.

After Crews (1998).

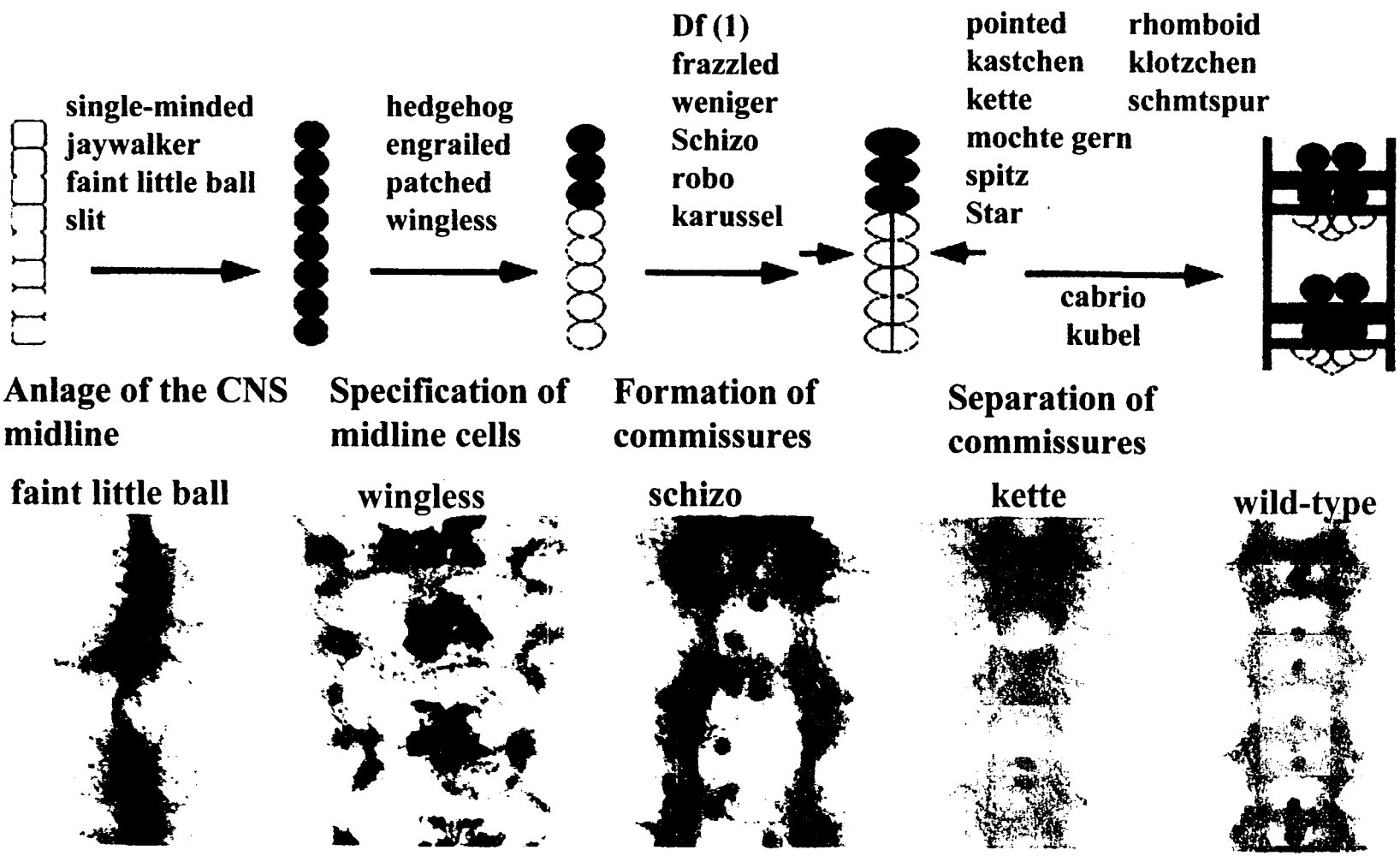


Figure 1.3 Genes controlling commissural development. The embryonic axon pattern in the ventral nerve cord develops in close relationship with midline glia and neuronal cells. Midline glia are shown in blue, ventral unpaired median (VUM) neurons in bright yellow, median neuroblast in pale orange and Rp1 neurons are indicated in gray. The formation of the CNS midline anlage by *single-minded* forges the specification of midline cells and formation and separation of commissural axons. Each stage is regulated by the groups of genes shown and the mutation or disruption of function of any one of these genes results in the phenotype shown in the panels below them. The 11 genes associated with the separation of commissures belong to the pointed group. Another group of 8 genes also associated with this stage in CNS midline establishment is known as the tramtrack group (not shown). Phenotypes depicted are of stage 15 embryos stained with monoclonal BP102. The midline glial cells are stained using the AA142 marker. Modified after Hummel et al., 1999.

Appendix for an elaboration on the embryonic development of the *Drosophila* CNS). It is the function of the *Drosophila sim* gene that orchestrates the behavior of the midline progenitor cells in such a manner (Thomas et al., 1988) and it is the expression of *sim* that is both sufficient and required in all steps of midline cell development (Nambu et al., 1991). To date, two direct interaction partners of *sim* have been reported. One is Dichaete (Fishhook), which associates with the PAS domain of Sim, is strongly expressed in the early neuroectoderm. Dichaete is required for proper differentiation of midline and lateral CNS cells (Nambu and Nambu, 1996; Sanchez Soriano and Russell, 1998). The other is a gene revealed as a partner of *sim* whose translational product directly interacts with the master regulator of *Drosophila* CNS development. Tango (Oshiro and Saigo, 1997; Sonnenfeld et al., 1997; Zelzer et al., 1997) associates with both the HLH and PAS domains of Sim (Ward et al., 1998) and is required for *Drosophila* embryonic development of CNS midline lineage.

I-1b Identification, Cloning and Characterization of Drosophila tango

As shown by sequence comparison of bHLH proteins between divergent species, the most amino terminal domain, the bHLH region, is often highly conserved with respect to the rest of the protein. In screening a *Drosophila* genomic library with a human Arnt bHLH probe, the cloning of a *Drosophila* Arnt-like gene was achieved through identification of positive clones restriction mapping (Sonnenfeld et al., 1997). The result, a single gene subsequently named "*tango*" (*tgo*). *tgo* is orthologous to Arnt both in sequence and structure (Hoffman et al., 1991); sequence analyses of the complete gene and corresponding embryonic cDNA clones suggest a high relation between *Drosophila tgo* and mammalian Arnt (Figure 1.4). Sequencing analysis of *tgo* genomic and cDNA clones exposed a small gene of 2.9kb with a single intron of 142 base pairs (bp) within the 5'-untranslated region (Figure 1.5). In comparison to the much larger and more complex exon-intron structure of the mammalian Arnt gene (Maltepe et al., 1997), the exon-intron structure of *tgo* is simple. *tgo* resides in the cytological vicinity of *neuralized* (Boulianne et al., 1991) in the same orientation. Analysis of cDNA clones revealed that the 5' end of the longest *tgo* cDNA clone is 328bp 3' to the *neuralized* gene (Sonnenfeld

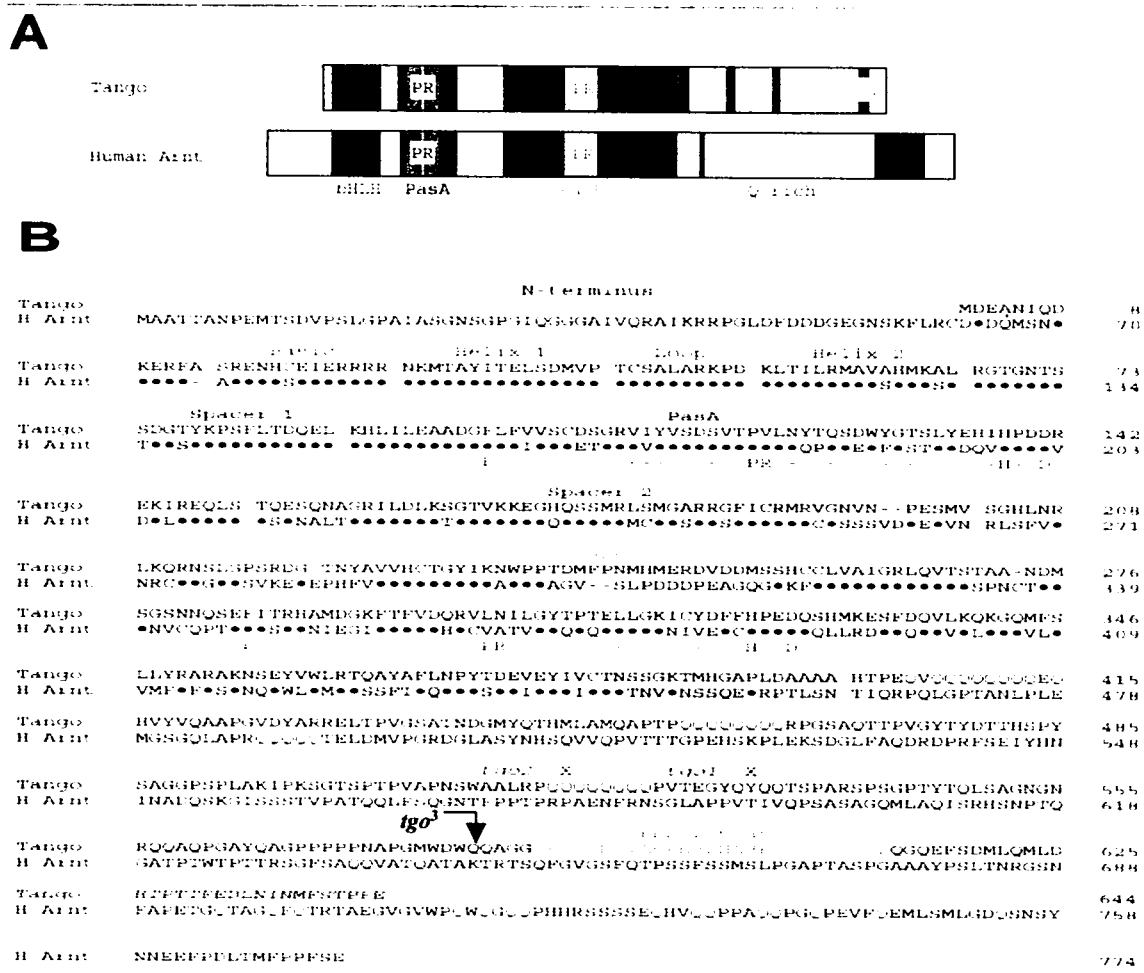


Figure 1.4 Drosophila *tango* is highly related to mammalian Arnt in both predicted structure and sequence.

(A) The sequence organization of the *Drosophila* Tango and human Arnt (H Arnt) proteins. Both block diagrams are depictions of cDNA clone sequences with associated important sequence motifs. The N-terminus is to the left. The bHLH domains (red) are followed by two PAS domains, Pas A (blue) and Pas B (green). Within each PAS domain is a Pas repeat (PR), shown in lighter colors. Housed in the carboxyl terminus are glutamine (Q)-rich repeats (purple). The Tgo protein is unique in that it contains a histidine-proline-rich region, the *paired repeat* (PRD) domain (cyan).

(B) Comparison of the protein sequences of Drosophila Tango and human Arnt (HArnt). Amino acid numbering is shown on the right. Sequence identities are indicated by dots and deletions by dashes. The spaces illustrate a difference in sequence regions. The two Pas repeats (shown below the sequence) are 44 amino acids long and flanked by invariant phenylalanine (F) and histidine (H) XX aspartic acid (D) residues. Three poly[glutamine] regions in the C-terminus are shown in purple. The Paired 7 (Prd7) *paired repeat* is shown is also shown in the C-terminus of Tgo in cyan. The premature translational stops (x) correspond to the *tgo*^{1}, *tgo*^{2} and *tgo*^{3} EMS mutations within the Tgo amino acid sequence are shown. After Sonnenfeld et al. (1997).

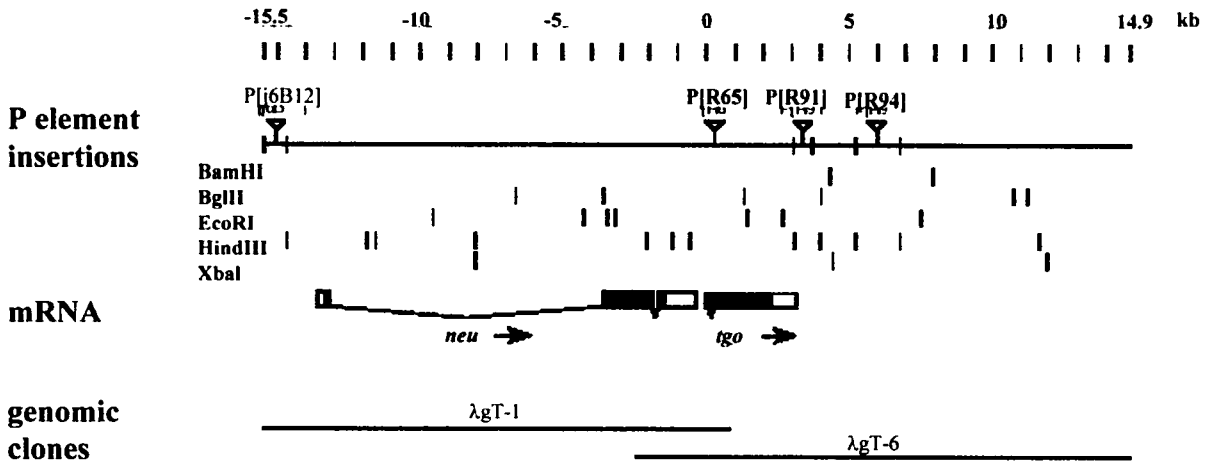


Figure 1.5 Structure of the *tango* genomic region. The genomic interval containing the *tango* (*tgo*) and *neuralized* (*neu*)genes are shown. The scale shown at the top is in kb with the number '0' denoting the 5'-end of the longest *tango* cDNA clone. The four P-element insertions that reside in this interval are also indicated; the location of P[R65] was determined by sequence analysis and the other three, P[j6B12], P[R91] and P[R94] were mapped by Southern hybridization of genomic DNA. The restriction enzyme cleavage sites of *Bam*H1, *Bgl*II, *Eco*RI, *Hind*III and *Xba*I are shown. cDNA analysis determined the structure of *neu* and *tgo* mRNAs. Transcriptional orientations (5' to 3') are indicated by arrows. Filled boxes are coding sequences, unfilled are translated regions and adjoining lines represent introns. λT-1 and λT-6 are two genomic clones spanning the 30.4kb interval. After Sonnenfeld et al. (1997).

et al., 1997), which has been mapped by polytene chromosome *in situ* hybridization to 85C (Boulianne et al., 1991). Like all bHLH-PAS proteins, the bHLH region is housed in the N-terminus of Tgo (see Figure 1.4). As mentioned above, the basic and HLH domains of bHLH-PAS transcription factors are required for DNA binding and dimerization, respectively (Mitchell and Tjian, 1989). The PAS domain of Tgo is a region that may confer specificity in protein interactions to regulate transcription (Zelzer et all., 1997). Between Tgo and Arnt, the bHLH regions are 92% identical and PAS domains 53% (see Figure 1.4). Although unrelated in primary sequence, the C-terminal regions of Tgo and Arnt share occurrence of glutamine repeats (18% in Tgo) and both are rich in proline (15% in Tgo). The glutamine-rich domains function as activation domains in mammalian Arnt (Jain et al., 1994; Li et al., 1994), *Drosophila sim* (Franks and Crews, 1994) and numerous other transcription factors (Mitchell and Tjian, 1989), including *tgo* (Sonnenfeld et al., 1997). As noted in the block diagram of Tgo in Figure 1.4, an interesting characteristic of the *Drosophila* Tgo carboxyl terminus is the presence of a his-pro-rich region (Frigerio et al., 1986) called the *paired repeat* (Oshiro and Saigo, 1997; Sonnenfeld et al., 1997). The present study involves characterization of this motif of the Tgo protein, revealing a possible requirement during early embryonic patterning and a later role during embryonic CNS midline formation.

The CME is an enhancer element for CNS midline transcription and this activity is dependent on *sim* function (Sonnenfeld et al., 1997; Crews, 1998). In addition to this *sim* requirement, CME transcription within the CNS midline is also dependent on *tgo* function. This is consistent with both theoretical consideration (Wharton et al., 1994) and experimental analysis (Swanson et al. 1995) predicting that the ACGTG core sequence of the CME serves as a binding site for heterodimers between *sim* and a *Drosophila* Arnt-like protein. Several lines of evidence are indicative of such a prediction. Ohshiro and Saigo (1997) and Sonnenfeld et al., (1997) have shown that the *tgo* gene is expressed in all embryonic cells. In most embryonic cells, *tgo* transcripts were found to exist at similar levels except for enhanced levels in the developing CNS and trachea (Sonnenfeld et al., 1997). Confocal imaging in the syncytial blastoderm embryo revealed that Tgo is found throughout the embryo and is exclusively cytoplasmic, localized at the apical ends

of the developing cells. Antibody staining revealed that nuclear accumulation occurred during gastrulation in the nuclei of the midline precursor cells (Ward et al., 1998). Embryos double-stained with anti-Tgo and anti-Sim show coincident Sim and Tgo nuclear localization in CNS midline cells during embryonic development. Sim and Tgo activate transcription of a multimerized CME (Figure 1.6), thereby providing direct evidence that Tgo and other bHLH-PAS proteins can form dimers and activate transcription through the CME (Sonnenfeld et al., 1997). *In vivo* analyses suggest genetic interactions between *tgo* and *sim* and show that CNS midline transcription and development are affected, as in transcription of the multimerized CME transgene (Sonnenfeld et al., 1997). It is likely that transcriptional activation *in vivo* by Sim::Tgo heterodimers is direct since both proteins contain powerful transcriptional activation domains (Franks and Crews, 1994; Sonnenfeld et al., 1997) and ectopic expression of *sim* activates gene expression and causes nuclear localization of Tgo (Emmons et al., 1999). A zygotic lethal *tgo* mutation produces a CNS axonal phenotype with disorganized commissures and thin longitudinal connectives, but there is no severe collapse of the axonal scaffold as in seen in null *sim* mutants (Sonnenfeld et al., 1997). It is believed that the less severe axonal phenotype of *tgo* mutants may be attributed to the use of hypomorphic *tgo* alleles. Additionally, gene dosage experiments suggest an *in vivo* interaction between Sim and Tgo, whereby the loss of a single copy of *sim* enhances the midline phenotype of *tgo* mutations, leading to a severe "*sim*-like" collapsed CNS phenotype (Sonnenfeld et al., 1997). The *in vivo* results combined above have also lead to speculation of an earlier requirement of *tgo*, one perhaps involving a maternal contribution. The current study involved isolation and *in vivo* analysis of this earlier contribution of *tgo* during embryonic patterning.

Isolation of three of the seven-*tgo* mutations was achieved by P-element and EMS mutagenesis using a two-step strategy. *tgo* maps to 85C on the third of four chromosomes in *Drosophila melanogaster* (Sonnenfeld et al., 1997). The first step to generating the *tgo* mutations was to isolate novel P-element insertions that mutated the *tgo* gene in or near it (Sonnenfeld et al., 1997). EMS-induced *tgo* mutations were then screened for lack of genetic complementation with the P-element insertional *tgo* strain.

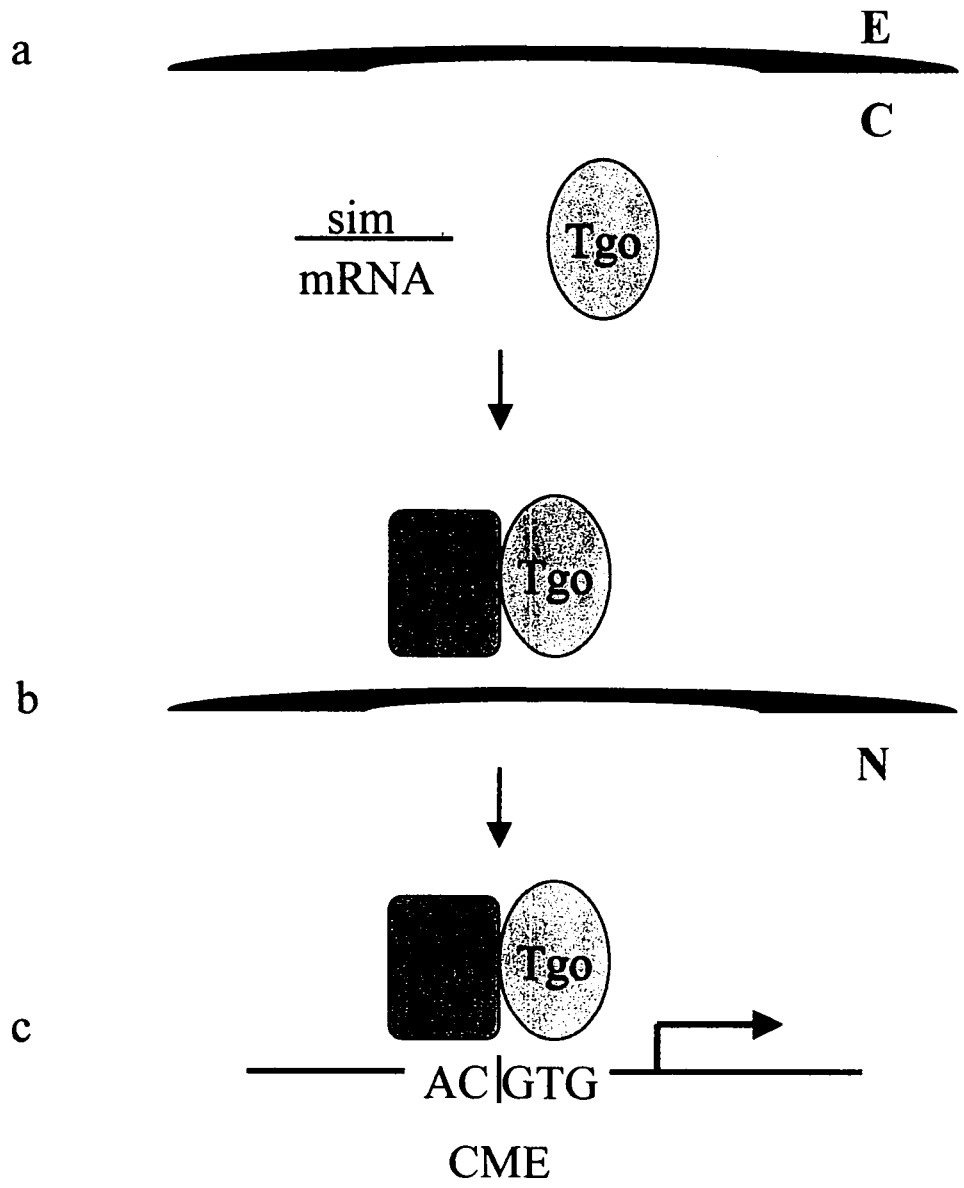


Figure 1.6 Ligand-independent regulation of bHLH-PAS protein function by Sim::Tgo.

(a) Tango (Tgo) resides in the cytoplasm of embryonic cells. Upon onset of neurogenesis, Single-minded (Sim) is transcribed in CNS midline cells.

(b) With the onset of midline establishment, Sim protein appears in the cytoplasm and will dimerize with Tgo through their respective PAS domains.

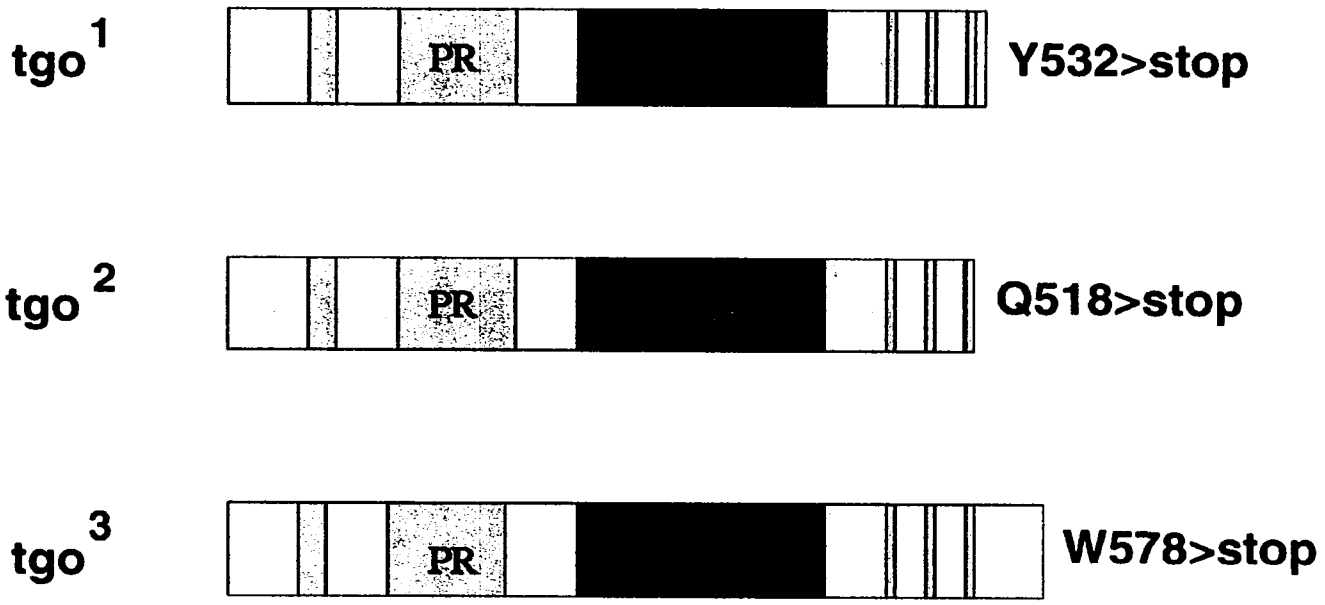
(c) The Sim::Tgo complex enters the nucleus, binds the central nervous system midline enhancer element (CME) and will activate the transcription of target genes regulating CNS midline development. On the CME, Sim will bind to the adenosine/cytosine (AC) and Tgo binds to guanine/tyrosine/guanine (GTG) half-site.

After Crews (1998)

EMS-induced *tgo* alleles were isolated and were then sequenced to identify the single base-pair change (see Figure 1.5). The tgo^1 mutant has a premature stop codon at amino acid 532 that deletes a glutamine-rich region, a proline-rich region and the *prd repeat* in the C-terminus of the protein (Figure 1.7). The tgo^2 mutant has a termination codon at aa518 that deletes another stretch of poly[glutamine] domain in addition to the region deleted in tgo^1. A premature stop codon at amino acid 578 in the tgo^3 allele, results in deletion of the *prd repeat* region. The *prd repeat* is not found in other Arnt proteins but found in transcriptional regulatory genes expressed early during *Drosophila* embryonic development, such as *paired* (*prd*) gene, *bicoid* (*bcd*) and *odd-skipped.* However, it is also found in a mammalian transcription factor, the rat class III POU domain protein Brain-2 (Li et al., 1993; see Discussion). Surprisingly, an absence of the *prd repeat*, in the germline does not result in female infertility as found with both tgo^1 and tgo^2. Infact, tgo^3 does not affect fertility or ovarian development (Sonnenfeld and Scanga, manuscript in process), but rather results in severe perturbation to early embryonic patterning. Therefore, based on the results described above, it is hypothesized that the *prd repeat* domain of *tgo* is required for regulating an early developmental event orchestrating embryonic patterning (see Statement of Problem).

Little is known about the *prd repeat* region. The *Drosophila paired* (*prd*) gene, a member of the pair-rule gene family, is involved in embryonic pattern formation along the antero-posterior (A-P) axis (Nüsslein-Volhard and Wieschaus, 1980; see next sub-chapter). *prd* is required in the regulation of downstream target gene expression, namely segment-polarity genes (see sub-chapter I-4), such as *engrailed* (*en*) (Weir et al., 1988). Earlier studies report that the Prd protein is comprised of three conserved motifs: a *homeodomain*, a *paired* domain and a *prd repeat* (Bopp et al., 1986; Frigerio et al., 1986). The *homeodomain* and *paired* domain are required for facilitating protein-protein interactions (Grueneberg et al., 1992) and DNA-binding (Treisman et al., 1991), respectively. The *prd repeat* is a 21-amino acid histidine-proline-rich sequence, similar to those found in *bicoid* and several other genes (Frigerio et al., 1986; see Discussion). The function of this domain remains unclear in these genes. However, in the C-terminal part of the Prd protein previous experiments have suggested that it may contribute to the

Figure 1.7 Three of the seven allelic forms or mutations of *tango* (*tgo*) have been sequenced. The N-terminus is to the left. *tgo*^{1} and *tgo*^{2} result from premature stop codons at tyrosine(Y) position 532 and glutamine (Q) position 518, respectively. Both yield sterile mutations. A premature stop codon at tryptophan (W) position 578 results in the deletion of the *paired repeat* domain in the C-terminus. This *tgo*^{3} mutation was used in this study to classify the requirement of the *paired repeat* during early embryonic CNS development and patterning. bHLH (basic and helix-loop-helix), PR (PAS repeats of the PAS domains A and B), glu-rich (glutamine-rich).



overall proline-rich transcriptional activation domain in the C-terminus of the Prd protein (Cai et al., 1994). A C-terminal Prd deletion of 121 amino acid including the *prd repeat* region results in loss of artificial *en* promoter activation (Han et al., 1989). Additionally, 74 amino acids from this region including the *prd repeat* were able to activate a heterologous (Sp1) DNA-binding domain in tissue culture cells. However in the context of the entire gene, it is unlikely that the Tgo *prd repeat* functions in a similar manner as to that of Prd since the sequences surrounding the Tgo *prd repeat* are not comparable to those surrounding the *prd repeat* of Prd (Cai et al., 1994; Sonnenfeld et al., 1997).

I-1c *The FLP-DFS Technique and GAL4-UAS System: genetic manipulations and their application to the study of tango during early embryonic patterning*

In this study, the generation of female germline mosaics and targeted gene expression allowed for analysis of the early embryonic role of *tgo*. The following is an explanation of each genetic manipulation as applied to elucidating the functional significance of *tgo* during early embryogenesis.

The Autosomal FLP-DFS Technique: the production of germline clones

The contributions of two different ovarian cell types enable the formation of the *Drosophila* egg: the nurse cell-oocyte complex is of germline origin and derived from the embryonic pole cells and the follicle cells originate from the embryonic mesoderm (Figure 1.8). Separation of these two cell types early on enables the generation of chimeras having different germline and follicle cell phenotypes (Figure 1.9) which in turn has been pivotal in conducting two methods of analysis. One, the presence of two cell types in the ovary allows tissue-specific analysis (germline versus somatic) of recessive female-sterile mutations. Saturation screens have provided a collection of female-sterile genes required during oogenesis and embryonic patterning (Perrimon et al., 1986). Second, germline chimeras detect the maternal-effect of recessive zygotic lethal mutations (Perrimon et al., 1984, 1989; Chou and Perrimon, 1992), like *tgo*. Maternal-effect genes can be identified by mutations that, when present in the mother do not damage her but have developmental effects on her progeny that cannot be rescued by

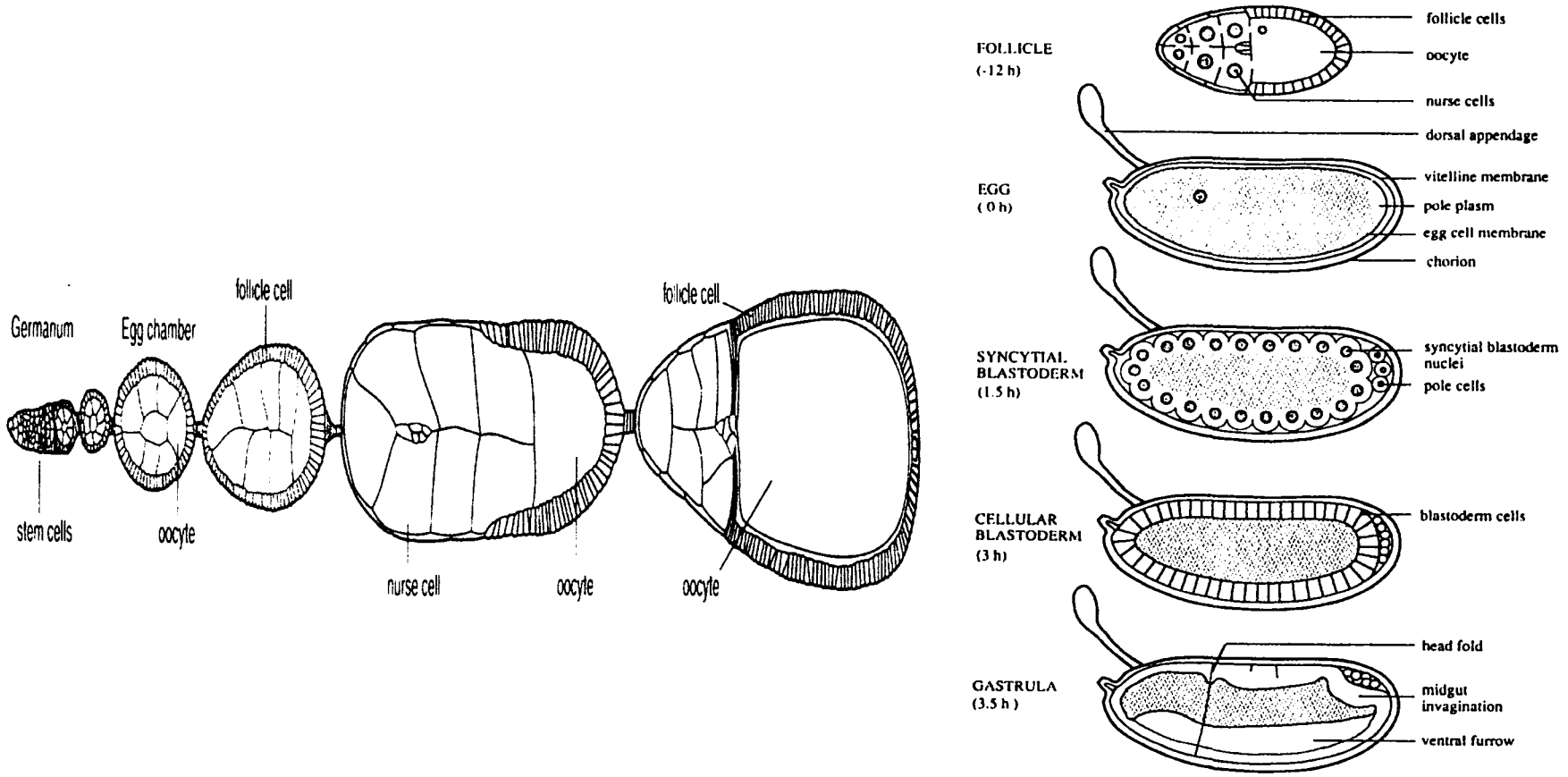


Figure 1.8 Egg development in *Drosophila*. Oocyte development begins in a germarium, with stem cells at one end. One stem cell undergoes four mitotic divisions to yield 16 cells with cytoplasm bridges between each other. One of these 16 cells will become the oocyte, the other 15 develop into nurse cells. The nurse cell-oocyte complex become enclosed by follicle cells and the resulting structure buds off from the germarium as an egg chamber. Subsequently generated egg chambers remain attached to each other at the poles. As the oocyte matures, the nurse cells expel proteins and RNAs into it while the follicle cells secrete the egg coverings. Both cell types degenerate at the completion of oogenesis. When the mature egg is laid (0h), it is enveloped by the vitelline membrane and chorion and is filled with cytoplasm. The pole plasm, a yolk-free mitochondria-enriched region, at the posterior end is the only distinctly specialized region of cytoplasm at this time. After fertilization, the zygotic nuclei go through a series of rapid mitotic divisions in the interior of the egg. Following nine divisions, most of the nuclei have migrated to the cortex to form the syncytial blastoderm (1.5h after egg laying, AEL). Precursors of the germline develop at this stage as the 3-4 nuclei that have entered the pole plasm to form the pole cells. The rest of the nuclei divide sequentially, giving rise to the ~5000 cells of the cellular blastoderm (3h AEL). Following cellularization, gastrulation (3.5h AEL) starts with invagination of the presumptive mesoderm through the ventral furrow, the development of the posterior midgut invagination that houses the pole cells and the appearance of the head fold. After St. Johnston and Nusslein-Volhard (1992).

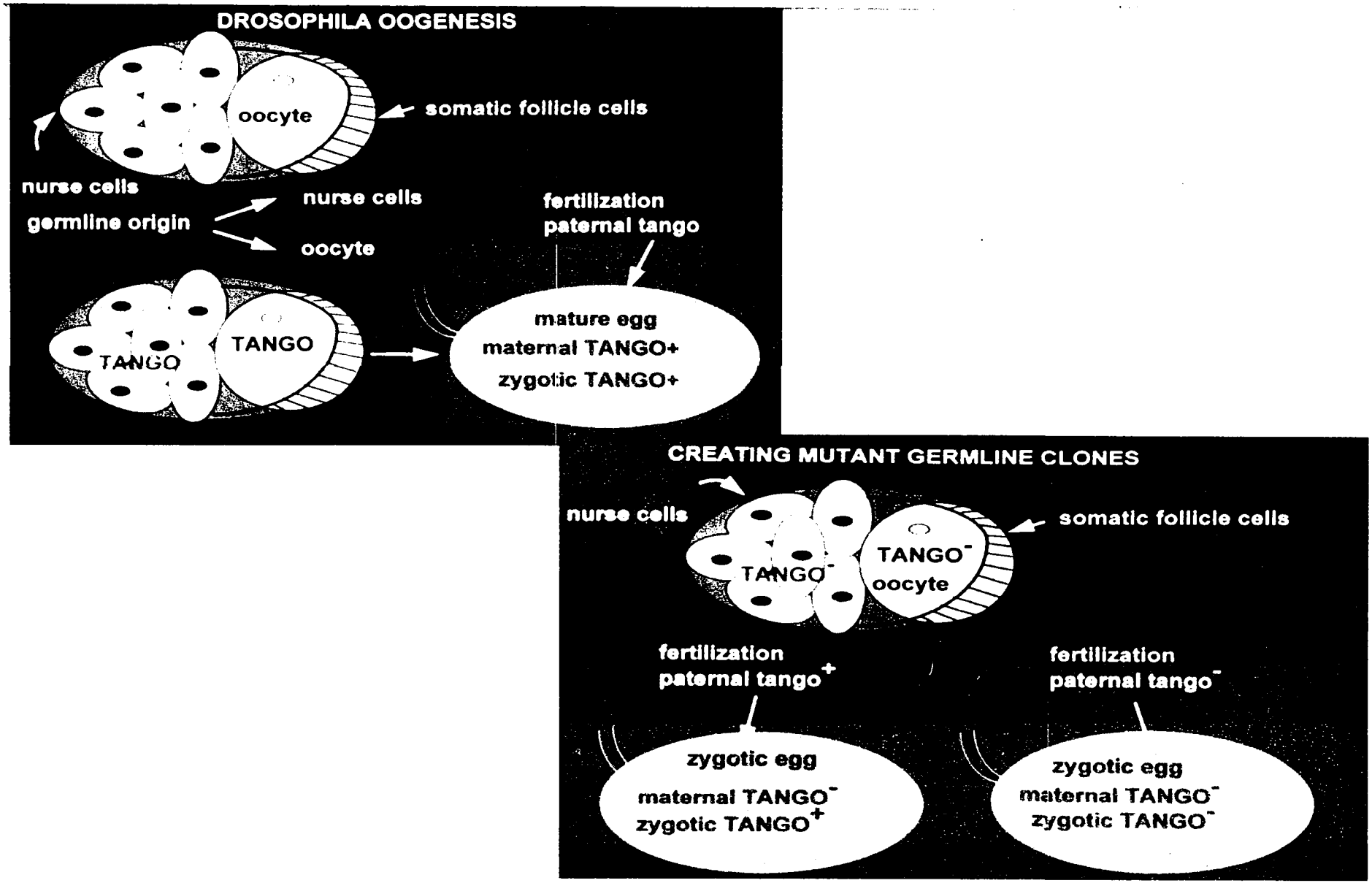


Figure 1.9 Oogenesis within the egg chambers of wild-type and the production of *tango*^{3} germline clone embryos. Top schematic: Oocyte development within wild-type *Drosophila* females. Both nurse cells and the oocyte have a supply of *tango* (*tgo*) transcripts and protein. The oocyte therefore carries the maternal complement to the zygotic *tgo* component. Fertilization of the oocyte supplies the paternal *Tgo*. Bottom schematic: Oocyte development within mosaic *tgo* females. Both nurse cells and the oocyte produce mutant *tgo* and all eggs deposited will develop in the absence of wild-type maternal *tgo* unless maternal *tgo* function is required for an early developmental process during *Drosophila* oogenesis and/or embryogenesis. If maternal *tgo* were required during an early embryonic process, for example, then this requirement could be investigated by analyzing development of the larva's cuticle.

paternal wild-type sperm (Nüsslein-Volhard and Wieschaus, 1980). Therefore, the roles of maternal genes during early patterning can be deduced from the effects of these maternal-effect mutations on the larva. Approximately 85% of the genes in *Drosophila* are zygotic lethals when mutated (Chou et al., 1993) and a direct approach to isolating the maternal function of these mutations is to analyze the phenotype of eggs derived from mosaic mothers that carry a homozygous mutant germline.

Two techniques have been reported in the production of the germline chimeras: pole cell transplantation and mitotic recombination (Wieschaus and Szabad, 1979). Both techniques are valued for yielding eggs derived from homozygous mutant cells that are distinguishable from the otherwise wild-type eggs. In the case of pole cell transplantation, mutant pole cells are transferred into an animal that does not develop germ cells. In the case of mitotic recombination, the most promising approach has been the use of germ-line-dependent dominant female-sterile (DFS) mutations that block egg laying but do not affect viability (Perrimon, 1984). The DFS technique consists of the production of germline clones in females heterozygous for the X-linked germline-dependent DFS mutation ovo^{D1}. Heterozygous mutations at the *ovo* locus lead to a defective female germline (the male is unaffected), wherein females homozygous for loss-of-function *ovo* alleles lack germline stem cell production (Oliver et al., 1987). A mitotic recombination event in the germ cells of females heterozygous for DFS results in the elimination of the DFS mutation in recombinant germline daughter cells. Subsequent production of homozygosity for the homologous chromosome leads to normal egg development. Using females *trans*-heterozygous for both the DFS mutation and a specific mutation, such as the zygotic lethal *tgo*, will result in simultaneous loss of the DFS mutation and homozygosity of the mutation following the mitotic exchange. The phenotypes of eggs and embryo derived from these germline clones can then be analyzed and the maternal-effect of a given zygotic lethal mutation during embryogenesis determined.

In implementing a germline clonal analysis, an important consideration is the frequency at which germline clones are recovered. X-ray irradiation followed by mitotic

recombination events induced in *ovo*^{D1} heterozygotes produced mosaic females among heterozygous *ovo*^{D1} females at a frequency of 5% (Perrimon, 1984). To increase this frequency, Chou and Perrimon (1992) devised the "FLP-DFS" technique by applying the properties of the yeast *flippase* (FLP) site-specific recombinase and its recombination targets (FRTs) to elicit site-specific recombination (Golic and Lindquist, 1989; Golic, 1991). Placed under the control of *hsp*70, the heat-inducible FLP recombinase gene recognizes and catalyzes site-specific recombination between homologous chromosomes at the level of the FRT sequences (Figure 1.10). The frequency of mosaics recovered from this system is nearly 100%. Germline clonal analysis not only allows classification of a zygotic lethal mutation and uncovers its role during *Drosophila* development, but it also allows for observation of a given gene's sufficiency during development in zygotically null embryos.

The GAL4-UAS System: targeted gene expression

When the product of a gene is required in a number of developmental processes or at several different times during development, its separate roles delineated by its various requirements throughout development can be identified and analyzed by restricting ectopic expression temporally or to specific cells or tissues (Brand et al., 1994). Ectopic expression of a gene can often induce a switch in cell fate, a manipulation thereby changing its characteristic pattern of gene expression consequently resulting perhaps in the activation of a gene in a cell in which it is not normally repressed. This could lead to at least three possible outcomes: (1) ectopic expression will have no effect on development; (2) ectopic expression will elicit a change in cell fate; (3) ectopic expression will force a change in neighboring cell fates. It is possible then to determine whether expression of a gene is necessary and sufficient to determine cell identity and whether its role is autonomous or not.

To date, there are a handful of different methods employable for ectopic expression in *Drosophila*, each with its merits and drawbacks. The first is to drive expression of a gene from tissue-specific promoters (Parkhurst and Ish-Horowicz, 1991), thereby restricting transcription to a defined subset of cells. However, the availability of cloned and

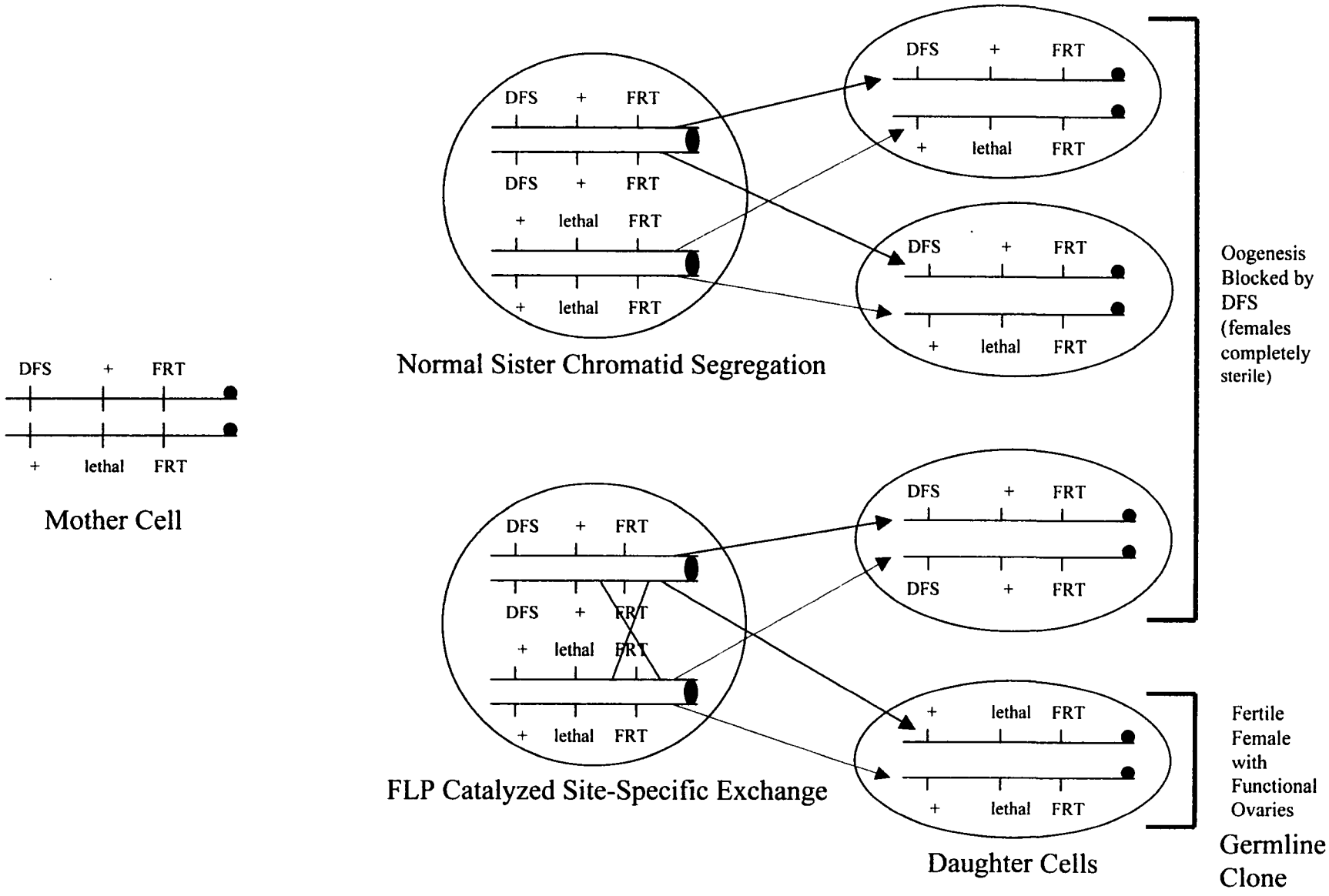


Figure 1.10 The FLP-DFS technique.
Chromosomal exchange that occurs in the euchromatin of a fly of genotype DFS + FRT/+ lethal FRT;FLP/+. The FRT insertion is located proximally to both DFS and the lethal mutation. Following heat shock induction, hsp70-FLP from another chromosome site can provide recombinase activity to catalyze site-specific chromosomal exchange at the position of the FRT sequences. FLP-catalyzed recombination results in 100% of females with lethal/lethal homozygous germline clones (lowest branch). Adapted from Chou and Perrimon (1996). Atrophic ovaries are shown as empty ovals and ovaries with developed ovarioles as filled ovals. FLP-recombinase target sequences (FRT). Dominant female sterile (DFS). Recessive zygotic lethal mutation (lethal).

characterized promoters directing expression in a desired pattern is limited. Moreover, toxic gene products may make it impossible to establish stable transgenic lines carrying the chimeric gene (Brand et al., 1994). The second method is to drive expression of a gene from a heat shock protein. Inducible expression at a specific point in development can result simply by heat shocking the transgenic animal (Struhl, 1985; Gonzales-Reyes and Morata, 1990; Manoukian and Krause, 1992). In addition to inducible expression throughout the organism, varying the temperature or duration of the heat shock (Manoukian and Krause, 1992) can monitor levels of ectopic expression. However, several disadvantages with this technique is that expression is ubiquitous, the heat shock promoter generates significant basal levels of expression and heat shocking can induce phenocopies (Petersen and Mitchell, 1987; Yost et al., 1990).

A third method to elicit ectopic expression relies on site-specific recombination orchestrated by the FLP/FRT system mentioned above in the generation of germline clones. The system is dependent on the generation of an inducible transgene (Struhl and Basler, 1993) consisting of a cloned FLP cassette and a transcriptional termination sequence bound by FRTs bound by a constitutive promoter and the sequence to be expressed. Following heat shock, the FLP cassette is excised and the transgene is expressed. The FLP technique is activated after a heat shock, like the heat shock method described above, but with this third method, the transgene is expressed throughout development because it is driven by a constitutive promoter and ectopic expression occur in clones of any cell type in the organism. Moreover, this directed misexpression varies from animal to animal since the clones are generated randomly (Brand et al., 1994).

