Chromatin regulators and the determination of embryonic polarity in Drosophila

by

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ABSTRACT

The gene *nanos* (*nos*) is required for the development of abdominal structures. This requirement is indirect, by Nos protein inhibiting the translation of the maternal Hunchback (Hb^{mat}) protein, which would otherwise repress the abdominal gap genes *knirps* (*kni*) and *giant* (*gt*). We have isolate mutations in at least five complementation groups that can suppress the nos phenotype. Mutations in different complementation groups interact with each other, suggesting that these mutations define a gene family. Mutations in one complementation group are allelic to the gene *Enhancer of zeste* (*E(z)*), which is a member of the Polycomb group (Pc-G) family. We show that *E(z)* function is required to maintain the repression of *kni* and *gt* that is initiated by the Hb^{mat} protein. Thus, *E(z)* is involved in the determination of their anterior boundaries of expression. A 1.8 Kb region of the *kni* promoter is sufficient for the regulation by Hb^{mat} and E(z). We further show that other Pc-g genes are also required for the repression of *kni* and *gt*. Because Pc-G genes are thought to maintain repressed transcriptional states by regulation at the level of chromatin structure, we propose that chromatin regulation is involved in the regulation of abdominal gap genes.

We also examine whether the trithorax group (trx-G) of genes, which are thought to antagonize the function of Pc-G products, may also have a role in gap gene regulation. Surprisingly, the effect of *trx* mutations on abdominal development is synergistic, and not antagonistic to that of Pc-G mutations. These results suggest a direct or indirect inhibitory role of trx-G genes on gap gene expression. Our results show that if the effect of *trx* is indirect, it is not mediated by *Kr* function. These data suggest that trx-G products may be involved in early events that affect gap gene expression.

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Lastly, this passage is for my faithful dog Bernard

For the evening has its social claims, pursuing which I may come back at midnight, with the last tram, or losing that am driven to return on foot, my head in a whirl of ideas and wine and smoke, full of roseate views of the world and of course long past the point of normal fatigue. And then the embodiment of that other, truer, soberer life of mine, my own hearthstone, in person, as it were, may come to meet me; not wounded, not reproachful, but on the contrary giving me joyous welcome and bringing me back to my own. I mean, of course, Bashan.

Thomas Mann, in A Man and His Dog

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CHAPTER I. INTRODUCTION TO THE DEVELOPMENT OF PATTERNING IN DROSOPHILA EMBRYOGENESIS

1. Foreword

The studies presented in this thesis refer to the development of pattern in the Drosophila embryo. In particular, these studies address the genetic interactions involved in the establishment and stabilization of the embryonic anteroposterior pattern.

As will be discussed below, embryonic patterning is initiated by localized maternal information present within the egg. Upon egg activation and fertilization, a cascade of events transduces this prepattern of positional information into regional patterns of zygotic gene expression. Interactions between maternal products and zygotic genes, and among the zygotic genes themselves contribute to the refinement and stabilization of the pattern. The main focus of my work has been the interface between maternal information, specifically that relayed by the maternal Hunchback morphogenetic protein gradient, and the activation of zygotic genes involved in the determination of the abdomen, namely, *Krüppel*, *knirps* and *giant*.

This chapter provides the necessary background for these studies. First, a general description of oogenesis and embryogenesis is presented. Then, the modes in which different sets of maternal and zygotic genes achieve embryonic patterning are described. Because a major topic of these studies is the maintenance of positional information once the original patterning molecules are no longer present, a description of different ways in which gene expression patterns are stabilized is also included. Finally, I present more detailed information, from studies in both Drosophila and yeast, on the modes of gene stabilization most relevant to these studies, namely, the imprinting of gene activity at the chromatin level.

2. Drosophila development: oogenesis and embryogenesis

In order to provide a background for this introduction, I will briefly describe the processes of oogenesis and embryogenesis, as well as the major landmarks of the cuticular pattern. For more comprehensive descriptions, see Spradling (1993) on oogenesis, Foe et al. (1993) on the early mitotic divisions, Costa et al. (1993) on gastrulation, Martinez Arias (1993) on the development of segmental structures, and Martinez Arias (1993) and Jürgens and Hartenstein (1993) for descriptions of the larval cuticle.

A) Oogenesis

Each of the two Drosophila ovaries contains about 16 ovarioles. Each ovariole contains an anterior region called the germarium, followed posteriorly by a string of six to seven egg chambers at progressively more advanced stages of development. Stem cells present at the anterior tip of the germarium divide to produce both more stem cells and cells committed to gametogenesis, the cystoblasts. Cystoblasts divide four times with incomplete cytokinesis. Because of this pattern of cell division, two of the resulting 16 cells are connected to their sister cells by four passages, called the ring canals. One of these two cells is chosen to become the future oocyte. The other 15 cells become nurse cells that will provide the oocyte with maternal products which will later be used by the embryo for growth and patterning (see below).

Shortly after the fourth cystocyte division, somatic cells present in the germarium begin to encircle the 16-cell cysts. These cells, called the follicle cells, divide a number of times and rearrange themselves over the ovary, to eventually produce structural elements of the egg membranes, as well as collaborate in the establishment of maternal patterning systems of the oocyte (see below).

Within the germarium, both the oocyte and the nurse cells appear to enter meiosis. While the oocyte becomes arrested at metaphase I, by the time the cysts exit the germarium (stage 2) the nurse cells have abandoned the meiotic cycle.

Stages 2 to 10 are characterized by the polyploidization of the nurse cells, the division of follicle cells and the beginning of vitellogenesis. In addition, during stages 9 and 10, the follicle cells carry out a number of migratory movements over the oocyte.

The production of maternal products by the nurse cells, as well as the uptake of yolk precursor particles from the haemolymph by the oocyte lead to the growth of the oocyte, so that by the beginning of stage 10 the oocyte is about the same size as the nurse cell complex. At stage 11 the nurse cells dump most of their contents into the oocyte, further increasing the size of the oocyte. The nurse cells then degenerate.

During the final day of oogenesis (stage 8 to 14), the follicle cells produce the two layers of the eggshell, the inner vitelline membrane and the outer chorionic membrane. This requires the production of specialized structures, such as the micropyle, an anterior protrusion with a pore that is the site of sperm entry, and the chorionic dorsal appendages, which are utilized for gas exchange. After production of the egg shell, the follicle cells surrounding the oocyte degenerate, leaving only the imprints of their cell membranes on the chorionic membrane.

B) Embryogenesis

Fertilization occurs in the uterus. Hydration of the egg during passage through the uterus triggers the completion of meiosis and the activation of protein synthesis. After pronuclear fusion, 13 rounds of nuclear divisions occur in the absence of cellular divisions. These divisions are also characterized by the absence of G phases, resulting in a rapid cycling between S and M phases.

The nuclei carry out a slow outward migration towards the periphery of the oocyte beginning at nuclear cycle 7. During cycle 9, a small number of nuclei reach the plasma membrane at the posterior pole of the oocyte. These nuclei are surrounded by plasma membrane and by nuclear cycle 10 form the pole cells, the germ cell precursors. The rest of the nuclei reach the periphery of the oocyte at nuclear cycle 10. Nuclei continue dividing at the periphery of the embryo during the syncytial blastoderm stages (cycles 10 to 13). These stages are characterized by the onset of gap and pair rule gene transcription (see below). During cycle 14, the plasma membrane surrounding the syncytial embryo begin to grow inward around the peripheral nuclei and by the end of this cycle have completely surrounded the nuclei: this is the cellular blastoderm stage.

After cellularization, the embryo begins the process of gastrulation. Nuclear divisions become asynchronous, with different cell populations dividing at different times. Prospective mesoderm and anterior midgut (endoderm) cells present along the ventral midline of the embryo invaginate through the ventral furrow, and prospective midgut and hindgut cells invaginate at the posterior pole of the embryo.

At the same time, the germ band elongates along the dorsal side of the embryo, effectively moving the posterior pole of the embryo moves anteriorly. At this time the embryo begins to be divided into parasegments by the activity of segment polarity genes (see below). In molecular terms the parasegmental boundaries are defined as the boundary between the expression of the segment polarity genes *wingless* and *engrailed.* Although considerable cell mixing occurs during this stage, no mixing occurs across the parasegmental boundary. The metameric units defined by these boundaries, the parasegments, do not correspond to the segments observed in the adult larvae but are instead out of register with them.

Germ band elongation occurs until the posterior end of the germ band reaches 75% egg length (EL- position from the posterior pole of the egg). During this so-called extended germ band stage, a visible metameric pattern of the embryo appears in the form of periodic grooves in the germ band. These grooves fall on the parasegmental boundaries of the embryo. During this stage, three visible indentations also appear anterior to the trunk

regions. These indentations, which are out of register with the parasegmental boundaries, are of segmental character.

After this stage the posterior end of the embryo begins to move posteriorly towards its original position: this is the so-called germ band retraction stage. During this stage the definite larval segments appear. Dorsal groves arise out of register with the ventrally located parasegmental boundaries. The position of these dorsal grooves coincides with the position of the tracheal slits, which are depressions that form in the anterior third of the parasegment. As a result, *engrailed*-expressing cells, the anterior most cells of each parasegment, are now the posterior most cells of each segment.

A number of morphogenetic events occur in the germ band extended embryo, including the separation of the imaginal disk primordia (which will give rise to adult structures during pupariation), dorsal closure, and head and tail morphogenesis.
In addition, head formation occurs through the series of complex morphogenetic events called head involution. This process involves the invagination of the anteriormost cells of the embryo, which will form the internal cephalopharyngeal structures.

C) The larval cuticle

Patterning in the mature embryo is most easily observable in the embryonic cuticle, which is secreted between 12 to 16 hours of development. Most maternal and zygotic screens for mutations that affect Drosophila embryonic development have therefore been based on the cuticular pattern. Therefore, I will here provide a brief description of the most prominent aspects of the larval cuticle.

The embryo consists of four general regions, an internal head, the thorax, the abdomen and the tail.

Head involution results in the internalization of most of the former head surface into the lining of a set of folds that are connected with each other and with the foregut. Upon deposition of a specialized cuticle, this structure becomes the cephalopharyngeal skeleton. A number of head structures derived from other regions of the fate map, such as the mouth hooks and the cirri, remain on the anterior surface of the embryo.

The thorax and the abdomen consist of three and eight segments, respectively. The ventral cuticle of each segment is characterized by the presence of denticles in the anterior half of the segment and a posterior smooth surface. On the dorsal side each segment has a pattern of fine dorsal hairs which differ slightly in each segment.

The thoracic ventral denticles are short and stubby, and are arranged in parallel rows. The abdominal denticle bands, on the other hand, are larger and hook-like. The

abdominal denticles are arranged in five rows (except for the first segment), which form a trapezoidal shape that becomes progressively less distinct in more posterior segments.

One of the most prominent structures of the tail region are the anal pads, which are placodes of naked tissue surrounding the anal opening. A single tuft of denticles lies posterior of the anal pads. On the dorsal surface, two protuberances bear the posterior spiracles. These spiracles are lined internally by a mesh of hairs called Filzkörper.

In addition to these structures, a number of sensory organs are present at precise locations on the surface of the embryo. These less obvious structures can also be used as landmarks of the cuticular pattern.

3. Maternal systems of patterning: localization of maternal information in the oocyte

Screens for maternal effect mutations have identified a large number of maternal genes required for embryonic patterning. Females mutant for these genes produce embryos with patterning defects irrespective of the embryonic genotype. The phenotypes caused by these mutations fall into four mostly non-overlapping classes, and thus these maternal genes have been classified into four groups.

One single group of genes is required for the determination of the dorsoventral axis. Null mutations in these genes cause either ventralization or dorsalization of the embryo, depending on the location of the gene within this genetic pathway.

The three other sets of genes are required for the development of structures along the anteroposterior axis: the so-called anterior, posterior, and terminal groups. Null mutations in these genes abolish the development of, respectively, anterior structures such the head and thorax, posterior structures such as the abdomen, and the anterior and posterior-most structures of the embryo.

Each of these sets of genes is required for the production and localization within the egg of a maternal product encoded by one of its members. Upon egg activation, the localized factor in each case directs the regional expression of zygotic genes.

This section (3) describes for each of these genetic pathways, the events that lead to the localization of the decisive maternal factors in the embryo. The following section (4) addresses how the information inherent in the localized factor is interpreted by the zygotic genome. For the sake of completeness, both sections include a description of patterning events along the dorsoventral axis. Nevertheless, dorsoventral patterning is not the main subject of this thesis and the reader may choose to skip these sections

For simplicity, henceforth embryos from females mutant for maternal-effect genes are referred to as "mutant embryos". Nevertheless, it should be kept in mind that this description refers to the genotype of the mother that produced the eggs (i.e. the type of maternal product inherited by the embryo), and not the genotype of the embryo itself.

A) The dorsal group of genes, a maternal system involved in dorsoventral axis determination

A single genetic pathway is required for the development of the dorsoventral axis (for reviews, see Chasan and Anderson (1993), Steward and Govind (1993)). This genetic pathway includes about 20 known maternal effect genes. For the majority of these genes, recessive mutations lead to dorsalized embryos which lack all lateral and ventral pattern elements and develop into hollow tubes of dorsal epidermis.

Recessive mutations in the genes *cactus*, *gürken*, *cornichon* and *Torpedo* lead to the opposite, ventralized, phenotype. Mutant embryos produce cuticles which lack dorsal and lateral pattern elements and are encircled by ventral denticle bands.

As with the terminal group in anteroposterior patterning, and in contrast to the anterior and posterior systems, this genetic pathway is not contained within the nurse cell-oocyte syncytium. Rather, patterning information is transmitted from the oocyte into the surrounding somatic cells and back into the oocyte. Since the oocyte and the follicle cells, which secrete the egg membranes, interact tightly in the earliest events of this pathway, mutations in genes involved in these events affect patterning of both the eggshell and the embryo. This common pathway branches into two different processes, one, which will not be described here in detail, required for the development of egg shell patterning and another, required for embryonic patterning.

Defining the D-V polarity of the egg and the embryo: signalling from the oocyte to the follicle cells

The earliest known steps within this pathway occur at stage 8 of oogenesis, when the oocyte nucleus moves, perhaps at random, to the future dorsal side of the oocyte. This asymmetrically located oocyte nucleus is thought to produce a signal required for the development of dorsal fates. This signal is thought to be encoded by the product of the gene *gurken* (Neuman-Silberberg and Schüpbach 1993). During oogenesis the gurken RNA becomes localized to the dorsal corner of the oocyte. *gurken* encodes a TGFa-like protein which is likely to be the secreted ligand of the Toll receptor in the follicle cells.

Recessive mutations in the genes *fs(1)K10*, *cappuccino*, *spire* and *squid* cause the mislocalization of the *gurken* transcript and therefore lead to the dorsalization of the both the egg chamber and the developing embryo (Haenlin et al. 1987; Manseau and Schüpbach 1989; Kelley 1993; Neuman-Silberberg and Schüpbach 1993).

The gene *squid* encodes a putative RNA binding protein (Kelley 1993). The Squid protein may interact with the gurken RNA and contribute to the localization of this signal to the dorsal side of the oocyte.

fs(1)K10 is transcribed by the oocyte nucleus and encodes a nuclear protein with a helix-loop-helix DNA binding motif (Prost et al. 1988; Haenlin et al. 1985). It is not yet known whether the fs(1)K10 product interacts directly with the gurken RNA or whether it regulates other factors required for gurken RNA localization.

The genes *capuccino* and *spire* also affect the formation of the germ plasm and cause in addition cellularization defects in the embryo (Manseau and Schüpbach 1989). Thus these two genes may be required for a more general function, perhaps related to the integrity of the oocyte cytoplasm.

Recessive mutations in *gurken*, *cornichon* and *Torpedo* lead to the ventralization of both the egg shells and the embryos (Schüpbach 1987; Price et al. 1989; Schüpbach and Wieschaus 1991; Ashburner et al. 1990). No molecular information is available on the nature of the protein encoded by *cornichon*. The Torpedo protein is required in the follicle cells and is thought to transduce the Gurken signal from the oocyte to the cytoplasm of the follicle cells (Schüpbach 1987; Price et al. 1989). *Torpedo*, a homologue of the epidermal growth factor receptor gene, encodes a transmembrane protein with a tyrosine kinase cytoplasmic domain.

Activation of the Torpedo receptor triggers a signal cascade likely similar to those associated with other receptor tyrosine kinases, such as those involved in Drosophila photoreceptor differentiation and patterning by the terminal group of maternal genes (see below).

Decision making in the follicle cells: branching of the egg shell and embryonic pathways

The activation of Torpedo in dorsal regions of the egg chamber triggers two independent pathways: the promotion of dorsal fates in the dorsal follicle cells, and the localization of an embryonic ventralizing activity in ventral regions of the egg chamber.

Little is known about the events that lead to the dorsalization of the egg chamber. The spindle group of genes is likely involved in this branch of the pathway, since mutations in these genes cause ventralization of the eggshell but do not affect the embryo

(Lindsley and Zimm 1990). Other genes involved in the determination of the egg shell dorsoventral axis may include genes found to be expressed in the dorsal follicle cells, such as a subset of chorion genes and genes presumably marked by enhancer trap constructs (Parks and Spradling 1987; Fasano and Kerridge 1988; Grossniklaus et al. 1989). Differential gene expression along the dorsoventral axis in the follicle cells likely leads to differential behaviors of these cells over the oocyte (see King 1970) and, ultimately, the dorsoventral asymmetry of the egg morphology.

The activation of the Torpedo-initiated pathway in the dorsal side of the egg chamber leads to the restriction of an embryonic ventralizing activity to the ventral region of the egg chamber. Recessive mutations in genes downstream of this branch of the pathway (with the exception of *cactus*, see below), lead to the dorsalization of the embryo, but not the egg shell.

Activation of Torpedo in dorsal regions could lead to the restriction to ventral regions of an embryonic ventralizing signal. This process may involve the repression in dorsal follicle cells of one or more of the somatic-dependent genes *pipe*, *nudel* and *windbeutel*, and, consequently, the localized activation of this gene(s) in ventral follicle cells by a default pathway (Anderson and Nüsslein-Volhard 1986; Schüpbach and Wieschaus 1989; Stein and Nüsslein-Volhard 1992). No information is available on the structure of these genes. Nevertheless, since *nudel* mutant females produce fragile eggshells, the Nudel product may be a more general component required for the integrity of the vitelline membrane.

Transmission of information back into the oocyte: activation and reception of a ventralizing signal.

Four germ-line dependent genes, *gastrulation defective*, *snake*, *easter* and *spätzle*, may link this ventrally restricted activity to the activation of the Toll receptor in the embryonic membrane. The protein products of these genes are likely translated in the oocyte and secreted into the vitelline membrane. This is suggested by their germ-line requirement (Seifert et al. 1987; Konrad et al. 1988b; Stein et al. 1991), the presence of a signal sequence, but no transmembrane domain in their coding region (DeLotto and Spierer 1986); Chasan and Anderson 1989; Chasan and Anderson 1993), and the presence of rescuing activity for mutations in these genes (not tested for gastrulation defective) in the perivitelline fluid (Stein and Nüsslein-Volhard 1992).

The genes *snake*, *easter* and *gastrulation defective* have sequence homologies to trypsin type serine proteases (DeLotto and Spierer 1986; Chasan and Anderson 1989; Chasan and Anderson 1993). This suggests that these genes may be part of a zymogen

cascade in which products are sequentially activated by protease cleavage. This cascade presumably leads to the activation of the Toll ligand.

A preactivated form of Easter lacking the "pro" domain causes a ventralized embryonic phenotype and is epistatic over loss-of-function dorsalizing mutations in *gastrulation defective*, *snake* and the somatic-dependent dorsalizing genes (Chasan et al. 1992). However, this preactivated Easter mutant protein is not epistatic over *spätzle* mutations. Thus Easter and Spätzle act downstream of the other dorsalizing genes. It is possible that the easter product activates Spätzle by cleavage and Spätzle acts as a ligand for the transmembrane Toll receptor (Hashimoto et al. 1988). Alternatively, Easter and Spätzle may be required in parallel for Toll activation.

A conserved intracellular pathway leads to a gradient of nuclear dorsal protein.

Activation of the Toll receptor in ventral regions of the embryo leads to the formation of a nuclear gradient of Dorsal protein with nuclear concentrations highest in ventral regions. This is achieved via an intracellular pathway involving the products of the genes tube, pelle and cactus.

tube codes for a protein with no sequence similarities to other proteins, although it is predicted to be cytoplasmic (Letsou et al. 1991). The Pelle protein encodes a putative serine/threonine protein kinase (Shelton and Wasserman 1993). Allele-specific genetic interactions between tube and pelle suggest that they encode proteins that interact directly (Chasan and Anderson 1993).

In contrast to the majority of mutations in the dorsal group of genes, loss-of-function *cactus* mutations lead to the ventralization of embryos (Schüpbach and Wieschaus 1989; Roth et al. 1991). Thus, cactus acts as a negative regulator of the ventralizing signal encoded by Dorsal.

The Cactus protein forms a cytoplasmic complex with the Dorsal protein via its ankyrin repeats and inhibits dorsal nuclear uptake and (Roth et al. 1991; Kidd 1992; Steward and Govind 1993). Genetic and biochemical experiments suggest a model in which Pelle phosphorylates the Dorsal protein in ventral regions of the embryo (Roth et al. 1991; Steward and Govind 1993). Phosphorylation of Dorsal in turn leads to the dissociation of Dorsal from Cactus and the translocation of Dorsal into the ventral nuclei.

The intracellular pathway Toll-Cactus/Dorsal seems to be homologous to that triggered by interleukin-1 (IL-1) in lymphocytes. In activated lymphocytes, the IkB/NFkB cytoplasmic complex dissociates and NF-kB is taken up into the nucleus (for review, see Lenardo and Baltimore 1989). The intracellular domains of Toll and the IL-1 receptor have

sequence homology (Schneider et al. 1991). IkB and Cactus also share sequence homology, including an acidic amino-terminal domain and ankyrin repeats, which are usually involved in protein-protein interactions, in their carboxy-terminus (Geisler et al. 1992; Kidd 1992). Finally, Dorsal and NF-kB belong to the rel family of transcription factors (Govind et al. 1992).

The similarities between these pathways suggest that they may act by very similar mechanisms. One potential difference between these homologous pathways may be in the event that triggers the dissociation of the inhibitor/nuclear factor cytoplasmic complex. While the dissociation and nuclear translocation of the dorsal protein appears to be triggered by the phosphorylation of the dorsal protein (Roth et al. 1991; Steward and Govind 1993), in the case of IkB/NFkB, complex dissociation and nuclear transport depend on direct phosphorylation of the inhibitor IkB (Lienhard-Schmitz et al. 1991).

The localized nuclear uptake of Dorsal protein in ventral regions produces a gradient of Dorsal nuclear localization. The resulting gradient of *dorsal* activity leads to the repression of zygotic genes that promote dorsal fates in ventral regions and the activation of zygotic genes that promote ventral fates in ventral regions (see below).

B) Maternal systems involved in anteroposterior patterning: the anterior group of genes

Localization and activation of the maternal anterior morphogen.

The anterior group of genes is required for the specification of the head and thoracic structures. In addition, the anterior genes are also required in conjunction with the terminal group of genes for the development of an acron, instead of a telson, in the anterior-most region of the embryo (for review, see Driever (1993)) .

The anterior group of genes consists of four members. Three of these, *exuperantia* (*exu*), *swalow* (*swa*) and *staufen* (*stau*), are required for the anterior localization in the oocyte of the mRNA of the fourth gene, *bicoid* (*bcd*) (Frohnhöfer and Nüsslein-Volhard 1987; Berleth et al. 1988).

The localization of bcd RNA occurs in several phases (for review, see St. Johnston et al. (1989)). bcd RNA can be observed localized as a ring at the anterior of the oocyte very early in oogenesis, at stages 5-6. The gene *exu* is required to maintain this localization of the bcd RNA at the stages 9-10a. During stages 10b-12, when the nurse cells pump large amounts of cytoplasm into the oocyte, the gene *swa* is required for the localization of the bcd RNA to the anterior cortex. After stage 12, bcd RNA is redistributed into a tight

cap at anterior and slightly dorsal positions. After fertilization, *stau* is required to maintain this final bcd RNA localization.

Localization of bcd RNA may involve interactions of the Exu, Swa and Stau products with the cytoskeleton. Studies using cytoskeletal inhibitor drugs showed that microtubules, but not microfilaments, are required for the establishment and maintenance of bcd RNA localization during oogenesis (Pokrywka and Stephenson 1991). These authors have suggested that *exu* is required for the association of bcd RNA to microtubules, while *swa* may be required more generally for a more stable microtubule network. The idea that *swa* may have a more general role in cytoskeletal organization is further supported by the fact that *swa* mutant embryos exhibit, in addition to head phenotypes, defects in cellularization and nuclear migration (Zalokar et al. 1975).

At least some of the components of this system may bind the bcd RNA directly. In particular, the predicted Stau protein contains several copies of a double stranded RNA binding motif (St. Johnston et al. 1991. Exu and Swa proteins have no significant homology to known proteins, except for a weak similarity in Swa to an RNA-binding motif (Chao et al. 1991; Macdonald et al. 1991; Marcey et al. 1991). The sequences in the bcd RNA required for its localization are present at the 3'end untranslated region of the RNA (Macdonald and Struhl 1988; Macdonald et al. 1993).

Upon egg activation, the bcd RNA starts to be translated forming a concentration gradient of Bcd protein with levels highest at the anterior end of the embryo. The simplest model predicts that this gradient is formed by synthesis from the localized RNA source, coupled with diffusion within the syncytial embryo and a constant rate of proteolytic degradation throughout the embryo. The bcd protein gradient regulates zygotic target genes in a concentration dependent manner (see below).

B) Maternal systems involved in anteroposterior patterning: the posterior group of genes

Embryos from females mutant for any of the eleven known posterior group of genes lack all abdominal segments. Nine of these genes, *cappuccino*, *spire*, *staufen*, *oskar*, *pipsqueak*, *vasa*, *valois*, *tudor*, and *mago nashi* are required for the formation of the pole plasm and polar granules at the posterior end of the egg. The polar granules are required for germ cell determination and thus mutations in these nine genes have an additional defect in that they do not form pole cells. The pole plasm is also the structure that serves to anchor the posterior determinant, the RNA of the tenth gene, *nanos*. The final member, *pumilio*, is required for *nos* function.

Assembly of the pole plasm, the anchor for nos RNA localization.

The nine genes required for pole plasm formation have been proposed to act in an ordered, hierarchical pathway, where the function and/or product of each gene are required for the stepwise assembly of the pole plasm (for review, see St. Johnson (1993).

The genes *capuccino*, *spire*, *staufen*, and perhaps *mago nashi*, are involved in the transport of the oskar mRNA to the posterior pole (Lehmann and Nüsslein-Volhard 1991; Manseau and Schüpbach 1989; Boswell et al. 1991). This is best shown by the fact that *capuccino*, *mago nashi*, *spire* and *staufen* are required for localization of endogenous oskar RNA to the posterior pole, but are not required for the phenotype caused by ectopic localization of oskar RNA to the anterior using the bcd 3' UTR localization signal (Ephrussi et al. 1991; Kim-Ha et al. 1991; Ephrussi and Lehmann 1992).

Mutations in *capuccino* and *spire* lead to additional phenotypes, such as the dorsalization of egg chambers, as well as defects in division and migration of cleavage nuclei in early embryos (Manseau and Schüpbach 1989). Thus these two genes may be required for more general functions, such as the organization of the cytoskeleton or intracellular transport.

On the other hand, *staufen* may have a more specific role in the transport of oskar RNA to the posterior pole. Staufen protein colocalizes with oskar RNA at all stages (Ephrussi et al. 1991; Kim-Ha et al. 1991 St. Johnston et al. 1991). First, both accumulate at the stage 2 oocyte. At stages 7-9, both of these products are found at the anterior margin of the oocyte, and at later stages, they are both found localized at the posterior pole of the oocyte. Staufen encodes a protein with sequence similarities to double stranded RNA binding proteins and thus it seems likely that Staufen acts as a chaperone protein during transport of osk RNA to the posterior pole (St. Johnston et al. 1991).

Oskar function is required for the localization of products of genes downstream of the pole plasm formation and abdominal development pathways, such as the Vasa protein and the nanos and pumilio RNAs (Hay et al. 1990; Lasko and Ashburner 1990; Wang and Lehmann 1991; Barker et al. 1992; Macdonald 1992). EM studies show that the Oskar protein, is localized to the polar granules (Dickinson and Lehmann, unpublished data).

Localization of osk mRNA is instrumental for the determination of pole plasm. Ectopic localization of oskar RNA to the anterior of the egg using the 3' UTR localization sequences from the *bicoid* gene leads to the formation of polar granules and competent germ cells at the anterior of the egg, as well as an ectopic anterior abdomen (Ephrussi and Lehmann 1992).

Mutations in *vasa*, *tudor* and *valois* do not affect oskar RNA posterior localization and thus interfere with steps downstream of oskar RNA localization (Ephrussi et al. 1991). The gene *pipsqueak* has recently been reported to also affect pole plasm formation by affecting, likely among a number of other genes, vasa mRNA levels (Siegel et al. 1993).

The Vasa protein, but not its RNA, is localized to the posterior pole of the embryo (Hay et al. 1988, Lasko and Ashburner 1990). Ultrastructural studies have shown that Vasa is found in the polar granules. Sequence analysis suggests that *vasa* encodes an ATP-dependent RNA helicase. Vasa protein may therefore interact with RNAs that localize to the posterior pole plasm, including the *nanos* and *pumilio* RNAs.

The gene *valois* is unique in that, although it appears to interfere with steps downstream of oskar RNA localization, it is not required for the function of the ectopically localized oskar (Ephrussi and Lehmann 1992). The significance of these results is unclear. Mutations in *valois* also affect cellularization in the early embryo (Schüpbach and Wieschaus 1986a). Thus *valois* may be involved in more general processes related to the cytoskeleton.

The *tudor* gene, on the other hand, appears to specifically affect polar granules (Boswell and Mahowald 1985; Schüpbach and Wieschaus 1986a). The fact that the size of polar granules in mutant correlates with the strength of *tudor* alleles, and the localization of Tudor protein to polar granules by EM studies, suggest that Tudor is an integral component of the polar granules (Boswell and Mahowald 1985; Bardsley et al. 1993).

During the assembly of the pole plasm, a number of interactions can be observed which differ from a linear assembly pathway. For example, the Oskar protein is required for the maintenance of its own RNA and the Staufen protein at the posterior pole (Ephrussi et al. 1991; Kim-Ha et al. 1991; St. Johnston et al. 1991), and the functions of *staufen*, *valois* and *tudor* are all required for the maintenance of Vasa localization during oogenesis or embryogenesis (Hay et al. 1988, Hay et al. 1988; St. Johnston et al. 1991). These interactions further suggest that components of the pole plasm interact with each other to form stable complexes that constitute the polar granules.

Pole plasm formation is important for anteroposterior patterning because the localization of the posterior determinant, the nanos RNA, depends on the formation of this specialized cytoplasm (Wang and Lehmann 1991; Wang et al., 1994). This localization occurs at the last stages of oogenesis and is directed by sequences present at the 3' end of the nanos RNA untranslated region (Gavis and Lehmann 1992).

The function of the Nanos protein gradient.

Upon egg activation, translation of the localized nanos RNA coupled with diffusion of protein anteriorly across the embryonic syncytium creates a Nanos protein gradient with levels highest at the posterior pole (Barker et al. 1992; Smith et al. 1992; Wang et al. 1994).

Nanos function is required for the activation of the abdominal gap genes *knirps* and *giant* (Rothe et al. 1989; Eldon and Pirrotta 1991; Kraut and Levine 1991a). Nanos, though, does not act on these genes directly, as is the case with the anterior determinant Bicoid and its targets genes. Rather, Nanos protein plays an indirect role, by translationally repressing maternal Hunchback, which would otherwise repress *knirps* and *giant* expression (Hülskamp et al. 1989; Irish et al. 1989a; Struhl 1989). The maternal hunchback RNA, which is found uniformly distributed in the unfertilized egg, is the only essential target of Nanos, since the abdominal phenotype caused *nanos* mutations is suppressed by the lack of maternal *hunchback* function.

Sequences in the 3' UTR region of the maternal hunchback RNA have been shown to be both necessary and sufficient for Nanos-dependent translational regulation (Wharton and Struhl 1989). A 11 base bipartite sequence, which occurs twice in this region, has been proposed to be the target of Nanos and has been termed the Nanos-responsive element (NRE).

The gene *pumilio* is also required, perhaps in an auxiliary role, for the Nanos-dependent translational repression of hunchback (Lehmann and Nüsslein-Volhard 1987a; Barker et al. 1992). *pumilio* is also required for adult viability and bristle determination, suggesting a more general function for this gene. The gene *pumilio* codes for a very large protein with eight tandem copies of a 36-amino acid repeat also found in several yeast and plant proteins of unknown function (Macdonald 1992; Barker et al. 1992; D. Barker, personal communication).

Translational repression of the maternal hunchback RNA by the Nanos protein gradient leads to a complementary gradient of maternal Hunchback protein with levels highest at the anterior of the embryo (Tautz 1988; Tautz and Pfeifle 1989). As discussed below, the Hunchback protein gradient regulates transcription of downstream zygotic genes in a concentration dependent manner.

D) Maternal systems involved in anteroposterior patterning: the terminal group of genes

Recessive mutations in terminal group genes affect embryonic structures corresponding to the anterior-most and posterior-most regions of the embryonic fate map. The anterior structures missing in these mutants define the acron, which includes the labrum, parts of the brain, and portions of the cephalopharyngeal skeleton. The posterior structures affected by these mutations define the telson, which includes structures posterior to the seventh abdominal segment (A7), such as A8, the posterior gut, the malpighian tubules, and the posterior spiracles.

The terminal group of genes includes seven maternal-effect genes: *torso*, *torso-like*, *fs(1)Nasrat*, *fs(1)polehole*, *trunk*, *corkscrew* and *l(1)polehole*. Recent screens for suppressors of *Torso* dominant alleles have identified a number of additional genes in this pathway (see below, for review, see Sprenger and Nüsslein-Volhard (1993)).

Production and localization of a terminal signal at both poles.

The pattern determinants of the anterior and posterior system are produced and localized within the confines of the nurse cell-oocyte syncytium. On the other hand, the terminal system, like the dorsoventral system, relies on a signal produced in the surrounding follicle cells which is stored within the egg vitelline membrane and transduced into the zygote after fertilization.

Transduction of this signal across the embryonic membrane is achieved via the product of the gene *Torso*. *Torso* encodes a membrane receptor with homology in its cytoplasmic domain to protein tyrosine kinases (Casanova and Struhl 1989; Sprenger et al. 1989). Autophosphorylation of Torso protein immunoprecipitated from embryonic extracts reveals that the Torso kinase activity is present only in a window of 1 to 2 hours after fertilization (Sprenger and Nüsslein-Volhard 1993). Thus Torso is activated, probably by an extracellular ligand, during the mid to late syncytial stages.

Torso dominant alleles have been particularly useful in the ordering by genetic epistasis of genes in this pathway. *Torso* dominant alleles produce constitutively active receptors which lead to the expansion of terminal structures at the expense of thoracic and abdominal regions (Klingler et al. 1988; Szabad et al. 1989. Four genes, *torso-like*, *trunk*, *fs(1)Nasrat* and *fs(1)polehole* do not suppress *Torso* dominant mutations and thus act upstream of *Torso* function (Ambrosio et al. 1989; Casanova and Struhl 1989; (Casanova and Struhl 1989; Perkins et al. 1992). Mutations in the genes *corkscrew* and *l(1)polehole*, on the other hand, suppress a *Torso* dominant mutation and thus act downstream of *Torso*.

One of the four genes that act upstream of *Torso* likely encodes a ligand for Torso receptor. Transplantation experiments have shown that this ligand is present in the perivitelline space, is difussible and limiting, and is locally produced at both ends of the embryo, where it appears to be sequestered by the Torso receptor (Stein et al. 1991).

fs(1)Nasrat and *fs(1)polehole* mutant females produce eggs that often collapse (Degelmann et al. 1990). These two genes may thus be involved in the integrity of the vitelline membrane that anchors this ligand, rather than encoding the ligand itself.

The gene *torso-like* could possibly encode the Torso ligand. *torso-like* is the only terminal group gene required in the somatically derived follicle cells and not the germ line (Stevens et al. 1990). In particular, *torso-like* function has been shown to be required in a few cells at each end of the egg chamber. This is in agreement with the expression of torso-like RNA specifically in follicle cells at the anterior and posterior ends of the growing oocyte (Savant-Bhonsale and Montell 1993). The Torso-like protein is predicted to be secreted, with leucine-rich regions that are similar to leucine-rich repeats that may mediate protein-protein and protein-lipid interactions. Ectopic expression of *torso-like* using a heat-inducible promoter produces phenotypes similar to those caused by constitutively active Torso. Thus, the Torso-like protein has all the properties expected of the localized, limited Torso ligand which is present in the perivitelline space.

Alternatively, the Torso ligand could be encoded by the germ line-dependent *trunk* gene (Casanova and Struhl 1993). Trunk protein could be secreted from the oocyte into the perivitelline space in an inactive form. Inactive Trunk could be locally converted into active ligand by the spatially restricted Torso-like protein.

Reception of the terminal signal and activation of an intracellular protein kinase cascade.

Activation of the Torso protein triggers an intracellular signalling cascade that includes the products of the genes *corkscrew* and *l(1)polehole*, and several other genes identified as suppressors of a constitutively active *Torso* allele, such as *Crk-like*, *Suppressor of sevenless* (*Sos*), *Ras-1* and *Dsor1* (Perrimon et al. 1984 Perrimon et al. 1985; Nishida et al. 1988; Ambrosia et al. 1989a; Ambrosia et al. 1989b); Perkins et al. 1992; Doyle and Bishop 1993; Lu et al. 1993; Tsuda et al. 1993). The precise epistatic relationship between these genes is unknown. Nevertheless, the similarity of this pathway to other receptor tyrosine kinase signal transduction pathways in yeast, C. elegans and vertebrates, as well as to photoreceptor determination in Drosophila, has provided a framework for understanding the events in this signalling cascade (Tsuda et al. 1993; Han

and Sternberg 1990; Simon et al. 1991; Bruder et al. 1992; Clark et al. 1992; Kyriakis et al. 1992; Williams et al. 1992).

The Crk-like protein may act as an adaptor between the activated receptor tyrosine kinase and the Ras protein. The Crk-like sequence contains SH2 and SH3 domains, which can mediate protein-protein interactions by binding to phosphotyrosine residues (Simon et al. 1993; Doyle and Bishop 1993; Margolis 1992).

The activation of Ras1 by the Torso receptor may also involve the function of the Son of sevenless (Sos) protein, a nucleotide exchange factor (Simon et al. 1991; Doyle and Bishop 1993). Sos activity leads to increases in the level of GTP-bound active Ras1. Eventually, Ras1 returns to its inactive, GDP-bound state by virtue of its GTPase activity.

The increased levels of active, GTP-bound Ras1 during signal transduction result in the activation of the product of the *l(1)polehole* gene, the serine-threonine kinase Drosophila Raf homologue (Raf-1) (Mark et al. 1987; Nishida et al. 1988, Sprenger et al., 1993). In mammals, Raf-1 is directly associated with receptor tyrosine kinases and acts as a kinase upon phosphorylation (Morrison 1990, App et al. 1991). Activation of Raf-1 leads to the phosphorylation of downstream targets in this cascade.

The gene *corkscrew* also modulates this pathway, but in a manner that remains unclear. The *corkscrew* gene, encodes a tyrosine phosphatase and has two SH2 domains (Perkins et al. 1992). Nevertheless, *corkscrew* mutations affect only a subset of the structures affected by a *Torso* null mutation. Thus, *corkscrew* does not relay the entire activity of the Torso signalling pathway but may only regulate the activity of a subset of its components.

Recently, mutations in Dsor1, the Drosophila homologue of the microtubule-associated protein (MAP) kinase activator, have been isolated as dominant suppressors of D-raf (Tsuda et al. 1993). Genetic epistasis studies suggest that Dsor1 acts downstream of both D-raf and corkscrew. This result predicts a MAP-kinase like molecule downstream of Dsor1, which could be encoded by DmERK-A (Biggs and Zipursky 1992)

This signal transduction cascade is likely to involve additional genes. Doyle and Bishop (1993) have identified, in addition to Sos and Ras1, five other complementation groups which, when mutated, act as suppressors of Torso and are thus likely involved in Torso signalling. Other genes may be involved in the negative regulation of this pathway. The *gap-1* gene may provide such negative signals, since mutations in *gap-1* enhance the activity of constitutive *Torso* alleles (Doyle and Bishop 1993)

Presumably, the end result of this signal transduction cascade is the localized phosphorylation, perhaps by Raf-1, of a transcription factor that activates terminal gap genes. This activity appears to be distributed as a gradient with highest levels at each pole,

and the activation of different downstream genes depends on different levels of terminal activity (see below).

4. Initiation of patterned zygotic gene expression: interpretation of maternal information gradients and its refinement via zygotic gene interactions

The maternal systems produce gradients of protein that will direct the patterning of different aspects of the embryo. These molecules act as morphogens, which are graded factors that induce different cellular responses at different threshold concentrations. At the molecular level, different cellular responses are produced by differential gene expression of cell fate-determining genes.

The establishment of patterned gene expression in the embryo utilizes both mechanisms that initiate and refine the gene expression domains and mechanisms that maintain and stabilize the already established pattern. For the sake of clarity these two different mechanisms will be discussed separately, in sections (4) and (5), respectively. Nevertheless, it should be kept in mind that throughout embryogenesis these two types of mechanisms often occur simultaneously, specially when considering different tiers of genes. It should also be noted that the distinction between cross-regulatory interactions that refine and those that stabilize a pattern is somewhat arbitrary, since in reality both of these sets of interactions likely contribute to both processes.

A) Patterning of zygotic dorsoventral genes

The differential nuclear translocation of Dorsal protein caused by the dorsal group of genes produces a gradient of *dorsal* activity with highest levels in ventral regions (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). Since the transcription factor Dorsal can only function when present in the nucleus, this gradient of dorsal nuclear localization leads to a gradient of dorsal activity with levels highest in ventral regions.

The graded Dorsal protein acts as a morphogen. Increasing levels of *dorsal* activity induce in cells the fates of progressively more ventral regions in the order: ammioserosa (dorsal midline cells, little or no *dorsal* activity required), dorsal epidermis (dorsolateral cells), neuroectoderm (ventrolateral cells), and mesoderm (mesoderm, high *dorsal* activity required). In embryos mutant for *dorsal*, ventral regions are deleted and dorsal and lateral regions are expanded, suggesting that the steepness of the gradient has changed due to the generally reduced *dorsal* activity (Anderson and Nüsslein-Volhard 1984). Mutations in the

recessive, ventralizing *cactus* alleles also seem to change the steepness of the gradient (Roth et al. 1991). Embryos mutant for *cactus* lack dorsal and dorso-lateral structures, but do not have an expanded mesoderm. Since Cactus normally inhibits translocation of the Dorsal protein to the nucleus, the shallower gradient of *dorsal* activity, as reflected in this phenotype, is consistent with an overall increase in nuclear Dorsal protein due to the lack of *cactus* function This and other data at the level of zygotic gene transcription (Rushlow et al. 1987) have suggested that Dorsal acts as a morphogen across the dorso-ventral axis.

Dorsal regulates ventral, lateral and dorsal zygotic genes. In ventral regions Dorsal activates genes such as *twist* and *snail*, which are required for the differentiation of the mesoderm (Jiang et al. 1991; Ip et al. 1992). Dorsal is also required for the activation of genes required for the more lateral neuroectodermal fates, such as *rhomboid*, *single minded* and *lethal of scute* (T3) (Bier et al. 1990; Kasai et al. 1992; Jiang and Levine 1993). In ventral regions, activation of the dorsal-promoting zygotic genes *zerknüllt*, *decapentaplegic* and *tolloid* appears to be a default state. The expression of these genes is restricted to dorsal regions by repression by Dorsal in ventral and lateral regions (Rushlow et al. 1987; St. Johnston and Gelbart 1987; Ip et al. 1991; Shimell et al. 1991) .

The activation of ventral and lateral genes and repression of dorsal genes by Dorsal is likely the result of direct transcriptional regulation. Binding sites for the Dorsal protein are found in the promoters of *twist*, *snail*, *rhomboid* and *zen* (for review, see Ip et al. (1992). The same sites have been shown to mediate Dorsal-dependent activation or repression depending on the target gene in which they are inserted (Ip et al. 1991; Jiang et al. 1992 Pan and Courey 1992). Thus additional sequences in the promoters of these genes, and likely binding by other transcription factors, determine the ultimate regulatory effect of Dorsal on transcription.

The affinities of the Dorsal binding sites are important for determining the threshold response. Strong Dl-binding sites in the *zen* promoter have a 5-fold higher affinity than Dl-binding sites in the *twist* promoter (Thisse et al. 1991; Jiang et al. 1991). Thus, in lateral regions, low levels of Dorsal can repress genes with high-affinity Dl-binding sites, such as *zen*, even though they are insufficient for the activation of genes with low-affinity binding sites, such as *twist*.

Activation in lateral regions (i.e. at low Dl levels) dependent on high affinity Dl-binding sites require cooperative interactions between Dl and bHLH transcriptional activators, such as *daughterless* and *twist* (Jiang and Levine 1993). Snail, which is a Zn-finger DNA binding protein, inhibits this cooperative interaction. This may be achieved by competitive binding to the bHLH binding sites (E boxes), although a more active repression mechanism that masks activation by Dl may also be involved. This mechanism

of repression by Snail may be used to restrict the expression of lateral genes such as *rhomboid*, contributing in this manner to the establishment of the mesoderm/neuroectoderm boundary.

The product of the dorsally expressed *decapentaplegic* (*dpp*) gene plays a major role in patterning in dorsal and lateral regions (Ferguson and Anderson 1992; Wharton et al. 1993). Gene dosage and RNA injection experiments have shown that progressively higher doses of Dpp promote the formation of progressively more dorsal fates: low Dpp levels cause ventral ectoderm pattern elements, intermediate Dpp levels produce dorsal ectoderm and high Dpp levels lead to the development of the ammioserosa.

Dpp encodes a member of the transforming growth factor beta (TGFb) family, and is most closely related to the bone morphogenetic factors (BMP) 2 and 4 (Padgett et al. 1987; Wozney et al. 1988). This homology and the fact that Dpp protein undergoes limited diffusion strongly suggests that Dpp is secreted and exerts its effects in the extracellular compartment (Panganiban et al. 1990).

Four genes, *tolloid* (*tld*), *shrew*, *screw* and *short of gastrulation* (*sog*), have been shown to exert a postrancriptional regulatory effect on *dpp* (Ray et al. 1991; Ferguson and Anderson 1992). *tolloid* encodes a Drosophila homologue of the vertebrate BMP-1, which forms a complex with BMP-2 and BMP-4 (Shimell et al. 1991 Wozney et al. 1988). These homologies suggest that Tld and Dpp form part of a multiprotein, extracellular complex and that the Tld protein modifies the activity of the Dpp protein. The *sog* gene, on the other hand, antagonizes Dpp activity in ventral regions, and may refine the Dpp extracellular gradient (Zussman et al. 1988; Shimell et al. 1991).

The *spitz* group of genes is required for the formation of ventrolateral (neuroectodermal) structures (Mayer and Nüsslein-Volhard 1988). This group includes the genes *spitz*, *Star*, *pointed* and *rhomboid*. The gene *spitz* codes for a transmembrane protein with the structural features of the transforming growth factor alpha (TGFa), a member of the epidermal growth factor (EGF) family (Rutlege et al. 1992). *Rhomboid* codes for a transmembrane protein with three to seven membrane spanning domains (Bier et al. 1990). This molecular information and the facts that *spitz* and *rhomboid* have similar phenotypes, and that some of these phenotypes are shared with mutations in the Drosophila EGF receptor (DER) suggest a model for the action of these genes (Rutlege et al. 1992). In this model, the spatially restricted Rho protein functions together with the more broadly distributed DER to potentiate the activation of the DER tyrosine kinase by the ubiquitous Spi ligand.

To the present, downstream targets of the dorsoventral patterning system have not been well studied. Some of these targets may be genes involved in anteroposterior

patterning. The Twist protein, for example, has been proposed to directly enhance the expression of Ubx in the mesoderm (Qian et al. 1993) These interactions help integrate the input from dorsoventral and anteroposterior patterning systems and lead to the complex set of structures observed in the mature larva.

B) Patterning of zygotic anteroposterior genes

Patterning along the anteroposterior axis is established by a series of tiers of zygotic expression. The first set of genes, the cardinal or gap genes, are expressed in broad domains. Gap genes determine the further subdivision of the embryo by controlling the segmentation genes, the pair rule and segment polarity genes. Simultaneously, gap gene products also regulate the expression of homeotic genes to determine the identity of each forming segment.

i) The cardinal genes: gap genes

The gap genes represent the first tier of zygotic anteroposterior expression. Six principal gap genes are known, *hunchback (hb), Krüppel (Kr), knirps (kni), giant (gt) tailless and huckebein (hkb)* (for reviews, see Hülskamp and Tautz (1991); Hoch and Jäckle (1993); Kornberg and Tabata (1993); Pankratz and Jäckle (1993)). In addition, the genes *orthodentical (otd), buttonhead (btd)* and *empty spiracles (ems)*, which act in the head region, have gap-like characteristics. All of these genes appear to be transcription factors: Hb, Kr and Btd have Zn-finger DNA binding motifs (Tautz et al. 1987; Rosenberg et al. 1986; Pankratz and Jäckle 1993); Kni and Tll have homologies to the Zn-finger DNA binding domain of steroid receptors (and Tll, but not Kni, has a weak homology in the steroid binding domain; (Nauber et al. 1988; Pignoni et al. 1990); Gt has a basic-leucine zipper region characteristic of DNA binding proteins (Mohler et al. 1989), and Otd and Ems are homeobox proteins (Dalton et al. 1989; Finkelstein and Perrimon 1990).

Mutations in gap genes create large gaps in the anteroposterior pattern. As might be expected, for each gene the regions of expression correlate with the fate map positions corresponding to the structures affected by these gaps. *hb* is expressed in the anterior 50% of the egg, and the absence of zygotic *hb* affects the development of head and thoracic regions (Lehmann and Nüsslein-Volhard 1987; Tautz et al. 1987; Tautz 1988). *Kr* is expressed as a single band in a central domain partially overlapping the *hb* domain (Gaul and Jäckle 1987). Accordingly, *Kr* mutations affect the development of the thorax and first five abdominal regions (Wieschaus et al. 1984b). *kni* is also expressed as a band, which is

just posterior to that of *Kr* (Rothe et al. 1989). Mutations in *kni* affect the formation of abdominal segments one through seven (Nauber et al. 1988). *kni* is also expressed in more anterior regions of the embryo, in domains overlapping the *hb* domain, although there is no obvious role for this domain of expression. *gt* is initially expressed in two domains, an anterior band overlapping the *hb* domain, and a posterior cap expressed just posterior to *kni* (Mohler et al. 1989). During the cellular blastoderm stage, the anterior band evolves into a three striped pattern and the posterior cap retracts into a band. Accordingly, mutations in *gt* have both anterior and posterior phenotypes, affecting the head and segments five through eight. Both *tailless* and *huckebein* are initially expressed as overlapping caps at each pole of the embryo, the *tll* expression domain being somewhat larger than that of *hkb* (Pignoni et al. 1990; Bronner and Jäckle 1991). Mutations in *tll* and *hkb* affect structures that originate in the acron and telson of the embryo (Strecker et al. 1986; Weigel et al. 1990). Finally, the head genes *otd*, *ems* and *btd* are expressed in progressively more posterior areas of the head region and are required for the development of those regions (Dalton et al. 1989; Cohen and Jürgens 1990; Finkelstein and Perrimon 1990).

The gap genes are regulated by the three sets of anteroposterior maternal genes. In general, the phenotype of a particular gap gene is similar to the phenotype of the maternal genes required for its activation (Finkelstein and Perrimon 1990). Thus,. mutations in *hb* and *bcd* both affect head and thoracic structures, which reflects the fact that *hb* is a major target for Bcd activation. In accord to the fact that Nos is required for the activation of *kni* and *gt*, mutations in *kni*, *gt* and *nos* all result in the deletion of abdominal segments. Similarly, *tll* and *hkb* are activated by the maternal terminal genes, and mutations in these maternal genes as well as *tll* and *hkb* affect terminal structures of the embryo.

The Bicoid morphogenetic gradient: regulation of anteriorly expressed genes.

The Bcd protein is distributed in an anteroposterior protein gradient and acts as a morphogen. This is indicated by experiments in which different *bcd* dosages lead to corresponding shifts in the embryonic fate map, as assayed by both morphological markers such as the head fold and the expression of marker genes such as *hb* and *even-skipped* (Driever and Nüsslein-Volhard 1988b; Struhl et al. 1989).

As mentioned above, the similarities between the *bcd* and *hb* mutant phenotypes suggest that the *hb* is a major target for Bcd. The Bcd protein gradient activates *hb* in the anterior half of the embryo. Indeed, the P2 promoter, which is used for the anterior *hb* zygotic expression, depends on *bcd* for its activation and contains Bcd-binding sites (Tautz et al. 1987; Driever and Nüsslein-Volhard 1989). These sites can confer Bcd-dependent

activation of heterologous genes in Drosophila and yeast (Driever and Nüsslein-Volhard 1989; Driever et al. 1989; Hanes and Brent 1989; Struhl et al. 1989).

The hb P2 promoter contains both low affinity and high affinity bcd binding sites (Driever and Nüsslein-Volhard 1989). In vivo studies of the expression conferred by fragments containing Bcd-binding sites suggest that the number and quality of these sites determine the threshold concentration of Bcd protein required for activation (Driever et al. 1989; Struhl et al. 1989).

It is still unclear how the rather shallow Bcd gradient can establish a sharp boundary of hb expression. Driever (1993) has pointed out that hb expression levels change 20 to 50 fold over its boundary of expression, while in the same region Bcd protein concentration changes only 2 to 3 fold. Presumably cooperative interactions between Bcd bound to the multiple promoter sites are responsible for this sharp threshold of activation. Alternatively, Bcd could be active only as dimers or multimers, and the Bcd monomer to multimer ratio could have a sharp dependence on Bcd concentration.

The existence of additional target genes for Bcd has been proposed since, in spite of their similarity, the *bcd* and *hb* mutant phenotypes are not identical. One or more of the anteriorly expressed genes *otd*, *ems* and *btd*, or the *gt* anterior domain of expression, may be such additional Bcd targets. The domains of expression of all of these genes are more anteriorly restricted than that of *hb*. In principle, this more anterior restriction could be mediated by a promoter with Bcd-binding sites of generally less affinity than those present in the *hb* promoter.

Recently the terminal group activity has been proposed to restrict activation by Bcd in anterior regions (Ronchi et al. 1993). This method of regulation may apply to anterior genes, such as *hb*, *otd* and the *sloppy paired1* locus, that are activated as a cap of expression and retract their anteriormost expression to evolve into a band. It may also be utilized in the regulation of genes that are activated somewhat later and appear as a band, such as *ems* and *sloppy paired2*. The fact that this effect is observed in reporter constructs whose expression is controlled solely by the Bcd binding site shows that this effect involves modification of the Bcd activity. The modification of Bcd is dependent on the activity of the serine/threonine protein kinase homologue D-raf, encoded by the terminal group gene *l(1)polehole* (see above). That Bcd may be modified through phosphorylation is further supported by the fact that Bcd protein is phosphorylated during early development in a process dependent on terminal activity. A hybrid Bcd protein carrying several copies of the transcriptional domain of yeast GCN4 did not respond to the terminal-dependent regulation, which suggests that the inhibition of Bcd activity occurs at the level of transcriptional activation and not DNA binding. Thus, the terminal group activity may

lead to Bcd phosphorylation. This modification in turn restricts the ability of Bcd to activate genes in the anteriormost regions, thus further refining the expression patterns of a number of head genes.

The Hunchback morphogenetic gradient and its organizing properties

There are two different sources of Hb protein that contribute to the Hb protein gradient: the maternal Hb (Hb^{mat}) protein gradient established by translational repression by Nanos of the hb^{mat} mRNA, and the zygotic Hb (Hb^{zyg}) protein gradient established by activation of Bcd in the anterior half of the embryo (see above). Although established by very different mechanisms, both gradients of Hb protein have similar distributions, i.e. high protein levels at the anterior of the embryo and low at the posterior.

The windows of temporal expression of the hb^{mat} and hb^{zyg} protein gradients overlap, but are not identical (Tautz 1988). The Hb^{mat} protein gradient appears very early at nuclear cycles 6 or 7 and can be observed (in *bcd* embryos, where Hb^{zyg} is not activated) to disappear during the cellular blastoderm stage (cycle 14). The Hb^{zyg} protein gradient appears later, during cycles 11 or 12, and the protein can be observed past the blastoderm stage until early gastrulation.

The hb^{mat} and hb^{zyg} RNA, although transcribed from different promoters, share the same coding sequence (Schröder et al. 1988). Thus, the Hb^{mat} and Hb^{zyg} protein gradients are formed by presumably an identical protein.

The spatially and temporally overlapping Hb^{mat} and Hb^{zyg} protein gradients as well as the identical primary sequence of the Hb^{mat} and Hb^{zyg} proteins suggest that the two gradients have similar properties. This is indeed the case, as both the Hb^{mat} and Hb^{zyg} gradients on their own can organize the zygotic gap gene pattern in a similar fashion (Hülskamp et al. 1990; Eldon and Pirrotta 1991; Kraut and Levine 1991a,Kraut and Levine 1991b; Kraut and Levine 1991b). These organizing properties are described below and the two Hb protein gradients will be jointly referred to as the Hb protein gradient.

The Hb protein gradient acts as a major organizer of gap gene transcription in the embryo. Hb regulates transcription of *Kr*, *kni* and *gt*. This regulation appears to be direct, since Hb-binding sites have been found in the promoter regions of these three genes (Hoch et al. 1991; Pankratz et al., 1992; M. Capovilla, personal communication).

High Hb protein levels repress transcription of *Kr* (Hülskamp et al. 1990; Struhl et al. 1992). Thus, the Hb protein gradient sets the anterior boundary of *Kr* expression at the position in which Hb levels become too high to allow *Kr* expression. On the other hand, moderate levels of Hb are required for the activation of *Kr*. Thus, the Hb protein gradient

also sets the posterior *Kr* boundary at the position in which Hb levels become too low to activate *Kr* transcription.

The concentration-dependent behavior of the Hb protein, acting as an activator at low concentrations and a repressor at high concentrations, is a property that it shares with the Kr protein (see below). This phenomenon has been partly mimicked in cultured Drosophila cells, where Hb activates transcription in a concentration dependent manner and this activation is lost at Hb concentrations above a certain threshold (Zuo et al. 1991). It is possible that high Hb levels titrate out a coactivator. Alternatively, Hb may multimerize in a concentration dependent manner and only the monomer can activate transcription, while the multimeric form may contribute to repression. This second mechanism appears to be involved in the dosage-dependent behavior of Kr as a regulator (see below).

Moderate levels of Hb also repress the expression of *kni* and *gt*. Thus the Hb gradient determines by repression the anterior boundaries of expression of these genes, at the positions where Hb protein levels are too high to allow their expression (Hülskamp et al. 1990 Eldon and Pirrotta 1991; Kraut and Levine 1991a, Kraut and Levine 1991b; Struhl et al. 1992). The expression of *kni* and *gt* appears to occur by default (Capovilla et al. 1992; Pankratz et al. 1992). In the case of *kni*, studies employing regions from the promoter fused to a reporter construct have shown that cis-acting sequences are required for the activation of these constructs (Pankratz et al. 1992). Additional regions of the *kni* promoter are required for Hb- and Tll-dependent repression (see below) and contain binding sites for these two proteins. The fact that activators and repressors act through different *kni* promoter regions suggests that repression can not be simply caused by competitive interference at the level of DNA binding, as has been proposed in other examples of gene regulation (see below).

These interactions allow the Hb protein gradient to organize the embryo into anterior (*hb*-expressing), middle (*Kr*-expressing) and posterior (*kni*- and *gt*- expressing) regions. Either the maternal Hb protein gradient on its own (as observed in *bcd* embryos) or the zygotic Hb protein gradients on its own (as tested in embryos from Hb mutant germ line clones) can organize the embryo in this manner.

Cross-regulatory interactions involving *Kr*, *kni* and *gt* constitute a self-organizing system that refines the pattern initiated by the Hb protein gradient. For example, the posterior *kni* boundary is determined by repression from Gt and Tll (Pankratz et al. 1989; Eldon and Pirrotta 1991), and the posterior *gt* boundary is determined by repression by Tll (Eldon and Pirrotta 1991; Kraut and Levine 1991b). In addition, the posterior *Hb* and *Kr* boundaries are refined at the late blastoderm stage by repression from Kr and Kni proteins, respectively (Gaul and Jäckle 1987; Gaul and Jäckle 1990)).

Cascading gradients

The picture of embryonic anteroposterior patterning that begins to appear is one in which patterning is not refined by a single morphogenetic gradient that acts along the entire length of the embryo, but by a series of cascading gradients each of which organizes sections of the embryo. Thus the anteroposterior bcd morphogenetic gradient activates the Hb^{zyg} gradient in the anterior half of the egg. Hb, in turn, also forms a morphogenetic gradient. Both Bcd and Hb contribute to the activation of *Kr* in a slightly more posterior domain. The expressed Kr protein is similarly capable of regulating genes in a concentration dependent manner, although these interactions appear to be more involved in the stabilization of the pattern than in its establishment (see below).

Activation of gap genes at both ends by the terminal activity

As described above, activation of the Torso tyrosine kinase receptor signalling cascade leads to the activation of an unknown transcription factor at both ends of the embryo. This presumably graded transcription factor may also been proposed to act as a morphogen, since different levels of terminal activity lead to the differential activation of downstream target genes and the formation of different cuticular structures (Casanova and Struhl 1989).

The terminal activator is required for the expression of at least *tll* and *hkb* as caps at each pole (*tll* anterior expression later resolves into a band) (Pignoni et al. 1990; Weigel et al. 1990; Brönner and Jäckle 1991). The boundaries of *tll* expression are further away from the poles than those of *hkb*. The regulation of these two genes by the unknown terminal activator has not been studied at the molecular level. Nevertheless, it seems plausible that the different extents of activation from the poles of *tll* and *hkb* are a consequence of differential affinities of their promoters to binding by the terminal activator.

This differential response to the graded terminal activity continues to organize the terminal pattern indirectly through the differential regulatory activity of its targets genes. This is the case for the posterior zygotic *hb* domain, which appears in the embryo after the establishment of the Bcd-dependent anterior *hb* domain. The posterior *hb* domain of expression is transcribed from the P1 *hb* promoter (the same used for transcription of the maternal hb mRNA) and appears as a cap that resolves into a stripe (Tautz 1988). The regulation of this domain as a stripe involves activation by Tll and repression by the more posteriorly expressed Hkb protein (Casanova 1990).

tll and *hkb* appear to be the only targets of the terminal activity at the posterior pole, since the posterior end of *tll, hkb* double mutant embryos resembles the phenotype caused

by lack of terminal activity (Weigel et al. 1990). This is not the case in the anterior of the embryo, however, and thus there are likely other target genes for the terminal system in the anterior pole.

The Tll protein codes for a Zn-finger DNA binding protein that belongs to the steroid receptor superfamily (Pignoni et al. 1990). This homology is primarily in the DNA binding domain, although weak homologies also exist in the ligand binding domain. Ectopic expression of Tll leads to ectopic *tll* activity, suggesting that if there is a Tll ligand, it is distributed throughout the embryo (Hoch et al. 1992).

Tll itself appears to form a morphogenetic gradient. This is suggested by the fact that different levels of *tll* activity lead to the determination of different structures (Strecker et al. 1988; Casanova 1990). This may be achieved by different levels of *tll* activity leading to the differential activation of downstream target genes, such as the seventh stripe of the pair rule gene *fushi tarazu* (*ftz*), and the homeotic gene *r-Abdominal B* (*r-Abd-B*) (Casanova 1990). The gene forkhead, which is expressed in a *tll*-dependent manner posterior to the seventh *ftz* stripe, may require for its activation levels of Tll slightly higher than *ftz*. Differential regulation to graded *tll* activity may also be responsible for the organization of the *caudal* and *r-Abdominal B* domains into different but partially overlapping domains (Sanchez-Herrero and Crosby 1988; Casanova 1990). Thus the putative graded terminal activator and Tll may constitute cascading morphogenetic gradients, as illustrated by the Bcd and Hb protein gradients (see above)

Tll also contributes to the pattern in non-terminal regions. Tll protein acts as a strong transcriptional repressor. Repression by the graded Tll domain contributes to the determination of the posterior boundaries of the abdominal gap genes *kni* and *gt* (Pankratz et al. 1989, Eldon and Pirrotta 1991; Kraut and Levine 1991b).

ii) The segmentation genes: pair rule genes

The rough subdivision of the embryo into broad gap gene domains is further refined by the pair-rule genes, which divide the embryo into one-segment units (for reviews, see Kornberg and Tabata (1993); Pankratz and Jäckle (1993)). Pair rule genes are expressed in seven stripes along the embryo. Mutations in these genes affect structures either even or odd numbered parasegments (Nüsslein-Volhard and Wieschaus 1980).

Patterning of the so called primary pair rule genes is achieved by the combinatorial effects of maternal and gap gene regulators. Primary pair rule gene products in turn regulate the expression of secondary pair rule genes. The of-register expression of pair rule genes in turn leads to the activation of the patterned segment polarity genes.

Activation of primary pair rule genes: stripe by stripe regulation of gene expression

The expression of primary pair rule genes is dependent only on maternal and gap gene factors and not on other pair rule genes (although some exceptions are beginning to appear, see Pankratz and Jäckle (1993)). The three known primary pair rule genes are *even-skipped* (*eve*), which encodes a homeodomain protein (Macdonald et al. 1986; Frasch et al. 1987), *hairy* (*h*), which encodes a helix-loop-helix DNA binding protein (Holmgren 1984), and *runt*, which encodes a nuclear protein with homology to the acute myeloid leukemia gene (Kania et al. 1990; Bae et al. 1993).

The promoters of primary pair rule genes appear to be composed of modules. Each of these modules responds individually to the combinatorial input of maternal and gap factors present in the region. The best studied example of these modules is that conferring the pattern of the second *eve* stripe (the "*eve* stripe-2 element"). Hb and Bcd activate this 480 bp element so that this element directs expression in an anterior region (Frasch et al. 1987; Stanojevic et al. 1991; Small et al. 1991). Expression in this domain is restricted to a band by the repressive action of the anterior Gt domain, which determines the anterior boundary of this stripe, and Kr, which determines its posterior boundary. This 480 bp region contains binding sites for Hb, Bcd, Kr and Gt proteins and these binding sites are required for repression (Stanojevic et al. 1989; Small et al. 1991; Stanojevic et al. 1991). Thus, regulation by these factors is likely to be direct. Binding sites for the repressors Kr and Gt overlap those of the activators Bcd and Hb, so that the mechanism of repression seems to involve competitive inhibition of DNA binding.

Another well studied example is the "*h* stripe-6 element". This 500 bp region confers activation by *kni* and *gt* in a broad domain in the posterior region (Howard and Struhl 1990; Pankratz et al. 1990; Riddihough and Ish-Horowicz 1991). The refining of the anterior and posterior boundaries of this stripe occurs by repression by the Kr and Tll domains, respectively (Pankratz et al. 1990; Pankratz et al. 1990). The presence of Kr and Kni binding sites suggests that, at least for these factors, regulation of this element occurs directly (Pankratz et al. 1990).

Other *eve* and *h* stripes appear to be regulated by separate modules in a similar fashion, although there are some exceptions to this general rule (for review, see Pankratz and Jäckle (1993)).

Regulation of secondary pair rule genes: simultaneous generation of striped patterns

The patterned expression of the secondary pair rule genes depends on the activity of the primary pair rule genes. These secondary pair rule genes are *fushi tarazu* (*ftz*), and *paired* (*prd*), which encode two homeodomain proteins (Paired also has a paired-box motif) (Kuroiwa et al. 1984; Weiner et al. 1984; Laughton and Scott 1984, Kilchherr et al. 1986), and *odd paired* and *odd skipped*, both of which encode Zn finger DNA binding proteins (Kornberg and Tabata 1993; Coulter et al. 1990).

The best studied example of regulation of a secondary pair rule gene is that for *ftz*. *ftz* is first ubiquitously expressed in the embryo. Subsequently this expression retracts from the poles, forming a domain spanning almost the entire embryo (Hafen et al. 1984). This activation appears to occur by default. The nearly uniform *ftz* pattern then evolves into a series of seven stripes. The resolution of this pattern may involve repression by *hairy* (Carroll and Scott 1986; Howard and Ingham 1986; Ish-Horowicz and Pinchin 1987), *odd skipped* (Ingham and Martinez Arias 1992) and *eve* (Frasch et al. 1988).

A 600 bp region just upstream of the *ftz* promoter is capable of conferring the complete seven-striped pattern (Hiromi et al. 1985; Hiromi and Gehring 1987). Thus this element has been called the "zebra" element. Deletion analysis of this element identified positive elements involved in expression in posterior regions of the embryo, as well as negative elements involved in the repression of *ftz* in posterior cells of odd-numbered parasegments (Dearolf et al. 1989). The Caudal protein activates the posterior-specific expression of *ftz*. Caudal protein is distributed as a gradient with levels highest at the posterior of the embryo (Macdonald and Struhl 1986). The Caudal protein binds the posterior-activating element, and has been shown to directly mediate activation by this element (Macdonald and Struhl 1986; Dearolf et al. 1989).

Another protein that has been found to bind the zebra element is Ftz-F1, a Zn-finger protein with structural similarity to steroid hormone receptors (Ueda et al. 1990; Lavorgna et al. 1991). Ftz-F1 binding sites are required for the activation of this element in more anterior stripes.

Ftz-F2, a Zn-finger protein encoded by the gene *tramtrack*, has also been found to bind to the zebra element (Harrison and Travers 1990; Brown et al. 1991). Ftz-F2 is required to repress *ftz* expression in the very early syncytial stages (beginning at the third nuclear cycle), perhaps by counteracting an early general activator (Brown et al. 1991). In addition, Ftz-F2 may act later at the germ band stage to repress *ftz* in the interstripe regions (Harrison and Travers 1990; Brown et al. 1991).

Thus, the seemingly simple zebra element is regulated by multiple inputs.

iii) The segmentation genes: segment polarity genes

Segment polarity genes are expressed as sets of fourteen stripes (although some are expressed uniformly or in very broad domains). Correspondingly, mutations in these genes affect the cuticular pattern of each segment, where a part of the pattern is deleted and replaced by a mirror image duplication of the remaining pattern (for reviews, see Ingham and Martinez Arias (1992); Martinez Arias (1993)). These genes fall into four classes on the basis of their phenotype. Embryos mutant for the wingless class (*wingless, armadillo, arrow, cubitus interruptus Dominant, disheveled, fused, gooseberry, hedgehog, porcupine, smoothered*) display deletions of the posterior portion of each segment with mirror image duplications of the remaining anterior pattern (which forms the denticles). The phenotype of embryos mutant for the naked class (*naked, zeste white 3/shaggy*) is the reverse of that of the wingless class: these mutations cause deletions of the anterior portion of the segment and duplications of the posterior portion (which lacks denticle bands, hence the name of these class). Mutations in the patched class of genes (*patched, costal*) are associated with small deletions of the anterior part of each segment and duplications of the segmental boundary. Mutations in a fourth class of genes, the engrailed class (*engrailed, branched*), lead to embryos covered with denticles.

The segment polarity genes are expressed during and after cellularization of the embryo, as opposed to gap and pair rule gene products, which act within a syncytium. Thus, while the members of the previous two groups of genes encode nuclear transcription factors, the segment polarity genes code for a variety of molecules involved in different aspects of cell determination and cell-cell signalling. Some segment polarity genes, such as *wingless* and *hedgehog*, act as extracellular signals (van den Heuvel et al. 1989; Gonzalez et al. 1991; Lee et al. 1992), and *patched* codes for receptor-like products (Nakano et al. 1989; Hooper and Scott 1989). Others, like *shaggy/zeste white3* and *fused* code for protein kinases and may be involved in intracellular signalling pathways (Preat et al. 1990; Bourouis et al. 1990). The protein encoded by *armadillo* is homologous to a protein found in adhesive junctions (Peifer and Wieschaus 1990). Finally, a number of segment polarity genes encode transcription factors, such as *engrailed* and *gooseberry* (which encode homeodomain proteins, Poole et al. 1985; Baumgartner et al. 1987), *Cubitus interruptus dominant* (which encodes a Zn-finger protein, Orenic et al. 1990), and *sloppy paired* (which contains a forkhead-type DNA binding motif (Orenic et al. 1990).

Activation of *engrailed* (*en*) and *wingless* (*wg*) is one of the best studied examples of the initiation of the segment polarity iterated pattern. *wg* is expressed in the posterior

cells of each parasegment, while *en* is expressed in an adjacent cell, the anterior cell of each parasegment (Kornberg et al. 1985; DiNardo et al. 1985; Baker 1987; van den Heuvel et al. 1989). Regulation of *en* depends on the products of the genes *ftz*, *eve*, *prd* and *opa*. Prd and Opa are required to potentiate this activation in alternate segments (DiNardo and O'Farrell 1987; Ingham et al. 1988). *ftz* and *eve* are expressed in alternate parasegments in a skewed bell shape pattern with levels highest in the anterior cell of each parasegment and lower levels trailing of in more posterior cells (Lawrence and Johnston 1989). Either Ftz or Eve can activate *en* expression and repress *wg* expression (Howard and Ingham 1986; DiNardo and O'Farrell 1987; Frasch et al. 1988). This has led to a model where a threshold level of activation by Ftz or Eve leads to the restriction of *en* expression to the anterior-most cell of the parasegment, while a lower threshold level for repression restricts *wg* expression to the posterior-most cell (Ingham and Martinez Arias 1992).

en and wg function are instrumental for the patterning of the segment. It has been proposed that *en* and *wg* act in part through a binary code, where *en* activity specifies cells that secrete the first row of denticles and *wg* activity specifies the naked cuticle (Bejsovec and Wieschaus 1993). In addition, a gradient of secreted Wg protein appears to be required for the generation of cell types corresponding to the remaining positional values, and the activity of this gradient is modulated by *patched*, *engrailed*, *naked* and *hedgehog* (Gonzalez et al. 1991; Bejsovec and Arias 1991; Bejsovec and Wieschaus 1993).

Aside from the input from the pair rule genes to initiate the pattern of segment polarity gene expression, there are complex interactions that further refine and maintain it. These interactions rely on the activity of the segment polarity genes themselves, which are present during gastrulation and germ band extension when the gap and pair rule regulators have decayed. Some of these interactions are described below in the section devoted to mechanisms of maintenance of gene expression.

iv) The selector genes: homeotic genes

Organization, expression and function of the homeotic genes

The homeotic genes are required for the different segment identities of the metamerized embryo. These genes are found in large complexes. In Drosophila there are two clusters of homeotic genes, the Antennapedia complex (ANTP-C, which includes *labial*, *Deformed*, *Sex combs reduced*, and *Antennapedia*) and the Bithorax complex (BX-C, which contains *Ultrabithorax*, *abdominal A* and *Abdominal B*) (for reviews Martinez Arias (1993); Morata (1993)). These two clusters are found together forming a single unit in other organisms, such as *C. elegans* (for review, see Burglin and Ruvkun (1993)), the

beetle Tribolium (Beeman 1987), and in vertebrates (for reviews, see McGinnis and Krumlauf (1992); Krumlauf (1993)).

A unusual feature of these clusters is that the order of these genes along the chromosome (proximal to distal) is the same as the order in which these genes are expressed along the anteroposterior axis of the embryo. This colinearity is also found in the other organisms mentioned above. Its significance is not well known, although it may be related to the regulation of these gene complexes at the chromatin level (see below).

After gastrulation, the expression of homeotic genes in the ectoderm show the following spatial pattern: *labial*, anterior to parasegment (PS) 0, *Deformed* in PS0/PS1, *Sex combs reduced* in PS2, *Antennapedia* in PS3 to PS13, *Ultrabithorax* in PS5 to PS12, *abdominal A* in PS7 to PS12, and *Abdominal B* in PS13 to PS15 (see Martinez Arias (1993)). In spite of the rather broad expression domains of some of these genes, they also exhibit segment specific modulation. For example, expression of *Antennapedia* (*Antp*)and *Ultrabithorax* (*Ubx*) is stronger in PS4,5 and PS6, respectively, than in other segments. The homeobox gene *caudal*, which is not contained within these clusters, also acts as a homeotic gene and is expressed in a domain in PS15 (Macdonald and Struhl 1986). Because there are more segments than homeotic genes, some segments must be determined by the combinatorial input of more than one homeotic genes (see, for example, Struhl et al. (1992)). It has been proposed that different segmental identities arise in "mosaic" segments with different combinations of cell populations expressing different homeotic genes (Peifer et al. 1987).

Mutations in homeotic genes result in transformations of one segment identity into another. In general, homeotic genes are expressed in the segments in which they are most required, as well as in more posterior segments. Thus, loss-of-function mutations of homeotic genes result in an "unmasking" of more anterior homeotic genes, which result in transformations to the identity of more anterior segments (Lewis 1978).

The down regulation of homeotic function by more posterior genes occurs at two levels. One is at the level of transcription, where posterior homeotic genes inhibit the expression of more anterior genes. In the example just mentioned, repression of *Antp* by BX-C products occurs partly at the level of *Antp* transcription (Hafen et al. 1984; Carroll et al. 1986). Trans repression between homeotic genes may help fine tune the amounts of homeotic products and thus influence their combinatorial effect on the determination of segment identity (Peifer et al. 1987). In addition, these interactions likely stabilize the pattern of homeotic expression (see below).

An additional level of regulation must be operating in the suppression of anterior homeotic activity by posterior homeotic genes. Ectopic expression of homeotic genes from

a heat shock promoter affects the identity of segments anterior to segments in which the homeotic gene is normally active, but fails to affect more posterior segments (Gibson and Gehring 1988; Gonzalez Reyes and Morata 1990; González-Reyes et al. 1990). Thus posterior homeotic genes still inhibit the activity of more anterior genes even when the latter are expressed by a promoter that is not sensitive to cross-regulation. This phenomenon has been termed phenotypic suppression.

Dosage experiments involving the simultaneous ectopic expression of several homeotic genes suggests a model in which posterior homeotic proteins inhibit the activity of more anteriorly expressed ones not by a strict functional hierarchy but by a quantitative competition among the homeotic proteins (Lamka et al. 1992). It has been suggested that phenotypic supression occurs by competitive inhibition at the binding sites of downstream target genes (Gibson and Gehring 1988; Gonzalez Reyes and Morata 1990).

The significance of phenotypic suppression remains obscure. Nevertheless, since the thoracic segments are likely to be more similar to an ancestral segment than the more posterior abdominal segments, it has been proposed that phenotypic suppression evolved as a way to determine more posterior identities in spite of the presence of homeoproteins conferring more anterior segment identities (Morata 1993). Moreover, this mechanism may be much more important in organisms such as vertebrates, where there seems to be no downregulation by other homeotic genes in posterior regions (see below).

In addition to homeotic genes, there are other genes which lead to homeotic transformations when mutated without affecting the expression of the homeotic genes per se. One such gene, *extradenticle*, may encode a cofactor required for the target specificity of homeotic proteins (Peifer and Wieschaus 1990) Another gene, *teashirt*, which encodes a protein with a Zn finger DNA binding motifs, is specifically required for the determination of PS3 and more generally for defining the basal identity of segments in the trunk region (Fasano et al. 1991; Roder et al. 1992). Recently, the Zn finger *spalt* gene has been proposed to inhibit teashirt expression and thus promote head identities (Kühnlein et al. 1994).

The homeotic genes all code for transcription factors containing the homeo-box DNA binding motif (reviewed in Levine and Hoey (1988). Thus they are likely to control segment identity by directly regulating transcription of downstream target genes. A number target genes for homeotic genes have been found through genetic and molecular techniques (reviewed by Morata (1993)). Some of these genes are transcription factors themselves. For example, the gene *empty spiracles*, which is required for the development of the posterior larval spiracles, codes for a homeodomain DNA binding protein and is directly activated by the Abdominal-B protein via cis acting sequences in its promoter (Dalton et al.

1989; Jones and McGinnis 1993). This suggests that there is a hierarchy of activation cascades downstream of the homeotic genes where additional transcription factors define subsegmental domain identities.

Initiation of boundaries of homeotic gene expression

The establishment and refinement of the pattern of homeotic gene expression has been shown to depend on multiple inputs from the gap genes and segmentation genes. In some instances, an involvement of maternal coordinate genes has also been observed (see below). In general, gap genes determine the boundaries of the broad domain in which homeotic genes can be expressed, and segmentation genes are used to regulate homeotic genes expression within each segment.

Genetic studies involving loss-of-function gap gene mutations and, in some cases, ectopic gap gene expression have suggested that gap genes initiate the boundaries of the domains in which homeotic genes can be active. Activation by Hunchback and the maternal morphogen Bicoid restrict the expression of *Deformed* to the anterior region, and the anterior Tailless domain determines by repression its anterior boundary (Jack and McGinnis 1990; Reinitz and Levine 1990). The *Antp* P1 promoter is activated by Kr (Irish et al. 1989b). The *Antp* P2 promoter, which is expressed in two bands in the anlagen of PS4 and the third thoracic segment, is activated by Hb and to some extent by Kr, and this activation may define the posterior boundary of expression from this promoter (Harding and Levine 1988; Irish et al. 1989b).

Regulation of *Ubx* is the best studied case of initiation of homeotic genes. The anterior boundary of the *Ubx* expression domain is established just anterior to PS5 by repression by Hb (White and Lehmann 1986; Irish et al. 1989b). The posterior *Ubx* boundary is determined at PS13 by repression from Tll (Reinitz and Levine 1990).

Promoter fusion constructs using promoter and the *Ubx* basal promoter have shown in vivo that segments form regulatory regions of *Ubx* (the bx, PBX and ABX regions) can confer on the *Ubx* basal promoter patterns with boundaries similar to that of the endogenous *Ubx* expression. The anterior boundaries of expression of these constructs have been shown to depend on repression by Hb, and in the case of the bx fragment, the posterior boundary has been shown to depend on *tll* function (Qian et al. 1991; Zhang et al. 1991; Müller and Bienz 1992). Hb-binding sites have been found in these three regions, and, in the cases bx and PBX subfragments, mutation of these sites have been shown to lead to derepression of expression similar to that produced by *hb* mutations (Qian et al. 1991; Zhang et al. 1991; Müller and Bienz 1992). Repression in anterior regions can be mimicked by Multiple Hb-binding sites inserted into an ubiquitous enhancer element from

the *Ubx* region (Zhang and Bienz 1992). Thus Hb is directly involved in the determination of the anterior Ubx boundary.

Gap genes have also been shown to regulate the expression of *Abdominal-B* (*Abd-B*). Activation of *Abd-B* requires *tll* activity, and its anterior boundary is determined by repression from the Kr gene product (Harding and Levine 1988). Studies from a fusion construct containing a fragment from the IAB5 control region driving the *Abd-B* promoter show that, in addition, repression by Kni and Hb downregulate the expression of these gene in PS10 and PS14, respectively (Busturia and Bienz 1993).

The pattern of homeotic expression is refined by regulation by pair rule gene products. The activity of *ftz* has been shown to activate homeotic genes in stripes within the domain of expression allowed by gap gene regulation, *Sex combs reduced* in PS2, *Antp* in PS4, *Ubx* in PS6 (Duncan 1986; Ingham and Martinez-Arias 1986). The bx and PBX fragments described above from the *Ubx* regulatory region, as well as the IAB5 fragment from the *Abd-B* regulatory region are expressed in even numbered parasegments and their activation requires *ftz* activity (Qian et al. 1991; Müller and Bienz 1991; Müller and Bienz 1992; Busturia and Bienz 1993).

Presumably, *eve* function has a similar role for the odd-numbered parasegments. This is supported by the fact that the ABX fragment from the *Ubx* regulatory region directs expression in odd number parasegments in an *eve*-dependent manner (Müller and Bienz 1992).

The regulation of homeotic genes by pair rule genes appears to have an important role in embryonic development. The requirement of *ftz* and *eve* activity for the activation of homeotic genes insures the precise register of parasegments, formed by the segmentation genes, and homeotic gene expression. In addition, because the *ftz* and *eve* expression pattern has an intrinsic polarity within a segment (a sharp anterior boundary at the parasegmental boundary and a gradual fading posteriorly) activation by these genes provides a similarly graded *Ubx* pattern within each segment (see, for example, Müller and Bienz (1992)).

The regulation of *Ubx* expression likely involves competitive inhibition between activators and repressors at the level of DNA binding. A 248 bp subfragment of the PBX region that directs expression in a striped pattern has been found to contain 3 Hb and 6 Ftz protein binding sites (Müller and Bienz 1992). In vivo testing of PBX-driven constructs with mutations in the Ftz and/or the Hb sites in vivo has shown that Ftz binding sites are required for expression in even parasegments and Hb-binding sites are required for the limits of this expression. Many of the binding sites for these two factors are found close to each other, which suggests that Ftz and Hb proteins directly compete for binding to the

PBX regulatory region. A similar arrangement of overlapping binding sites for activators and repressors is found in the bx enhancer region of *Ubx*, and in this case, competitive binding to the promoter has been mimicked in vitro (Qian et al. 1993). Competitive inhibition between positive and negative transcription factors has been proposed to contribute to the sharpening of Ubx boundaries near the parasegmental borders.

Segment polarity genes have also been shown to modulate the expression of homeotic genes. *engrailed*, for example contributes to the down regulation of *Ubx* in posterior compartments (Martínez-Arias and White 1988). The expression of segment polarity genes is in turn modulated in a segment specific manner under the influence of the homeotic genes (Martinez Arias 1993). Thus the final stages of segment differentiation may involve the interplay between segment polarity and homeotic genes.

Recently, alternative modes in homeotic gene patterning have been reported (for review, see Morata (1993)). Of particular interest is the regulation of homeotic patterning in the endoderm, which forms after the expression of gap and segmentation genes has subsided. Patterning in the endoderm requires inductive signals, such as decapentaplegic and wingless, that are secreted from the adjacent visceral mesoderm (Immergluck et al. 1990; Panganiban et al. 1990; Reuter et al. 1990).

v) Homeotic genes in other animal species

Homeotic genes homologous to the ANTP/BX complexes in Drosophila have been found in a wide variety of organisms, from unsegmented Hydra and nematode species to vertebrates (Beeman 1987; Shenk et al. 1993; McGinnis and Krumlauf 1992; Burglin and Ruvkun 1993; Krumlauf 1993). The structure of these complexes is very similar to that in Drosophila, which suggests that they are all derived from a common ancestral complex (incidentally, in Drosophila, but not in other organisms studied, this complex has split into the two subclusters, the Antp-C and Bx-C). This suggests that these genes have been utilized and conserved throughout evolution for the regional patterning of the embryonic axis. Here I will briefly describe the organization and known function of Homeotic clusters in the nematode *C. elegans* and the mouse as models for homeotic function in unsegmented organisms and vertebrates, respectively.

The *Caenorhabditis elegans* homeobox gene cluster

The Hox cluster in C. elegans contains five genes arranged in the order *ceh-13*, *lin-39*, *mab-5*, *egl-5* and *ceh-23* (Bürglin et al. 1991; Kenyon and Wang 1991; Wang et al. 1993). Sequence homologies suggests that these genes are homologous to the genes in the

Drosophila homeotic clusters: *ceh-13* is a *labial* homologue, *lin-39* seems to be related to an ancestor of *Deformed* and *Sex combs reduced*, *mab-5* may be related to an ancestor of *Antp*, *Ubx* and *abd-A*, and *egl* is most similar to *Abd-B*. *ceh-23* is most similar to *ems*, which in Drosophila is not part of the homeotic cluster (although it is functionally related to it, see above), but is also present at the end of vertebrate homeotic clusters.

The order the genes in the chromosome is similar to the order of their homologues in Drosophila and vertebrates (although in C. elegans the order of *ceh-13* and *lin-39* has been inverted). As in Drosophila, this order is colinear with the domains along the embryonic axis that express and require these genes.