A method devised by Brand and Perrimon (1994) to direct gene expression in *Drosophila* overcomes many of the drawbacks presented in the other methods outlined above. The GAL4-UAS system (see Materials and Methods, Figure 2.7) allows for the selective activation of any cloned gene in a wide variety of cell- and tissue-specific patterns. This method of inducible ectopic expression was used in this study to drive both ubiquitous and targeted misexpression of *tgo* very early during embryogenesis (see Results 3.3). This manipulative technique is invaluable for several reasons. First, the system allows

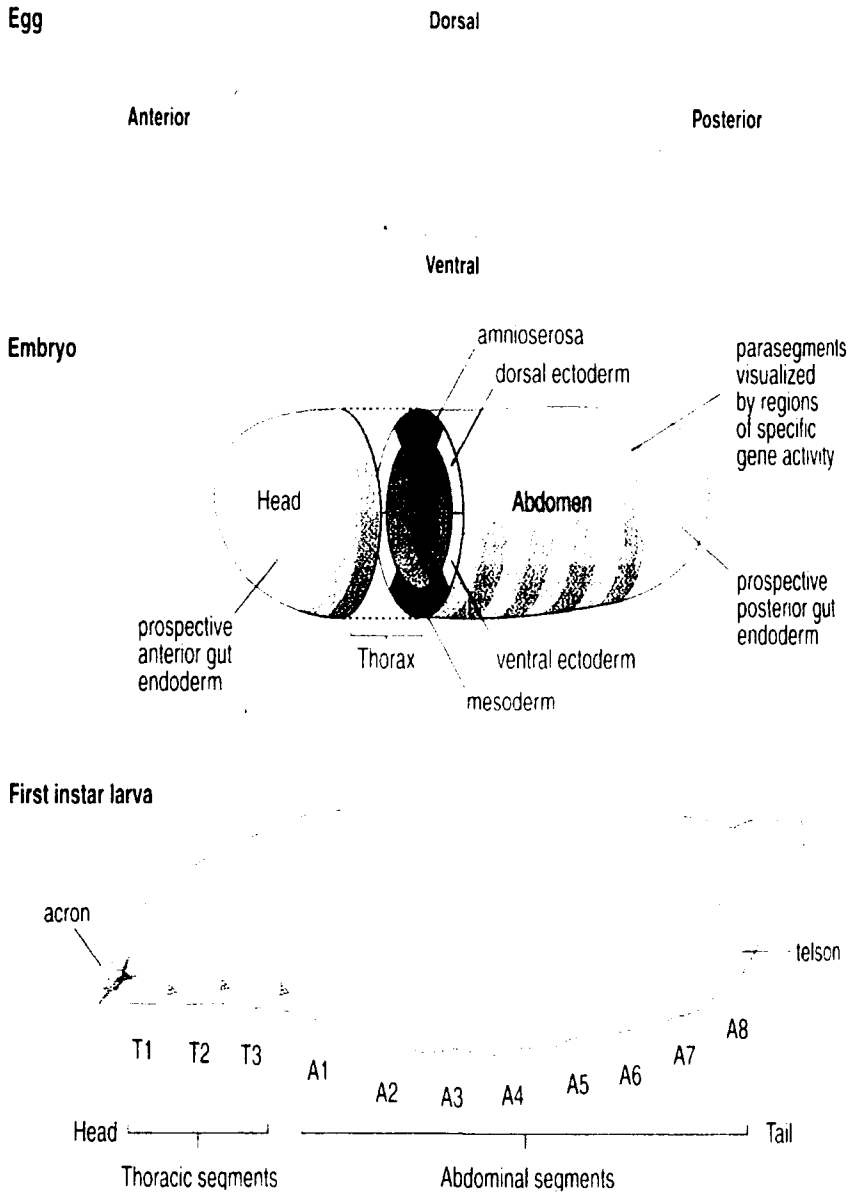
for the construction of individual strains in which ectopic expression of the target gene (gene of interest) can be directed to different tissues or cells. Second, two distinct transgenic lines are established, separating the target gene from its transcriptional activator. This ensures the viability of the parental lines; only by crossing the two lines is the target gene turned on and the phenotypic consequence of misexpression (including lethality) can be studied in the progeny. Lastly, the GAL4-UAS system is designed so that the target can be activated in different cell and tissue types by crossing a single line carrying the target gene to a library of "driver lines", activator-expressing lines. These driver lines can direct the expression of each new target gene in numerous distinct patterns. The GAL4-responsive target gene is subcloned behind a tandem array of five optimized GAL4 binding sites referred to as the upstream activating sequence (UAS) and upstream of the SV40 transcriptional terminator.

I-2 Morphogenetic Gradients and the Establishment of Axial Polarity

I-2a Specification of the Antero-Posterior Axis

Development from a single egg cell into a multicellular organism is dependent on both the fates of many cell types and their organization into elaborate patterns. As has been recognized by embryologists since the early part of this century, many organisms, including *Drosophila melanogaster*, contain localized regions of cytoplasm that direct the formation of specific parts of the embryonic pattern (St. Johnston and Nüsslein-Volhard, 1992). However, for successful progression of *Drosophila* embryogenesis from one of homogeneous beginnings found in the egg to that of larval segmental compartmentalization, not only must cell fate be established accordingly, but also provision of a prepattern for subsequent development must be accounted for. Maternally encrypted signals regulate both requirements; four localized maternal signals define the basic organization and polarity of the two major embryonic axes, antero-posterior (A-P) and dorso-ventral (D-V) (Figure 1.11). These maternal signals provide positional information in the egg and control the spatial pattern of zygotic gene expression, ultimately determining the developmental fate of individual cells. Messenger ribonucleic

Figure 1.11 The body plan is patterned along two separate axes.
The antero- posterior and dorso-ventral axes are at right angles to each other. Laid down initially in the egg, the dorso-ventral axis is divided into four region in the early embryo: mesoderm (red), ventral ectoderm (yellow), dorsal ectoderm (orange) and amnioserosa (an extra-embryonic membrane, green). The ventral epidermis and neural tissue originate from the ventral ectoderm, the epidermis from the dorsal ectoderm. The antero-posterior axis becomes divided into various regions that develop into the head, thorax and abdomen. Once the broad body regions become defined,segmentation begins. The future segments can be observed as transverse stripes by staining for specific gene activity (10 of the 14 are marked here). The embryo develops into a segmented larva.The hatched larva displays the 14 stripes, or parasegments, converted into thoracic (T1-T3) and abdominal (A1-A8) segments (see figure 1.22). Different segments are characterized by the pattern of bristles and denticles on the cuticle. Specialized terminal structures, the acron and telson, develop at the head and tail ends, respectively. After Wolpert, 1998.



acids (mRNAs) from the mother are transferred from within the nurse cell/oocyte syncytium into the developing oocyte and becomes localized accordingly (Berleth et al., 1988). Signaling between the oocyte and surrounding somatic follicle cells during oogenesis initiates the establishment of the A-P axis in *Drosophila melanogaster* (Rongo and Lehmann, 1996; Deng and Bownes, 1998). First, a posterior fate is induced (Deng and Bownes, 1998). Activation of the epidermal growth factor receptor (EGFR) pathway is elicited within predetermined terminal follicle cells upon their receiving a signal originating from the oocyte called Gurken (Grk), a transforming growth factor-alpha homologue. Subsequent to the sending of an unidentified signal back to the oocyte, there is a reorganization of the oocyte cytoskeletal polarity required for proper localization of maternal determinants and the establishment of the A-P body axis (Deng and Ruohola-Baker, 2000). This correct spatial deposition within the developing oocyte determines both the A-P polarity of the oocyte itself and the subsequent embryo (Deng and Bownes, 1998). In a time during development where no signs of morphological cellular specification exists, how do the maternal factors organize amongst themselves to lay down along the establishing axes and establish the "blueprint" from which subsequent factors base their roles upon? How is a precise hierarchy of zygotic gene activity established by the regulation of what the mother provides her embryo? Although not as simple as the tossing of a coin, the answers begin with a decision made between heads and tails.

I-2b Terminal Localized Centers of Activity in the Drosophila Egg

Studies performed by the groups of Frohnhofer et al. (1986), Nüsslein-Volhard et al. (1987) and Struhl (1989) report two centers of activity as sources of morphogenetic gradients. They present evidence for terminal organizing centers with long-range influences. Through experimental embryology and developmental genetics, the collective data illustrates a prelocalization of morphogenetic determinants. These morphogenetic determinants in turn orchestrate the polarity and pattern of the embryo.

To isolate the factors providing spatial information to the developing embryo, information collected from various experimental manipulations of the *Drosophila* egg provided much insight into the system properties of embryonic pattern formation. Classical embryologists sought answers to four main problems: (i) the number of factors exclusively involved in pattern formation, (ii) the spatial distribution of these factors, (iii) the connection between the resultant pattern and the factors, and (iv) characterization of the interaction or interdependence of different factors. The customary approach used in collecting this kind of data was to mechanically manipulate the egg cell such that an altered pattern resulted. Nüsslein-Volhard et al. (1987) performed such experiments through procedures of egg pricking and transfer of the cytoplasm, showing that the *Drosophila* egg contains localized cytoplasmic determinants. Pricking at the anterior pole of the syncytial blastoderm primarily generated embryos with reduced head structures and thoracic defects, including an anterior shift of the segmented pattern (Frohnhofer et al., 1986; Nüsslein-Volhard et al., 1987). In removing cytoplasm from the posterior end, the resulting larvae showed abdominal deletions, but the most posterior somatic region of the embryo, the telson, showed little to no signs of defect. These cytoplasm leak experiments suggested that there are factors localized at both the anterior and posterior egg poles required for the development of respective embryonic regions. Moreover, studies involving the transfer of anterior cytoplasm to any position along the A-P axis, resulted in an induction of anterior development and the suppression of posterior structural formation, including most abdominal segments (Nüsslein-Volhard et al., 1987). With the transplant of posterior pole plasm to the anterior pole, phenotypes were indistinguishable from those having resulted from removal of anterior plasm, suggesting inhibition of anterior factors by posterior ones (Nüsslein-Volhard et al., 1987). Additional studies involved the transplantation of posterior pole plasm into the anterior pole void of anterior pole plasm, which lead to induction of a complete posterior abdominal end with reversed polarity at the anterior and a phenotype referred to as bicaudal. Comparable results were obtained by transplanting anterior cytoplasm, which suppressed posterior end development. However on the opposite side of that same coin, it could be said that not only is there a suppression of hallmark posterior structural development, but rather an inability of the anterior activity to induce posterior segmental

identity. In response to this, a study conducted by Struhl (1989) provides supporting evidence to the idea of an existing posterior morphogen system opposing the anterior one. The anterior morphogen, *bicoid* elicits a directly instructive influence on head and thoracic patterning via changes in its concentration along the embryonic A-P axis. In contrast, Struhl (1989) found that the primary role of the posterior morphogen *nanos* is to permit abdominal patterning by eliminating the expression of a *bicoid*-regulated ubiquitous repressor, *hunchback*, from the posterior half of the embryo. Struhl (1989) found that ubiquitous expression of the repressor led to no posterior development, a phenotype similar to that of homozygous *nanos* embryos. In this study, Struhl also found that in removing *nanos* activity from the embryo, along with the repressive activity of hunchback, embryos were actually viable and produced normal larvae and adults with normal abdominal patterns. These data lead Struhl to suggest that the anterior and posterior morphogen systems may specify the subdivision of the *Drosophila* body into distinct anterior and posterior patterning domains dependent on their immediate effects on subsequent downstream target genes such as *hunchback*, thus making heads from tails. As presented in further detail in the next sub-chapter, *bicoid*, *hunchback* and *nanos* are all crucial in establishing the antero-posterior axis and patterning of the developing embryo.

Collectively, the studies of Frohnhofer et al. (1986), Nüsslein-Volhard et al. (1987) and Struhl (1989) provided supporting evidence towards the possible existence of opposing anterior and posterior morphogen systems. With this came the myriad of genes, their products and interdependent interactions manifested in the elaborate hierarchy of embryonic polarity and subsequent segmental identification.

I-2c Three Classes of Maternal Genes Specify the Antero-Posterior Axis in Drosophila

The fertilized *Drosophila* egg initiates polarity and patterning within a three-hour period following fertilization into a segmentally organized larva. The complexity of larval morphology that results is in stark contrast to the almost homogenous egg and is telling in that what lies beneath is more than just a proliferative cell mass; the egg cell must contain spatial cues guiding early developmental decisions. At this first stage of cellular

development, all somatic cells are formed and the blastoderm is comprised of approximately 5000 cleavage nuclei synchronously separated by cell membranes growing in the egg membrane. Yet amidst the homogeneity of this stage, the beginning of segmental differentiation is concomitant to the spatially restricted expression of embryonic segmentation genes (Nüsslein-Volhard and Wieschaus, 1980; see sub-chapter I-4). The identity of these segmentation genes involved in establishing the anlagen of various larval organs and tissues from the blastodermal sheet of cells were discovered and the defects observed in the recovered mutants classified into specific phenotypic classes. A schematic blastoderm fate map indicating the areas from which the various larval body regions form and one of a wild-type larva can be seen in A2 of the Appendix.

Differences along the A-P axis before egg fertilization are created by maternal gene expression. It is these differences that distinguish the future head and posterior ends of the larvae, including the segmental units in between. Saturation mutagenesis experiments were conducted to identify the genes responsible for creating the larval segmental patterning. Termed the maternal coordinate genes, they are classified into groups responsible for the development of one of the three embryonic regions, anterior, posterior or terminal and act independent of one another (Figure 1.12). Mutations of coordinate genes exert both inductive and polarizing activities on larval patterning and are classified into three global phenotypes: those that affect anterior regions, comprised of the head and thoracic segments; those affecting the posterior region consisting of the abdominal segments; and those that affect both terminal regions, consisting of two morphologically nonsegmented ends (the acron and telson) (Nüsslein-Volhard et al., 1987). As defined previously (sub-chapter I-1, pg.9) females that carry such mutations lay eggs morphologically intact, however the development of ectodermal derivatives shows the underlying result of patterning defects. As highlighted in a review published by St. Johnston and Nüsslein-Volhard (1992), several relevant conclusions can be taken from such observations. First, the number of genes that are specifically involved in the regulation of positional information in the developing egg must be quite small. Second, because mutations of maternal-effect genes affect either the A-P pattern or the D-V pattern, never both, the two body axes must be established independently. Finally,

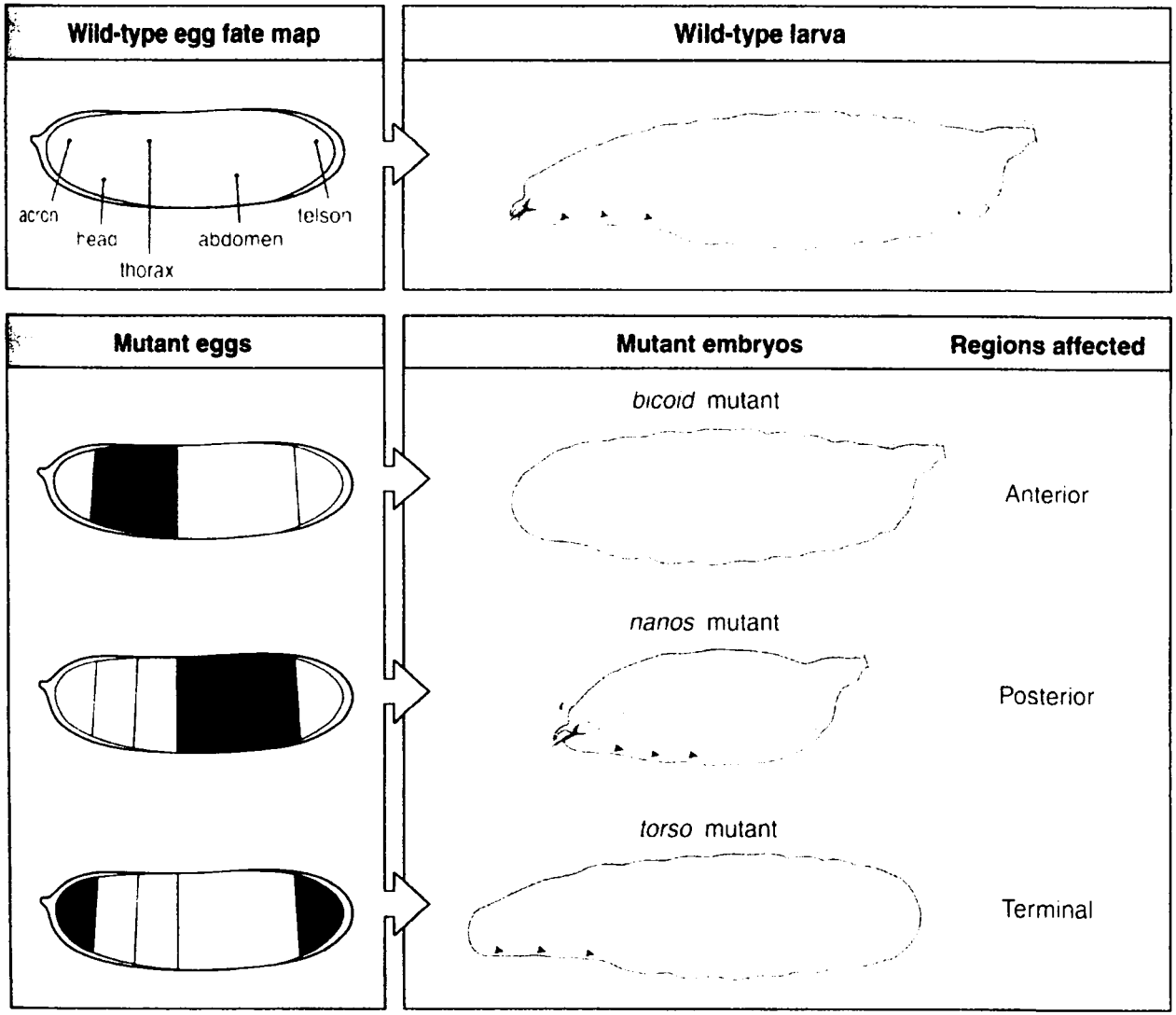


Figure 1.12 Mutational effects in the maternal gene system. Mutations in maternal-effect genes result in deletions and abnormalities in anterior, posterior or terminal structures. The wild-type fatemap illustrates which regions of the egg yield particular regions and structures in the larva. Affected regions in mutant eggs resulting in lost or altered structures in larva are shaded in red. In *bicoid* mutants, there is a partial loss of anterior structures and appearance of an ectopic posterior structure, the telson, at the anterior end. *nanos* mutants are missing a large part of the posterior region. Both the acron and telson fail to develop in *torso* mutants. After Wolpert, 1998.

compared to the number of genes involved in the specification of the body axes, the number of embryonic phenotypes observed is much smaller. Essentially then, genes can be grouped into particular classes based on which parts of the embryo they affect; the common phenotype generated by mutations in the genes of one class suggests that these genes act along a common pathway to specify a specific part of the embryonic pattern. It is this dogma in which the current study has based its classification of the *Drosophila tango* gene. The following subchapter takes a look at the maternal genes required in polarizing the A-P axis as they function through their established morphogenetic gradients.

I-3 Maternal Genes Establish the Drosophila Antero-Posterior Body Axis

The cellular blastoderm is a hotbed of genetic activity, where the activity of one gene is dependent on the activity of another and then another and so on. The deposition of maternal genes into her newborns is where this inter-dependent hierarchy of regulation begins. The products of four maternal genes in particular-*bicoid* (*bcd*), *hunchback* (*hb*), *nanos* (*nos*) and *caudal* (*cad*)-become distributed along the antero-posterior (A-P) axis and are essential in establishing it. The A-P axis of the *Drosophila* embryo is also specified by a set of maternal-effect genes that define the terminal boundary regions belonging to a maternal system that is discrete, but not necessarily mutually exclusive, of the anterior and posterior organizing centers. That is, factors of the terminal system may function synergistically with factors of the other two maternal systems, namely, the anterior system.

Anterior End Development

I-3a A-P Polarity is Initiated by the Anterior Determinant, bicoid

Two properties must be active for a maternal system to contribute to the specification of the embryonic pattern (review, St. Johnston and Nüsslein-Volhard, 1992). First, there must be the localization of some factor of the system in order to establish the initial

asymmetric signal. Second, this established signal must result in the production of a transcription factor that in turn regulates zygotic target genes. In the specification of the anterior system, both properties define the products of the *bicoid* (*bcd*) gene. *bcd* is of special interest in understanding the maternal-effect of *tgo* during early embryonic patterning due to its unique organizing effect on *Drosophila* anterior development (Frohnhofer and Nüsslein-Volhard, 1986) and unifying measures between the anterior and terminal *Drosophila* maternal systems (Schaeffer et al., 2000). Located within the homeotic gene *Antennapedia* complex (HOX-C) between *zerknult* and *Deformed* (Randazzo et al., 1993), *bicoid* is expressed maternally in response to a general transcription factor encoded by the gene *serendipity delta* (*sry* δ) in the nurse cell/oocyte syncytium (Payre et al., 1994). The *bicoid* protein (Bicoid, Bcd) consists of four exons, wherein the first one is found at the N-terminal end and consists of a region homologous to the alternating histidine-proline *paired* (*prd*) *repeat* domain. The *prd repeat* motif is housed within the carboxyl terminal end of Tgo, Paired and is encoded by several other genes expressed during early development (Frigerio et al., 1986; see Chapter 4, Discussion). Following the *prd repeat* in the amino-terminal region, the third exon encodes a homeodomain within its amino-terminal portion, with no more than approximately 40% amino acid identity to any other known homeobox sequences (Frigerio et al., 1986). Following the homeodomain in the second half of the third exon is a region of repetitive glutamines called M- (McGinnis et al., 1984) or opa-repeats required for transcriptional activity (Wharton et al., 1985). With the exception of the homeobox, M-repeat and *prd repeat*, there has been no significant homologies to other sequenced genes detected (Berleth et al., 1988).

The anterior pattern of the *Drosophila melanogaster* embryo is established by a small number of maternal-effect genes: *bicoid*, *exuperentia* (*exu*), *swallow* (*swa*) and *staufen* (*stau*). Both *bcd* and *exu* show anterior defects only, whereas head defects are only one aspect of the *swa* and *stau* phenotypes (see Martinez-Arias, 1993). Other maternal genes have been isolated for exhibiting an anterior phenotype, however these genes are primarily involved in determining the oocyte and establishing oocyte polarity (Manseau and Schupbach, 1989) and therefore will not be considered here. Of particular interest to

this study is *bcd* and its influence on embryonic development immediately following fertilization, specifically in assigning blastodermal cell fate by determining both anterior structural formation and entire embryonic polarity. All eleven *bcd* alleles are strictly maternal, each with a penetrance of 100% but different in the extent of anterior defects they generate (Frohnhofer and Nüsslein-Volhard, 1986). In general, embryos mutant for *bcd* fail to produce head and thorax: amorphic *bcd* alleles completely lack head and thorax development and form an ectopic telson (posterior terminal end structure) at the anterior end whereas weaker *bcd* alleles lack proper anterior and thoracic structural development to varying degrees (Nüsslein-Volhard and Wieschaus, 1980). Therefore, two types of defects define the *bcd* phenotype; loss of anterior structures due to shrinkage of the anlagen and abdominal segmentation defects (Frohnhofer et al., 1986). Moreover, several lines of evidence demonstrated an anterior-to-posterior morphogenetic role for Bcd (Frigerio et al., 1986; Berleth et al., 1988; St. Johnston et al., 1989) as a dosage-sensitive gene (Frohnhofer et al., 1986; Berleth et al., 1988). Cytoplasmic transplantation experiments involving injection of synthesized wild-type *bcd* (bcd^+) RNA into various parts of the *Drosophila* embryo prior to three hours after egg laying resulted in the formation of ectopic head and thoracic structure, with the most anterior pattern elements forming closest to the site of injection (Frohnhofer et al., 1986) (Figure 1.13). Not only had this line of evidence revealed the long-range organizing effects of bcd^+ activity on the A-P pattern, but also importantly, it was seen that the actual shape of the Bcd gradient defines the polarity of anterior patterning in an inductive manner. This defined *bcd* as a determinant of anterior development. Notably, when discussing morphogenetic gradients, it is important to keep in mind the possible variability within the established concentration slope. Often within the *Drosophila* model system, the function of a morphogen along its gradient is interpreted much like the schematic presented in Figure 1.14. Although perhaps depicted as a constant and somewhat linear function of the morphogenetic gradient, a recent report challenges this perception. A profile quantitative analysis of Bcd conducted by Houchmandzadeh et al. (2002) cites a significant variability from one wild-type embryo to another in the formation of the Bcd gradient, with no significant effect on the distribution of another established morphogenetic gradient present in the syncytial blastoderm known as Hunchback (Hb) (a *bcd* target gene and A-P

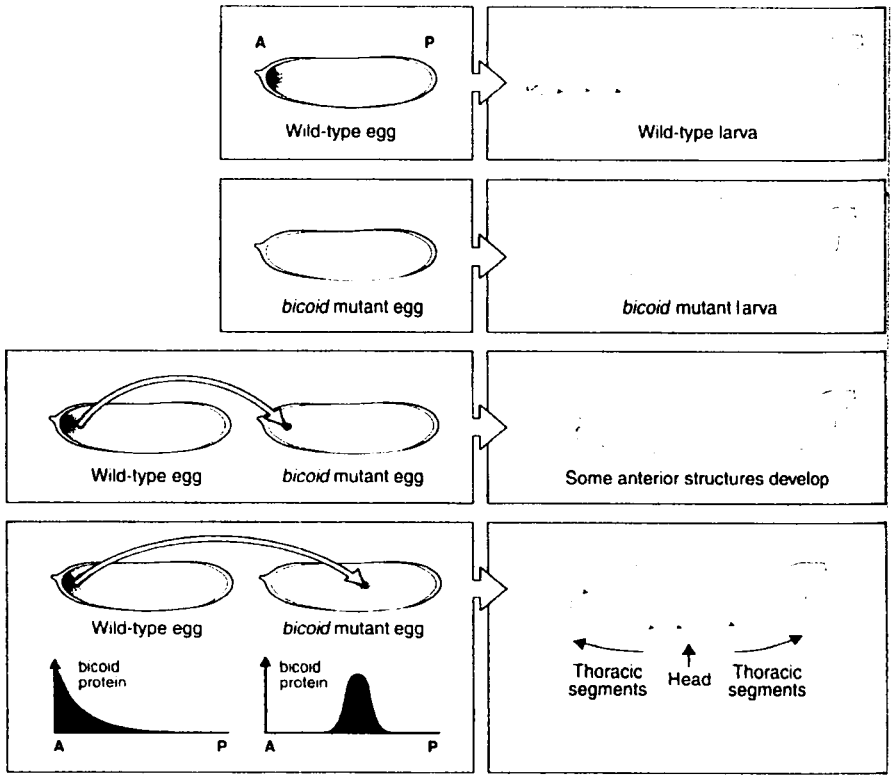


Figure 1.13 The development of anterior structures is dependent on the bicoid gene. Embryos whose mothers lack the *bicoid* gene lack anterior regions (second row panels from the top). Partial anterior structure development occurs at the site of injection with the transfer of anterior cytoplasm from wild-type embryos to *bicoid* mutant embryos (third row panels). Transfer of wild-type anterior cytoplasm to the middle of a *bicoid* mutant egg or early embryo results in ectopic head structure development at the site of injection, flanked on both sides by thoracic-type segments (bottom panels). The anterior cytoplasm establishes a gradient of *bicoid* protein with the highest concentration at the site of injection. After Wolpert, 1998.

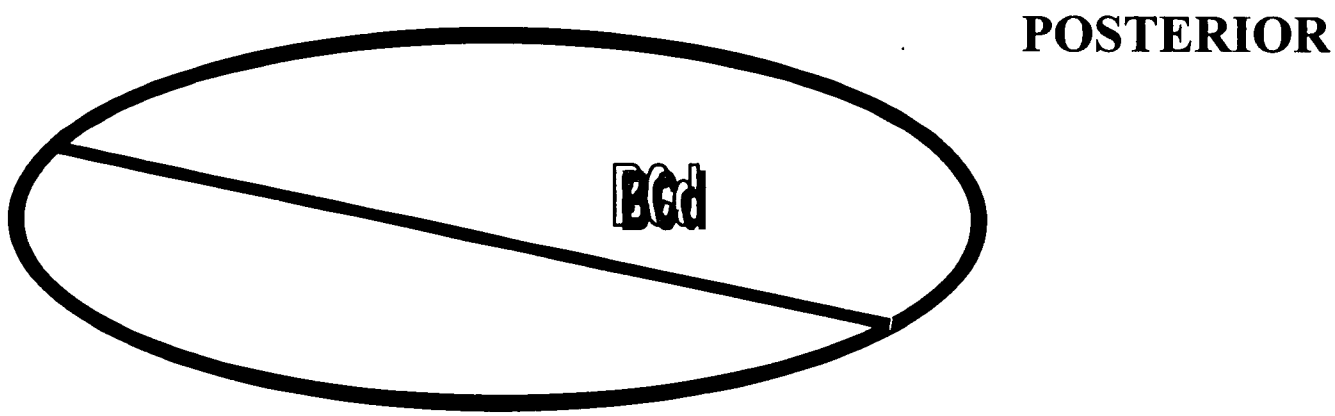


Figure 1.14 The interpretation of morphogenetic gradients. Downstream morphogenetic activity may be believed to exist as a linear and faithful function dependent on earlier established gradients, perhaps visualized much like the schematic on the right. Consistent with this view, the gradient of Hb is a function of the previously established Bcd gradient. A report conducted by Houchmandzadeh et al. (2002) challenges this interpretation.

axis determinant; see next section). A maintained positional readout in targeted protein distribution profiles following manipulation of a previously established gradient may be attributed to a noise-filtering mechanism that may be a general property of morphogenetic systems and required for the precise elaborate patterning of the developing embryo (Patel and Lall, 2002). Houchmandzadeh et al., (2002) support this proposal by suggesting that the distribution of Hb may be influenced by filtering correction mechanisms that both normalize the variability in Bcd gradients and import conserved positional information.

Nonetheless, regardless of how the regulatory role of *bcd* is analyzed, a development apex is certainly reached: *bcd* is necessary for the establishment of anterior-to-posterior structural development due to its maternally derived morphogenetic properties. Remember that for a maternal system to play part in the specification of the embryonic pattern, it must act on two properties, one of which is to provide a localized signaling factor present asymmetrically in the developing embryonic system and the other property involves the regulation of downstream target genes. The role of maternal *bcd* during embryogenesis does not stop at its requirement in anterior development. The maternal-effect of *bcd* is globally consequential, setting the stage for head-to-tail formation through provision of positional information for other maternal genes (such as *hunchback*, for example) and segmentation genes (such as gap genes) involved in the establishment of A-P polarity.

I-3b Synergy between the Bicoid and Hunchback Morphogens is Essential in the Organization of Anterior Body Patterning

Proteins containing homeobox domains are thought to act as transcription factors primarily required to bind directly to specific DNA sequences to activate or repress transcription (Jaynes and O'Farrell, 1988; Han et al., 1989). *bcd* is an example of such homeodomain-containing transcription factors responsible for positive or negative regulatory interactions. The first zygotic group of genes thought to respond to maternally provided spatial cues are the gap genes (Figure 1.15, see next sub-chapter). Several

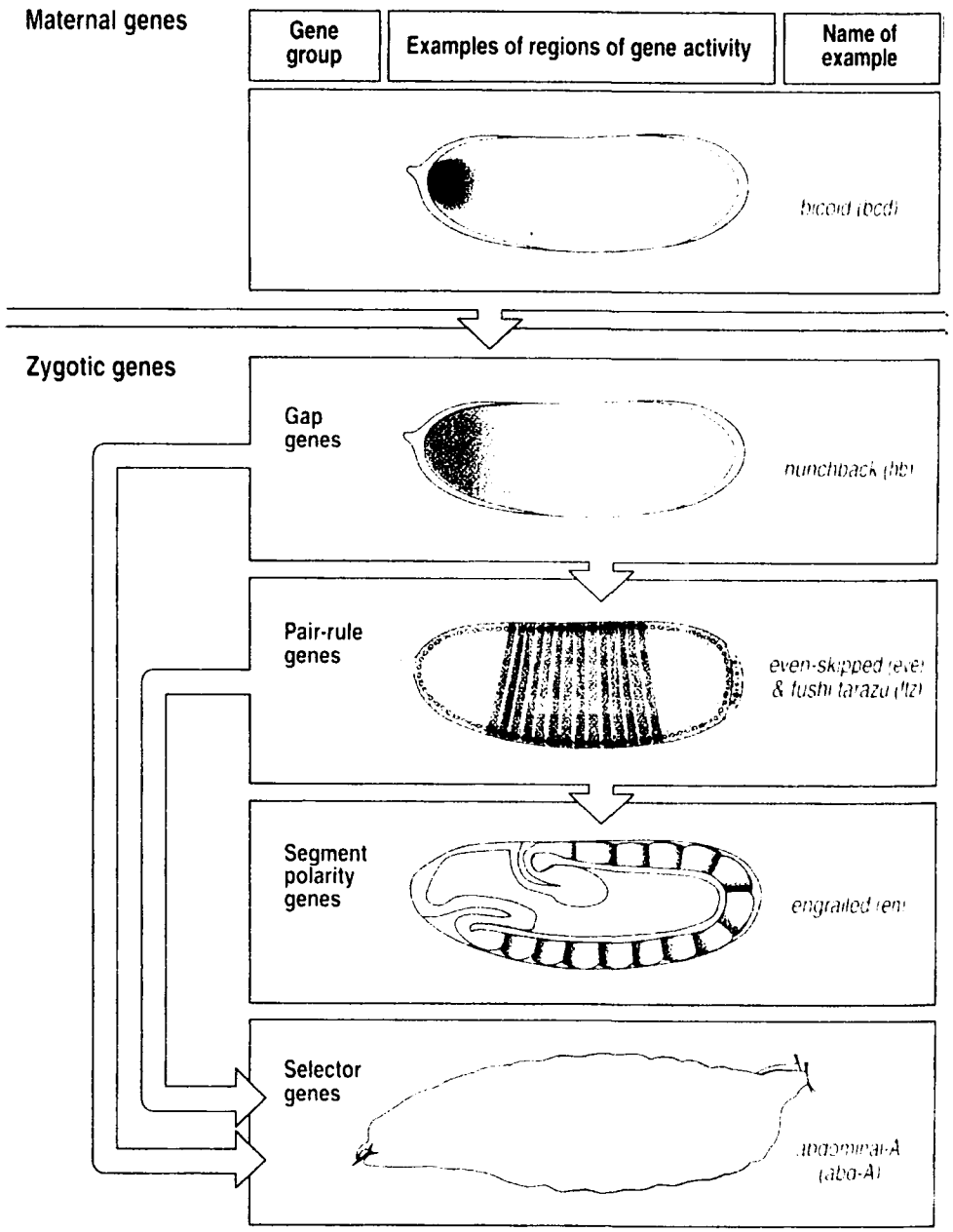


Figure 1.15 The sequential expression of different sets of gene establishes the body plan along the antero-posterior axis. Following fertilization, there is translation of maternal gene products laid down in the egg. Maternal gene products such as *bicoid* mRNA provide position information which activates the zygotic genes. There are four main classes of zygotic genes acting along the antero-posterior axis: the gap genes, the pair-rule genes, the segment polarity genes and the selector, or homeotic, genes. The gap genes demarcate regional differences leading to the expression of a periodic pattern of gene activity by the pair-rule genes, which define the parasegments and establishes a "blueprint" of segmentation. Elaboration of this pattern is performed by the segment polarity genes and segmental identity is determined by the selector genes. After Wolpert, 1998.

studies report that *bcd* determines the A-P axis and defines head and thoracic structures by differentially directing the patterned expression of gap genes. Gap genes contain promoters that respond to the concentration gradient of *bcd* through *bcd*-binding sites with variable affinities (Driever et al., 1989; Struhl et al., 1989). Members of this class of genes include those restricted to the most anterior region of the embryo where *bcd* concentration is at its highest and contains presumably weak *bcd*-binding sites, such as the head gap genes *orthodenticle* (*otd*), *empty spiracles* (*ems*) and *buttonhead* (*btd*). Other gap genes include those requiring a low threshold level of *bcd* for their activation, such as *Krüppel* (*Kr*), *knirps* (*kni*) and *hunchback* (*hb*) (Driever and Nüsslein-Volhard, 1989). *hb* is a likely target of direct spatial control under *bcd* (Nüsslein-Volhard and Wieschaus, 1980; Lehmann and Nüsslein-Volhard, 1987) and is also required for the proper establishment of the anterior half of the embryo (Tautz et al., 1987, 1988; Driever and Nüsslein-Volhard, 1989). Encoding a zinc finger protein, *hb* is expressed maternally as well as zygotically and its maternal mRNA is ubiquitously distributed at the time of egg deposition (Tautz et al. 1987). The maternal and zygotic *hb* mRNAs encode identical proteins that share the same spatial expression domain in the anterior end of the early embryo, consistent with the ability of wild-type *hb* or zygotic *hb* (hb^{zyg}) in compensating for maternal *hb* (hb^{mat}) (Tautz et al., 1987). The two gradients are almost redundant: hb^{mat} is dispensable for A-P patterning and activation of hb^{zyg} by *bcd* almost is (Hulskamp et al., 1989; Wimmer et al., 2000). Compensation for the absence of hb^{mat} can be provided by a paternal copy of hb^{zyg}, however embryos lacking both hb^{mat} and hb^{zyg} exhibit an anterior cuticular phenotype of greater severity compared to zygotic mutants alone (Lehmann and Nüsslein-Volhard, 1987). This suggests that hb^{mat} plays a role in anterior development (Lehmann and Nüsslein, 1987). Initially, the ubiquitous expression of hb^{mat} develops into a pattern that mirrors that of the early *bcd*-dependent hb^{zyg} pattern (Tautz, 1988). Hb^{mat} functions as a bone fide morphogen capable of patterning the thorax and abdomen through its regulation of gap gene expression in the middle and posterior regions of the embryo (Hulskamp et al., 1990; Struhl et al., 1992). This was concluded in studies showing that hb^{zyg} can compensate for the lack of hb^{mat} and reciprocally, hb^{mat} known for its role in specifying positional values in a concentration-dependent manner, can in part compensate for the loss of *bcd* and hb^{zyg}

(Hulskamp et al., 1990; Struhl et al., 1992). Translation of hb^{mat} mRNA is inhibited in the posterior half of the embryo by the activity of the posterior group gene *nanos* (*nos*) (see following section). Interestingly, the genetic removal of hb^{mat} has been shown to lead to the complete rescue of the *nos* mutant phenotype. In this exceptional circumstance, where the influence of maternal *hb* transcripts is removed by mutation thereby obviating a requirement for *nos*, normal embryonic patterning becomes dependent on the formation of an *hb* gradient under *bcd* control (Hulskamp et al., 1990). Therefore to monitor anterior patterning, in addition to establishing normal polarity to the developing embryo, *bcd* alone cannot enable such provisions. Not only is this telling with regards to the purpose of *nos* in its primary role in blocking the production of Hb protein in the posterior half of the embryo (Struhl, 1989; Hulskamp et al., 1989) but also there appears to be a concerted effort in ensuring that *hb* activity remains high in the anterior of the early embryo and not diffuse into the posterior end. This mechanism at work once again highlights the functional significance of morphogenetic gradients and the positional information they carry in establishing the A-P axis. The evidence combined above suggests that *hb* has an instructive rather than permissive role, one that is *bcd*-dependent, in establishing the entire body plan (Simpson-Brose et al., 1994). As is the case for many early-acting genes during *Drosophila* embryogenesis, this instructive role of Hunchback is believed to function as both an activator and repressor in establishing segmental identity in the *Drosophila* embryo (Pankratz and Jackle, 1993). However, even with direct molecular proof of a concentration-dependent function, it would be difficult to precisely determine at which point in development of the embryo a factor could switch from activation to repression of target gene expression. This problem remains unresolved in the Hb study (Pankratz and Jackle, 1993).

Posterior End Development

I-3c nanos Permits Expression of Genes Directing Abdominal Development in its Role as the Posterior Determinant

In the patterning of the A-P body axis, post-transcriptional regulation is essential in regulating the production of key developmental regulators from maternally supplied RNAs. As described in section I-3a, *bicoid* (*bcd*) is required in the establishment of the embryonic longitudinal axis in Drosophila in part through its interaction with *hunchback* (*hb*). Additionally, establishment of the embryonic A-P axis is under the control of another localized determinant, *nanos* (*nos*) (Figure 1.16; Driever and Nüsslein-Volhard, 1989). Just as it is essential that *bcd* protein be localized to the anterior of the embryo for the establishment of A-P polarity, so too is the restriction of Nos protein to the posterior end in establishing proper axial patterning (Driever and Nüsslein-Volhard, 1989; Wang et al., 1994). Both *bcd* and *nos* are synthesized maternally and translation of *bcd* and *nos* mRNAs localized to their respective pole leads to opposing protein gradients in the early syncytial embryo. Bcd is translated in the anterior pole diffuses to form an anterior-to-posterior gradient and Nos becomes translated at the posterior pole forming a posterior-to-anterior gradient (Driever and Nüsslein-Volhard, 1989; Gavis and Lehmann, 1992; Wang et al., 1994). Despite these similarities in the earliest establishment of the *bcd* and *nos* protein gradients, the two function along eminently different mechanisms. In the anterior half of the embryo, Bcd activates zygotic transcription of anterior-specific genes, including *hb* (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989; Driever, 1993), whereas Nos represses translation of the uniformly distributed maternal *hb* RNA in the posterior (Tautz, 1988; Tautz and Pfeifle, 1989). Sites in *hb* mRNA that regulate repression of maternal transcripts of *hb* by *nos* have been isolated in the 3' untranslated region (UTR) (Murata and Wharton, 1995). They are known as Nanos Response Elements (NREs) and presumably serve to recruit Nos to in turn inhibit some component of the translation machinery (Murata and Wharton, 1995). Furthermore, as the *bcd* 3'UTR also contains an NRE, it is thereby essential that *nos* activity be limited to the posterior half of the embryo in order to permit anterior end development; ectopic Nos in

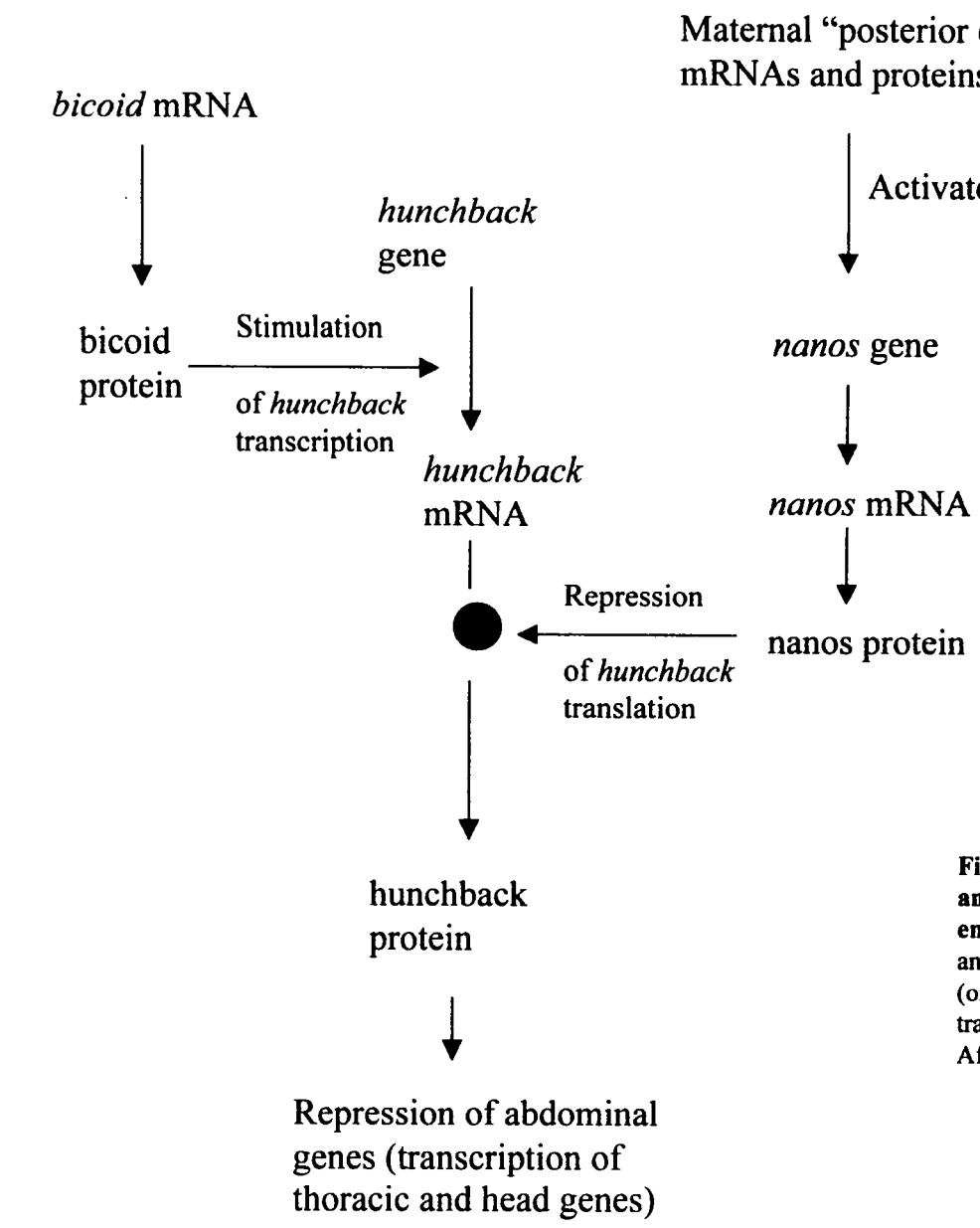


Figure 1.16 Schematic flowchart for the action of *bicoid* and *nanos* proteins in specifying the antero-posterior axis of the embryo. *bicoid* activates the transcription of *hunchback* in the anterior region where Nanos is not present to inhibit its expression (orange). In the posterior region (gray), *nanos* represses *hunchback* translation, thereby enabling the expression of abdominal genes. After Gilbert (1991).

the anterior half leads to translational repression of both *bcd* and *hb*, consequently resulting in suppression of head and thorax development (Gavis and Lehmann, 1992). Genetic experiments have shown that *nos* is required during embryogenesis principally to suppress hb^{mat} translation: homozygous nos is embryonic lethal, however embryos double mutant in both nos and hb^{mat} are viable (Hulskamp et al., 1989; Struhl, 1989). This much needed repression of hb^{mat} in the posterior end of the developing embryo permits proper development by allowing nos to direct expression of abdominal-specific genes (Clark et al., 2002). Rather than be solely responsible in providing a morphogen for the spatial organization of the abdomen, the primary function of the posterior system is to promote proper development through its interaction with maternal systems, such as *bcd* (Hulskamp et al., 1990; Struhl et al., 1992; see section I-3e).

I-3d caudal is an Activator of Abdominal Segmentation Genes Through Previously Established Maternal Systems, bicoid and hunchback

Shortly following translation of *bcd* mRNA, a second homeodomain protein under *bcd* control, *caudal* (*cad*) (Mlodzik et al., 1985), accumulates in an opposing posterior-to-anterior protein gradient much like Nos (Macdonald and Struhl, 1986; Driever and Nüsslein-Volhard, 1989; Mlodzik et al., 1990). Antibodies directed against the Cad protein along with mutations in the *cad* gene revealed Cad nuclear localization and an A-P gradient throughout the embryo during most of the syncytial blastoderm stage (Macdonald and Struhl, 1986). *cad* activity is required for proper posterior end development (Macdonald and Struhl, 1986), whereas ectopic expression of *cad* in the anterior of the embryo causes deleterious effects of head and thoracic development, illustrating the need for restricted spatial expression of factors active immediately following fertilization. *cad* has both a maternal and zygotic phase of expression. Maternal cad mRNA is controlled translationally by the anterior maternal system. For example, the repression of maternal *cad* by *bcd* has been shown as crucial in establishing A-P polarity and normal patterning in the developing embryo (Macdonald and Struhl, 1986). Binding assays, transgene and mutant analyses have shown that *cad* mRNA translation becomes repressed through a 3′ UTR-bound Bcd unit that interferes with the assembly of the initiation complex (Niessing et al., 2002). Moreover, the function of the

abdominal *cad* domain was studied in the absence of normal *bcd* and *hb* expression. By introducing an artificial Hb gradient into embryos lacking *bcd* and *hb* activity, an ectopic zygotic *cad* domain in the more anterior region resulted. Using this system, Schulz and Tautz (1995) showed that the *cad* domain activates expression of abdominal gap segmentation genes *knirps* (*kni*) and *giant* (*gt*), thereby implicating a role for *cad* during early embryonic patterning likely regulated by hb activity. Embryos lacking cad^{mat} are viable, however the zygotic *cad* component, spatially restricted to the posterior end of the pre-cellular blastoderm (Schulz and Tautz, 1995) is essential for embryonic viability (Macdonald and Struhl, 1986).

Terminal End Development

I-3e The Terminal Maternal System is Linked to the Activity of bicoid in Establishing Proper Antero-Posterior Polarity

The third determinant system for A-P axial development in the *Drosophila* embryo consists of the terminal class of genes. The terminal system is responsible for specifying the patterns at both ends of the embryo. As it is not affiliated with localized transplantable cytoplasmic factors, the terminal system is distinctly different from either the anterior or posterior systems (Sprenger and Nüsslein-Volhard, 1993). Therefore, the existence of the terminal system, required independently of and in addition to the anterior and posterior systems, was not revealed by classical embryological studies as reported earlier in this introduction (section I-2), but rather, through analyses of mutant phenotypes obtained in genetic screens. Three genes, *torso*, *torsolike* and *trunk* were defined as significant to establishing the terminal ends of the developing embryo.