Mutations in these genes lead to the transformation of cell identities in different embryonic regions as evidenced by their altered patterns of cell division and migration and the structures that they form. Many of the cell fate changes produced by loss-of-function mutations are, like in Drosophila, posterior to anterior transformations, although transformations in the opposite direction are also observed (reviewed by Burglin and Ruvkun (1993)).

lin-39, *mab-5* and *egl-5* are required for the identity of central, posterior and tail regions (Kenyon 1986; Chisholm 1991); Clark et al. 1993; Wang et al. 1993). Accordingly, constructs carrying the promoter regions of these genes driving *lac Z* expression show that these genes are expressed in overlapping regions. These regions of expression correspond to the domains affected by their respective mutations. A similar construct carrying the *ceh-13* promoter shows that this gene is also expressed in a spatially restricted manner (Burglin and Ruvkun 1993). *ceh-23* also has a pattern of expression reminiscent of its fly and vertebrate homologues (Wang et al. 1993). Genetic studies show that, as in Drosophila, a combination of genes in regions of overlap of homeotic gene expression is important for certain cell identities (Clark et al. 1993; Wang et al. 1993).

The mouse homeobox gene cluster

In vertebrates, like in Drosophila, duplication of the genes within clusters has led to complexes with more genes than the presumed ancestral homeotic cluster, which is likely more similar in its structure to the *C. elegans* homeotic cluster (for reviews, see McGinnis and Krumlauf (1992); Burglin and Ruvkun (1993); Krumlauf (1993)). The vertebrate clusters as a whole have in addition undergone a process of repeated duplications, so that in vertebrates there are, not one, but four clusters of homeotic genes.

Like in other organisms, there is a colinearity between the position of vertebrate homeotic genes in the cluster and the domain along the axis in which they are expressed. Expression of vertebrate homeotic genes is less region specific than the corresponding

expression of Drosophila homeotic genes. While the latter often have anterior and posterior restrictions in their domains of expression, vertebrate homeotic genes are expressed in a contiguous domain that extends from an anterior boundary to the posterior end of the embryo. This arrangement, coupled to the phenomenon of posterior prevalence (see below), suggests that a particular gene acts in its anterior region of expression, where it is not coexpressed with other genes of the cluster. Because there are four such clusters, the identity of each segment may be defined by the combinatorial effect of the "unique" genes from each cluster. Alternatively, different clusters may contribute to the specification of different cell types within the segment which together interact to give the segment its particular identity.

Genetic studies involving gene "knock-out" and ectopic expression experiments have shown that mouse homeotic genes have properties that are remarkably similar to those of Drosophila homeotic genes (reviewed by Krumlauf (1993)). In short, loss of function mutations lead to posterior to anterior transformations and, at least in some cases, ectopic expression of genes confers a more posterior identity to more anterior segments. Similar to the phenotypic suppression of Drosophila (see above), the posterior-most expressed gene confers the identity of the segment and can not be overridden by ectopic expression of a more anteriorly expressed gene. This phenomenon is referred to as "posterior prevalence".

Drosophila and mouse homeotic genes may be similar not only in structure and organization, but also in some aspects of their regulation and function. The mouse *Deformed* (*Dfd*) homologue *Hox4b*, for example, appears to be controlled, like the Drosophila *Dfd*, by autoregulation (Regulski et al. 1991; Propperl and Featherstone 1992), and mouse and Drosophila *Dfd* cis acting regulatory sequences can partially substitute for each other in their respectively heterologous environment (Awgulewitsch and Jacobs 1992; Malicki et al. 1992). The homeobox proteins themselves may also be similar in function: ectopic expression of both the *Antp* and *Dfd* mouse homologue proteins can partially recreate the transformation phenotypes produced by the ectopic expression of their Drosophila counterparts (Malicki et al. 1990; McGinnis et al. 1990). Thus some elements in the upstream and downstream regulatory network of Drosophila and mouse genes have been conserved throughout evolution.

Nevertheless there are likely important differences in homeotic regulation and function between Drosophila and vertebrates. As opposed to Drosophila, where the presence or absence of a homeotic product transform segment identities in opposite directions, in the mouse the precise dose of the homeotic product may also be important for segment identity. This is suggested by the fact that both loss-of-function and ectopic expression of *Hoxc8* produce posterior to anterior transformations (Le Mouellic et al. 1992;

Pollock et al. 1992). In addition, the regulation of mouse homeotic products is likely very different from that in Drosophila, given that it occurs in a cellular environment, as opposed to the Drosophila syncytium.

Recently, some advances have occurred in the understanding of the regulation of homeotic gene expression. Mutations in the Retinoic acid receptor γ (RARγ) have been found to produce phenotypes similar to those of *Hox-b4* mutations (Lohnes et al. 1993; Ramirez-Solis et al. 1993). This suggests that *Hox-b4* is a major target of a pathway involving the retinoic acid and RARγ. The idea that retinoic acid regulates homeotic genes further supported by studies where exogenous retinoic acid leads to shifts in Hox expression in a concentration and time dependent manner (Morriss-Kay et al. 1991 Marshall et al. 1992; Conlon and Rossant 1992).

A gene that is known to directly regulate homeotic gene expression is *Krox-20*, which encodes for a Zn-finger transcription factor (Gilardi et al., 1991). *Krox-20* expression is restricted to rhombomeres 3 and 5, and mutations in *Krox-20* lead to the reduction or elimination of these segments (Wilkinson et al. 1989, Schneider-Maunoury et al. 1993). Endogenous and ectopic *Krox-20* protein drives the expression from a *Hox-2b* promoter in the mouse embryo, and this activation is dependent on the presence of Krox-20-binding sites in the *Hoxb2* promoter region (Sham et al. 1993). Thus, direct activation by Krox-20 is used in vivo to upregulate expression of *Hoxb2* in the third and fifth rhombomeres.

Clearly, given the obvious different morphologies between Drosophila and vertebrates, the targets of homeotic products in each system must also be different. As mentioned above, and specially in vertebrates, little is currently known about these target genes.

5. Stabilization of transcriptional states in Drosophila

Gene expression patterns are often initiated by regulatory products that are transiently expressed. In many cases, the pattern that is initially established persists in a stable manner even after the transient regulators are no longer present. A clear example is the lasting regulated expression of segment polarity and homeotic genes during embryogenesis, even though the gap and pair rule gene products that initiated their expression decay shortly after the beginning of gastrulation. Although not all cases of maintenance of gene expression are as obvious as in this example, molecular genetic analysis suggests that, in other cases, more subtle maintenance phases occur and are important for the stabilization of gene expression patterns. An understanding of the

importance of these stabilizing interactions is essential for the appreciation of the results presented in this thesis. This section describes different ways in which these maintenance phases are achieved.

A) Stabilizing cross-regulatory interactions

Here, I describe cross-regulatory interactions that maintain or stabilize established patterns of gene expression. I have divided these interactions into two groups: interactions whose disruption leads to an observable destabilization of gene expression boundaries (essential interactions), and those without an obvious effect but which are inferred to provide robustness and stability to the patterning system (redundant interactions).

Although cases for both essential and redundant stabilizing interactions can be found in all tiers of zygotic gene expression, I provide examples mainly from the tier of gap gene regulation. Part of this bias reflects the fact that cross-regulatory interactions among gap genes are more thoroughly understood than those among other sets of genes. In addition, it is this tier of zygotic gene expression that is most relevant to the studies presented in this thesis. In these examples, it is useful to remember the order of the main domains of gap gene expression along the anteroposterior axis, from anterior to posterior: anterior *hb*-*Kr*-posterior *kni*-posterior *gt* (in addition, the anterior *gt* domain overlaps that of *hb*)

This description does not intend to be exhaustive, but rather provide a flavor for the use and value of cross-regulatory interactions.

i) Essential cross-regulatory interactions

An important stabilizing element in gap gene patterning is the product of the gene *Krüppel* (also, see below). The anterior boundary of the posterior domain of *gt* expression is initiated by the Hb protein gradient. In *Kr* mutants, this boundary is initiated at its normal position, but shifts anteriorly at the late cellular blastoderm stage (Eldon and Pirrotta 1991; Kraut and Levine 1991b; Struhl et al. 1992). Thus, Kr acts as a negative regulator of *gt* to stabilize the anterior *gt* boundary.

Another example of stabilizing cross-regulation is the interdependence of *en* and *wg* expression in adjacent sets of cells through an extracellular signalling process (see below).

As already described, cross regulatory interactions between homeotic genes are also important to maintain their patterns of expression. In general, a homeotic gene tends to repress the expression of other homeotic genes that are normally expressed more anteriorly.

The functional significance of this interaction has been challenged since the ectopic expression of homeotic genes using a heat shock promoter does not override the activity of more posterior homeotic genes (Gibson and Gehring 1988; Gonzalez Reyes and Morata 1990; González-Reyes et al. 1990). (As described above, this phenomenon has led to the concept of phenotypic suppression). Nevertheless, in these experiments the ectopic homeotic product may have been induced too late to exert an effect. Experiments where the heat shock is administered at earlier stages show that ectopic homeoproducts can indeed influence the identity of more posterior segments (Lamka et al. 1992) . This suggests that homeotic cross-regulation is likely important at early stages for the stabilization of homeotic gene patterns.

ii) Redundant cross-regulatory interactions

A genetic interaction is redundant when other interactions can provide a similar function. The interactions can be equally important, in which case a similar (or no) effect results from disrupting either one, whereas disruption of both produces a strong effect. Perhaps more commonly, some interactions may be stronger than others. In this case eliminating the stronger interactions may lead to phenotypes, while disrupting the weaker interactions may not. In some of the cases described below, the stronger interaction(s) of the redundant set of unequal strength has already been described as what appears to be the main component in pattern initiation.

It has been proposed that redundant interactions accumulate during evolution to increase the stability of biological systems, which are inevitably subject to internal and external fluctuations (Tautz 1992). Redundant interactions may provide "fall-back" positions on which to rely in case the primary interaction fails. In addition, a set of redundant regulators may act in a combinatorial fashion and provide a more robust mechanism of gene/patterning regulation.

Numerous redundant interactions have been reported to occur during early zygotic gene patterning (see for review, Hülskamp and Tautz (1991)). For example, either Bcd or Hb proteins alone can activate *Kr* expression (Hülskamp et al. 1990). The level of *Kr* expression in mutants suggests that Hb contributes more to the overall activation of *Kr* than Bcd. The activation conferred by these two products appears to be direct (Hoch et al. 1991; Hoch et al. 1992).

Kr expression is also subject to multiple negative regulation. Ectopically expressed Gt can act as a repressor of *Kr* (Eldon and Pirrotta 1991; Kraut and Levine 1991b). In an otherwise wild-type background, however, mutations in *gt* do not lead to changes in *Kr*

(Gaul and Jäckle 1987; Eldon and Pirrotta 1991; Kraut and Levine 1991b). Similarly, ectopic Tll can represses *Kr* expression, although *Kr* expression is normal in *tll* mutants (Gaul and Jäckle 1987; Klingler et al. 1988); Hoch et al. 1992). Thus, both Gt and Tll have the potential to repress *Kr* expression, thereby stabilizing its boundaries of expression. In both cases, the interactions are likely to be direct, since Gt- and Tll-binding sites are found in the *Kr* promoter (Hoch et al. 1992; Capovilla et al. 1992; Pankratz and Jäckle 1993). Nevertheless, as explained above, the *Kr* boundaries are primarily determined by other factors, such as Bcd, Hb and Kni.

Another example of redundant negative regulation is the repression of *kni* by Kr. In *Kr* mutants, the posterior domain of *kni* expression is not altered, while in hb^{zyg} mutants it is shifted anteriorly (Pankratz et al. 1989; Hülskamp et al. 1990). When the embryos are doubly mutant for hb^{zyg} and *Kr*, the anterior *kni* boundary shifts further anteriorly (Hülskamp 1991). Thus, although the anterior *kni* boundary is mainly determined by repression by Hb, repression by Kr is also likely to be involved in the determination of this boundary. Repression by Kr may be responsible for the stabilization of the anterior *kni* boundary that is initiated by Hb in a manner analogous to the effect of Kr on *gt* as described above. In the case of *kni*, however, regulation by other factors is strong enough so that single *Kr* mutations do not have an observable effect on *kni* expression boundaries.

Two general observations support a role for redundant interactions in the patterning process. First, the strength of both redundant and non redundant interactions are modulated in accordance with the spatial positions of the genes involved. For example, in the cross-regulatory interactions between Kr, kni and gt, the stronger repressive interactions are those between genes that are not directly adjacent to each other (i.e. Kr and gt), while immediate neighbors (Kr-kni and kni-gt) interact only weakly. The fact that redundant interactions accommodate to this rule suggests that they provide real regulative roles which are modulated by evolution.

In addition, as pointed out by Pankratz and Jäckle (1993), even if when a gene is mutated there are no changes in the boundaries of expression of other genes, it is possible that the product of the first gene regulates the other genes at locations in the embryo other than their boundary positions. Molecular information on the *Kr* promoter can be used to exemplify this point. The CD1 element from the *Kr* upstream region, which can confer *Kr* expression patterns to heterologous genes, contains binding sites for Hb, Bcd, Kni, Tll and Gt proteins (Hoch et al. 1990; Jacob et al. 1991; Hoch et al. 1992). Genetic studies show that the anterior boundary of *Kr* is determined by Hb repression (Hülskamp et al. 1990; Struhl et al. 1992), while mutating *tll* function has no effect on *Kr* (Gaul and Jäckle 1987). Thus, just anterior to the *Kr* anterior boundary, the CD1 element is likely occupied and

repressed primarily by Hb protein. At the anterior tip of the embryo, however, where the *hb* expression domain normally retracts (Tautz 1988), binding and repression by Tll could be much more important. Interestingly, Tll binding sites overlap most of the binding sites which mediate activation of Bcd, which is present at high concentrations in this anterior region (Hoch et al. 1992). Thus, the Tll protein, which is seemingly unimportant in the regulation of *Kr*, may be important in counteracting the activation of *Kr* by Bcd in anteriormost regions.

iii) The Kr protein gradient: a stabilizing morphogenetic gradient?

The Kr protein, distributed in a bell-shaped domain that is activated in the middle of the embryo by Hb and Bcd, has properties reminiscent of a morphogenetic gradient. Nevertheless, the ability of Kr to regulate genes in a concentration dependent manner appears to be involved mainly in secondary interactions, i.e. in the refinement and/or stabilization of boundaries. Most of these interactions have been described in the above sections. Anteriorly, high levels of Kr repress *hb* and contribute to the refinement of its posterior boundary. Posteriorly, Kr may have a dual function, acting both as an activator and a repressor of genes. High and moderate levels of Kr appear to be involved, respectively, in the stabilization of the anterior boundaries of the posterior *kni* and *gt* domains, which are initiated by the Hb gradient. In addition, low levels of Kr may be required to activate *kni*, and thus contribute to the stabilization of the posterior *kni* boundary.

The idea that Kr acts as an activator of *kni* at low concentrations was proposed when *Kr* mutations were found to decrease the levels of posterior *kni* expression (Pankratz et al. 1989). The significance of this finding remains unclear, since these results could be simply explained indirectly, by the fact that Kr is required to inhibit Gt, which in turn is a repressor of *kni* (Capovilla et al. 1992). Nevertheless, some data suggests that Kr may indeed act as a transcriptional activator at low concentrations. Deletion of a *kni* promoter region containing Kr-binding sites leads to decreased expression of a reporter gene (Pankratz and Jäckle 1993; this report has the caveat that this region is large and may be regulated by other factors). More appealing support for this idea is provided by the fact that single Kr-binding sites have been found to mediate reporter gene activation at low Kr concentrations in tissue culture cells (Sauer and Jäckle 1991). Thus Kr can mediate trancriptional activation.

In the last study, it was found that, while Kr-binding sites mediate activation at low Kr concentrations, higher concentrations of Kr lead to transcriptional repression. More

recent studies have found that this behavior depends on Kr dimerization: at low Kr concentrations Kr monomers activate transcription, and at high concentrations Kr dimerizes and acts as an repressor through the same target sequences (Sauer and Jäckle 1993). It is not known whether the similar dosage-dependent behavior of the Hb protein (see above) depends on a similar mechanism. These studies support the idea that Kr, like Hb, may act as a concentration-dependent regulator of gap gene expression in the embryo.

iv) Redundancies at the level of patterning systems

Redundancy can also be observed at the level of patterning systems. In Drosophila, perhaps one of the best examples of this type of redundancy are the maternal systems that determine anteroposterior polarity: the *bcd*-dependent Hb^{zyg} and the *nos*-dependent Hb^{mat} morphogenetic gradients. As described above, each of these gradients can on their own organize the embryo into the major gap gene pattern *Kr-kni-gt*.

In this case, the Hb^{mat} protein gradient appears to provide the weaker input. The levels of Hb^{mat} protein are lower, so that its long-range effects are weaker. As a consequence, boundaries of *kni* and *gt* determined by the Hb^{mat} protein gradient alone are shifted anteriorly with respect to the wild-type borders (and determination of the anterior *Kr* boundary by repression, which requires high Hb protein levels, cannot be attained) (Hülskamp et al. 1990; Struhl et al. 1992). Moreover, while the Hb^{zyg} protein gradient is essential for proper gap gene patterning, elimination of the entire Hb^{mat} patterning system does not have major consequences on this process (Hülskamp et al. 1989; Hülskamp et al. 1990; Irish et al. 1989a; Struhl 1989; this thesis). Presumably, the apparently dispensable function provided by the Hb^{mat}-mediated system is nevertheless important for the robustness of the patterning process, a topic which will be further developed in this thesis.

B) Autoregulation

Autoregulation can be defined as the control of the activity of a gene by its own product. Here, I will provide a brief description of autoregulatory processes that occur at the level of gene transcription.

i) Positive autoregulation.

Direct positive autoregulation

The activation of a gene by its own product can provide a means to maintain the expression of a gene after its original activators are no longer present. This process may be particularly important for the regulation of segment polarity and homeotic genes, whose expression patterns persist much longer than the molecules that initiate them. In addition, as demonstrated for *eve*, an autoregulatory loop can enhance the activity of a gene and, under some circumstances, contribute to the refinement of expression patterns.

A 100 bp sequence located about 5 Kb upstream of the *eve* promoter can confer a *eve*-like pattern to a heterologous promoter (Harding et al. 1989; Jiang et al. 1991). This expression is dependent on *eve* function, and thus this sequence is an autoregulatory element. The minimal autoregulatory element contains two Eve-binding sites; these have been shown to be essential for autoregulation in vivo.

Autoregulation of *eve*, aside from contributing to the maintenance of *eve* expression, has been proposed to mediate the sharpening of the anterior *eve* boundary (Warrior and Levine 1990; see also Ingham and Martinez Arias 1992). When they first appear, *eve* stripes have a symmetrical bell-shape. Each *eve* stripe later adopts a skewed shape with a sharp anterior boundary and a gradually decreasing posterior tail. *eve* stripes are overlapped on different sides by stripes of *hairy* and *runt* expression. Negative regulation by Runt in the posterior end of each *eve* stripe, coupled with rapid turnover of Eve gene products may lead to a skewing of the stripe towards the anterior. This tendency would then be enhanced and stabilized by autoregulation of *eve* expression above a certain threshold level of Eve protein.

A similar process may be used for the regulation of *ftz* in the alternate parasegments. The *ftz* gene contains a cis-acting autoregulatory element (Hiromi and Gehring 1987). Autoregulation of *ftz*, coupled with repression by a different set of factors may also be implicated in the sharpening of the anterior *ftz* boundaries (Ingham and Martinez Arias 1992).

Autoregulatory loops have also been described for homeotic genes. As mentioned above, a 920 bp element 5 Kb upstream of the *Dfd* promoter contains four Dfd-binding sites required for autoregulation in vivo (Kuziora and McGinnis 1988; Regulski et al. 1991; González-Reyes et al. 1992). Similarly, autoregulatory elements for the expression of labial in different tissues have been found in the labial promoter (Chouinard and Kaufman 1991; Tremml and Bienz 1992).

Finally, the presence of binding sites for their respective gene product has been reported for several genes, including *hb* (Stanojevic et al. 1989), and *Ubx* (Beachy et al. 1988; Irvine et al. 1993), and may be indicative of direct autoregulatory interactions. In particular, Ubx has been shown to act as a direct positive transcriptional activator of its own promoter (Johnson and Krasnow 1990).

Indirect positive autoregulation

In some cases, autoregulation has been shown to occur indirectly and depend on extracellular signalling. It has been proposed that indirect autoregulation which involves cellular interactions provides a way to coordinate cell fate decisions in the context of cell populations (Bienz 1992).

Extracellular signalling may mediate, at least partially, *Ubx* autoregulation in the visceral mesodern. In this tissue, *Ubx* activity is indirectly required for its own expression. *Ubx* activity is required for the activation of an extracellular signal encoded by *decapentaplegic* (*dpp*) (Reuter et al. 1990). Dpp is in turn required for the activation of *wingless* in the adjacent cells (Immergluck et al. 1990). Both Dpp and Wg activity feeds back on the *Ubx*-expressing cells and is required to maintain *Ubx* expression (Panganiban et al. 1990; Thüringer and Bienz 1993).

It has been proposed that the indirect autoregulation of *Ubx* in the visceral mesoderm may be similar to a phase of maintenance of *engrailed* expression (Bienz 1992). In the latter case, *en* autoregulation is driven via interactions with adjacent *wg*-expressing cells. *Hedgehog*, which encodes an extracellular signalling molecule, is expressed in the *en*-expressing cells, and is required for *wg* activation (Lee et al. 1992). The Wg signal completes the feed back loop by acting on the *en*-expressing cells to maintain *en* expression (Martinez-Arias et al. 1988; DiNardo et al. 1988).

Multiple phases of regulation

Many genes are likely to use multiple modes of regulation during different phases of their expression. The gene *engrailed (en)* provides a good example of this phenomenon (Heemskerk et al. 1991). The initial activation of *en* depends on pair rule gene products. Once initiated, *en* expression becomes dependent on *wg* activity by the indirect autoregulatory loop. Later, a requirement for autoregulation persists, but it is no longer dependent on *wg* activity and may be direct. In the final stage, *en* is no longer dependent on its own function and may be maintained by the activity of more global factors such as the trithorax group genes (see below).

ii) Negative autoregulation

Negative autoregulation, in which a product represses its own expression, can be utilized in two ways. First, it can stabilize levels of expression through a negative feedback loop. Second, it can decrease or completely eliminate its own gene expression.

Ubx again provides one of the best studied examples (Irvine et al. 1993). *Ubx* activity is required to repress the expression of a *lacZ* gene fused to the 35 Kb *Ubx* upstream regulatory region. Moreover, varying the dosage of *Ubx* from 0 to four copies affects the expression of the *Ubx-lacZ* construct as well as the endogenous *Ubx* in a manner consistent with a negative autoregulatory loop.

This mechanism leads to an adjustment in the expression of *Ubx* when its gene dosage is changed. For example, lowering the dose of *Ubx* to half of the wild-type level reduces *lacZ* or the endogenous Ubx expression to a lesser extent, presumably because of a relief in negative autoregulation. Conversely, additional doses of *Ubx* have less effect on *lacZ* or *Ubx* expression than expected. The fact that *Ubx* doses near wild-type levels have an observable effect on expression suggests that this mechanism is used in vivo to stabilize the levels of *Ubx* expression.

The same study shows that autoregulation of *Ubx* is required for the spatial modulation of its expression. Absence of endogenous *Ubx* leads to ectopic expression of both *Ubx* and the *Ubx-lacZ* construct. Implicit in this mechanism is the idea that cells that are dependent on *Ubx* function to turn off *Ubx* expression did indeed express *Ubx* initially. Thus the temporal and spatial patterns of *Ubx* expression are modulated by Ubx itself.

C) Maintenance at the level of chromatin regulation

An additional level of maintenance of transcriptional regulation is mediated by two sets of genes, the Polycomb-group genes and the trithorax-group genes. Although not conclusive, the studies in Drosophila, as well as in other systems, including the Drosophila regulators of heterochromatin (modifiers of position effect variegation) and the yeast SPT/SIN and SWI/SNF families, have led to the idea that the Polycomb and trithorax families are involved in chromatin regulation (for reviews, see Paro (1990); Bienz (1992); Kennison and Tamkun (1992); Winston and Carlson (1992); Kennison (1993)). Here, I will present a summary of the information available on three Drosophila and one yeast gene families.

i) The Polycomb group of genes

The Polycomb group (Pc-G) of genes was originally identified by the homeotic phenotypes they produce when mutated. The Polycomb family includes the genes *Additional sex combs* (*Asx*, Jürgens 1985; Sinclair et al. 1992), *Enhancer of zeste/polycombeotic* (*E(z)*, Kalisch and Rasmuson 1974; Shearn et al. 1978; Wu et al. 1989; Jones and Gelbart 1990; Phillips and Shearn 1990) *extra sex combs* (*esc*, Struhl 1981)), *pleiohomeotic* (*pho*, Duncan 1982)), *Polycomb* (*Pc*, Lewis 1978; Duncan and Lewis, 1982), *Polycomb like* (*Pcl*, Duncan 1982; Breen and Duncan 1986), *polyhomeotic* (*ph*, Jürgens 1985; Dura et al. 1987), *Posterior sex combs* (*Psc*, Jürgens 1985; Adler et al. 1991), *Sex combs extra*, (*Sce*, Breen and Duncan 1986), *Sex combs on midleg* (*Scm*, Jürgens 1985), and *super sex combs* (*sxc*, Breen and Duncan 1986). On the basis of synergistic phenotypic interactions which result in homeotic transformations, it has been estimated that there are a total of about 40 genes that belong to this family (Jürgens 1985). In addition, the gene *Suppressor of zeste 2* (*Su(z)2*) may be loosely classified in this family. Although it does not promote homeotic transformations, it is located in the same complex as the *Psc* gene, and genetic and biochemical data suggests that it interacts with other Pc-G genes products (Adler et al. 1989); Rastelli et al. 1993; Chapter 3 of this thesis; see below).

Phenotypes of Pc-G genes

Mutations in Pc-G genes produce homeotic transformations. Homozygous mutant embryos for the prototype gene, *Polycomb*, for example, exhibit strong transformations of all segments into the eighth abdominal segment (Lewis 1978; Duncan and Lewis, 1982). This embryonic "Pc" phenotype is produced by the ubiquitous derepression of homeotic genes and the posterior dominance of *Abdominal-B*, the homeotic gene expressed in the eigth abdominal segment (Struhl 1981; Jürgens 1985; Struhl and White 1985; (Struhl and Akam 1985; Dura and Ingham 1988; Glicksman and Brower 1990; Jones and Gelbart 1990; McKeon and Brock 1991; Simon et al. 1992). In the adult, Pc-G mutations also produce homeotic transformations, which also result from ectopic expression of homeotic genes (Struhl 1981; Duncan 1982; Busturia and Morata 1988; Glicksman and Brower 1990; Jones and Gelbart 1990; Martin and Adler 1993).

The phenotype exhibited by null mutations in different Pc-G genes is variable. This variability depends, at least in part, on the different maternal contribution of these genes. For example, Pc itself has very little maternal contribution, which is consistent with the strong zygotic phenotype exhibited by these embryos (Haynie 1983; Lawrence et al. 1983).

Embryos mutant for *Asx*, *Pcl*, *Psc*, *Sce* and *Scm* all die as embryos or first instar larvae and exhibit weaker homeotic transformations, a phenotype which is enhanced in the absence of their respective maternal products (Breen and Duncan 1986, Martin and Adler 1993). Other Pc-G genes, such as *E(z)* and *pho*, provide enough maternal information to reach more advanced stages of development, such as early pupae and pharate adults, respectively. The absence of maternal product E(z) or Pho product, though, leads to embryonic lethality due to homeotic transformations (Breen and Duncan 1986; Jones and Gelbart 1990); Phillips and Shearn 1990). In the case of *esc*, maternal product provides most of its required function: *esc* homozygotes from heterozygous mothers reach to be viable adults, and homozygous mutant females produce embryos with very strong homeotic transformations (Struhl 1981). The gene *ph* seems to be strongly required both maternally and zygotically (Dura et al. 1988; see below).

Except for *esc*, which may be specific for the regulation of homeotic genes (Moazed and O'Farrell 1992; Chapter 3 of this thesis) Pc-G genes likely regulate other target genes. Some of these targets appear to be the segmentation genes. Mutations in *ph*, *Pc*, *Scm*, *Pcl*, *Psc*, *pho* and *E(z)* are required for the repression of the segment polarity gene *engrailed* in posterior cells of parasegments (Dura and Ingham 1988; Smouse et al. 1988; Busturia and Morata 1988; Moazed and O'Farrell 1992). Mutations in *Pcl* and *Asx* lead to pair rule-like phenotypes, and in the case of *Asx* this phenotype is associated with ectopic expression of *even-skipped* (Breen and Duncan 1986; Sinclair et al. 1992). Similarly, *ph* mutant embryos have abnormal patterns of *eve* and *ftz* expression in the nervous system (Dura et al. 1988).

In addition, some Pc-G genes, such as *ph*, *E(z)* and *pho*, exhibit very pleiotropic phenotypes. Lack of zygotic *ph*, besides causing homeotic transformations, leads to the absence of ventral thoracic and abdominal derivatives (Dura et al. 1987). In addition, *ph* mutant embryos show misrouting of axonal pathways in the CNS (Smouse et al. 1988; Smouse and Perrimon 1990). Embryos that lack only the maternal Ph product show very poor cuticular development, and if they are also mutant for zygotic *ph* function, arrest development at the blastoderm stage (Dura et al. 1988). Mutations in *E(z)* lead to small disc and oogenesis phenotypes (Jones and Gelbart 1990; Phillips and Shearn 1990). Embryos from homozygous *pho* mutant germ line clones rarely develop a cuticle, and in such cases, exhibit extensive segmentation defects (Breen and Duncan 1986). It is possible that these phenotypes result from the misregulation of unknown targets in backgrounds mutant for these Pc-G genes. Alternatively, some Pc-G genes may be required for chromosomal integrity and, therefore, cell division.

Mutations in a subset of Pc-G genes modify the repression of white by the Zeste^1 product. The Zeste^1 product, encoded by an aberrant allele of the gene *zeste* (see below), represses *white* transcription, apparently by forming protein aggregates on the *white* promoter (Chen and Pirrotta 1993). Loss-of-function mutations in the genes *E(z)* and *Su(z)2*, *Psc* and *Scm* suppress the zeste^1 phenotype (Wu et al. 1989; Jones and Gelbart 1990). Thus the products of these genes are thought to be required for the repression of white by the Zeste^1 product

The varied phenotypes produced by different Pc-G gene mutations suggest that, although by definition they all affect homeotic gene regulation, different batteries of Pc-like genes may act on distinct target genes.

Mode of action of Pc-G genes

Pc-G products have been shown to be required for the maintenance of the boundaries of homeotic gene expression (Struhl and Akam 1985; Glicksman and Brower 1990; Jones and Gelbart 1990; McKeon and Brock 1991; Simon et al. 1992; Zhang and Bienz 1992). The initial boundaries of expression, which are established by the spatially restricted gap gene products (see above), appear normal in Pc-G gene mutants. Nevertheless, at the germ band extended stage, these boundaries are no longer maintained and ectopic homeotic gene expression occurs. The defect in homeotic regulation appears shortly after the gap gene products have decayed. Thus, Pc-G genes are required to stabilize the repressed states of homeotic gene expression initiated by gap genes.

In the case of *engrailed*, Pc-G genes appear to also have a role in the maintenance of a repressed state. This is suggested by the fact that, in Pc-G mutant embryos, *engrailed* expression is initially normal, and expands into ectopic areas at later stages (Moazed and O'Farrell 1992).

The Pc protein contains a 37 aa stretch with homology to the HP1 Drosophila protein (Paro 1990). HP1 is the product of the *Su(var)205* locus, a modifier of position effect variegation (PEV, see below) which is thought to be an integral component of heterochromatin (Eissenberg et al. 1990; Eissenberg et al. 1992; James and Elgin 1986; see below). This domain of similarity has been termed the "chromodomain" (Paro 1990), and has been shown to be required for binding to chromosomes (Messmer et al. 1992). The presence of the chromodomain in proteins from both the Pc-G and PEV genes has led to the proposal that Polycomb and, by extension, the products of other Pc-G genes promote the formation of stably repressed states of gene expression by organizing a "heterochromatin-like" higher order structure (Paro 1990).

This idea is further supported by a number of genetic studies that show functional similarities between Pc-G and PEV genes. First, the Pc-G and PEV sets of genes functionally overlap. This is shown by the fact that some Pc-G genes act as PEV modifiers, and some PEV modifiers exhibit Pc-G phenotypes (Reuter and Spierer 1992). Second, in both systems the extent of inactivation is sensitive to the dosage of a family of dominant repressors (the Pc-G and the Su(var) genes) and dominant activators (the trx-G and the E(var) genes)(see below). Third, constructs in euchromatin carrying the regulatory regions of *ph* driving a mini-*white* gene exhibit a PEV-like eye color variegation phenotype (Fauvarque and Dura 1993). This variegation phenotype is sensitive to, at least, *ph* and *Pc* function, but it is not affected by traditional PEV modifiers. Thus, Pc-G genes confer on genes a clonally inherited expression pattern that is functionally very similar to the heterochromatinization that produces PEV.

The promotion of a heterochromatin state by Pc-G gene proteins may involve the formation of large, multi-subunit complexes. This was first suggested by the similar phenotypes caused by mutations in these genes, the sensitivity of these phenotypes to dosage imbalances, and the apparent synergistic action of these genes (Jürgens 1985; Kennison and Russell 1987; Kennison and Tamkun 1988). Recently, biochemical studies have corroborated this idea. The Pc and Ph proteins are associated within large multiprotein complexes which contain about 10-15 proteins (Franke et al. 1992).

Further supporting the idea that Pc-G genes act through multiprotein chromatin structures, Pc-G gene proteins (Pc, ph, Psc and Su(z)-2) bind to salivary gland polytene chromosomes at largely overlapping sets of about 50-100 different sites (Zink and Paro 1989; DeCamillis et al. 1992; Rastelli et al. 1993). In the case of Psc and Su(z)-2, binding to chromosomes has been shown to depend on *E(z)* function (Rastelli et al. 1993). Thus, Pc-G genes bind as complexes at specific chromosomal locations and this binding is dependent on Pc-G function (at least that of *E(z)*).

Some of the chromosomal bands bound by Pc-G proteins correspond to loci that have been shown to be regulated by Pc-G genes, such as the Antp and Bx complexes. Regulatory regions from the Antp and Bx complexes, when inserted at new locations in the genome, serve as new Pc and Ph binding sites (DeCamillis et al. 1992; Zink et al. 1991). Thus Pc-G proteins directly interact with their target regulatory chromosomal region.

It is not clear which Pc-G gene if any, directly binds DNA. Attempts to show binding activity of Pc protein and Ph protein, which contains a single Zn finger motif (DeCamillis et al. 1992), to naked DNA have been unsuccessful (Franke et al. 1992). Other candidates for direct DNA binding are the products of the genes *Su(z)2* and *Psc*, which are homologous to each other and to the murine oncogene *bmi* (Brunk et al. 1991;

van Lohuizen et al. 1991). These proteins contain at their amino-terminus a cysteine-rich region which has been proposed to be a novel type of Zn finger.

Recently, the sequence of E(z) has shown that it contains at its C-terminus a 53 aa region with homology to the C-terminal region of the gene *trithorax* (Mazo et al. 1990; Jones and Gelbart 1993). This motif is included within the region of greater similarity between trx and its human homologue, ALL-1/Hrx, which has been implicated in acute leukemias (Djabali et al. 1992; Gu et al. 1992; Tkachuk et al. 1992). Both E(z) and Trx have, in addition, cysteine rich domains which may possibly have Zn finger structure. This finding is particularly interesting given that *trithorax* is a genetic antagonist of Pc-G genes that is required for the maintenance of active states. Jones and Gelbart (1994) speculate that this domain may allow E(z) or Trx to interact with a common target, and that binding of each product may organize the assembly of either a repressive, Pc-G complex, or an activating, trx-G complex.

Regions from the Bx-C can confer onto reporter genes stable patterns of expression that have been shown to depend on Pc-G function (Müller and Bienz 1991; Zhang and Bienz 1992; Busturia and Bienz 1993; Simon et al. 1993). Simon et al. (1993) , have shown that the elements required for initiation and maintenance of homeotic gene boundaries are separable. A 6.8 Kb fragment from the *abx* region appears to have the initiation elements necessary to establish proper boundaries of expression, but lack maintenance elements necessary to maintain those boundaries during germ band extension. The juxtaposition of this abx fragment to a 11.5 Kb fragment from another Bx-C region, *iab-3*, which on its own can maintain boundaries of expression, provides elements sufficient for maintained restriction of expression at different positions along the anteroposterior axis. The maintained restricted expression by the combination of fragments is dependent on Pc-G gene function. These results suggest that the Pc-G responsive elements (PREs) are not specialized for particular parasegments. Rather, they provide a general maintenance function to restrict expression initially specified by gap and pair-rule gene products.

Recently, a formaldehyde-crosslinking/immunoprecipitation technique has allowed mapping, to a 1 Kb resolution, of the association of Pc protein to a 350 Kb region in cultured cells (Orlando and Paro 1993). Pc protein is found broadly associated with the control regions of *Ubx* and *abd A*, and in some cases, it appears to be enriched in regions known to regulate homeotic gene expression. Importantly, *Ubx* and *abd A* are inactive in these cells, while the *Abd B* gene, which is expressed in these cells, is largely devoid of Pc protein. This correlation strongly supports the idea that Pc-G genes are involved in the repression of inactive chromosomal domains. (A similar correlation had been observed in

salivary glands with short *Antp* P1 promoter constructs which appear to be subject to position effects. In this case, the presence of a new Pc binding site correlated with the inactivity of the gene construct located at that site (Zink et al. 1991).

The finding that Pc protein is associated with large regions of the Bx complex agrees well with the idea that Pc-G are involved in the assembly of a "heterochromatin-like" state that relies on a multimeric process. It has been proposed that one reason why homeotic genes tend to be found in conserved clusters is because extended regulatory regions may facilitate the formation and/or increase the stability of such multimeric complexes (Bienz 1992, Orlando and Paro 1993).

It is still unclear how these heterochromatin-like complexes may be established. Pc-G proteins may recognize protein-DNA complexes initiated by gap and segmentation gene repressors and, perhaps by interacting with additional DNA sites, nucleate the formation of a multimeric Pc-G/DNA complex. This complex would be maintained in spite of cell division because of its self-assembling properties. Alternatively, it has been suggested (Orlando and Paro 1993) that independent chromatin domains may facilitate the nuclear spatial or temporal compartmentalization of origins of replication, which in turn may provide appropriate microenvironments for the replication of Pc-G/DNA complexes.

Recently, mutations in *C. elegans*, such as in the gene *polyarray*, and in the mouse, such as the in the mouse homologue of *Psc*, the *bmi* gene, have been found to produce phenotypes consistent with derepression of homeotic genes (C. Kenyon and R. Krumlauf, personal communications). Thus, regulation of homeotic genes by Pc-G-like genes may be have been widely conserved through evolution.

ii) The trithorax group of genes

Mutations in genes in the trithorax group (trx-G) produce phenotypes that are consistent with a general loss of activity of genes in the Antp and Bx-C complexes. The group is named after its first member, *trithorax* (*trx*) (Ingham 1983, Ingham 1984). Later, screens for modifiers of homeotic gene function led to the isolation of mutations in about a dozen loci, including *trx*, that have been included in this family (Kennison and Tamkun 1988). These genes are *kismet*, *Su(Pc)37D*, *Brista, devenir*, *brahma*, *kohtalo*, *verthandi*, *l(3)87Ca*, *urdur*, *moira*, *osa*, *skuld* and *sallimus*. The genes *absent, small or homeotic disks (ash)1*, *ash2* and *female sterile (1) homeotic* (*fsh*) are included in this family because of their trx-like phenotype and their interactions with other trx-G genes (Shearn 1989; Haynies, 1989). Another gene included in this family is *zeste*, a nonessential gene that

facilitates transcriptional activation, perhaps by allowing distant cis-acting regions to interact with each other (for reviews, see Wu and Goldberg (1989); Tartof and Henikoff (1991); see also Chapter 3 Addendum, Section L).

The members of the trx-G family have not been characterized as extensively as the Pc-G gene family. Somatic clones of *trx*, *kismet* and *moira* produce patches of adult tissue with homeotic transformations (Ingham 1985, Kennison and Tamkun 1988). Leaky alleles of *ash1* and *ash2* lead to adult transformations phenotypes similar to those associated with *trx* mutations. *fsh* has a strong maternal component: homozygous *fsh* mutant mothers produce embryos with homeotic transformations even if the sperm carries additional *fsh* doses (Gans et al. 1975; Forquignon 1981; Digan et al. 1986). However, *fsh* is also required zygotically for larval and pupal development.

Homozygosity for *trx* leads to embryonic lethality. Nevertheless, these embryos exhibit only weak homeotic transformations. This mild phenotype, which is independent of maternal Trx product (Ingham 1983), is surprising, especially considering the strength of *trx* mutations in the adult, and the fact that early embryos contain abundant trx message (Mozer and Dawid 1989) Thus *trx* function is not essential in the expression of genes in the Antp and Bx complexes. Nevertheless, the fact that, *trx* mutations suppress the embryonic phenotype caused by *esc* mutations (Ingham 1983), and that *trx* mutations affect embryonic homeotic gene expression (Breen and Harte 1991; Breen and Harte 1993), show that *trx* is involved in the expression of at least some homeotic genes in the embryo.

The fact that mutations in trx-G genes act as genetic antagonists of Pc-G genes in the regulation of homeotic genes (Ingham 1983; Kennison and Tamkun 1988; Shearn 1989; Tamkun et al. 1992; Jones and Gelbart 1993), suggests that trx-G genes have a function that is similar, but opposite to that of Pc-G genes. The trx-G genes act at the level of activation of homeotic gene expression: *trx* and *brahma* mutations have been shown to reduce the levels of homeotic gene expression in the larvae and the adult (Ingham 1985; Mazo et al. 1990; Breen and Harte 1991; Tamkun et al. 1992,). Together, these findings have suggested the notion that the function of trx-G genes may be analogous to that of Pc-G genes in gene repression, that is in maintaining the activated states initiated by transient gene products.

Recently, *brahma* has been found to be homologous to the SNF2/SWI2 yeast gene (Tamkun et al. 1992). The fact that the yeast SNF/SWI genes are thought to activate gene transcription by counteracting chromatin suggests a similar function for *brm* and other trx-G genes in antagonizing chromatin regulation by Pc-G gene products (for reviews, see Kennison (1993); Kennison and Tamkun (1992); Winston and Carlson (1992), also see below). Antibodies against SWI1 and SWI3 recognize proteins in Drosophila extracts

(Peterson and Herskowitz 1992), which suggests the existence of other SNF/SWI Drosophila homologues which might correspond to trx-G genes. Interestingly, members of the SNF/SWI family have been shown to be required in yeast for the activation of target genes by the Drosophila Bicoid and Fushi tarazu proteins (Peterson and Herskowitz 1992; Laurent and Carlson 1992). Together, these data suggest that the SNF/SWI and trx-G genes are homologous families of genes and that their function has been conserved through evolution.

It has been proposed that SNF/SWI genes have a dual role in the activation of genes. Some of the proteins encoded by these genes act by relieving repression induced by the histone group, and others have a more direct role in activating the transcriptional machinery (Laurent and Carlson 1992; see below). Such a dual role may also exist in the trx-G: the Brahma protein, which contains conserved motifs found in helicases (Tamkun et al. 1992), may be involved in helicase activity. On the other hand, the Trx protein, which contains Zn finger motifs and stretches of acidic residues (Mazo et al. 1990) may be more directly involved in transcriptional activation.

The SNF/SWI genes have been proposed to form multimeric complexes (Laurent et al. 1991; Peterson and Herskowitz 1992; see below). This suggests that trx-G gene products, like the Pc-G gene proteins, may also form multiprotein structures. As mentioned above, the similar domain present in both *E(z)* and *trx* has led to the proposal that these two genes may be important for the nucleation of either Pc-G or trx-G multimeric complexes onto a common target. The type of complex assembled would in turn determine the chromatin state of the gene.