Localized mRNAs, such as *bcd* and *nos*, dictate the function of the anterior and posterior systems respectively, whereas the terminal system depends on the spatially targeted production of an extracellular ligand that activates the receptor tyrosine kinase Torso (Tor) (see Sprenger and Nüsslein-Volhard, 1993; Casanova and Struhl, 1993). The Tor protein is uniformly distributed in the cytoplasmic membrane surrounding the early

syncytial embryo (Stevens and Nüsslein-Volhard, 1991), yet it is only active at the anterior and posterior termini. This spatial delineation is a product of a localized ligand molecule and all cytoplasmic components of the terminal signal transduction pathway evenly distributed in the embryo (Figure 1.17) (see Sprenger and Nüsslein-Volhard, 1993). Therefore, the function of Tor involves receiving a spatial signal external to the cytoplasm and transmitting it inward to initiate a signal transduction cascade triggered to elicit local activation of target genes. Disruption in *tor* function results in head and tail defects rescuable by a local injection of *tor* mRNA into both the anterior and posterior regions of the oocyte early in syncytial stages (see Sprenger and Nüsslein-Volhard, 1993). This implies that local expression of tor in the anterior and posterior regions is required and sufficient for development of all terminal structures. The gene *torsolike* (*tsl*) is expressed and required in somatic follicle cells at both prospective poles of the maturing oocyte and takes part in generating the localized terminal signal (Martin et al., 1994). Another terminal class gene, *trunk*, isolated for its requirement in the female germline, is believed to encode the extracellular Tor ligand (Casanova et al., 1995). Mutations in any one of *tor*, *tsl* or *trunk* results in identical phenotypes affecting both polar ends of the embryo: most anterior structures lost such as the labrum and dorsal bridge and lack of all structures posterior to the seventh abdominal segment (A7) (Schupbach and Wieschaus, 1986a).

In the posterior end of the *Drosophila* embryo, local activation of *tor* regulates expression of terminal gap genes (Martin et al., 1994), while in the anterior, terminal gap genes are activated in an additive manner by the *tor* pathway and the anterior determinant, *bcd* (Schaeffer et al., 2000). Several lines of evidence support this link between the two maternal systems. First, a complete lack of *bcd* activity results in total loss of head and thoracic structures, replaced by posterior terminal ones (recall section I-2a). However, hypomorphic *bcd* alleles only show disruption in complete anterior structural development, such as failure to form labrum and dorsal bridge a phenotype similar to terminal mutants (Frohnhofer et al., 1986). Furthermore, similar phenotypes have been observed in *exu* or *swa* mutant embryos where *bcd* mRNA fails to localize anteriorly, leading to a reduction of *bcd* activity (Frohnhofer et al., 1987). Therefore, a lack of

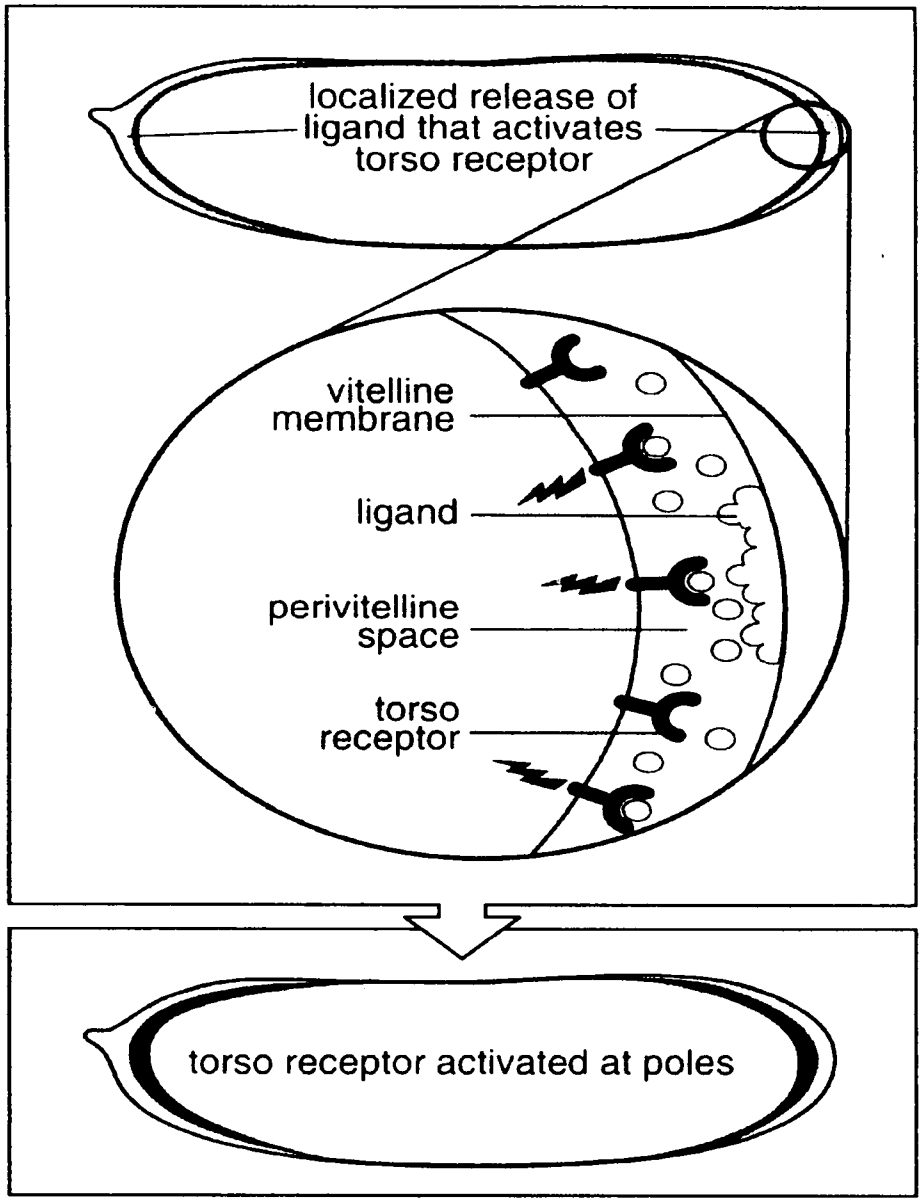


Figure 1.17 The terminal regions of the embryo are in part specified by the gene *torso*. The receptor protein encoded *torso* is ubiquitously distributed throughout the egg plasma membrane. Its ligand is localized at each pole of the egg in the vitelline membrane. Subsequent to fertilization, the ligand is released and diffuses across the perivitelline space to activate the *torso* receptor protein at the ends of the embryo. After Wolpert, 1998.

terminal activity and hypomorphic *bcd* situations appear to result in very similar anterior phenotypes, thereby suggesting that the anterior and terminal maternal systems converge or share common targets. Secondly, most Bcd targets expressed as an anterior cap at the anterior pole of the early syncytial embryo later retract from the tip (Ronchi et al., 1993), an action dependent on the terminal system in order for proper anterior development to occur (Janody et al., 2000). Several additional studies imply a convergence of the anterior and terminal maternal systems in establishing the A-P axis of the developing *Drosophila* embryo. *tor exu* double mutants show a phenotype that is more severe than that seen in homozygous mutants for either *tor* or *exu*, with a strong enhancement of the anterior phenotype exhibiting no head structure formation. This thereby suggested an additive role of the *tor* pathway in the establishment of anterior development by *bcd* (Schupbach and Wieschaus, 1986b). Also, expression analysis of the common target gene *huckbein* (*hkb*) also revealed an additive but independent function of the terminal and anterior systems, as either *tor* or *bcd* alone can activate *hkb*, but together they enhance *hkb* expression at the anterior pole (Bronner and Jackle, 1996). In addition to the collection of studies reported above, Bronner and Jackle (1996) propose a potential coupling between the anterior and terminal maternal systems during early *Drosophila* embryogenesis, one that lies in the derivation of possible shared target genes and the concerted effort of establishing proper axial and pattern formation. The potential connection between the two systems became of special interest to the current study in considering the role of *tgo* in establishing proper antero-posterior polarity.

I-4 Zygotic Genes Pattern the Early Embryo

I-4a The Segmentation Genes

As detailed in previous sub-chapters, maternal factors function along gradients that serve as messengers of positional information instructing the establishment of axial fate in the developing zygote. It is these gradients that are important for the earliest patterning events in *Drosophila* embryogenesis (see St. Johnston and Nüsslein-Volhard, 1992). Transcription factors encoded by *bicoid* (*bcd*) and the maternal component of *hunchback*

(hb^{mat}) are distributed along the A-P axis as gradients with high levels in the anterior regions (Driever and Nüsslein-Volhard, 1988; Tautz, 1988; see Introduction, section I-2a,b). These gradients initiate a hierarchy of genetic interactions eliciting sharp on/off choices in response to positional instruction. This leads to regionalization of target gene expression, resulting in the subdivision of the embryo into 14 segmental primordia, or parasegments, where each behaves as an independent developmental unit under the control of a given set of genes (Figure 1.18; see Pankratz and Jackle, 1993). For example, *bcd* has been shown to be a concentration-dependent activator of zygotic *hunchback* (hb^{zyg}) expression in the anterior half of the embryo (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989; Simpson-Brose et al., 1994). Also, it has been shown that the hb^{mat} gradient marks specific boundaries of target gene expression in posterior regions (Hulskamp et al., 1990; Struhl et al., 1992). In both cases, it is believed that thresholds of protein concentration delimit positions of gene expression boundaries. In this way, nuclei at specific positions within the gradient are exposed to different concentrations of these factors and respond by activating or repressing given sets of genes.

The segmentation genes responsible for establishing segmental identity of the developing embryo within the first three hours of embryogenesis are classified into three groups depending on the regions of the segmenting body plan affected (Nüsslein-Volhard and Wieschaus, 1980). Among the first targets of maternal gradients are the gap genes, the first group of zygotic patterning genes expressed in patterns of one or two broad bands at exact positions along the A-P axis. Mutations of the gap genes delete regions along the A-P axis associated with the given gap gene expressed. Next, the pair-rule genes subdivide the broad gap gene domains into segments. Mutations of this group of genes often delete portions of every segment. Last, the segment polarity genes function to maintain particular repeated structures within each segment. Mutations in the segment polarity genes result in a portion of each segment to be deleted and replaced by a mirror-image structure of another portion of the segment. Mutations in any one gene belonging to any one of these three groups are embryonic lethal and the disrupted A-P pattern is canvassed by ectodermal derivatives most easily seen on the ventral surface of the

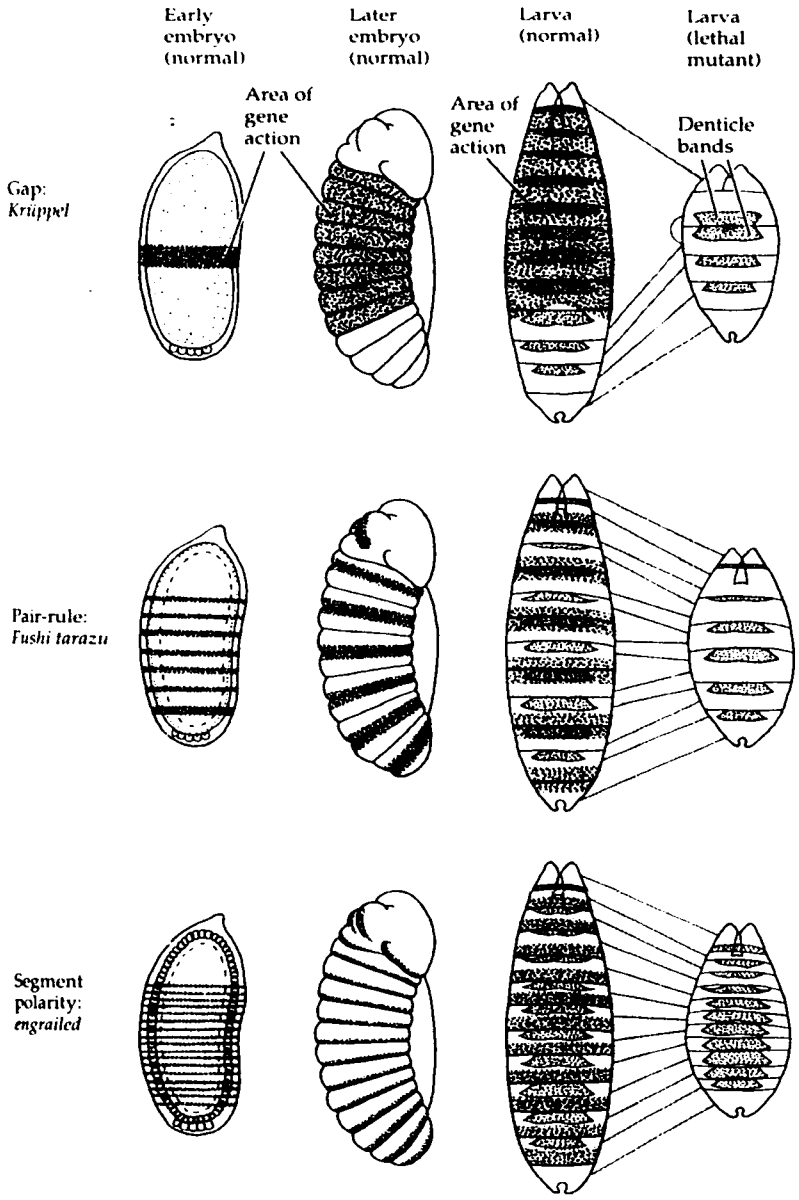


Figure 1.18 Three types of segmentation pattern mutants. The cleavage-stage embryo is depicted on the left side of the figure. The shaded regions represents that in which the classified gene exemplified in each group is normally transcribed in wild-type embryos. The embryos shown to the right of the cleavage-stage embryo in each classification show the areas in color deleted when these mutants develop. The table below shows several major Loci affecting segmentation pattern in *Drosophila* After Gilbert (1991)..

TABLE 2
Major loci affecting segmentation pattern in *Drosophila*

Category	Loci
Gap genes	*Krüppel (Kr)*
	knirps (kni)
	hunchback (hb)
	giant (gt)
	tailless (tll)
	huckebein (hkb)
Pair-rule genes (primary)	*hairy (h)*
	even-skipped (eve)
	runt (run)
Pair-rule genes (secondary)	*fushi tarazu (ftz)*
	odd-paired (opa)
	odd-skipped (sdd)
	sloppy-paired (slp)
	paired (prd)
Segment polarity genes	*engrailed (en)*
	wingless (wg)
	cubitus interruptus^{D} (*cb*^{D})
	hedgehog (hh)
	fused (fu)
	armadillo (arm)
	patched (ptc)
	gooseberry (gsb)

zygote. The *Drosophila* larva secretes a variety of cuticular processes and sensory structures that provide markers of their position and polarity (Ingham, 1991). The anterior half of each segment is characterized by a belt of denticles generated by the epidermal cells of each of the thoracic and abdominal segments. The posterior half, on the other hand, is comprised of smooth or 'naked' cuticle. These groups of segmentation genes are responsible for either interpreting the basic framework of maternally derived positional information or elaborating on it, thereby defining each region of the developing embryo from head to tail.

I-4b Gap Genes Divide the Antero-Posterior Axis into Broad Regions: Krüppel and its role in establishing and initiating embryonic patterning

Several zygotic mutations affect structures in the anterior part of the head or the anterior abdomen (Wieschaus et al., 1984). Mutagenesis screens detected genes that when mutated caused pattern deletions spaced at regular intervals along the length of the larva. Isolated from these screens was a small number of loci unrelated to any repeated homology system in the embryo and identified for their resulting single large gaps in the segment pattern (Figure 1.19; Wieschaus et al., 1984). *Krüppel* (*Kr*) is one of the several genes associated with this class of mutations resulting in the deletion of consecutive segments. Expressed in both an anterior end region and a broad domain in the center of the embryo, its difference in spatial expression is indicative of its developmental requirements. The anterior-most expression of Kr is associated with Malpighian tubule development (Gaul and Wiegel, 1990) and the circumferential band of eight to ten nuclei in the center of the blastoderm embryo, termed the Kr central domain (Knipple et al., 1985), is affiliated with abdominal segmental patterning (Nüsslein-Volhard and Wieschaus, 1980). This domain of Kr was of particular interest in this study given the results observed in the germline clonal analysis and subsequent segmentation protein distribution. *Kr* mutants display a pattern deletion roughly corresponding to the position of the early belt of central domain expression, although as seen in homozygous amorphic *Kr* mutants, a larger region is absent from the segmental pattern in slightly older embryos. This thereby suggests that *Kr* may affect cells in which it is not expressed

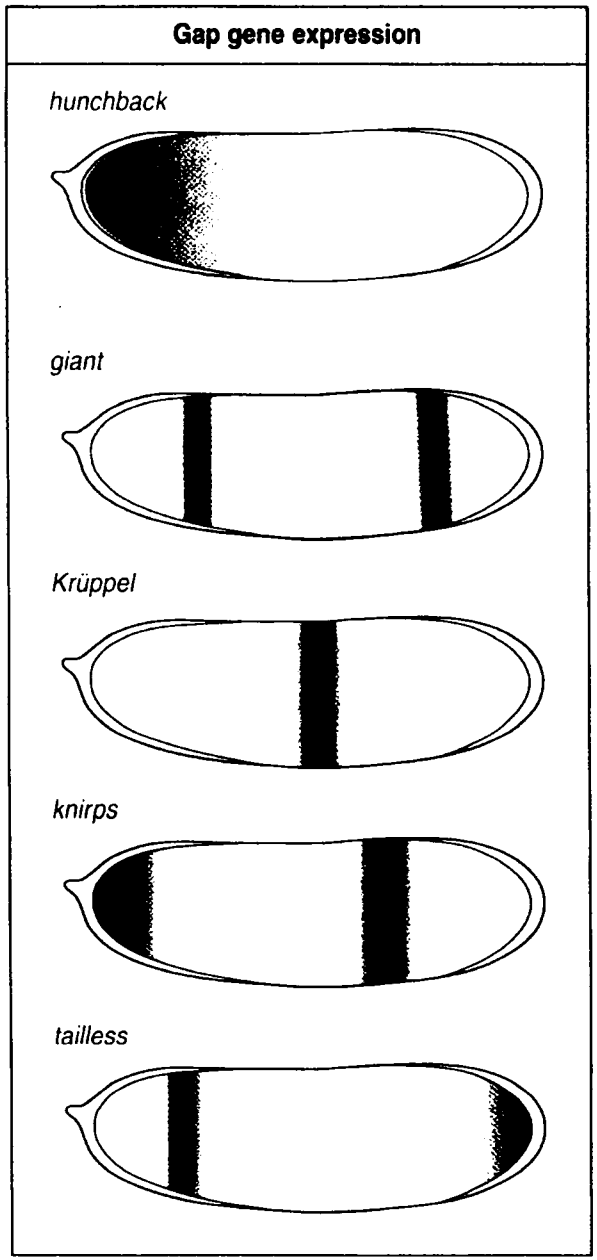


Figure 1.19 The expression patterns of gap genes *hunchback*, *Kruppel*, *giant*, *knirps* and *tailless* in the pre-cellular blastoderm. The expression of gap genes along the antero-posterior axis is regulated by the concentration of ***bicoid*** and ***hunchback*** proteins, together with interactions among the gap genes themselves. The expression pattern of transcription factors spanning the antero-posterior axis and delimits broad regions. After Wolpert, 1998.

(Scott and O'Farrell, 1986). Embryos homozygous for amorphic *Kr* alleles result in deletion of all three thoracic and five abdominal segments that are replaced by partially duplicated posterior abdominal segments with reversed polarity (Nüsslein-Volhard and Wieschaus, 1980; Wieschaus et al., 1984).

Distributed along a concentration gradient (Stanojevic et al., 1989; Pankratz et al., 1990), the requirement of *Kr* is strictly zygotic, as revealed by morphological results stemming from genetic studies, such as mutational analyses and saturation mutagenesis (Nüsslein-Volhard and Wieschaus, 1980; Wieschaus et al., 1984). There are no effects of maternal Kr dosage on the *Kr* phenotype, nor does homozygosity of *Kr* hinder germ cells from making normal eggs proficient of normal embryonic development when fertilized by wild-type sperm as per the definition of maternal-effect genes (see Introduction; Wieschaus et al., 1984). Kr is indeed regulated by maternal factors, although in a manner that is different from, for example, that reported for earlier-acting genes, such as *hunchback* regulation by maternal Bicoid (Bcd) and Nanos (Nos). Maternal Bcd, Nos and Tor all inhibit Kr transcription (see Pankratz and Jackle, 1993). For instance, in *bcd* mutants the anterior border of the central Kr domain shifts anteriorward, implicating a repressive role of *bcd* in establishing the anterior border of the central Kr expression domain (Gaul and Jackle, 1987). However, the proper establishment of the Kr central domain is also dependent upon activation of *Kr* by maternal-effect genes, such as *bcd* and *hb* (Hulskamp et al., 1990) (Figure 1.20). The synergistic relationship between *bcd* and hb^{mat} mentioned earlier for its importance in the establishment of proper embryonic axial development and embryonic patterning is exemplified in the activation of Kr; together *bcd* and hb^{mat} serve as positive regulators of *Kr* expression (Hulskamp et al., 1990). In their genetic analysis, Hulskamp et al., (1990) submit that the anteriorward shift of Kr in *bcd* mutants may be due to the anterior shift of a *bcd*-dependent, yet identified, negative regulator of *Kr* expression. These results imply that perhaps *bcd* and *hb* functions as redundant activating systems in the specification of *Kr* expression and that there may therefore be one or several other factors acting to regulate Kr activity. In addition to being one of the first zygotic genes to respond to initial cues provided by the maternal gradients, *Kr* encodes a transcription factor, thereby implicating its involvement in

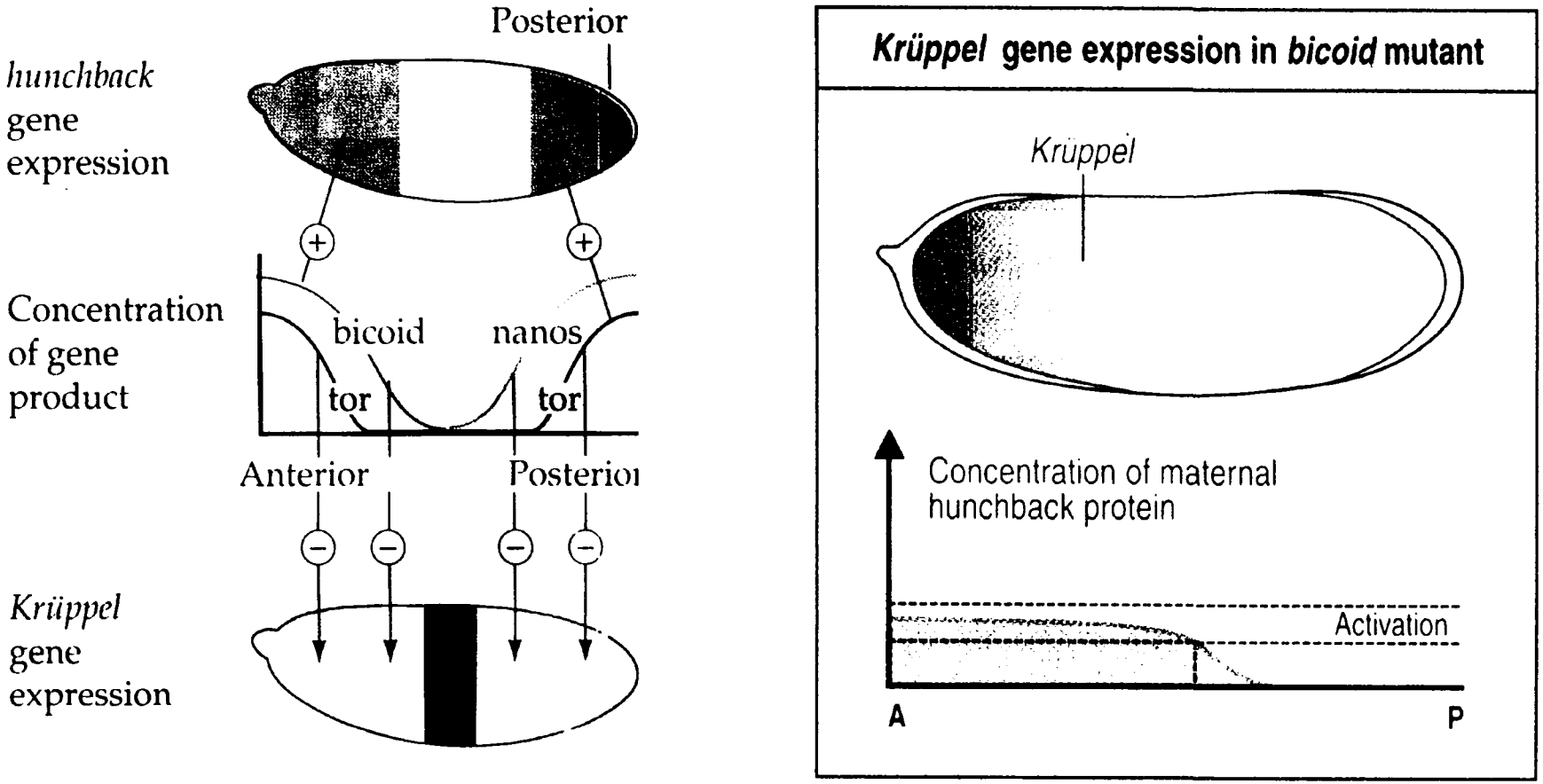


Figure 1.20 Maternal-effect gene control of gap genes hunchback and *Kruppel*. The expression of hunchback protein is activated by *bicoid* protein and repressed by *nanos* protein, thereby restricting *hunchback* expression to the anterior half of the embryo. *Kruppel* is regulated rather differently; *bicoid*, *nanos* and *torso* proteins all inhibit *Kruppel* transcription. In *bicoid* mutants, *Kruppel* is activated at the anterior end of the embryo, giving an abnormal pattern. Mutants lacking the *bicoid* gene also lack zygotic *hunchback* gene expression and only maternal *hunchback* is present in the anterior end of the embryo at relatively low levels, as seen in the figure on the left. Ultimately, the concentration levels in the middle of the embryo of *bicoid*, *nanos* and *torso* are low and allow *Kruppel* transcription as seen in the figure on the right. After Gaul and Jackle (1990).

various auto-regulatory and repressive interactions between neighboring gap expression domains during the refinement of borders (Knipple et al., 1985; Wu et al., 1998). *In vitro* studies report autoregulatory control of *Kr* as a transcriptional repressor in the central region of the blastoderm embryo and as an activator of target genes external to the central domain where the concentration of Kr gradually decreases (Sauer, 1993). Furthermore, as with the expression domains of other gap genes, Kr expression pattern is believed to overlap with the expression domains of neighboring gap genes. With the overlapping of mature gap expression domains at their edges, each nuclei along the A-P axis becomes further defined by the concentration and combinations of gap proteins it accumulates (Wu et al., 1998) (Figure 1.21). It is this internetwork of overlapping gap and mostlikely maternal proteins that sets the stage for the next group of genes in the segmentation hierarchy, the pair-rule genes.

I-4c Pair-Rule Genes Delimit the Periodic Pattern of Early Embryogenesis: even-skipped and its role in initiating and establishing embryonic patterning

The pair-rule genes are characterized by their striking mutant phenotype in which loss of gene function results in pattern deletions of alternate segments or parts of segments. Similar to gap genes, the precise region of deletion within the affected segments is characteristic of each pair-rule gene (see Figure 1.18; Nüsslein-Volhard and Wieschaus, 1980). Much like the requirement of overlapping gap gene activities in building contiguous blocks of segments, overlapping series of pair-rule gene activities appear to be needed in assigning sets of alternate segments within the presumptive trunk region of the embryo (Ingham et al., 1985; Macdonald and Struhl, 1986; Grossniklaus et al., 1992). The gap protein gradients, in combination with the maternal protein gradients that still persist, provide the signals for directing the expression of the pair-rule genes (see Pankratz and Jackle, 1993). However, there is evidence derived from epistatic analysis that suggests the existence of a hierarchy within the pair-rule class itself. This is because pair-rule expression is not only altered in maternal and gap gene mutants but also in other pair-rule mutants as well (Carroll and Scott, 1986). These results gave rise to the grouping of pair-rule genes into two subclasses, the primary and secondary pair-rule

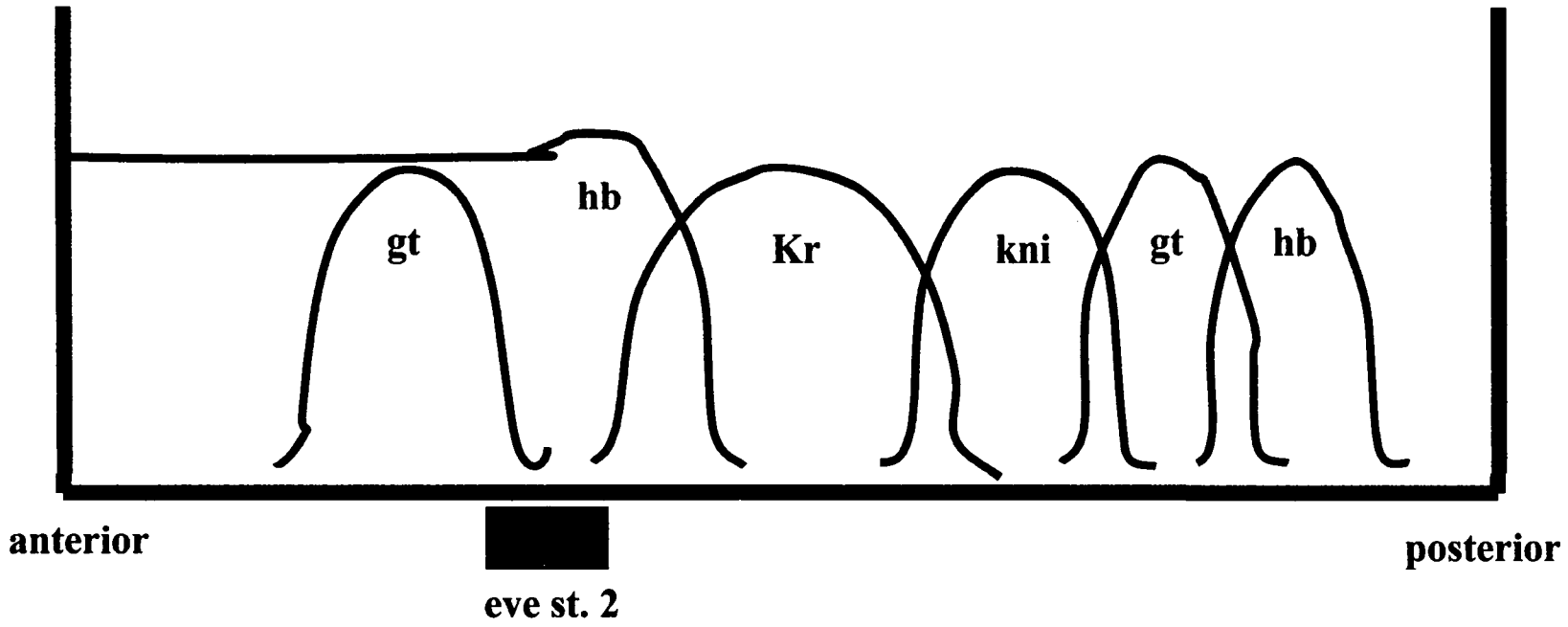


Figure 1.21 Schematic representation of the overlapping gap gene expression patterns in blastoderm stage embryos. One possibility regarding mechanisms at work in the hierarchical system of segmentation genes is the relationship between genes of the same group. For example, each gap gene expression domain may serve as a source for a diffusion gradient that establishes the position of target gene expression borders, such as the second stripe of *even-skipped* (eve st.2), based on protein concentration. After Wu et al. (1998).

genes. The primary pair-rule genes derive their periodicity from the maternal and gap genes, whereby the secondary pair-rule genes derive their periodic pattern contingent on or in concert with the primary pair-rule genes. *even-skipped* (*eve*) is an example of a primary pair-rule gene.

eve is unique among the pair-rule genes in that hypomorphic alleles delete alternating segments while null mutants display complete loss of segment boundaries within middle body regions (Nüsslein-Volhard et al., 1987). *eve* encodes a homeodomain transcription factor during *Drosophila* segmentation required for activation of the segment polarity gene *engrailed* (*en*) (Macdonald and Struhl, 1986); see following section) and for strict organization of odd-numbered parasegments (Manoukian and Krause, 1992). The combined activity of *bcd*, zygotic *hb*, *Kr* and *giant* (*gt*) transcribes *eve* into seven broad symmetric stripes along the A-P axis, subsequently refined into narrow stripes that correspond cell-for-cell with the odd-numbered parasegment boundaries (Goto et al., 1989; Small et al., 1991) (Figure 1.22). In embryos lacking *bcd* and hb^{zyg} functions, the second stripe of *eve* ("*eve* stripe2"), the best characterized pair-rule stripe element, is absent or its level is greatly reduced, indicating the requirement of these two factors in the activation of this stripe element. In *Kr* mutants, the stripe expands posteriorly, assigning a requirement for *Kr* in establishing the posterior border by repression; in *gt* mutants, stripe-2 expression shifts anteriorly, implicating a requisite of *gt* activity normally to establish the anterior border also by repression. Thus, a function of the working genetic hierarchy, the pair-rule parasegmental organization resulting from mutual activation and repression is achieved through their response to different concentrations and combinations of the gap gene transcription factors in each independent stripe formed (Figures 1.23 and 1.24). Molecular studies have revealed multiple binding sites for each of the different factors (Bcd, Hb, Kr and Gt) within the *cis*-regulatory element required for *eve* stripe2 expression, implicating their direct involvement in controlling this stripe element (Stanojevic et al., 1989; Small et al., 1991). The refinement of *eve* striping may be attributed to *eve* autoregulation through direct combinatorial interactions with various nuclear factors (Jiang et al., 1991) and/or indirectly under the direction of intermediary genes (Manoukian and Krause, 1992).

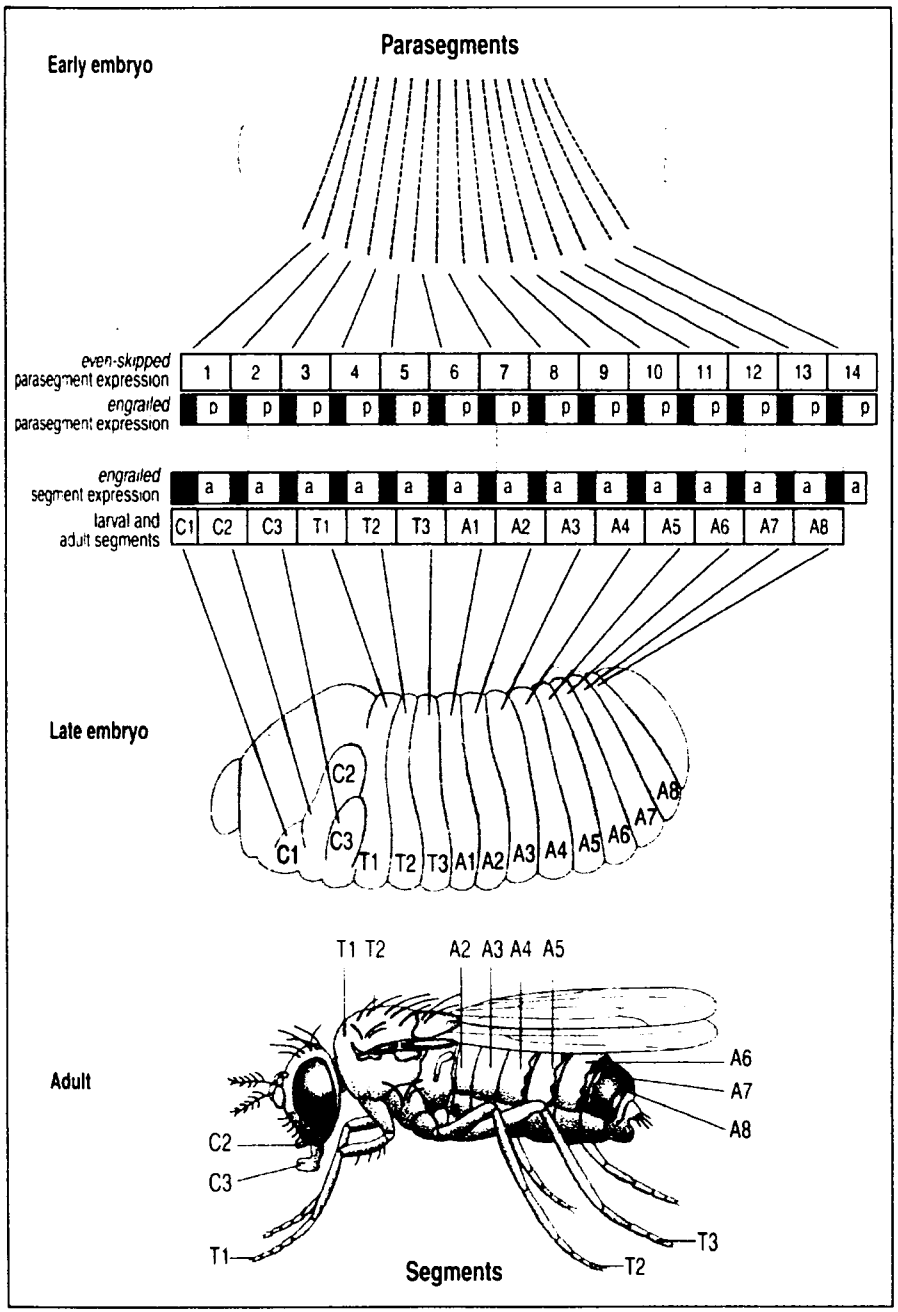


Figure 1.22 The relationship between parasegments and segments in the early and late embryo. Initially, pair-rule genes are expressed in the embryo as stripes in alternating parasegments. For example, *even-skipped* (yellow) is expressed in odd-numbered parasegments and the segment polarity selector gene *engrailed* (blue), which defines the anterior margin of each parasegment, is expressed in the anterior domain of every parasegment. Each larval segment is comprised of the posterior region of one parasegment and the anterior region of the next. The parasegmental domains are equal in width to future segments, but are out of register. Therefore, segments are offset from the original parasegments and *engrailed* becomes expressed in the posterior region of each segment. a (anterior compartment); p (posterior compartment); C1, C2 and C3 represent segments that fuse to form the head region; T (thoracic segments); A (abdominal segments). After Wolpert, 1998.

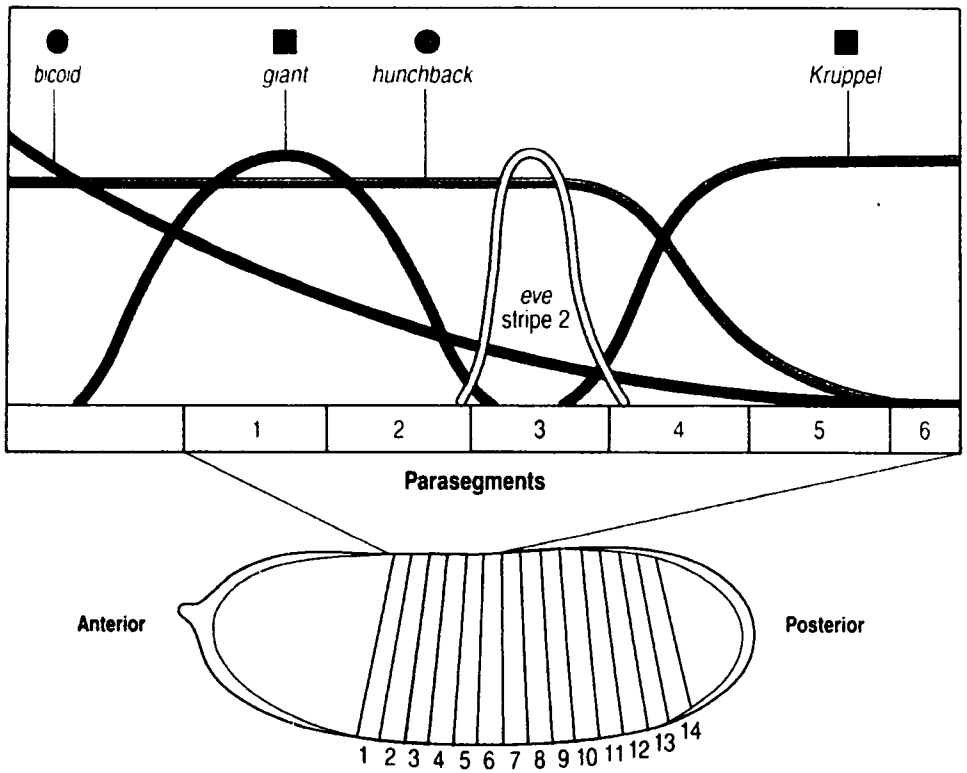


Figure 1.23 The specification of the second even-skipped stripe by gap gene proteins. The variable concentrations of transcription factors encoded by gap genes *hunchback*, *giant* and *Kruppel* localizes *even-skipped* at a very specific point along their gradients in parasegment 3. Activation of *even-skipped* in a broad domain is mediated by both *bicoid* and *hunchback*, however repression by *giant* and *Kruppel* proteins respectively form the anterior and posterior borders. After Wolpert, 1998.

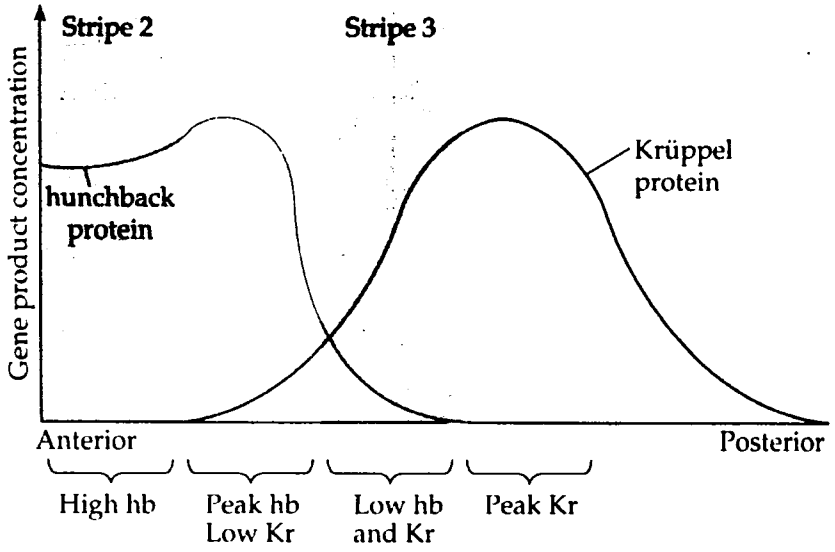


Figure 1.24 Model for the formation of the second andthird stripes of even-skipped. The *hunchback* and *Kruppel* proteins are expressed in broad overlapping domains. *even-skipped* transcription may be activated by local concentrations of Hunchback and Kruppel, while other concentration of these proteins may act to repress *even-skipped* activity. For example, stripe 2 coincides with cells having high Hunchback concentrations but lacking Kruppel whereas stripe 3 occurs in cells expressing low levels of Hunchback and high levels of Kruppel. After Stanojevic et al. (1989).

Such interactions not only include those eliciting activation of the gene, but as it seems to be an established developmental mechanism at work in promoting proper embryonic patterning, *eve* spatial expression is also regulated by negative or repressive activity of regulatory factors, such as those mentioned above. Moreover, the latter suggestion proposes that the regulatory requirement of a gene within the syncytial blastoderm may change dramatically within very short periods of time, perhaps even locally (Manoukian and Krause, 1992).

I-4d Segment Polarity Genes Circumscribe Compartmentalization: engrailed and its role in establishing and initiating embryonic patterning

As noted previously, in the cuticle of every segment in the *Drosophila* embryo is characterized on the ventral surface by an anterior half of denticles and a posterior smooth region; the dorsal surface displays an intricate pattern of hairs with subtle segmental differences. Coincidentally, the pattern of the insect cuticle is representative of the complexity of the epidermal cells that secrete it and is an expression of their interactions during development (Ingham and Martinez-Arias, 1992). The segment polarity genes are classified as a distinct group of mutations that disrupt the A-P polarity of the segments and the ventral pattern of the cuticle in every segment. They elicit this activity through variable deletions of the posterior region of each segment associated with mirror image duplications of the denticle belts from the remaining region (Nüsslein-Volhard and Wieschaus, 1980). Dissimilar to the gap and pair-rule genes, which mainly encode RNA- and DNA-binding proteins and are members of transcriptional cascades, segment polarity genes have a much less restricted range of function. They encode a variety of proteins, including diffusible factors, membrane proteins and those involved in cell signaling (Ingham and Martinez-Arias, 1992). Activated in part to respond to pair-rule gene expression, they are expressed in 14 transverse stripes, one stripe corresponding to each parasegment (Howard and Ingham, 1986). One segment polarity gene isolated for its dependence on pair-rule activity, *engrailed* (en) is of particular interest for its overall establishment of A-P embryonic patterning.

en encodes a homeodomain-containing transcription factor responsible for recognizing specific DNA sequences and regulating the expression of several target genes (Desplan et al., 1985; Serrano et al., 1997). Immunofluorescence studies localized *en* protein accumulation in the nucleus of 14 evenly spaced domains in the cellular blastoderm that become the anterior part of each parasegment (DiNardo et al., 1985), supporting its role as a nuclear regulator (Maschat et al., 1998). Anterior *en* cells underlie naked cuticle whereby the posterior *en* cells underlie first row denticles. Therefore, *en*-expressing cells can adopt either the smooth cell fate or a first row denticle fate, thereby assuming its role as a selector gene possibly functioning as a binary developmental switch (Kornberg et al., 1985). Indicative of its varying role during early embryonic patterning, the phenotype of *en* null mutants depicts pleiotropic aberration of segmentation throughout the animal (Kornberg et al., 1981).

en also encodes a domain that confers repressive activity to heterologous DNA-binding proteins in a variety of systems (Jaynes and O'Farrell, 1991; Han and Manley, 1993; John et al., 1995). Therefore, as expected, the activity of several genes, such as *cubitus interruptus* (*ci*) (Schwartz et al., 1995) and *wingless* (*wg*) (Heemskerk et al., 1991) are repressed by *en* activity during *Drosophila* development. In addition to its role as a repressor however, *en* is also involved in target gene activation (Serrano and Maschat, 1998). The question then became, how could a molecularly characterized repressor activate transcription? Proposed by Manoukian and Krause (1993) in explaining transcriptional activation by the repressive activity of *eve* and later elaborated by Smith and Jaynes (1996), one possibility is that *en* may initiate transcriptional activity by repressing a repressor via an intermediate. This idea is supported by recent evidence presented by Alexandre and Vincent (2003). These three groups of investigators propose that En may confer activating ability via the activity of true activators and via recruitment of cofactors or intermediates (Pinsonneault et al., 1997). These cofactors would be responsible for the activation by En of positive targets in the appropriate context (reviewed by Mannervik et al., 1999). These studies argue that since En harbors a robust repressor domain, one or several cofactor(s) mask this domain and recruit an activation function. Given the importance of repressive activity in the appropriate context in

substantiating proper embryonic polarity and patterning, proposal of this 'repressor of repressors' mechanism may be a favorable consideration in other similar studies of early embryonic patterning.

I-5 Statement of Problem

tango (*tgo*) has been previously isolated as a *Drosophila* bHLH-PAS master transcriptional regulator of cell identity in the midline of the central nervous system (CNS) and tracheal/respiratory network. In these tissues, *tgo* has been shown to heterodimerize with a region-specific partner such as Single-minded (Sim), Trachealess (Trh) or Spineless (Ss) (Sonnenfeld et al., 1997; Emmons et al., 1999). These heterodimers translocate to the nucleus to bind their target site, ACGTG. When multerimerized and fused to lacZ, the target site is activated in regions containing these nuclear heterodimers (Sonnenfeld et al., 1997). Activation of the target site is therefore consistent with the transcriptional activity of *tgo* bHLH-PAS heterodimers.

Unpublished reports have suggested an early developmental role for *tgo*, one perhaps regulated by one of its five recognized motifs. The purpose of this project was to further characterize the zygotic role of *tgo* during CNS development and identify its maternal contribution during early embryonic patterning. The role of the *paired* (*prd*) repeat domain, or tgo^3, during both developmental periods was the focus of this study. Two lines of evidence suggested that *tgo* may have an earlier developmental role, one maternally regulated. First, *in situ hybridization* and immunohistochemical analysis localized both *tgo* transcripts and protein in pre-cellular blastoderm embryos (Oshiro and Saigo, 1997; Sonnenfeld et al., 1997; Zelzer et al., 1997). Second, zygotic homozygous and hemizygous mutations in multiple *tgo* alleles do not produce CNS and tracheal phenotypes as severe or as penetrant as those observed by mutations in partners of *tgo*, including *sim* and *trh* and *ss* (Sonnenfeld et al., 1997; Emmons et al., 1999). These results are contrary to the heterodimeric partnership of Tgo with Sim and Trh in establishing the CNS midline and tracheal tubule network in *Drosophila*, respectively (Oshiro and Saigo, 1997; Sonnenfeld et al., 1997). The relatively weak zygotic *tgo* phenotypes may, in part, be the result of maternal *tgo* gene product enabling normal development in the absence of zygotic *tgo* function.

The C-terminal part of Tgo houses a region rich in histidines and prolines, known as the *paired* (*prd*) *repeat* domain. Deleted by a premature stop codon in tgo^3 mutations

(Sonnenfeld et al., 1997), solid consensus with respect to the function of the *prd repeat* has yet to be established. A protein database query in the present work shows that the *prd repeat* is encoded by several transcriptional regulatory proteins required for early developmental functions in both invertebrates and vertebrates (see Discussion). Additionally, the present body of work suggests and early role for the *prd repeat* of *tgo* during *Drosophila* embryonic patterning.

My hypothesis was:

embryonic CNS development (zygotic-effect) - if *tgo* defects can be complemented by *tgo* alleles carrying mutations affecting other aspects of the *tgo* function, then the CNS phenotype should reflect the pleiotropy that may result from a mutational analysis of the *prd repeat* domain in combination with other *tgo* mutants

embryonic patterning (maternal-effect) - if deletion of the *prd repeat* region results in the repression of early segmentation markers, then perhaps *tgo* (*prd repeat*)is normally required in the activation of targeted early-acting segmentation genes, and/or

- if deletion of the *prd repeat* region results in activation of early segmentation markers, then perhaps *tgo* (*prd repeat*) is normally required in repressing the activity of targeted early-acting segmentation genes

Ectopic expression of segmentation markers with a reduction or loss of the *prd repeat* function may reflect a requirement of the *prd repeat* to act as a repressor of transcriptional activity during the early stages of pattern establishment. A reduction of segmentation marker expression would be expected to result where the *prd repeat* is involved in activating transcriptional activity. The regulation of early *Drosophila* embryonic patterning by *tgo* may depend on the functional requirement(s) of the C-terminal part of the Tgo protein. Such requirements may be contingent upon interaction of Tgo with other bHLH-PAS or non-bHLH-PAS factors encoded by maternal coordinate genes and/or gap segmentation genes present in the anterior and/or terminal regions of

the developing embryo. Moreover, Tgo may function as an essential cofactor whose requirement is dictated by spatially restricted factors present in the anterior and/or terminal regions of the pre-cellularized embryo. A model suggesting the mechanism of *tgo* function during embryonic patterning is proposed. It should be noted that this proposal, although prompted by the results contained in this body of work, should be considered hypothetical and would require further investigation beyond that presented here for validation.

In order to address these questions, I conducted two independent studies. The first study involved characterizing the zygotic role of *tgo* through an inter-allelic analysis of embryonic CNS development using an antibody to reveal the CNS axon scaffold and HRP/DAB histochemistry. An inter-allelic analysis was conducted to classify defects of *tgo* that could be complemented by the alleles of *tgo* may be carrying mutations affecting other aspects of the *tgo* function (consider the motifs of the Tgo protein, see Figures 1.4 and 1.7). In the second study, I conducted two *in vivo* analyses to investigate the early function of the *tgo prd repeat*. The first analysis involved production of tgo^3 germline clones whereby the effect of reduced maternal *tgo* product was studied on the development of the larva's cuticle. Subsequent analysis of segmentation gene expression in both embryos homozygous mutant for maternal tgo^3 and embryos ectopically expressing various *tgo* transgenes involved staining with early segmentation gene markers within the first three hours of development. This second study was performed in order to identify the early developmental pathways requiring maternal *tgo* function during embryonic patterning.

II. MATERIALS AND METHODS

2.1 Fly Stocks and Genetics

Flies were raised on a cornmeal/molasses/yeast/agar medium with both Tegosept and propionic acid at 25°C. The following stocks were obtained from the Bloomington *Drosophila* Stock Center at Indiana University: P(*ovo*^{D1})3R FRT82B/Sb, Btub85Dss/TM3, Sb; *yw* P[*ry*$^{+}$, hsFLP22Sb]; and TM3/Cxd; *yw*; P[*ry*$^{+}$, hs-*neo*, FRT82], P[w^{+}]88C, NM (Xu and Rubin, 1993). Isolation of *tgo*^{1} through *tgo*^{3} is described in chapter 1 (Sonnenfeld et al., 1997). *tgo*^{5} through *tgo*^{7} are EMS-induced alleles isolated by Emmons et al. (1999). *tgo*^{4} has yet to be sequenced. *nanos*-Gal4 drives expression under control of the *nanos* (*nos*) promoter and was obtained from Norbert Perrimon (Department of Genetics and Howard Hughes Medical Institute, Harvard Medical School). *tgo* transgenes P[UAS*tgo*^{full}], P[UAS-*tgo*$^{\Delta b}$] and P[UAS-*tgo*$^{\Delta C}$] were obtained from Stephen Crews (Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill).

2.2 Embryo Fixation

Embryos were collected twice daily or prior to three hours after egg laying (AEL) as required on apple juice agar-filled petri plates containing a small smear of live yeast paste (Fleischmann's Dry) to encourage egg laying. Before use, these stored plates were left at room temperature for approximately one hour to allow restoration of axon morphology and microtubule repolymerization.

Embryos were collected from apple juice-agar plates and immersed in 50% commercial bleach for ten minutes to remove the outer chorion layer. Dechorinated embryos were collected onto a nitex sieve and rinsed with distilled water. Once blotted dry on a kimwipe, the nitex sieve holding the dechorinated embryos was immersed in a scintillation vial containing a fixation solution of 45% 1 x PBS (phosphate buffered saline), 5% formaldehyde (1:1 ratio) overlayed by a heptane layer.

Embryos were fixed for thirty minutes at the interface between the heptane and bottom aqueous layer containing the fixative. The heptane at this interface ensures permeabilization of embryos to allow entry of the fixative and subsequent antibodies.

The bottom aqueous layer was removed, replenished with fresh methanol and the solution was shaken vigorously for approximately twenty seconds in order to remove the vitelline layer surrounding the embryos. Devitellinated embryos fell to the bottom into the methanol layer and were transferred to a disposable plastic tube using a pasteur pipette. Traces of heptane were removed by three subsequent methanol washes. Embryos were either stored at 4°C for a maximum of two weeks or used immediately.

2.3 Immunohistochemistry

In proceeding with antibody staining, the methanol was replaced with PBT (1XPBS containing 0.1% Triton X-100). Embryos were then washed for one hour in PBT on a rotator before incubating in 100ul of PBT containing 10uL of normal goat serum (NGS) for thirty minutes to block nonspecific antibody binding. A specified dilution of primary antibody (see Figure 2.1) was added to the block solution and embryos were incubated at 4°C overnight. The primary antibody was recycled, and any residual antibody was washed away from the embryos with PBT for 1-1 1/2 hours. The washed embryos were blocked again in PBT containing NGS for thirty minutes. The secondary antibody (either goat anti-mouse IgG or goat anti-rabbit conjugated to horseradish peroxidase) was added to the blocking solution and embryos were incubated for two hours at room temperature. The secondary antibody was then washed out with PBT for two hours or overnight on a rotator.

Antibody reactivity was visualized by incubating embryos in 200ul PBT containing 100ul 0.3 mg/ml diaminobenzadine (DAB). To this solution, 3ul of 3% hydrogen peroxidase were added and the reaction proceeded until the background staining in the embryos began to darken. Dilution with PBT stopped the reaction and waste DAB was neutralized in bleach. Embryos that were processed for single antibody labeling were dehydrated through a series of consecutively increasing ethanol concentrations (50%, 70%, 95%, and 100%). To preserve their staining, embryos were transferred to methyl salicylate to be stored indefinitely.

2.4 Antibodies

The following antibodies were used: BP102 (Developmental Studies Hybridoma Bank, DSHB; Fujita et al., 1982) to visualize formation of embryonic CNS axons at a 1:20 dilution; rabbit anti-β-galactosidase (βgal) (DSHB; Bruns et al., 1979) at a 1:100 dilution to reveal balancer-containing embryos (see below); rabbit anti-Krüppel (Kr) (Knipple et al., 1985; a gift from Sean Carroll and Kathy Vaccaro) at a 1:3 dilution to identify early gap segmentation; 2B8 anti-Even-skipped (Eve) (DSHB; Frasch et al., 1987) for observation of early pair-rule patterning at a 1:100 dilution; 4D9 anti-Engrailed/Invected (En) (DSHB; DiNardo et al., 1985) to examine early segmentation at a dilution of 1:3 and monoclonal anti-Tango (Tgo) (Sonnenfeld et al., 1997) was used to identify early expression at a dilution of 1:1. See Figure 2.1.

2.5 Light and Fluorescence Microscopy

Embryo fixation and staining were according to standard protocols (Patel, 1994). Embryo staging was according to Campos-Ortega and Hartenstein (1985) (Figure 2.2). Mutant *tgo* embryos were identified from lines carrying a balancer chromosome containing the P[ubx-lacZ] insertion and stained with anti-β-galactosidase (β-gal) (Promega). To unambiguously identify *tgo* homo- and heteroallelic mutants, all progeny were stained with β-gal to identify balancer-containing embryos of the inter-allelic analysis.

Immunofluorescence using anti-Krüppel (Kr) and anti-Engrailed (En) was performed using FITC conjugated secondary antibodies. Fluorescently labeled embryos were mounted in 4% n-propyl gallate to inhibit photobleaching and analyzed on a Zeiss Axiovert 100TV confocal microscope. Optical sections of 1μm were collected in line average mode. Anti-Eve staining was carried out by HRP immunohistochemistry and was analyzed on a Zeiss Axioskop. All figures were processes with Adobe Photoshop software.

Antibodies		Dilution of primary	Source	Reference
Primary	Secondary			
BP102	IgG	1:20	mouse	Fujita et al. (1982)
βgal	IgG	1:100	rabbit	Bruns et al. (1979)
Kr	IgG	1:3	rabbit	Knipple et al. (1985)
2B8 Eve	IgG	1:1000	mouse	Frasch et al. (1987)
4D9 En	IgG	1:3	mouse	DiNardo et al. (1985)
Tgo	IgG	1:1	mouse	Sonnenfeld et al. (1997)

Figure 2.1 Antibody Chart.

These primary antibodies were used in the visualization of embryonic CNS axons, balancer lines, early embryonic patterning and early Tango localization. Respective secondary antibodies, primary antibody dilutions and animal sources are also displayed. IgG was conjugated to either HRP or FITC.

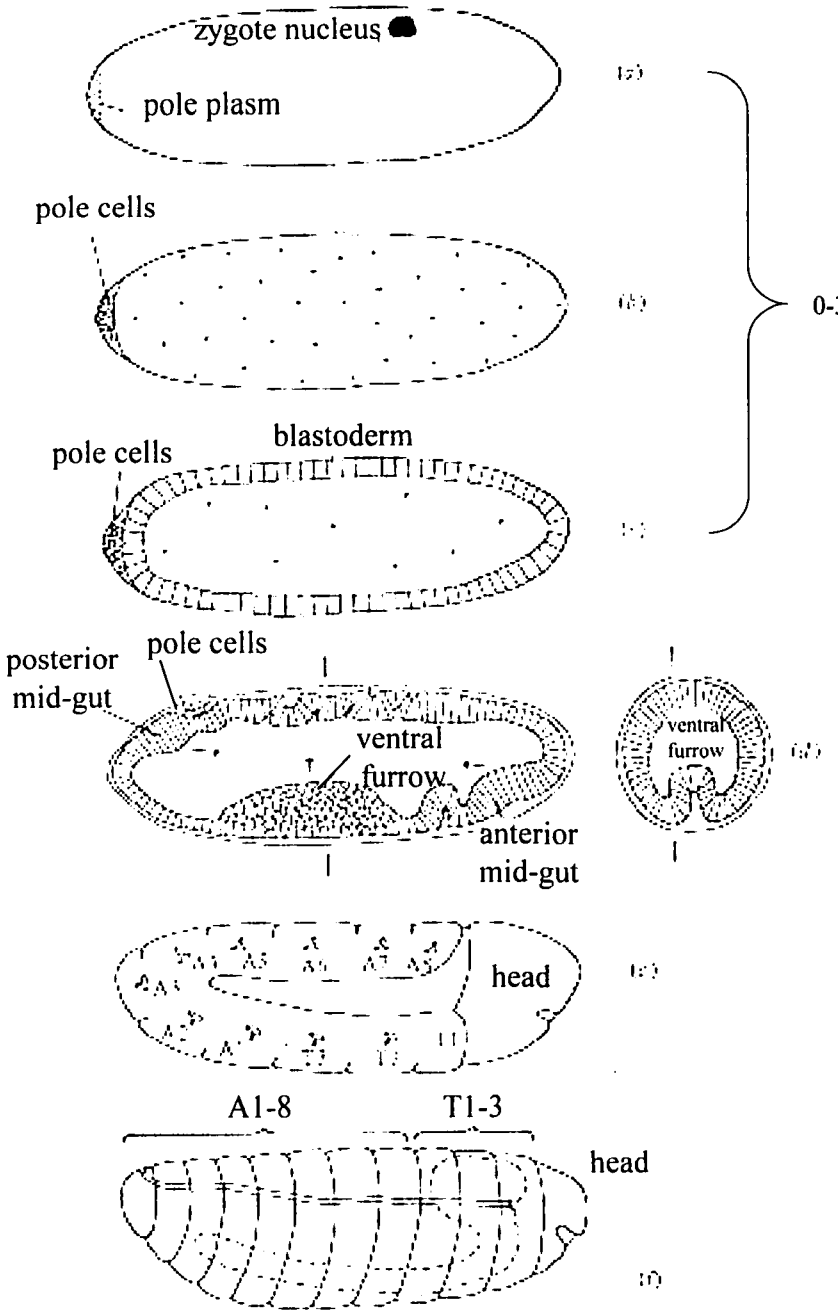


Figure 2.2 Embryonic development of *Drosophila melanogaster*.

(a) Zygote, (b) cleavage, (c) cellular blastoderm, (d)gastrulation, (e) maximum germ band extension, (f) after dorsal closure, whereT1-3 indicates thoracic segments, A1-8 abdominal. Modified from Campos-Ortega and Hartenstein (1985).

2.6 Inter-allelic Analysis of *tgo*^{3} during CNS axonal formation

To characterize the role of *tgo*^{3} in axonal formation during CNS development, homozygotes of the *tgo*^{3} allele and other *tgo* alleles, *tgo*^{4} and *tgo*^{5} (Sonnenfeld et al., 1997; Emmons et al., 1999) were stained with the monoclonal antibody (mAb) BP102. Additionally, trans-heterozygotes of *tgo*^{3} with *tgo*^{2} (Sonnenfeld et al., 1997), *tgo*^{4} and *tgo*^{5} were generated to observe axonal development during CNS formation and the interaction between two given genetic pathways required for proper CNS establishment in *Drosophila* (Sonnenfeld et al., 1997). A representation of the genetics behind the selection of mutants during both homozygous and trans-heterozygous pairings can be seen in the Punnett square schematic in Figure 2.3.

Classification of defects in axonal CNS development for both homozygous and trans-heteroallelic analyses was of variable expressivity (extent to which a genetic defect is expressed) and so was narrowed down into four phenotypic classes: fused, collapsed, neurogenic and stalled. The fused phenotypic class refers to commissural development and the proper separation of anterior and posterior commissural axons. Phenotypic classification of a loss of essential repulsive cues between longitudinal tracts is manifested in the collapsed phenotype. The neurogenic phenotype is classified as having a hyperplasic CNS along with over-growth of associated axons (see Figure 2.4 after Boulianne et al., 1991). Finally, the stalled phenotype refers to reductions in commissural and longitudinal connectives spanning the midline and A-P axis, respectively. In all analyses, stage 15 embryos were fixed and subsequently stained with mAb BP102 and anti-β-galactosidase (anti-βgal) (recall Figure 2.1, antibody chart) following the protocol outlined in section 2.4 of this chapter and subsequently analyzed following the procedure described in section 2.3 of this chapter detailing procedure of immunohistochemistry.

2.7 Production of Germline Mosaics using the FLP-DFS Technique

Germline clones were generated using the dominant female-sterile autosomal-FLP-DFS technique (Chou and Perrimon, 1992; Chou et al., 1993; Perrimon et al., 1996). Females, carrying a source of flippase, were of the genotype P[*ry*$^+$; hsFLP22]; P[*ry*$^+$; hs-*neo*;

Figure 2.3 The Making of Homozygotes and Trans-Heterozygotes: The Punnett Square, *tgo*^{3} homozygotes and *tgo*^{3}/*tgo*^{4} transheterozygotes.

The Punnett square is based on Mendel's Law of Segregation, which states:

> The two members of a gene pair separate from each other into the gametes, so that one-half of the gametes carry one member of the pair and the other one-half of the gametes carry the other member of the gene pair.
>
> Suzuki et al., 1986

The parental generation, or P, carries the source of the gene of desire, in this case, the mutated form of tango, *tgo*^{3}. Additionally, parent flies will also carry selection markers known as balancers. Balancer chromosomes maintain a gene (which is lethal when homozygous) in permanent heterozygous condition. The balancer chromosomes contain deficiencies or duplications to prevent crossing over and thereby minimize the pairing between homologous chromosomes during meiosis. Therefore, by this definition, it can be understood that the inheritance of balancers from both parents is fatal. Balancers are also useful in that they may be used to determine on which chromosome a genetic unit resides. For example, TM3 is a third chromosome balancer carrying a dominant marker such as Stubble (Sb). Therefore *tgo*/TM3 would implicate *tgo* to be located on the third chromosome. This is useful information when assaying a homozygous recessive lethal mutation in order to trace a given genetic unit subsequent to the crossing of mates because the marker would allow the following of genotypes.

Take for example, the generation of *tgo*^{3} homozygotes. To study the effect of *tgo*^{3} on CNS axonal development, virgin females (written on the right side of a cross) and males (on the left) carrying the *tgo*^{3} mutation over the balancer chromosome TM3 and also containing the reporter (P[Ubx-lacZ]) were crossed. Placing female gametes along one side of the Punnett square and the male gametes along the other side and combining them will allow observation of F1 (first filial generation) zygotes. In the staining of embryos, a convenient way to isolate homozygous *tgo*^{3} mutants, almost by default, is by staining embryos already stained for CNS axonal development (having used mAb BP102) with anti-β-galactosidase (anti-βgal). Anti-βgal will identify tgo heteroallelic embryos containing the Ubx balancer; homozygous *tgo*^{3} embryos do not contain the balancer chromosome due to loss of it during meiosis.

A. Collection of homozygous embryos

tgo^3/P[Ubx-*lacZ*] x *tgo*^{3}/P[Ubx-*lacZ*]

♀ \ ♂	*tgo*^{3}	P[Ubx-*lacZ*]
tgo^3	$\frac{tgo^3}{tgo^3}$	$\frac{tgo^3}{\text{P[Ubx-}lacZ\text{]}}$
P[Ubx-*lacZ*]	$\frac{tgo^3}{\text{P[Ubx-}lacZ\text{]}}$	$\frac{\text{P[Ubx-}lacZ\text{]}}{\text{P[Ubx-}lacZ\text{]}}$

B. Collection of trans-heterozygous embryos

tgo^3/P[Ubx-*lacZ*] x *tgo*^{4}/P[Ubx-*lacZ*]

♀ \ ♂	*tgo*^{3}	P[Ubx-*lacZ*]
tgo^4	$\frac{tgo^3}{tgo^4}$	$\frac{tgo^4}{\text{P[Ubx-}lacZ\text{]}}$
P[Ubx-*lacZ*]	$\frac{tgo^3}{\text{P[Ubx-}lacZ\text{]}}$	$\frac{\text{P[Ubx-}lacZ\text{]}}{\text{P[Ubx-}lacZ\text{]}}$

Stain embryos with BP102 and anti-βgal to identify CNS commissural development and to unambiguously identify *tgo* mutants, respectively.

FRT82B], *tgo*/TM3 and were mated to males carrying both the dominant female sterile mutation ovo^{D1} and flippase target sites (FRTs) (P[ovo^{D1}]; P[ry^{+}; hs-*neo*, FRT82B]). Approximately 30 parental lines (hsFLP x ovo^{D1}) for both control and experimental (germline clones) were established in total and parents of each cross were transferred to fresh media continuously until the eventual decline in number of eggs laid. This resulted in an approximate total of 90 parental lines established (three transfers per cross). The number of independent rounds of analysis (where "independent round" is defined as the number of cuticle preparatory and subsequent analysis sessions) was approximately 56 for both control and experimental groups. The same method of establishment was followed for wild-type cuticle samples, crossing only w^{118} females to w^{118} males and continuing parental line transfer from approximately 25 crosses established. A genetic schematic of the FLP and FRT constructs and the scheme of mating can be seen in Figures 2.4 and 2.5, respectively.

Mitotic recombination was induced at FRT sites by heat shocking the progeny (Brand et al., 1994; see figure 1.10). Heat shock performed for production of germline clones was carried out upon progeny of experimental and control groups for 90 minutes at 37^0C during third instar stages in the vials in which they were laid (Chou et al., 1993). Heterozygous *tgo* females of the genotype hsFLP22/+; P[ry^{+}; hs-*neo*; FRT82B] *tgo*/ TM3; P[ovo^{D1}], P[ry^{+}; hs-*neo*; FRT82B] were collected and crossed to wild-type (w^{118}) males. Embryos were collected for at least one week and aged at room temperature for at least three to four days and subsequently analyzed as described below.

2.8 Characterization of *tgo* Germline Clones

Cuticle preparations were made from unhatched larvae (tgo^{3glc} embryos) derived from females mutant for tgo^3 and from control females according to Wieschaus and Nüsslein-Volhard (1986). Unhatched larvae were dechorinated in 50% bleach, the vitelline membranes removed by agitation in 1:1 methanol/heptane, followed by a wash in methanol. Upon evaporation of methanol, larvae were cleared in Hoyer's medium/lactic acid (4:1) (refer to the Appendix for the recipe) for 24 hours at 65^0C (Wieschaus and Nüsslein-Volhard, 1986). Cuticle samples were flattened overnight and subsequently

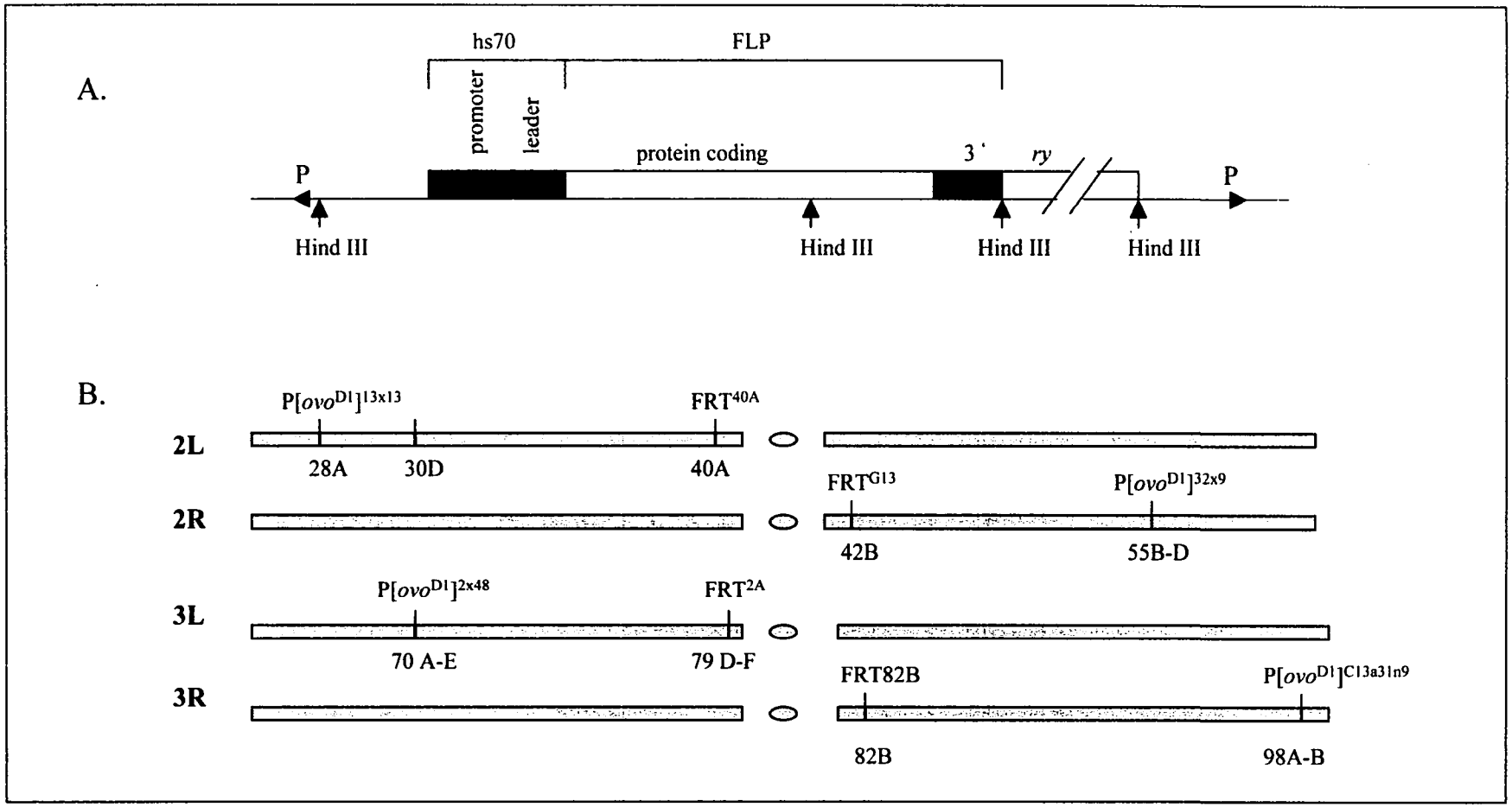


Figure 2.4 Schematic Diagram of FLP and FRT Constructs to Generate Germline Clones.

(A) P[ry, hsFLP]. The hsp70-FLP fusion gene (hsFLP) is diagrammed. Cloned into a P element vector, also carrying *rosy* (*ry)* as an eye marker for germline transformation, hsFLP was designed and constructed by Golic and Lindquist (1989).

(B) Structure of the P[ovo^{D1}] FRT chromosomes. The location of FRT and P[ovo^{D1}] insertions are shown respectively. The left arm of chromosome 2 (2L) P[ovo^{D1}] insertions were designed and constructed by Chou et al., (1993) and those of 3L by Chou and Perrimon (1996).

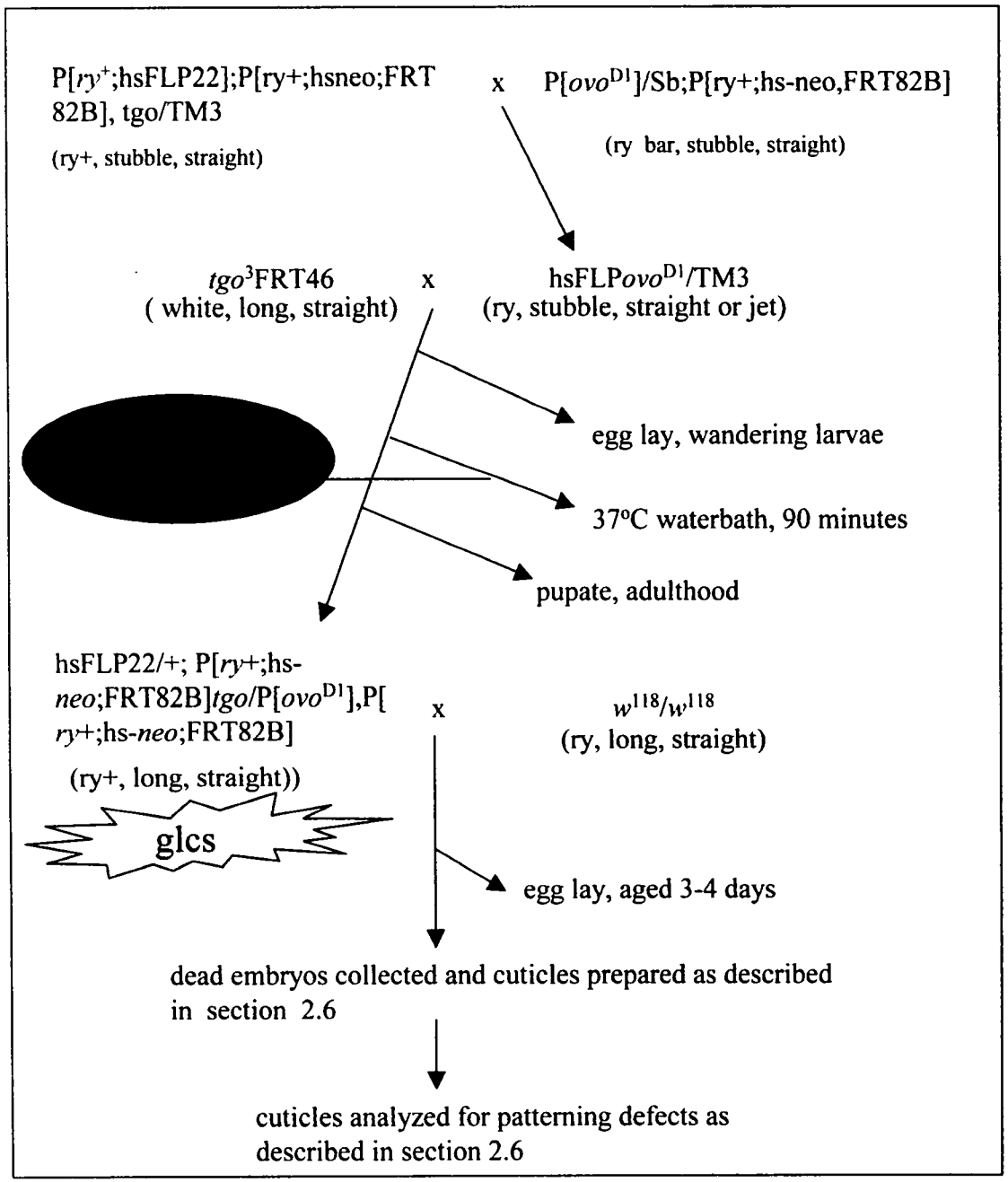


Figure 2.5 Genetic Flowchart for the Production of Germline Clones.

Virgin females are depicted on the left side of each cross, males on the right. The direction of arrows leads to progeny collected from the cross above. The dominant markers of flies such as eye colour, bristles and wing shape are written in parentheses. Site-specific mitotic recombination is induced by the FLP-FRT system (Golic and Lindquist, 1989)(see Figure 2.4). The fly line, *tgo*^{3}FRT46, carries the source of *tgo*^{3} and FRT, which is the site of recognition for the flippase enzyme in their male mates. In addition to promoting site-specific chromosomal exchange, *ovo*^{D1}, an X-linked germline-dependent dominant female sterile (DFS) mutation, was used as a selection against the occurrence of germline recombination events; only germ cells that do not have the DFS mutation led to egg formation. For simplification, males collected from the first (parental) cross are depicted as carriers of the flippase source (regulator of the induced mitotic recombination event), ovoD1 (as per above) and their selection balancer marker (TM3 or stubble bristles). These eggs were left to pupate and the hatched virgin females, *tgo*^{3} females, were crossed to wild-type (w^{118}) males. These *tgo*^{3} females carry the result of the induced homologous recombination (glcs), thereby containing "mosaic ovaries". Once crossed to w^{118}, *tgo*^{3glc} mutations were characterized for patterning defects by:

(1) cuticle analysis and,

(2) characterization of segmentation gene expression (see section 2.6).

analyzed for their ventral denticle band and naked cuticle patterns as compared to both wild-type and segmentation gene cuticular patterns on a Zeiss Axiophot microscope using phase contrast imaging. Images were captured using a Sony DXC 950 camera and Northern Exposure software. Figures were compiled using Adobe Photoshop.

Cuticles from the experimental group germline clones (glcs) were classified as either having severe or intermediate defects as compared to control and wild-type. Severe germline clones were classified as those exhibiting disrupted anterior-posterior patterning, in which both characteristic anterior and posterior structures failed to form, such as the cephalopharyngeal skeleton and both Filzkorper and posterior spiracles, respectively. Additionally, germline clones were analyzed for proper denticle band formation, a reflection of segmental development. Intermediate germline clones were classified as those that had mild disruption in the development of both the above mentioned anterior and posterior structures, including disruption in denticle band formation. The intermediate group of germline clones did not have entire structural nor segmental deletions; their cuticle development did not seem to be as severely disrupted compared to the "severe" group. A schematic of the blastodermal fate map is seen in A2 of the Appendix.

To further characterize tgo^{3} mutations, tgo^{3glc} embryos were stained for segmentation gene expression using both anti-Krüppel (anti-Kr) and anti-Even-Skipped (anti-Eve), HRP antibodies independently.

2.9 Quantitative analysis of cuticles and segmentation

Classification of germline clone embryos following both cuticle preparation and immunohistochemical analysis also involved independent quantification of associative phenotypes for each group.

Slides containing tgo^{3glc} cuticles baked overnight were viewed using phase contrast optics and classified as having a phenotype comparable to wild-type or having either severe or intermediate defects as described in the previous section. Using Northern Exposure

Software, cuticles were projected onto a computer screen and viewed under 40x amplification in order to count the denticle hairs, one by one manually with the naked eye. Each embryo contains approximately eleven denticle bands, three thoracic and eight abdominal segments. Each denticle hair of each developed denticle band in both wild-type, control and experimental (both intermediate and severe) groups were counted and recorded. Once completed, an average number of denticle hairs was tabulated by totaling the number of denticle hairs counted for each segment for each embryo and dividing by the total number of embryos in the sample group. To observe a summary of this quantification analysis, a graph was made using Microsoft Excel software, plotting the average number of denticle hairs per embryo versus genotype. The total larvae sample size for wild-type, control and tgo^{3glc} counts from which denticle hairs were counted was 30, 30 and 21 respectively.

Defects in *Kr* expression were noted as aberrant from controls within the central domain of Kr expression, whether it was an increase or decrease in width span of the Kr stripe (Figure 2.6). Defects in Eve expression patterning along the anterior-posterior axis in tgo^{3glc} embryos were classified defective where Eve striping was stunted, partially missing or absolutely lacking in the abdomen and posterior end of the embryo in comparison to the control group (as described below in section 2.10) were recorded. tgo^{3glc} embryos were subjected to fluorescence staining of both anti-Kr and anti-Eve independently and the number of embryos exhibiting a disruption in expression pattern aberrant to that observed in wild-type and control samples (also described in section 2.10). Also recorded were the number of embryos that did not exhibit any change in Kr and Eve expression patterns. A percentage of the phenotype was calculated from the total number of embryos counted in the given sample, where the sample size of tgo^{3glc} embryos stained with anti-Kr was 20 and those independently stained with anti-Eve was 21.

2.10 Expression and Ectopic Analysis of *tgo* dominant negatives

The GAL4-UAS gene targeting system (Brand and Perrimon, 1993) (Figure 2.7) was used to ectopically express various *tgo* transgenes, including P[UAS-$tgo^{\Delta b}$], P[UAS-

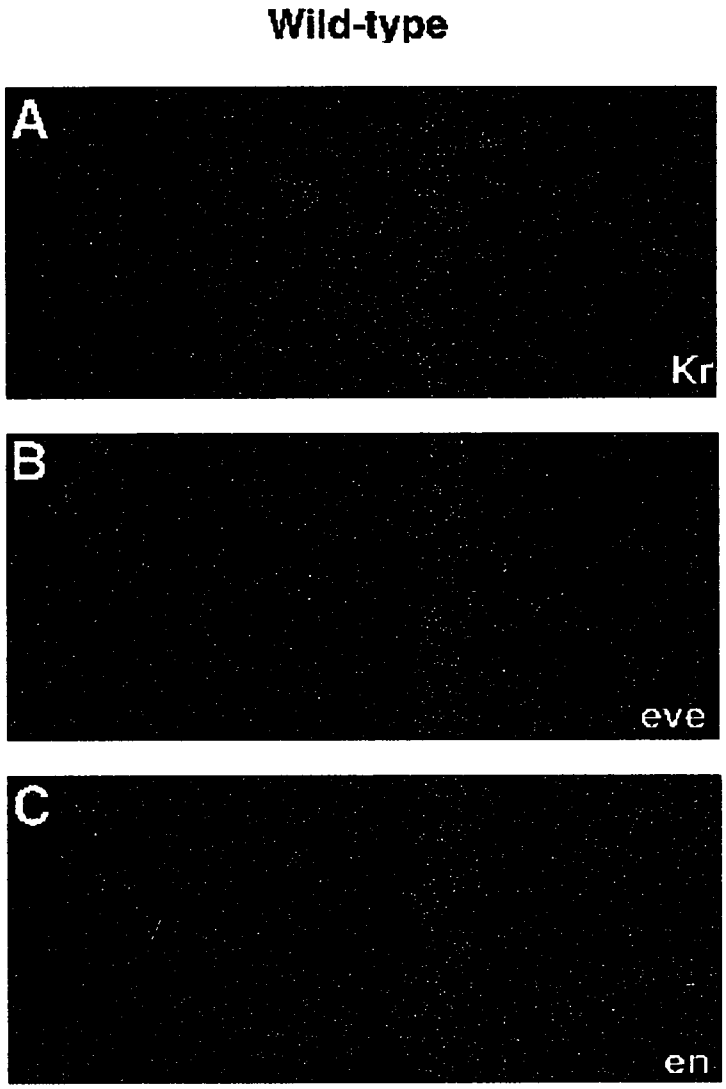


Figure 2.6 Segmentation Gene Expression.

There are four main classes of zygotic genes acting along the antero-posterior axis, the gap genes, the pair-rule genes, the segment-polarity genes and the selector, or homeotic genes. Mutational effects observed during the *tgo*^{3} germline clone analysis and later in embryos expressing various *tgo* deletions (section 2.8) included analysis for patterning defects by characterization of segmentation gene expression. Characterization of *Krüppel*(*Kr*), *even-skipped* (*eve*) and *engrailed* (*en*) segmentation gene expression was conducted using determined dilutions and known secondary antibodies (see Figure 2.1, antibody chart). The gap genes, example *Kr*, define embryonic regionalization resulting in a periodic pattern of gene activity by the pair-rule genes, as, for example, *eve*. Segment-polarity genes, such as *en*, elaborate on this template of segmental pattern (see Chapter 1, sub-chapter 4). These embryos have been stained with anti-Kr (A), anti-Eve (B) and anti-En (C) in wild-type blastoderm embryos using FITC conjugated secondary antibodies.

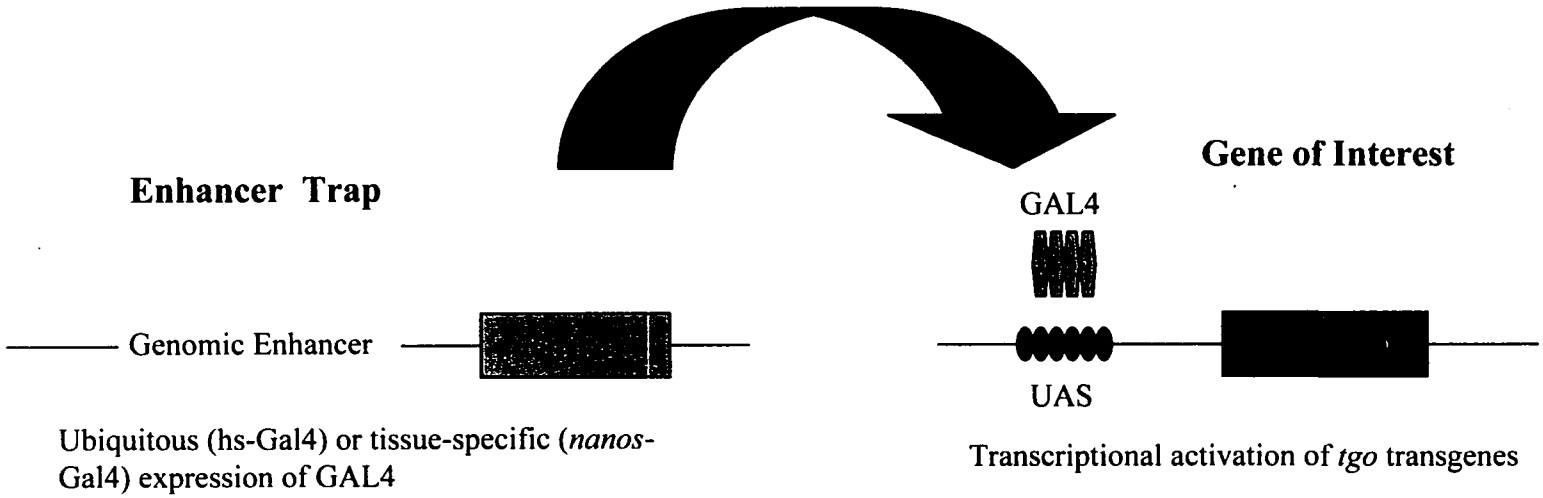


Figure 2.7 The GAL4-UAS system.

This method for directing gene expression in *Drosophila* allows the ubiquitous or directed expression of a gene from a heat shock promoter or the use of tissue-specific promoters, respectively. As described in Chapter 1, the GAL4 gene is randomly inserted into the genome, in turn creating "driver lines" where GAL4 expression is driven from numerous different genomic enhancers. A GAL4-dependent target gene is developed by subcloning any sequence of interest (Gene X, $tgo^{\Delta b}$ for example) behind GAL4 DNA-binding sites called upstream activating sequences (UAS). Otherwise silent, the target gene is activated in a cell- and tissue-specific pattern in the presence of the GAL4 protein. Activation of the target gene occurs by the mating of flies carrying the target (UAS-*gene X)* to flies expressing GAL4 (Enhancer Trap GAL4 in the diagram above). In the progeny of this cross, UAS-Gene X is activated in cells where GAL4 is expressed, thereby allowing the analysis of directed misexpression on development.

tgo$^{\Delta C}$], P[*tgo*^{full}] and the control P[UAS-*lacZ*] during precellular blastoderm embryogenesis. Female flies carrying the hsp70-Gal4 or *nanos*-Gal4 (*nos*-gal4) drivers on the second chromosome were crossed to male flies carrying the various UAS-*tgo* deletions (Figure 2.8). Embryos were collected from the crosses for the first three hours after egg laying (AEL). Embryos carrying hsp70-Gal4 express Gal4 ubiquitously due to induction by the *Drosophila* hsp70 gene after heat shock (Rubin and Spradling, 1982). *nos*-Gal4 embryos target UAS-*tgo* transgene expression to *nos*-expressing cells in the posterior end of the precellular embryo.

The heat shocking time varied with respect to the promoter source: hs-Gal4 embryos received a one hour heat shocking session in a 37°C water bath, whereas *nos*-Gal4 embryos were heat shocked five minutes in a 37°C water bath to provide a surge of *nos* activity. *nos* is constituitively active irrespective of heat shocking and therefore *nos* product is at a basal level in the embryo (Brand et al., 1994). However, a surge of heat shock was provided to ensure functionally relevant expression levels (Manoukian and Krause, 1992). Collection plates were sealed with parafilm and floated in the waterbath for the allotted time and given 30-minute recovery periods at room temperature. Embryos were then fixed and subsequently subjected to immunohistochemical procedures as described in section 2.3.

In a study to ubiquitously express the *tgo* transgenes (P[UAS-*tgo*^{full}, P[UAS-*tgo*$^{\Delta b}$] and P[UAS-*tgo*$^{\Delta C}$]), embryos were collected from the following crosses, female genotype on the left:

(1) hs-Gal4 x P[UAS*lacZ*] (2) hs-Gal4 x P[UAS-*tgo*^{full}]

(3) hs-Gal4 x P[UAS-*tgo*$^{\Delta b}$] (4) hs-Gal4 x P[UAS-*tgo*$^{\Delta C}$]

In a study of targeted misexpression of *tgo* transgenes, males carrying (P[UAS-*tgo*^{full}, P[UAS-*tgo*$^{\Delta b}$] and P[UAS-*tgo*$^{\Delta C}$]) were crossed to females carrying *nos*-Gal4. A genetic scheme using the Gal4-UAS system can be seen in a flowchart presented in Figure 2.9. In this figure, ubiquitous expression can be followed in blue and targeted expression in red.

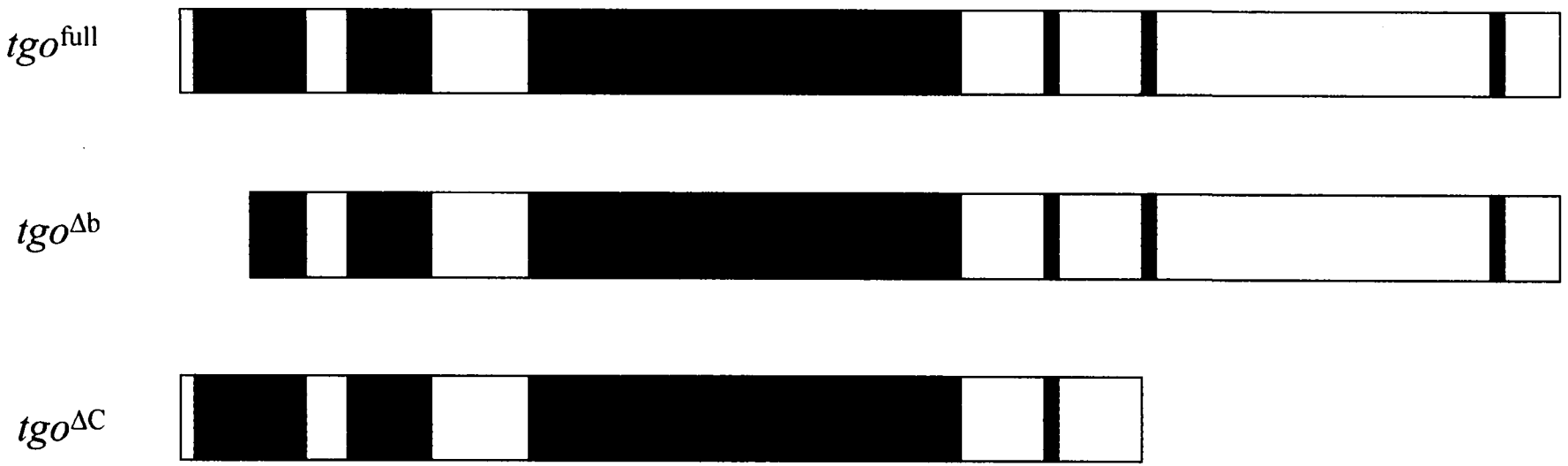


Figure 2.8 Block schematics of the *Drosophila* Tango transgenes used in the ectopic expression analyses in this study. These block diagrams are representative of the domains included in the *tango* (*tgo*) transgenes tgo^{full}, $tgo^{\Delta b}$ and $tgo^{\Delta C}$. The sequence organization of Tango from cDNA clone sequences are shown with important sequence motifs. The N terminus is to the left. The bHLH domains are shown in red and the PAS domains, PasA and PasB, each with a Pas repeat, have been subdivided and are shown in dark purple and green, respectively. The C-terminal regions contain glutamine-rich amino acid stretch (light purple) and a histidine-proline-rich *paired repeat* (cyan). tgo^{full} is represented as the entire cDNA clone (like that seen in figure 1.4). The basic region in the N terminus is deleted and shown as $tgo^{\Delta b}$ (the block is shown as half to suggest the remaining HLH domain). A C-terminal truncated *tgo* transgene missing the paired repeat and part of the glutamine-rich stretch is represented as $tgo^{\Delta C}$. The constructs were used to generate stable transgenic fly lines via P-element transformation and used in this study to analyze the role of *tgo* during embryonic patterning prior to three hours after egg laying.

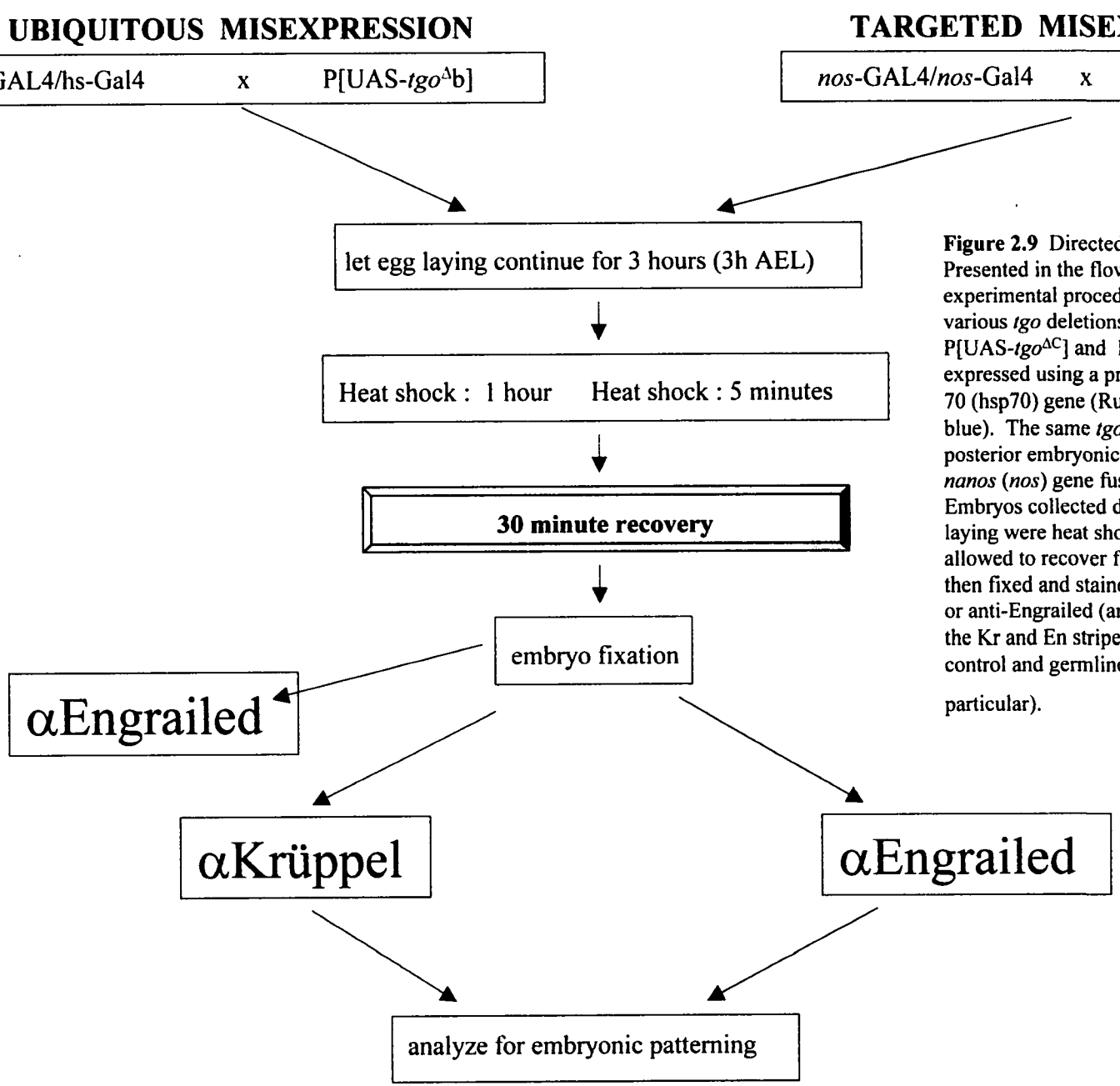


Figure 2.9 Directed expression of *tgo* deletions. Presented in the flowchart are examples of two experimental procedures followed to misexpress various *tgo* deletions. Tgo transgenes P[UAS-$tgo^{\Delta b}$], P[UAS-$tgo^{\Delta C}$] and P[UAS-*tgo*full] were ubiquitously expressed using a promoter of the heat shock protein 70 (hsp70) gene (Rubin and Spradling, 1982; boxed in blue). The same *tgo* transgenes were targeted to posterior embryonic segments using a promoter of the *nanos* (*nos*) gene fused to Gal4 (boxed in red). Embryos collected during the first three hours of egg laying were heat shocked for specified times and then allowed to recover for 30 minutes. Progeny were then fixed and stained with anti-Kruppel (anti-Kr) or anti-Engrailed (anti-En) accordingly. Once stained, the Kr and En stripes were examined in comparison to control and germline clonal mutants (Kr staining in particular).