Recently, the product of the modifier of PEV, *E(var)3-93D* has been reported to exhibit trx-like phenotypes when mutated and appears to be involved in the positive regulation of homeotic genes (Dorn et al. 1993). The product of *E(var)3-93D* is found at a large number of loci, including the Antp and Bx complexes, and in many cases these sites correlate with less condensed interband regions. E(var)3-93D contains at its N-terminus a region of homology with the transcriptional regulators Tramtrack and the products of the Broad complex. In addition these proteins also contain a Zn finger motif. Thus *E(var)3-93D* may be a common component of the trx-G and PEV genes involved in the determination of open chromatin domains.

Not all the genes above are directly involved in transcriptional regulation. The *fsh* gene, for example, encodes a transmembrane protein, and thus likely acts upstream of events that may lead to transcriptional regulation (Haynes et al. 1989).

iii) Position effect modifiers and the mass action model

Position effect variegation (PEV) is a phenomenon observed when an autonomous gene is translocated to a position near heterochromatin (for reviews, see, Henikoff (1990); Reuter and Spierer (1992)). Clonal inactivation of the gene leads to a variegated phenotype. A classical example is the variegation in the eye of *white* expression.

PEV of white can be enhanced or suppressed (leading to a greater number of cells with inactive and active gene expression, respectively), by a number of environmental factors, including temperature, inhibitors of histone metabolism, the amount of heterochromatin in the genome (such as the presence of Y chromosome) and genetic modifiers.

With the assumption that PEV modifiers might encode genes involved in chromatin structure or metabolism, several screens for dominant suppressors or enhancers of PEV have been carried out (Reuter and Wolf 1981; Sinclair et al. 1983; Locke et al. 1988; Sinclair et al. 1989; Wustmann et al. 1989; Sinclair et al. 1992). These screens have led to the isolation of about 120 modifier of PEV loci.

These modifier mutations can be classified into four groups. About 10 genes suppress PEV when present in only one dose (haplo-suppressors) and enhance PEV when present in additional copies (e.g. triplo-enhancers).

This particular type of dosage dependence on PEV is consistent with a model in which these PEV genes encode repressive heterochromatin components. The clonal inactivation of the variegated gene would be a result from the gradual spreading of multimeric heterochromatin complexes along the chromosome according to the chemical law of mass action (Locke et al. 1988).

The cloning of some of these genes has been reported, and these results agree with this basic model. The predicted product of the *Suvar(3)7* gene contains 5 widely spaced Zn fingers which could help package chromatin into heterochromatin (Reuter et al. 1990). *Suvar(2)5* encodes a protein, HP1, previously known to be associated with heterochromatin (James and Elgin 1986; Eissenberg et al. 1990). As mentioned above, the HP1 protein has a region of homology shared with the Polycomb protein. The gene *modulo* encodes a lineage-specific protein that directly binds DNA (Krejci et al. 1989). Thus, the Modulo protein may act as an anchor for the heterochromatin complex. The sequence of *Suvar(231)* suggests that it may interact with DNA and the cytoskeleton (Reuter and Spierer 1992). Thus molecular information for genes in this class is consistent with them being structural components of heterochromatin.

The idea that all integral components of heterochromatin belong in this first class of PEV genes (haplo-suppressors, triplo enhancers) is nevertheless, likely an oversimplification. This is suggested by the fact that deletions of part of the histone cluster lead to the suppression of PEV, but its duplication does not cause enhancement of PEV (Moore et al. 1983).

A second class of PEV modifier mutations, which includes about 10 genes, consists of haplo-enhancer, triplo-suppressor loci. These genes may be involved in maintaining active chromatin domains. One member of this class may be the gene *E(var)3-93D*, which exhibits trithorax-like phenotypes and contains a region of homology to other transcriptional regulators (Dorn et al. 1992).

The last two categories consist of genes that have haploinsufficient but not triplodominant effects (about 75 enhancers and 25 suppressors). These genes may encode chromatin components that are less dosage-dependent, as in the example of the histone cluster, or, alternatively, enzymes involved in the metabolism and modification of chromosomal proteins. One such modifying factor may be the predicted protein phosphatase encoded by *Suvar(6)* (Dombrandi et al. 1989).

As mentioned above, the similarities between the modifiers of PEV and the Pc/trx sets of genes, and the fact that they appear to share common components, has led to the suggestion that both these two pairs of genes may act through common mechanisms.

iv) The yeast SPT/SIN and SWI/SNF systems: a model for the regulation by Polycomb and trithorax groups of genes

Several lines of work involving transcriptional regulation of a number of yeast genes have converged into the study of two sets of genes that affect chromatin structure (for review, see Winston and Carlson (1992)). One set includes the *SPT/SIN* genes, which encode histone and non-histone chromatin repressors. The other set includes the *SWI/SNF* genes, which are required for transcriptional activation and are thought to counteract the repressive action of the *SPT/SIN* genes.

The SWI/SNF group of genes consists of *SWI1*, *SWI2/SNF2*, *SWI3*, *SNF5* and *SNF6*. All five genes have been shown to be required for the normal transcription of a number of genes, including *ADH2*, *GAL1*, *SUC2*, *INO1* and *HO* (see Peterson and Herskowitz (1992)).

These genes are required for maximal activation of transcription by other activator proteins which have DNA-binding activity. For example, activation by GAL4 and the Drosophila protein Fushi tarazu is reduced in the absence of *SWI1* function (Peterson and

Herskowitz (1992)), and activation by lexA-GAL4 and LexA-Bicoid fusions have been shown to depend on *SWI2/SNF2*, *SNF5* and *SNF6* (Laurent and Carlson 1992). Recently, the human homologue of *SWI2/SNF2* and brahma, *hbrm*, has been found to cooperate with the glucocorticoid receptor in transcriptional activation (Muchardt and Yaniv 1993). Interestingly, it is not required for activation of a number of other factors.

Studies of the activation properties of SNF protein that are tethered to the DNA via the LexA DNA binding domain show that SNF2, SNF5 and SNF6 are likely to be directly involved in transcriptional activation (Laurent et al. 1991; Laurent and Carlson 1992). Activation by the lexA-SNF2 and LexA-SNF5 fusions requires the function of *SWI1*, *SNF5*, *SNF6*. These results show that even when these two genes are tethered to DNA they are functionally dependent on other genes in the group. The lexA-SNF6 fusion, on the other hand, has considerable activity in the absence of other *SWI/SNF* genes (see below).

This mutual requirement may reflect that these genes act as a multimeric complex. This has been suggested by the similar phenotypes of single and multiple mutant combinations, by the fact that all these five genes regulate the same set of target genes, and by the reduction in SWI3 protein stability that is observed in *swi1* and *swi2* mutants (Peterson and Herskowitz 1992).

The SPT/SIN group of genes include *SPT4*, *SPT5*, *SPT6*, *SPT11*, *SPT16*, *SIN1* and *SIN2*. The identity of some of these genes suggests that gene regulation by the *SPT/SIN* genes involves repression by chromatin. The *SPT1*, *SPT2* and *SIN2* genes encode the histone proteins H2A, H2B and H3, respectively (Clark-Adams et al. 1988; Winston and Carlson 1992). *SIN1* encodes a protein similar to High mobility group 1 (HMG1) proteins and has been shown to nonspecifically bind DNA (Kruger and Herskowitz 1991).

The SPT4, SPT5 and SPT6 proteins appear to also form a complex. First, mutations in all three genes alter transcription in similar ways. Second, some recessive mutations in different complementation groups fail to complement. Third, the genotypes are sensitive to gene dosages. Four, SPT5 and SPT6 proteins coimmunoprecipitate (Nasmyth and Stillman 1987).

The fact that mutations in *SPT/SIN* genes suppress mutations in the *SWI/SNF* genes suggests that SWI/SNF proteins activate transcription by counteracting repression by chromatin components (Sternberg et al. 1987; Nasmyth and Stillman 1987; Estruch and Carlson 1990; Neigeborn and Carlson 1984; Neigeborn et al. 1986; Hirschhorn et al. 1992). The sequence of *SWI2/SNF2* is particularly revealing, since this protein contains a motif characteristic of helicases (Laurent and Carlson 1992; Davis et al., 1992). In its

human homologue, Hbrm, this motif has been shown to be required for the potentiation of the glucocorticoid-dependent transcriptional activation (Muchardt and Yaniv 1993). This suggests that SWI2/SNF2 may be involved in changing the conformation of DNA in order to reposition nucleosomes and facilitate binding of transcriptional activation factors.

The results of LexA-SNF fusion proteins described above have led to the proposal that *SWI/SNF* genes have two types of functions (Laurent and Carlson 1992). Proteins such as SNF2 and SNF5, which when tethered to DNA via the LexA protein require other *SNF/SWI* genes, may be involved in relieving repression by the histone group. On the other hand, the SNF6 protein, which activates independently of other *SWI/SNF* genes in the same system, may have a more direct role in transcriptional activation. Alternatively, it is also possible that SNF6 produces an activating effect indirectly, by changing the local chromatin conformation.

Several studies have shown that *SWI/SNF* and *SPT/SIN* genes affect chromatin structure (Matallan et al. 1992; Hirschhorn et al. 1992). These studies have found differences in the chromatin accessibility of the *SUC2* promoter in wild-type and *swi/snf* mutant strains. Hirschhorn et al. (1992) have further shown that mutations in *SPT11* and *SPT12* (H2A and H2B), which suppress the SNF phenotype, restore the wild-type accessibility pattern in *swi2/snf2* or *snf5* mutant backgrounds. The MNase cleavage patterns are consistent with nucleosomes being present on the *SUC2* promoter in *swi/snf* mutants, and absent or positioned differently in wild type or *swi/snf, spt* double mutants.

A similar change in chromatin accessibility in *swi/snf* mutants was obtained when the TATA box is deleted from the *SUC2* promoter. The fact that the change in chromatin structure is independent of transcriptional activity suggests that this change precedes the binding of transcription factors such as TFIID. Thus this study provides compelling evidence for a causal relationship between *swi/snf* mutations and chromatin structure.

As mentioned above, the analogy between the repressive-activating systems in yeast (*SPT/SIN-SWI/SNF* genes) and Drosophila (Polycomb-trithorax groups of genes), together with the fact that *SWI2/SNF2* and *brahma*, a trithorax group gene, are homologous, has suggested that these two systems have similar functions and mechanisms of action.

6. Introduction to Chapters and Appendixes

In this thesis I specifically address the regulation of the abdomen-promoting gap genes, *knirps* and *giant*, by the maternal Hunchback (Hb^{mat}) protein gradient. Chapter 2 presents the screens for suppressors of *nanos* (*nos*) that were carried out with the purpose of isolating interacting products within the *nos/*hb^{mat}*/*abdominal gap genes genetic pathway, as well as a summary of the genetic characterization of suppressor-of-*nos* mutations.

Chapter 3 consists of an article that is in print (Genetics, April 1994), which in itself is a good summary of much of the work presented in this thesis. This article presents the genetic characterization of the suppressor-of-*nos* mutations allelic to the Polycomb group gene *Enhancer of zeste E(z)*. The basis for the suppression of the nos phenotype is investigated, as well as the possible role of *E(z)* in the wild type embryo. We conclude that *E(z)* is required for the maintenance of the repression of *knirps* and *giant* which is initiated by the Hb^{mat} protein, and that therefore *E(z)* function contributes to the determination of anterior *kni* and *gt* boundaries of expression. In addition, we show that other Polycomb group genes are involved in this process, which suggests that the domains of expression of *kni* and *gt* are regulated at the level of chromatin.

Chapter 4 presents a series of studies that show that trithorax group genes, which are genetic antagonists of Polycomb group genes, interact with the gap gene machinery to affect the expression of abdominal gap genes. Nevertheless, in the case of abdomen formation, the effect of trx group genes on the nos phenotype is synergistic, not antagonistic, to that of Polycomb group genes. An interpretation of these unexpected results is difficult. The basis for this phenomenon is at the present unknown, although an indirect effect through *Kr* has been ruled out.

The appendixes present "side stories" of work carried out during my program of studies. The first two appendixes describe the genetic characterizations of two other suppressor-of-*nos* mutations. Appendix A presents genetic studies carried out with the son^{29} mutation, which is the strongest suppressor-of-*nos* mutation that is not allelic to *E(z)*. In addition, this appendix describes a screen for genes that interact with son^{29} and *E(z)*, and reports the isolation of the second site modifier gene, *Sufsi-1*.

Appendix B presents the genetic characterization and preliminary cloning of a P-element induced suppressor of nos mutation, $Pson^{49}$.

Appendix C refers to a topic unrelated to the bulk of this thesis, the isolation of second site suppressor of the Bicaudal-D mutation. The mutations isolated define several

complementation groups, some unknown and some in already known genes. These genes may be involved in the determination of the polarity of the oocyte.

A clarification on nomenclature: The "nos phenotype" refers to the lack of abdominal segmentation produced by mutations in *nanos* (nos). The "son phenotype", on the other hand, refers to the suppression of the nanos phenotype exhibited by Pc-G mutations, which allow the formation of abdominal segments in the absence of *nos* function.

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CHAPTER II. Screens for suppressors of *nos* identify interacting products in the abdominal segmentation pathway

ABSTRACT

Wild-type *nanos* (*nos*) function is required for the expression of abdomen-promoting gap genes *knirps* and *giant*. This requirement is indirect, by the Nos protein inhibiting the translation of the maternal Hunchback (Hb^{mat}) protein, which otherwise would repress transcription of *knirps* and *giant*. We reasoned that we could isolate mutations in interacting genes within this pathway by screening for suppressor-of-*nos* (*son*) mutations. Loss-of-function mutations in a gene could act as suppressors of *nos* if the wild-type gene is required for the production, stability, or function of the Hb^{mat} protein. In addition, *son* mutations could consist of gain-of-function alleles of genes involved in the activation of *knirps* and *giant*. Here, I describe screens for suppressor-of-*nos* (*son*) mutations, as well as the initial genetic characterization of the identified *son* mutations. Aside from their common son phenotype, the identified *son* genes themselves form a family of interacting genes which appear to be involved in processes such as morphogenesis and oogenesis.

INTRODUCTION

Embryos mutant for *nanos* (*nos*) lack the posterior domain of expression of the abdomen-promoting gap genes *knirps* (*kni*) and *giant* (*gt*) and therefore do not develop abdominal segments. The requirement for *nos* function on transcription of *kni* and *gt* is not direct, but rather occurs through a double negative mechanism. Nos is required to inhibit in the prospective abdominal region translation of the maternal hunchback (Hb^{mat}) repressor protein (Tautz 1988; Wang and Lehmann 1991), which otherwise would repress transcription of *kni* and gt (Hülskamp et al. 1989; Irish et al. 1989a; Struhl 1989; Eldon and Pirrotta 1991; Kraut and Levine 1991a; Kraut and Levine 1991b).

The indirect requirement of *nos* for abdomen formation suggested that it should be possible to isolate mutations that suppress the *nos* phenotype. Such mutations could in principle restore abdomen segmentation in *nos* mutant embryos in a number of ways. First, suppressor-of-*nos* (*son*) mutations may affect the levels of the Hb^{mat} protein. This could be achieved by mutations that affect the production, transport into the oocyte, or translation of the hb^{mat} mRNA. Second, *son* mutations could be mutations in the Hb^{mat} protein itself, which would reduce its activity. Nevertheless, this type of mutations may be

difficult to isolate, since *hb* is required zygotically (Lehmann and Nüsslein-Volhard 1987b). Fourth, *son* mutations may reduce the function of corepressor factor(s) required to act in conjunction with the Hb^{mat} protein. Fifth, *son* mutations may create overactive alleles of sofar unknown transcriptional activator(s) of *kni* and *gt* which can override repression by Hb^{mat}. Finally, in what may be a less likely scenario, *son* mutations may activate an alternate pathway which bypasses the abdominal requirement for *kni* and *gt* activation.

These multiple possibilities suggested that the isolation of *son* mutations could be useful in identifying additional proteins involved in this genetic pathway. Moreover, a number of scenarios predict that loss-of-function mutations, which are the type of mutations most easily produced, could behave as *son* mutations. Thus we undertook a number of screens for suppressors of *nos*.

In order to maintain the screens for *son* mutations as simple as possible we opted for an F1 screen, in which mutations would have to produce a dominant son phenotype (see Figure 2.1). In order increase the possibilities of isolating such dominant son mutations, the screen was carried out in a sensitized genetic background that is heterozygous for *hb*. Maternal heterozygosity of *hb* causes a slight alleviation of the *nos* phenotype, allowing the embryos to produce one or two, but rarely three, abdominal segments (Hülskamp et al. 1989; Irish et al. 1989a; Struhl 1989). In this genetic background, half of the Hb^{mat} protein is produced (see Chapters III and IV). This reduced Hb level appears to be near the threshold of hb^{mat} activity required for repression of *kni* and *gt*. Thus embryos from *hb nos / + nos* females are likely more sensitive to fluctuations in the abdominal gap gene activation pathway caused by one mutant *son* copy.

Suppressor and enhancer screens have become a useful tool in the identification of interacting genes in a genetic pathway. Such screens are based solely on function and not phenotype, and are not subject to biases which may appear when analyzing mutations which on their own have unexpected phenotypes. Dominant suppressor screens, in particular, may identify a late (or maternal) function of genes that are required at earlier steps in development. In addition, dominant suppressor screens are usually genetically simpler, and this allows the screening of larger number of mutational events.

Suppressor screens have been used successfully in a number of systems. Such screens are most useful in unicellular organisms, where large numbers of mutations can be easily screened (see for example, Stearns and Botstein (1988)). The nematode *C. elegans* is also particularly amenable to suppression screens, due to, again, the ease of scoring a large number of individuals. In addition, the ability of *C. elegans* to self-fertilize greatly facilitates the isolation of recessive suppressors (see, for example, Herman (1988)

In Drosophila, highly selective dominant suppression and enhancer screens have also been effective in the identification of interacting genes. Fuller and coworkers identified genes coding for microtubule associated proteins as second-site noncomplementers (enhancers) of mutations in tubulin subunits (reviewed in Fuller et al. (1989). Kennison and Tamkun (1988) have isolated mutations in the Polycomb and trithorax groups of genes as enhancers and suppressors, respectively, of mutations that cause a low level of ectopic homeotic expression. A number of laboratories have isolated mutations in structural or modifying chromatin proteins in screens for suppressors and enhancers of position effect variegation (for review, see Reuter and Spierer (1992). As a final example, screens for suppressors of activated components of receptor tyrosine kinase cascades required for photoreceptor cell differentiation and embryonic patterning have identified further genes involved in these signalling pathways (Simon et al. 1991; Doyle and Bishop 1993; Tsuda et al. 1993).

This chapter describes screens for suppressor of *nanos* (son) mutations using either the point mutagen ethyl-methane-sulfonate (EMS) or P-element mutagenesis. In addition this chapter describes the initial genetic characterization of the isolated *son* mutations.

MATERIALS AND METHODS

Genetic analysis and strains: All genetic tests were carried out at 25°C, and only the progeny from the first four days of laying were analyzed. Embryos were allowed to develop cuticle structures (24 hrs at 25°C) and the number of abdominal segments was scored directly under a dissecting scope (the embryos being cleared by a film of oil). The *nos* allele used was nos^{L7} (lack of function with respect to the abdomen formation function, although it retains some function, (Lehmann and Nüsslein-Volhard 1991). In all screens the *nos* chromosome was isogenized prior to mutagenesis. The *hb* allele used was hb^{7M} (null, Lehmann and Nüsslein-Volhard 1987; Tautz 1988).

Screens for EMS induced suppressors of *nos*: The screens for dominant suppressors of *nos* were carried out as diagrammed in Figure 2.1a. Homozygous nos^{L7} males homozygous were mutagenized with either 35mM or 45mM ethyl-methane-sulfonate (EMS). The F1 *nos* females were in addition heterozygous for hb^{mat}. In principle, a reduction in hb^{mat} dosage should help in the isolation of dominant and partially dominant *son* mutations. Embryos from F1 *hb nos / nos* females never reach adulthood due to the lack of proper abdominal development. If these females carry a dominant *son* mutation, they produce embryos that form complete abdomens and become fertile adults. One half of

these adult progeny should carry the dominant suppressor mutations. We therefore individually backcrossed up to 10 individual males with the *hb nos* tester stock to test for dominant suppression of the *nos* phenotype (Figure 2.1b). In sequential crosses, the same males were also mated to females carrying appropriate balancers to establish balanced stocks on the first, second and third chromosomes (Figure 2.1b).

A pilot screen of 1,842 F1 * *nos / hb nos* females was first carried out and led to the isolation of the allele $E(z)^{son3}$. Later, two larger screens, of 7,530 and 3,842 F1 females from experiments using 35mM and 45mM EMS concentrations, respectively, were carried out. Because the initial characterization of $E(z)^{son3}$ had shown that the son phenotype is temperature sensitive, these larger screens were carried out at a constant temperature of 25°C, as opposed to the pilot screen, which had been conducted at room temperature. The higher temperature appears to have increased the number of putative son lines with respect to the pilot screen, although, perhaps by chance, the final frequency of isolated strong *son* mutations is similar. Table 2.1 summarizes the results from these three experiments.

An estimate of the efficiency of mutagenesis was obtained by scoring for newly induced mutations in visible marker genes in the F1 progeny. Newly induced *curled* (*cu*) mutations were identified in trans to the *hb nos* chomosome, which carries *cu*, and newly

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Figure 2.1. A) A screen for suppressors of *nos*. * denotes induced mutations on the chromosome. *TM3* is a third chromosome balancer. B) Strategy to determine the chromosomal location of the suppressor mutation. Half of the F2 progeny should carry the dominant *son* mutation. Up to 10 single F2 males were sequentially crossed to females of the genotypes indicated. i) A cross to *hb nos / TM3* females allows retesting for the son phenotype. Five F3 *hb nos / nos* females were tested individually for the son phenotype. These crosses allow to roughly locate the chromosomes which carry the *son* mutations. If the *son* mutation is on the X or third chromosome, all F3 hb nos / nos females should carry the suppressor. If the son mutation is on the second or fourth chromosomes, only half of the F3 females should show the son phenotype. Further, if this cross yields only Sb^+ (non-*TM3*) flies, this indicates that the F2 tester male carried the mutagenized *nos* chromosome rather than the *hb nos* chromosome. ii) A cross to p^p / *TM3* females allows the construction of third chromosome balanced stocks in the next generation. iii) A cross to + / *CyO* (a second chromosome balancer), will bring the mutagenized second chromosomes or a wild-type second chromosome in trans to the balancer in the next generation. If from the retest in (i) it appears that the *son* mutation may be located on the second chromosome, additional second chromosomal lines were established and tested separately for the son phenotype. iv) A cross to an attached X stock immediately balances the X chromosome. If the retest in (i) indicates that the son mutation could be on the X or third chromosome and further retesting of a third chromosomal balanced stocks (see ii) did not show that the son mutations is on the third chromosome, further crosses were carried out to test whether the *son* mutation was located on the X chromosome. Because of its small size, no attempt was made to balance possible mutations on the fourth chromosome.

Figure 2.1

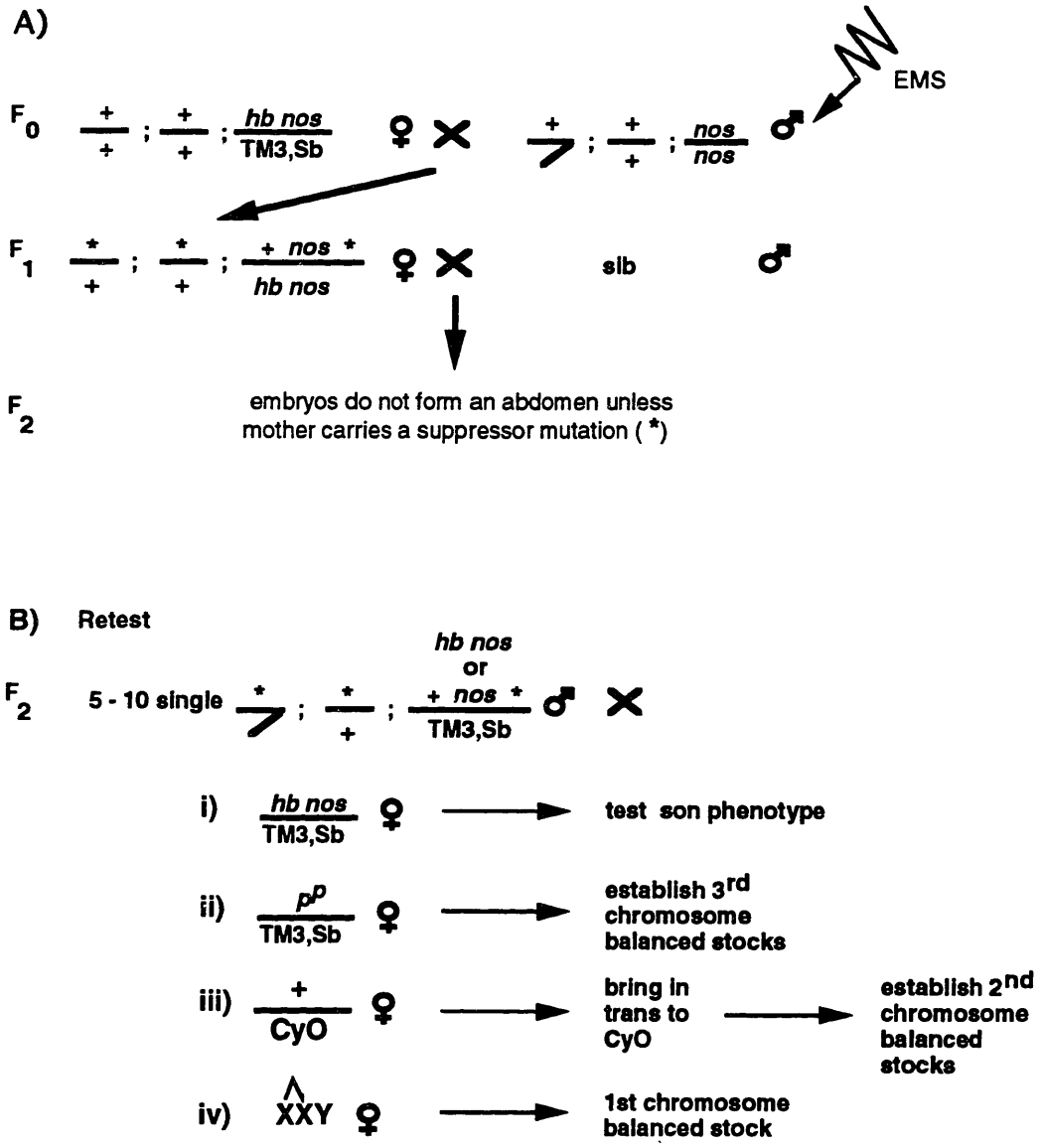
A)
EMS
F0
hb nos
TM3,Sb
nos
nos
F1
+ nos *
hb nos
sib
F2
embryos do not form an abdomen unless
mother carries a suppressor mutation (*)
B) Retest
F2
5 - 10 single
hb nos
or
+ nos *
TM3,Sb
i)
hb nos
TM3,Sb
test son phenotype
ii)
p^p
TM3,Sb
establish 3rd chromosome balanced stocks
iii)
+
CyO
bring in trans to CyO
establish 2nd chromosome balanced stocks
iv)
XXY
1st chromosome balanced stock

Table 2.1. Screens for suppressors of *nos*

Mutagen		Total # females screened	Estimated hit frequency (hits/locus/ chromosome)	# of selected females (1) (%of total)	# of retested lines (2)	# lines saved (3)	# mapped mutations (4)
EMS(pilot)(5)	35mM	1,842	n. d.	2 (0.1%)	1 (0.05%)	1 (0.05%)	1 (0.05%)
EMS	35 mM	7,530	1.7	33 (0.4%)	21 (0.2%)	16 (0.2%)	6 (0.06%)
	45mM	3,482	n. d.	≤38(≤1%)(6)	u. k. (6)	2 (0.06%)	1 (0.03%)
multi - p[w+]		15,612	0.4	55 (0.4%)	24 (0.2%)	15 (0.1%)	2 (0.01%)

(1) Females from pools of 4 which produced higher than background numbers of embryos with abdominal segments were individually separated. The number is this column is the number of separated females that led to any adult progeny.

(2) Number of lines with fertile offspring, which could be backcrossed to a *hb nos* stock.

(3) Number of lines with apparently positive tests, which were saved as appropriate stocks.

(4) Number of lines which have been genetically mapped. In practice, these mutations are those that cause the strongest and most consistent son phenotypes.

(5) The pilot screen was carried at room temperature and not at 25°C, which enhances the son phenotype. Hence this screen yielded a proportionately smaller number of putative *son* lines.

(6) Exact number is unknown.

induced *pink* (*p*) alleles in trans to the p^p allele on the balancer (TM3). Out of 7,530 flies scored for *cu* and p^p in the 35mM EMS experiment, 9 new *cu* alleles and 17 new p^p alleles were observed. This represents an average hit frequency for loss-of function mutations of 1.7 hits per locus per 1000 chromosomes. Assuming a similar hit frequency in both the 35mM and 45mM experiments one can predict that out of the total 12,854 half genomes screened we induced, on average, 22 loss-of-function mutations per locus (1.7 hits per locus per chromosome x 12,854 chromosomes).

A screen for P-element induced *son* mutations: The screen for P-element induced *son* mutations was a modification of the screens for EMS induced *son* mutations that allowed mobilization of p[w$^+$] elements from a multi-p[w$^+$] X chromosome (Bier et al. 1989) in the F_0 males (Figure 2.2). A *w* mutant background was used to follow the mobilized p[w$^+$] elements.

An estimate of the efficiency of mutagenesis was calculated using the frequency of recovery of newly induced *cu* alleles. Of the total number of mutagenized third chromosomes (15,612), seven new *cu* mutations were induced. This suggests an approximate hit frequency of 0.4 hits / 1000 chromosomes. Nevertheless, this estimate is only approximate since P-elements are known to vary widely with respect to the frequency of insertion at different loci and to our knowledge no information is available with regard to the insertion frequency at the *cu* locus.

Fifty five (0.4% of the total screened) single flies produced adult progeny. Males from twenty four (0.2% of the total screened) of these vials were fertile and were backcrossed to *yw, hb nos / TM3* flies in order to test for the son phenotype. Balanced lines were established on the third chromosomes, and where appropriate (as indicated by the retests), on the first and second chromosomes. Those third chromosome lines which produced homozygous viable adult females were tested for the son phenotype in a wild-type hb^{mat} background. Whenever appropriate, w^+ and w^- flies from the same line were tested separately in order to determine whether the new son mutation was associated with a p[w$^+$] P-element. In those lines where the son effect did not seem to correlate with the third chromosome, further crosses were carried out to establish first and/or second chromosome balanced stocks.

Genetic mapping of *son* and *Pson* mutations: Genetic mapping was carried out with multiple factor crosses. The multiply marked chromosomes used for mapping *son* mutations were: for mutations on the third chromosome, "*neuple*" (*th st ri roe* p^p *cu sr* e^s) and "*rucuca*" (*ru h th st cu sr* e^s *ca*), and for mutations on the second chromosome (i.e. *Pson49*), the multiply marked *al dp b pr c px sp* chromosome. The initial mapping located the mutations to a particular interval on the chromosome. Further

tests using a greater number of chromosomes with recombination events within the appropriate interval allowed a more precise mapping of these mutations. Only results from the most relevant recombinant lines are described below. Results from other recombinant lines are always consistent with the reported map position.

E(z)$^{son1-3}$: *son^{3}* was isolated in the pilot screen, and its initial mapping was carried out prior to the isolation of the other *E(z)^{son}* alleles. The original chromosome that carried the *son^{3}* mutation was also lethal. In 17 recombinant lines, both the lethality and the *son* phenotype were unseparable and mapped within the *hairy-thread* interval (3-26.5 to 43.2). This suggested that the *son* mutation was lethal. This notion was confirmed when we identified two additional *son* mutations, *son^{1}* and *son^{2}*, both of which are lethal in trans to *son^{3}* (see also Chapter III). *son^{1}* and *son^{2}* were more finely mapped within the *h-th* interval using their associated lethality in trans to the *son^{3}* mutation. This indicated that *son^{1}* and *son^{2}* mapped to the same genetic position at 3-31.5 and 3-30.9, respectively (number of recombinant lines used: 47 and 66, respectively).

son^{29}: *son^{29}* was mapped using the *neuple* chromosome and is very closely associated with the visible marker *radius incompletus* (*ri*, map position: 3-46.8): the *son* phenotype was inseparable from *ri* in 47 recombination events within the *th-ri* interval (a distance of 3.6 map units) or in 17 recombination events within the *ri-p* interval (a distance of 1.2 map units). 21 of these recombination events were used to map the female sterile phenotype of *son^{29}* in trans to *E(z)^{son3}* (15 and 6 in the *th-ri* and *ri-p* intervals, respectively). In all cases tested female sterility cosegregated with the son phenotype and the *ri* marker.

Finer mapping was performed by isolating recombinants within the *Wrinkled* (*W*, map position: 3-46.0) - *ri* and *ri- p^{p}* intervals from *son^{29} W^{+} ri^{+} p^{p+} / W ri p^{p}* females. In 6 recombination events within the *r-p^{p}* interval *son^{29}* always cosegregated with *ri*. On the other hand, in 2 of 7 recombination events within the *W-ri* interval, *son^{29}* was separable from the *ri* locus. This indicates that *son^{29}* maps closely, and distal to the *ri* locus, at about 3-46.6.

Testing *son^{29}* in trans to deficiencies in the region did not reveal any effects on viability, visible adult phenotypes or fecundity (deficiencies used: *Df(3L)in61*, *Df(3L)ri^{XT106}*, *Df(3L)ri^{XT104}* and *Df(3L)ri^{XT1}*, which together span the cytological region from 76F-77D, and 77D to 78A, these deficiencies may not overlap at the 77D region). It is unknown whether the distal end of this interval covers the genetic position of *son^{29}*, at 46.6 (for reference see Lindsley and Zimm 1992).

son^{59,126}: The *son* phenotype as well as the recessive lethal and visible wing phenotypes of *son^{59}* and *son^{126}* were mapped using a *rucuca* chromosome. The wing

phenotype associated with the son^{59}/son^{59} and son^{59}/son^{126} genotypes mapped very close to *claret* (*ca*, map position: 3-100.7) on the son^{59} chromosome: 5 son^{59}, *ru h th st cu sr es* ca^+ recombinant lines in trans to the son^{126} allele showed the wing phenotypes. Four of these lines also showed the wing phenotype in trans to son^{59} (the last line may not have shown it due to variability in the phenotype and a small sample size (n=14)). The reciprocal recombinant chromosomes, carrying the entire originally mutagenized chromosome except for the *ca* region, did not exhibit wing defects in trans to son^{126} (not tested in trans to son^{59}). In addition, a lethal phenotype associated with the son^{126} mutation also maps in the *ca* region (7 lines with only the ca^+ region were lethal). The sterility of son^{59}/son^{126} females has not been mapped. Nevertheless, it is possibly associated with the same mutations, since this phenotype is exhibited by both son^{59} homozygotes and son^{59}/son^{126} transheterozygotes. However, the chromosomes used in this experiment are not recombinant lines and it is also possible that both of these chromosomes carry female sterile mutations that do not complement each other.

The *son* phenotype of son^{59} and son^{126} could not be properly mapped. Specifically, for both mutations all types of recombinant lines exhibited a low and variable degree of segment formation. It is possible that the son phenotype of son^{59} and son^{126} is synthetic and caused by more than one mutation. An additional mutation that contributes to the son phenotype of these chromosomes may be closely associated with the *stripe* (*sr*, map position: 3-62.0) gene. This region could not be tested in recombinant lines, since the *sr* marker was used to follow the *nos* mutation. It is however equally possible that the penetrance of the son phenotype associated with the recombinant chromosomes is too low to allow mapping in the *hb nos / nos* background.

son^{72} and son^{108}: Due to the low penetrance of the son phenotype caused by these two mutations, attempts to genetically map them using the *rucuca* chromosome were unsuccessful.

$Pson^{82}$: The son effect and the lethality of $Pson^{82}$ were mapped using the *rucuca* chromosome to within the *sr-ca* interval at 3-77 (number of recombination events tested within this interval: 30 (sr^+ ca^-) and 9 (sr^- *ca*). In all cases tested (n= 24) the son effect and the lethality cosegregated).

$Pson^{49}$: The w^+ marker associated with the $Pson^{49}$ mutation was mapped using the *al dp b pr c px sp* multiply marked chromosome to 2-16. (28/29 dp^+ *b* and 4/23 *dp* b^+ are w^+). The son phenotype correlates with the presence of the p[w^+] element.

In situ hybridization to salivary chromosomes: Performed essentially as in Laverty (1990). The probe used was a biotinylated pC4bgal plasmid (Thummel et al. 1988), which contains *LacZ* sequences.

RESULTS

Screen for EMS-induced suppressor of *nos(son)* mutations

The screens for EMS induced son mutations are diagrammed in Figure 2.1. Out of a combined total of 12,845 F1 * *nos / hb nos* females screened, 19 lines (0.1% of the total) behaved after backcrossing to a tester *hb nos* chromosome as if they may carry a *son* mutation and were therefore saved as balanced stocks.

These 19 putative suppressors stocks were retested by crossing balanced males with *hb nos* heterozygous females and testing for the son phenotype in *hb nos / * nos* progeny females. The results with 8 third chromosome lines (0.06% of the total number of half genomes screened) which showed a significant suppression-of-*nos* phenotype are shown in Table 2.2. This dominant phenotype is strongest in the sensitized hb^{mat} heterozygous background, but for many of these mutations can also be observed in the presence of a wild-type dose of hb^{mat}. The mutation son^{59} is regarded as a mutation in a *son* gene in spite of its negligible effect in this particular test because of its apparent allelism with another *son* mutation, son^{126} (see below).

The *son* complementation groups

Seventeen putative suppressors from the 35 mM EMS experiment were tested for allelism in an *inter se* complementation matrix. In this analysis, most of the transheterozygous combinations showed no interaction (i.e. transheterozygotes showed no deleterious effect on viability and females produced embryos without any obvious phenotype, aside from the background *nos* phenotype). Some interactions were observed between a number of *son* mutations. The data from this complementation matrix, along with the genetic mapping of the son phenotype and other associated phenotypes (see Materials and Methods for details), has allowed grouping of EMS induced son mutations in the following complementation groups:

***son*$^{1\text{-}3}$**:son^1, son^2 and son^3 are lethal *in trans* to each other. son^1 and son^2 map to the same position at approximately 3-31, and son^3 has been mapped to the *hairy -thread* interval (3-26.5 to 43.2), suggesting that these mutations are allelic.

$son^{1\text{-}3}$ were cytologically mapped by testing for non-complementation against deficiencies in the region. A set of deficiencies, including two small deletions, $E(z)^{65}$ and $E(z)^{66}$, which uncover only the *haywire* (*hay*) and *Enhancer of zeste* (*E(z)*) transcription

Table 2.2. Suppression of the *nos* phenotype by *son* mutations.

genetic background (1)	*nos / nos*		*hb nos / + nos*	
	% rescue (2)	n	% rescue (2)	n
+ / +	0	338	2	160
son^{1} / +	11	156	76	186
son^{2} / +	6	160	42	145
son^{3} / +	13	895	88	179
son^{59} / +	0	128	3	220
son^{126} / +	0	141	28	184
son^{29} / +	46	109	63	172
son^{72} / +	0	173	8	253
son^{108} / +	0	192	14	167

(1) "+" denotes the wild-type copy of the particular suppressor mutation.

(2) % of embryos with ≥3 abdominal segments.

units, failed to complement son^{1-3} mutations (Table 2.3). When tested singly, *hay* mutations are viable in trans to these alleles. On the other hand, son^{1} and son^{3} are not viable in trans to *E(z)* mutations, and $son^{2}/E(z)^{null}$ transheterozygous are viable but males exhibit ectopic sex combs on the second and third legs, a phenotype associated with *E(z)* mutations (Jones and Gelbart 1990) see also Chapter III Addendum). Thus, these three mutations are all alleles of the essential gene *Enhancer of zeste (E(z))* (see below) and will be referred in the text as $E(z)^{son1}$, $E(z)^{son2}$ and $E(z)^{son3}$.

***son*^{29}:** son^{29} homozygous flies are viable and fertile. The son phenotype associated with son^{29} (as well as its female sterile interaction with $E(z)^{son}$ alleles, see below) map at 3-46.6. Deficiencies which span the corresponding cytological region (76F to 78A) were tested in trans to son^{29} but failed to show any effect on viability or fertility (see Materials and Methods). Similarly, these deficiencies did not show the female sterile interaction in trans to $E(z)^{son3}$. Nevertheless, these results do not necessarily imply that son^{29} is not uncovered by these deficiencies since the nature of the son^{29} mutation is unknown.

Appendix A describes the genetic properties of son^{29} in more detail, along with further attempts to determine its identity and function.

***son*^{59,126}**: son^{59}/son^{59} and son^{59}/son^{126} transheterozygotes are viable and exhibit a high frequency of wing defects. I observed various degrees of blistering (where the two sides of the wing blade are disattached) and a reduction in wing size (see Table 2.4). These flies also show occasional thoracic bristle defects, such as kinks, although these were less frequent and more variable. In addition, son^{59}/son^{59} and son^{59}/son^{126} females lay a high frequency (about 80%) of apparently unfertilized eggs. son^{126}/son^{126} homozygotes are lethal.

The lethal and wing phenotypes associated with these two *son* mutations map to the same location, closely associated with *claret* (3-100.7). These results suggest that son^{59} and son^{126} are alleles of a gene essential for viability wing morphogenesis and perhaps fertility that is closely associated with *ca*.

***son*^{72} and *son*^{108}** : Neither of these two mutations shows interactions with any other *son* mutation. They are included in this discussion because of their effect, albeit small, on the nos phenotype (Table 2.1). Due to the low penetrance of their son phenotype, attempts to genetically map these mutations using the *rucuca* chromosome were unsuccessful.

Table 2.3. Complementation mapping of *son*$^{1-3}$ alleles.

Chromosomes tested (1)	Phenotype of transheterozygotes (2)	n
Deficiencies:		
Df(3L)Ez2 vs. ***son*^{1}**	lethal	74
Df(3L)Ez3 vs. ***son*^{1}**	lethal (1/4)	64
Df(3L)Ez6 vs. ***son*^{1}**	lethal	25
Df(3L)Ez6 vs. ***son*^{2}**	viable 22 v., # with e-s-c: n.d.	52
Df(3L)Ez6 vs. ***son*^{3}**	lethal	44
Df(3L)lxd6 vs. ***son*^{1}**	lethal	20
Deletions of both *E(z)* and *hay*		
E(z)^{65} *vs.* ***son*^{1}**	lethal (1/4)	61
son^{1} *vs.* ***E(z)*^{65}**	lethal (3)	249
son^{2} *vs.* ***E(z)*^{65}**	viable 167 v., 62 of 82 males with e-s-c	400
son^{3} *vs.* ***E(z)*^{65}**	lethal	303
E(z)^{66} *vs.* ***son*^{1}**	lethal (1/4)	34
Individual *E(z)* mutations		
son^{1} *vs.* ***E(z)*^{63}**	lethal	493
son^{2} *vs.* ***E(z)*^{63}**	viable 180 v., 40 of 60 males with e-s-c	634
son^{3} *vs.* ***E(z)*^{63}**	lethal	416
E(z)^{64} *vs.* ***son*^{1}**	lethal	36
E(z)^{64} *vs.* ***son*^{3}**	lethal	36
Individual *hay* mutations		
nc2 vs. ***son*^{3}**	viable 37 v.	116
nc2^{DEB12} *vs.* ***son*^{3}**	viable 51 v.	132
nc2^{rv1} *vs.* ***son*^{3}**	viable 29 v.	94
nc2^{rv3} *vs.* ***son*^{3}**	viable 54 v.	99

(1) X vs Y: X/Balancer males x **Y/Balancer females**. Other deficiencies in the *h-th* interval that did not uncover the *son*$^{1-3}$ mutations are not included.

(2) Absence of transheterozygous individual, which should be 1/3 (except were indicated by "1/4", where it should be 1/4) of the total offspring, is indicated as "lethal". In "viable" combinations the number of viable (v.) individuals and of individuals with ectopic sex combs (e-s-c) on the second or third thoracic legs is indicated. n.d.= not determined.