Following antibody staining, embryos for each genotype were classified as wild-type or defective using a Zeiss Axiophot light microscope. Double-blind counts were conducted in studies analyzing *engrailed* expression patterns. Double-blind counts involved independent and separate recordings of the number of embryos displaying aberrant *engrailed* staining as conducted by M. Sonnenfeld and myself. Number counts were totaled per antibody stain for each genotype as a function of embryos displaying a defective phenotype. Error analysis involved a derivation of the confidence level seen in A4 of the Appendix. Classification involved grouping embryos stained with anti-Krüppel (anti-Kr) and anti-Engrailed (anti-En) into categories with wild-type or alterations in patterning along the anterior-posterior axis. For example, embryos stained with anti-Kr were classified as defective if the Kr staining was either thicker or thinner in the central Kr domain of expression. Embryos stained with anti-En were classified as defective if En staining was (a) stunted, leaving either small or big clusters of En-expressing cells along the anterior-posterior axis, and/or (b) partially missing in the posterior end, and/or (c) absolutely lacking in the posterior end.

III. RESULTS

3.1 Zygotic *tango* Inter-allelic Complementation Analysis in the Embryonic CNS Shows Variable Phenotypes

During development of the neurons and glia comprising the midline of the *Drosophila* embryonic central nervous system (CNS), Tango (Tgo) dimerizes with Single-Minded (Sim) (Sonnenfeld et al., 1997) and these bHLH-PAS master regulatory genes activate CNS midline gene expression (Nambu et al., 1990, 1991; Sonnenfeld et al., 1997; Oshiro et al., 1997). The formation of CNS axons in various *tgo* mutant alleles was examined to phenotypically classify homozygous and trans-heterozygous *tgo* function during embryonic development. To gather some insight into whether the function of the *paired* (*prd*) *repeat* of *tgo* participates with other *tgo* functional domains, I analyzed embryos heterozygous for tgo^3 and other *tgo* alleles.

Embryos were placed into four phenotypic classes. One classification, termed the "fused" commissure phenotype, is associated with improper separation of anterior and posterior commissural axons whereby the posterior boundary of the anterior commissure and anterior boundary of the posterior commissure do not separate from each other (Stemerdinks and Jacobs, 1997). Another, referred to as the "collapsed" axonal phenotype, results from a loss of repulsive cues, thereby exhibiting one longitudinal tract spanning from anterior to posterior in the embryo, similar to those of homozygous *single-minded* and *slit* mutants (Nambu et al., 1990). A hyperplasia of the CNS and over-growth of associated CNS axons characterize the third group, the "neurogenic" phenotype (Boulianne et al., 1991). Lastly, the "stalled" axonal phenotype is characterized by reductions in commissural and longitudinal axons across the midline and between the ventral nerve cord segments and local broader CNS segments. All other ambiguous disruptions of CNS development visualized were classified as nervous system (NS) defects.

To examine homozygous and inter-allelic *tgo* function during embryonic development, previously isolated EMS-induced *tgo* mutant alleles, tgo^2, tgo^4, and tgo^5 (Sonnenfeld et al., 1997; Emmons et al., 1999) were used to characterize the role of tgo^3 in the formation

of CNS axons. Crossing flies heterozygous for tgo^3 and a third chromosome balancer (P [Ubx-lacZ, TM3] to male flies heterozygous for *tgo* and the balancer generated trans-heterozygotes. Embryos were collected and subjected to fixation and immunostained for CNS axonal development as described in Materials and Methods, section 2.2. Embryos were stained with BP102 to visualize the axons and with anti-βgal to unambiguously identify homozygous and trans-heterozygous embryos. In whole-mount preparations of wild-type and mutant embryos, I detected distinct deviations from the wild-type staining pattern in all mutant samples (Fig. 3.1). A percentage of each phenotypic class was observed in each of the mutant embryonic groups (Figure 3.1I), consistent with a variably expressive *tgo* CNS phenotype.

3.1a Wild-type Embryonic CNS Axonal Phenotype

The *Drosophila* embryonic CNS is comprised of a brain and ventral nerve cord consisting of fourteen ganglia.; each ganglia is comprised of glia and approximately 400 neurons, some of which group together to form axon bundles (Crews, 1998). Some of these neurons extend axons that bundle together into groups of fascicles, each containing an average of 10-15 axons. These fascicles form the longitudinal connectives that rest on either side of the CNS midline (Fig. 3.1A, arrowheads) and the two segmentally repeated commissures that cross the midline (Fig. 3.1A, arrows). Successful establishment of the embryonic ventral nerve cord relies on the sequential step-wise requirement of genetic activity and cell interactions shown schematically in figure 1.3 in Chapter 1. Elimination or alteration of the required genetic activity of any one of the genes grouped in any one of the several stages leading to the proper formation of the axonal scaffold result in developmental disruption of the axon commissures in a predictable manner (Klämbt et al., 1991). Previous studies report and suggest why several phenotypes, also observed in this study, may occur (see Discussion).

3.1b Homozygous tgo Mutations

Several reports suggest that weak phenotypes, such as the fused commissure phenotype, imply defects in midline glial differentiation. Enhancer trap studies have shown an absence or loss of two pairs of midline glia (midline glia middle and midline glia

Figure 3.1 Zygotic *tgo* alleles show pleiotropic effects during CNS development.

The CNS phenotype of *tgo* alleles is variably expressive. All homozygous or trans-heterozygous embryos are stage 15 and are stained for CNS axonal development using the mouse monoclonal antibody BP102. Anterior is to the top, and the ventral midline surface is facing in all panels.

A. A wild-type embryo revealing the CNS axon scaffold, with formation of commissural (arrows) and longitudinal (arrowheads) processes.

B. In mutant tgo^3 embryos, the ventral nerve cord is narrower than the wild-type scaffold. The fused commissures are shorter (arrow) and longitudinal connectives are thin (yellow arrowhead) or missing entirely (blue arrowhead). This is referred to as the fused commissure phenotype..

C. A tgo^4 homozygous mutant embryo, showing a less severe collapsed CNS phenotype, in which the commissural and longitudinal processes are collapsed at the middle (arrow) and some of the neuromeres are fused such that a single narrow connective (arrowhead) links adjacent neuromeres.

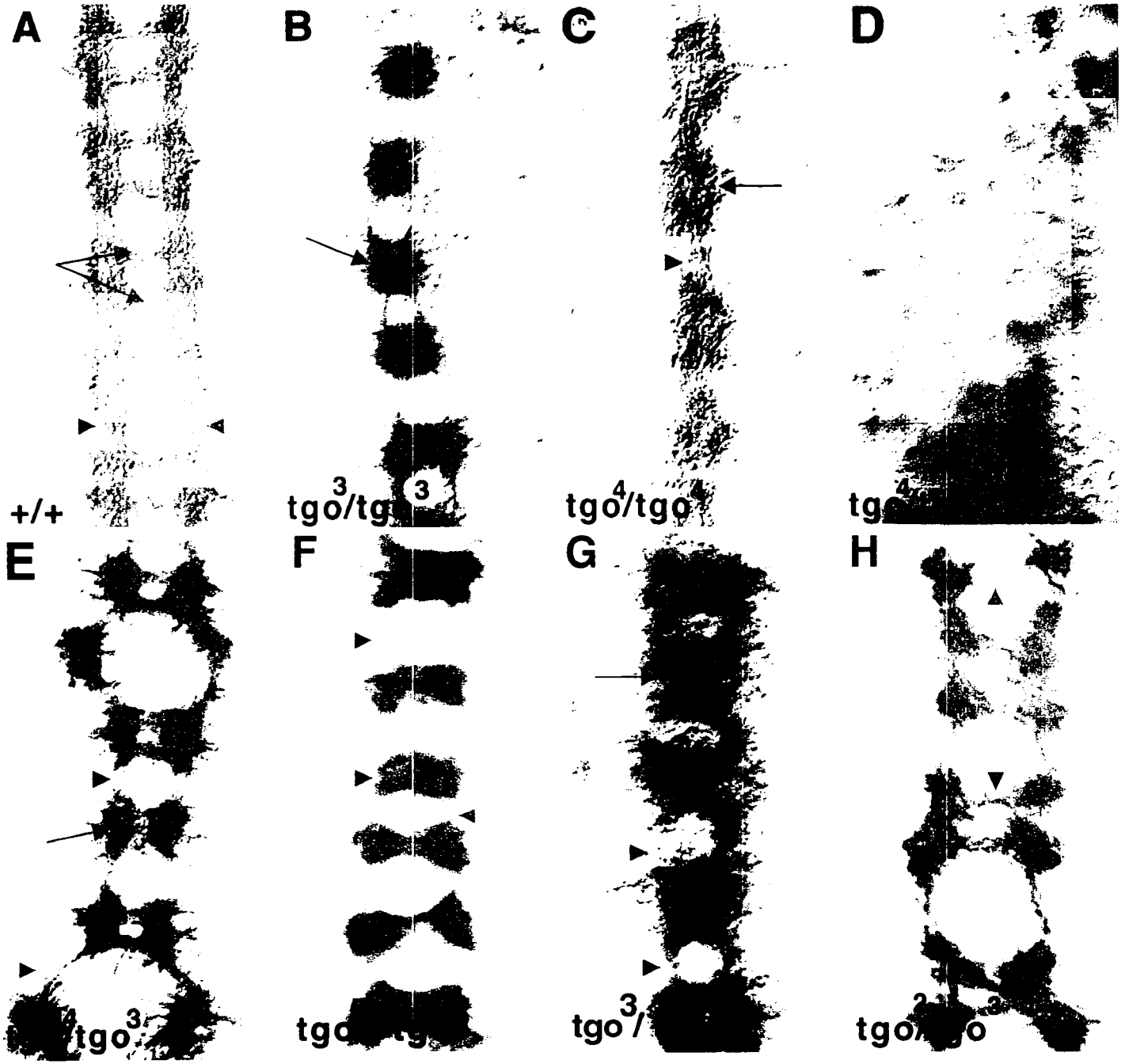
D. A tgo^4/tgo^3 trans-heterozygous embryo displaying a neurogenic phenotype, comparable to that seen in *neuralized* homozygous mutant embryos, such as *neuralized.* A hyperplasia of CNS axons and the ventral nerve cord is typically observed.

E. A tgo^4/tgo^3 mutant embryo displaying a stalled commissure phenotype. Here, the commissures are disorganized and shortened to different extents compared to wild-type (arrow). The longitudinal connectives, if not interrupted, are thin (black arrowhead), giving way to a local bulbous nervous system in some segments (red arrowhead).

F. tgo^5 homozygous mutants show fused commissures (black arrowhead) and longitudinal connectives are seen as reduced in bundle thickness (green arrowhead) or lacking entirely (red arrowhead).

G. A tgo^3/tgo^5 trans-heterozygous embryo showing a combination of fused anterior and posterior commissures (arrow) and a reduction in bundle thickness of longitudinal connectives (arrowheads). This phenotype is an example of one classified under NS defects.

H. A tgo^2/tgo^3 heterozygote displaying the stalled phenotypes of greater severity when compared to E. There is a reduction in the number of commissural fibers in some neuromeres (black arrowhead) or poor midline traversal (red arrowhead). This staining was provided by earlier works performed by M. Sonnenfeld.


A
B
C
D
+/+
tgo3/tg3
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tg4
E
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tgo/tgo

anterior), suggesting disruption in the normal determination or early differentiation of these glial cells (Klämbt et al., 1991, Sonnenfeld et al., 1997; Hummel et al., 1999). The fusion of anterior and posterior commissural axons was observed in homozygous tgo^3, tgo^4 and tgo^5 mutant embryos (Fig. 3.1, B, F and G). With no space between the anterior and posterior commissures, embryos homozygous for tgo^3 mutations exhibit the most severe of fused commissure phenotypes (Hummel et al., 1999; Fig. 3.1B, arrow). Also, homozygous tgo^3 mutants show a reduction in thickness (Fig. 3.1B, yellow arrowhead) or complete lack (Fig. 3.1B, blue arrowhead) of longitudinal connectives. Embryos homozygous for tgo^5 mutations also display fused commissures (Fig. 3.1F, black arrowhead) and a reduction of longitudinal connective thickness if at all developed (Fig. 3.1F, green and red arrowheads, respectively). Exemplified in homozygous tgo^4 mutants (Fig. 3.1C) the commissures are eliminated and the left and right halves of the ganglia are fused, comparable to commissural development seen in the *sim* phenotype (Crews et al., 1988; Thomas et al., 1988).

3.1c Trans-Heterozygous tgo Mutations

There are a wide variety of phenotypes associated with all trans-heterozygous combinations, implying that these alleles may not display null phenotypes. Antibody staining with the monoclonal antibody BP102 on control w^{118} embryos did not reveal the classified axonal phenotypes; the approximately 300-embryo sample size primarily displayed successful wild-type axonal development (Fig. 3.1A). A small percentage (2%) of all *tgo* trans-heterozygotes display the neurogenic phenotype (Fig. 3.1D; Fig. 3.1I), where there is a hyperplasia of CNS neuroblasts comparable to that seen in homozygous *neuralized* embryos (Boulianne et al., 1991). Approximately 30% of trans-heterozygotes are associated with reductions in the number of both commissural axons crossing the midline (Fig. 3.1E, arrow), when compared to wild- type. Less severe phenotypes show longitudinal connectives spanning between ventral nerve cord segments (Fig. 3.1E, arrowheads). Both these tgo^4/tgo^3 and tgo^3/tgo^2 heterozygotes differ from the homozygous phenotypes of each allele; tgo^2 homozygous mutants qualitatively show similar phenotypes to tgo^1 and tgo^3 homozygotes, but are quantitatively more severe, thereby suggesting tgo^2 to be hypomorphic (Sonnenfeld et al., 1997). tgo^3/tgo^5 trans-

heterozygote embryos show a mixture of fused anterior and posterior commissures (Fig. 3.1G, arrow) and stalled longitudinal axons (Fig. 3.1G, arrowheads). In this study, these phenotypes sharing various classifications, were grouped under NS defects. Heterozygous tgo^2/tgo^3 mutants display the stalled phenotype, comparable to that observed in tgo^4/tgo^3 heterozygotes, but of greater severity (Fig. 3.1H). Both commissures are absent in some neuromeres (Fig. 3.1H, red arrowhead) or if formed, are reduced in thickness (Fig. 3.1H, black arrowhead). Nonetheless, there is clearly a reduction in the number of commissural fibers. It has been suggested that a reduction in the number of axons crossing the midline corresponds with the reduction in the number of midline glial cells (Hummel et al., 1999). The BP102 antibody was useful to analyze for CNS phenotypic abnormalities and additional studies involving the use of midline glial markers would verify these mutations as affecting the number of midline glial cells,

3.1d Quantitative Analysis of CNS Axonal Phenotypic Classes in tgo Homozygous and Trans-Heterozygous Embryos

Quantification of the CNS axon phenotypes in *tgo* heteroallelic embryos is displayed in Figure 3.1I. Phenotypes observed were variable within each genotypic group, as described above, but all groups contained given percentages of each of the phenotypes including collapsed, commissureless, neurogenic, stalled and ambiguous NS defects. An average sample size of 576 embryos was screened for each genotype and data are plotted as a percentage of embryos displaying CNS midline defects. On average, embryos within each genotypic analysis displayed 14 fused commissure, 39 collapse, 15 neurogenic, 41 stalled phenotypes.

Homozygous tgo^3 embryos exhibit each of the four phenotypic classifications outlined above, including variable NS defects. Homozygous tgo^4 embryos interestingly show a significantly greater percentage of the commissureless phenotype, calculated at 24% of wild-type, followed by 4% exhibiting a neurogenic phenotype and 2% for both collapsed and NS defect phenotypes. tgo^4/tgo^4 homozygous embryos did not display the stalled phenotype. 9.3% of homozygous tgo^5 embryos primarily show a collapsed phenotype.

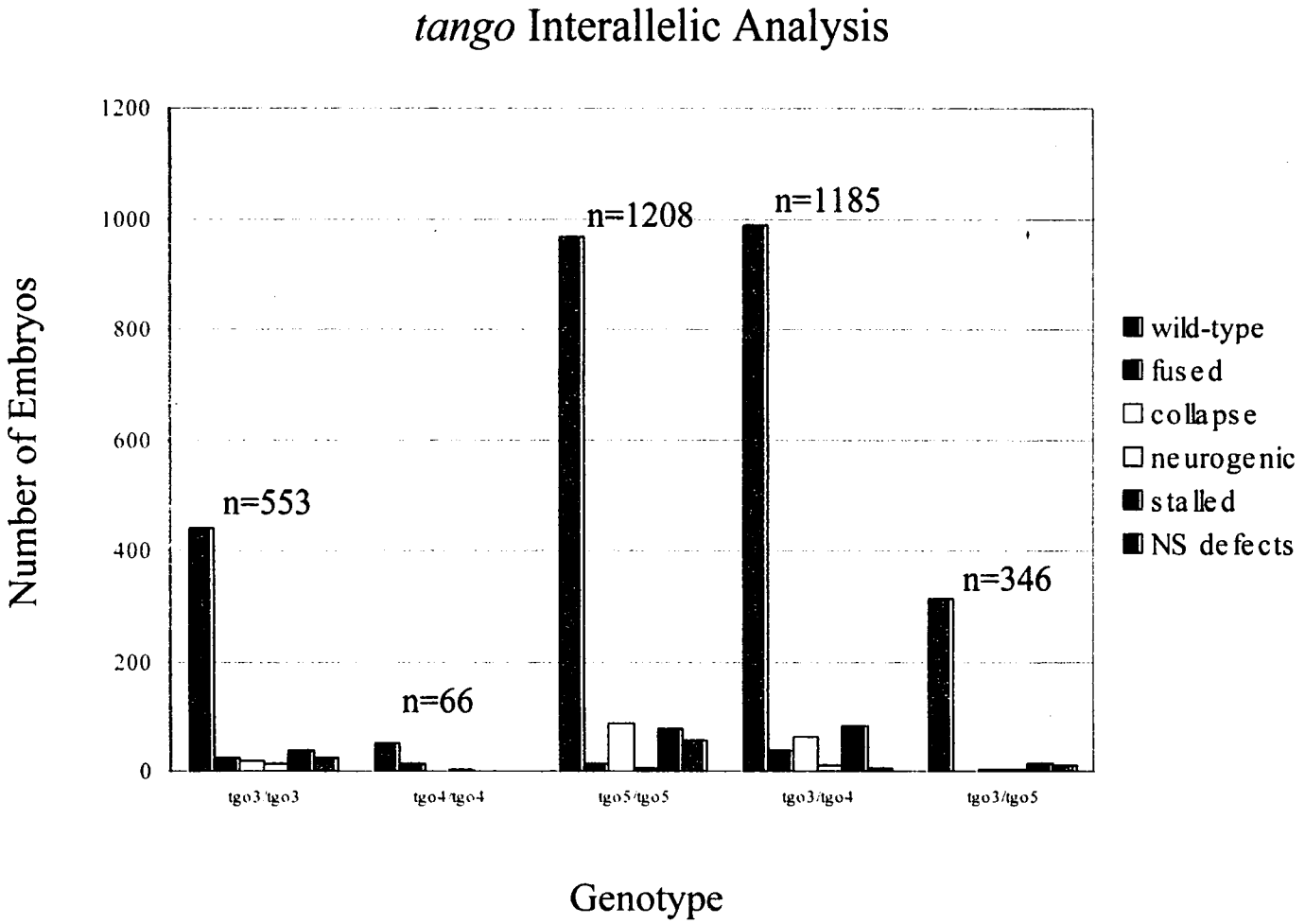


Figure 3.11 The *tgo* CNS phenotype is highly expressive.
Quantification of homozygous and trans-heterozygous axonal phenotypes observed in stage 15 embryonic CNS midline development. Vertical bars are confidence intervals of the number of embryos showing a CNS midline phenotype (n) classified in one of five groups [fused, collapse, stalled or nervous system (NS) defects]. The fused phenotype was observed as a fusion of the anterior and posterior commissures. A collapse of the axonal scaffold resulted in fusion of the longitudinal connectives along the midline with collapse of the anterior and posterior commissures. The neurogenic phenotype is associated with an overgrowth of CNS neuroblasts at the midline at the expense of epidermal tissue. A hesitation of midline axons properly crossing the midline during axon scaffold establishment is affiliated with the stalled axonal phenotype. Classification was made possible through the use of mAb BP102 as compared to wild-type.

The stalled phenotype was observed in 8.2% of embryos, NS defects in 5.8%, commissureless in 1.2% and the neurogenic phenotype was seen in 0.4% of embryos.

Heterozygous tgo^4/tgo^3 embryos primarily display a stalled phenotype in 10.4% of embryos. The collapsed phenotype is seen in 7.5% of embryos, both commissureless and NS defective in 2.1% and the neurogenic phenotype in 0.9% of embryos. Heterozygous tgo^3/tgo^5 embryos exhibit the stalled phenotype in 4.4% of embryos, NS defects in 3.5% and both collapse and neurogenic in approximately 1.0% of embryos. The commissureless phenotype was not observed in tgo^3/tgo^5 embryos.

The *tgo* CNS phenotypes classified in my inter-allelic analysis are not as penetrant as that observed in embryos mutant for *single-minded* (Thomas et al., 1988; Crews et al., 1988; Sonnenfeld et al., 1997). This may be due to a rescuing effect provided by maternal *tgo*, one promoting proper establishment of *Drosophila* CNS midline development. Embryos in the inter-allelic study were either homozyogous or trans-heterozygous zygotic lethals for the isolated alleles of *tgo*, leaving the maternal component to plausibly mask the severity of the zygotic *tgo* CNS phenotype. Additionally, the CNS pleiotropy observed could be the result of overlapping functions of the various Tgo protein motifs and/or due to a possible interaction between Tgo and other bHLH-PAS, non-bHLH-PAS or *prd repeat*-containing proteins expressed during CNS development or earlier.

The remainder of this thesis deals with testing the hypothesis that maternal *tgo* plays an important role during earlier embryonic patterning.

3.2 Maternal *tango* is required for early embryonic patterning

Characterization of the embryonic phenotypes associated with the tango maternal effect: a brief introduction to cuticle preparation and segmentation analyses

The ventral ectoderm, from which the ventral epidermis is derived, also produces the central nervous system (CNS). It was thereby interesting to observe whether the tgo^3

mutation affects only the CNS, or whether is also elicits corresponding defects in the ventral epidermis. Maternal factors direct the earliest patterning events in the developing *Drosophila* embryo (Simpson-Brose et al., 1994), defects of which can be seen on the protective cuticle derived from the ventral epidermis (Nüsslein-Volhard and Wieschaus, 1980). Therefore, to study the *in vivo* role of maternal *tgo* during embryogenesis, I generated homozygous *tgo* mutations in the female germline. Since mutations in maternal genes display morphological aberrations from normal development during early morphogenesis (Mayer and Nüsslein-Volhard, 1988), development was analyzed in embryos during the first three hours after egg laying (AEL). Therefore, the maternal role of *tgo* was characterized by analyzing embryonic phenotypes associated with a reduced maternal tgo^3 function through developmental analysis of cuticle patterns and segmentation gene expression (Figures 3.2.1 and 3.2.2, respectively).

3.2.1 Cuticular analysis

Cuticular phenotypes of embryos derived from control embryos

3.2.1a Control larvae

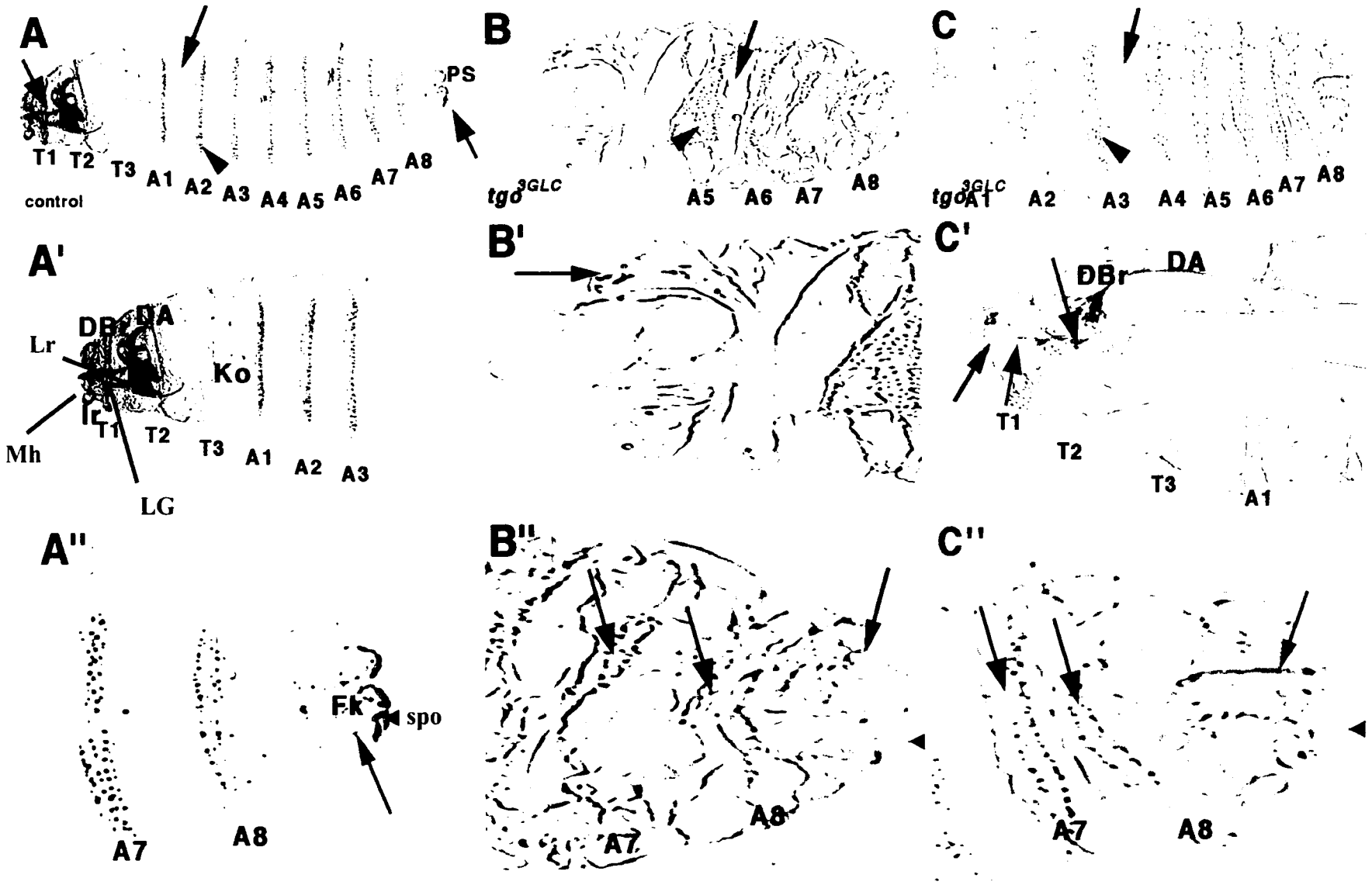
The epidermal cells start to secrete a cuticle at approximately twelve hours after egg laying (Martinez Arias, 1993). Approximately fourteen hours AEL, the denticles can be observed as protrusions of plasma membrane from the most anterior cells of every segment (Pesacreta et al., 1989).

The cuticle of a control larva, much like wild-type (Fig. 3.2.1A) displays a well-defined antero-posterior (A-P) and dorso-ventral (D-V) polarity of pattern elements manifested in a segmental arrangement of denticle bands and naked cuticle. Proper A-P and D-V axial establishment is revealed by the formation of anterior mouthparts and the Filzkorper in the posterior end (Lohs-Sharadin et al., 1979a). The most anterior region of each segment in both thoracic and abdominal segments (T1-A8) is blanketed with denticle hairs (Fig. 3.2.1A, arrowhead). The denticle hairs alternate with naked cuticle (Fig. 3.2.1A, arrow). These segmentally arranged rows of denticle bands are variable in

Figure 3.2.1 Maternal tgo is required for embryonic development.

Larval cuticle pattern of wild-type and *tgo*^{3} maternal mutant (*tgo*^{3glc}) embryos. Anterior is to the left and details of the ventral surface are shown. Phase contrast images are shown of cuticles of wild type (A, A′, A′′) and *tgo*^{3glc} larvae (B-C′′). Scale 1mm= 6.65μm.

(A) Ventral surface of a wild type cuticle. Its cuticle pattern shows the characteristic segmental arrangement of hairs (arrowhead) and naked cuticle (arrow) from anterior to posterior. Formation of the cephalopharyngeal head skeleton (red arrow) in the anterior end and the Filzkorper and posterior spiracles (green arrow) in the posterior end are associated with proper A-P axial development. Note the trapezoidal shape of denticle bands two to seven (A2-A7). **(A′)** Details of the anterior segments, showing the three thoracic segments and the first three of eight abdominal segments. The Keilin's organ (Ko), dorsal arm (DA), dorsal bridge (DBr), the lateralgraten (LG), labrum (Lr) and mouthhook (Mh) are characteristic of the cephalopharyngeal skeleton. Position of each denticle band (arrowhead), polarity of denticle hairs and naked cuticle (arrow) within each denticle band are comparable to wild-type. **(A′′)** Details of the cuticular larval tail reveal denticle hair organization of segments A7-A8, the Filzkorper (Fk) (arrow) and the spiracular opening (spo) (arrowhead). **(B)** A larval cuticle derived from a *tgo*^{3glc} female showing a severe phenotype. There is a loss of all anterior structures and thoracic segments T1-A3. Portions of A5-A8 remain. Posterior structures are also missing. Both denticle hairs (arrowhead) and naked cuticle (arrow) been formed. Note the shorter A-P length of the mutant (compare with A). **(B′)** Anterior enlargement of B. Loss of thoracic segments and the first abdominal denticle is consistent with a loss of the Keilin's organ. Remnants of a head skeleton are what remains of the cephalopharyngeal skeleton (arrow). **(B′′)** Details of the posterior segments of a *tgo*^{3glc} severe embryo. Portions of A7-A8 denticle belts remain (red arrows). There is a loss of both the Filzkorper (black arrow) and spiracular openings (arrowhead). **(C)** An embryo derived from a *tgo*^{3glc} female showing an intermediate phenotype. Development of abdominal segments A2 through A8 ane poorly developed posterior structures are shown. Denticle hairs (arrowhead) and naked cuticle (arrow) form. The anterior portion of the cuticle is not shown. **(C′)** Close-up view of the anterior region of the cuticle in C. Both the DA DB of the head skeleton are present, but the LG(green arrow), Lr (red arrow) and MH (black arrow) do not form properly. **(C′′)** A close-up of the posterior portion of the cuticle in C. The terminal pole of an intermediate *tgo*^{3glc} embryo shows poor development of the Filzkorper (arrow) and posterior spiracles (arrowhead). Abdominal segments A7-A8 form as seen in the severe germline clone phenotype (red arrows).


A
T1 T2 T3 A1 A2 A3 A4 A5 A6 A7 A8
PS
control
B
tgo3GLC
A5 A6 A7 A8
C
tgo3GLC
A1 A2 A3 A4 A5 A6 A7 A8
A'
Lr
DB
DA
Ko
Mh
T1
T2
T3
A1
A2
A3
LG
B'
C'
DBr
DA
T1
T2
T3
A1
A''
Fk
spo
A7
A8
B''
A7
A8
C''
A7
A8

morphology (see Chapter 1, sub-chapter 1.4, section c). In the thorax, denticle hairs are short and stubby, whereas in the abdomen they are variable in texture and take on a trapezoidal shape that becomes less distinct in the most posterior segments. On average, a wild-type embryo contains approximately 3500 denticle hairs, comparable to the control, which is approximately 3100 (see section 3.2.1d and Fig. 3.2I).

The process of head involution results in mouth formation inside the animal (see Martinez-Arias, 1993) (Fig. 3.2.1A′). The anterior region contains a non-segmented area known as the acron, but the most obvious anterior end structure in control and wild-type larvae is the cephalopharyngeal skeleton (Fig. 3.2.1A, A′). This structure is a composite of highly modified segments, consisting of the prothorax with structural elements dorsal arm (DA), dorsal bridge (DB), and lateralgraten (LG) and the pseudocephalon which is comprised of the labrum (Lr) and mouth hook (Mh). Also notable in the preparation is the Keilin organ (Ko), three sensory hairs characteristic of the ventral thoracic segments and seen between segments T3 and Al (Mayer and Nüsslein-Volhard, 1988) (Fig. 3.2.1A′). On average, the number of denticle hairs that develop in the thoracic region in a wild-type embryo is 1055 (+/- 197) which is comparable to the 947 (+/- 54) denticle hairs found in control embryos.

Similar to the anterior end, the tail region of the embryo also consists of several highly modified segments and a rudimentary non-segmental end called the telson (Lohs-Shardin, et al., 1979a; Nüsslein-Volhard et al., 1987). Two proturberances characterized as the posterior spiracles extend form the dorsal surface and a mesh of spiracular hairs, named the Filzkorper (Fig. 3.2.1A′′, Fk, arrow) lead up to the spiracular opening (Fig. 3.2.1A′′, spo, arrowhead). These hairs are fine in structure and were difficult to capture under the microscopy used.

Cuticular phenotypes of embryos derived from tgo^3 germline clones

tgo^3 germline clone (tgo^{3glc}) embryos were generated using standard genetic procedures (Golic and Lindquist, 1989) and the cuticles of these embryos were prepared as described

(see Materials and Methods). Embryonic populations derived from females carrying tgo^3 germline clones showed variability in their cuticle phenotypes. For example, 57% showed a phenotype classified as 'severe' which defined a loss of entire body parts as described in more detail below. A second phenotype was observed in the remaining 43% of the tgo^3 germline clones and was classified as 'intermediate' and is described subsequently. Unlike control larvae, tgo^{3glcs} embryos show a reduction or entire deletion of larval body segments.

3.2.1b The tgo^{3glc} *severe phenotype*

The cephalopharyngeal skeleton is absent in tgo^{3glc} embryos, and this correlates with the loss of thoracic segments and the first five abdominal segments (Fig. 3.2.1B). This severe phenotype leaves a hole in place of the cephalopharyngeal skeleton in the blastoderm layer and remnants of head skeletal structures (Fig. 3.2.1B′, arrow). The naked cuticle between remaining denticle belts seems undisturbed and intact (see Fig. 3.2.1B, arrow). The tgo^{3glc} severe phenotype also includes a lack of Filzkorper development (Fig. 3.2.1B″, black arrow) and an absence of posterior spiracle formation (Fig. 3.2.1B′′, arrowhead). The seventh and eighth abdominal denticle belts manage to develop in the severe germline clones, although they form poorly (Fig. 3.2.1B″, red arrows). The severe tgo^{3glc} phenotype is comparable to the cuticular phenotype displayed by amorphic *bcd* alleles and hypomorphic gap gene mutants (eg. *Kr*).

The impression of a hole in the blastoderm layer is similar to the pole hole phenotype (Degelmann et al, 1986), with the distinction of occurring in the anterior rather than posterior terminal end of the embryo. Observed in cellularized embryos lacking terminal activity, the pole hole phenotype characteristically gives the impression of a hole in the blastoderm layer due to nuclei closest to the pole (sex) cells eventually being pushed into the yolk, where they become indistinguishable from yolk nuclei. This phenotype is observed in loss-of-function alleles of *torso*, *trunk* and *torsolike* ((Sprenger and Nüsslein-Volhard, 1993). The pole hole phenotype does not result from cell death, but rather is believed to be the consequence of a shift in the fate map whereby subterminal regions become expanded and terminal regions are lost. This shift in fate map can be visualized

at the blastoderm stage by the change in the expression domains of the gap and pair rule genes (Casanova and Struhl, 1989). The present study does not allow for a similar conclusion to be drawn of the tgo^3 allele; it is not known if the severe phenotype observed is a result of a shift in embryonic fate map. However, subsequent analysis of segmentation protein distribution may suggest a shift in fate map visualized at the blastoderm stage (see section 3.2.2).

3.2.1c The *tgo^3glc intermediate phenotype*

In a less severe tgo^{3glc} phenotype, thoracic and abdominal denticle belts develop in contrast to the severe phenotype, maintaining proper denticle hair and naked cuticle formation (Fig. 3.2.1C, arrow and arrowhead respectively). In embryos of the intermediate phenotype, an effort in dorsal arch and dorsal branch formation is made, even though development of the labrum is poorly formed and the lateralgraten and mouth hook are lacking (Fig. 3.2.1C′, red and black arrows respectively) when compared to the control (Fig. 3.2.1, see A′). The terminal end of embryos with intermediate phenotypes show formation of the denticle hairs of posterior belts in greater number than what is seen in embryos with a severe phenotype (Fig.3.2.1C′′, red arrows). However, the Filzkorper fails to entirely form into the proturberances seen in the control (Fig. 3.2.1C′′, black arrow) and the spiracular opening also fails to properly form (Fig. 3.2.1C′′, arrowhead).

3.2.1d Quantitative analysis of denticle formation

A quantitative analysis was performed on denticle development in cuticles extracted from larvae derived from control and tgo^{3glc} females, as compared to wild-type (Figure 3.2I). Sample sizes for wild-type, control and tgo^{3glc} groups were 30, 30 and 21 larvae, respectively. The total number of denticle hairs was counted in each specimen and the average number of total denticle hairs for wild-type embryos was found to be 3494 (+/- 1061), which is comparable to the control average number of total denticle hairs, of 3135 (+/- 431). In contrast, cuticles from embryos derived from mutant tgo^{3glc} females showing a severe phenotype yield an average of 554 (+/- 72) denticle hairs, an

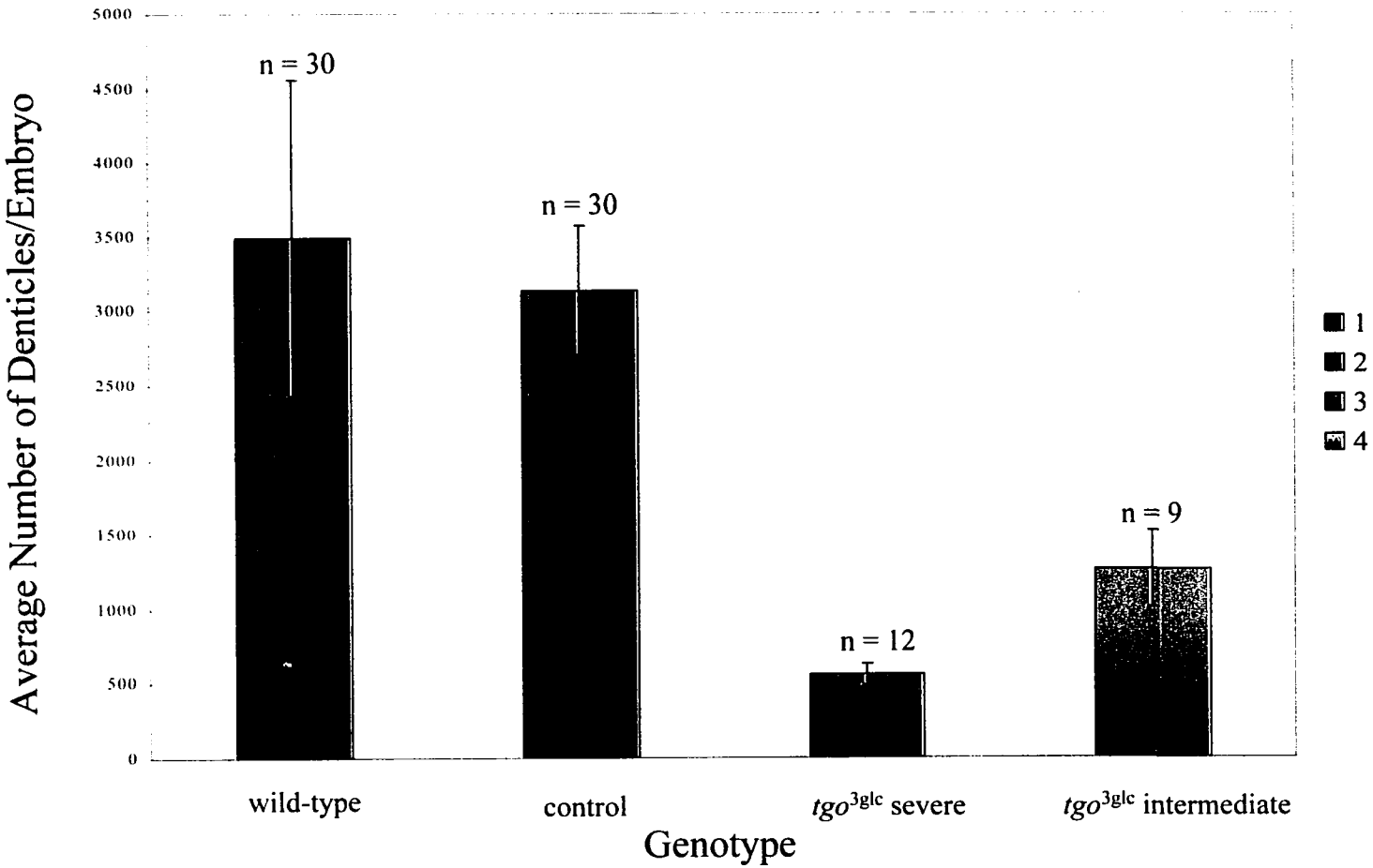


Figure 3.21 Reduction of maternal *tgo* function correlates with drastic loss in denticle hairs.
Quantitative analyses of denticle development after the reduction of maternal tgo^3 show consistent denticle hair loss with tgo^{3glc} embryos losing entire body segments compared to wild-type and control larvae. Vertical bars are standard deviations of the average number of denticle hairs manually counted following germline clonal analysis in the total number (n) of larvae prepared. The analysis of variance revealed a significant effect for group of average number of denticles/embryo. The null hypothesis would be rejected $F(3,77)=35.354$, $p=0.001$. Thus it can be concluded that there is at least one difference among the groups. Pre-planned comparisons which are orthogonal were also tested. Under the null hypothesis the contrast = 0, the alternative hypothesis indicates the contrast is not equal 0. Contrast one compared control with wild-type was not significant ($t(77)=-1.34$, $p>0.05$). A second contrast comparing the severe group with control was significant ($t(77)= -7.90$, $p<0.05$). A third contrast comparing the intermediate group with control was also significant ($t(77)=5.16$, $p<0.05$). The null hypothesis could be rejected indicating that removal or decrease in maternal tgo^3 function is consistent with a drastic loss in the average number of denticle hairs/embryo.

approximate 62% reduction in the average number of hairs compared to control groups. The average number of total denticles counted in embryos displaying the intermediate phenotype was 1265 (+/- 258). The loss in denticle hair number is consistent with the loss of body segments found in the cuticle preparations described in section 3.2.1b-c.

3.2.2 Analysis of Segmentation Gene Expression

Genes involved in establishing the segmental organization of the *Drosophila* larval body have been identified through saturation mutagenesis experiments (Jurgens et al., 1984; Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984a). Mutations in these experiments were generated by chemically-induced mutagenesis to identify the maternal and zygotic gene products involved in specific events in pattern formation of the embryo. The assumption underlying these screens is that the expression of genes that encode "decision making" function is restricted to the corresponding developmental stage. These large-scale genetic screens have identified genes with functions in oogenesis via screening for female sterility, in addition to isolating zygotic patterning genes instrumental in controlling specific embryonic decisions via screens for embryonic lethal mutations. The protein products of these isolated genes are localized in distinct regions of the blastoderm embryo and specify different cell fates in a position-dependent manner. The cuticle phenotype associated with mutant maternal *tgo* function is comparable to patterning defects observed in embryos homozygous for mutations in maternal coordinate or gap segmentation genes (Nüsslein-Volhard and Wieschaus, 1980). To determine whether maternal *tgo* function is required during early embryogenesis for the process of segmentation, the spatial distribution of the gap protein Krüppel (Kr) (Knipple et al., 1985) and pair-rule protein Even-skipped (Eve) (Frasch et al., 1987; Frasch and Levine, 1987) was independently analyzed in embryos derived from control and mutant tgo^3 females.

3.2.2.a Spatial Distribution of Tgo During Early Embryogenesis

The subcellular localization of Tgo has been previously characterized in the CNS midline and trachea during later embryogenesis (Ward et al., 1998). To address the early role of *tgo*, it was therefore important to determine whether the *tgo* protein product was present during the first three hours of egg laying. *tgo* transcripts have been identified prior to three hours after egg laying (AEL) (Sonnenfeld et al., 1997; Oshiro and Saigo, 1997; Ward et al., 1998). To examine the spatial and temporal aspects of Tgo function in the first few hours AEL, Tgo subcellular localization and distribution were analyzed in wild-type embryos stained with a monoclonal Tgo antibody (Sonnenfeld et al., 1997; Ward et al., 1998). In 30 cellular blastoderm embryos, Tgo shows an even distribution from anterior to posterior in the embryo (Fig. 3.2.2A). This contrasts with the localized expression patterns of most segmentation genes (Pankratz and Jackle, 1993). A closer examination of Tgo distribution within the cellular blastoderm shows that there is a punctate nuclear localization present in many cells (Fig. 3.2.2A′, arrowheads). However, there also exists what seems to be cytoplasmic localization as well (Fig. 3.2.2A′, arrows). This suggests that Tgo may be present either in the cytoplasm or nucleus during early embryogenesis. Propidium iodide studies need be conducted to confirm nuclear subcellular localization of Tgo.

3.2.2b Analysis of Krüppel ad Engrailed Spatial Distribution in Control and tgo Maternal Mutant Embryos

A polyclonal Kr antibody was used to visualize the spatial and temporal distribution of the Kr protein in embryos derived from control and tgo^{3glc} females. In addition to its requirement in the formation of thoracic and anterior abdominal segments (Gaul et al., 1987), the Kr gene has additional expression domains not correlating with its function as a segmentation gene (reviewed in Pankratz and Jackle, 1993). As is the case for most segmentation genes, the distribution of Kr in the early embryo is regulated by the normal distribution of adjacent partially overlapping domains of segmentation gene activity (Nüsslein-Volhard and Wieschaus et al., 1980; Wieschaus et al., 1984). Both the anterior and posterior borders of the Kr domains are defined by the actions of various other

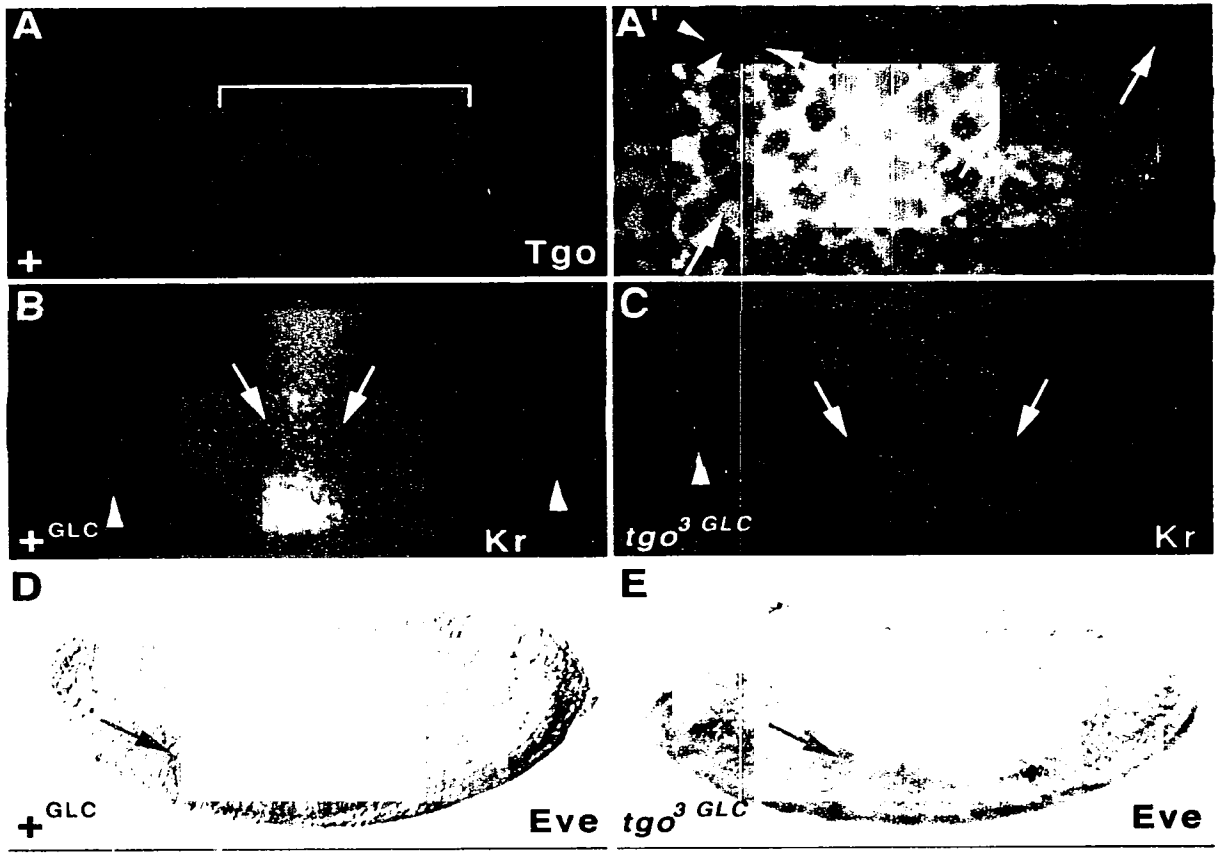


Figure 3.2.2. Spatial patterns of Tgo, Kr and Eve proteins prior to three hours AEL. Whole-mount blastoderm embryos are shown with the anterior end to the left. Photos were taken under confocal (A-C) and light microscopy (D, E). These 3 hour embryos were stained with anti-Tgo (A,A'), anti-Kr (B, C) and anti-Eve (D,E). (A) a wild-type embryo showing an evenly distributed ubiquitous distribution of Tgo spanning the A-P axis. (A') magnification of bracketed area in A suggesting both cytoplasmic and nuclear Tgo localization in wild-type. (B) *tgo*3glc control stained for Kr expression pattern showing Kr staining comparable to wild-type (see figure 3.3.1). (C) 43% of tgo^{3glc} embryos stained for *Kr* expression pattern show an expansion of both anterior and posterior Kr borders. Sight anterior end staining is observed in embryos B and C, consistent with the role of *Kr* in Malphigian tubule development (Pankratz and Jackle, 1993). (D) tgo^{3glc} control stained with anti-Eve displaying the periodicity of Eve expression in seven stripes along the A-P axis. (E) 45% of tgo^{3glc} embryos stained for anti-Eve displaying aberrant Eve striping along the A-P axis show a loss of Eve striping in the abdomen and complete loss of Eve expression in the posterior end. Scale 1mm=6.67um

segmentation genes: the anterior border is governed by synergistic action of *bcd* and *hunchback* (*hb*) and will expand in the absence of this regulation (Jackle et al., 1986; Gaul and Jackle, 1987), and the posterior border is established by the combined action of *bicoid* (*bcd*), *hunchback* (*hb*), *knirps* and *giant* (Jackle et al., 1987; Gaul and Jackle, 1991).

In all 40 whole–mount control embryos analyzed, Kr protein was detected within the nuclei of cells that form an "hour-glass figure" stripe in the middle of the embryo, roughly equidistant from each of the embryonic poles and along the anterior-posterior (A-P) axis (Fig. 3.2.2B, arrow). Contrary to the hourglass figure observed in control embryos, tgo^{3glc} embryos show expansion of the anterior and posterior borders of Kr distribution, although anterior expression persists and seems slightly dispersed (Fig.3.2.2C, arrows and arrowhead respectively). The distinct Kr central domain expression pattern is gone, and Kr staining appears broadened. Despite the alterations, the location of the stripe does remain centralized along the A-P axis. This phenotype was observed in 43% of 20 tgo^{3glc} embryos stained with anti-Kr, with the remaining percentage appears to be similar to wild-type. The expanded *Kr* expression pattern observed in tgo^{3} germline clones suggests that normally, maternal *tgo* may act to repress *Kr* expression required during early embryonic patterning, thereby having a role in restricting it to its hallmark central domain. This function of *tgo* may in part depend on the activity of its *prd repeat* domain. Additional studies investigating the levels of *tgo* transcript and protein stability in both wild-type and *tgo* mutants (including tgo^{3} germline clones) would provide evidence and comparison of *tgo* product functionality with respect to where it becomes localized. *tgo* transcript and protein levels are expected to be reduced in *tgo* mutants compared to levels in wild-type and control embryos.

Subdivision of the A-P axis into segmental units is also regulated by the pair-rule gene, *even-skipped* (*eve*) (Frasch et al., 1987). Combined with the activity of other pair-rule genes, the seven stripe pattern of the Eve protein, observed as early as the blastoderm stage, is indicative of proper cell fate establishment and segmentation (Small, 1992). This wild-type expression pattern of Eve is comparable to that seen in all 40 control

germline cellular blastoderm embryos analyzed (Fig. 3.2.2D). However, in 21 tgo^{3glc} embryos stained for *eve* expression there is a drastic reduction in the number of Eve stripes and aberrations in the remaining stripe pattern compared to that in control embryos (Fig. 3.2.2E). Of the 52% tgo^{3glc} embryos exhibiting defects, 55% showed stunted and/or partially missing eve striping along the A-P axis and 45% showed complete loss in Eve striping in the posterior end of the blastoderm embryo. The reduction in Eve striping suggests a requirement of maternal *tgo* function, perhaps dependent on the activity of its *prd repeat* motif, in activating *eve* expression. In conclusion, the combined data above show that maternal *tgo* function is required for proper distribution of both Kr and Eve segmentation proteins during early embryogenesis, a function perhaps dependent on the action of the *prd repeat* domain.

3.3 The *tango paired repeat* domain is required for proper embryonic patterning

Ectopic Expression of tango Deletions During Drosophila Embryonic Segmentation.

The fate of a cell is largely determined by its characteristic pattern of gene expression (Brand et al., 1994). Therefore, the ability to manipulate transcriptional activity in a directed fashion through induction by ectopic expression of a gene is a powerful tool to analyze that gene's role in development. The basis of this directed manipulation rests on the expression of a transcriptional activator or repressor in a different cell or different time from where and when it would be normally expressed during development. In turn, this enables an examination of the given activator or repressor on subsequent expression of a target gene or on a particular developmental process, such as segmentation. The GAL4-UAS system (Brand and Perrimon, 1993) was used to manipulate the expression of two *tgo* transgenes during the first three hours of development. To analyze whether segmentation was proceeding properly, embryos were stained independently with antibodies that recognize expression of Krüppel (Kr) (Knipple et al., 1985) and Engrailed (En) (Kornberg et al., 1985; Fjose et al., 1985) segmentation genes. Two of the *tgo* transgenic fly lines contain deletions in different locations in the *tgo* sequence: one is

missing 3′ sequences encoding the transactivation domain and *prd repeat* in the C-terminal end (UAS-*tgo*$^{\Delta C}$), while the other is missing the basic DNA binding domain in the N-terminus (UAS-*tgo*$^{\Delta b}$). The third *tgo* transgene contains a full-length *tgo* coding region (UAS-*tgo*^{full}).

Specifically, I have used GAL4-directed transcription to express the *tgo* transgenes under the control of two different promoters. Flies carrying hsGal4 were crossed to those carrying the various UAS-*tgo* transgenes. Embryos were collected from this cross and transgene expression induced by heat shock. Embryos were then stained with antibodies to analyze segmentation. Under regulation of the heat shock promoter (*hsp*70) (Rubin and Spradling, 1982), *tgo*$^{\Delta C}$ and *tgo*$^{\Delta b}$ and *tgo*^{full} would be expressed ubiquitously throughout the first three hours of embryogenesis and analyzed for their effects on both Krüppel (Kr) and En protein expression patterns. Secondly, the *tgo* deletions were expressed under the direction of the *nanos* (*nos*) promoter (Clark et al., 2000) by crossing flies carrying *nos*-Gal4 to those carrying the UAS-*tgo* transgenes. In this study, *nos* was selected for its restricted expression in the posterior end of the embryo (Nüsslein-Volhard et al., 1987; Lehmann and Nüsslein-Volhard, 1991).

3.3a Ubiquitous Expression of tgo Transgenes

The effect of tgo transgene activity on Kr expression, a gap segmentation gene

A polyclonal Kr antibody was used to observe the spatial and temporal localization patterns of Kr protein within wild-type embryos (Fig. 3.3.1A). Kr is prominently localized within a striped domain several cells wide in the middle of the embryo (Fig. 3.3.1A, black arrows). Control embryos ubiquitously expressing UAS*lacZ* (hsGal4UAS*lacZ*) show a Kr localization pattern similar to that in wild-type embryos (Fig. 3.3.1B).

Embryos ectopically expressing a C-terminal deletion of *tgo* (P[UAS-*tgo*$^{\Delta C}$]) in the first three hours after egg laying show one of either two opposite effects: (1) an expansion of

the Kr stripe compared to controls, similar to that observed in *tgo*^{3} glcs stained with anti-Kr, or (2) a reduction in the Kr stripe compared to that of control embryos. Approximately 47% of embryos (n=151) ubiquitously expressing P[UAS-*tgo*$^{\Delta C}$] showed a phenotype (Fig. 3.3I). In 49.3% of these embryos, the stripe was expanded beyond its normal boundaries (Fig. 3.3.1C), while in 57.7%, the Kr stripe was reduced (Fig.3.3.1D). Combined, these data suggest that the *tgo* C-terminal deletion show dominant negative effects during early gap segmentation stages of embryogenesis. Suggestion as to why there may be what seems to be two opposite effects of C-terminal end deletions of *tgo* on early segmental identity is explained in a hypothetical model proposed in Chapter 4 (Discussion).

In embryos ubiquitously expressing full-length *tgo* (P[UAS-*tgo*^{full}]), 38% showed one of the two Kr defects (Fig. 3.3I). This percentage is higher than that observed in the control group, a result not expected in this study. The implication of this is not well understood, and would require repeated and further experimentation. Embryos ectopically expressing the *tgo* basic region deletion (P[UAS-*tgo*$^{\Delta b}$]) did not show as consistently higher penetrance of aberrations as those observed in UAS-*tgo*$^{\Delta C}$ embryos stained for segmentation gene marker expression (anti-Kr, including anti-En, see below). The percentage of defective embryos in populations expressing P[UAS-*tgo*$^{\Delta b}$] is similar to that of control embryos (Fig. 3.3I). An explanation for the observation of defects in both wild-type and control embryos, including embryos ubiquitously expressing P[UAS-*tgo*$^{\Delta b}$], is that perhaps there are variable levels of *tgo* expression (either transcript or protein) present due to an instability of heat shocked product. Nonetheless, the consistently lower penetrance of defects associated with ubiquitous expression of the basic region *tgo* deletion compared to the percentage observed in embryos ubiquitously expressing P[UAS-*tgo*$^{\Delta C}$] implies a lack of dominant negative effects associated with the P[UAS-*tgo*$^{\Delta b}$] transgene. Moreover, this suggests little or no requirement of the DNA-binding activity of Tgo in regulating early *Drosophila* embryonic patterning.

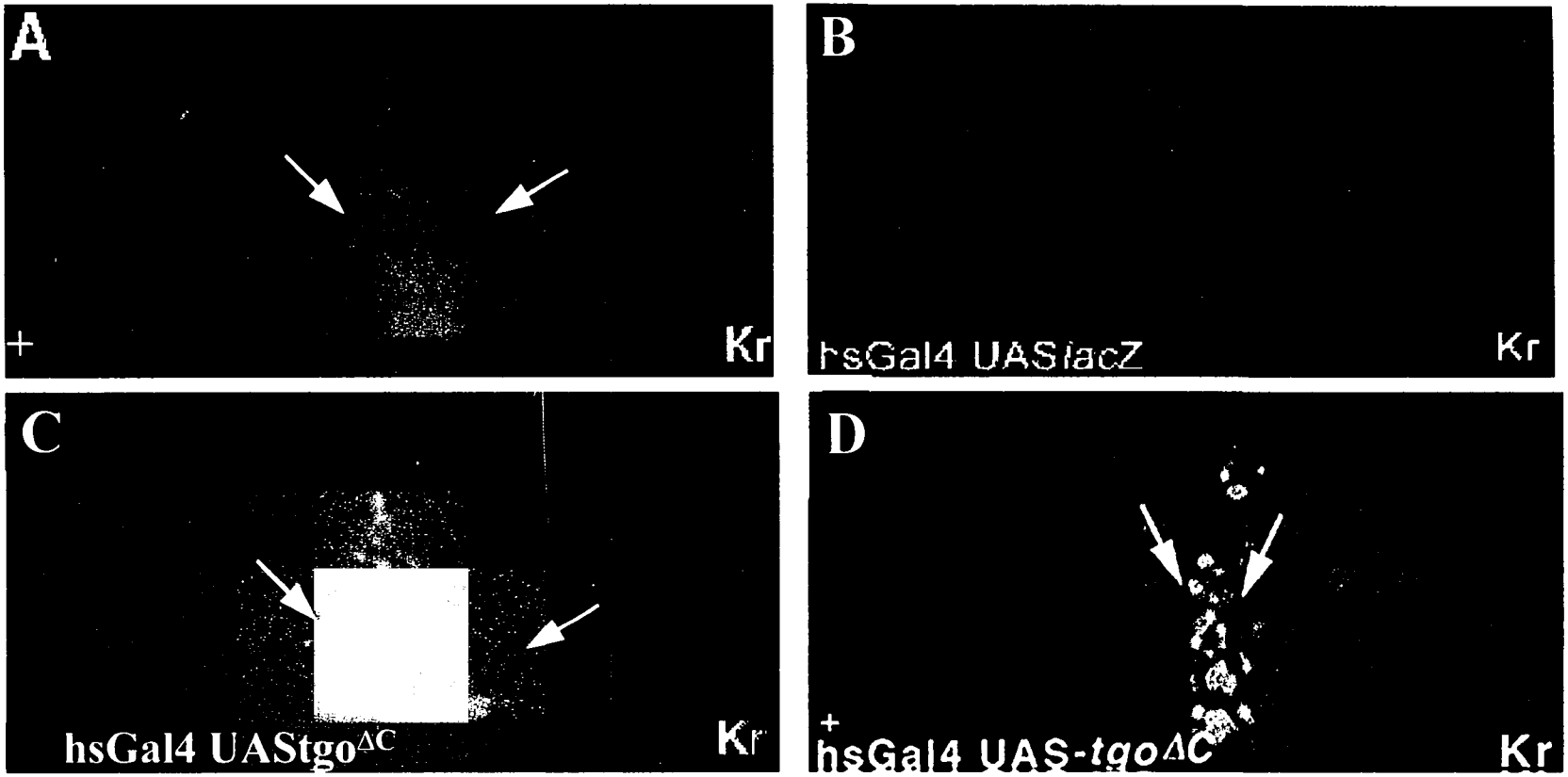


Figure 3.3.1 Ubiquitous misexpression of *tgo* transgenes show defects in the Kr gap gene expression pattern. Cellular blastoderm embryos are shown with the anterior end to the left. Heat shocking was induced using the hsp70 promoter and subjecting 3 hour old embryos to a 37^{0}C waterbath for 30 minutes. (A) a wild-type blastoderm stained with anti-Kr showing the normal Kr distribution in its central domain of expression (arrows). (B) Kr expression pattern in a control P[UAS-lacZ] embryo showing comparable Kr expression pattern. (C) 49.3% of P[UAS-*tgo*$^{\Delta C}$] embryos displaying improper Kr expression show an expansion of what seems to be both anterior and posterior Kr borders. (D) 57.7% of P[UAS-*tgo*$^{\Delta C}$] embryos showing aberrant Kr striping display what seems to be a reduction of both anterior and posterior Kr borders. Scale 1mm=5.00um.

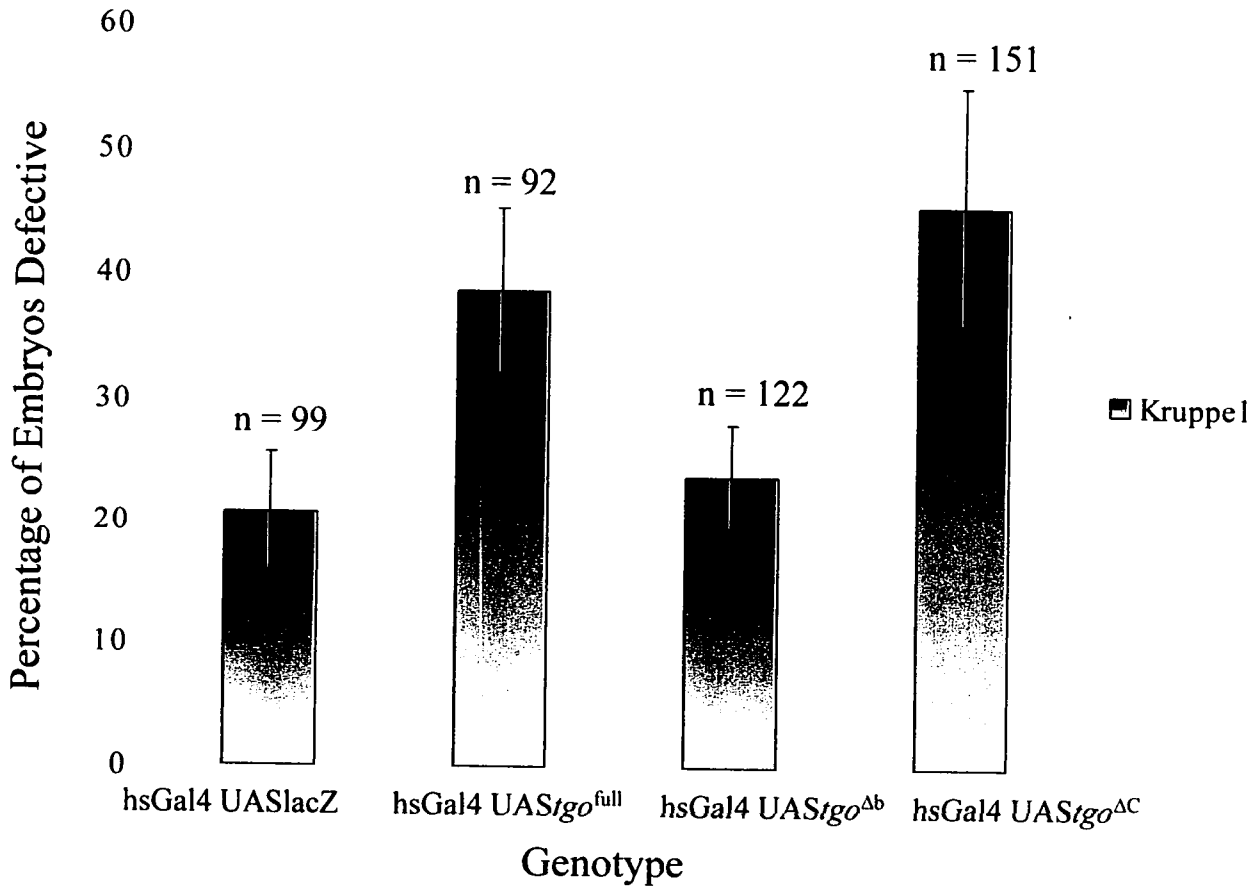


Figure 3.3I Ubiquitous expression of the C-terminal *tgo* transgene shows a consistently higher penerance of aberrant Kr expression pattern. Quantified analyses of percentage of embryos ubiquitously expressing P[UAS-*tgo*lacZ], P[UAS-*tgo*^{full}], P[UAS-*tgo*$^{\Delta b}$] and P[UAS-*tgo*$^{\Delta C}$] showing improper Kr spatial distribution. The vertical bars are confidence interval of the percentage of embryos showing aberrant Kr expression pattern compared to wild-type in the total number (n) of embryos prepared. The analysis of variance revealed a significant effect for group on percentage of embryos defective. Chi-square analysis was used because the dependent variable was categorical (looking at the percentage of mutants thus requiring non-parametric analysis). The chi-D value obtained ($\chi^2(3)=19.462$, $p<0.001$) shows significant effect for at least one group of percentage of embryos defective. Therefore further analyses were run to look at specific contrasts between the three experimental groups (P[UAS-*tgo*^{full}], P[UAS-*tgo*$^{\Delta b}$], P[UAS-*tgo*$^{\Delta C}$]) and control (P[UAS-lacZ]). Contrast one compared tgofull and *tgo*$^{\Delta b}$ with control was unexpectedly significant ($\chi^2(1)=6.601$ (which does challenge an expected rejection of the null hypothesis), $p<0.05$ and not significant ($\chi^2(1)=0.403$, $p=0.52$), respectively. A second contrast compared *tgo*$^{\Delta C}$ and control was significant ($\chi^2(1)=13.833$, $p<0.001$). Considering the second contrast, the null hypothesis could therefore be rejected indicating that ubiquitous expression of *tgo*$^{\Delta C}$ is consistent with significant defects compared to control.

The effects of tgo transgene expression on engrailed, a segment-polarity gene

In wild-type embryos, Engrailed is expressed in 14 evenly spaced segmental stripes (Kornberg et al., 1985; Fjose et al., 1985) (Fig. 3.3.2A). Consistent (Fig. 3.3.2A, arrows) and full-spanning (Fig. 3.3.2A, bracket) stripes indicate proper En localization along the A-P and D-V axis of the developing embryo. Comparable En striping was observed in control embryos ubiquitously expressing P[UAS*lacZ*] induced by hsGal4 in 0-3 hour embryos (Fig. 3.3.2B).

In contrast however, the distribution of En was altered in embryos ubiquitously expressing P[UAS-$tgo^{\Delta C}$]. In a total sample of 128 embryos ubiquitously expressing the C-terminal deletions of *tgo*, 46.9% showed defects in En expression compared to 27% in control embryos expressing P[UAS*lacZ*] (Fig. 3.3II). Of these embryos ubiquitously expressing P[UAS-$tgo^{\Delta C}$] and showing defects, 22.7% of them showed a stunted expression of En striping, 30.5% showed partial stripe loss in the posterior end and 46.8% showed a complete loss of En striping in the posterior end along with stunted En striping spanning the A-P axis. This last, most severe phenotype is depicted in Figure 3.3.2C.

En protein distribution was not disturbed in most embryos ubiquitously expressing P[UAS-tgo^{full}], nor was it aberrant in embryos ubiquitously expressing P[UAS-$tgo^{\Delta b}$]. Quantitative analysis showed that only 21.2% of embryos expressing P[UAS-tgo^{full}] (n=85) and 26.9% of embryos expressing P[UAS-$tgo^{\Delta b}$] (n=104) showed variations in En patterning (Fig. 3.3II). Neither group displayed a complete loss of En striping in the posterior end of the embryo as that observed in P[UAS-$tgo^{\Delta C}$]-expressing embryos (Fig. 3.3.2C, arrow). These defective embryos primarily displayed slightly stunted En striping, leaving clusters of En-expressing cells similar to those seen in embryos ubiquitously expressing P[UAS$tgo^{\Delta C}$] (Figure 3.3.2C, arrowheads). The small percentage of defects observed in embryos ubiquitously expressing P[UAS-tgo^{full}] was comparable to the percentage of defects observed in control embryos (Fig. 3.3II). Additionally, the small percentage of defects seen in embryos ubiquitously expressing P[UAS-$tgo^{\Delta b}$] suggests

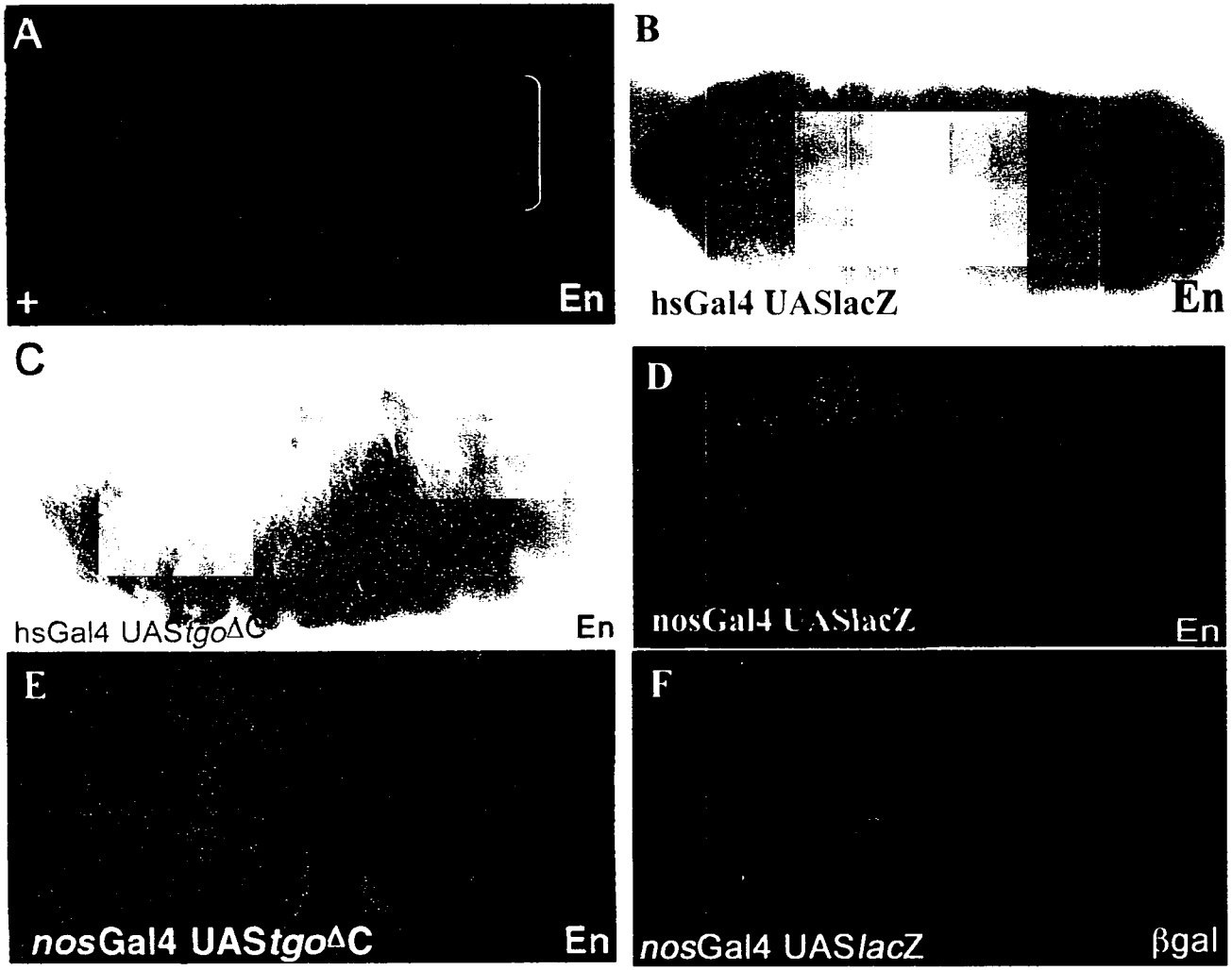


Figure 3.3.2. Ubiquitous and targeted misexpression of *tgo* transgenes show defects in the En segment polarity gene expression pattern. Cellular blastoderm embryos are shown with the anterior end to the left. Heat shock was induced using a 37^{0}C waterbath and followed protocol stated in Materials and Metods (Chapter2). Photos were taken using confocal (A, D-F) and light (B,C) microscopy. The embryo in panel B is shown with the ventral surface facing and embryos in panels A, C-F are sagital. (A) the spatial distibution of En in a wild-type blastoderm shows a series of periodic stripes along the A-P axis. (B) Comparable En patterning to a wild-type blastoderm is seen in the control. (C) 46.9% of embryos ubiquitously expressing P[UAS- *tgo* $^{\Delta C}$] showed reduction of En striping in the abdomen and loss of En stripes in the posterior end. (D) a control blastoderm for *nos*-directed Gal4 activity shows comparable En expression to that in wild-type (compare to A). (E) 43.9% of embryos targeting P[UAS-*tgo*$^{\Delta C}$] to *nos*-expressing cells in the posterior end show stunted En striping in the abdomen and loss of En expression in the posterior end. (F) a control embryo for the efficiency of the Gal4-UAS system using βgal detection of the lacZ reporter highlights directed reporter activity to *nos*-expressing cells in the posterior end of the blastoderm embryo. Results in embryos shown in panels C and E are consistent with results observed in *tgo*^{3glc} embryos stained for Eve expression pattern (see figure 3.3.2). Scale 1mm=6.17um.

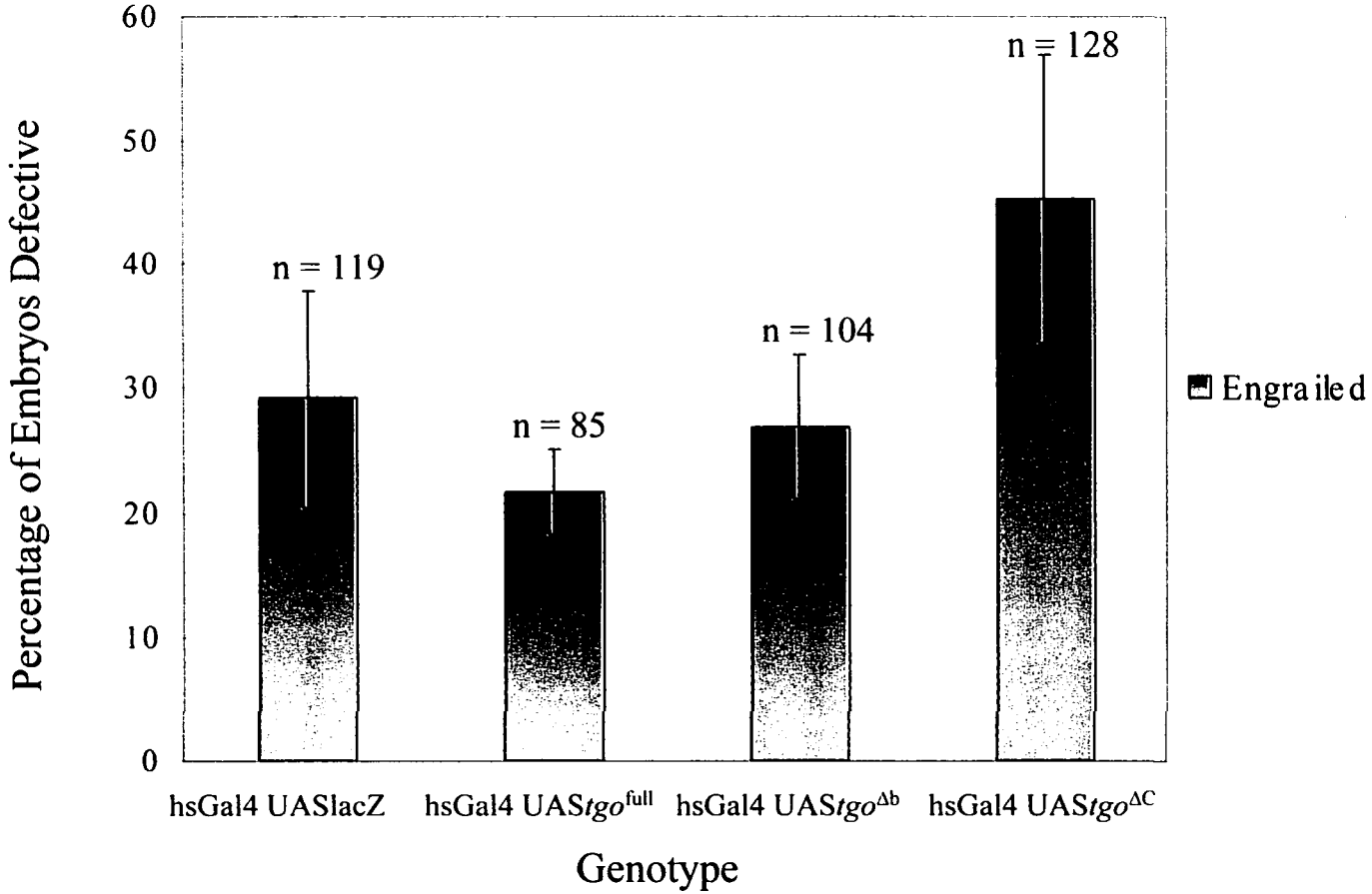


Figure 3.3II Ubiquitous expression of the C-terminal *tgo* transgene shows a consistently higher penetrance of aberrant En expression pattern. Quantified analyses of the percentage of embryos ubiquitously expressing P[UAS-*tgo*lacZ], P[UAS-*tgo*^{full}], P[UAS-*tgo*$^{\Delta b}$] and P[UAS-*tgo*$^{\Delta C}$] showing improper En spatial distribution. The vertical bars are confidence intervals of the percentage of embryos showing aberrant En expression pattern compared to wild-type in the total number (n) of embryos prepared. The analysis of variance showed a significant effect for group on percentage of embryos defective. Chi-square analysis wsa used because the dependent variable wsa categorical (looking at percentage of mutants therefore requiring non-parametric analysis). The chi-D value obtained (χ^2=(3)=19.461, p<0.001) shows significant effect for at least one group of percentage of embryos defective. Therefore further analyses were conducted to look at specific contrasts between the three experimental groups (P[UAS-*tgo*^{full}], P[UAS-*tgo*$^{\Delta b}$] and P[UAS-*tgo*$^{\Delta C}$]) and control P[UAS-lacZ]). Contrast one compared P[UAS-*tgo*^{full}] and P[UAS-*tgo*$^{\Delta b}$] with control was not significant (χ^2(2)=1.437, p=0.49). A second contrast compared P[UAS-*tgo*$^{\Delta C}$] and control was significant (χ^2(1)=8.760, p<0.05). The null hypothesis could be rejected indicating that ubiquitous expression of P[UAS-*tgo*$^{\Delta C}$] is consistent with significant aberrant defects compared to control.

that the DNA-binding domain exerts no effect on the proper establishment of embryonic segmentation.

3.3b Targeted Misexpression of the tgo Transgenes: The Effects Observed by nanos-Induced Expression

To examine the consistency of the transgene-induced phenotypes from early heat shock procedures, the *tgo* transgenes were expressed using a *nanos*-Gal4 (*nos*Gal4) driver. During wild-type embryogenesis, *nos* is required for proper spatial and temporal development of posterior parasegments (Driever and Nüsslein-Volhard, 1989). The *nos*Gal4 driver was therefore used to direct *tgo* transgene expression to posterior parasegments. Flies carrying *nos*Gal4 were crossed to those carrying UAS*tgo* transgenes. Embryos were collected from this cross, were stained with anti-Engrailed (anti-En) and En expression patterns were analyzed using *nos*Gal4;P[UASlacZ] as the control group (Fig. 3.3.2D). Additionally, the expression pattern of *nos*Gal4 was analyzed using UAS*lacZ* as a control. Female flies carrying *nos*Gal4 were crossed to male flies carrying UAS*lacZ* and their progeny were stained with monoclonal anti-β-galactosidase (anti-βgal). βgal staining was observed in the posterior end of these embryos, consistent with the normal *nos* expression pattern (Nüsslein-Volhard et al., 1987; Lehmann and Nüsslein-Volhard, 1991) (Fig.3.3.2F).

Similar to the ubiquitous expression study, the targeting of P[UAS-$tgo^{\Delta C}$] transgene expression to *nos*-expressing cells in the posterior end was associated with a phenotype. Double-blind counts of 139 embryos targeting P[UAS-$tgo^{\Delta C}$] to the *nos* expression domain resulted in 48.2% of these embryos exhibiting various defects in *en* expression compared to that in only 26.7% of control embryos (n=120) (Fig. 3.3III). Approximately 25.1% of $tgo^{\Delta C}$-expressing embryos displayed stunted En striping, 31.0% showed partial loss of En patterning in the posterior end and 43.9% showed a drastic reduction in the number of En stripes in the posterior end of the embryo (Fig.3.3.2E). In each group showing partial or complete loss of En patterning in the posterior end, most if not all embryos also exhibited stunted En striping along the A-P axis. Therefore, the effects of

expressing P[UAS*tgo*$^{\Delta C}$] ubiquitously or in a restricted pattern are similar and the results are consistent. These effects are expected if considering that the C-terminal end sequence of Tgo, including the *prd repeat*, is required during early embryonic patterning (see Statement of Problem). Aberrant engrailed patterning in 26.0% of embryos (n=127) targeting the P[UAS-*tgo*$^{\Delta b}$] transgene to *nos*-expressing cells in the posterior end was similar to that observed with the targeted expression of P[UAS*lacZ*] and P[UAS-*tgo*^{full}] independently (Fig. 3.3III). In embryos directing P[UAS-*tgo*$^{\Delta b}$], P[UAS*lacZ*] and P[UAS-*tgo*^{full}], the severe phenotype depicted in Figure 3.3.2E was not seen in the percentage of defective embryos observed in any of these sample groups. Compared to the percentage of embryos expressing P[UAS-*tgo*$^{\Delta C}$] to *nos*-expressing cells in the posterior end, it is possible that the DNA-binding domain of *tgo* may not be required in the correct establishment of segmental identity during the first three hours after egg laying.

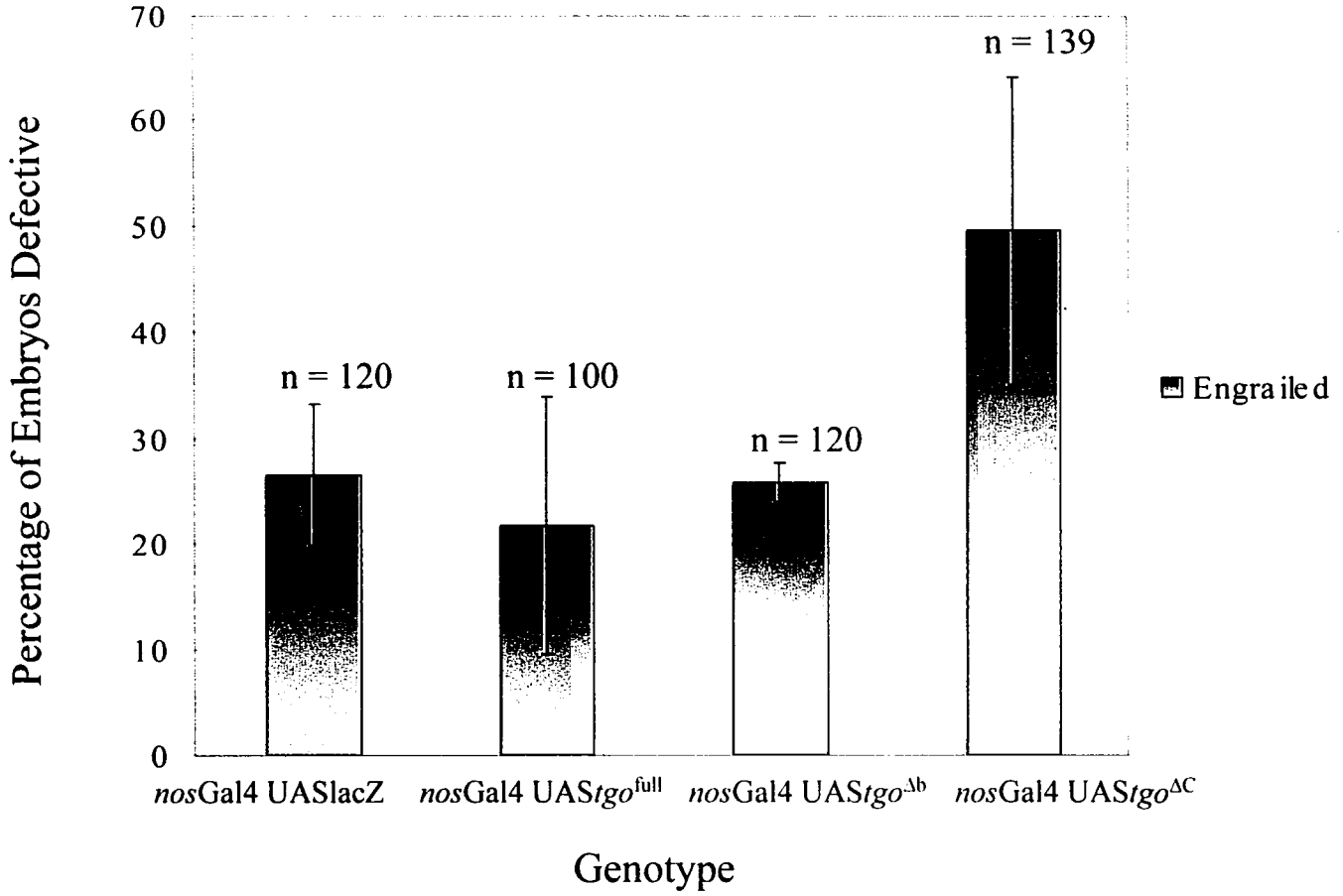


Figure 3.3III Targeted expression of the C-terminal *tgo* transgene shows a consistently higher penetrance of aberrant En expression pattern. Quantified analyses of the percentage of embryos targeting expression of PUAS-*tgo*lacZ], P[UAS-*tgo*^{full}], and P[UAS-*tgo*$^{\Delta b}$] and P[UAS-*tgo*$^{\Delta C}$] to *nos*-expressing cells in the posterior end of the blastoderm embyro showing improper En spatial distribution. The vertical bars are confidence intervals of the percentage of embryos showing aberrant En expression pattern compared to wild-type in the total number (n) of embryos prepared. The analysis of variance revealed a significant effect for group on percentage of embryos defective. Chi-square analysis was used because the dependent variable was categorical (looking at the percentage of mutants therefore requiring non-parametric analysis). The chi-D value obtained ($\chi^2(3)=26.176$, $p<0.001$) shows significant effect for at least one group of percentage of embryos defective. Therefore further analyses were run to look at specific contrasts between the three experimental groups (P[UAS-*tgo*^{full}], P[UAS-*tgo*$^{\Delta b}$], P[UAS-*tgo*$^{\Delta C}$]) and control (P[UAS-lacZ]). Contrast one compared P[UAS-*tgo*^{full}] and P[UAS-*tgo*$^{\Delta b}$] was not significant ($\chi^2(2)=1.094$, $p=0.58$). A second contrast compared P[UAS-*tgo*$^{\Delta C}$] and control was significant ($\chi^2(1)=12.648$, $p<0.001$). The null hypothesis could be rejected indicating that targeted expression of P[UAS-*tgo*$^{\Delta C}$] is consistent with significant defects compared to control.

IV. DISCUSSION

Using powerful *Drosophila* genetic techniques, I have dissected the maternal and zygotic functions of the *tango* (*tgo*) gene. To this end, the *prd repeat* domain seems to be an important player in dictating roles of Tgo that may be independent from its previously characterized roles in bHLH-PAS pathways. Deletions of the C-terminal *prd repeat* domain, as in the tgo^3 allele, result in disruption of central nervous system (CNS) midline development. As earlier studies have shown, *tgo* mutant embryos using the tgo^1 allele show CNS midline defects consistent (although less penetrant) with Tgo being a dimerization partner of Single-minded (Sim) (Sonnenfeld et al., 1997). In the current study, involving an inter-allelic genetic complementation and CNS phenotypic analysis of tgo^3, the monoclonal antibody BP102 was used to examine the requirement of the *prd repeat* of *tgo* during CNS axonal development for indication of midline defects. The presence of pleiotropic *tgo* mutant CNS phenotypes suggests that the role of *tgo* during CNS establishment is both intricate and multi-faceted. Consistent with the prospect of exerting several independent effects, *tgo* mutants are seen as abnormal in their general embryonic morphology, distinct from *single-minded* (*sim*) defects (Sonnenfeld et al., 1997). This implies that perhaps Tgo may be involved in processes mediated by bHLH-PAS or non-bHLH-PAS factors. As is often the case, eukaryotic molecules function at multiple times and in different pathways during embryogenesis, thereby resulting in the difficult task of assessing the function of a gene due to the pleiotropy that may result from its mutant phenotypes. In this way, zygotic functions important for the development of the embryo may be masked if the associated gene is also maternally expressed, in that the maternal component can exhibit rescuing effects on zygotic phenotypes. I have attempted to identify an uncharacterized regulatory role of *tgo* by generating germline clones and using heat-shock-inducible *tgo* transgenes to elucidate the early requirement of *tgo* during the first three hours of *Drosophila* development. Analysis of cuticle preparations from embryos collected from mosaic tgo^3 females revealed a deletion of thoracic, abdominal and terminal body segments of either severe or intermediate severity. The results were consistent with those found in examination of early segmentation protein distribution patterns in tgo^3 germline clones and embryos ectopically expressing a *tgo* C-terminal deletion (UAS-$tgo^{\Delta C}$). By monitoring the

expression of various segmentation genes in the blastoderm embryo, I observed phenotypes suggesting that *tgo* may function during early embryonic patterning.

Overall, the phenotypes observed in the CNS inter-allelic and germline clonal analyses shed light on how Tgo may function in each respective process in wild-type embryos. The pleiotropic nature of the *tgo* CNS mutants observed in this study might be due to a conglomeration of overlapping functions of the domains it encodes. *tgo* may elicit its possible requirement in establishing A-P polarity and proper segmentation during early embryonic development through an interaction with other factors regulating early development. The latter hypothesis has evolved into a more elaborate interpretation of the results presented in the analysis of the role of *tgo* during early embryonic patterning. This is in light of the given analyses performed in this body of work and the proposal of mechanisms possibly underlying early embryogenesis during this period of time in development by other bodies of work. Therefore, I have addressed the zygotic role of *tgo* during embryonic CNS development and its maternal role during embryonic patterning independent of one another.

4.1 *tango* may function along a common biological pathway required for embryonic survival

In this study, a collection of EMS-induced *tgo* alleles was used in inter-allelic complementation and CNS phenotypic analysis to classify defects in *tgo* that could be complemented by alleles carrying mutations possibly affecting other aspects of *tgo* function. These alleles, giving rise to embryonic recessive lethal phenotypes, were isolated through independent EMS mutageneses and as such, may denote missense mutations. It was therefore important to elaborate on the relationship between and among them. Phenotypic analysis of CNS axonal development in a selection of *tgo* homo- and hetero-allelic mutants enabled general classification within each genotypic group. However, genetic distinction between the different aspects of the embryonic CNS phenotypes was difficult in that each inter-allelic analysis produced the same or similar phenotypes, although to varying frequencies.

The highly expressive *tgo* CNS phenotype suggests that the function of *tgo* during CNS development is intricate, involving several independent roles in directing the establishment of the axon scaffold. I was interested in associating the different mutant axonal phenotypes to specific defects in the CNS midline and subsequently, to the sequential development of the CNS axon pattern (refer to figure 1.3). Each genotype analyzed exhibited a consistent spectrum of axonal phenotypic effects in CNS stage 15 embryos, with the exception of tgo^4/tgo^4 and tgo^3/tgo^5embryos independently. Embryos displayed all four effects classified in this body of work, namely fused anterior-posterior commissures (Klämbt et al., 1991; Stemerdink and Jacobs, 1997), a collapse of the axonal scaffold (Thomas et al., 1988), a hyperplasia of the nervous system or neurogenic phenotype (Boulianne et al., 1991) and phenotypes exhibiting stalled axonal development across the midline. Also included was a group of nervous system defects to acknowledge those phenotypes of arbitrary classification. Knowing that tgo^1 and tgo^2 individually affect embryonic cellular differentiation (Sonnenfeld et al., 1997), I wanted to see whether tgo^3 mutations also affect similar developmental events.

The stalled phenotype

Displayed in heterozygous tgo^4/tgo^3 and tgo^2/tgo^3 embryos analyzed for CNS axonal development in Figure 3.1, the phenotype exhibited is a thin extension of longitudinal connectives to adjacent segments in addition to poor traversal of commissural fascicles across the midline in some segments. This phenotype was classified as "stalled", in reference to a stunted and disorganized appearance of commissural axons and expanded connectives between adjacent segments, resulting in a "hesitation" of commissures crossing the midline. The stalled phenotype was observed in each inter-allelic analysis, with the exception of homozygous tgo^4 mutants. In some segments, the nervous system becomes bulbous, sometimes exhibiting local expansion of segments and associated extension of the longitudinal connectives. In the most severe cases, there is a reduction in the number of commissural tracts traversing the midline (Fig. 3.1H). In less severe phenotypes, the commissures are shorter compared to wild-type (Fig. 3.1E). A similar variability in the length and/or number of commissural tracts has also been documented in mutations in genes belonging to the *spitz* group (*spitz*, *Star*, *pointed*, *rhomboid*). These

studies have suggested that the reason for the variability may lie in a reduction or lack in the number of midline neurons (Hummel et al., 1999). Moreover, the midline has been defined as a source of both attractive (Tessier-Lavigne et al., 1988; Mitchell et al., 1996) and repulsive (Kidd et al., 1999) signaling. Therefore, in contending with a reduction or absence of commissural tracts crossing the midline, an important consideration would be growth cone guidance and extension. An intriguing proposal involves a change in cell fate by key cells accountable for providing or responding to the important signals for commissural guidance, or alternatively, by mutations in genes encoding necessary components of the signaling process (Seeger et al., 1993). Consequently, a change in cell fate or mutation in genes regulating proper midline cell differentiation would result in an inability to promote cues of attraction and repulsion required for correct commissural guidance across the midline. In light of this proposal, given the reduction in the number of commissures crossing the midline in the stalled phenotype, perhaps *tgo* plays a role in the signaling pathway conferring the proper guidance of commissural axons during midline development. Whether this role involves cellular differentiation or provision of attractive or repulsive cues is not known, however analysis of the other phenotypes observed in the inter-allelic study suggest that *tgo* could conceivably be involved in cellular differentiation. tgo^4 mutants do not display the stalled phenotype, an exception that may be due to what motifs are affected by the tgo^4 mutation. For example, if tgo^4 were to be sequenced and found in the HLH domain of the Tgo protein, it would be then hypothesized that Tgo, although capable of DNA-binding, would lose its heterodimeric abilities. Therefore, in keeping with the possibility that tgo may have a role during commissural axonal guidance, it may be that the domains of Tgo affected by the tgo^4 mutation may not be required.

As referenced above, mutations that may result in a reduction in the number of midline neuronal cells have been developmentally associated with variability in the number of commissural tracts. However a reduction in the number of midline glial cells have been documented to result in a fused anterior-posterior commissure phenotype (Hummel et al., 1999).

The fused commissure phenotype

Formation of the elaborate cytoarchitecture of a mature nervous system is dependent upon the initial establishment of a simple pattern, or scaffold. These pathways are subsequently followed by axons from neurons that later arise (Jacobs and Goodman, 1989). With the establishment of the mature axonal tract, the scaffold is no longer needed and the cells that initially contributed to the scaffold are either removed or differentiate into glial support cells. The midline glia (MG) of the *Drosophila* ventral nerve cord are transient and function to establish the position and morphology of the commissural axon tracts of the nervous system during mid-embryogenesis. These cells also produce proteins such as D-netrins and Commissureless, both implicated in growth cone guidance (Seeger et al., 1993). Separation of the anterior and posterior commissures is in part accomplished during commissural tract morphogenesis, when a subset of the MG migrate and interpose themselves between axon fascicles crossing the midline (Klämbt et al., 1991). Little is known about the genetic basis of determination and early differentiation of the MG (Stemerdink and Jacobs, 1997). Rather, more is understood about the genetic basis of MG survival. Genes of the *Drosophila* epidermal growth receptor (DER) and *spitz* groups are not required for early MG functions. However, when function of these genes is lacking, the MG fail to migrate. This leads to failure in separation between the anterior and posterior commissural tracts and subsequent apoptotic death of all MG (Klämbt et al., 1991; Sonnenfeld and Jacobs, 1994). A striking phenotype associated with each inter-allelic combination of this study, with the exception of heterozygous tgo^3/tgo^5 mutant embryos was a fusion of segmental commissures. The exception may be attributed to embryos exhibiting two or more of the four classifiable phenotypes as arbitrary nervous system defects. Within the fused commissure class of CNS mutations, there seems to be distinction with respect to affected axonal morphology. The terminal CNS phenotype of homozygous tgo^3 embryos is manifested in the fusion of segmental commissures, with no space remaining between anterior and posterior commissures. However, disruption of axon morphology is extended to the longitudinal connectives, whereby a reduction of bundles is observed (Fig. 3.1B). This is comparable (although more severe) to phenotypes observed in *Star* mutant embryos described by Mayer and Nüsslein-Volhard (1988) and similar to mutants of the *pointed* group, which

includes some members of the *spitz* group mutants mentioned above (*spitz* and *Star* for example) (Hummel et al., 1999). Homozygous tgo^5 embryos display a fused commissural phenotype similar to that observed in *pointed, rhomboid* double mutant embryos; fused anterior-posterior commissures and reduced axon bundle thickness in the longitudinal connectives (Klämbt et al., 1993).