(3) Two individuals without the dominant markers present in the balancers were observed. It is unclear whether these are *son*^{1}*/E(z)65* escapers or individuals carrying a balancer chromosome, but not exhibiting the *Ser* marker (which in some backgrounds is not completely penetrant).

Interactions between *son* mutations

In addition to noncomplementation of alleles within complementation groups, a number of interactions were observed between alleles of different complementation groups.

The son^{29}-$E(z)^{son}$ female sterile interaction: transheterozygous combinations between son^{29} and $E(z)^{son1-3}$ revealed a strong female sterile interaction (see Appendix A).

The $son^{59,126}$-$E(z)^{son2}$ wing, bristle defect interaction: transheterozygotes between son^{59} and son^{126} and the allele $E(z)^{son2}$ exhibit similar wing and bristle defects as those observed for son^{59}/son^{59} and son^{59}/son^{126} flies. This interaction is specific to the $E(z)^{son2}$ mutation, since it is observed only at a very low degree with $E(z)^{son1}$ or $E(z)^{son3}$ (Table 2.4).

The genetic interactions within and between *son* complementation groups are summarized in Figure 2.2. These data suggest that the gene products encoded by at least three different genes ($E(z)^{son1-3}$, son^{29} and $son^{59,126}$) interact in a process related to the *nos* phenotype and in additional processes such as oogenesis and bristle and wing morphogenesis.

Screen for P-element induced suppressor-of-*nos* (*Pson*) mutations

A screen for suppressors of *nos* was also carried out using P-element induced mutagenesis (see Figure 2.3). The ammunition chromosome used was an X chromosome with five P-elements (multi-p[w^+], Bier et al. 1989). P-element induced mutagenesis has the advantage over EMS mutagenesis in that genes can be molecularly tagged by the insertion of the P-element at locations usually adjacent to the mutated gene. The P-element used had additional features. First, they contain "plasmid rescue" sequences, which contain bacterial origins of replication and drug resistance genes and allow the easy cloning of adjacent genomic fragments. These constructs can also act as "enhancer-traps", i.e. they

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Figure 2.2. Genetic interactions between *son* alleles. *son* mutations are grouped as three different genes, $E(z)^{son}$, $son^{59,126}$ and son^{29}, according to complementation and mapping data. Arrows indicate genetic interactions, which are abbreviated as follows: L, lethality; S, female sterility; wg, br; wing and thoracic bristle defects. son^{59} and son^{126} mutations interact specifically with $E(z)^{son2}$. son^{29} interacts with the three $E(z)^{son}$ alleles. The sterile interaction associated with $son^{59,126}$, has not been mapped and may be associated with additional mutations in these chromosomes.

Figure 2.2

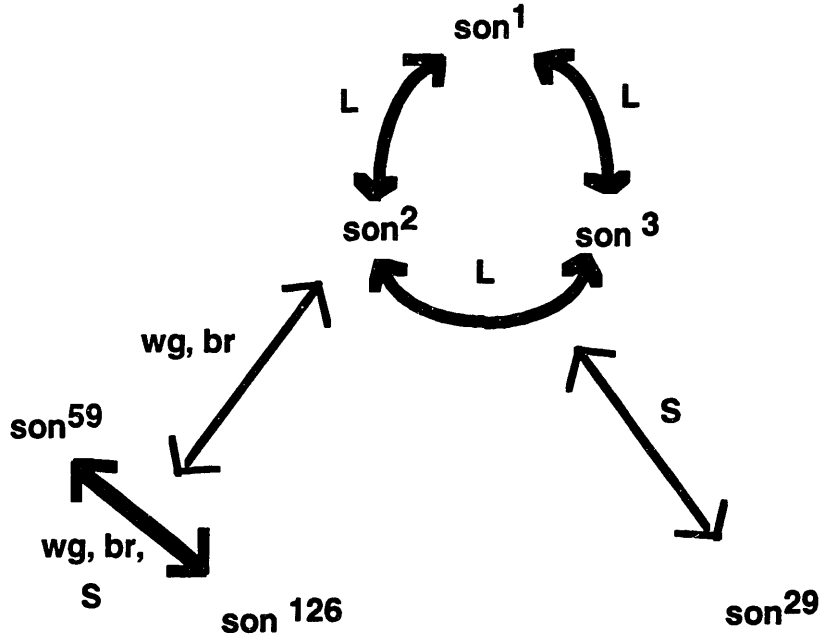


Table 2.4. *son*^{59,126} interact with themselves and *E(z)*^{son2} to produce a wing phenotype.

zygotic genotype (1)	% with defective wings	n
son^{59} / *son*^{59}	46	35
son^{59} / *son*^{126}	52	122
son^{126} / *son*^{126}	LETHAL	
son^{59} / *E(z)*^{son1}	0	59
son^{59} / *E(z)*^{son2}	17	109
son^{59} / *E(z)*^{son3}	1	122
son^{126} / *E(z)*^{son1}	0	51
son^{126} / *E(z)*^{son2}	20	50
son^{126} / *E(z)*^{son3}	4	68

(1) Recombinant chromosomes of *son*^{59}, *son*^{126} and *E(z)*$^{son1-3}$ were used in this experiment, such that only a small portion of the original mutagenized chromosome is retained in the tester chromosomes.

carry a weak promoter fused to a *lacZ* reporter gene which can reflect the activity of nearby enhancers. 15,612 F1 flies of the genotype *y w* p[w$^+$] */ y w ; hb nos / nos* were screened for suppression of the *nos* phenotype in a manner similar to that implemented in the EMS induced screen.

After the first test for the *son* phenotype, 15 selected lines were considered candidates for carrying suppressors. These lines were tested for potential lethality and sterility in trans to mutations in previously known *son* genes, such as *E(z)* (tester alleles *Ez6* and $E(z)^{son3}$) and $son^{59,126}$ (tester allele, son^{126}). These tests did not reveal any noncomplementation or other interactions between these putative *Pson* mutations and the previously isolated son genes *E(z)* and $son^{59,126}$.

A final round of testing for dominant son phenotypes led to the selection of the *Pson* mutations presented in Table 2.5.

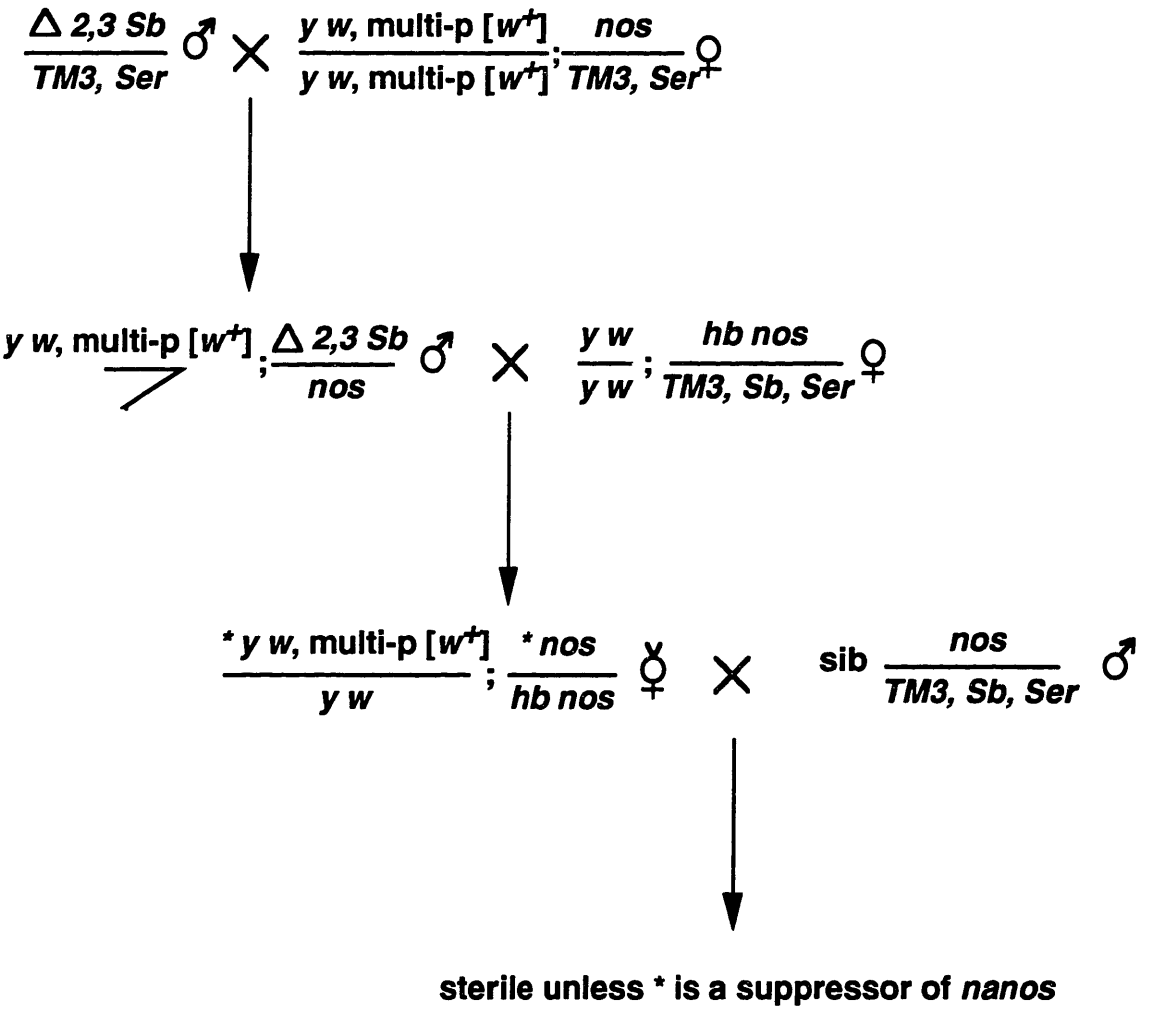
***Pson*^{49} :** $Pson^{49}$ is associated with a p[w$^+$] insertion in cytological band 26A9 (Figure 2.4A, B). This P-element insertion seems to be responsible for the son phenotype of this chromosome, as well as reduced viability and fertility of homozygotes (see Appendix B).

***Pson*^{82}:** $Pson^{82}$ is a w^- line which exhibits a moderate son phenotype and is homozygous lethal. The son effect and the lethality of $Pson^{82}$ both map at 3-77.

***Pson*^{33} and *Pson*^{56}:** These two lines have a son phenotype which appears to be associated with the original multi-p[w$^+$] chromosome. Cytological detection of the P-elements in the $Pson^{56}$ line suggests that neither excision of the original P-elements nor new P-element insertions have occurred in this chromosome (Figure 2.4D, compare to C; this has not been determined for $Pson^{33}$).

Thus the mutation $Pson^{56}$, as well as $Pson^{82}$ (which, as stated above, is not associated with a w^+ insertion), may have been induced either spontaneously or by P-element induced DNA rearrangements. Induction of mutations not associated with a p[w$^+$] element has been previously observed in screens that use this and other ammunition chromosomes (see, for example, Bier et al. 1989). In the screen carried out by Bier et al., there was a preselection for p[w$^+$] insertions in the autosomes, so that all chromosome tested carried a new p[w$^+$] element. In the son screen presented here, no such preselection for new insertion was carried out in order to maintain it as a simple F1 screen. This may account for the higher frequency of mutations not associated with a p[w$^+$] insertion.

Figure 2.3


Δ 2,3 Sb / TM3, Ser ♂ × y w, multi-p [w+] / y w, multi-p [w+] ; nos / TM3, Ser ♀
y w, multi-p [w+] ; Δ 2,3 Sb / nos ♂ × y w / y w ; hb nos / TM3, Sb, Ser ♀
* y w, multi-p [w+] / y w ; * nos / hb nos ♀ × sib nos / TM3, Sb, Ser ♂
sterile unless * is a suppressor of nanos

Potential synergistic effects between different *son* and *Pson* mutations

A mutant background with a highly penetrant son phenotype would be extremely useful to study of the nature of the son phenotype at both the genetic and molecular level. The strongest dominant *son* mutations are homozygous lethal, which excludes the possibility of simply homozygosing these mutations as a way of obtaining a stronger effect. Somewhat surprisingly, the only strong *son* allele that is homozygous viable, son^{29}, does not appear to increase markedly in its penetrance when homozygosed (see Appendix A). Therefore, allelic combinations between some of the strongest suppressors were tested for their son phenotype in the hope that simultaneously mutating several *son* genes would lead to a stronger son effect. Several transheterozygous combinations of the mutations $E(z)^{son3}$, son^{29}, $Pson^{82}$ and son^{126} were tested and the results are presented in Table 2.6. In general the presence of two *son* mutations causes stronger son phenotype than that conferred by single mutations. However, none of the combinations tested in a genetic background with the normal hb^{mat} dosage exhibited a son phenotype exceeding 50% penetrance. As expected, reducing the dosage of hb^{mat} by half further enhanced the son phenotype. Nevertheless, one would like to study the effect of the *son* mutations independently of a reduction in the hb^{mat} product and therefore none of these transheterozygous combinations appeared to be suitable for studies that require a highly penetrant mutant background.

More penetrant *son* genetic backgrounds have been obtained by combining various *E(z)* alleles (described in Chapter III).

Figure 2.3. A screen for P-element induced suppressors of nos. Delta 2,3 is a constitutive source of transposase, which induces mobilization of P-elements (Robertson et al. 1988). The screen was carried out in a background mutant for *white* (*w*) in order to genetically follow the P-elements, which carry a w^+ minigene. The *y w*, multi-p[w+] chromosome carries multiple P-elements (Bier et al. 1990). *TM3* is a third chromosome balancer, *Sb* and *Ser* are dominant visible markers, *y* is a recessive visible marker. The F1 sibling males were selected for the *Ser* marker to insure that the source of transposase was no longer present in the following generations. Retesting and localizing the *Pson* mutations was in general as described in the legend of figure 2.1B, except for the following changes: a) all stocks were *y w*. b) F2 surviving males carrying the *TM3, Sb, Ser* balancer were backcrossed to *hb nos / TM3, Ser* (not *Sb*) females. This allowed retesting for the son effect and generating a third chromosome balanced stock in a single cross (so that step (ii) in Figure 2.1B was not needed).

Table 2.5. Summary of P-element induced suppressor-of-*nos* mutations

son background	Dominant effect				Recessive effect		w^+-associated?
	nos / nos		*hb nos / + nos*		*nos / nos*		
	% rescue(1)	n	% rescue(1)	n	% rescue(1)	n	
controls							
wild type (2)	0	158	13	163	0	158	n.a.
$E(z)^{son3}$ (2)	n.d.		80	127	n.a.		n.a.
Pson mutations							
$Pson^{49}$	5	348	35	277	40	5	on II
$Pson^{82}$	0	145	42	129	n.a. (lethal)		no
$Pson^{33}$	0	215	5	250	6	809	n.k. (3)
$Pson^{56}$	0	275	35	131	7	285	no (4)

(1) % of embryos with ≥3 abdominal segments.

(2) Controls in a *y w* background. wild type : no suppressor (negative control); $E(z)^{son3}$: *son* phenotype of a previously isolated *son* mutation (positive control).

(3) son phenotype is associated with multi-p[w^+] chromosome. It is not known whether it is associated with a P-element mobilization event.

(4) son effect is associated with multi-p[w^+] chromosome, but does not appear to be caused by a P-element mobilization event.

Table 2.6. Son phenotype of embryos from females transheterozygous mutant for two *son* genes.

son tranheterozygous combination	*nos / nos*		*hb nos / + nos*	
	% rescue (1)	n	% rescue (1)	n
$y^+ w^+ / y^+ w^+$ (wild-type) background (2)				
$E(z)^{son3} / +$	9	341	48	390
$E(z)^{son3} + / + son^{126}$	22	172	67	238
$son^{29} / +$	39	406	n.d.	
$son^{29} + / + son^{126}$ (3)	4	190	n.d.	
$y\ w / y^+ w^+$ background (2)				
$E(z)^{son3} / +$	7	341	74	402
$E(z)^{son3} + / + Pson^{82}$	32	232	93	44
$son^{29} / +$	49	301	n.d.	
$son^{29} + / + Pson^{82}$	50	504	n.d.	

(1) % of embryos with ≥3 abdominal segments.

(2) Since the *yw* background (present in *Pson* mutations) enhance penetrance of the son phenotype, the crosses have been grouped accordingly.

(3) In this transheterozygous combination the son^{126} mutation weakens the enhancement of the son^{29} mutation. This experiment has not been repeated and the significance of this particular result is unclear.

CONCLUSIONS

A summary describing the better characterized EMS and P-element induced son mutations is presented in Table 2.7. From the results of this chapter, it seems appropriate to emphasize a number of points:

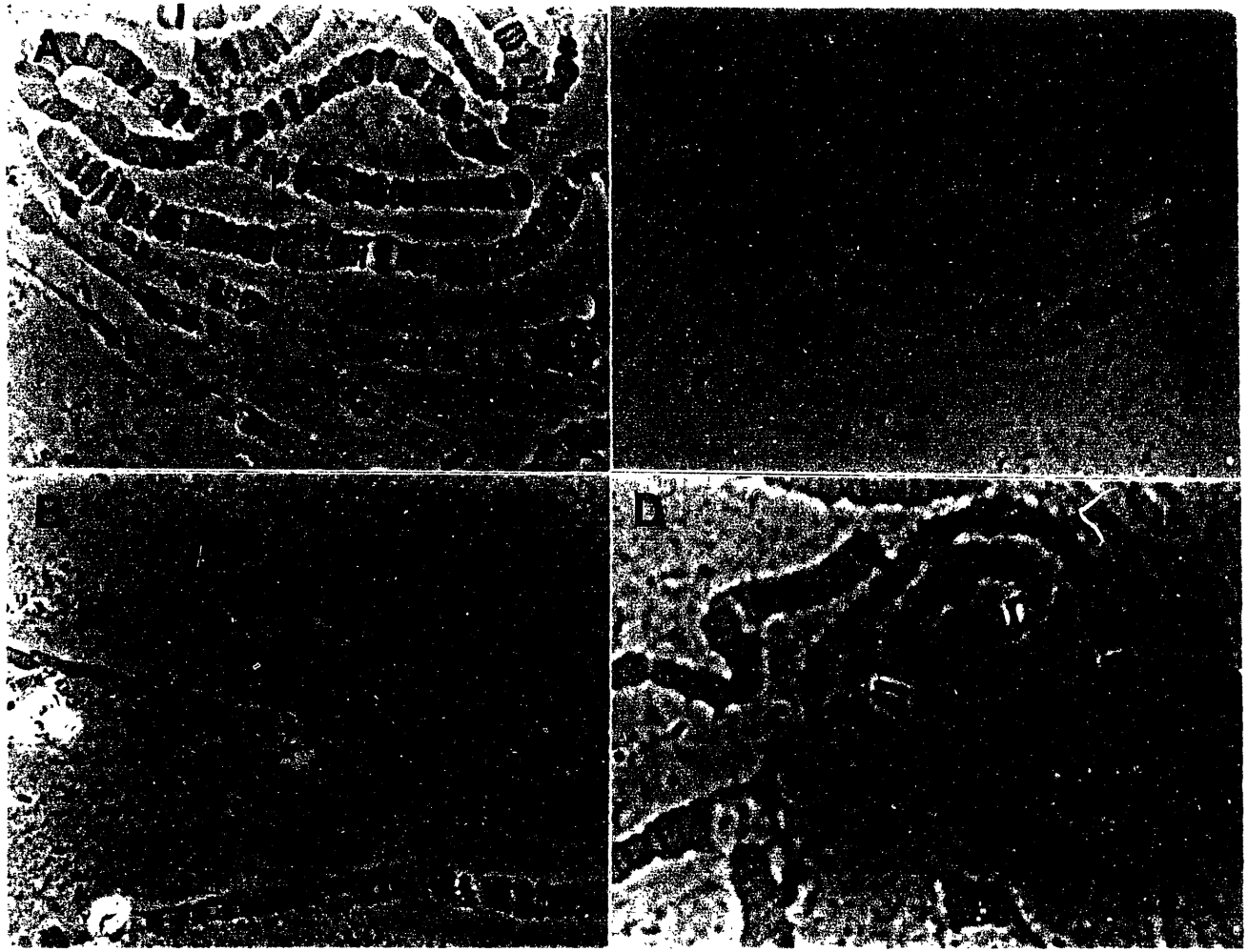
a) The son phenotype is in general enhanced by heterozygosity for *hb*. This is in accordance with the expectation that *son* mutations will affect hb^{mat} production and/or function (see Introduction). In a hb^{+} background, on the other hand, most *son* mutations do not have a strong dominant *son* phenotype and would likely not have been isolated in a F1 screen. Thus, sensitizing the genetic background is important to isolate mutations that cause semidominant effects.

b) Even though the screen was designed in principle to isolate mutations on all chromosomes, most of the suppressors isolated are located on the third chromosome. It is likely that this bias is due to the way in which the screens were designed, such that the mutagenized third chromosome is balanced and can be followed, while the other chromosomes are unbalanced. In addition, the assignment of a mutation to a particular chromosome is difficult because it depends on testing a number of single females (see legend of Figure 2.1) and the son phenotype is somewhat variable from female to female. Thus, son mutations in chromosomes that are unbalanced may be difficult to recover. Similarly, in a different screen, the only recovered dominant suppressors of the *Bicaudal-D* mutation were on the second chromosome, where *Bicaudal-D* maps (see Appendix C). That *son* mutations will only be more likely recovered when they map to a marked chromosome is also shown by the finding that *son* mutations isolated in chromosomes other than the third were linked to a p[w^{+}] marker.

c) *son* mutations in particular genes were identified with a much lower frequency than presumed loss-of-function mutations in the marker genes *cu* and *p*. For *E(z)*, for example, we isolated one $E(z)^{son}$ mutation for every seven *cu/p* loss-of-function alleles. In the case of *E(z)* this discrepancy can be explained by the nature of the $E(z)^{son}$ mutation. Indeed, $E(z)^{son}$ mutations are gain-of-function alleles (see Chapter III), which would be expected to be induced at a lower frequency than loss-of-function alleles. Moreover, *E(z)*

Figure 2.4. In situ hybridization to salivary chromosome of mutant *Pson* lines using sequences present in the P-element. A, B) Second chromosome in $Pson^{49}$ line: hybridization signal in polytene band 26A. This result agrees with the genetic position of the inserted P-element at 2-16. C, D) X-chromosome in the $Pson^{49}$ (C) and $Pson^{56}$ (D) lines. In both lines, hybridization signals are found in chromosomal regions 1, 9 and 18, which correspond, respectively, to the elements B/E, C and A of Bier et al. (1989). The D element, which should be in region 3, has likely excised before mutagenesis.

Figure 2.4



null alleles have only a weak son phenotype (see Chapter III), and thus *E(z)* null mutations would likely not have been selected in our screens. It is possible that some of the other *son* mutations isolated in these two screens are also unusual gain-of-function mutations, although this remains to be proven.

d) Many of the *son* mutations interact in transheterozygous combinations to affect processes such as oogenesis, and wing and bristle morphogenesis. Thus, these genes may form a family of interacting products that act together in processes related not only to early segmentation gene regulation but also to other aspects of development. One of the *son* genes, *E(z)*, is a known member of the Polycomb group of genes. The genes in this group share in common the requirement for proper homeotic gene regulation, although many Pc-G genes have pleiotropic phenotypes. Interestingly, mutations in other Pc-G genes also show a son phenotype (see Chapter III). Thus it is tempting to speculate that the *son* mutations isolated in these two screens and the Pc-G genes are functionally related. Indeed, *E(z)* may not be the only *son* gene which produces homeotic phenotypes, since the head involution and segmentation defects found among the progeny of son^{29} / $E(z)^{son}$ females and from $Pson^{49}$ homozygous females, resemble phenotypes produced by weak homeotic transformations (see Appendixes A and B).

Table 2.7. Summary of selected EMS and P-element induced *son* mutations.

Denomination	son phenotype	other characteristics	location (1)
$E(z)^{son1\text{-}3}$	strong	$E(z)^{son1\text{-}3}$ are gain-of function (2). $E(z)^{son1,3}$ are lethals over nulls. $E(z)^{son2}$ is viable over nulls. Interactions of $E(z)^{son1\text{-}3}$ with son^{29} and of $E(z)^{son2}$ with $son^{59,126}$	3-34.0 67E3-4
son^{29}	strong	viable. Interaction with $E(z)^{son1\text{-}3}$ (female sterility).	3-46
$son^{59,126}$	weak-moderate	son^{59} homozygotes viable. son^{126} homozygotes lethal. Also affects on wings and bristle morphology and fertility. Interaction with $E(z)^{son2}$ (wing and bristle defects).	3-100
$Pson^{49}$	moderate	reduced viability and sterility. Allele is likely not a null. Associated with p[w^+]	2-16 26A9
$Pson^{82}$	moderate	lethal	3-77

(1) First term indicates genetic location (chromosome and map position), second term, when known, indicates cytological position.

(2) See Chapter III.

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Chapter III. A Role of Polycomb Group genes in the regulation of gap gene expression in Drosophila

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A Role of Polycomb Group Genes in the Regulation of Gap Gene Expression in Drosophila

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ABSTRACT

Anteroposterior polarity of the Drosophila embryo is initiated by the localized activities of the maternal genes, *bicoid* and *nanos*, which establish a gradient of the *hunchback* (*hb*) morphogen. *nanos* determines the distribution of the maternal Hb protein by regulating its translation. To identify further components of this pathway we isolated suppressors of *nanos*. In the absence of *nanos* high levels of Hb protein repress the abdomen-specific genes *knirps* and *giant*. In *suppressor-of-nanos* mutants, *knirps* and *giant* are expressed in spite of high Hb levels. The suppressors are alleles of *Enhancer of zeste* (*E(z)*) a member of the Polycomb group (Pc-G) of genes. We show that *E(z)*, and likely other Pc-G genes, are required for maintaining the expression domains of *knirps* and *giant* initiated by the maternal Hb protein gradient. We have identified a small region of the *knirps* promoter that mediates the regulation by *E(z)* and *hb*. Because Pc-G genes are thought to control gene expression by regulating chromatin, we propose that imprinting at the chromatin level underlies the determination of anteroposterior polarity in the early embryo.

ESTABLISHMENT of pattern along the anteroposterior axis in Drosophila is initiated by maternal gene products which are synthesized during oogenesis. These maternal gene products direct the spatial expression of gap genes which are transcribed from the embryonic genome and whose products are expressed in large, overlapping domains [reviewed in HÜLSKAMP and TAUTZ (1991)]. Precise transition from maternal to zygotic control of gene expression is critical for the initiation and maintenance of a stable pattern of gap gene expression.

Transition between maternal and zygotic information along the anteroposterior axis is in part achieved by the transition from a concentration gradient of maternally derived Hunchback protein (Hb^{mat}) to a gradient of zygotic Hb protein (Hb^{zyg}) expressed by the embryo. The maternal gene *nanos* (*nos*) establishes the maternal Hb protein gradient (see Figure 1A). *nanos* RNA is synthesized during oogenesis and becomes localized to the posterior pole of the mature oocyte (WANG and LEHMANN 1991). After fertilization a posterior to anterior concentration gradient of Nanos protein emanates from the local RNA source (BARKER *et al.* 1992). *Hb* RNA is also synthesized during oogenesis and is distributed uniformly throughout the freshly laid egg. Nanos protein is a repressor of *hb* translation and thereby establishes a concentration gradient of Hb^{mat} complementary to that of Nos (TAUTZ 1988; HÜLSKAMP *et al.* 1989; IRISH *et al.* 1989a; STRUHL 1989; TAUTZ and PFEIFLE 1989; WANG and LEHMANN 1991; WHARTON and STRUHL 1991). Zygotic expression of *hb*, on the other hand, is controlled by the transcription factor *bicoid* (*bcd*). Like *nos*, *bcd* RNA is synthesized during oogenesis and is localized within the oocyte, but to the anterior pole (FROHNHÖFER and NÜSSLEIN-VOLHARD 1986; BERLETH *et al.* 1988). *Bcd* RNA translation results in an anterior to posterior concentration gradient of Bcd protein (DRIEVER and NÜSSLEIN-VOLHARD 1988). This protein, in turn, activates *hb* and other genes in the anterior half of the embryo in a concentration-dependent manner (SCHRÖDER *et al.* 1988; TAUTZ 1988; DRIEVER and NÜSSLEIN-VOLHARD 1989; DRIEVER *et al.* 1989; STRUHL *et al.* 1989).

Thus, both the anterior morphogen *bcd* and the posterior determinant *nos* achieve, by different mechanisms, a similar end result: the formation of an anterior to posterior gradient of Hb protein. Although there are a large number of additional regulatory interactions between maternal signals and gap genes, and between gap genes themselves [reviewed in HÜLSKAMP and TAUTZ (1991); see also ELDON and PIRROTTA (1991), KRAUT and LEVINE (1991a,b), CAPOVILLA *et al.* (1992), and STRUHL *et al.* (1992)], the Hb protein gradient stands out as a major organizer of the embryonic gap gene expression pattern. Hb protein can act both as a transcriptional activator and repressor, and the Hb protein gradient determines the expression domains of gap genes thereby dividing the embryo into anterior (*hb*-expressing), middle (*Krüppel* (*Kr*)-expressing) and posterior (*knirps* (*kni*)- and giant (*gt*)-expressing) regions (see Figure 1A) (HÜLSKAMP *et al.* 1990; KRAUT and LEVINE

1991a,b; STRUHL *et al.* 1992). A gradient of either Hb^{mat} or Hb^{nos} protein, which are identical in primary sequence (TAUTZ *et al.* 1987), is sufficient on its own to organize the embryo into this basic (*Kr-kni-gt*) gap gene pattern (although only Hb^{nos} protein attains the high levels necessary for *Kr* repression) (HÜLSKAMP *et al.* 1990; STRUHL *et al.* 1992).

Changes in the distribution of Hb disrupt normal embryonic patterning. This is demonstrated most directly in embryos from *nos* mutant females in which Hb^{mat} is translated throughout the embryo. Uniformly high levels of Hb^{mat} repress transcription of the abdomen-specific gap gene *kni* and *gt* and therefore these embryos lack abdomen. Since Hb is the major repressor of gap gene expression in *nos* mutants (HÜLSKAMP *et al.* 1989; IRISH *et al.* 1989a; STRUHL 1989), we reasoned that additional genes required for the production or the activity of the Hb^{mat} protein could be identified as suppressors of *nos* (Figure 1).

We report here the identification and characterization of three such *suppressor-of-nos* (abbreviated *son*) mutations. We show that these mutations are alleles of the previously characterized gene *Enhancer of zeste* (*E(z)*) (JONES and GELBART 1990; PHILLIPS and SHEARN 1990). We investigated the role of *E(z)* in the determination of the anteroposterior pattern and conclude that *E(z)* is required to maintain transcriptional repression of the gap genes *kni* and *gt* once repression has been initiated by the Hb^{mat} protein.

MATERIALS AND METHODS

Nomenclature: Throughout the text we refer to embryos from mutant females as "mutant embryos" which describes their maternal and not their zygotic genotype. Marker mutations and balancer chromosomes are described in LINDSLEY and ZIMM (1992). Staging of embryos is as in FOE and ALBERTS (1983).

Screen for suppressors of *nos*: hb^{7M} is a protein null (LEHMANN and NÜSSLEIN-VOLHARD 1987; TAUTZ 1988), nos^{L7} behaves as a loss-of-function mutation with respect to abdomen formation although it still retains *nos* function required for oogenesis (LEHMANN and NÜSSLEIN-VOLHARD 1991). $hb^{7M}nos^{L7}/TM3$ females were crossed to nos^{L7} homozygous males mutagenized with either 35 mM or 45 mM ethyl methylsulfonate (EMS). F_1 $hb^{7M}nos^{L7}/nos^{L7}$ females were tested in groups for producing hatching embryos. Single females were retested and lines were established from the F_2 males. We tested 12,854 half genomes, *i.e.*, *hb nos/nos* females (9,372 from the 35 mM batch and 3,482 from the 45 mM batch). To determine the efficiency of mutagenesis we also screened for newly induced *p* alleles and *cu* alleles. The estimated yield of our mutagenesis is about 1.7 hits per locus per 1000 chromosomes at 35 mM (not determined for 45 mM).

Genetic analysis and strains: Suppression of the *nos* phenotype by all suppressors including the *E(z)* mutations is stronger at higher temperatures and is observed more frequently in the layings of younger females. Therefore, to assure consistency in different experiments, all genetic tests were carried out at 25° (except where noted), and only the progeny from the first four days of laying were analyzed. Embryos were allowed to develop cuticle structures (24 hr at 25°) and the number of abdominal segments was scored either directly under a dissecting scope (the embryos being cleared by a film of mineral oil) or as cuticle preparations embedded in Hoyer's medium (WIESCHAUS and NÜSSLEIN-VOLHARD 1986).

$E(z)^{son}$ mutations are alleles of *Enhancer of zeste* (synonym: *polycombeotic*).

Map position: son^1 and son^2 map within 5 map units of *E(z)* (3–34.0), son^1 to the 3–26.5 to 3–43.2 interval.

Associated phenotypes: the $E(z)^{son}$ alleles, like other *E(z)* alleles are larval lethal when *trans*-heterozygous. The alleles $E(z)^{son1}$ and $E(z)^{son3}$ are lethal in *trans* to *E(z)* null alleles or deficiencies. $E(z)^{son2}$ is viable and fertile in trans to those alleles but males exhibit ectopic sex combs on the second and third legs, a phenotype associated with *E(z)* mutations (WU *et al.* 1989; JONES and GELBART 1990; PHILLIPS and SHEARN 1990). In addition, these three alleles, like antimorphic *E(z)* alleles, act as strong suppressors of the *zeste-white* interaction (data not shown) (JONES and GELBART 1990).

Complementation: a *P* element carrying only the *E(z)* transcription unit (JONES and GELBART 1993) suppresses the lethality associated with the $E(z)^{son}$ alleles (zygotic genotypes tested: $E(z)^{son3}/E(z)^{05}$, $E(z)^{son1}/E(z)^{son3}$, and $E(z)^{son2}/E(z)^{son3}$), and reduces their *suppressor-of-nos* effect (maternal genotypes tested: in a hb^{mat} heterozygous background, $E(z)^{son2}/+$ and $E(z)^{son3}/+$; in a wild-type hb^{mat} background, $E(z)^{son1}/E(z)^{son3}$ and $E(z)^{son2}/E(z)^{son3}$). Thus these gain-of-function (g-o-f) alleles are antimorphic ("poison").

E(z) alleles are referred to according to LINDSLEY and ZIMM (1992): *Df(3L)Ez6*, $E(z)^{05}$, $E(z)^{06}$ (deficiencies, $E(z)^{06}$ partial deletion, weakly antimorphic, JONES and GELBART 1993), $E(z)^{03}$, $E(z)^{04}$ (nulls, $E(z)^{04}$ slightly antimorphic); $E(z)^{01}$ (temperature sensitive, see legend of Table 1), $E(z)^{n0}$, $E(z)^{1}$ (g-o-f, suppressor and enhancer of the *zeste-white* interaction, respectively).

Suppressor of zeste-2 (Su(z)-2) complex mutations [except where otherwise stated (see BRUNK *et al.* 1991)]: $Su(z)2^{1\,b8}$, *Df(2R)vg-B* [deletions of *Psc*, *Su(z)2* and *Su(z)2-Distal* (*Su(z)2-D*)]; *Su(z)-2*: $Su(z)2^{1\,b7}$ (null), $Su(z)2^{1}$ (g-o-f, suppressor of the *zeste-white* interaction); *Posterior sex combs*: Psc^{1445} (hypomorph, possibly maternal-effect g-o-f [see ADLER *et al.* (1989)], Psc^{24}, Psc^{1433} [null and g-o-f, respectively (C.-T. WU and M. HOWE, personal communication)] Psc^{1} (g-o-f, suppressor of the *zeste-white* interaction); *Su(z)-2-D*: $Su(z)3^{1}$(g-o-f, suppressor of the *zeste-white* interaction). Other Pc-G mutations are *Additional sex combs*, Asx^{D1} [g-o-f (SINCLAIR *et al.* 1992)]; *extra sex combs*, esc^{2} (null), esc^{10} (deletion) (STRUHL 1981) [recovery of *esc* null embryos was essentially as described in STRUHL and AKAM (1985)]; *pleiohomeotic*, pho^{c} (hypomorph) (DUNCAN 1982); *Df(4)G* (deletion) (BREEN and DUNCAN 1986); *Polycomb*, *Df(3L)Pc* (deletion) (HAYNIE 1983); *Polycomblike*, Pcl^{D5} (null) (BREEN and DUNCAN 1986); *polyhomeotic*, ph^{503} (null) (DURA *et al.* 1987); *Sex combs extra*, Sce^{D1} (nature unknown) (BREEN and DUNCAN 1986); *Sex combs on midleg*, Scm^{D1} (null) (BREEN and DUNCAN 1986); *super sex combs*, sxc^{1} (null) (INGHAM 1984). The partial loss-of-function allelic combination ph^{410}/ph^{br} (DURA *et al.* 1987) did not show significant suppression of the *nos* phenotype.

Pole cell transplants: Ovo^{D1} females were used as hosts for pole cell transplants as described in LEHMANN and NÜSSLEIN-VOLHARD (1987). Ovo^{D1} causes degeneration of the female germ line (OLIVER *et al.* 1987). Donor embryos were derived from the following crosses: (1) $E(z)^{son1}nos$/TM3 × $E(z)^{son3}nos$/TM3 to obtain $E(z)^{son1}nos/E(z)^{son3}nos$ clones; (2) $E(z)^{son2}nos$/TM3 × $E(z)^{son3}nos$/TM3 to obtain $E(z)^{son2}$ $nos/E(z)^{son3}nos$ clones ($nos = nos^{L7}$). The genotype of the transplanted pole cells was determined by the phenotype of the progeny.

$E(z)^{son1}nos/E(z)^{son3}nos$ germ cells did not lead to adult progeny but their genotypes were inferred by the rescue of the *nos*

phenotype and/or other associated defects. At 25°, 8/16 fertile females (presumably $E(z)^{ion1}nos/E(z)^{ion1}nos$) produced very few, abnormal eggs, most of which did not form cuticles. At 18° 3/4 fertile adult females (presumably $E(z)^{ion1}nos/E(z)^{ion1}nos$) laid eggs which developed into larvae (71 larvae/147 eggs). All embryos formed six to eight abdominal segments. Most of the developed embryos (69/71) failed to hatch and none reached adulthood. Many (84%, $n = 32$) showed head involution defects, similar to those observed in embryos with mild homeotic transformations [see, for example, JÜRGENS (1985)].

$E(z)^{ion2}nos/E(z)^{ion3}nos$ germ line clones: 8/25 fertile females exhibited phenotypes similar to those observed in females carrying $E(z)^{ion1}nos/E(z)^{ion1}nos$ germ line clones. Two females produced embryos which reached adulthood. In these cases the genotype of the transplanted pole cells was unambiguously identified.

The additional phenotypes associated with these *E(z)son* germ line clones, such as egg laying defects, were also observed when similar germ line clones were obtained in the presence of functional *nos* product ($E(z)^{ion1}nos^+$). A more detailed description of these germ line clone results will appear elsewhere.

Analysis of expression patterns: $E(z)^{ion2}/E(z)^{o1}$ females were grown at 25° and eggs were collected from young females. Mutant backgrounds: nos^{L7} homozygotes, bcd^{E1} homozygotes, gt^{1A} hemizygotes, tsl^{D35}/tsl^{D34} *trans*-heterozygotes.

Whole-mount *in situ* hybridization with digoxigenin-labeled RNA probes for *kni*, *gt*, *Kr* and *lac Z* was performed as described in GAVIS and LEHMANN 1992.

The transgenes which contain the *kni* promoter-*lac Z* fusions were kindly provided by M. PANKRATZ and H. JÄCKLE (PANKRATZ *et al.* 1992). Males carrying the transgene were crossed to females of the appropriate genotype. Two insertion lines led to identical results.

Whole-mount antibody staining was performed as in GAVIS and LEHMANN (1992). The rabbit anti-Hb antibody, a gift from G. STRUHL, was diluted 1:50 and preadsorbed against 4–18-hr embryos. Biotinylated secondary antibody (from Vector Laboratories) was diluted 1:15 and preadsorbed against 8–14-hr embryos.

RESULTS

Identification of *Enhancer of zeste* alleles as suppressors of *nanos*: Embryos that lack functional Nos product lack all eight abdominal segments. In the absence of both Nos and Hb^{mat} embryonic patterning can proceed normally if *hb* is expressed zygotically (HÜLSKAMP *et al.* 1989; IRISH *et al.* 1989; STRUHL 1989). Thus, the major role of Nos during early embryogenesis is to establish the Hb protein gradient. We therefore reasoned that further components of the *nos-hb* regulatory pathway could be identified as *suppressor-of-nos* mutants (Figure 1). To sensitize the selection system we searched for suppressor mutants in a background heterozygous for *hb*. This reduction of the maternal *hb* gene dosage weakens the *nos* mutant phenotype, such that one to three abdominal segments are formed in the progeny of *hb nos*/+ *nos* females (Table 1) (HÜLSKAMP *et al.* 1989; IRISH *et al.* 1989; STRUHL 1989). We screened for EMS-induced mutations that allowed embryos from *hb nos*/+ *nos* females to develop into adults (Figure 1). After screening approximately 13,000 F_1 females, five suppressor mutations were isolated. These mutants cause a strong dominant suppression of the *nos* phenotype in a maternal background heterozygous for *hb*, and to a lesser extent, they also suppress the *nos* phenotype in a maternal background of normal *hb* dosage (Table 1).

Two of the suppressor mutations represent single hits in as yet unidentified genes and will be described elsewhere. Three other mutations are alleles of the gene *Enhancer of zeste* (*E(z)*) (JONES and GELBART 1990; PHILLIPS and SHEARN 1990) (see MATERIALS AND METHODS for details). We refer to these three mutations collectively as the $E(z)^{ion}$ alleles and individually as $E(z)^{ion1}$, $E(z)^{ion2}$ and $E(z)^{ion3}$. Mutations in *E(z)* have previously been analyzed in detail and *E(z)* has been shown to play a role in regulation of expression of homeotic genes in the Antennapedia and Bithorax gene complexes. Moreover, *E(z)* is required for proliferation of imaginal disc cells and the development of egg chambers past early stages of oogenesis (JONES and GELBART 1990; PHILLIPS and SHEARN 1990). Our results suggest that *E(z)* function is also involved in establishing the abdominal anlagen in the early embryo.

$E(z)^{ion}$ alleles are specific for abdomen formation: Suppression of the *nos* phenotype by *E(z)* alleles depends on the maternal genotype, thus *nos* embryos form a normal abdomen only when the *E(z)* mutations are present in the mother. Newly synthesized zygotic *E(z)* gene product provided by the paternal genome has no effect on the *nos* mutant phenotype (data not shown). Thus, consistent with a role in the regulation of expression of the first tier of segmentation genes, functions affected by the $E(z)^{ion}$ mutations are provided only maternally. In contrast, homeotic transformations which affect a later stage in the segmentation process are caused by lack of maternal *E(z)* function, but they also depend on the embryonic genotype (JONES and GELBART 1990; PHILLIPS and SHEARN 1990).

$E(z)^{ion}$ mutations are semidominant, and suppression of the *nos* phenotype is much stronger in embryos from *nos* females homozygous for $E(z)^{ion}$ (see Table 1). Table 1 shows the dominant maternal effect of $E(z)^{ion}$ alleles compared with other *E(z)* alleles that had been isolated previously on the basis of other phenotypes. Although many of the previously known alleles, including *E(z)* deficiencies, have a significant dominant effect, none of them can suppress the *nos* phenotype to the same extent as the $E(z)^{ion}$ alleles. Thus the $E(z)^{ion}$ alleles are gain-of-function mutations that appear to code for aberrant proteins (see also MATERIALS AND METHODS).

To determine whether the phenotype of the gain-of-function $E(z)^{ion}$ alleles reflects a requirement of wild-type *E(z)* function for early pattern formation, we tested the effect of *E(z)* loss-of-function allelic combinations in a *nos* mutant background. Since *E(z)* null mutations are homozygous lethal, we used the temperature sensitive allele $E(z)^{ts1}$ which has significantly reduced *E(z)* activity at the restrictive temperature (JONES and GELBART 1990).