In all of these mutations, it has been reported that the MG do not migrate between the anterior and posterior commissures, resulting in a failure to separate them and subsequent apoptosis of the cells (Klämbt et al., 1991; Sonnenfeld and Jacobs, 1994). Since the fused commissure phenotypes observed in the *tgo* inter-allelic study are comparable to those of *pointed* (including *spitz*) null mutations, it is therefore perhaps reasonable to correlate the occurrence of similar underlying events associated with the fused phenotype in *tgo* CNS mutants. Therefore, *tgo* CNS mutations may elicit a failure of MG migration in enabling separation of the axon bundles of the anterior and posterior commissures. As previously mentioned, a method to test this hypothesis in the *tgo* CNS midline mutants could involve a similar approach taken by Klämbt et al., (1991) by using glial markers in tracing the behavior of midline cells. It is important however to consider another mechanism that when disrupted causes a fused commissure phenotype. In the midline, repulsive cues have at least three distinct roles: to prevent ipsilateral axons from entering the midline, to pioneer commissural axons through the midline, and to prevent commissural axons from crossing the midline more than once (Rajagopalan et al., 2000). In *Drosophila*, an example of such a midline repellent fulfilling each of these functions is Slit. In *slit* mutants, both ipsilateral and contralateral axons enter the midline and never exit it, resulting in a collapse of the axon scaffold similar to the *sim* phenotype (Kidd et al., 1999; see below). These errors in axonal projection demonstrate that Slit is required for both preventing ipsilateral axons from entering the midline and disallowing commissural axons from lingering at the midline. Moreover, *Drosophila* embryonic CNS stained with mAB BP102 showed that the loss of *roundabout* (*robo*), an axon guidance receptor of the immunoglobulin superfamily for Slit, causes severe ectopic midline crossing at the midline, thickening of the commissures and reduction in the longitudinal connectives between segments (Simpson et al., 2000). The phenotype of *robo* mutants

therefore is similar to the fused commissure phenotype observed in the inter-allelic *tgo* study. This similarity could suggest that, as is possibly the case in *slit* mutants, the complete absence of the repulsive signal at the midline results in axons being attracted to it but never able to leave (Simpson et al., 2000)

The collapsed phenotype

Commissural connections in the CNS of different species develop in very similar ways. Initially, commissural growth cones grow towards the midline and once crossing it, travel along tracts along the ipsilateral side. In the vertebrate nervous system, the floor plate houses a specialized set of ectodermal cells at the ventral midline of the neural tube that has a role in commissure formation and specification of cells in the adjacent neuroectoderm (Taniguchi et al., 2002). The floor plate cells acquire their identity via *sonic hedgehog* inductive signaling from the underlying notochord and in turn, the floor plate and notochord induce cell fate in the neuroectoderm (Yamada et al., 1991). The role of the *Drosophila* CNS midline cells in inducing cell fate in the adjacent ventral neuroectoderm is similar to the role of the specialized ectodermal cells of the vertebrate floor plate. Within *Drosophila*, axonal patterning is regulated by attractive and repulsive cues provided by the midline cells and so, in this regard, the midline cells, much like the floor plate cells in vertebrates, act as an organizing center within the developing CNS (Pielage et al., 2002). Apoptosis of the *Drosophila* midline cells prior to complete commissure formation generates a phenotype wherein there is a loss in equilibrium between cues of attraction and repulsion at the midline. The resultant CNS phenotype is a failure in commissural axon tract development and a fusion of the longitudinal axon connectives at the midline due to a loss of repulsion (Crews et al., 1988; Thomas et al., 1988; Klämbt et al., 1991; Kidd et al., 1999). This phenotype, classified as a collapse of the axon scaffold, is a hallmark of homozygous *sim* mutants and comparable to that observed in each inter-allelic analysis of this study (see Fig. 3.1C; Fig.3.1I). Tracing the fate of midline cells in *sim* null mutants revealed that the mutant cells do not characteristically meet at the ventral midline at gastrulation (Nambu et al., 1991). Coincidentally, none of the subsequent events affiliated with CNS midline development occur, including failure to undergo a series of proliferative synchronized divisions and an

inability to differentiate nerve cell precursors into neurons and glia and take their appropriate positions in the developing CNS (Nambu et al., 1993). Collapse of the axon scaffold has also been documented in *slit* embryos, where there is no cell death occurring at the midline like in *sim* mutants, but rather the midline cells are displaced and do not appear to properly differentiate (Sonnenfeld and Jacobs, 1994). Time course analysis of axon pathway development reveals that in *sim* and *slit* mutant embryos, there is enough differentiation of midline cells to enable one or both commissures to form (albeit abnormally), only to eventually collapse and disappear (Klämbt et al., 1991). Both genes are active at the onset of commissure formation (Klämbt et al., 1991), but as stated previously, Tgo directly interacts with Sim to modulate CNS development (Sonnenfeld et al., 1997). Knowing this, it is possible that the collapse phenotype observed in the series of inter-allelic analyses of this study may be attributed to a failure in cell-type specification or differentiation in the initial stages of midline development in the ventral nerve cord, perhaps attributable to cell death rather than displacement. Again, midline cell markers could be used to trace the course of action taken by midline cells in *tgo* CNS mutants.

The neurogenic phenotype

Development of the CNS in *Drosophila melanogaster* begins with the formation of neuroblasts in the ventral neurogenic region of the embryo. Once committed to becoming a neuronal precursor, a cell will instruct neighboring cells to enter epidermogenesis through an inhibitory process that involves intercellular communication (Campos-Ortega, 1988). In *Drosophila*, a group of embryonic lethal mutations known as the neurogenic mutants have been isolated for their inability to elicit this process of lateral inhibition. Neurogenic mutants all share a common phenotype, that is, a hyperplasia of neural tissue in the ventral region with no recognizable axon pattern at the expense of epidermal structures (for review, see Campo-Ortega, 1988; Campos-Ortega and Jan, 1991). This phenotype was observed in each inter-allelic analysis performed in this study, exemplified in tgo^4/tgo^3 embryos in Figure 3.1D. *tgo* resides in the cytological vicinity of 85C, mapping to a site immediately adjacent to a member of the neurogenic family of genes, *neuralized* (*neu*); *neu* has been mapped by polytene chromosome *in situ*

hybridization to 85C (Boulianne et al., 1991) and is oriented upstream of *tgo* (Sonnenfeld et al., 1997). Gene dosage studies indicate that *neu* interacts with several other neurogenic genes in the specification of neuroblast versus epidermoblast cell fate (de la Concha et al., 1988). Homozygous *neu* mutants exhibit a severely hyperplasic nervous system (Boulianne et al., 1991), comparable to the hyperplasia observed in the *tgo* inter-allelic analyses. Therefore, in observing that the neurogenic phenotype in each inter-allelic *tgo* analysis is comparable to the *neu* phenotype, perhaps *tgo* and *neu* function along similar pathways, share common targets or elicits synergistic regulation involved in CNS midline development. To test if Tgo and Neu interact, several methods could be employed; genetically, a gene dosage study could be conducted comparing *tgo/neu* heterozyotes to homozygous *tgo* and *neu* CNS phenotypes independently, and molecularly, a yeast-II hybrid analysis and/or a GST pull-down assay could show direct interactions.

The observed phenotypes in this study of *tgo* inter-allelic activity indicate that the sequential development of commissures and connectives is controlled by different and possibly overlapping gene functions. The results suggest key roles for both midline glia and midline neurons in the formation of commissures in each segment of the *Drosophila* CNS. Disruption in any one of the key roles should lead to a predictable phenotype (Klämbt et al., 1991). Most phenotypes catalogued in this study document two groups of irregularities. One, those mutations exhibiting a reduction in the number of commissural tracts and/or longitudinal connective axons, such as fused and stalled and secondly, those showing a elimination of complete commissural development, such as collapsed and neurogenic. In reference to previous studies conducted, investigating the correlation of axon pattern phenotypes and the different midline cell phenotypes, it is possible that during CNS midline development, *tgo* may also affect embryonic cellular differentiation. The embryonic phenotypes of tgo^1 and tgo^2 have been previously described for the CNS midline and trachea and are associated with defects in the differentiation of cells in both tissues (Sonnenfeld et al., 1997). The premature translational stop in tgo^2 is located 60 amino acids towards the amino terminus, deleting a proline-rich region, a poly [glutamine] stretch and the *prd repeat*. It is therefore likely that the protein product

produced by *tgo*^{2} lacks considerably more amino acids than any protein product produced by *tgo*^{3}. With deletion of the C-terminal domains, including the glutamine-rich transactivation region, the protein product of *tgo*^{2} may function as a dominant-negative capable of heterodimerization and DNA-binding, but unable to initiate transcriptional activity to promote the proper given developmentally regulated event. The *tgo*^{3} allele was isolated by its lack of genetic complementation with *tgo*^{2} after EMS mutagenesis and given the molecular nature of these alleles, it is tangible to assume that their lack of complementation is due to defects attributable to the 3' region of the gene. Defects in the formation of CNS axons and in the differentiation of midline neurons were detected using various neuronal markers (Sonnenfeld, personal communication), suggesting that the *tgo*^{3} mutation affects zygotic processes similar to those affected by *tgo*^{1} and *tgo*^{2} alleles. In light of the pleiotropic nature of phenotypes not only within but among the *tgo* inter-allelic analyses conducted perhaps in addition to or mutually exclusive of overlapping gene functions, *tgo* may interact with a ubiquitous factor or factors required in embryonic cell-type specification. Investigative techniques could begin by performing a yeast-two hybrid screen to isolate potential partners of *tgo* in specifically regulating midline cell differentiation and subsequent commissural formation during CNS development.

In summation, the *tgo* alleles analyzed seem to affect most aspects of the zygotic phenotype to the same extent. As implied by the similar embryonic phenotypes, the inter-allelic analysis reveals a lack of genetic complementation among EMS-induced *tgo* alleles. This discerns a requirement of each *tgo* allele in promoting embryonic survival. The difficulty in genetically distinguishing between the different aspects of the embryonic CNS axonal phenotypes of *tgo* trans-heterozygous embryos suggests that the same pathway is used or a convergence of pathways are used by *tgo* in mediating diverse zygotic biological pathways during axon scaffold formation.

4.2 Implications of Tango cytoplasmic and nuclear localization prior to three hours after egg laying

Staining of *Drosophila* embryos with an antibody generated against Tgo indicates that Tgo is present in all embryonic cells prior to three hours after egg laying. Tgo seems to

be predominantly localized in the cytoplasm of most cells, however there appears to be strong accumulation in the nuclei of others. The working model with regards to Tgo expression patterns in the developing embryo is that Tgo is localized to the cytoplasm in cells lacking other bHLH-PAS dimerization partners and with their appearance, dimerization will occur and the complex will translocate to and accumulate in the nucleus (Ward et al., 1998). Ultimately, it is believed that nuclear Tgo correlates with the presence of bHLH-PAS::Tgo heterodimers (Ward et al., 1998). Combined data from Sonnenfeld et al., (1997) and Ward et al., (1998) respectively showing that *Drosophila* Tgo can dimerize with Sim, Trh and Similar (Sima) and that the nuclear localization of Tgo is dependent on the presence of dimerization with another bHLH-PAS protein provide two important correlative criteria with respect to sites of nuclear Tgo localization (Ward et al., 1998). One, these *Drosophila* results demonstrate that the nuclear localization of Tgo correlates with the presence of dimerization-competent bHLH-PAS proteins such as Sim and Trh. Second, sites of nuclear Tgo localization correlate with cells in which Tgo is functional. Furthermore, these results have implications for the function of the mammalian orthologue of Tgo, Arnt, such that sites of Arnt nuclear localization in the mammalian embryo most likely correlate with cell types in which bHLH-PAS::Arnt heterodimeric complexes regulate transcription (Ward et al., 1998). Supportive in vivo analysis of bHLH-PAS::Arnt interactions is yet required, particularly between mammalian Sim proteins (Ema et al., 1997) and Arnt (Ward et al., 1998). However, an important difference between Arnt and Tgo lies in the presence of a nuclear localization sequence in Arnt. Unlike Tgo, Arnt can localize in the nuclei of cultured cells in the absence of any known bHLH-PAS protein (Eguchi et al., 1997). Conceivably then, Arnt-related physiological processes in mammals may be more complicated than in *Drosophila*.

To date, *Drosophila* studies of Tgo localization suggest that Tgo does not form transcriptionally active homodimers during embryogenesis: Tgo immunostaining studies show that Tgo concentrates within the nuclei of cells in which it is part of a heterodimeric complex (Ward et al., 1998) and Tgo dimerizes with Sim and Trh to control transcription within the CNS midline and tracheal system, respectively (Sonnenfeld et al., 1997).

Mammalian Arnt has been shown to form functional homodimers both *in vitro* and in cell culture (Sogawa et al., 1995; Sonnenfeld et al., 1997), however biochemical studies have not been performed to determine whether Tgo can form functional homodimeric complexes. Nonetheless, the data put forth by Ward et al., (1998) imply that the DNA binding ability and transcriptional regulatory activity of Tgo during embryogenesis are not the result of Tgo homodimerization. Yet, this does not exclude the possibility of Tgo homodimer formation in the cytoplasm, leading to other novel Tgo regulatory or functional roles distinct from its role as a nuclear transcriptional regulator.

Prior to three hours after egg laying in *Drosophila*, most likely within the developmental time period in which a maternal contribution would be required, Tgo was found in both the cytoplasm of some cells and in the nuclei of others. Embryos derived from mothers bearing germline clones of tgo^3 showed disrupted embryonic segmentation and overall anterior-posterior polarity. This may demonstrate a unique role of Tgo during embryogenesis correlative to its cytoplasmic localization during possible maternal Tgo requirement, however it should be considered that a cytoplasmic role of Tgo might not be functional on its own. A hypothesis of Ward et al., (1998) is that the function of cytoplasmic Tgo may be dependent upon its dimerization with other bHLH-PAS proteins. One implication of this hypothesis may be a concurrent nuclear role of Tgo, a notion that is agreeable with the punctate anti-Tgo immunostaining results presented in this study (Figure 3.2.2). An interesting future analysis could include the staining of *Drosophila* embryos derived from mosaic *tgo* females prior to three hours after egg laying (AEL) to observe any aberrations of Tgo localization compared to the wild-type expression pattern. If both cytoplasmic and nuclear staining is altered, or reduced as would be expected, then a dual cytoplasmic and nuclear role of Tgo may be required during the maternal regulation of pre-blastoderm ectodermal patterning. Altered or reduced Tgo distribution would also be expected if the localization of Tgo prior to three hours AEL is dependent on the function or interactions of its C-terminal domains, such as the transactivation and/or *prd repeat* domains. For example, this effect would then be expected in embryos ubiquitously expressing $tgo^{\Delta C}$ stained for Tgo distribution prior to three hours AEL, as it is lacking both the transactivation and *prd repeat* domains. Tgo distribution in embryos

ubiquitously expressing $tgo^{\Delta b}$ would not be expected to change from wild-type distribution, consistent with the ectopic expression studies and segmentation protein distribution analyses revealing no role for the DNA-binding ability of Tgo during embryonic patterning (Results 3.3).

An additional implication of the hypothesis suggested by Ward et al., (1998) might be the presence of cytoplasmic bHLH-PAS proteins dictating an early role for *tgo* during embryonic patterning. Speculation as to what patterning pathways may require cytoplasmic *tgo* for their function can be based on the tgo^3 mutant phenotype and expression studies performed, but the ubiquitous distribution of Tgo in blastoderm embryos must also be considered. Based on the tgo^3 germline clonal phenotype, perhaps the best candidate pathways requiring cytoplasmic *tgo* activity are *bicoid* and *hunchback* since both are needed for proper development of anterior body parts and Kr striping. However, since closer examination shows uniformly high levels of Tgo in the cytoplasm and lower levels in the nuclei of the majority of cells, it is possible that the segmentation function of *tgo* relies on the specificity dictated by other interacting proteins that control segmental information. Regardless of cytoplasmic or nuclear localization of Tgo, isolation of potential bHLH-PAS partners, or non bHLH-PAS proteins such as Bcd or Odd, in the embryo prior to three hours AEL may include performing a yeast-II hybrid interaction assay. This could be carried out using the coding sequence of Tgo containing the bHLH and PAS domains fused to a reporter gene such as LexA as a bait protein. Verification of cytoplasmic and nuclear Tgo localization may include double staining for Tgo with SH2 or PTB (Settle et al., 2003) and propidium iodide, respectively.

4.3 *Transcriptional regulatory genes contain similar paired repeats*

Two studies conducted within this body of work provide evidence suggesting a significant *in vivo* function for the Tgo *prd repeat*. First, germline clones mutant for the histidine-proline-rich region and 3' sequences were analyzed for cuticular patterning defects. This clonal analysis revealed a surprising role for the Tgo *prd repeat* in the establishment of anterior-posterior axial polarity. Second, ubiquitous and targeted misexpression of a C-terminal Tgo deletion, prior to three hours after egg laying (AEL)

resulted in the disruption of pattern formation. This truncated form of Tgo was generated by the deletion of the transactivation domains as well as the *prd repeat* and 3' sequences, thereby disclaiming the loss of *prd repeat* function solely responsible for the associated patterning defects. Interestingly, ectopic expression of an N-terminal deletion of Tgo missing the basic DNA-binding domain prior to three hours AEL displayed no effect on embryonic patterning. Taken together, these data suggest that the function(s) associated with Tgo C-terminal sequences are independent of the DNA-binding ability of Tgo.

To gather further insight into potential *prd repeat* function, a protein database query was conducted using BLASTP. This enabled identification of other molecules, of either vertebrate or invertebrate conservation, containing *prd repeat* sequences. The *prd repeat* differs from the more familiar *paired box* and it is exclusive to transcriptional regulatory genes (Figure 4.1). The *prd repeat* of Tgo shares the highest identity at the amino acid level with the *prd repeat* regions of mammalian Sox1 (48.8%), *Drosophila* Odd-skipped (Odd) (48.1%) and Bicoid (Bcd) (44.4%). The *prd repeat* of Tgo is also homologous to *Drosophila* Paired (Prd) (40.7%), mammalian Brain-2 (33.3%) and a smaller percentage identity with *Drosophila* Ecdysone Eip 74E (22.2%). Given the role of Bcd in establishing anterior-posterior polarity and segmentation, in addition to its contribution to anterior end development, the homology shared between the *prd repeats* of Tgo and Bcd are of particular interest since the areas affected by maternal tgo^3 mutations are similar to those affected in *bcd* mutants.

bcd regulates the activities of anterior gap genes and embryos derived from females containing amorphic *bcd* alleles lack all head structures and form an ectopic telson at the anterior end. Hypomorphic *bcd* alleles, however, develop reduced head structures and defects in the thoracic region (Frohnhofer et al., 1986; Nüsslein-Volhard et al., 1987), a phenotype comparable to that observed in embryos from females mutant for tgo^3. In light of these results, the lack of duplicated telson in tgo^3 germline clones may be due to tgo^3 being a weak allele, incapable of alone providing instructive signaling during development. Is it therefore possible that Tgo and Bcd function along a shared developmental pathway in regulating early embryonic patterning? Perhaps of immediate

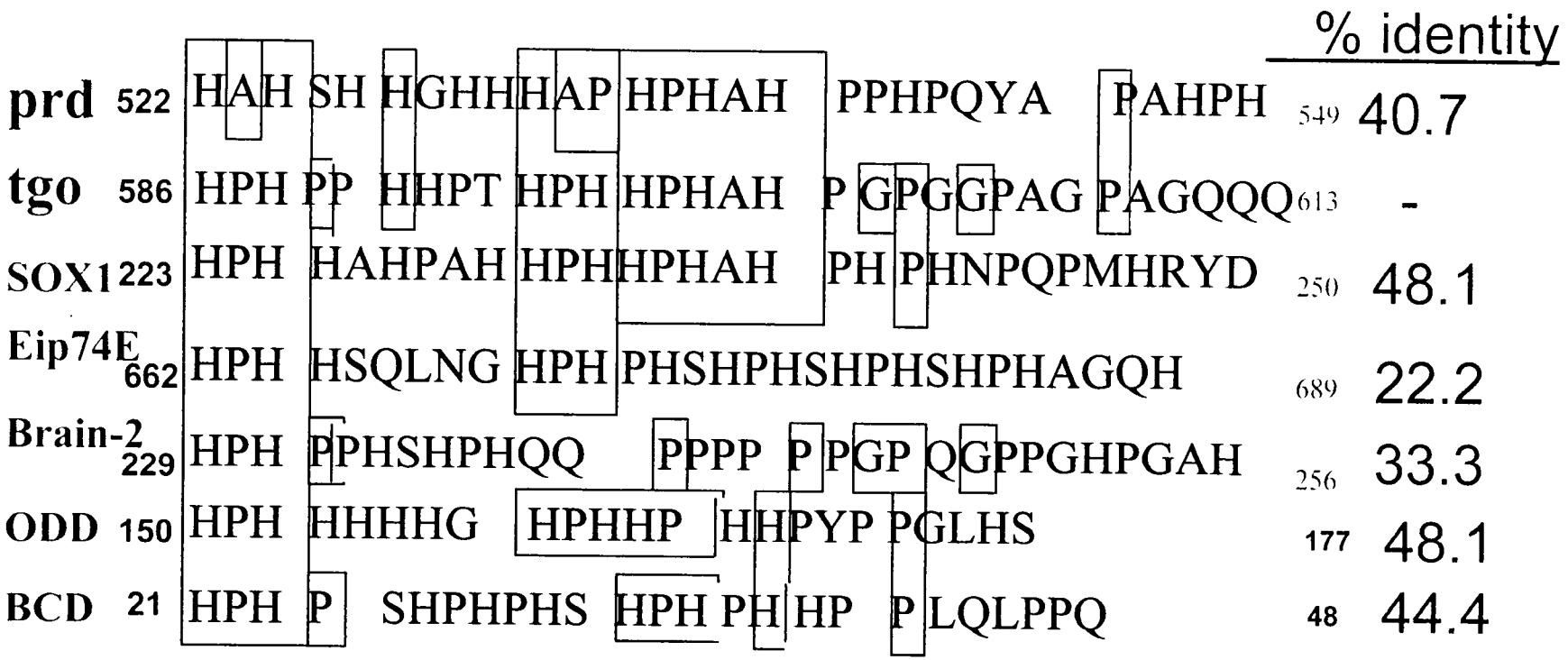


Figure 4.1 ***paired repeat*** **sequence identity.** A current protein query was conducted using the BLASTP program and the *paired* (*prd*) *repeat* of Tgo (Altshul et al., 1997). Six proteins involved in transcriptional activation show identity with the Tgo *prd repeat*. These proteins include *Drosophila* Paired (40.7%), mouse Sox 1 (48.1%), Eip 74E (22.2%), rat Class III Pou domain Brain-2 (33.3%), Odd-Skipped (48.1%) and Bicoid (44.4%). The association between the *prd repeat* and transcriptional regulatory proteins may be relevant given the importance of this domain to early developmental processes in both invertebrates and vertebrates.

interest is questioning if this comparable effect on anterior and thoracic development is conducted in partnership as, for example, through a direct interaction. Alternatively, the regulatory effects of Tgo and Bcd on these two anterior-posterior regions may be mutually exclusive. *bcd* mRNA encodes a 489-amino acid homeodomain-containing protein. Unlike Tgo, the histidine-proline-rich *prd repeat* of Bcd is found in the amino-terminal end of the protein (Frigerio et al., 1986; see Driever, 1993). In the anterior of *Drosophila* embryos prior to three hours AEL, Bcd is both cytoplasmic and nuclear (Paryre et al., 1994). At this time in development, Tgo is also cytoplasmic and nuclear. Additionally, prior to three hours AEL, the *Drosophila* embryo is a polynucleated blastoderm. These criteria contend with the possibility that Tgo and Bcd would be capable of interacting. It is however unlikely that possible interaction includes regulation of the anterior-posterior Bcd gradient by Tgo since Tgo itself is not distributed throughout the embryo along a concentration gradient. Therefore, it is reasonable to assume that the expression of Tgo and its role in establishing anterior-posterior polarity and anterior-thoracic development is subsequent to the establishment of the Bcd morphogenetic gradient along the developing anterior-posterior axis.

The discrete function of the *prd repeat* and its mechanistic pathway remain elusive. However, various reports provide several intriguing points that are noteworthy. Transient transfection assays of a truncated Prd protein product, including deletion of its 21-amino acid *prd repeat* resulted in a loss of target gene activating ability, raising the possibility that the *prd repeat* may be functioning as a transcriptional activation domain (Han et al., 1989). Ectopic expression assays using *prd repeat* deletion mutants provide partial support of this claim in their report that the *prd repeat* contributes to the overall activity of the proline-rich transcriptional activation domain in the C-terminal of the *Drosophila* Prd protein (Cai et al., 1994). As described in Chapter 1 (Introduction), 74 amino acids from this region, in addition to the *prd repeat* alone, activated a heterologous (Sp1) DNA-binding domain in tissue culture. However, the sequences surrounding the Tgo *prd repeat* are dissimilar to the proline-rich transactivation domain of Prd. It is therefore possible that the *prd repeat* of Tgo does not function in a similar context with respect to the overall gene function (Cai et al., 1994; Sonnenfeld et al., 1997). Nonetheless, it

would be interesting to see if the activity of the histidine-proline-rich domain of Tgo contributes to its specificity of function and if so, what this means in the context of *tgo* function during early embryonic patterning. Furthermore, the required activation of the proline-rich domain has been shown to support combinatorial regulation of refined expression patterns of segmentation gene products by the Prd protein (Cai et al., 1994). Also, combinatorial interactions between Pou domain and Sox proteins with bHLH-PAS proteins further highlight the association between *prd repeats* and transciptional regulatory proteins (Boube et al., 2000; Zelzer and Shilo, 2000). Moreover, yeast II hybrid analysis has shown that the *prd repeat* of the *Drosophila* NK-1 homeobox specifically interacts with the *prd repeat* of Prd (Kim et al., 1995), therefore implying that the *prd repeat* can function as a protein interaction domain. These findings, as well as those reported in this study reflecting the potential requirement of *prd repeat* function during embryonic segmentation, provide supporting evidence for an early role for *Drosophila tgo* independent from its later role in bHLH-PAS interactions. The associations between the *prd repeat* of Tgo and those of the proteins isolated by the database search are relevant when considering that each protein isolated by the query are required for a given respective early developmental function.

4.4 *tango* mutations are associated with segmentation defects

Prior to fertilization, maternal gene expression establishes morphogenetic differences in the egg along the anterior-posterior axis. These differences dictate the future head and posterior ends through the establishment of three global parts of the *Drosophila* larval body: the anterior region comprised of the head and thoracic segments, the posterior region consisting of the abdominal segments and the terminal region consisting of two morphologically nonsegmented ends. Following fertilization these genes, termed maternal coordinates, hierarchically regulate the expression of a small number of zygotic genes known as the segmentation genes, responsible for patterning the embryo grouped into three classes including gap, pair-rule and segment polarity genes (St. Johnston and Nüsslein-Volhard, 1992). In light of germline clonal analysis of tgo^3 mutations and targeted early expression of a tgo C-terminal deletion (UAS-$tgo^{\Delta C}$), the classification of *tgo* within this hierarchy became of interest. In removing both maternal and zygotic Tgo

function, phenotypes of greater severity than those of zygotic *tgo*^{3} mutants alone were observed in *tgo*^{3} germline clones (Sonnenfeld and Scanga, submitted). The absence of maternal *tgo* results in embryonic lethality, therefore the level of heterozygous Tgo protein provided by the zygotic component alone could not support completely proper development, as it is unable to rescue the *tgo*^{3} maternal effect. Furthermore, *tgo*^{3} germline clone mutations do not affect oocyte development (Sonnenfeld, personal communication). Combined, these observations classify *tgo* as a maternal gene (St. Johnston and Nüsslein-Volhard, 1992). Embryos from mosaic *tgo*^{3} females or expressing *tgo*$^{\Delta C}$ prior to three hours after egg laying (AEL) show a deletion of thoracic, abdominal and/or terminal body segments, comparable to both *bicoid* (*bcd*) and gap gene phenotypes. Moreover, the germline clonal and ectopic expression analyses were consistent with early segmentation protein distribution patterns. These studies have provided observation of phenotypic variability enabling classification and speculation of the mechanism of *tgo* function during early embryonic patterning. Aberrant *even-skipped* expression within embryos mutant for maternal *tgo*^{3} and disruption in *engrailed* expression in embryos ectopically expressing UAS-tgo$^{\Delta C}$ were expected when considering the concept of hierarchical regulation within the classification of segmental specification. Therefore, discussion and explanation of a proposed model will deal with both maternal coordinates and gap segmentation genes whose proteins initiate the hierarchy of embryonic patterning.

The roles of maternal genes can be deduced from the consequences of maternal-effect mutations on the larva. The loss of maternal *tgo*^{3} activity results in two discernable cuticular phenotypes, severe and intermediate. The existence of two distinct phenotypes associated with reduced maternal *tgo* activity could be a consequence of different biochemical mechanisms. Additionally, a reason for observing a *tgo*^{3} germline clone intermediate phenotype may be attributable to the level of heterozygous Tgo protein in the absence of maternal Tgo leading to a minimal effort of rescuing the patterning phenotype. Severe germline clones exhibit deletion of all head and most anterior end cuticular structures, in addition to a loss of posterior end structural elements, whereas the intermediate germline clones show defects in each of these body regions but to a much

lesser degree (see figure 3.2.1). The hole existing in the anterior end of severe cuticular phenotypes may be due to failed head involution. Phenotypes of severe and intermediate germline clones are both comparable to phenotypes observed *bcd* and *Krüppel* mutants. Perhaps then, the developmental pathway in which *tgo* functions may be a part of or interact with the pathway(s) in which maternal coordinate and gap segmentation genes function, namely those regulating anterior and/or terminal regional development. This sort of relationship between *tgo* and maternal coordinate or gap segmentation genes may occur through a shared interacting factor or synergistically. One candidate gene for this shared pathway could be *bcd*. Embryos of weaker *bcd* alleles exhibit a continuous decrease of wild-type *bcd* activity, only showing pattern defects primarily in the anterior region (Wieschaus and Nüsslein-Volhard, 1980; Nüsslein-Volhard et al., 1987), much like the intermediate cuticular phenotype of tgo^3 germline clone embryos.

However, significantly different from the intermediate phenotype is the severe tgo^3 germline clone phenotype, which is comparable to amorphic *bcd* cuticular phenotypes. Embryos of strong *bcd* alleles approximate the complete loss of wild-type function, completely lacking the head and thorax (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1987). Moreover, severe tgo^3 germline clones show a lack in posterior end structural development; a phenotype also observed in mutants of terminal class genes, such as *torso* for example (see Sprenger and Jackle, 1993). The lack of both anterior and posterior end cuticular structures is most likely indicative of improper development of axial polarity and that factors required for their normal development are missing. Also, the tgo^3 germline clone severe cuticular phenotype is comparable to that of embryos homozygous for gap genes, such as *Krüppel* (*Kr*) and *hunchback* (*hb*), lacking both thorax and anterior abdomen. Gap genes consist of the first group of zygotically active genes that are necessary for development of segmental organization in the embryo. Gap genes are regulated by maternal coordinates such as *bcd* (see Pankratz and Jackle, 1993). The classification of gap segmentation genes was originally based upon the cuticle phenotypes of mutations in the genes *hunchback* (*hb*), *Kr* and *knirps* (*kni*) in which contiguous segments were missing or defective (Nüsslein-Volhard and Wieschaus, 1980). Therefore, the failure to regulate Kr striping in maternal tgo^3 embryos

may be the result of reduced gap gene function. Collectively, the above suggests that *tgo* may function as or along a similar pathway as a maternal coordinate gene or gap segmentation gene. For example, *tgo* may operate along a common pathway shared by *bcd* or gap genes like *Kr* or *hb* in specifying axial polarity and overall embryonic patterning. This assumption is based on the belief that the classification of genes required during early embryonic developmental processes is contingent upon which body parts of the embryo they affect (see St. Johnston and Nüsslein-Volhard, 1992). How this interaction occurs remains unknown, however considering that both Tgo and Bcd contain a *paired* (*prd*) *repeat* domain for instance (see figure 4.1), could proper anterior development, overall embryonic patterning and axial establishment could occur through a prd::prd interaction? Additional studies would need to be conducted (such as a yeast-II hybrid for example) in order to not rule this possibility out. Another possible interaction between Tgo and Bcd may be indirect and dependent upon formation of a shared cofactor in the thorax, abdomen and terminal embryonic domains (see below).

The examination of Kr protein distribution in tgo^3 germline clones revealed a wider domain of expression compared to control embryos (see figure 3.3.1C). Furthermore, both ubiquitous and targeted misexpression of UAS-$tgo^{\Delta C}$ also revealed a wider domain of Kr expression, but of the sample analyzed, almost half of the mutant population in each analysis exhibited a reduction in Kr expression pattern (see figure 3.3.1D). In all likelihood, the concerted activity in generating the Kr expression domain by *bcd* and *hb* (Hulskamp et al., 1990) persists. The proper formation of both the anterior and posterior borders of the *Kr* expression domain does indeed fail, however it is not completely removed. Moreover, expansion or reduction of the anterior border is not independent from an expression or reduction of the posterior one. In other words, the anterior and posterior Kr borders of the central domain of expression either expand beyond the wild-type expression domain "together", or they reduce "together". From what could be observed the anterior border did not shift anteriorly while the posterior one stayed in place. This is an important distinction to note as both borders, although set by different neighboring genetic interactions, respond to varying levels of concentration gradients and therefore molecularly different mechanisms. The establishment of the anterior Kr border

is believed to occur through the repressive activity of Hb, as the central domain of Kr expression expands anteriorly in embryos lacking zygotic *hb* (maternal *hb* activity alone can activate the expression of *Kr*) (Gaul and Jackle, 1987). Alternatively, the posterior border of *Kr* expression can in principle, in part, form passively taking into account the eventual decrease in Bcd and Hb concentration threshold levels required to activate *Kr* in the more posterior regions of the embryo (Gaul and Jackle, 1991). Therefore, perhaps there is a function of Tgo required outside of the Kr expression domain, in non-Kr expressing cells, that is regulating the wild-type Kr expression pattern. If either one of the anterior or posterior Kr borders were displaced independent of the other, consideration of Tgo regulatory effects on *Kr* expression would include genetic interactions at the displaced Kr border establishing their placement. The activities of *hb* and other gap genes such as *knirps* and *giant* (Gaul and Jackle, 1989) should be considered in view of the function of Tgo in establishing the borders of Kr expression in the central domain. Supplementary studies could be conducted whereby the expression pattern of each of these genes is analyzed in tgo^{3glc} embryos and embryos ectopically expressing P[UAS-$tgo^{\Delta C}$] compared to respective controls. However, such considerations should not be independent of previously established morphogenetic gradients and the positional information they carry in eliciting concentration-dependent interactions. For example, immunological studies could be directed to analyze Bcd distribution in both tgo^{3glc} and embryos ectopically expressing P[UAS-$tgo^{\Delta C}$] compared to controls.

Throughout embryonic development, gene expression patterns determine cell fates. The first step of this process for the antero-posterior axis of the *Drosophila* embryo depends on a spatial gradient of the maternal morphogen Bcd. Positional information encoded within this gradient becomes transmitted to downstream gap genes (Driever and Nüsslein-Volhard, 1988; Driever and Nüsslein-Volhard, 1988; Struhl et al., 1988), such as *Kr* for example. Germline clonal analysis revealed an unanticipated role for *tgo* in segmentation. As noted earlier, in addition to resembling weak *bcd* phenotypes, the phenotypes associated with loss of maternal *tgo* function are comparable to those caused by mutations in the gap genes that cause the deletion of broad contiguous segmental units of the developing embryo prior to cellularization (2-2.5 hours AEL). Based on this

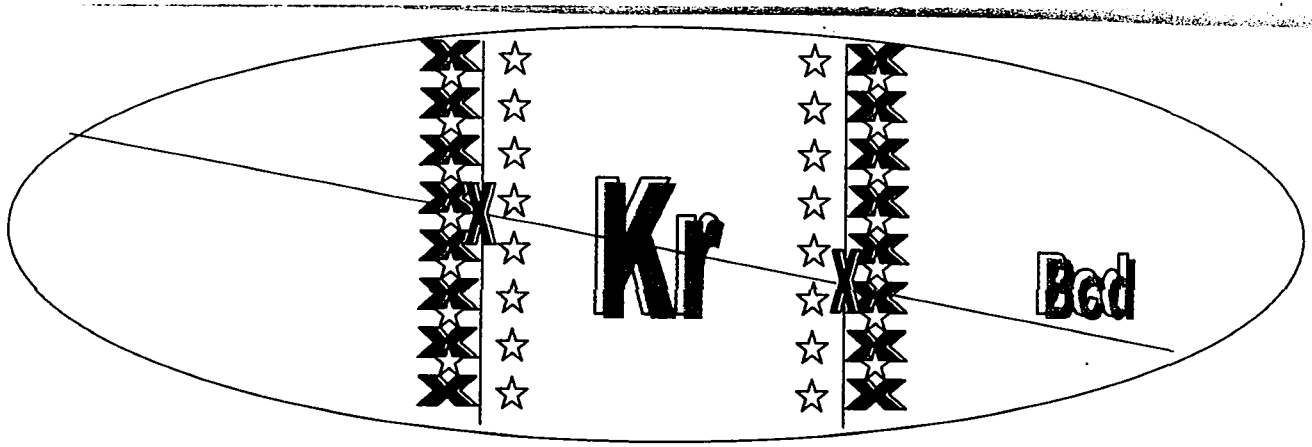
cuticular phenotype and considering subsequent antibody analyses investigating Kr expression patterns, *tgo*, particularly the *prd repeat* region, thus appears to be necessary for the expression or perhaps function of one or more maternal coordinate genes and/or gap genes. The question then raised is, if Tgo is indeed required in segmental specification during early embryonic patterning, along what mechanistic pathway does it elicit such regulatory effects? Unlike Kr, Eve, En and most other segmentation proteins, Tgo is uniformly expressed in the early embryo. This suggests that perhaps Tgo functions as an essential cofactor or modifier of Kr or other segmentation proteins (Daubresse et al., 1999). Or perhaps Tgo, within the appropriate context (Cai et al., 1994) could act by recruiting a specific set of cofactors (Pinsonneault et al., 1997). The primary structure of Tgo shows glutamine-rich regions (Sonnenfeld et al., 1997). The C-terminal deletion of Tgo used in this study, $Tgo^{\Delta C}$, not only lacks the *prd repeat* domain housed in the C-terminal end of the protein, but it also is missing part of the glutamine-repeat motif. Perhaps this transactivation domain, required in mediating proper CNS midline development through transcriptional activation (Sonnenfeld et al., 1997; Ward et al., 1998; Emmons et al., 1999) could be required in early embryogenesis to activate *Kr* expression. Consistent with this notion, the reduction in *Kr* expression in embryos ubiquitously expressing UAS-$tgo^{\Delta C}$ would therefore be expected. However, also seen in these embryos was an expansion of *Kr* expression. How then, could a molecularly characterized activator possibly be involved in repressing transcription? One possibility is that, since no clear repression domain is recognizable in Tgo (Sonnenfeld et al., 1997), perhaps negative targets (targets eliciting repression of transcriptional activity for example) of Tgo during segmentation can recruit cofactors that provide repressive activity (Smith and Jaynes, 1996; Manoukian and Krause, 1993; Alexandre and Vincent, 2003). In this way Tgo, perhaps through its *prd repeat* motif, may then direct its action as a repressor during early embryonic segmentation, whether it is the cofactor itself or whether it demands recruitment of other cofactors, as a member of a repressor complex unit. The following is a reflection of a hypothetical model proposed in light of the genetic and immunological studies conducted in this body of work, in addition to consideration of previous studies conducted during the time period in which segmental identity becomes established in *Drosophila*.

The question of how *Drosophila tgo* may regulate repressive activity during embryonic segmentation deals with the important issue of how regulatory proteins control cell fate in negative as well as positive modes. There are several modes of action by which a regulatory protein can mediate repression. One model proposes that a regulatory protein could directly bind target gene DNA and repress in association with cofactors. In this model, Tgo would complex with target genes and corepressors. Another model suggests that the regulatory protein interact with positively acting regulatory proteins to prevent their function, rather than directly bind target gene DNA. A final model is that a regulatory protein elicits indirect repressive activity by activating the transcription of genes encoding repressive factors. DNA binding and transcriptional activation by the regulatory protein would be required according to this model. Figure 4.2 is a schematic of a possible mechanism of the regulatory function of *tgo* during early embryonic patterning through a repressor complex conferring similarity to both the first and last models proposed above. According to this model, wild-type Tgo allows for proper Kr spatial expression as it is working in adjacent, normally non-*Kr* expressing, domains by repressing Kr expression. However, with loss of wild-type Tgo activity, for example with the expression of a C-terminal deletion of Tgo including both transactivation domains and the *prd repeat* region, the repression normally defining non-*Kr* expressing cells adjacent to the wild type Kr domain seems to become lifted, thereby allowing *Kr* expression in cells where it may normally be repressed.

Transcription of gap genes outside of their normal expression domains, such as that seen with Kr expression in ectopically expressed *tgo*, is not a surprising result when considering a report by Cadigan et al. (1994a,b). This study reports that ectopic expression of the pair-rule gene *sloppy-paired* results in phenotypes similar to hypomorphic and amorphic *even-skipped* mutants, and they also observed similar segmentation defects in embryos mutant for maternal *kismet* (a homeotic gene). But why is there also a reduction in *Kr* expression observed in embryos mutant for maternal *tgo* and embryos ubiquitously expressing $tgo^{\Delta C}$? A reduction in *Kr* expression is expected in both of these cases once considering that the loss of wild-type *tgo* function and the dominant negative form of Tgo interferes with the regulatory function Tgo may exert as

Figure 4.2

A.



non-*Kr* expressing cells

ubiquitous cofactor (Tgo?)

Bcd threshold levels defining *Kr* expression

Regulation of Kr expression by Tgo may depend on its interaction with factors of previously established morphogenetic gradients and recruited cofactors. This schematic of a wild-type blastoderm embryo (anterior is to the left) depicts the Kr expression domain, its adjacent domains free of Kr protein (red x), a ubiquitous cofactor (star) and the anterior to posterior gradient of Bcd (blue line). This model proposes the possibility that *Kr* expression may depend on Tgo functioning, as either an activator or a repressor as required, synergistically with a maternal coordinate (and/or gap) protein. In keeping with this, between these thresholds of Bcd, perhaps some other factor is competing for Kr regulation. Another possibility is that the repressive activity of *tgo* is repressed in this central domain of Kr expression (see B. below). Combined, germline clonal analysis and ectopic expression studies suggest that both the *prd repeat* and transactivation domains of Tgo may be required in the regulation of *Kr* by *tgo* during the first three hours of *Drosophila* embryogenesis. Determining whether this regulation is direct or indirect would need additional investigation.

B.

Tgo protein

interacting protein (contains prd repeat?)

cofactor (ubiquitous?)

Kr in non-*Kr* expressing cells

Tgo may be part of a repressor in regulating *Kr* expression. In the wild-type blastoderm, regardless of whether Tgo (T) is acting as a ubiquitous cofactor or whether it recruits a specific set of factors through an interaction with negative targets, it may allow the proper expression of *Kr* due to repressive activity in neighboring expression domains. However, without proper *tgo* function, the repressive activity it may take part in exerting on *Kr* expression could become lifted. Therefore, in the appropriate context (consider A. above), the result can be expression of *Kr* where it is normally not expressed (ie. where it is normally repressed).

both a repressor and activator of *Kr* gene expression. Also, as noted earlier, *Kr* expression is not completely abolished and this may be due to possible competition between $Tgo^{\Delta C}$ and endogenous Tgo proteins for the same interacting (cofactor) protein(s). Figure 4.3 proposes potential scenarios of Tgo regulation of *Kr* expression as a possible repressor and activator of the Kr gap gene expression pattern. including a test of the hypothetical model proposed via these scenarios. These scenarios suggest that Tgo functions as a member of a repressor complex in normally non-*Kr*-expressing cells. As a member of a repressor complex, *tgo* would normally act through the repressive activity of a negative target gene providing or recruiting cofactors that in turn themselves may ultimately result in repressive action on *Kr* expression. This regulation would be maintained unless in the given appropriate context (example, disrupted function of a member of the repressor complex) the repressive action of the negative target normally recruited along this pathway is repressed ("repression of a repressor"), thereby permitting normal Kr expression.

This figure also proposes an approach to test the legitimacy of the potential scenarios presented. Since the present study is the first to suggest a requirement of *tgo* function during early embryonic patterning, there is need of defining Tgo as a potential cofactor and/or isolating potential targets of Tgo in order to validate the proposed repressor complex model suggested above. It is important to note that the proposed hypothetical model is contingent upon known targets of *tgo* regulation. To isolate potential direct targets, future studies would include a yeast-II hybrid interaction assay supplemented by GST pull-down assays using the *prd repeat* exclusively. However, initial studies to determine the repressive regulatory ability of the *prd repeat* of Tgo prior to any knowledge of *tgo* targets could involve investigation of the transcriptional abilities of the prd repeat using the GAL4-UAS interaction system in combination with a heterologous promoter system in *Drosophila* SL2 culture cells. Such a study could follow on the heels of first determining the transcriptional ability of the *prd repeat* via a luciferase or CAT transcriptional assay, comparing its independent ability to promote transcriptional ability compared to the other domains encoded by Tgo (Figure 4.4). This sort of study would complement those conducted by Cai et al. (1995). To investigate the transcriptional

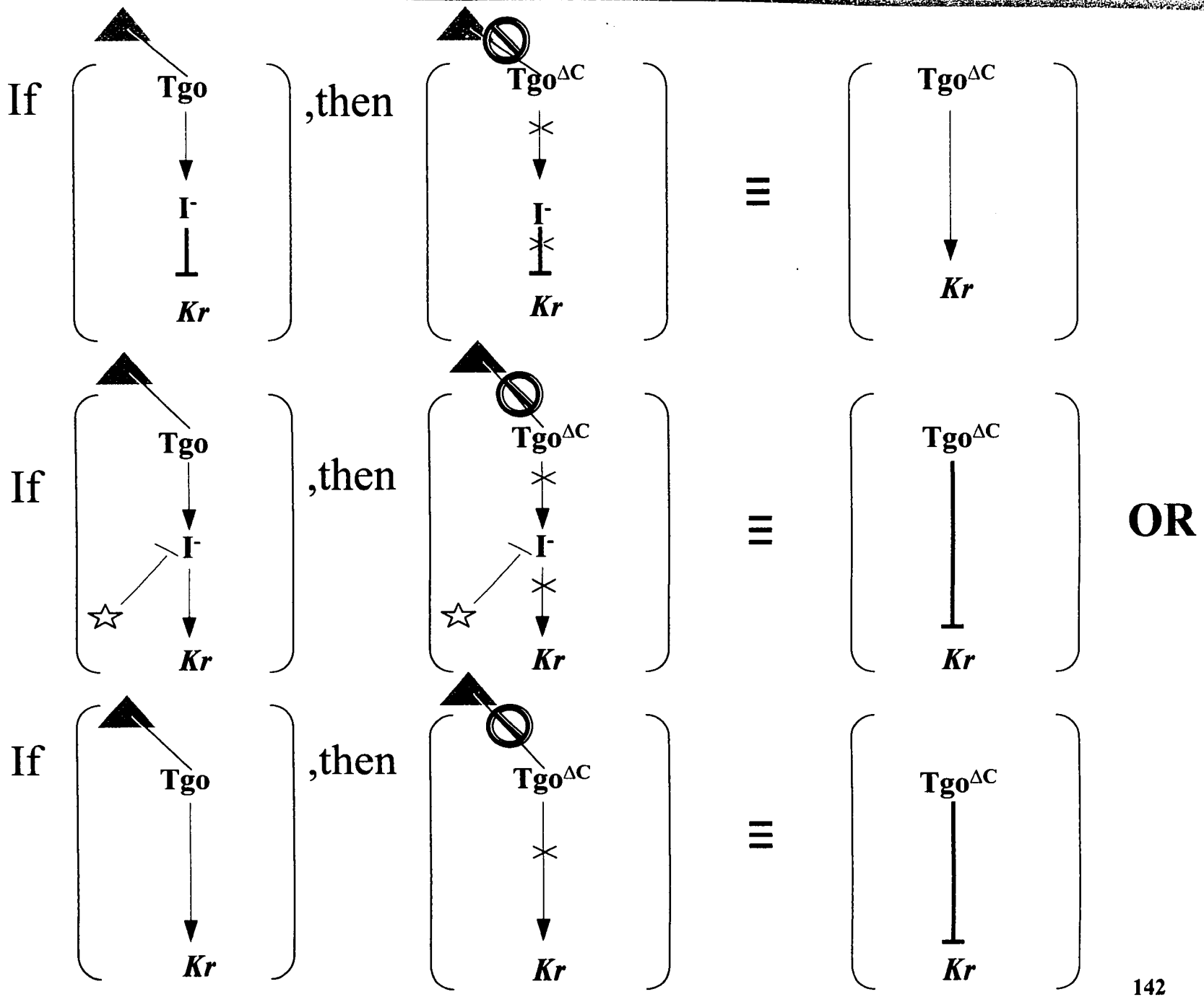
Figure 4.3 Possible effects of Tgo lacking the *prd repeat* domain. The direction of arrows does not imply a presumed epistatic relationship.

Non-Kr cells If Tgo protein interacts with a *prd repeat*-containing factor that normally recruits the activity of a negative target gene in cells external but adjacent to the central Kr domain leading to the repression of *Kr* expression in the embryonic regions through recruitment of cofactors that provide a repressive action, then removal of wild-type *tgo* function involved in this regulation should result in loss of recruitment ability and therefore a removal in the usual repression of *Kr* expression. In the ectopic expression studies where a truncated form of Tgo ($Tgo^{\Delta C}$) was used, this removal of repressive activity could also be attributed to an interference of Tgo function as a member of the repressor complex (see Figure 4.2 proposal). The net result expected would be ectopic *Kr* expression. Since these results were observed in experiments involving removal of the *prd repeat*, and in the case of ectopic analyses part of the transactivation domain, it is possible that repression of Kr expression by Tgo may rely in part on the function of these C-terminal domains of Tgo.