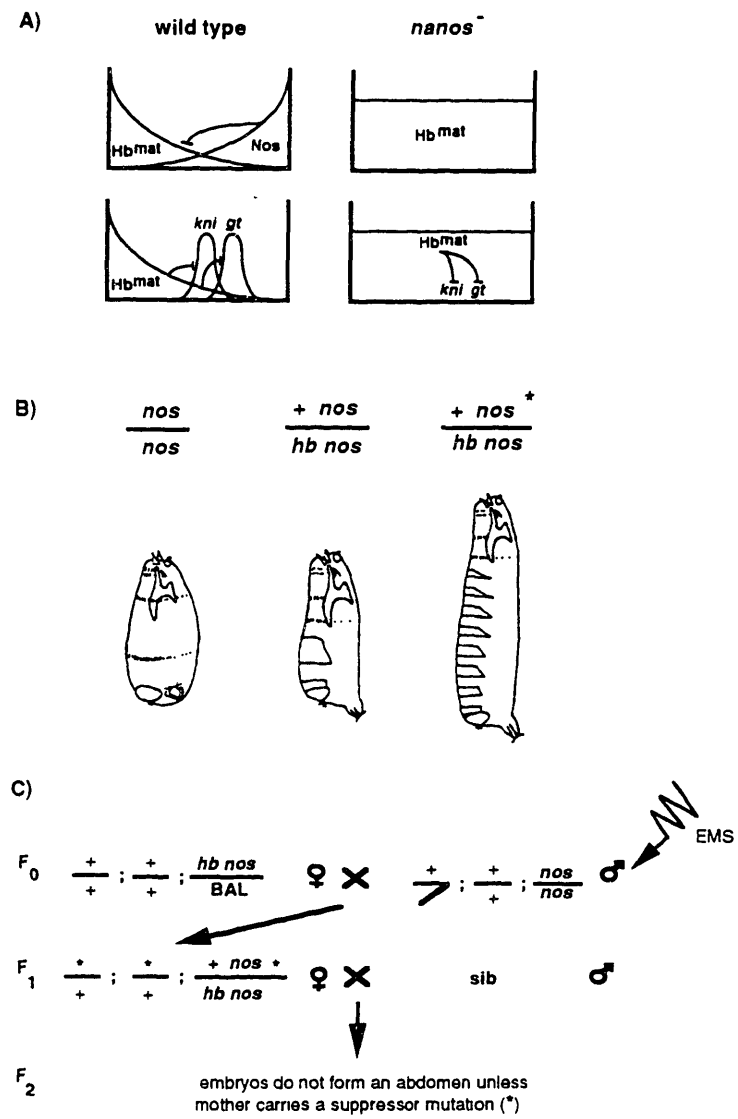

FIGURE 1.—Screen for suppressors of *nanos*. (**A**) Regulatory interactions at the early cleavage stage (top diagrams) and at the syncytial/cellular blastoderm (bottom diagrams) in embryos from wild-type and *nos* mutant females. In wild-type embryos the Nos protein represses translation of hb^{mat} RNA (top, left). The concentration of Hb determines the anterior boundaries of *kni* and *gt* expression (bottom, left). In embryos that lack functional Nos protein, the hb^{mat} RNA is translated throughout the egg (top, right). High levels of Hb^{mat} repress *kni* and *gt* transcription in the prospective abdominal region and thus no abdominal development occurs (bottom, right). (**B**) Phenotypes of embryos from *nos* mutant females. *Nos* mutant embryos lack abdominal segments, a reduction of the hb^{mat} gene dosage (+ *nos*/*hb nos*) weakens the *nos* phenotype. We identified suppressors of *nos* in this "sensitized" background (+ *nos* */*hb nos*). In principle, we should also recover new *hb* alleles, however, this is difficult since *hb* is also required zygotically (LEHMANN and NÜSSLEIN-VOLHARD 1987). (**C**) Screen for dominant suppressors of *nos*.

We found that homozygosity or hemizygosity for $E(z)^{61}$ at semipermissive (25°) and restrictive (29°) temperatures causes a significant suppression of the *nos* phenotype (Table 1, Figure 2C). We conclude that the wild-type maternal *E(z)* product is required for repression of abdominal development in a *nos* mutant background.

The phenotypes of *E(z)* null mutations suggest multiple requirements for *E(z)* wild-type product(s) at different stages of development (JONES and GELBART 1990; PHILLIPS and SHEARN 1990). In contrast, the $E(z)^{ion}$ mutations are specific for abdomen formation. This specificity of the $E(z)^{ion}$ alleles is demonstrated most clearly by the phenotype of embryos derived from germ line cells mutant for the two strongest $E(z)^{ion}$ alleles ($E(z)^{ion1}$ and $E(z)^{ion3}$). Since these alleles are lethal in trans, we generated females whose germ line is homozygous for *nos* and *trans*-heterozygous for these alleles by pole cell transplants (see MATERIALS AND METHODS). Embryos from such females frequently develop a complete set of abdominal segments, but do not display the strong homeotic transformations characteristic of mutations in *E(z)* and other Pc-G genes (Table 1, Figure 2B). On the other hand, embryos from $E(z)^{61}/Df(E(z))$ females develop only some abdominal segments but show very strong homeotic transformations (Table 1, Figure 2C). This specificity of the $E(z)^{ion}$ alleles is inconsistent with a model in which different levels of wild-type *E(z)* activity are required for different functions of *E(z)*. Rather, we

TABLE 1

***E(z)* mutations suppress the *nos* abdominal phenotype**

E(z) maternal genotype[a]	Maternal genotype: *nos/nos* — Percent rescue[b]	*n*	*hb nos/+ nos* — Percent rescue[b]	*n*
+ +	0	338	2	160
$E(z)^{son1}$/+	11	156	76	186
$E(z)^{son2}$/+	6	160	42	145
$E(z)^{son3}$/+	13	895	88	179
Df(3L)Ez6/+	0	525	9	768
$E(z)^{s5}$/+	0	356	2	473
$E(z)^{s6}$/+	0	253	10	229
$E(z)^{s1}$/+	0	361	9	293
$E(z)^{s4}$/+	0	575	12	353
$E(z)^{1}$/+	0	297	1	122
$E(z)^{s6}$/+	0	562	4	318
$E(z)^{s1}$/+	0	619	8	251
$E(z)^{s1}/E(z)^{s1}$ (25°)	2	216	ND	
(29°)	6	84	ND	
$E(z)^{s1}/E(z)^{s5}$ (25°)	24	270	ND	
(29°)	38	39	ND	
$E(z)^{son2}/E(z)^{s1}$	95	366	ND	
$E(z)^{son1}/E(z)^{son3c}$	100	29	ND	
$E(z)^{son2}/E(z)^{son3d}$	100	14	ND	

Tests were performed in a genetic background with a full or half dosage of hb^{mat} (*nos/nos* and *hb nos/+ nos* columns, respectively). All tests are at 25° except where otherwise stated. The temperature-sensitive allele $E(z)^{s1}$ retains some wild-type activity even at the "restrictive" temperature (29°) since suppression of the *nos* phenotype is stronger in hemizygotes ($E(z)^{s1}/E(z)^{s5}$) than in homozygotes [see also JONES and GELBART (1990)]. For a description of alleles see Materials and Methods.

[a] A "+" refers to the wild-type *E(z)* copy (*i.e.*, $E(z)^{+}$).

[b] Percent of embryos with ≥3 abdominal segments.

[c] From germ line clones. Test performed at 18°; 86% of the embryos scored had 8 abdominal segments.

[d] From germ line clones; 50% of the embryos scored had 8 abdominal segments.

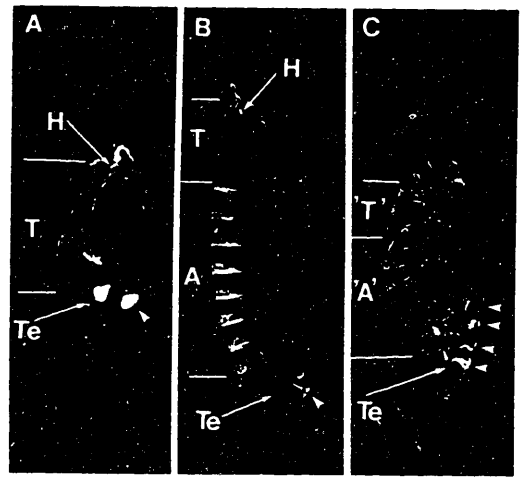

FIGURE 2.—Suppression of the *nanos* phenotype by *E(z)* mutations. (**A**) *Nos* embryos form a normal head and thorax but lack abdominal segments. (**B**) *E(z)* mutations suppress the *nos* phenotype. Embryo from $E(z)^{son1}nos/E(z)^{son3}nos$ germ line with a complete abdomen. (**C**) Embryo with suppressed *nos* phenotype from $E(z)^{s1}nos/E(z)^{s5}nos$ female at 29°. All segments show strong homeotic transformations toward an A8 identity. Note that the *E(z)* mutant combination in (B) shows no obvious homeotic transformations. Filzkörper (arrowheads) unstretched in (A), stretched in (B), repeated in more anterior segments in (C). Darkfield optics. Anterior up, ventral left. H, head skeleton; T, thorax; A, abdomen; Te, telson; 'T' and 'A' are thoracic and abdominal regions; all segments transformed into an A8 identity.

propose that the E(z) protein is a complex molecule and that the $E(z)^{son}$ mutations affect a specific function of this protein.

***E(z)* is required for maintenance of gap gene repression:** To understand how $E(z)^{son}$ mutations alter the *nos* phenotype, we studied the expression patterns of gap genes in embryos derived from *E(z) nos* double mutant females. As a source of mutant *E(z)* embryos we used females of the genotype $E(z)^{son2}/E(z)^{s1}$, which is a viable allelic combination that strongly suppresses the *nos* phenotype even in the presence of the normal maternal *hb* gene dosage (Table 1). As observed previously, *nos* mutant embryos fail to express the gap genes *kni* and *gt* in the prospective abdominal region (in Figure 3, compare E, F with B, C, respectively) (ROTHE *et al.* 1989; ELDON and PIRROTTA 1991; KRAUT and LEVINE 1991a). In contrast, *E(z)* mutant embryos express these gap genes in spite of the absence of *nos* function (Figure 3, H and I). Thus, *E(z)* wild-type function is required for the repression of abdomen-specific gap genes.

E(z) mutations suppress the abdominal phenotype of embryos derived from females mutant for *oskar* and *vasa* alleles, which cause a failure to localize *nos* RNA (data not shown; WANG *et al.* 1994). Likewise, *E(z)* mutations suppress the abdominal defect of embryos from females which lack *nos* RNA and protein (data not shown) (WANG *et al.* 1994). This indicates that *E(z)* mutations act downstream of *nos* function and thus must affect a function involved in the production, the stability, or the activity of the Hb^{mat} protein. Using anti-Hb antibody staining of embryos, we have not detected any significant effect of *E(z)* mutations on either the distribution or the levels of the uniformly distributed Hb^{mat} protein present in *nos* mutants (data not shown, see also Figure 4, D and I). Thus, *E(z)* most likely acts downstream of, or in conjunction with Hb^{mat}.

It has been proposed that *E(z)* and other Pc-G genes are required for the maintenance but not for the initiation of the repressed state of homeotic genes (STRUHL and AKAM 1985; GLICKSMAN and BROWER 1990; JONES and GELBART 1990; MCKEON and BROCK 1991; SIMON *et al.* 1992). To determine whether *E(z)* is also required for the maintenance of transcriptional repression of *kni* and *gt*, we compared the time when these two genes are first expressed in wild-type embryos to the timing of their expression in embryos from *E(z) nos* double mutant females. The posterior expression of both *kni* and *gt* is first detectable in *E(z) nos* mutant embryos at the mid-

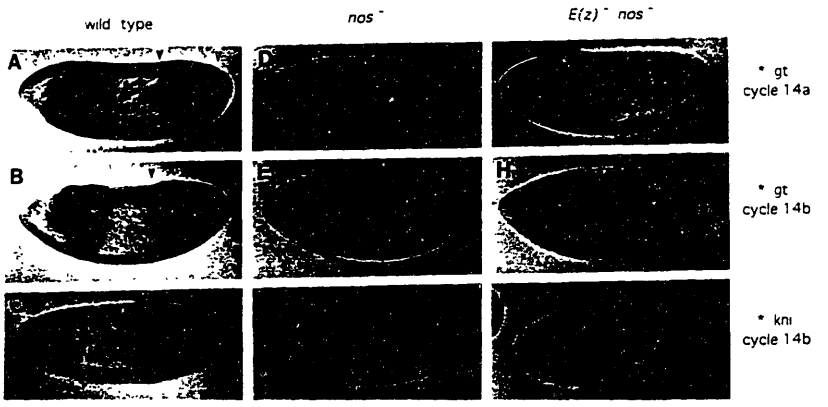

FIGURE 3.—*E(z)* mutations allow abdominal gap gene expression in the absence of *nos* function. Expression patterns of *kni* and *gt* RNA in embryos from wild-type, *nos* and *E(z) nos* females. Only the posterior-most domain of expression of these two genes (flanked by arrowheads) is required for abdomen formation and depends on *nos* function. In wild-type embryos *kni* and *gt* posterior expression is initiated at nuclear cycles 11–12 (MOHLER *et al.* 1989; ROTHE *et al.* 1989; ELDON and PIRROTTA 1991; KRAUT and LEVINE 1991a) [shown in (A) at cycle 14a]). Posterior expression is undetectable at early stages in *E(z) nos* embryos [shown in (G) at cycle 14a], but becomes detectable at a later stage [shown in (H), cycle 14b]. (Data shown only for *gt*, although a similar delay in expression is observed for *kni* in *E(z) nos* embryos.) Weak expression of *gt* in *nos* embryos (E) (KRAUT and LEVINE 1991a) is variable and low compared with wild-type and *E(z) nos* embryos. The posterior domains of both *kni* and *gt* are shifted slightly posteriorly in *E(z) nos* embryos. This may be due to incomplete penetrance of the *E(z)* mutations, and/or partial repression of *kni* and *gt* by *Kr* gene activity. In *nos* and *E(z) nos* embryos *Kr* is activated more posteriorly than normal due to the high concentration of Hb^{mat} protein in the abdominal region (data not shown). This suggests that activation of *Kr* by Hb is not affected by *E(z)* mutations. Nomarski optics of whole-mount *in situ* hybridizations. Anterior left, dorsal up.

cellular blastoderm stage, at least one nuclear cycle delayed with respect to the onset of wild-type expression (in Figure 3, compare B, H with A, G, respectively). The time at which *kni* and *gt* are expressed in *E(z) nos* embryos roughly coincides with the disappearance of the Hb^{mat} protein (TAUTZ 1988) (our own observations). Thus, our results are consistent with a role for *E(z)* in the maintenance of the repressed state initiated by the Hb^{mat} protein.

Role of *E(z)* in anteroposterior patterning: In wild-type embryos Nos protein emanates from the posterior pole and generates a complementary distribution of Hb^{mat} protein. At the syncytial blastoderm stage, when the maternal Hb^{mat} protein is no longer detectable, hb^{zyg} is transcribed in the anterior half of the embryo under the control of *bcd*. The concentration of either Hb^{mat} or Hb^{zyg} along the anteroposterior axis establishes the anterior boundaries of *kni* and *gt* expression (HÜLSKAMP *et al.* 1990; ELDON and PIRROTTA 1991; KRAUT and LEVINE 1991a,b; STRUHL *et al.* 1992). Since *E(z)* is required for the continued repression of these gap genes in *nos* mutant embryos, we asked whether *E(z)* might also be required in wild-type embryos for the proper positioning of the anterior boundaries of *kni* and *gt* expression. In embryos that are mutant for *E(z)*, but are otherwise wild-type, the anterior boundaries of *kni* and *gt* are normal (data not shown). This result could imply that the E(z) product is required for the maintenance of gap gene boundaries established by Hb^{mat} but is dispensable if anteriorly expressed genes, such as Hb^{zyg}, are activated by *bcd*.

Therefore, to determine whether *E(z)* is required for the determination of the anterior boundaries of *kni* and *gt* initiated by the Hb^{mat} gradient, we tested the effect of *E(z)* in *bcd* mutant embryos. In these embryos, the only source of anteroposterior polarity is the Hb^{mat} gradient. We find that *E(z) bcd* double mutant females produce embryos in which the anterior boundaries of *kni* and *gt* expression are shifted anteriorly (compare Figure 4, G, H with B, C, respectively). Thus, *E(z)* is required for the proper formation of the anterior boundaries of *kni* and *gt* expression by the Hb^{mat} protein gradient.

The new boundaries of *kni* and *gt* expression in *E(z) bcd* double mutant embryos are determined solely by cross-regulatory gap gene interactions. The anterior and posterior boundaries of *gt* in *E(z) bcd* mutant embryos depend upon repression by *tailless* (*tll*) and other terminal gap genes (compare Figure 4G with 5A). Negative regulation by terminal gap genes also determines the anterior border of *kni* (compare Figure 4H with 5B). The posterior boundary of *kni* in *E(z) bcd* embryos is negatively controlled by *gt* (compare Figure 4H with 5C).

The effect of *E(z)* mutations on *kni* and *gt* transcription is not observed when these genes are first expressed: anterior boundaries of expression in *E(z) bcd* embryos are established at positions similar to those found in *bcd* embryos. Later, at the cellular blastoderm stage, *kni* and

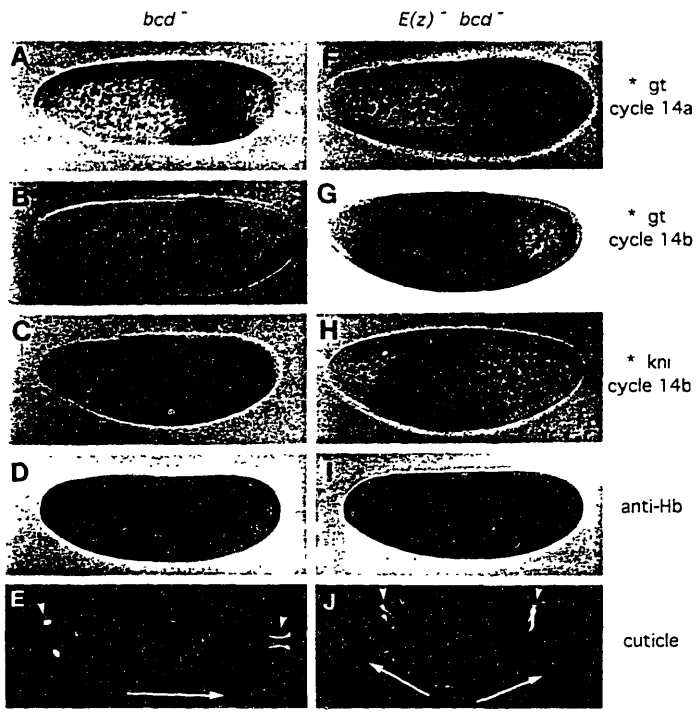

FIGURE 4.—*E(z)* maintains the anterior boundaries of *kni* and *gt* expression in *bcd* embryos. (**A–C, F–H**) *gt* and *kni* RNA expression in whole-mount embryos from *bcd* and *E(z) bcd* mutant females. Until nuclear cycle 14a the anterior boundaries of *gt* and *kni* are similar in *bcd* and *E(z) bcd* mutant embryos (A, F data shown for *gt*). At cycle 14b, however, these boundaries have shifted anteriorly in *E(z) bcd* embryos (B, G and C, H). The embryo in (G) shows the most extreme effect of *E(z)* mutations on *gt* expression. In less extreme cases *E(z) bcd* embryos show a gap of variable extent within the *gt* expression domain (not shown). This gap is likely caused by hb^{mat}-dependent activation of *Kr*, a mutual repressor of *gt* (HÜLSKAMP *et al.* 1990; ELDON and PIRROTTA 1991; KRAUT and LEVINE 1991b; STRUHL *et al.* 1992). The variability in the expression of *gt* is likely due to the incompletely penetrant *E(z)* background. The expression patterns of *Kr* and *kni* are similar and within a single embryo the patterns of *kni* and *Kr* are roughly complementary to that of *gt* (data not shown). It is therefore probable that expression of *Kr* affects *gt* expression in embryos that display the less extreme phenotype and thereby indirectly leads to activation of *kni* (H) (CAPOVILLA *et al.* 1992). (**D, I**) Antibody staining at a pre-pole-bud stage shows no detectable difference in Hb^{mat} protein levels in *bcd* and *E(z) bcd* embryos. *E(z)* does not affect the levels but rather the activity of Hb^{mat} protein: in embryos from *bcd* mutant females heterozygous for a protein null, hb^{7M}, the levels of Hb^{mat} are lower than in *E(z) bcd* embryos even though abdominal pattern duplications are not observed (see below, data not shown). Hb is detected by anti-Hb antibody. Nomarski optics. Anterior left, dorsal up. (**E, J**) Cuticular phenotypes of larvae from *bcd* and *E(z) bcd* females. (E) *Bcd* mutant larvae lack head and thoracic structures and form a second telson with unstretched Filzkörper (left arrowhead). (J) *E(z) bcd* larvae develop two short abdomens in mirror image and stretched Filzkörper at both ends (arrowheads). The anterior abdomen of *E(z) bcd* embryos is usually shorter than the posterior one; this asymmetry may be caused by the off-centered expression of *kni* which overlaps with a domain of residual *Kr* activity (H, not shown). Arrows indicate anterior to posterior polarity of abdomens. Darkfield optics. Anterior left, ventrolateral view.

gt expression is expanded anteriorly in *E(z) bcd* embryos (in Figure 4, compare B, G with A, F, respectively). This suggests that the relative concentration of Hb^{mat} protein initiates the restrictions of *kni* and *gt* expression. At the cellular blastoderm stage, when Hb^{mat} protein is no longer detectable, *E(z)* function is required to maintain a stably repressed state.

***Hb* and *E(z)* act on the same *cis*-acting sequences in the *kni* promoter:** Our experiments suggest that *E(z)* and *hb* act in conjunction to restrict expression of *kni* and *gt* within the prospective abdominal region. To analyze a possible molecular interaction between the two gene products we asked whether sequences within the *kni* promoter, known to be required for abdomen-specific expression, contain sequences required for Hb^{mat} and *E(z)* mediated regulation. A 1.8-kbp fragment of the *kni* promoter fused to the *lac Z* reporter gene confers abdomen specific expression that is identical to that of the endogenous *kni* gene (PANKRATZ *et al.* 1992) (see Figure 6A). This region contains at least five potential binding sites for the Hb protein (PANKRATZ *et al.* 1992).

To determine whether this reporter construct responds to repression by Hb^{mat} we first examined the expression of the transgene in the progeny of *nos* and *bcd* mutant females. Like the endogenous *kni* expression,

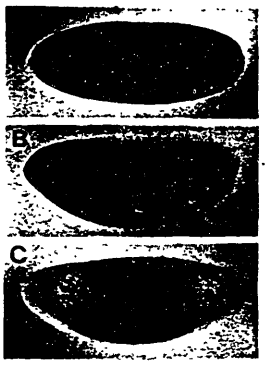

FIGURE 5.—Regulation of *gt* and *kni* expression by gap gene interactions in *E(z) bcd* embryos. Expression patterns of *kni* and *gt* RNA in *E(z) bcd tsl* and *gt, E(z) bcd* embryos. (**A**) *gt* is ubiquitously expressed in *E(z) bcd tsl* embryos (cf. Figure 4G). This embryo shows the most extreme effect of *E(z)* mutations on *gt* expression in this background. The anterior expansion of the *gt* domain is less extreme in other *E(z) bcd tsl* embryos. (**B**) The anterior boundary of *kni* expression expands anteriorly in *E(z) bcd tsl* embryos (*cf.* Figure 4H). The expression pattern in (A) represents a more extreme situation than that in (B) (see legend of Figure 4). (**C**) *kni* expands posteriorly in a *gt, bcd* embryo (*cf.* Figure 4H).

lac Z RNA is absent from the abdominal region in embryos from *nos* mutant females (Figure 6B) and is expressed as a single band in embryos from *bcd* mutant females (Figure 6D). We then asked whether these sequences are also sufficient to confer *E(z)* dependent regulation. We find that *kni-lac Z* is expressed in the prospective abdominal region in embryos from *E(z) nos* females (Figure 6C) and that the domain of expression is expanded anteriorly in embryos from *E(z) bcd* females (Figure 6E). Thus, the regulation of the transgene is identical to that of the endogenous posterior *kni* domain. We conclude that this region of the *kni* promoter contains all sequences required for *E(z)*-dependent transcriptional repression.

Other Polycomb group genes are also involved in the maternal to zygotic transition of gene expression: Based on the similar homeotic phenotypes of Pc-G genes, it has been proposed that their gene products act in conjunction. To determine whether these genes also interact during the maternal-zygotic transition of gene expression we tested mutations in additional Pc-G genes for suppression of the *nos* phenotype.

We first determined whether mutations in any of the known Pc-G genes show a dominant maternal effect similar to that of *E(z)*. Mutations in the genes *Additional sex combs, Polycomb, Polycomblike, polyhomeotic, Sex combs on midleg* and *Sex combs extra* do not show any significant dominant suppression of the *nos* phenotype (data not shown; for these and other Pc-G genes, see MATERIALS AND METHODS for specific alleles used and references). We did, however, detect significant dominant suppression of the *nos* phenotype by mutations in the *Su(z)2* complex (*Su(z)2-C*) (Figure 7A, Table 2). Interestingly, deletions of the entire complex, which includes the genes *Posterior sex combs (Psc), Suppressor of zeste 2* (*Su(z)2*) and *Suppressor of zeste 2-D* (*Su(z)2-D*) show significantly stronger suppression than single mutations in any of the genes. Thus, reducing the dosage of more than one *Su(z)2-C* genes may imbalance a multicomponent "repression complex," or alternatively, the genes within the *Su(z)2-C* may be partially redundant in function. In addition, we detected dominant suppression of *nos* by mutant alleles of the gene *pleiohomeotic* (*pho*) (Table 2, see below).

Since mutations in most Pc-G genes lead to homozygous lethality, it is not simple to determine the recessive maternal effect of these genes. Nevertheless, we were able to test two genes for which homozygous mutant allele combinations are viable: null alleles of the gene *extra sex combs* (*esc*) and a hypomorphic allele of *pho*. Embryos from *nos* mutant females, which also lack maternal and zygotic *esc* product, do not form segments in the abdominal region (Figure 7C). Since a loss-of-function *E(z)* background results in suppression of the *nos* phenotype, whereas a null *esc* background has no effect, we conclude that *esc* is not involved in the repression of gap genes by Hb^{mat}. On the other hand, embryos from *nos;pho* females can form a complete set of segments in the abdominal region (Figure 7B). Thus, in addition to *E(z)* several Pc-G genes are required for the repression of gap genes by Hb^{mat}. We conclude that maintenance of gap gene boundaries, like the maintenance of homeotic gene boundaries, may involve several Pc-G group gene products.

DISCUSSION

Screens for dominant suppressors or enhancers of specific mutations have proven to be very successful for identifying interacting products. This is especially applicable to unicellular organisms where a large number of individuals can be tested to detect rare events (see, for example, STEARNS and BOTSTEIN 1988). In higher eukaryotes such as Drosophila, where the number of individuals that can be screened is limiting, highly selective suppressor and enhancer screens have been successfully used for the identification of microtubule-associated proteins (reviewed in FULLER *et al.* 1989), activators and repressors of homeotic genes (KENNISON and TAMKUN 1988), and products involved in signal transduction during photoreceptor cell determination (SIMON *et al.* 1991) or during embryonic patterning (DOYLE and BISHOP 1993).

We carried out a screen for suppressors of the *nos* phenotype and discovered that *Enhancer of zeste* (*E(z)*), a member of the Polycomb group of genes (Pc-G), is a negative transcriptional regulator of the abdomen-specific gap genes *knirps* (*kni*) and *giant* (*gt*). Our genetic analysis indicates that *E(z)* maintains the proper

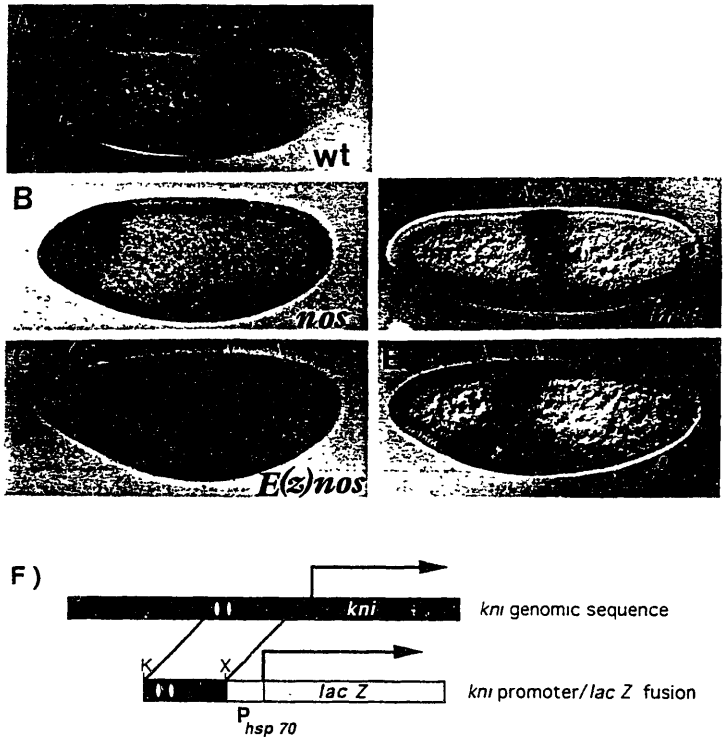

FIGURE 6.—A 1.8-kbp fragment of the *kni* regulatory region fused to *lac Z* faithfully mediates regulation by hb^{mat} and *E(z)*. (A–E) Whole-mount *in situ* hybridization showing expression from the *kni-lac Z* fusion construct in (F). Posterior domain of expression is marked by arrowheads. (A) Expression of the transgene in a wild-type embryo (*cf.* Figure 3C). (B) *Nos* embryo lacks the posterior domain of expression (*cf.* Figure 3F). (C) *E(z) nos* embryo expresses the transgene in the posterior domain (*cf.* Figure 3I). (D) In *bcd* embryos the anterior domain is missing and the posterior domain is shifted slightly anteriorly (*cf.* Figure 4C). (E) In *E(z) bcd* embryos the posterior domain is shifted further anteriorly than in *bcd* embryos (*cf.* Figure 4H). Ectopic expression in the ventral region is likely due to a mesoderm enhancer element present in the *ry* gene, which is part of the *P* element transformation vector (DOYLE *et al.* 1989). (F) Schematic diagram showing the *kni/lac Z* fusion construct. A 1.8-kbp region of the *kni* regulatory region fused to a basal heat shock promoter directs *lac Z* expression [KX construct from PANKRATZ *et al.* (1992)]. The double ovals mark a cluster of at least five potential binding sites for Hb protein (PANKRATZ *et al.* 1992). All embryos at cycle 14b, Nomarski optics, anterior left, dorsal up.

anterior boundaries of *kni* and *gt* expression once the initial domains of expression have been set according to the concentration gradient of the repressor Hunchback (Hb). Thus, the suppression-of-*nos* screen uncovered an involvement of Pc-G genes in the early patterning of the embryo.

***E(z)* is required for repression of gap genes:** We have studied the effect of *E(z)* mutations in embryos from *nos* and *bcd* mutant females. In *nos* embryos translation of hb^{mat} RNA is deregulated resulting in high levels of Hb^{mat} protein throughout the prospective abdominal region (TAUTZ 1988; WANG and LEHMANN 1991). Hb^{mat} in turn represses transcription of the gap genes *kni* and *gt*, inhibiting abdominal development (HÜLSKAMP *et al.* 1989; IRISH *et al.* 1989a; STRUHL 1989; ELDON and PIRROTTA 1991; KRAUT and LEVINE 1991a,b). We show that *E(z)* mutations lead to expression of *kni* and *gt* in the prospective abdominal region of *nos* embryos. It is this effect of *E(z)* mutations that constitutes the basis for suppression of the *nos* phenotype in the presence of Hb^{mat}. In *bcd* embryos, hb^{bcd} is not expressed, and the Hb^{mat} protein gradient is the major organizer that determines the anterior boundaries of *kni* and *gt* (HÜLSKAMP *et al.* 1990; STRUHL *et al.* 1992). We show that the E(z) product is required for the proper maintenance of these boundaries.

What is the molecular basis for the effect of *E(z)* mutations on transcriptional repression of *kni* and *gt*? *E(z)* mutations, as any other *suppressor-of-nos* mutation, may in principle affect either the production or the activity of the Hb^{mat} protein. We were unable to detect any significant reduction in the levels of Hb^{mat} in embryos from *E(z)* females. Thus, *E(z)* does not seem to affect the production of Hb^{mat} protein. In addition, the effects of *E(z)* mutations in embryos are only apparent when the Hb^{mat} protein is no longer detectable. We therefore conclude that *E(z)* affects a subsequent step of gene regulation that involves the stable maintenance of a transcriptionally repressed state.

In theory, *E(z)* mutations could affect the levels of Kr, which would then alter the boundaries of *kni* and *gt*. This idea seems unlikely, however, since the effects of *E(z)* mutations are more extreme than those caused by a lack of Kr: first, *Kr* mutations do not restore abdominal pattern in *nos* embryos (KRAUT and LEVINE 1991a), and second, the anterior border of *gt* is shifted further anteriorly in *E(z)bcd* embryos than in *Kr;bcd* double mutant embryos [this report and STRUHL *et al.* (1992)]. We favor the idea that *E(z)*, like hb^{mat}, directly affects *kni* and *gt* expression. This is consistent with the finding that the phenotype of *E(z)* mutations most closely resembles that of deleting hb^{mat} (HÜLSKAMP *et al.* 1989, 1990; IRISH *et al.* 1989a; STRUHL 1989).

We have mapped *cis*-acting sequences required for *E(z)* mediated repression to a small fragment in the *kni* regulatory region that contains binding sites for Hb pro-

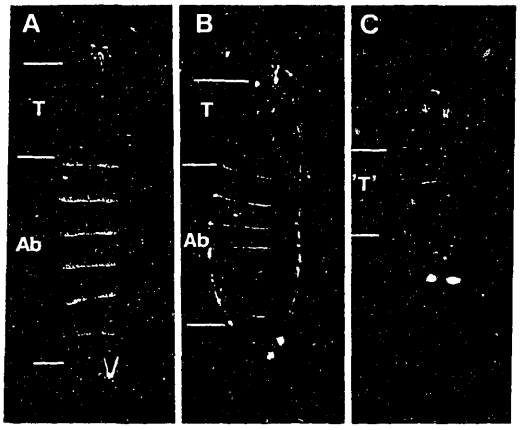

FIGURE 7.—Suppression of the *nos* phenotype by other Polycomb group genes. Cuticle preparations of larvae derived from females mutant for *nos* and various Pc-G group genes. (**A**) Embryo from a *nos* mutant female that is also heterozygous for a deficiency of the entire *Su(z)2* complex. (**B**) Embryo from a *nos;pho* double mutant female. *Nos;pho* embryos show variable degrees of homeotic transformations (not shown). (**C**) Strong *nos* phenotype in *esc*; *nos* embryo; note complete transformation of the three thoracic segments ('T') into an A8 identity. Darkfield optics. Anterior up, ventral view. T, thorax; Ab, abdomen.

TABLE 2

Suppression of *nos* abdominal phenotype by Pc-G genes

Pc-G maternal genotype[a]	Maternal genotype: *nos/nos* — Percent rescue[b]	*nos/nos* — *n*	*hb nos/+ nos* — Percent rescue[b]	*hb nos/+ nos* — *n*
Wild type	0	594	0	614
Df(Su(z)2-C)/+[c]	2	266	27	445
$Psc^{1}/+$	0	352	0	382
$Psc^{1445}/+$	2	350	7	256
$Psc^{1431}/+$	0	651	12	532
$Psc^{r24}/+$	0	132	0	632
$Su(z)2^{1}/+$	0	52	3	316
$Su(z)2^{167}/+$	0	866	0	689
$Su(z)3^{1}/+$	0	212	0	266
Df(4)G/+	0	378	5	587
$pho^{r}/+$	0	455	10	659
pho^{1}/pho^{r}[d]	89	18	ND	
esc^{2}/esc^{10}	0	181	ND	

Tests were performed in a genetic background with a full or half dosage of hb^{mat} (*nos/nos* and *hb nos/+ nos* columns, respectively). All tests are at 25° except where otherwise stated. For a description of alleles see MATERIALS AND METHODS.

[a] A "+" refers to the wild-type copy of the particular Pc-G gene tested.

[b] Percent of embryos with ≥3 abdominal segments.

[c] Data shown is for the deficiency $Su(z)2^{159}$. A similar result was obtained using the deficiency *Df(2R)vg-B*.

[d] Test performed at 18°.

tein (PANKRATZ *et al.* 1992). It is possible that *E(z)* and other Pc-G genes interact with the DNA region present in this transgene. Alternatively, binding sites for E(z) may be distributed throughout the genome and interactions between these sites and specific sequences bound by Hb may be required for stable repression.

Our conclusion that the E(z) product, and other Pc-G products (see below), are required for the transcriptional repression of gap genes agrees well with the previously postulated role of *E(z)* as a transcriptional repressor of the *white* and *engrailed* genes (JONES and GELBART 1990; MOAZED and O'FARRELL 1992) and of homeotic genes (JONES and GELBART 1990; PHILLIPS and SHEARN 1990; SIMON *et al.* 1992). Similar to its role in gap gene regulation, a function of Pc-G genes has been implied in the maintenance of *engrailed* repression in anterior cells within each segment (MOAZED and O'FARRELL 1992) and in the maintenance of ordered expression of homeotic genes along the anteroposterior axis (STRUHL and AKAM 1985; GLICKSMAN and BROWER 1990; JONES and GELBART 1990; MCKEON and BROCK 1991; SIMON *et al.* 1992; ZHANG and BIENZ 1992).

The determination of anterior boundaries of gap gene expression by Hb^{mat} appears analogous to the later determination of anterior boundaries of homeotic gene expression by gap genes (WHITE and LEHMANN 1986; HARDING and LEVINE 1988; IRISH *et al.* 1989b; REINITZ and LEVINE 1990; QIAN *et al.* 1991; ZHANG *et al.* 1991; ZHANG and BIENZ 1992; BUSTURIA and BIENZ 1993). In both cases transient, spatially restricted repressors initiate boundaries of expression and those boundaries persist in a Pc-G-dependent process after the original repressors are no longer present (Figure 8). The difference between the two processes is that in the blastoderm embryo additional independent regulators (*e.g.*, Hb^{og}) obviate the absolute requirement for the Pc-G function in the determination of gap gene boundaries. Regulators like Hb^{og} may not require a Pc-G-dependent maintenance function because they are present throughout the time that *kni* and *gt* are expressed. In the later embryo at the extended germ band stage, no similar redundant mechanisms exist for the proper regulation of homeotic gene boundaries in the absence of Pc-G genes.

Polycomb group genes and the chromatin link: The Pc-G genes are estimated to comprise about 40 genes (JÜRGENS 1985), of which only about a dozen are known. Here, we show that mutations in several other Pc-G genes, such as *pho*, and genes in the Su(z)2-complex, can also act as suppressors of *nos*. We speculate that these and perhaps other Pc-G genes are involved in the negative regulation of gap genes by Hb^{mat}. At least one Pc-G gene, *esc* is clearly not required for this process. *Esc* may be specific for homeotic regulation, as it is also not required for regulation of *engrailed* (MOAZED and O'FARRELL 1992).

It is likely that other Pc-G genes are involved in the repression of gap genes but were not identified in our screen which selected for rare dominant gain-of-function mutations. A rigorous test for a role of maternal Pc-G genes in gap gene regulation will require testing the effect of homozygosity for null mutations in these genes on the *nos* phenotype. These tests will require

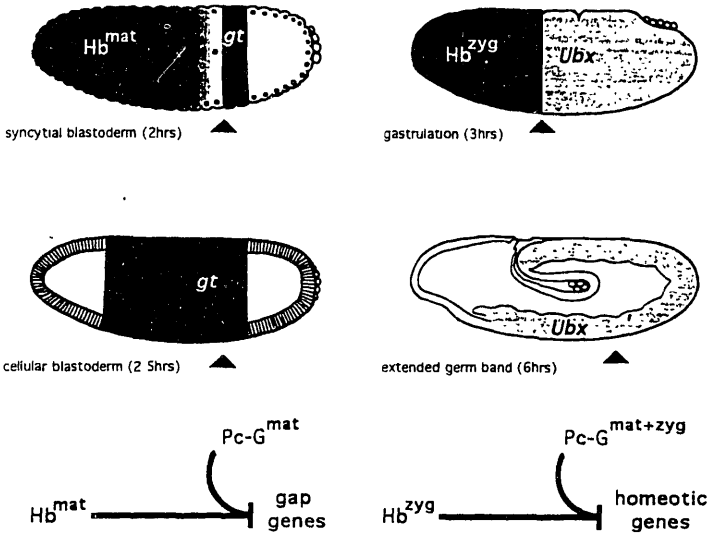

FIGURE 8.—Polycomb group genes are required for two anteroposterior patterning pathways during embryogenesis. (Left side) Model for gap gene regulation by Hb^{mat} and Pc-G genes as proposed in this article (top, syncytial stages; bottom, cellular blastoderm stage). The situation depicted is that in *bcd* mutant embryos, where the only source of anteroposterior polarity is the hb^{mat} gradient. During the syncytial stages Hb^{mat} establishes the boundaries of gap genes such as *gt* (shown) and *kni*. By the end of the blastoderm stage, when Hb^{mat} is no longer detectable, the original boundaries remain at their original positions in wild-type embryos (arrowhead), but are not maintained in Pc-G mutant embryos (as drawn). (Right side) Model for homeotic regulation by gap genes such as hb^{zyg} and Pc-G genes as previously proposed (top, beginning of gastrulation stage; bottom, germ band extended stage). Gap gene products (*e.g.*, Hb^{zyg}) are present until the early gastrulation stages, and establish the boundaries of homeotic genes such as *Ubx*. In wild-type embryos these boundaries remain at their original locations (arrowhead in the extended germ band embryo shown) when gap gene products are no longer present. In Pc-G mutant embryos, though, these boundaries are not properly maintained at these stages. In both models, we propose that at an early stage a specific repressor is present in a spatially restricted manner where it initiates boundaries of gene expression. At a later stage, when the initial repressors are no longer present, Pc-G gene products are required to maintain those boundaries. In embryos that lack Pc-G gene activity gene expression occurs indiscriminately throughout the embryo (the remaining gene boundaries are determined by cross-regulatory interactions among zygotic genes–see text).

generation of germ line chimeras since the function of most Pc-G genes is required at different times during development and thus mutations in these genes are homozygous lethal.

It has been proposed that Pc-G genes may be involved in the formation of a condensed, or "closed" chromatin structure that is less accessible to transcription factors [reviewed in PARO (1990), KENNISON and TAMKUN (1992), WINSTON and CARLSON (1992) and KENNISON (1993)]. Promotion of a stably repressed chromatin state may involve the formation of large multi-subunit complexes composed of several Pc-G gene products. This was first suggested by the similar homeotic phenotypes caused by mutations in these genes, the sensitivity of these phenotypes to dosage imbalances and the apparently synergistic action of these genes (JÜRGENS 1985; KENNISON and RUSSELL 1987; KENNISON and TAMKUN 1988). Recently, biochemical studies have corroborated this idea (ZINK and PARO 1989; DECAMILLIS *et al.* 1992; FRANKE *et al.* 1992; RASTELLI *et al.* 1993; MARTIN and ADLER 1993). Our results suggest that the Pc-G repression machinery may also regulate gap gene expression.

Redundant gradients in AP axis determination: The discovery that *nos* function is dispensable for abdomen formation in the absence of functional Hb^{mat} product raises the question about the importance of the *nos*-dependent patterning system. In particular, Hb^{zyg}, which is activated by the anterior morphogen *bicoid*, can determine the anterior boundaries of *kni* and *gt* and thus fully compensate for the lack of Hb^{mat} (HÜLSKAMP *et al.* 1990; STRUHL *et al.* 1992). At present we can only speculate about the evolution of these two maternal systems that can independently specify the proper positioning of gap gene expression domains along the anteroposterior axis.

The finding that the maintenance of repression of both gap genes and homeotic genes utilizes Pc-G products suggests an ancestral scenario for the determination of the anteroposterior axis in insects. In this model, a local source of *nos* would establish a complementary concentration gradient of Hb. Hb would then regulate both the subdivision (*e.g.*, gap gene pattern) and identity (*e.g.*, homeotic gene expression) of the first embryonic regions. A Pc-G gene dependent process would maintain this prepattern throughout embryogenesis. Indeed, homeotic genes are known to respond to regulation by the Hb^{mat} protein (IRISH *et al.* 1989b; ZHANG *et al.* 1991). Further comparative molecular studies will help

clarify the evolutionary history of the "*nos-hb*-Pc-G" system of anteroposterior patterning.

It has been proposed that redundant systems of positional information may be favored through evolutionary time, since independent overlapping functions would make the patterning process more resilient to external or internal fluctuations (TAUTZ 1992). In this context, a role of Pc-G genes in the determination of transcriptional state of gap genes suggests that the Hb^{mat} gradient may organize the embryo along the anteroposterior axis by "imprinting" the promoters of gap genes with a particular chromatin conformation. This implies that cell fate determination along the anteroposterior axis is regulated at the chromatin level, by reducing access to the promoters of posterior-promoting gap genes in anterior regions. A precedent for spatial imprinting of genes has been reported recently in mouse muscle cells (DONOGHUE *et al.* 1992). In the Drosophila embryo, such a chromatin scaffold would provide a robust base for further refinement by the overlying network of zygotic gene interactions.

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ADDENDUM TO CHAPTER III

This section contains a number of studies which are referred to, but not described in detail, in Chapter III (Genetics **136**: 1341-1353, April, 1994).

MATERIALS AND METHODS

Except where specifically described, materials (including fly strains) and methods are as in Chapter III.

Genetic analysis and strains: The *vasa* and *oskar* alleles used were: vas^{D1} (strong, Lehmann and Nüsslein-Volhard 1991), vas^{PD} (partial loss-of-function, Schüpbach and Wieschaus 1986), osk^{166} (strong, Lehmann and Nüsslein-Volhard 1986). The *hunchback* class V alleles used were hb^{9K49} and hb^{9K59} (Lehmann and Nüsslein-Volhard 1987a). The *knirps* alleles used were: kni^{IL}, kni^{FC}, kni^{IID} (strong), kni^{IIV}, kni^{14B} (weak); the *Krüppel* allele used was Kr^{1} (deficiency); the *zeste* alleles used were: z^{a} (amorph), and z^{1} (gain-of-function)(for references see Lindsley and Zimm 1992).

Production of $E(z)^{son}$ germ line clones: Ovo^{D1} females were used as hosts for pole cell transplants as described in Lehmann and Nüsslein-Volhard (1987a). Ovo^{D1} causes degeneration of the female germ line (Oliver et al. 1987). Donor embryos were derived from the following crosses:

Experiment (A) - Creation of $E(z)^{son1}/E(z)^{son3}$ clones: $E(z)^{son1}$ *nos / TM3, Ser* females x $E(z)^{son3}$ */ TM3, Sb* males.

Experiment (B) - Creation of $E(z)^{son2}/E(z)^{son3}$ clones: $E(z)^{son2}$ *nos / TM3, Ser* females x $E(z)^{son3}$ */ TM3, Sb* males.

Note on experiments (A) and (B): Mutations in the gene *nos* are completely recessive, and thus this *nos* heterozygous background should be equivalent to wild-type background.

Experiment (C) - Creation of $E(z)^{son1}$ *nos* / $E(z)^{son3}$ *nos* clones: $E(z)^{son1}$ *nos / TM3, Ser* females x $E(z)^{son3}$ *nos / TM3, Sb* males.

Experiment (D) - Production of $E(z)^{son2}$ *nos* / $E(z)^{son3}$ *nos*: $E(z)^{son2}$ *nos / TM3, Ser* females x $E(z)^{son3}$ *nos / TM3, Sb* males.

Adult females derived from the transplanted hosts were mated to various types of males. In most cases these males carried chromosomes with the recessive markers *scarlet* and *ebony* which are also present in the $E(z)^{son}$-carrying chromosome. This allows to genetically distinguish the $E(z)^{son}$-carrying chromosomes in the progeny of these females.

(Presence of the *TM3* balancer chromosome, can also be distinguished in this progeny by the dominant visible marker *Stubble* (*Sb*) and *Serrated* (*Ser*)).

In many cases these males were in addition heterozygous for the mutation $E(z)^{son3}$, which would lead to the production of embryos which are mutant for both maternal and zygotic *E(z)*.

Temperature sensitive period for the son phenotype during oogenesis: Females of the genotype $E(z)^{son3}$ *nos/hb nos* were grown at either 18°C or 25°C. During early adulthood, these females were shifted to 25°C or 18°C, respectively, and eggs were collected every two hours at 25°C, or every four hours at 18°C (at 25°C development is about one half as fast as that at 18°C (Ashburner 1989)). Embryos were allowed to age and were scored for the presence of abdominal segments.

Temperature sensitive shift of $E(z)^{61}$ *nos* / $E(z)^{65}$ *nos* embryos: $E(z)^{61}$ *nos* / $E(z)^{65}$ *nos* females ($E(z)^{61}$ is a temperature sensituve alelle, $E(z)^{65}$ is a null allele) were grown and mated to wild-type males in egg laying cages at the semi-permissive temperature of 25°C. Embryos from these females were collected at 30 minute intervals and then shifted to the restrictive temperature of 29°C. Eggs collected in this manner should contain embryos at developmental stages earlier than nuclear cycle 4. This is earlier than the stage at which the Hb^{mat} protein gradient forms (nuclear cycle 6-7), which is the earliest time in which *E(z)* function would expected to be required for gap gene regulation. Nevertheless, because of retention of fertilized eggs within females, a fraction of these embryos are likely at older developmental stages even if collected within this time interval. This fraction of embryos may be past the ts stage and thus may obscure an early ts period.

Expression of gap genes in *E(z)* mutant, but otherwise wild-type embryos: Expression of the gap genes *Kr*, *kni* and *gt* was detected by in situ hybridization using digoxigenin-labelled RNA probes. The expression patterns of embryos at the late cellular blastoderm stage were then digitized and averaged using the NIH image program as described in Barker et al. (1992), except that the embryos were visualized with Nomarsky (light) optics. In addition, the intensity profiles have not been inverted, and thus the highest intensity of RNA expression corresponds to the lowest point in the profiles as presented in Figure 3.14. The numbers of wild-type (control) and *E(z)* mutant embryos used were, respectively, for *Kr* expression, 6 and 8; for *kni* expression, 11 and 6 ; and for *gt* expression, 7 and 5. For each gene expression pattern, T-analysis at a 95% confidence level was perfomed using data points collected from individual embryos. Data was collected in two different ways: i) One set of data consists of the staining intensity at a fixed position where the anterior boundary of expression was approximately half-maximal, standarized to the total intensity differential in the region. Egg lengths (EL) positions used

were 42% EL for *Kr* expression, 55% EL for *kni* expression, and 63% EL for *gt* expression. Staining intensity values were converted to the fraction: [((intensity of the experimental value at the fixed EL) - (background intensity near the center of the embryo)) / ((highest intensity in the domain of expression) - (background intensity near the center of the embryo))]. ii) A second set of data consisted of the EL position at half-maximal intensities (correcting for the background intensity), i.e. EL position at [(((highest intensity in the domain of expression) - (background intensity near the center of the embryo)) / 2) + (background intensity near the center of the embryo)]. For each gene expression pattern, statistical analysis of both sets of data yielded similar results.

Effect of son and Pc-G mutations on the zeste-white interaction: These experiments used the $z^1\ w^{is}$ allele, which is particularly sensitive to alterations in *E(z)* function (Jones and Gelbart 1990). To test each mutation (*m*) the following cross was performed: + / Y ; *m / TM3* males x $z^1\ w^{is}$ / $z^1\ w^{is}$; *Df(3L)Ez6 / TM3* females. 3 - 5 day old male progeny of the genotype $z^1\ w^{is}$ / Y ; *m / TM3* were scored for eye pigmentation. In each cross, sibling males of the genotype $z^1\ w^{is}$ / Y ; *Df(3L)Ez6 / TM3* served as a control for direct comparison with the tested mutation. $z^1\ w^{is}$ / Y ; $E(z)^{son2}$ / *Df(3L)Ez6* males were obtained in the same cross as $z^1\ w^{is}$ / Y; $E(z)^{son2}$ / *TM3* males. In all cases except for the *Df(3L)Ez6*-carrying chromosome, the chromosome tested in the z-w assay carried a *nos* mutation and was the same that was used to test for the son phenotype.

RESULTS AND DISCUSSION

Lethal phase of $E(z)^{son}$ alleles

In the process of identifying $son^{1\text{-}3}$ mutations as alleles of *Enhancer of zeste* (*E(z)*), it was of interest to study the lethal phenotype of these three alleles and compare it to that of previously known *E(z)* alleles. Therefore, the lethal phases of $E(z)^{son}$ mutations in transheterozygous combinations with each other and with $E(z)^{63}$, a loss-of-function (lof) allele, and $E(z)^{65}$, a deficiency of *E(z)*, were determined in two different manners.

First, flies that carried *E(z)* mutations in trans to a *TM6B* third chromosome balancer were crossed to each other. This balancer carries the dominant marker *Tubby* (*Tb*), which allows us to distinguish transheterozygous larvae and pupae by their Tb^+ phenotype. The results shown in Table 3.3 indicate the stage up to which *E(z)* allelic combinations can support development. This stage is an upper limit since dying Tb^+ individuals were also observed at earlier stages. A control cross showed that $E(z)^{63}/E(z)^{65}$ individuals can reach early pupal stages. This is consistent with previously

reported data (Jones and Gelbart 1990). $E(z)^{son1}/E(z)^{lof}$ died at approximately the same stage. In trans to $E(z)^{63}$ or $E(z)^{65}$, $E(z)^{son2}$ and $E(z)^{son3}$ supported development to later stages: $E(z)^{son2}$ up to viable adults and $E(z)^{son3}$, at least in trans to $E(z)^{65}$, to the late puparium stage. This is consistent with $E(z)^{son2}$ and $E(z)^{son3}$ alleles retaining some wild-type *E(z)* function (see Chapter III). Because transheterozygotes for *E(z)* deficiencies do not survive to the pupal stage (Jones and Gelbart 1990), $E(z)^{son1}$ may also retain some residual wild-type *E(z)* activity.

In the second set of crosses flies which carried *E(z)* mutations in trans to a wild-type third chromosome were intercrossed. The observed lethality in such crosses should only be produced by *E(z)* mutations. These data show that *E(z)* mutations do not cause embryonic lethality (there is a dominant maternal-effect lethality associated with the $E(z)^{65}$ chromosome; it is unclear whether this is caused by $E(z)^{65}$ or another mutation on that chromosome). In most cases where the *E(z)* allelic combination is lethal, the percent of surviving adult progeny is less than the expected 75%. This may reflect a dominant deleterious effect of *E(z)* mutations on viability. Surprisingly, progeny from the cross between the $E(z)^{63}$ and $E(z)^{65}$ alleles are the least viable. This may indicate a high background lethality associated with heterozygosity and homozygosity for E(z) null mutations. Crosses involving these $E(z)^{lof}$ alleles and $E(z)^{son}$ alleles exhibit a lesser extent of zygotic lethality. This result may again indicate that the $E(z)^{son}$ alleles retain wild-type *E(z)* function.

Table 3.3.
(1) "+" indicates a chromosome with wild-type *E(z)*, t indicates a third chromosome as indicated in (2) and (3). Boldface indicates maternal genotype.
(2) *t* =third chromosomal balancer *TM6B*, which carries the dominant marker *Tabby* (*Tb*). Transheterozygous mutant larvae and pupae can be distinguished by their Tb^+ phenotype from siblings which carry the Tb marker.
(3) Flies were crossed in egg laying cages and the development of the progeny was followed in apple juice plates supplemented with yeast. The stage written is the latest stage in which transheterozygous (Tb^+) progeny was observed. Stages were designated by using the following criteria: early prepupa: white or brown pupal case, no recognizable eye or wing formation; late prepupa: eye and wings begin to be visible; late puparium: eye color is red. This stages correspond roughly to stages P2, P4 and P14, respectively (Ashburner, 1989).
(4) *t* =third chromosome from the Canton S wild-type stock. Transheterozygote larvae and pupae cannot be distinguished from siblings carrying wild-type chromosomes.
(5) Flies were crossed in egg laying cages. Indicated is the % of progeny that did not hatch from the egg case (embryonic lethal).
(6) One hundred first instar larvae that had hatched in the cross (see (5)) were transferred to food vials. Indicated is the number of individuals that led to pupae and eclosed adults.
(7) Control cross: "+" is a third chromosome from the Canton S wild-type stock.
(8) Control cross for the last three rows: "+" is a third chromosome with the nos^{L7}, *st e* background chromosome in which the $E(z)^{son}$ mutations were induced.

Table 3.3. Lethal phase of *E(z)^{son}* alleles

Genetic cross (1) (Maternal genotype in **bold**)	Transheterozygotes marked (t=*TM6,Tb*) (2) Latest stage with live transheterozygotes(3)	Transheterozygotes unmarked (t=wild-type chromosome) (4) embryonic lethality (5)		surviving progeny (of 100) (6)	
		%	n	# pupae	# adults
+/t x +/t (7)	eclosed	0	211	98	97
+/t x +/t (8)	eclosed	0	359	90	90
E(z)^{65} x E(z)^{63}/t	early prepupa	19	293	46	34
+/t x E(z)^{65}/t	n.d.	38	222	n.d.	n.d.
E(z)^{son1}/t x E(z)^{63}/t	early prepupa	0	305	74	54
E(z)^{son1}/t x E(z)^{65}/t	early prepupa	33	329	68	50
E(z)^{son2}/t x E(z)^{63}/t	eclosed	0	317	90	82
E(z)^{son2}/t x E(z)65/t	eclosed	36	318	75	57
E(z)^{son3}/t x E(z)^{63}/t	early prepupa	0	312	75	63
E(z)^{son3}/t x E(z)^{65}/t	late puparium	42	397	74	55
E(z)^{son1}/t x E(z)^{son2}/t	late prepupa	0	383	91	63
E(z)^{son1}/t x E(z)^{son3}/t	early prepupa	0	309	80	68
E(z)^{son2}/t x E(z)^{son3}/t	late puparium	0	345	88	64

In summary, both of these experiments show that $E(z)^{son1}$ and $E(z)^{son3}$ mutations, like other lethal *E(z)* mutations, are larval/pupal lethal, while $E(z)^{son2}$ retains enough function to allow development to adulthood. These results are consistent with the idea that son^{1-3} mutations are alleles of *E(z)* which retain wild-type activity in the order $E(z)^{son2} > E(z)^{son3} > E(z)^{son1}$.

Production of germ line clones mutant for $E(z)^{son}$

Before the identification of the mutations son^{1-3} as alleles of the gene *Enhancer of zeste*, an attempt was carried out to discern the wild-type function of this then unidentified complementation group. In particular, since these mutations had a maternal effect on the nos phenotype, it was of interest to test the maternal effect of heteroallelic combinations for these mutations in a wild-type background. It was also of interest testing whether in a *nos* mutant background these semi-dominant suppressors of nos exhibited a stronger, or even complete, son phenotype. Because these mutations were lethal in trans to each other, both of these experiments required transplants of germ line clones mutant for $E(z)^{son}$ alleles into wild-type embryos (see Materials and Methods). A summary of the production of these germ line clones is presented in Tables 3.4 and 3.5.

$E(z)^{son}$ clones in a wild-type background. $E(z)^{son1}$ / $E(z)^{son3}$ germ line clones: Most the egg laying females which could carry a germ line of this genotype were tested at 25°C. Only two of these females produced a relatively large number of eggs. When embeded in Hoyer's medium, most of these eggs appeared unfertilized (109/110). One egg case contained an embryo which had developed a cuticle and had apparent head involution and dorsal closure defects. After 5 days of laying at 25°C these females were transferred to 29°C to observe their egg laying phenotype at this temperature. Again, most of the egg cases appeared unfertilized (47/49). One embryo showed head involution defects and another appeared to have arrested development during gastrulation. Both at 25°C and at 29°C, many of the eggs also had an abnormal morphology, being smaller and rounder than wild type, and sometimes having a flacid appearence. After a five more days at 29°C days, dissection of these females revealed that they did not have the normal array of developmental stages found in wild-type ovaries but rather appeared to have degenerated and exhibited a stringy appearance. This phenotype has also been described for temperature sensitive *E(z)* alleles by Jones and Gelbart (1990) and Phillips and Shearn (1990).

$E(z)^{son2}$ / $E(z)^{son3}$ germ line clones: Most ot the $E(z)^{son2}$ / $E(z)^{son3}$ germ line clones were tested at 25°C. Two females carrying $E(z)^{son2}$ / $E(z)^{son3}$ clones had been

Table 3.4. Production of *E(z)^{son}* germ line clones of in wild-type and *nos* mutant backgrounds.

Experiment	Total # injected	# hatched (% of total)	# pupae (% of total)	# eclosed (% of total)	# of tested females (% of total)	# of egg laying females (% of total, % of tested females)
A	588	356 (61%)	308 (52%)	295 (50%)	131 (22%)	18 (3.1%, 14%)
B	571	400 (70%)	312 (54%)	307 (53%)	121 (21%)	31 (5.4%, 26%)
C	570	334 (59%)	293 (51%)	291 (51%)	123 (21%)	20 (3.5%, 16%)
D	540	352 (65%)	269 (50%)	262 (49%)	111 (21%)	29 (5.3%, 26%)

(A) Cross to obtain donor pole cells: *E(z)^{son1} nos / TM3* females x *E(z)^{son3} / TM3* males. Used to obtain *E(z)^{son1} / E(z)^{son3}* germ line clones.

(B) Cross to obtain donor pole cells: *E(z)^{son2} nos / TM3* females x *E(z)^{son3} / TM3* males. Used to obtain *E(z)^{son2} / E(z)^{son3}* germ line clones.

(C) Cross to obtain donor pole cells: *E(z)^{son1} nos / TM3* females x *E(z)^{son3} nos / TM3* males. Used to obtain *E(z)^{son1} nos / E(z)^{son3} nos* germ line clones.

(D) Cross to obtain donor pole cells: *E(z)^{son2} nos / TM3* females x *E(z)^{son3} nos / TM3* males. Used to obtain *E(z)^{son2} nos / E(z)^{son3} nos* germ line clones.

Table 3.5. Number of germ line clones produced of each *E(z)^{son}* genotype.

Experiment (1)	Tempera-ture	# females tested	# of egg laying females of a particular genotype				
			E(z)^{son}/ TM3 (2)	*E(z)^{son}/ E(z)^{son}* (3)	Putative *E(z)^{son}/ E(z)^{son}* (4)	Not deter-mined (5)	*ovo^{D1}* revertant or other(6)
"*E(z)^{son1} /*	25°C	129	9	0	2	7	0
E(z)^{son3}"	18°C	2	0	0	0	0	0
"*E(z)^{son2} /*	25°C	85	11	10	2	2	2
E(z)^{son3}"	18°C	36	3	1	0	0	0
"*E(z)^{son1} nos /*	25°C	75	7	0	1	7	1
E(z)^{son3} nos"	18°C	48	1	0	3	0	0
"*E(z)^{son2} nos /*	25°C	86	19	2	3	1	3
E(z)^{son3} nos"	18°C	25	0	0	1	0	0

(1) The experiment shown correspond, in the same order, as experiments A-D in Table 2.

(2) Females carrying this genotype were distinguished by the presence in their progeny of the dominant marker *Stubble* present in the *TM3* balancer.

(3) Females carrying this genotype were distinguished by the presence in their progeny of the recessive markers *scarlet* and *ebony* present in the *E(z)^{son} (nos)* chromosomes and the absence of the dominant marker *Stubble* present in the *TM3* balancer

(4) Females carrying this genotype produced ≥ 10 eggs. Nevertheless, these eggs did not develop into adult offspring, and therefore their genotype could not be genetically proven. These females are assumed to carry *E(z)^{son}* transheterozygous germ line clones because of the presence of egg and embryonic phenotypes similar to those observed in the genetically proven *E(z)^{son}* transheterozygous clones (column 5), and, in the *nos* mutant background, the presence of embryos with partially rescued abdominal phenotypes.

(5) Females in this column produced ≤ 10 apparently unfertilized eggs. It is unclear whether these females carry *E(z)^{son}* germ line clones or whether the egg production observed comes from leakiness in the *ovo^{D1}* mutation.

(6) Females in this column produced adult flies without the recessive markers associated with the *E(z)^{son}* mutations or the *Sb* marker associated with the *TM3* balancer chromosome. Therefore, the germ line of these females is assumed to carry a mutated *ovo^{D1}* mutation that no longer confers dominant female sterility.

mated to males wild type for the *E(z)* mutation. About 21% (n=359) of the egg cases appeared to be unfertilized. Of the embryos that developed a cuticle, 21% (n=281) did not hatch. Some (fraction not determined) of these embryos exhibited head defects which could be caused by head involution defects. Thus $E(z)^{son2}$ / $E(z)^{son3}$ germ line clones exhibit some degree of maternal-effect sterility (38% (n=359) of the egg cases did not develop into hatching larvae). The oogenesis and embryogenesis phenotypes caused by the $E(z)^{son2}$ / $E(z)^{son3}$ genotype are milder than those found in $E(z)^{son1}$ / $E(z)^{son3}$ clones (Table 3.6). This is consistent with $E(z)^{son2}$ retaining more wild-type function than $E(z)^{son1}$ (see section above). Eight females carrying $E(z)^{son2}$ / $E(z)^{son3}$ clones had been mated to $E(z)^{son3}$ / + males. The progeny from these females exhibited a degree of embryonic lethality higher than when females with a similar germ line were mated to wild-type males (Table 3.7). Control females carrying $E(z)^{son2}$ / *TM3* or $E(z)^{son3}$ / *TM3* germ lines mated to males of the same genotype show that a zygotic $E(z)^{son}$ mutant genotype per se does not cause embryonic lethality. Thus, the embryonic lethality depends on an $E(z)^{son}$ maternal genotype and is enhanced by being in addition zygotically mutant for $E(z)^{son}$. Ovaries from females carrying $E(z)^{son2}$ / $E(z)^{son3}$ germ line clones were dissected and had no apparent abnormalities.

Adult $E(z)son^{(2or3)}$ / + progeny from $E(z)^{son2}$ / $E(z)^{son3}$ germ lines exhibited a high frequency of wing defects such as disruptions of the LII and LIII wing veins (16/80) and reduction in wing size (2/80) (Figure 3.9). No wing defects were found in progeny from control $E(z)son^{(2or3)}$ / *TM3* germ lines. Thus the effects of the maternal background mutant for $E(z)^{son}$ can be observed as late as the development of adult structures.

$E(z)^{son}$ clones in a *nos* mutant background. These clones showed phenotypes similar to those described above in a nos^{+} background, such as egg laying phenotypes, head involution defects, and the presence of wing defects in the adult progeny. Here, I will emphasize the son phenotype conferred by these genotypes.

$E(z)^{son1}$ *nos* / $E(z)^{son3}$ *nos* germ cells did not lead to adult progeny but their genotypes were inferred by the rescue of the *nos* phenotype and/or other associated defects. At 25°C, eight females produced very few, abnormal eggs, most of which did not form cuticles. We presume that these females carried $E(z)^{son1}$ *nos* / $E(z)^{son3}$ *nos* germ line clones. At 18°C 3/4 fertile adult females (presumably $E(z)^{son1}$ *nos* / $E(z)^{son3}$ *nos*) laid eggs which developed into larvae (71 larvae/147 eggs). All embryos formed six to eight abdominal segments (86% of the embryos scored (n=29) had 8 abdominal segments). Most of the developed embryos (69/71) failed to hatch and none reached adulthood. Many

Table 3.6. *E(z)^{son2} / E(z)^{son3}* germ line clones exhibit weaker oogenesis and maternal effect embryonic lethality *E(z)^{son1} / E(z)^{son3}* germ line clones

germ line genotype x genotype of males (1)	% of total eggs that appear unfertilized	% embryonic lethality (2)	% of total infertility (3)	Total number of eggs
E(z)^{son1} / E(z)^{son3} *x E(z)^{son3} / +* (4)	99	100	100	110
E(z)^{son2} / E(z)^{son3} *x + / +* (5)	21	21	38	359
E(z)^{son2} / E(z)^{son3} *x E(z)^{son3} / +* (5)	12	44	48	1540

(1) "+" indicates the wild-type *E(z)* copy, which in this experiment was present on a *rucuca* chromosome (*rucuca* is a multiply marked chromosome which is wild type for *E(z)*).

(2) % of embryos that form a cuticle that do not hatch.

(3) % of total number of eggs that do not lead to hatching larvae (i.e. apparently unfertilized + embryonic lethals).

(4) Results from two females mated to *E(z)^{son3} / rucuca* males.

(5) Results from two females mated to *rucuca / rucuca* males.

(6) Results from eight females mated to *E(z)^{son3} / rucuca* males.

Table 3.7. The maternal effect embryonic lethality caused by *E(z)^{son}* mutations is enhanced by zygotic *E(z)^{son}* mutations.

germ line genotype x genotype of males (1)	% embryonic lethality (2)	Total number of eggs
E(z)^{son2} / E(z)^{son3} x *+ / +* (3)	21	359
E(z)^{son2} / E(z)^{son3} x *E(z)^{son3} / +* (4)	44	1540
E(z)^{son2} / TM3, Sb x *E(z)^{son3} / +* (5, 6)	0.6	527
E(z)^{son3} / TM3, Ser x *E(z)^{son3} / +* (5, 7)	1.8	623

(1) "+" indicates the wild-type *E(z)* copy, which in this experiment was present on a *rucuca* chromosome.

(2) % of embryos that form a cuticle that do not hatch.

(3) Results from two females mated to *rucuca / rucuca* males.

(4) Results from eight females mated to *E(z)^{son3} / rucuca* males.

(5) *E(z)^{son2} / TM3* and *E(z)^{son3} / TM3* germ lines could be distinguished by the *Sb* and *Ser* markers in their progeny (see Methods of this section).

(6) Results from three females mated to *E(z)^{son3} / rucuca* males.

(7) Results from three females mated to *E(z)^{son3} / rucuca* males.

(84%, n=32) showed head involution defects, similar to those observed in embryos with mild homeotic transformations (see, for example, Jürgens (1985)).

E(z)^{son2} nos / E(z)^{son3} nos germ line clones: 8/25 fertile females exhibited phenotypes similar to those observed in females carrying *E(z)^{son1} nos / E(z)^{son3} nos* germ line clones. Two females produced embryos with a high degree of abdominal rescue (50% of the embryos scored (n=14) had 8 abdominal segments). Many of these embryos reached adulthood. In these cases the genotype of the transplanted pole cells was unambiguously identified by the genetic markers observed in the progeny.

In summary, transheterozygous combinations for *E(z)^{son}* alleles exhibit, aside from a very strong son phenotype, a number of additional phenotypes. Some of these defects, such as the oogenesis defects and maternal effect embryonic lethality, have been previously described using temperature sensitive *E(z)* alleles. Thus *E(z)* is likely involved in these processes. Other phenotypes, such as the egg morphology defect and the maternal-effect adult wing phenotypes have not been reported and may be caused by the gain-of-function character of these alleles.

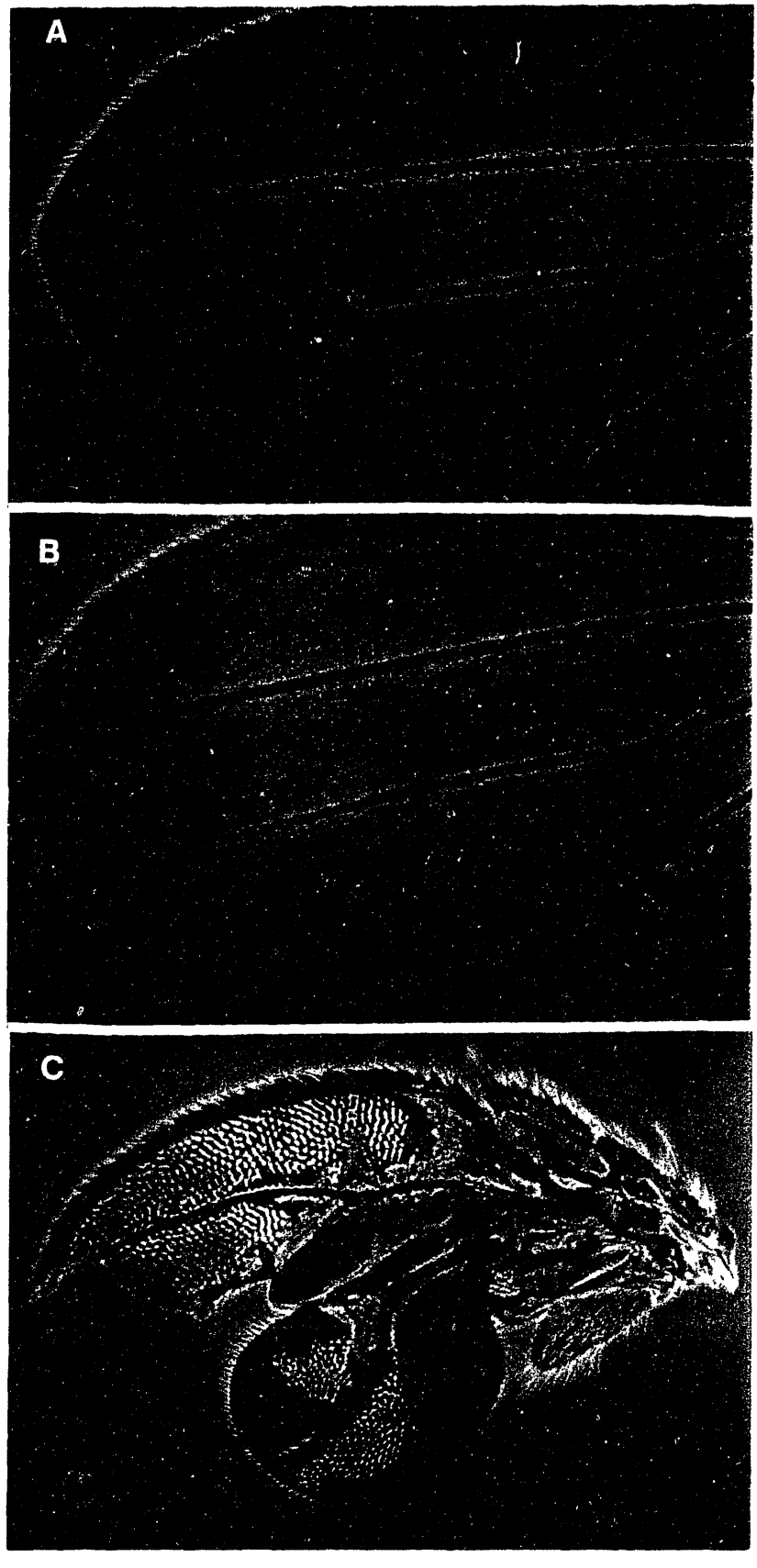
In Chapter III we discussed the fact that *E(z)^{son}* heteroallelic germ lines, while exhibiting a very strong son phenotype, caused relatively weak, if any, homeotic phenotypes. This suggests that the *E(z)^{son}* gain-of-function products preferentially interfere with gap gene repression.

Suppression of *E(z)^{son}*-associated phenotypes with extra *E(z)$^{+}$* copies.

The complementation of a phenotype with wild-type copies of a particular gene constitutes strong genetic evidence for allelism of the mutations producing the phenotype to the wild-type gene. In addition, the effect of extra wild type gene copies can provide information on the nature of the mutations. Therefore, I tested the effect of extra doses of *E(z)* on two phenotypes associated with *E(z)^{son}* mutations, namely, the lethality and the son phenotype induced by heteroallelic combinations. The wild-type *E(z)* copy was carried within a P-element construct that only contains the *E(z)* transcription unit (p[E(z)], see Jones and Gelbart (1993))

Figure 3.9. *E(z)^{son}* germ line clones cause a maternal effect wing phenotype in the adult progeny. A) Wild-type wing from *E(z)^{son} / +* siblings. B, C) Wing phenotypes exhibited by adult progeny form *E(z)^{son2} / E(z)^{son3}* germ line clones: interrupted LII and LIV wing veins (B), and small wings, possibly with a wing to haltere homeotic transformation.

Figure 3.9



Suppression of the lethality associated with *E(z)^{son}* mutations. The heteroallelic combinations *E(z)^{son3} / Df(3L)Ez*, *E(z)^{son1} / E(z)^{son3}* and *E(z)^{son2} / E(z)^{son3}* are lethal (see Table 2.3). Extra doses of *E(z)* suppress the lethality associated with all these genotypes (Table 3.8). Interestingly, for a particular genotype there is a tendency to obtain less progeny which carry two p[E(z)] constructs than one p[E(z)] construct (observed in crosses C and D).

Reversion of the son phenotype. The effect of extra *E(z)* copies was tested on both the dominant son phenotype and the recessive son phenotype. Table 3.9 shows that extra *E(z)* copies decrease the dominant phenotype induced by *E(z)^{son}* mutations in a *hb* heterozygous background. Surprisingly, the presence of the *CyO* balancer chromosome used in these crosses also decreases the son phenotype. It is unknown whether this effect is due to the presence of specific mutations on the *CyO* chromosome or by non-specific effects caused by balancer chromosomes in general. As in the rescue of the *E(z)^{son}* associated lethality, two p[E(z)] copies tend to exhibit a weaker reversion effect than only one p[E(z)] copy.

Table 3.10 shows that one extra *E(z)$^{+}$* copy greatly reduces the strong son phenotype confered by two *E(z)^{son}* heteroallelic combinations in a wild-type *hb^{mat}* background. In addition, the oogenesis and embryonic phenotypes associated with this *E(z)^{son}* maternal genotype are also alleviated. For example, at 25°C *E(z)^{son1}/E(z)^{son3}* clones are mostly unable to produce eggs that can be fertilized and develop into embryos (see above). At a lower temperature the egg laying defect is alleviated and 84% of the embryos that develop cuticles exhibit head involution defects. *E(z)^{son1}/E(z)^{son3}* females that carry one *E(z)$^{+}$* copy, on the other hand, can produce eggs that develop into embryos at 25°C, only 25% (n=16) of which show head defects.

In conclusion, the fact that P-element constructs carrying only the wild-type *E(z)$^{+}$* transcription unit can rescue both the lethality and the maternal-effect phenotypes (including the son phenotype) associated with *E(z)^{son}* mutations strongly argues that the *E(z)^{son}* mutations are *E(z)* alleles.

In Chapter III we show that the *E(z)^{son}* mutations have a stronger son phenotype than *E(z)^{null}* mutations. Thus the *E(z)^{son}* mutations behave as gain-of-function (g-o-f) alleles with respect to the son phenotype. Here I show that additional wild-type *E(z)* copies revert the son phenotype caused by these g-o-f mutations. This indicates that *E(z)^{son}* alleles are antimorphic in nature, that is, that they may code for aberrant products that interfere with the wild-type E(z) function (at least in the process of gap gene regulation).

Table 3.8. Complementation of the lethality caused by *E(z)^{son}* mutations with extra wild-type *E(z)* copies.

Genetic cross A (1)	
E(z)^{son3} / TM3 females x *p[E(z)] / CyO ; Df(3L)Ez6 / TM3* males	
Genotype of progeny	number of progeny
+ / CyO ; (Df(3L)Ez6 or E(z)^{son3}) / TM3	36
p[E(z)] / + ; (Df(3L)Ez6 or E(z)^{son3}) / TM3	37
+ / CyO ; E(z)^{son3} / Df(3L)Ez6	0
p[E(z) / + ; E(z)^{son3} / Df(3L)Ez6	22
Genetic cross B (1)	
p[E(z)] / CyO ; E(z)^{son3} / TM3 females x *p[E(z) / CyO ; E(z)^{son1} / TM3* males	
Genotype of progeny	number of progeny
p[E(z)] / CyO ; E(z)$^{son(1\ or\ 3)}$ / TM3	17
p[E(z)] / CyO ; E(z)^{son1} / E(z)^{son3}	24
p[E(z)] / p[E(z) ; E(z)$^{son(1\ or\ 3)}$ / TM3	14
p[E(z)] / p[E(z)] ; E(z)^{son1} / E(z)^{son3}	9
Genetic cross C (1)	
p[E(z)] / CyO ; E(z)^{son3} / TM3 females x *p[E(z) / CyO ; E(z)^{son2} / TM3* males	
Genotype of progeny	number of progeny
p[E(z)] / CyO ; E(z)son(2 or 3) / TM3	12
p[E(z)] / CyO ; E(z)^{son2} / E(z)^{son3}	41
p[E(z)] / p[E(z) ; E(z)$^{son(2\ or\ 3)}$ / TM3	12
p[E(z)] / p[E(z)] ; E(z)^{son2} / E(z)^{son3}	13

(1) In these experiments, the chromosomes carrying *E(z)$^{son1-3}$* and *Df(3L)Ez6* carried in addition a *nos^{L7}* mutation. The heteroallelic combinations *E(z)^{son3} / Df(3L)Ez6*, *E(z)^{son1} / E(z)^{son3}*, and *E(z)^{son2} / E(z)^{son3}* are 100% inviable (see Table 1).

Table 3.9. Extra $E(z)^{+}$ copies revert the revert the dominant son phenotype caused by $E(z)^{son}$ mutations (hb^{mat} heterozygous background).

Maternal *E(z)* complement (1)	$E(z)^{son}$ maternal genotype (*hb nos / nos* background)			
	$E(z)^{son2}$ / +		$E(z)^{son3}$ / +	
	% rescue (2)	n	% rescue (2)	n
+ / CyO	18	574	26	352
p[E(z)] / CyO	10	1269	9	0.13
p[E(z)] / +	26	1010	58	1868
p[E(z)] / p[E(z)]	31	287	65	373
+ / + (3)	42	145	88	179

(1) Females tested for the data in rows 1 and 3 were the progeny of the cross: *p[E(z)] / CyO* ; $E(z)^{son}$ *nos / TM3* x *+ / + ; hb nos / TM3.* Females tested for the data in rows 2 and 4 were the progeny of the cross: *p[E(z)] / CyO* ; $E(z)^{son}$ *nos / TM3* x *p[E(z)] / CyO ; hb nos / TM3.* A + indicates a wild type chromosome that carries an $E(z)^{+}$ copy. *CyO* is a chromosomal balancer used in the cross. For unknown reasons, the presence of this balancer appears to suppress the son phenotype.

(2) % embryos with ≥ 3 abdominal segments.

(3) Data is from Table 2.1 and is included for comparison.

Table 3.10. Extra *E(z)*$^{+}$ copies revert the revert the recessive son phenotype caused by heteroallelic *E(z)*^{son} combinations (full *hb*^{mat} dose).

Maternal *E(z)* complement (1)	*E(z)*^{son} maternal genotype (*nos / nos* background)			
	E(z)^{son1} / *E(z)*^{son3}		*E(z)*^{son2} / *E(z)*^{son3}	
	% rescue (2)	n	% rescue (2)	n
+ / + (3)	100	29	100	14
p[E(z)] / + (4)	35	133	20	300

(1) Data is from germ line clone experiments and is included for comparison.

(2) % of embryos with ≥ 3 abdominal segments.

(3) Females of these genotypes were produced with the crosses *E(z)*$^{son(1\ or\ 3)}$ */ TM3* males x *p[E(z)] / CyO; E(z)*^{son3} *nos / TM3* females.

(4) Data collected at 25°C.

Interestingly, both in the reversion of lethality and the son phenotype, there seems to be a tendency for two extra wild-type *E(z)* copies to have a smaller reversion effect than one extra *E(z)* copy. It is possible that, at least in these backgrounds already "sensitized" with the presence of $E(z)^{son}$ products, too much E(z) product actually interferes with *E(z)* function.

Variables that influence the son phenotype

During the characterization of the son phenotype, it became apparent that the penetrance of this phenotype was influenced by a number of different factors. The following is a discussion on the factors that have been observed to influence the son phenotype.

Temperature dependency of the son phenotype. The penetrance of the son phenotype caused by different *son* alleles increases with temperature (Table 3.11). At 18°C *son* mutations have no detectable effect on the nos phenotype. Nevertheless, this relatively low temperature is not incompatible with the son phenotype since the strong $E(z)^{son1}/E(z)^{son3}$ combination produces a highly penetrant son phenotype at 18°C (see above and Chapter II). At 29°C, the son phenotype is most penetrant, but heterozygosity for hb^{mat} also leads to a son phenotype at this temperature. At 25°C, the effect of son mutations in a hb^{mat} heterozygous background is high in comparison with the alleviation of the nos phenotype by the hb^{mat} heterozygous background. The higher signal to noise ratio of this assay at 25°C is one reason why most tests for the son phenotype are carried out at this temperature (the other reason is practical, since at 29°C flies tend to be less healthy and males become sterile).

The temperature sensitivity of the son phenotype is observed with all son mutations tested and even in a wild-type (son^+) background. This indicates that the temperature sensitivity of the son phenotype is not caused by the temperature sensitivity of a particular son allele but that it is caused by a *E(z)*-independent process that affects abdomen formation.

To obtain information about the nature of the son phenotype, I determined the temperature sensitive period during oogenesis for the dominant son phenotype exhibited by $E(z)^{son3}$ *nos/hb nos* females (see Materials and Methods). Temperatures of 18°C and 25°C were chosen for this experiment because of the absence of a dominant son phenotype at 18°C, and a strong son phenotype at 25°C. Because Drosophila ovaries contain strings of egg chambers at different stages of development, eggs that are collected at different times

Table 3.11: Temperature sensitivity of the son phenotype

son maternal genotype[1]	Maternal genotype					
	nos / nos % rescue [2] (n)			*hb nos / nos* % rescue [2] (n)		
	18°C	25°C	29°C	18°C	25°C	29°C
+/+	0 (73)	0 (303)	n.d.	0 (180)	2 (160)	70 (103)
E(z)^{son1}/+	0 (51)	2 (54)	32 (49)	0 (4)	91 (80)	74 (53)
E(z)^{son2}/+	0 (270)	3 (347)	35 (23)	0 (10)	17 (169)	77 (39)
E(z)^{son3}/+	0 (51)	11 (152)	52 (25)	n.d.	88 (179)	98 (80)
son^{59}/+	0 (101)	0 (93)	0 (60)	0 (67)	3 (330)	69 (170)
son^{126}/+	0 (72)	0 (218)	7 (57)	n.d.	49 (167)	44 (76)

(1) A "+" represents the corresponding wild-type allele.

(2) % of embryos with ≥ 3 abdominal segments.

after the temperature shift come from egg chambers that were at progressively earlier stages in oogenesis at the time of the shift. The duration of each stage in Drosophila oogenesis has been previously calculated (see Ashburner 1989), and thus it is possible to correlate the ts period with particular stages of oogenesis. Figure 3.10 shows that the ts period for the son phenotype spans a broad window between about 10 and 70 hours after the shift. This implies that this process is temperature sensitive during a broad period that approximately lasts from stage 2 in oogenesis, when the nurse cells begin polyploidization to stage 10, just before the nurse cells pour their contents into the oocyte.

Time since balanced stock was established. The dominant son phenotype has decreased in some of the son stocks as they have been passaged for a number of generations. For example, *E(z)^{son3} nos / nos* females exhibit weaker son phenotypes in experiments carried out at later times after the establishment of the stock (Figure 3.11A). This effect may be due to the accumulation of modifier mutations that increase the viability of heterozygous *son* stocks.

These modifier mutations do not affect the stronger son phenotype when in addition to the *son* mutation a *hb* mutation is introduced by crossing (Figure 3.11B). Indeed, the son phenotype exhibited by chromosomes carrying both *E(z)^{son}* and *hb* alleles is weaker than that exhibited by a transheterozygous combination of the same mutations (*E(z)^{son3} hb / + +* and *E(z)^{son3} + / + hb* genotypes produce 48% (n=390) and 88% (n=179) embryos with ≥ 3 abdominal segments, respectively). These observations underscore the need for outcrossing when carrying out suppressor-of-*nos* tests.

Age of females. In general one observes that the first layings from females carrying a *son* mutation exhibit a stronger son phenotype than later layings. This effect is also observed in *hb nos / nos* females even in the absence of *son* mutations. Figure 3.11B exemplifies this phenomenon in a *E(z)^{son3} nos / hb nos* background.