Kr cells If a negative target gene normally recruited by Tgo to restrict *Kr* expression is repressed by a cofactor or intermediate within a given concentration threshold of an interacting factor (example Bcd, see Figure 4.2 A. above), the usual repressive action of Tgo through its negative target becomes repressed. This thereby permits normal *Kr* expression. Therefore removal of wild-type Tgo activity, and perhaps more importantly *prd repeat* function and/or the ability to transcriptionally activate, should result in an inability of activating the components or proper function of the repressor complex, resulting in a net effect of inhibiting *Kr* expression. Of course, there is consideration that within the given concentration threshold of Bcd in the central Kr domain, Tgo could directly activate *Kr* expression while maintaining its involvement in a repressor complex in adjacent, non-Kr domains. However, keep in mind the syncytial state of the developing embryo during the first three hours AEL, upstream regulatory effects on *tgo* activity, and also the independent deleterious effects of the tgo^{3} EMS mutation and the Tgo C-terminal dominant negative on early segmental patterning. Direct activation does not seem to take into account domains outside of the normal Kr protein distribution, contrary to the repressor complex model proposed.

Test To test its repressive function, a form of Tgo that can only function as a repressor could be engineered by removing its transactivation (glutamine-rich) region and replacing it by a characterized repressor domain (thus making reTgo). Because no clear repressor domain is recognizable in Tgo (Sonnenfeld et al., 1997), the model proposed presumes that negative targets can recruit cofactors that can provide a repressive function (Mannervik et al., 1999). This test is assuming that the regulatory effect of *tgo* on embryonic patterning is irrespective of its DNA-binding ability, as suggested by ectopic and targeted misexpression studies conducted using UAS-$Tgo^{\Delta b}$. Considering the possibility that *tgo* could regulate segmental identity prior to three hours AEL through the repressive action of a negative target gene, the results of this test are expected to match those observed in the ectopic expression analysis upon staining for Kr protein distribution. An alternate test is proposed in the text.

Figure 4.3


Non-Kr cells
If
Tgo
I-
Kr
,then
Tgo^ΔC
I-
Kr
≡
Tgo^ΔC
Kr
Kr cells
If
Tgo
I-
Kr
,then
Tgo^ΔC
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Kr
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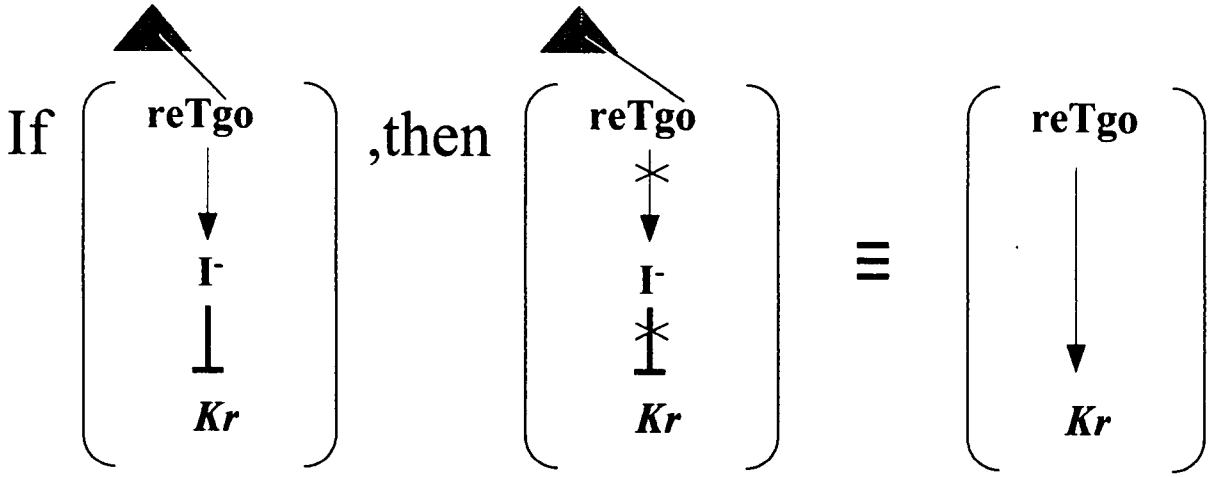
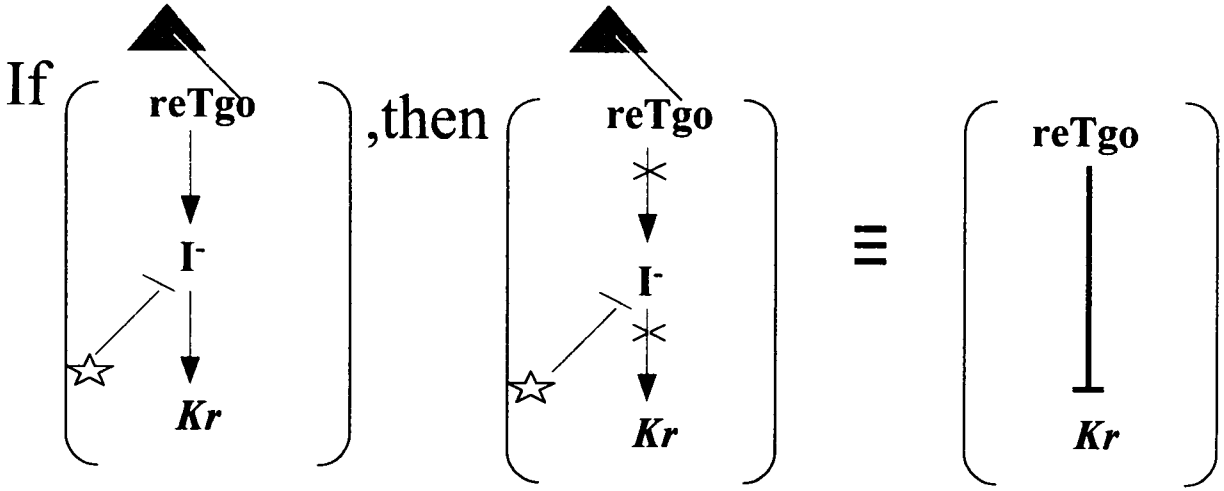
Test

Non-Kr cells



Kr cells



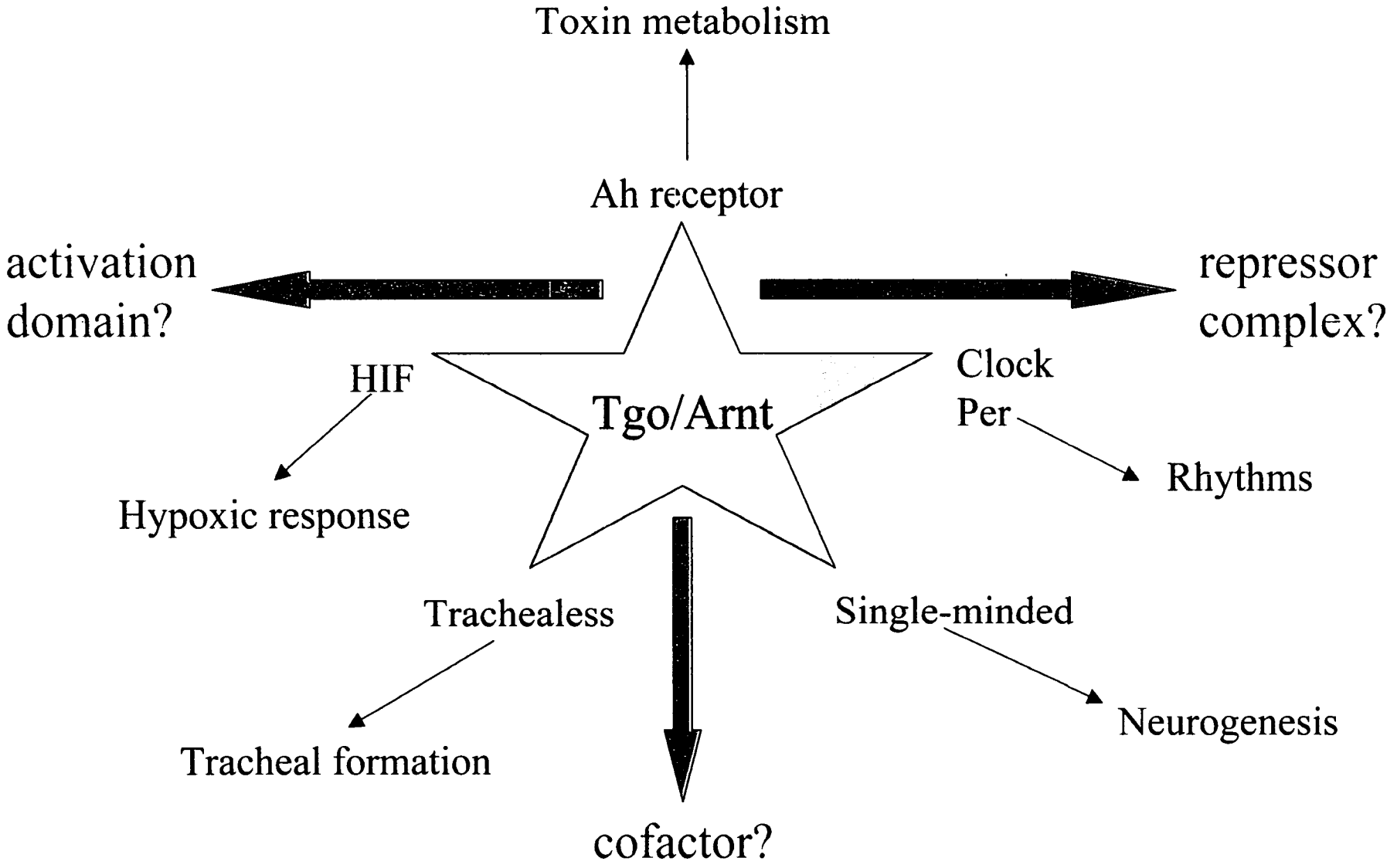
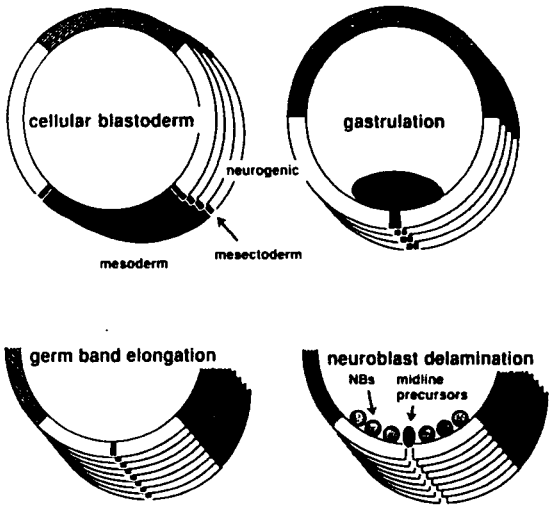


Figure 4.4 Array of Tgo/Arnt interactions. The diagram shows the proteins Tgo/Arnt dimerizes with in regulation of various developmental and physiological processes. The *in vivo* relationship between Tgo/Arnt, Clock and Per remains unknown. Shown in green are speculative means in which Tgo/Arnt may function in regulating early embryonic development. As hypothesized in this study, Tgo/Arnt may function as a cofactor and/or as a member of a repressor complex in establishing proper segmental identity. The *prd repeat* of Tgo may function, in part, as a transcriptional activation domain, as per transfection studies conducted by Cai et al. (1995). Modified after Crews (1998).

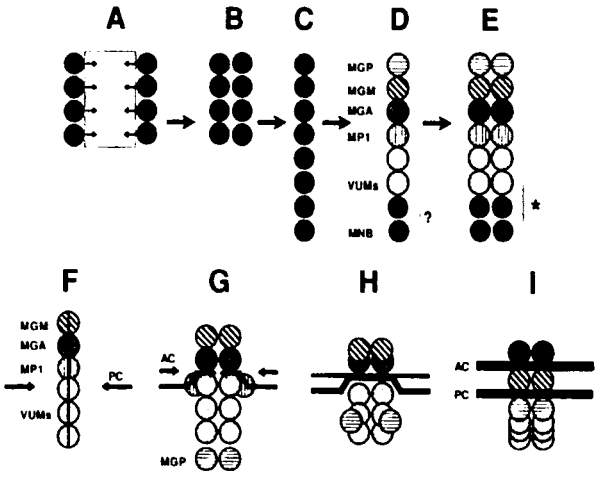
abilities of the prd repeat of Tgo, four constructs should be generated: one containing the *prd repeat* sequence downstream of Gal4 (C1), one encoding the UAS sequences (C2), a third encoding Gal4 fused to a reporter gene (such as luciferase) downstream of a known repressor (C3) and a fourth construct much like the third but containing the promoter region of a known activator (C4). C3 and C4 independently transfected with the C2 would serve as control luciferase values to co-transfection of C1 with both C2 and C3 or C4. In other words, there would essentially be two experimental groups. One would involve a measure of transcriptional activity dictated by the *prd repeat* (C1) on a characterized repressor (C3), such as the gap gene knirps (Gaul and Jackle, 1989). Increase or decrease in transcriptional activity compared to its control would suggest an activating or repressing regulation, respectively, of the *prd repeat* of Tgo on a known repressor. The same sort of experimental analysis would be conducted involving a characterized activator, such as *tgo* (Ohshiro and Saigo, 1997; Sonnenfeld et al., 1997). All transfection assays would be normalized for luciferase activity by using a βgal vector. This study would further characterize the role of the *prd repeat* of Tgo and bring understanding of its functionality during early developmental processes one step closer to reaching a consensus.

APPENDIX

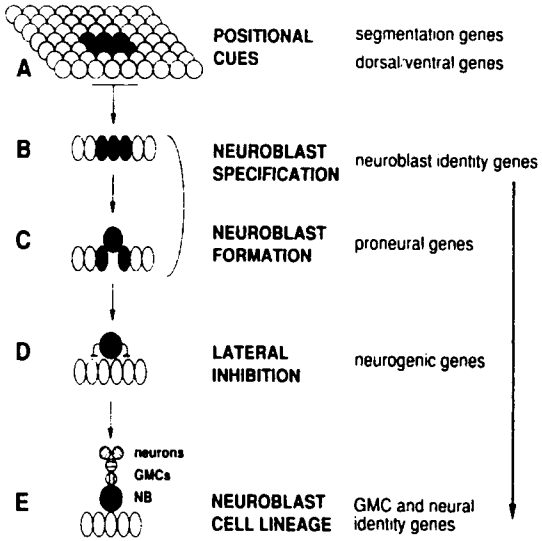
A1. An overview of embryonic *Drosophila* neurogenesis



Development of the neurogenic region and ventral midline. A series of four schematic cross sections of embryos from four successive developmental stages depicts the development of the midline. Dorsal side is up. During the cellular blastoderm stage, the midline precursorcells, or mesectodermal cells shown in black, flank the mesodermal anlage and separate the presumptive mesoderm from the presumptive neurogenic ectoderm. During gastrulation, the four midline precursors per segment become juxtaposed at the ventral midline in rows of two as mesodermal cells invaginate. As germ-band elongation proceeds, the mesoectodermal cells form a row of eight midline precursor cells per segment. Delamination as neuroblasts from the ventral ectoderm into the same layer occurs prior to division of these precursor cells into neurons and glial cells of the ventral midline. The remaining gap created by this process is later filled by the rearrangement of epidermal cells. After Klambt et al., 1991.

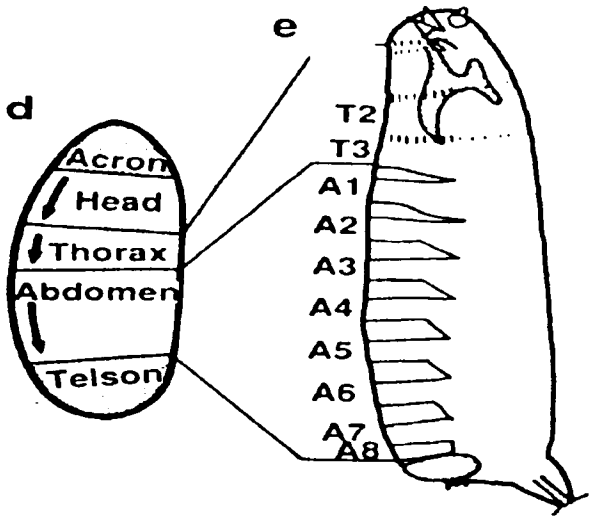


Development of the midline and axon commissures. A-E is explained in the above diagram. (F-I) Development of the two axon commissures occurs through a series of discrete steps, which involved (F) the pioneering of the posterior commissure (PC), (G) pioneering of the anterior commissure (AC), (H) migration of the midline glia and (I) the resulting separation of the anterior and posterior commissures. In H, the separation of the anterior and posterir commissures results from migration of the MGM glia. After Goodman and Doe, 1993.



A1 contd.

Formation of neuroblasts, ganglion mother cells and neurons. (A) Positional information is provided to clusters of four to six cells (dark gray) by cues (light gray) In the neurogenic ectoderm. In the anterior-posterior axis, the pair-rule and segment polarity genes may encode some of these cues; genes encoding D/V cues have yet to be identified. (B,C) Depending on their position in the neurogenic region, each cluster of four to six ectodermal cells expresses one or more proneural genes (gray) at a specific time. One cell among this cluster becomes the neuroblast. (D) The enlarging neuroblast inhibits all other cells of the cluster from developing into neuroblasts and these cells lose expression of both proneural and neurogenic genes (white). (E) The neuroblast initiates a stem cell lineage to generate a series of ganglion mother cells (GMCs). The GMCs divide to form pairs of neurons and together, the GMCs and neurons become specified. After Goodman and Doe, 1993.



A2. Schematic fate map of the *Drosophila* embryo. The schematic on the left depicts areas from which various regions of the larval body will develop. The arrows represent the polarity of the pattern. The schematic on the right is of a wild-type larva, showing distinct structural landmarks, such as the acron and telson which areshaded. T, thorax; A, abdomen. After Nusslein-Volhard et al., 1987.

A3. Hoyer's recipe. Add 30g of gum arabic (acacia powder) to 50ml of distilled water in a 400ml beaker, stir overnight until completely dissolved. Under continuous stirring, add 200g of glycerol. Centrifuge the mixture until the mountant is clear and devoid of debris (generally 3h to overnight at 12000*g*).

A4. Confidence interval. Any confidence interval has two components: an interval computed from the data and a confidence level denoting the probability that the method produces an interval covering the parameter. I used a confidence level of 95% in my ectopic expression studies. The general definition of a confidence interval for an unknown parameter (p) and the confidence level (C) is: a level C confidence interval for a parameter x is an interval computed from sample data by a method that has probability C of producing an interval containing the true value of p. The confidence interval is to estimate an unknown parameter and indicating how accurate the estimate is and how confident we are that the result is correct. Therefore, in using a 95% confidence interval, 95% of the intervals will contain the true parameter value. The interval often has the form of sample data +/- a margin of error. The equation used was:

$$\bar{x} \pm t^* \frac{s}{\sqrt{n}}$$

$\bar{x}$ = mean

s = standard error

n = degrees of freedom (n-1)

t* = upper (1-C)/2 critical value for the standard normal distribution.

V. REFERENCES

Alexandre C, Vincent JP. 2003. Requirements for transcriptional repression and activation by Engrailed in *Drosophila* embryos. Development 130:729-39.

Battyre, R., Stevens and Jacobs, J.R. 1999. Axon repulsion from the midline of the *Drosophila* CNS requires *slit* function. Development 126: 2475-2481.

Berleth T, Burri M, Thomas G, Bopp D, Richstein S, Frigerio G, Noll M, Nusslein-Volhard C. 1988. The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. EMBO 7:1749-56.

Bopp D, Burri M, Baumgartner S, Frigerio G, Noll M. 1986. Conservation of a large protein domain in the segmentation gene *paired* and in functionally related genes of Drosophila. Cell 47:1033-40.

Boube, M., Llimargas, M. and Casanova, J. 1999. Cross-regulatory interactions among tracheal genes support a co-operative model for the induction of tracheal fates in the *Drosophila* embryo. Mech. Dev. 91; 271-278.

Boulianne GL, de la Concha A, Campos-Ortega JA, Jan LY, Jan YN. 1991. The *Drosophila neurogenic* gene *neuralized* encodes a novel protein and is expressed in precursors of larval and adult neurons. EMBO J 10:2975-83

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

Brand, A.H., Manoukian, A.S., and Perrimon, N. 1994. Ectopic expression in *Drosophila*. In "Method in Cell Biology: (Academic Press) v. 44 pp.635-654.

Bronner G, Jackle H. 1996. Regulation and function of the terminal gap gene *huckebein* in the *Drosophila* blastoderm. Int J Dev Biol 40:157-65.

Burbach KM, Poland A, Bradfield CA. 1992. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Proc Natl Acad Sci U S A 89:8185-9.

Cadigan KM, Grossniklaus U, Gehring WJ. 1994. Localized expression of *sloppy paired* protein maintains the polarity of *Drosophila* parasegments. Genes Dev 8:899-913.

Cai, J., Lan, Y., Appel, L.F. and Weir, M. 1994. Dissection of the Drosophila Paired protein: Functional requirements for conserved motifs. Mech. Dev. 47: 139-150.

Campos-Ortega, A.J. and Hartenstein, V. 1985. The embryonic development of Drosophila melanogaster. New York: Springer-Verlag.

Campos-Ortega JA. 1988. Cellular interactions during early neurogenesis of *Drosophila melanogaster*. Trends Neurosci 11:400-5.

Campos-Ortega JA, Jan YN. 1991. Genetic and molecular bases of neurogenesis in *Drosophila melanogaster*. Annu Rev Neurosci 14:399-420.

Carroll SB, Scott MP. 1986. Zygotically active genes that affect the spatial expression of the *fushi tarazu* segmentation gene during early *Drosophila* embryogenesis. Cell 45:113-26.

Casanova J, Furriols M, McCormick CA, Struhl G. 1995. Similarities between trunk and spatzle, putative extracellular ligands specifying body pattern in *Drosophila*. Genes Dev 9:2539-44.

Chou, T.B. and Perrimon, N. 1992. Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. Genetics 131:643-653.

Chou, T.B., Noll, E. and Perrimon, N. 1993. Autosomal P[*ovo*D1] dominant female-sterile insertions in *Drosophila* and their use in generating germ-line chimeras. Development 119: 1359-1369.

Clark IE, Dobi KC, Duchow HK, Vlasak AN, Gavis ER. 2002. A common translational control mechanism functions in axial patterning and neuroendocrine signaling in *Drosophila*.

Coumailleau, P., Poellinger, L., Gustafsson, J.A. and Whitelaw, M.L. 1995. Definition of a minimal domain of the dioxin receptor that is associated with hsp90 and maintains wild-type ligand binding affinity and specificity. J. Biol. Chem 270: 25291-25300.

Crews, S.T. 1998. Control of cell lineage-specific development and transcription by bHLH-PAS proteins. Genes Dev. 12: 607-620.

Crews, S.T., Thomas, J.B. and Goodman, C.S. 1998. The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product. Cell 52: 143-151.

Dalby B, Pereira AJ, Goldstein LS. 1995. An inverse PCR screen for the detection of P element insertions in cloned genomic intervals in *Drosophila melanogaster*. Genetics 139:757-66.

Daubresse G, Deuring R, Moore L, Papoulas O, Zakrajsek I, Waldrip WR, Scott MP, Kennison JA, Tamkun JW. 1999. The *Drosophila kismet* gene is related to chromatin-remodeling factors and is required for both segmentation and segment identity. Development 1999126:1175-87.

Deng WM, Bownes M. 1998. Patterning and morphogenesis of the follicle cell epithelium during *Drosophila* oogenesis. Int J Dev Biol 42:541-52.

Deng WM, Ruohola-Baker H. 2000. Laminin A is required for follicle cell-oocyte signaling that leads to establishment of the anterior-posterior axis in *Drosophila*. Curr Biol 10:683-6.

Desplan C, Theis J, O'Farrell PH. 1985. The *Drosophila* developmental gene, *engrailed*, encodes a sequence-specific DNA binding activity. Nature 318:630-5.

Dolwick, K.M., Swanson, H.I. and Bradfield, C.A. 1993. *In vitro* analysis of Ah receptor domains involved in ligand-activated DNA recognition. Proc Natl Acad Sci USA 90: 8566-8570.

Driever, W. 1993. Maternal control of anterior development in the *Drosophila* embryo. In the Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1-70.

Driever W, Nusslein-Volhard C. 1989. The *bicoid* protein is a positive regulator of *hunchback* transcription in the early *Drosophila* embryo. Nature 337:138-43.

Driever W, Thoma G, Nusslein-Volhard C. 1989. Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the *bicoid* morphogen. Nature 340:363-7.

Eguchi H, Ikuta T, Tachibana T, Yoneda Y, Kawajiri K. 1997. A nuclear localization signal of human aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor 1beta is a novel bipartite type recognized by the two components of nuclear pore-targeting complex. J Biol Chem 272:17640-7.

Ema M, Sogawa K, Watanabe N, Chujoh Y, Matsushita N, Gotoh O, Funae Y, Fujii-Kuriyama Y. 1992. cDNA cloning and structure of mouse putative Ah receptor. Biochem Biophys Res Commun 184:246-53.

Emmons, R, B., Duncan, D., Estes, P.A., Kiefel, P., Mosher, J.T., Sonnenfeld, M., Ward, M.P., Duncan, I. And Crews, S.T. 1999. The Spineless-Aristapedia and Tango bHLH-PAS proteins interact to control antennal and tarsal development in *Drosophila*. Development 126: 3937-3945.

Fjose A, McGinnis WJ, Gehring WJ. 1985. Isolation of a homoeo box-containing gene from the *engrailed* region of *Drosophila* and the spatial distribution of its transcripts. Nature 313:284-9.

Franks, R.G and Crews, S.T. 1994. Transcriptional activation domains of the Single-minded bHLH protein are required for CNS midline development. Mech. Dev. 45:269-277.

Frigerio, G., Burri, M., Bopp, D., Baumgartner, S. and Noll, M. 1986. Structure of the segmentation gene *paired* and the *Drosophila* PRD gene set as part of a gene network. Cell 47: 735-746.

Frohnhofer HG, Lehmann R, Nusslein-Volhard C. 1986. Manipulating the anteroposterior pattern of the *Drosophila* embryo. J Embryol Exp Morphol 1986 Oct;97 Suppl:169-79.

Gaul, U. and Jackle, H. 1987. Pole region-dependent repression of the *Drosophila* gap gene Kruppel by maternal gene products. Cell 51: 549-555.

Gaul U, Jackle H. 1990. Role of gap genes in early *Drosophila* development. Adv Genet 27:239-75.

Gaul U, Weigel D. 1990. Regulation of Kruppel expression in the anlage of the Malpighian tubules in the *Drosophila* embryo. Mech Dev 33:57-67.

Gavis ER, Lehmann R.. 1992. Localization of *nanos* RNA controls embryonic polarity. Cell 71:301-13.

Gilbert. 1991. The genetics of pattern formation in *Drosophila*. Developmental Biology. 3rd ed. Sinauer Associates, Inc. 650-669.

Golic KG, Lindquist S. 1989. The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. Cell 59:499-509.

Golic, K.G. 1991. Site-specific recombination between homologous chromosomes in *Drosophila*. Science 252: 958-961.

Gonzalez-Reyes A, Morata G. 1990. The developmental effect of overexpressing a Ubx product in *Drosophila* embryos is dependent on its interactions with other homeotic products. Cell 61:515-22.

Goto T, Macdonald P, Maniatis T. 1989. Early and late periodic patterns of *even-skipped* expression are controlled by distinct regulatory elements that respond to different spatial cues. Cell 57:413-22.

Grossniklaus U, Pearson RK, Gehring WJ. 1992. The *Drosophila sloppy paired* locus encodes two proteins involved in segmentation that show homology to mammalian transcription factors. Genes Dev 6:1030-51.

Grueneberg DA, Natesan S, Alexandre C, Gilman MZ. 1992. Human and *Drosophila* homeodomain proteins that enhance the DNA-binding activity of serum response factor. Science 257:1089-95.

Hahn, M.E. 1998. The aryl hydrocarbon receptor: A comparative perspective. Comp. Biochem. and Physiol. Part C 121: 23-53.

Han K, Levine MS, Manley JL. 1989. Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. Cell 56:573-83.

Han K, Manley JL. 1993. Transcriptional repression by the *Drosophila even-skipped* protein: definition of a minimal repression domain. Genes Dev 7:491-503.

Hankinson O. 1995. The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol 35:307-40.

Heemskerk J, DiNardo S, Kostriken R, O'Farrell PH. 1991. Multiple modes of engrailed regulation in the progression towards cell fate determination. Nature 352:404-10.

Hoffman EC, Reyes H, Chu FF, Sander F, Conley LH, Brooks BA, Hankinson O. 1991. Cloning of a factor required for activity of the Ah (dioxin) receptor. Science 252:954-8.

Houchmandzadeh B, Wieschaus E, Leibler S. 2002. Establishment of developmental precision and proportions in the early *Drosophila* embryo. Nature 415:798-802.

Howard K, Ingham P. 1986. Regulatory interactions between the segmentation genes *fushi tarazu*, *hairy*, and *engrailed* in the *Drosophila* blastoderm. Cell 44:949-57.

Hulskamp M, Schroder C, Pfeifle C, Jackle H, Tautz D.. 1989. Posterior segmentation of the Drosophila embryo in the absence of a maternal posterior organizer gene. Nature 338:629-32.

Hulskamp M, Pfeifle C, Tautz D. 1990. A morphogenetic gradient of *hunchback* protein organizes the expression of the gap genes Kruppel and knirps in the early *Drosophila* embryo. Nature 346:577-80.

Hummel T, Schimmelpfeng K, Klambt C. 1999. Commissure formation in the embryonic CNS of *Drosophila*. Dev Biol 209:381-98.

Ingham PW, Baker NE, Martinez-Arias A. 1985. Regulation of segment polarity genes in the *Drosophila* blastoderm by *fushi tarazu* and *even-skipped*. Nature 331:73-5.

Ingham PW. 1991. Segment polarity genes and cell patterning within the *Drosophila* body segment. Curr Opin Genet Dev 1:261-7.

Ingham PW, Martinez Arias A. 1992. Boundaries and fields in early embryos. Cell 68:221-35.

Jacobs JR, Goodman CS. 1989. Embryonic development of axon pathways in the Drosophila CNS. I. A glial scaffold appears before the first growth cones. J Neurosci 9:2402-11.

Jain., S., Dolwick, K.M., Schmidt, J.V. and Bradfield, C.A. 1994. Potent transactivation domains of the Ah receptor and the Ah receptor nuclear translocator map to their carboxyl termini. J. Biol. Chem. 269: 31518-31524.

Janody F, Reischl J, Dostatni N. 2000. Persistence of Hunchback in the terminal region of the *Drosophila* blastoderm embryo impairs anterior development. Development 127:1573-82.

Jaynes JB, O'Farrell PH. 1988. Activation and repression of transcription by homoeodomain-containing proteins that bind a common site. Nature 336:744-9.

Jaynes JB, O'Farrell PH. 1991. Active repression of transcription by the *engrailed* homeodomain protein. EMBO J 10:1427-33.

Jiang J, Hoey T, Levine M. 1991. Autoregulation of a segmentation gene in *Drosophila*: combinatorial interaction of the even-skipped homeo box protein with a distal enhancer element. Genes Dev 5:265-77.

John A, Smith ST, Jaynes JB. 1995. Inserting the Ftz homeodomain into engrailed creates a dominant transcriptional repressor that specifically turns off Ftz target genes *in vivo*. Development 121:1801-13.

Jurgens, G., Wieschaus, E., Nusslein-Volhard, C. and Kluding, H. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila* melanogaster II. Zygotic loci on the third chromosome. *Wilhelm Roux 325s Arch.Dev.* Biol 193, 283-295.

Kidd, T., Bland, K. and Goodman, C.S. 1999. Slit is the midline repellant for the Robo receptor in *Drosophila*. Cell 96: 785-794.

Klambt, C., Jacobs, J.R. and Goodman, C.S. 1991. The midline of the Drosophila central nervous system: A model for the genetic analysis of cell fate, cell migration and growth cone guidance. Cell 64: 801-815.

Klambt C. 1993. The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. Development 117:163-76.

Knipple DC, Seifert E, Rosenberg UB, Preiss A, Jackle H. 1985. Spatial and temporal patterns of Kruppel gene expression in early *Drosophila* embryos. Nature 317:40-4.

Kornberg T, Siden I, O'Farrell P, Simon M. 1985. The *engrailed* locus of *Drosophila*: in situ localization of transcripts reveals compartment-specific expression. Cell 40:45-53.

Lamb P, McKnight SL. 1991. Diversity and specificity in transcriptional regulation: the benefits of heterotypic dimerization. Trends Biochem Sci 1991 16:417-22.

Larkin P, Baehr W, Semple-Rowland SL. 1999. Circadian regulation of iodopsin and clock is altered in the retinal degeneration chicken retina. Brain Res Mol Brain Res 70:253-63.

Lehmann R, Nusslein-Volhard C. 1987. *hunchback*, a gene required for segmentation of an anterior and posterior region of the *Drosophila* embryo. Dev Biol 119:402-17.

Li P, He X, Gerrero MR, Mok M, Aggarwal A, Rosenfeld MG. 1993. Spacing and orientation of bipartite DNA-binding motifs as potential functional determinants for POU domain factors. Genes Dev 7:2483-96.

Li, H., Dong, L. and Whitlock, J.P. 1994. Transcriptional activation function of the mouse Ah receptor nuclear translocator. J. Biol. Chem. 269: 28098-28105.

Lohs-Schardin, M., Cremer, C. and Nusslein-Volhard, C. 1979. A fate map for the larval epidermis of *Drosophila melanogaster*: localized cuticle defects following irradiation of the blastoderm with an ultraviolet laser microbeam. Dev. Biol. 73: 239-55.

Ma Q, Whitlock JP Jr. 1997. A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Biol Chem 272:8878-84.

Ma Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ. 1998. *neurogenin1* is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 20:469-82.

Macdonald PM, Struhl G. 1986. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. Nature 324:537-45.

Mannervik M, Nibu Y, Zhang H, Levine M. 1999. Transcriptional coregulators in development. Science 284:606-9.

Maschat F, Serrano N, Randsholt NB, Geraud G. 1998. engrailed and polyhomeotic interactions are required to maintain the A/P boundary of the *Drosophila* developing wing. Development 125:2771-80

Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC. 1997. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature 386:403-7.

Manoukian AS, Krause HM. 1992. Concentration-dependent activities of the *even-skipped* protein in *Drosophila* embryos. Genes Dev 6:1740-51.

Manoukian AS, Krause HM. 1993. Control of segmental asymmetry in *Drosophila* embryos. Development 118:785-96.

Manseau LJ, Schupbach T. 1989. The egg came first, of course! Anterior-posterior pattern formation in *Drosophila* embryogenesis and oogenesis. Trends Genet 5:400-5.

Martin JR, Raibaud A, Ollo R. 1994. Terminal pattern elements in *Drosophila* embryo induced by the *torso-like* protein. Nature 367:741-5.

Martizez Arias A, Baker NE, Ingham PW. 1988. Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. Development 103:157-70.

Mayer, U. and Nusslein-Volhard, C. 1988. A group of genes required for pattern formation in the ventral ectoderm of the *Drosophila* embryo. Genes Dev 2: 1496-1511.

McGinnis W, Levine MS, Hafen E, Kuroiwa A, Gehring WJ. 1984. A conserved DNA sequence in homoeotic genes of the *Drosophila* Antennapedia and bithorax complexes. Nature 308:428-33.

Mitchell PJ, Tjian R. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245:371-8.

Mitchell KJ, Doyle JL, Serafini T, Kennedy TE, Tessier-Lavigne M, Goodman CS, Dickson BJ. 1996. Genetic analysis of Netrin genes in *Drosophila*: Netrins guide CNS commissural axons and peripheral motor axons. Neuron 17:203-15.

Mlodzik M, Gibson G, Gehring WJ. 1990. Effects of ectopic expression of caudal during *Drosophila* development. Development 109:271-7.

Moffett P, Reece M, Pelletier J. 1997. The murine Sim-2 gene product inhibits transcription by active repression and functional interference. Mol Cell Biol 17:4933-47.

Moore, D.S. and McCabe, G.P. 1997. Introduction to the practice of statistics. 2nd ed. W.H.Freeman and Company, New York. 429-441; 46-51.

Murata Y, Wharton RP. 1995. Binding of pumilio to maternal *hunchback* mRNA is required for posterior patterning in Drosophila embryos. Cell 80:747-56.

Murre C, Bain G, van Dijk MA, Engel I, Furnari BA, Massari ME, Matthews JR, Quong MW, Rivera RR, Stuiver MH. 1994. Structure and function of helix-loop-helix proteins. Biochim Biophys Acta 1218:129-35.

Nambu, J.R., Franks, R .G., Hu, S. and Crews, S.T. 1990. The *single-minded* gene of *Drosophila* is required for the expression of genes important for the development of CNS midline cells. Cell 63: 63-75.

Nambu, J.R., O. Lewis, J., Wharton, K.A. Jr. and Crews, S.T. 1991. The *Drosophila single-minded* gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. Cell 67: 1-20.

Nambu JR, Lewis JO, Crews ST. 1993. The development and function of the Drosophila CNS midline cells. Comp Biochem Physiol Comp Physiol 104:399-409.

Nambu JR, Chen W, Hu S, Crews ST. 1996. The *Drosophila melanogaster* similar bHLH-PAS gene encodes a protein related to human *hypoxia-inducible factor 1* alpha and *Drosophila single-minded.* Gene 172:249-54.

Nambu PA, Nambu JR. 1996. The *Drosophila fish-hook* gene encodes a HMG domain protein essential for segmentation and CNS development. Development 122:3467-75.

Niessing D, Blanke S, Jackle H. 2002. Bicoid associates with the 5'-cap-bound complex of *caudal* mRNA and represses translation. Genes Dev 16:2576-82.

Nusslein-Volhard, C. and Wieschaus, E. 1980. Mutations affecting segment number and polarity in Drosophila. Nature 287: 795-801.

Nusslein-Volhard, C., Wieschaus, E. and Kluding, H. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila* melanogaster I. Zygotic loci on the second chromosome. *WilhelmRoux 325sArch.Dev.* Biol 193, 267-282.

Nusslein-Volhard C, Frohnhofer HG, Lehmann R.. 1987. Determination of anteroposterior polarity in *Drosophila.* Science 238:1675-81.

Ohshiro, T. and Saigo, K. 1997. Transcriptional regulation of breathless FGF receptor gene by binding of TRACHEALESS/dARNT heterodimers to three central midline elements in *Drosophila* developing trachea. Development 124: 3975-3986.

Oliver B, Perrimon N, Mahowald AP. 1987. The *ovo* locus is required for sex-specific germ line maintenance in *Drosophila.* Genes Dev 1:913-23.

Pankratz MJ, Jackle H. 1990. Making stripes in the *Drosophila* embryo. Trends Genet 6:287-92.

Pankratz, M.J. and Jackle. H. 1993. Blastoderm Segmentation. In the Development of *Drosophila melanogaster.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1-70.

Parkhurst SM, Ish-Horowicz D. 1991. Mis-regulating segmentation gene expression in Drosophila. Development 111:1121-35.

Patel NH, Lall S. 2002. Precision patterning. Nature 415:748-9.

Payre F, Crozatier M, Vincent A. 1994. Direct control of transcription of the *Drosophila* morphogen *bicoid* by the *serendipity delta* zinc finger protein, as revealed by *in vivo* analysis of a finger swap. Genes Dev 8:2718-28.

Perrimon N, Mohler D, Engstrom L, Mahowald AP. 1986. X-linked female-sterile loci in *Drosophila melanogaster*. 1986. X-linked female-sterile loci in *Drosophila* melanogaster. Genetics 113:695-712.

Perrimon N, Engstrom L, Mahowald AP. 1989. Zygotic lethals with specific maternal effect phenotypes in *Drosophila melanogaster*. I. Loci on the X chromosome. Genetics 121:333-52.

Perrimon N, Lanjuin A, Arnold C, Noll E. 1996. Zygotic lethal mutations with maternal effect phenotypes in *Drosophila melanogaster*. II. Loci on the second and third chromosomes identified by P-element-induced mutations. Genetics 144:1681-92.

Perrimon, N. 1998. Creating mosaics in Drosophila. Int. J. Dev. Biol. 42:243-247.

Petersen NS, Mitchell HK. 1987. The induction of a multiple wing hair phenocopy by heat shock in mutant heterozygotes. Dev Biol 121:335-41.

Petersen RB, Lindquist S. 1989. Regulation of HSP70 synthesis by messenger RNA degradation. Cell Regul 1:135-49.

Pielage J, Steffes G, Lau DC, Parente BA, Crews ST, Strauss R, Klambt C. 2002. Novel behavioral and developmental defects associated with *Drosophila single-minded.* Dev Biol 249:283-99.

Pinsonneault J, Florence B, Vaessin H, McGinnis W. 1997. A model for *extradenticle* function as a switch that changes HOX proteins from repressors to activators. EMBO J 16:2032-42.

Pollenz RS, Sattler CA, Poland A. 1994. The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. Mol Pharmacol 45:428-38.

Probst MR, Fan CM, Tessier-Lavigne M, Hankinson O. 1997. Two murine homologs of the *Drosophila single-minded* protein that interact with the mouse aryl hydrocarbon receptor nuclear translocator protein. J Biol Chem 272:4451-7.

Rajagopalan S, Nicolas E, Vivancos V, Berger J, Dickson BJ. 2000. Crossing the midline: roles and regulation of Robo receptors. Neuron 2000 28:767-77.

Randazzo FM, Seeger MA, Huss CA, Sweeney MA, Cecil JK, Kaufman TC. 1993. Structural changes in the *antennapedia* complex of *Drosophila* pseudoobscura. Genetics 134:319-30.

Ronchi E, Treisman J, Dostatni N, Struhl G, Desplan C. 1993. Down-regulation of the Drosophila morphogen bicoid by the torso receptor-mediated signal transduction cascade. Cell 74:347-55.

Rongo C, Lehmann R. 1996. Regulated synthesis, transport and assembly of the *Drosophila* germ plasm. Trends Genet 12:102-9.

Rubin GM, Spradling AC. 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science 218:348-53.

Sauer F, Jackle H. 1993. Dimerization and the control of transcription by Kruppel. Nature 364:454-7.

Schaeffer V, Killian D, Desplan C, Wimmer EA. 2000. High *bicoid* levels render the terminal system dispensable for *Drosophila* head development. Development 127:3993-9.

Schulz C, Tautz D. 1995. Zygotic *caudal* regulation by *hunchback* and its role in abdominal segment formation of the *Drosophila* embryo. Development 121:1023-8.

Schupbach T, Wieschaus E. 1986. Germline autonomy of maternal-effect mutations altering the embryonic body pattern of *Drosophila*. Dev Biol 113:443-8.

Schwartz C, Locke J, Nishida C, Kornberg TB. 1995. Analysis of *cubitus interruptus* regulation in *Drosophila* embryos and imaginal disks. Development 121:1625-35.

Scott MP, O'Farrell PH. 1986. Spatial programming of gene expression in early Drosophila embryogenesis. Annu Rev Cell Biol 2:49-80.

Settle M, Gordon MD, Nadella M, Dankort D, Muller W, Jacobs JR. 2003. Genetic identification of effectors downstream of Neu (ErbB-2) autophosphorylation sites in a Drosophila model. Oncogene 22:1916-26.

Seeger M, Tear G, Ferres-Marco D, Goodman CS. 1993. Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. Neuron 10:409-26.

Serrano N, Brock HW, Maschat F. 1997. beta3-tubulin is directly repressed by the engrailed protein in *Drosophila*. Development:2527-36.

Serrano N, Maschat F. 1998. Molecular mechanism of polyhomeotic activation by Engrailed. EMBO J 17:3704-13.

Simpson JH, Kidd T, Bland KS, Goodman CS. 2000. Short-range and long-range guidance by *slit* and its Robo receptors. Robo and Robo2 play distinct roles in midline guidance. Neuron 28:753-66.

Simpson-Brose M, Treisman J, Desplan C. 1994. Synergy between the hunchback and *bicoid* morphogens is required for anterior patterning in *Drosophila*. Cell 78:855-65.

Small S, Kraut R, Hoey T, Warrior R, Levine M. 1991. Transcriptional regulation of a pair-rule stripe in *Drosophila*. Genes Dev 5:827-39.

Smith ST, Jaynes JB. 1996. A conserved region of *engrailed*, shared among all *en-*, *gsc*, *Nk1-*, *Nk2-* and *msh*-class homeoproteins, mediates active transcriptional repression in vivo. Development 122:3141-50.

Sogawa K, Nakano R, Kobayashi A, Kikuchi Y, Ohe N, Matsushita N, Fujii-Kuriyama Y. 1995. Possible function of Ah receptor nuclear translocator (Arnt) homodimer in transcriptional regulation. Proc Natl Acad Sci U S A 92:1936-40.

Sonnenfeld MJ, Jacobs JR. 1994. Mesectodermal cell fate analysis in Drosophila midline mutants. Mech Dev 46:3-13.

Sonnenfeld, M., Ward, M., Nystrom, G., Mosher, J., Stahl, S. and Crews, S. 1997. The *Drosophila tango* gene encodes a bHLH-PAS protein that is orthologous to mammalian Arnt and controls CNS midline and tracheal development. Development 124: 4583-4594.

Soriano NS, Russell S. 1998. The *Drosophila* SOX-domain protein Dichaete is required for the development of the central nervous system midline. Development 125:3989-96.

Sprenger F. and Nusslein-Volhard. 1993. The Terminal System of Axis Determination in the Drosophila embryo. In the Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1-70.

Stanojevic D, Hoey T, Levine M. 1989. Sequence-specific DNA-binding activities of the gap proteins encoded by *hunchback* and *Kruppel* in *Drosophila*. Nature 341:331-5.

Stemerdink C, Jacobs JR. 1997. Argos and Spitz group genes function to regulate midline glial cell number in *Drosophila* embryos. Development 124:3787-96.

St. Johnston, D. and Nusslein-Volhard, C. 1992. The origin of pattern and polarity in the Drosophila embryo. Cell 68: 201-219.

St Johnston D, Driever W, Berleth T, Richstein S, Nusslein-Volhard C. 1989. Multiple steps in the localization of bicoid RNA to the anterior pole of the *Drosophila* oocyte. Development 107 Suppl:13-9.

Struhl G. 1989. Differing strategies for organizing anterior and posterior body pattern in *Drosophila* embryos. Nature 338:741-4.

Struhl G, Johnston P, Lawrence PA. 1992. Control of *Drosophila* body pattern by the hunchback morphogen gradient. Cell 69:237-249.

Struhl G, Basler K. 1993. Organizing activity of wingless protein in *Drosophila*. Cell 72:527-40.

Taniguchi E, Toyoshima-Morimoto F, Nishida E. 2002. Nuclear translocation of plk1 mediated by its bipartite nuclear localization signal. J Biol Chem 277:48884-8.

Tautz D. 1988. Regulation of the *Drosophila* segmentation gene *hunchback* by two maternal morphogenetic centres. Nature 1988 332:281-4.

Tautz D, Pfeifle C. 1989. A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma 98:81-5.

Tessier-Lavigne M, Placzek M, Lumsden AG, Dodd J, Jessell TM. 1988. Chemotropic guidance of developing axons in the mammalian central nervous system. Nature 336:775-8.

Thomas, J.B., Crews, S.T. and Goodman, C.S. 1998. Molecular genetics of the *single-minded* locus: A gene involved in the development of the *Drosophila* nervous system. Cell 52: 133-141.

Treisman J, Harris E, Wilson D, Desplan C. 1992. The homeodomain: a new face for the helix-turn-helix? Bioessays 14:145-50.

Wang, C.L., Dickinson, K. and R. Lehmann, R. 1994. The genetics of *nanos* localization in *Drosophila*. Dev. Dynam. 199: 103-115.

Ward, M.P., Mosher, J.T.. and Crews, S.T. 1998. Regulation of *Drosophila* bHLH-PAS protein cellular localization during embryogenesis. Development 125: 1599-1608.

Weir MP, Edgar BA, Kornberg T, Schubiger G. 1986. Spatial regulation of *engrailed* expression in the *Drosophila* embryo. Genes Dev 2:1194-203.

Wharton KA, Yedvobnick B, Finnerty VG, Artavanis-Tsakonas S. 1985. *opa*: a novel family of transcribed repeats shared by the Notch locus and other developmentally regulated loci in *D. melanogaster*. Cell 40:55-62.

Wharton RP, Struhl G.. 1991. RNA regulatory elements mediate control of *Drosophila* body pattern by the posterior morphogen *nanos*. Cell 67:955-67.

Whitlock JP Jr, Okino ST, Dong L, Ko HP, Clarke-Katzenberg R, Ma Q, Li H. 1996. Cytochromes P450 5: induction of cytochrome P4501A1: a model for analyzing mammalian gene transcription. FASEB J 10:809-18.

Wieschaus E, Szabad J. 1979. The development and function of the female germ line in *Drosophila melanogaster*: a cell lineage study. Dev Biol 68:29-46.

Wieschaus, E. and Nusslein-Volhard, C. and Jurgens, G. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaste r*III.Zygotic loci on the X chromosome and fourth chromosome. *WilhelmRoux 325s Arch.Dev.* Biol 193, 296-307.

Wieschaus E, Nusslein-Volhard C, Kluding H. 1984. *Kruppel*, a gene whose activity is required early in the zygotic genome for normal embryonic segmentation. Dev Biol 104:172-86.

Wimmer EA, Carleton A, Harjes P, Turner T, Desplan C. 2000. Bicoid-independent formation of thoracic segments in *Drosophila*. Science 287:2476-9.

Wolpert. 1998. Development of the *Drosophila* body plan. Principles of Development. Oxford University Press, New York. 126-161.

Wu X, Vakani R, Small S. 1998. Two distinct mechanisms for differential positioning of gene expression borders involving the *Drosophila* gap protein *giant*. Development 1998 :3765-74.

Xu, T. and Rubin, G.M. 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development 117: 1223-1237.

Yamada T, Placzek M, Tanaka H, Dodd J, Jessell TM. 1991. Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. Cell 64:635-47.

Yost HJ, Petersen RB, Lindquist S. 1990. RNA metabolism: strategies for regulation in the heat shock response. Trends Genet 6:223-7.

Zelzer, E., Wappner, P. and Shilo, B.Z. 1997. The PAS domain confers target gene specificity of *Drosophila* bHLH-PAS proteins. Genes and Dev. 11: 2079-2089.

Zelzer E, Shilo BZ. 2000. Interaction between the bHLH-PAS protein Trachealess and the POU-domain protein Drifter, specifies tracheal cell fates. Mech Dev;91:163-73.