During the first experiments after the identification of the *E(z)^{son3}* mutation the age dependency of the phenotype was not observed. Thus, the son phenotype does not seem to be intrinsically dependent on the age of the female (this can also be observed in the progeny of *E(z)^{son3} nos / nos* females - Figure 3.11A). The son phenotype caused by mutations in the same stock tested at later times did, however, show a dependency on the age of the females. Thus, the accumulation of genetic modifiers in the stock carrying son alleles may have enhanced this age-dependent phenomenon.

Practical consequences for experimental procedures. The effect on the son phenotype by genetic and non-genetic factors causes a practical problem when comparing strengths of different *son* genotypes. To minimize the effect caused by these variables,

data quantifying the son phenotype reported in these studies come from tests which, except where otherwise stated, conform to the following guidelines:

a) The female flies used grew, and were tested at, a constant temperature of 25°C.

b) Quantitative comparison of the son phenotype confered by different genotypes were made using chromosomes (either those carrying newly induced mutations or recombinant chromosomes) that had been recently created (less than 3 passages of the stocks). In the cases when tests were repeated, all mutant stocks involved in the comparison were retested.

c) Only embryos from the first four days of layings were scored for the son phenotype.

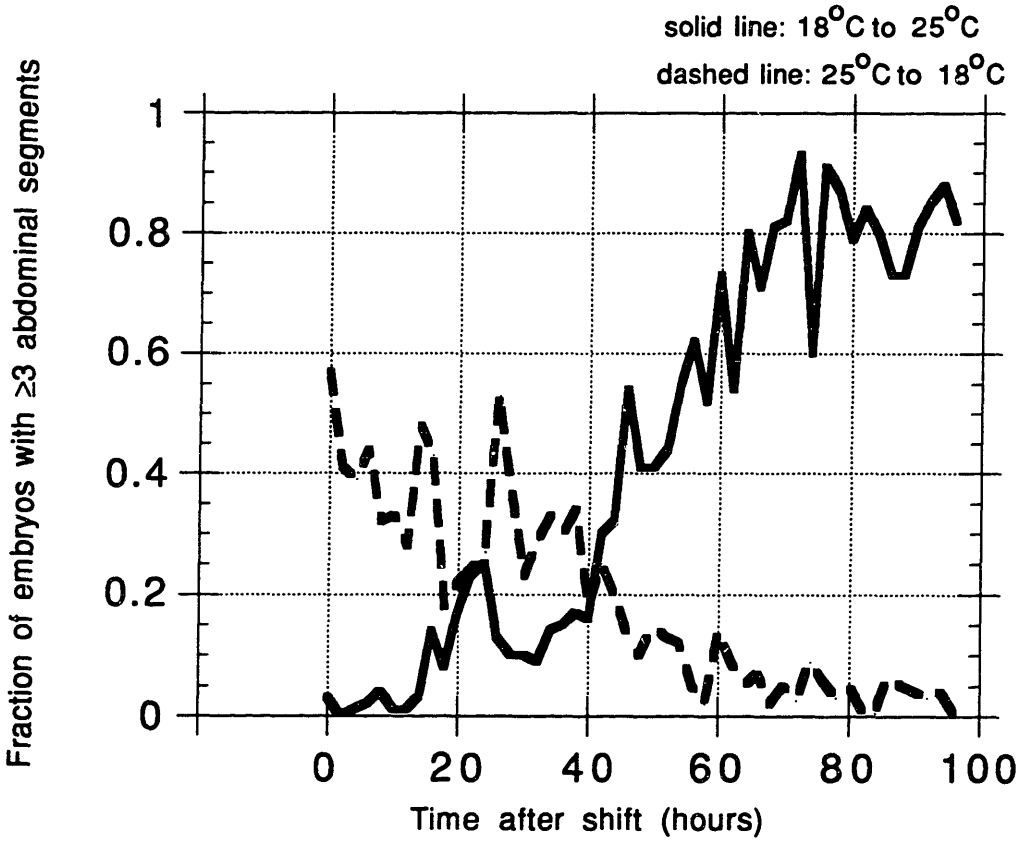
The effect of temperature on the son phenotype is reminiscent of the effect of temperature on variegation. Higher temperatures increase both the suppression of nos and the suppression of variegation. Since suppression of nos and suppression of variegation may have a similar molecular basis (see Chapters I and III), it seems possible that the temperature sensitivity for both of these processes may have similar causes.

It has been proposed that the effect of temperature on variegation occurs by affecting developmental rate, i.e. that higher developmental rates suppress variegation (Hartmann-Goldstein 1967). This is also supported by the fact that variegation is enhanced by increased crowding in the food medium, which decreases developmental rates. In more specific terms, this would mean that higher developmental rates lead to a higher level of gene transcription of genes regulated at the chromatin level.

Similarly, the son phenotype, which we argue involves derepression of chromatin-regulated genes, may also be affected by some process related to developmental rate. This hypothesis may help explain the puzzling result that, while *E(z)* appears to act in the early embryo, a son-independent process related to the son phenotype has a temperature sensitive period during oogenesis (there is also an *E(z)*-dependent ts period during early embryogenesis, see below). It is possible that the son phenotype depends, directly or indirectly, on the developmental rate of the early embryo, which would in turn depend on the accumulation of maternal products during oogenesis.

Figure 3.10. Temperature sensitive period of the son phenotype. *E(z)^{son3} nos / hb nos* females were grown at either 18°C or 25°C. At the time indicated as 0 hours in the graph, each group of females was shifted to 25°C or 18°C, respectively. The temperature sensitive period for the son phenotype, which spans from about 10 to 70 hours prior to egg deposition, corresponds to approximately stages 2 to 10 in oogenesis.

Figure 3.10. Temperature shift of *E(z)*^{son} *nos / hb nos* females



The dependency on the age of females for suppression may also indicate that eggs produced by younger females have a maternal content that give them a different character (e.g. lead to a higher developmental rate of the embryo) than eggs produced by older females. The fact that eggs produced early and late have different properties has also been observed in layings from females carrying maternal effect mutations, which often exhibit stronger phenotypes when laid by older females.

In summary, the son phenotype is modified by genetic and non-genetic factors. The non-genetic factors can be external (e.g. temperature) or internal (e.g. age of the females). The exact nature of all of these factors is unclear. At least in some of these cases, the effect on the son phenotype appears to originate during oogenesis. Thus processes that occur during oogenesis can affect the outcome of developmental decisions that occur in the early embryo.

The son phenotype depends on maternal, but not zygotic, E(z) product

The regulation of homeotic genes by Pc-G gene products involves in most cases both maternal and zygotic Pc-G contribution. Females mutant for *E(z)*, for example, have a maternal effect homeotic phenotype (Jones and Gelbart 1990; Phillips and Shearn 1990; see also Chapter III, Figure 2C) which is enhanced when these females are mated to *E(z)* mutant males. It was therefore of interest to determine whether both maternal and zygotic E(z) product may be also involved in the regulation of the gap genes *kni* and *gt*.

First, $E(z)^{son3}$-carrying males were crossed to appropriate females and tested for the son phenotype (Table 3.12). A paternal $E(z)^{son3}$ copy does not suppress the abdominal phenotype of embryos from *hb nos / nos* females. Nor does paternally introduced $E(z)^{son3}$ enhance the maternal effect son phenotype of $E(z)^{son3}$ *nos/ hb nos*

Second, *nos* females that in addition were hemizygous for the temperature sensitive allele $E(z)^{61}$ were crossed, at the restrictive temperature, with either wild type males or $E(z)^{61}$ hemizygous males. The extent of abdomen formation is very similar in the progeny

Figure 3.11. Dependence of the son phenotype on both the age of the females and the age of the balanced stocks. Data labelled 10/89, 1/91 and 3/93 correspond to experiments carried out at the corresponding months and year. A) Results from $E(z)^{son3}$ *nos/ nos* females. The son phenotype decreases in strength in older stocks. B) Results from $E(z)^{son3}$ *nos/ hb nos* females. Then son phenotype enhanced by hb^{mat} heterozygosity does not decrease in strength, but becomes more dependent on the age of the female.females. Thus the $E(z)^{son3}$ allele can only produce the son phenotype when introduced maternally.

Figure 3.11

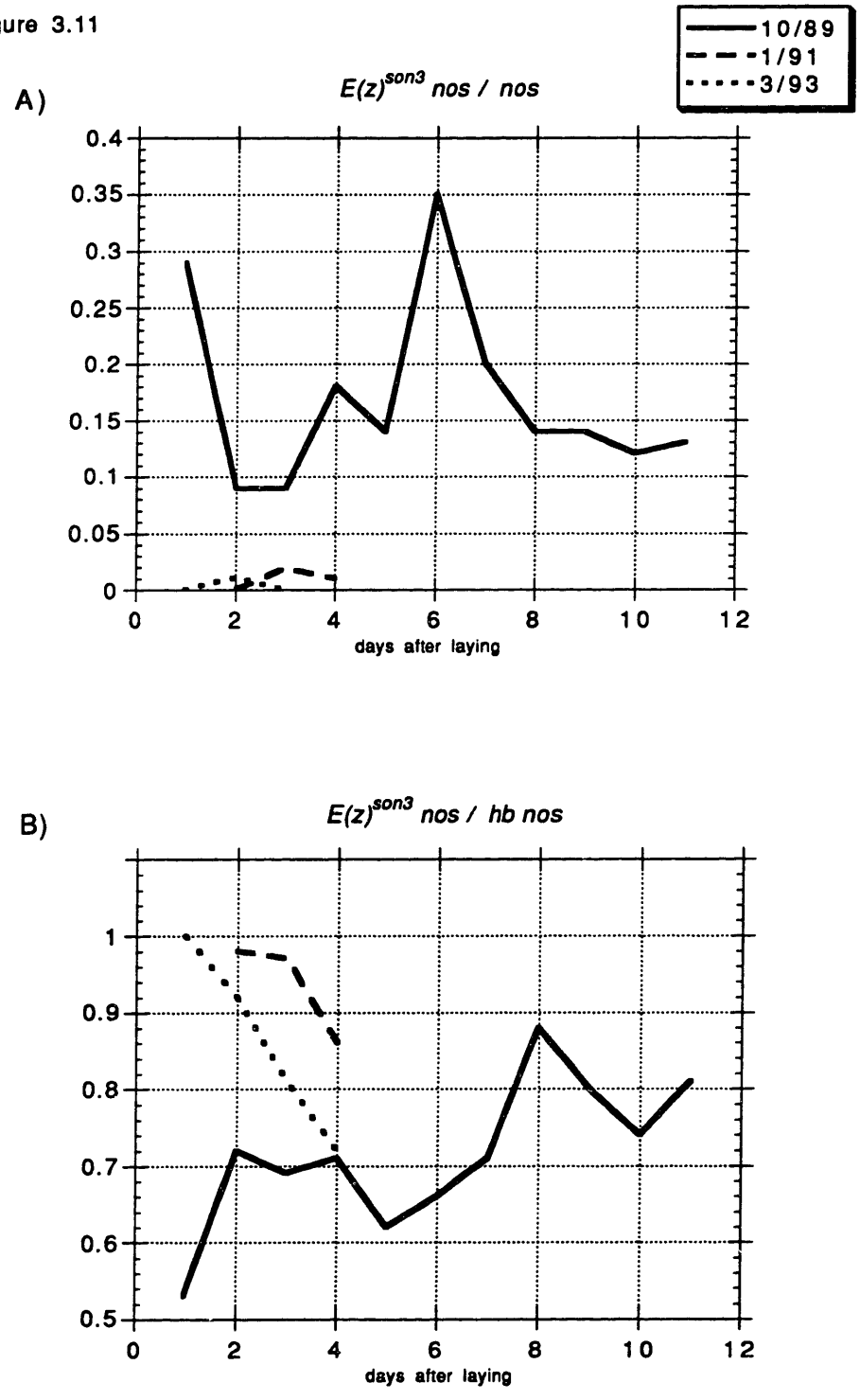

A)
E(z)son3 nos / nos
10/89
1/91
3/93
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
days after laying
B)
E(z)son3 nos / hb nos
1
0.9
0.8
0.7
0.6
0.5
0
2
4
6
8
10
12
days after laying

Table 3.12. *E(z)^{son3}* does not produce or enhance the son phenotype when introduced through the father

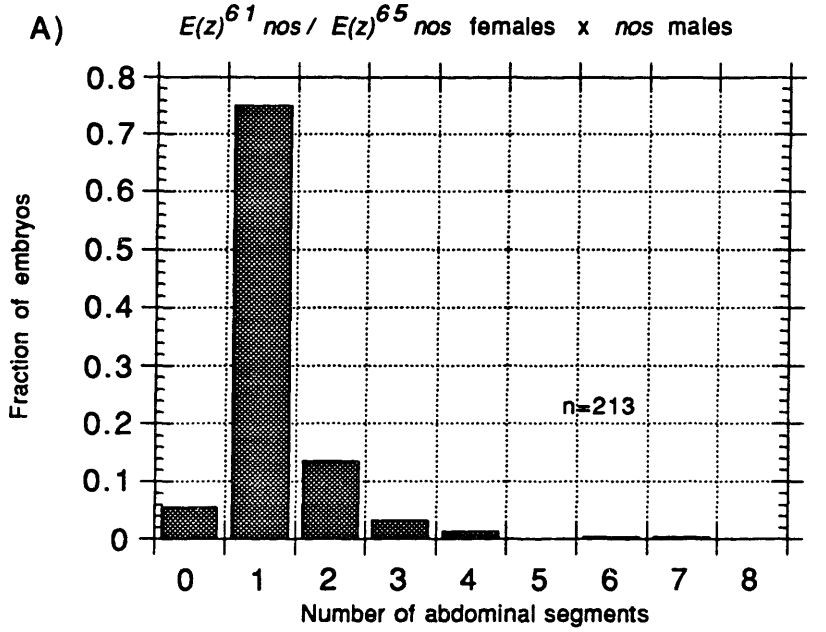
Paternal genotype (1)	Maternal genotype			
	hb nos / nos		*E(z)^{son3} nos / hb nos*	
	% rescue (2)	n	% rescue (2)	n
+ / TM3	4	166	81	97
E(z)^{son3} / TM3	4	189	65	89

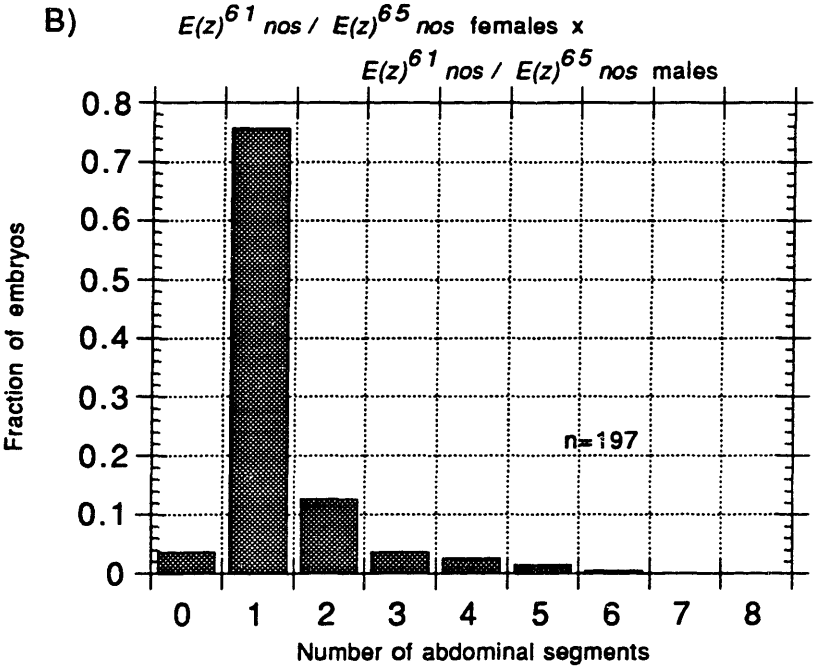
(1) "+" is the wild-type *E(z)* copy. Both chromosomes tested (+ and *E(z)^{son3}*) contained in addition the mutation *nos^{L7}*.

(2) % of embryos with ≥ 3 abdominal segments.

Figure 3.12. Paternal E(z) product is not involved in the son phenotype. Embryos from *E(z)^{61} / E(z)^{65}* females were collected at the restrictive temperature for the *E(z)^{61}* allele. The profiles of abdominal segment formation are very similar whether the females had been mated to wild-type or *E(z)^{61} / E(z)^{65}* males.

Figure 3.12


A)
E(z)61 nos / E(z)65 nos females x nos males
Fraction of embryos
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
n=213
0 1 2 3 4 5 6 7 8
Number of abdominal segments


B)
E(z)61 nos / E(z)65 nos females x
E(z)61 nos / E(z)65 nos males
Fraction of embryos
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
n=197
0 1 2 3 4 5 6 7 8
Number of abdominal segments

from both crosses (Figure 3.12). This indicates that zygotic E(z) product does not affect the suppression of the nos phenotype confered by the maternal *E(z)* mutations.

Both of these results argue that the repression of *kni* and *gt* depends only on the E(z) product already present in the egg. This is consistent with gap genes being part of the first tier of zygotically expressed genes. Apparently, zygotic E(z) product is not produced early enough to regulate the genes in this first tier of zygotic expression. Although this has not been proven, for similar reasons, we assume that the zygotic products of other Pc-G genes also do not contribute to the regulation of *kni* and *gt*.

Summary of mutations that affect the nos phenotype

The following are complete tables for the tested son phenotype conferred by mutations in *E(z)* and in other Pc-G genes. Many of these results have already been presented in Chapter III. Here, only results not previously shown will be discussed.

Suppression-of-*nos* by *E(z)* alleles. Table 3.13 has a complete list of the son phenotype confered by *E(z)* mutations in trans to wild-type *E(z)* copies and to other *E(z)* mutations. $E(z)^{son2}$ confers a weaker son phenotype in trans to the deficiency $E(z)^{65}$ than in trans to the temperature sensitive $E(z)^{61}$ allele, even at semi-restrictive temperatures. This is unexpected since $E(z)^{61}$ appears to retain more wild-type function than the deficiency (compare, for example, the effect of $E(z)^{61}/E(z)^{61}$ with $E(z)^{61}/E(z)^{65}$). This discrepancy may indicate that the $E(z)^{son2}/E(z)^{61}$ combination has an allele-specific synthetic effect that produces a particularly strong son phenotype.

Suppression-of-*nos* by mutations in other Pc-G genes. Table 3.14 has a complete list of the son phenotype caused by mutations in Pc-G genes other than *E(z)*. The table has been divided in two groups, those with genes which show no detectable effect on the son phenotype, and those with genes that show an effect.

Mutations in the gene *Polycomb* do not significantly enhance the son phenotype confered by *son* mutations. The homeotic transformations produced by mutations in Pc-G genes are particularly sensitive to haploinsuficiency for the *Polycomb* locus (see, for example, Kennison and Russell (1987)). I therefore tested whether a similar effect could be observed in the case of the son phenotype. Table 3.15 shows that maternal haploinsufficiency for *Pc* does not significantly enhance the son phenotype conferred by $E(z)^{son1\text{-}3}$ and son^{29}, another *son* mutation (compare to Chapter III, Table 2). These results indicate that if *Pc* is involved in the regulation of gap genes, the requirement for its product is less dosage dependent than in the case of homeotic gene regulation.

Table 3.13. *E(z)* mutations suppress the *nos* abdominal phenotype

E(z) Maternal Genotype (1)	Maternal Genotype			
	nos / nos		*hb nos / + nos*	
	% rescue (2)	n	% rescue (2)	n
+/+	0	338	2	160
$E(z)^{son1}$ / +	11	156	76	186
$E(z)^{son2}$ / +	6	160	42	145
$E(z)^{son3}$ / +	13	895	88	179
Df(3L)Ez6 / +	0	525	9	768
$E(z)^{65}$ / +	0	356	2	473
$E(z)^{66}$ / +	0	253	10	229
$E(z)^{63}$ / +	0	361	9	293
$E(z)^{64}$ / +	0	575	12	353
$E(z)^{1}$ / +	0	297	1	422
$E(z)^{60}$ / +	0	562	4	318
$E(z)^{61}$ / +	0	619	8	251
$E(z)^{61}$ / $E(z)^{61}$ (25°C)	2	216	n.d.	-
(29°C)	6	84	n.d.	-
$E(z)^{61}$ / $E(z)^{65}$ (25°C)	24	270	n.d.	-
(29°C)	38	39	n.d.	-
$E(z)^{son2}$ / $E(z)^{65}$ (25°C)	31	216	n.d.	-
(29°C)	44	78	n.d.	-
$E(z)^{son2}$ / $E(z)^{61}$	95	366	n.d.	-
$E(z)^{son1}$ / $E(z)^{son3}$ (3)	100	29	n.d.	-
$E(z)^{son2}$ / $E(z)^{son3}$ (4)	100	14	n.d.	-

(1) a "+" represents the wild-type *E(z)* gene.

(2) % of embryos with ≥ 3 abdominal segments.

Table 3.14. Suppression of *nos* abdominal phenotype by Pc-G genes

Pc-G Maternal Genotype (1)	Maternal Genotype			
	nos / nos		*hb nos / + nos*	
	% rescue (2)	n	% rescue (2)	n
wild type	0	594	0	614
Pc-G genes which do not exhibit dominant son phenotypes				
Asx^{D1} / +	0	398	0.5	753
esc^{10} / +	0	89	2	201
esc^{2} / esc^{10}	0	181	n.d.	-
Pc_5 / +	0	296	2	517
Pcl^{R5} / +	0	227	3	281
ph^{501} / +	n.d.	-	0	966
ph^{410} / ph^{br}	0	149	n.d.	-
Sce^{D1} / +	0	406	0	657
Scm^{D1} / +	0	420	0.9	212
Scx^{1} / +	0	351	0	426
Pc-G gene which exhibits no dominant or recessive son phenotype				
esc^{10} / +	0	89	2	201
esc^{2} / esc^{10}	0	181	n.d.	-

Table 3.14 (continuation)

Pc-G genes which exhibit a dominant and / or recessive son phenotype				
Df(Su(z)2-C) / + (3)	2	266	27	445
Psc^{1} / +	0	352	0	382
Psc^{1445} / +	2	350	7	256
Psc^{1433} / +	0	651	12	532
Psc^{e24} / +	0	132	0	632
$Su(z)2^{1}$ / +	0	52	3	316
$Su(z)2^{1.b7}$ / +	0	866	0	689
$Su(z)3^{1}$ / +	0	212	0	266
Df(4)G / +	0	378	5	587
pho^{c} / +	0	455	10	659
pho^{c} / pho^{c} (4)	89	18	n.d.	-

(1) A "+" represents the wild-type copy of the corresponding gene.

(2) % of embryos with ≥ 3 abdominal segments.

Table 3.15. Heterozygosity for *Pc* does not significantly enhance the son phenotype conferred by *son* mutations

son Maternal Genotype (1)	*Maternal genotype*	
	Pc5 nos / + nos	
	% rescue (2)	n
+/+	0	296
E(z)son1 / +	0	347
E(z)son2 / +	0	524
E(z)son3 / +	0	531
son29 / +	56	723

(1) A "+" represents the wild-type copy of the corresponding gene.

(2) % of embryos with ≥ 3 abdominal segments.

In summary, mutations in some Pc-G genes, but not in others, exhibit dominant and/or recessive effects on the *nos* phenotype. The son phenotypes exhibited by some Pc-G genes indicate that their products are likely involved in gap gene regulation. The absence of dominant son phenotype is not easily interpretable. Given the weak dominant effect confered by *E(z)* null and loss-of-function mutations, it is possible that mutations in some of these Pc-G products do not exhibit a dominant son phenotype but would cause a recessive son phenotype. In the case of *extra sex combs* (*esc*), embryos which entirely lack Esc product can be easily obtained, and an involvement of this gene in the regulation of gap genes has been ruled out. For other Pc-G genes, the creation of germ line clones homozygous mutant for these genes will be required in order to rigorously test their role in gap gene regulation.

Epistasis analysis of *E(z)* and other genes in the pathway

In order to genetically locate the effect of *E(z)* within the pathway that leads to abdominal development, I carried out epistasis analysis using double mutant combinations of mutations in *E(z)* and other genes in this pathway.

Supression of the abdominal phenotype caused by mutations in genes upstream of nos localization and by other *nos* mutations. $E(z)^{son3}$ was tested for epistatis over mutations in the genes *vasa* and *oskar*. These two genes are required for formation of the pole plasm and therefore embryos from females mutant for them are defective in both abdomen and pole cell formation. Since nos embryos have no defects in pole plasm assembly, suppressors of *nos* would be expected to act downstream of this process. Indeed, $E(z)^{son3}$ suppresses the abdominal defect of embryos from *vasa* and *oskar* females (Table 3.16). The adult progeny from these females were sterile. Thus *E(z)* mutations specifically rescue the abdominal defect caused by mutations that cause defects in pole plasm assembly.

It was also of interest to test whether the son phenotype requires allele-specific interactions involving the nos^{L7} allele, which is not a null allele (Lehmann and Nüsslein-Volhard 1991). Thus $E(z)^{son3}$ was tested for the son phenotype in backgrounds hemizygous for other *nos* alleles. $E(z)^{son3}$ suppresses the nos phenotype produced by all *nos* mutations tested, which include null alleles such as nos^{RD} and nos^{BN} (Lehmann and Nüsslein-Volhard 1991; Gavis and Lehmann 1994)(Table 3.16). Thus E(z) mutations bypass the *nos* requirement for abdomen formation.

Female mutant for strong *vasa* and *nos* alleles have additional oogenesis phenotypes such as the production of few eggs, which in the case of *vasa* lack dorsal appendages

Table 3.16 *E(z)^{son3}* suppresses the abdominal phenotype caused by mutations in genes upstream of *nanos* and by other *nanos* mutations

Maternal Genotype (1)	Maternal Genotype			
	nos / nos		*hb nos / + nos*	
	% rescue (2)	n	% rescue (2)	n
vas^{D1} / vas^{PD}; + / +	0.4	2934	34	525
vas^{D1} / vas^{PD}; E(z)^{son3} / +	24	480	94 (3)	174
osk^{166} / Df(3R)^{pxT103} (4)	n.a.		0.4	758
E(z)^{son3} osk^{166} / Df(3R)^{pxT103}	n.a.		73 (5)	236
osk^{166} / Df(3R)^{pxT26} (4)	n.a.		0.6	483
E(z)^{son3} osk^{166} / Df(3R)^{pxT26}	n.a.		72 (6)	246
nos^{L7} / Df(3R)^{Dlx43} (7)	0	413	n.d.	
+ nos^{L7} / E(z)^{son3} Df(3R)^{Dlx43}	11	290	50	150
nos^{BN} / Df(3R)^{Dlx43}	0	94	n.d.	
+ nos^{BN} / E(z)^{son3} Df(3R)^{Dlx43}	35	196	97	43
nosRD / Df(3R)^{Dlx43}	0	100	n.d.	
+ nosRD / E(z)^{son3} Df(3R)^{Dlx43}	16	38	48	41
nos^{RW} / Df(3R)^{Dlx43}	81	873	n.d.	
+ nos^{RW} / E(z)^{son3} Df(3R)^{Dlx43}	70	248	n.d.	

(1) "+" indicates the wild-type copy of the appropiate gene.

(2) % of embryos with ≥3 abdominal segments.

(3) Ten male progeny from these females were crossed to wild-type females and found to be sterile.

(4) *Df(3R)^{pxt103}* and *Df(3R)^{pxt26}* delete the *hb* and *osk* genes.

(5) 17 male and 12 female progeny from these females were crossed to wild-type flies and found to be sterile.

(6) Three male and seven female progeny from these females were crossed to wild-type flies and found to be sterile.

(7) *Df(3R)Dl^{x43}* deletes the *nos* gene.

n.a. = not applicable; n.d. = not determined.

(Lehmann and Nüsslein-Volhard 1991). It is unclear whether the egg production phenotype observed in *vasa* females is due to a requirement of *vasa* for production of *nos* during early oogenesis. The presence of a $E(z)^{son3}$ mutation in these females is not associated with a significant increase in the number of eggs produced by *vasa* and *nos* mutant ovaries (Table 3.17). This suggests that *E(z)* does not regulate downstream targets of *nos* during oogenesis.

An increase in egg production of *vasa* mutant ovaries is associated with *hb* heterozygosity (Table 3.17). This suggests that *vasa* and *hb* may be part of a common pathway during early oogenesis. Nevertheless, the egg production defect caused by *nos*, are not alleviated by *hb* heterozygosity. Completely removing *hb* function in a germ line mutant for strong *nos* alleles would clarify whether *vasa*, *nos* and *hb* may be part of a pathway during oogenesis required for egg production.

On the other hand, the ventralized egg phenotype associated with *vasa* mutations did not appear to be significantly suppressed by either the *hb* or $E(z)^{son3}$ mutations, which suggests that this phenotype is unrelated to *nos*, *hb* and *E(z)*.

In conclusion, these data suggest a role for *hb* , but not *E(z)*, in a *vas-* (and perhaps *nos-*) dependent pathway in oogenesis.

Enhancement of the son phenotype by other *hb* alleles. The enhancement of the son phenotype conferred by *hb* mutations would not be expected to be specific to the hb^{7M} allele, since this allele is a protein null. Indeed, a deficiency for *hb* also enhances the son phenotype (Table 3.18). Thus, this enhancement is caused by a reduction in the *hb* dosage.

Lehmann and Nüsslein-Volhard (1987a) described a type of *hb* alleles that caused zygotic phenotypes that are stronger than those caused by a hb^{null} mutations. Testing of these so called class V alleles for their enhancement of the son phenotype showed that these alleles also produce a stronger dominant son phenotype than *hb* nulls. This can be observed both in *E(z)* wild-type and $E(z)^{son}$ mutant backgrounds (Table 3.18). Thus class V *hb* alleles appear to code for dominant negative products that interfere with the function of wild-type *hb* copy.

The son phenotype requires zygotic gap genes. The abdominal phenotype observed in embryos from *nos* females occurs because of transcriptional repression of the gap genes *kni* and *gt*. *E(z)* mutations could suppress the *nos* phenotype by reactivating the expression of these gap genes, or by activating an unknown parallel pathway that can promote abdominal development. In order to distinguish these two possibilities I tested whether $E(z)^{son}$ mutations could suppress the abdominal phenotype caused by lack of *kni* function.

Table 3.17. Mutations in *hb*, but not in *E(z)* suppress the egg production defects associated with strong *vasa* alleles.

Maternal genotype (1)	# of eggs(2)	# of females	# eggs/day/female	% ventralized eggs
vas^{D1}/vas^{D1} background (3)				
+ +/+ +	17	19	0.3	94
$E(z)^{son3}$ + /+ +	9	21	0.14	67
+ hb^{7M} / + +	573	23	8.3	100
$E(z)^{son3}$ + / + hb^{7M}	458	29	5.3	98
$nos^{RC}/Df(3R)D^{x43}$ background				
+ +/+ +	32	6	1.3	
$E(z)^{son3}$ + /+ +	1	9	0.03	n.a.
$E(z)^{son3}$ + / + hb^{7M}	1	5	0.07	n.a.
$nos^{RD}/Df(3R)D^{x43}$ background				
+ +/+ +	100	6	4.2	
$E(z)^{son3}$ + /+ +	38	6	2.1	n.a
$E(z)^{son3}$ + / + hb^{7M}	41	6	2.3	n.a.
$nos^{91}/Df(3R)D^{x43}$ background				
+ +/+ +	22	7	0.79	
$E(z)^{son3}$ + /+ +	3	6	0.17	n.a.
$E(z)^{son3}$ + / + hb^{7M}	1	5	0.07	n.a.

(1) *vasa* or *nos* mutant bacground are as indicated. In the rows with data, the genotype with respect to *E(z)* and *hb* are indicated in this same order.

(2) # of eggs produced in the first three days of laying for *vasa* mutant females and the first four days of laying for *nos* mutant females.

(3) The $E(z)^{son3}$-carrying chromosome in rows 2 and 4 also contained the nos^{L7} allele. To control for a possible effect of the *nos* mutation, females in rows 1 and 3 were also heterozygous for this mutation.

Table 3.18. Suppression of the nos phenotype by different *hb* alleles.

nos mutant background						
Maternal *hb* genotype (1)	Maternal *E(z)* genotype (1)					
	+/+		*E(z)^{son2} / +*		*E(z)^{son3} / +*	
	% rescue (2)	n	% rescue (2)	n	% rescue (2)	n
+/+	0	338	n.d.	-	n.d.	-
hb^{7M} / +	0	1081	20	278	96	275
Df(hb) / + (3)	n.d.	-	n.d.	-	66	15
hb^{9K49} / +	1	223	25	407	81	487
hb^{9K59} / +	70	632	82	378	99	306

(1) "+" indicates the wild-type allele.

(2) % of embryos with ≥ 3 abdominal segments.

(3) *Df(hb)* is *Df(3R)p^{XT103}*.

Embryos homozygous for strong *kni* alleles were tested for their degree of abdominal segmentation in maternal genotypes containing one copy of $E(z)^{son3}$ ($E(z)^{son3}$ / +), or a combination of $E(z)^{son3}$ and *hb* heterozygosity ($E(z)^{son3}$ + / + *hb*). These two maternal genotypes, specially the second one, suppress the abdominal defects caused by *nos* mutations. Nevertheless, these genotypes do not suppress the abdominal phenotype caused by strong *kni* alleles (see Table 3.19). This indicates that *E(z)* acts upstream of *kni* by reactivating the normal pathway that leads to abdominal development.

In summary, $E(z)^{son}$ mutations are epistatic to *nos* and its upstream genes, interact with *hb* and require the expression of the zygotic gap gene *kni*. Since *nos* mutant embryos rescued by *E(z)* mutations form structures which are dependent on *gt* function and since *kni* and *gt* appear to be coordinately regulated, we presume that *gt* function is also required for the son phenotype. These genetic results locate the site of action of *E(z)* downstream of *nos* and upstream of the activation of zygotic gap genes. In Chapter III we show that *kni* and *gt* are expressed in *E(z) nos* mutant embryos, which is in agreement with these results. Furthermore, we locate the site of *E(z)* action with respect to hb^{mat}, by determining that *E(z)* does not affect the levels of Hb^{mat}. Thus together the molecular and the genetic data converge in a model in which *E(z)* is required to act in conjunction with the Hb^{mat} protein to repress the gap genes *kni* and *gt*.

An *E(z)*-dependent temperature sensitive period during early embryogenesis is consistent with *E(z)*-dependent gap gene regulation.

We have proposed that *E(z)* mutations cause the son phenotype because *E(z)* is required to repress gene expression of the abdomen-promoting gap genes. This model predicts that using the temperature sensitive (ts) allele $E(z)^{61}$ one might be able to observe an *E(z)* dependent ts effect on the son phenotype during the early stages of embryogenesis.

Control embryos from *nos* females which were wild-type for *E(z)* function and had been shifted to the restrictive temperature during early embryogenesis showed a small increase in abdominal segmentation (about 20% of the embryos shift from the "0 segments" to the "1 segment" category- compare, in Figure 3.13, (A) and (B)). Temperature sensitivity for abdomen formation during early embryogenesis has been reported and may be related to the function of the gene pumilio (Lehmann and Nüsslein-Volhard, 1987b). Embryos from $E(z)^{61}$ *nos* / $E(z)^{65}$ *nos* females that had been similarly treated exhibited a larger increase in segment formation. Specifically, about 60% of these embryos shifted

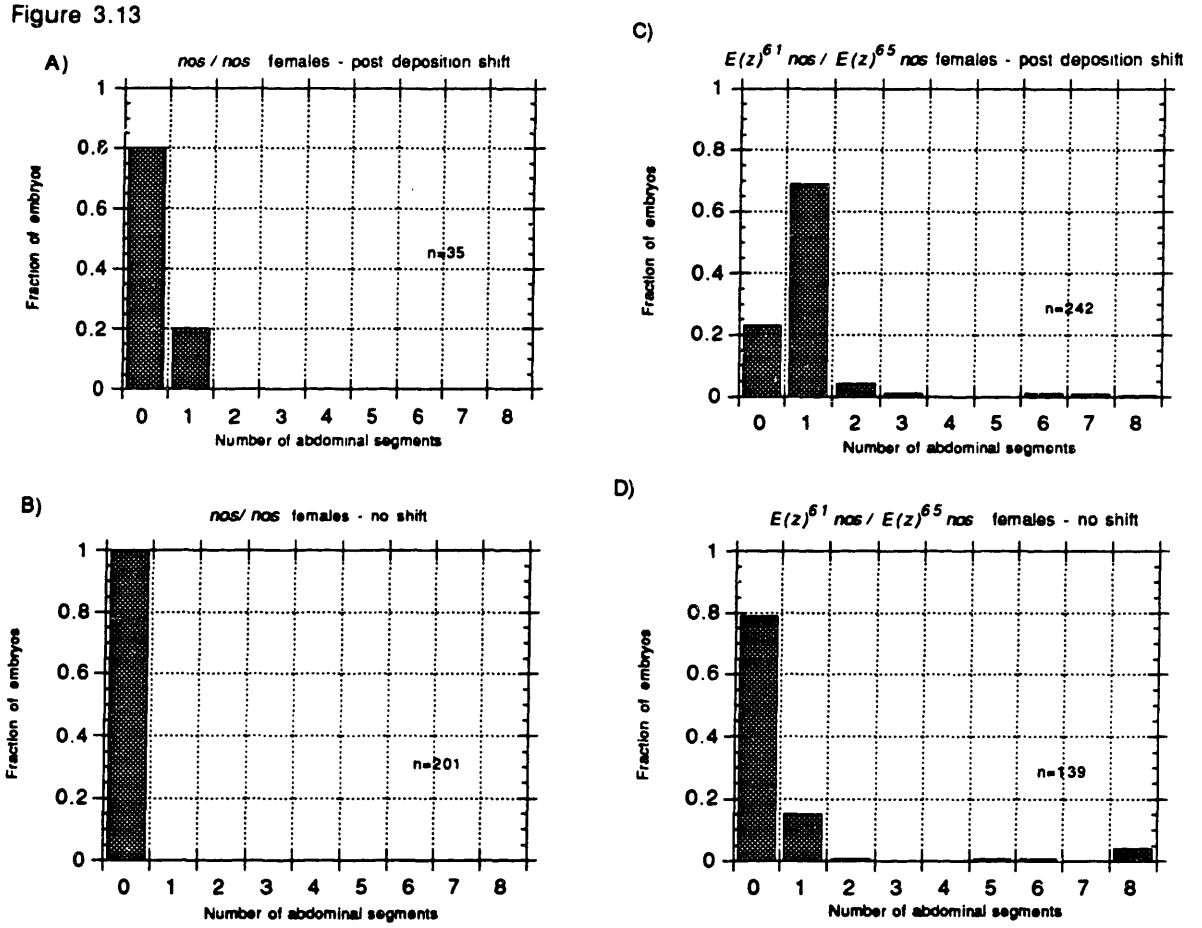
Table 3.19. Mutations in *E(z)* and *hb* can not rescue the abdominal phenotype caused by strong *kni* mutations.

Maternal genotype (1)	Zygotic genotype			
	kni^{1L} homozygotes		kni^{FC} homozygotes	
	average # of abdominal segments (2)	n	average # of abdominal segments (2)	n
+ + / + +	2	34	2	22
$E(z)^{son3}$ + / + +	2	131	2	56
$E(z)^{son3}$ + / + *hb*	2	127	2	104

(1) "+" indicates the wild-type copy of the corresponding gene.

(2) All embryos scored had two abdominal segments.

Figure 3.13


A)
nos / nos females - post deposition shift
Fraction of embryos
n=35
Number of abdominal segments
C)
E(z)61 nos / E(z)65 nos females - post deposition shift
Fraction of embryos
n=242
Number of abdominal segments
B)
nos/ nos females - no shift
Fraction of embryos
n=201
Number of abdominal segments
D)
E(z)61 nos / E(z)65 nos females - no shift
Fraction of embryos
n=139
Number of abdominal segments

from the "0 segments" category to categories with one or more abdominal segments (in Figure 3.13, compare panels (C) and (D)).

Thus, although there is also a small *E(z)*-independent temperature dependence for the formation of abdominal segments, there appears to be a significant *E(z)*-dependent temperature sensitivity during early embryogenesis. This result is consistent with the above described model for *E(z)* function in early embryogenesis.

Is *E(z)* involved in transcriptional regulation of *knirps* and *giant* in wild-type embryos?

In Chapter III we show that *E(z)* is required for the maintenance of the anterior boundaries of *kni* and *gt* that are initiated by the Hb^{mat} protein gradient. The embryos used in these experiments were mutant for *bcd*. This prevented the activation of many anteriorly expressed genes which depend on *bcd* for their activation, including the zygotic *hb* expression, so that the only source of anteroposterior polarity in these embryos is the Hb^{mat} protein gradient

The effect of *E(z)* mutations in *bcd* mutant embryos, and thus on the determination of boundaries by Hb^{mat}, was very clear. It is less clear what role *E(z)* may have in the determination of gap gene boundaries in the presence of the normal set of regulatory interactions that occur in wild-type embryos. In particular, since the Hb^{mat} protein gradient has been shown to be nonessential, it is possible that *E(z)* function is also dispensable for proper gap gene regulation.

The following two studies attempt to obtain genetic evidence for a role of *E(z)* function in the transcriptional regulation of gap genes in wild-type embryos.

Expression of gap genes in embryos mutant for *E(z)* but otherwise wild type. The $E(z)^{son2}$ / $E(z)^{61}$ heteroallelic combination was used as the *E(z)* mutant background to try to detect possible differences. In embryos from *nos* and *bcd* mutant females, this *E(z)* mutant combination confers strong phenotypes detectable both at the cuticular and the gap gene expression levels.

Embryos from $E(z)^{son2}$ / $E(z)^{61}$ females were collected under conditions similar to

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Figure 3.13. Post-deposition temperature shift of embryos from *nos* and *E(z) nos* females. Embryos from *nos/nos* or $E(z)^{61}$ *nos*/$E(z)^{65}$ *nos* females were collected at 25°C at 30 minute intervals and immediately shifted to 29°C (the restrictive temperature for $E(z)^{61}$, $E(z)^{65}$ is a null). For each genotype, control samples of embryos which were not shifted to 29°C were also scored. A and B) About 20% of the embryos form one field of denticles upon shifting to the higher temperature. C and D) About 60% of the embryos form a higher number of abdominal segments at the restrictive temperature for $E(z)^{61}$.

those carried in the studies involving *nos* and *bcd* mutant backgrounds. Expression of the gap genes *Kr*, *kni* and *gt* was detected by in situ hybridization using digoxigenin-labelled RNA probes and analized as described in the Materials and Methods. The average profiles of about 5 to 11 embryos are plotted in Figure 3.14. Statistical analysis of the data points at the anterior boundaries of *kni* expression suggest that these boundaries are different in wild-type and mutant embryos (see Materials and Methods). This difference is, though, very small (about 2% EL). Similar analysis suggests that the anterior boundaries of *Kr* and *gt* are not statistically different.

Suppression of weak *kni* alleles by $E(z)^{son}$ and hb^{mat} mutations. Above we show that strong *kni* alleles are completely epistatic to $E(z)^{son}$ mutations. This indicated that *kni* acts downstream of *E(z)* in the pathway that leads to abdominal segmentation. In principle, the abdominal phenotype produced by weak *kni* mutations could behave differently, i.e. it may be suppressed by $E(z)^{son}$ mutations. This would occur if the defect produced by the partially defective *kni* allele can be alleviated by increased concentrations of the mutant Kni product. In order to test whether *E(z)* and / or hb^{mat} mutations may affect the levels of Kni product I tested the effect of these mutations on the abdominal phenotype caused by two weak *kni* alleles, kni^{IIV} and kni^{14B}.

Embryos mutant for each one of these weak *kni* alleles in trans to the strong *kni* alleles kni^{IL}, kni^{IID} and kni^{FC} were produced from wild-type, *hb* / +, $E(z)^{son3}$ / + and $E(z)^{son3}$ + / + *hb* females. Preliminary studies showed that the abdominal phenotype caused by kni^{IIV} is not affected in any of these backgrounds (Figure 3.15), while that caused by kni^{14B} seemed to be affected.

A larger scale experiment was repeated with kni^{14B} / $kni^{(IL\ or\ FC)}$ embryos. Figure 3.16 shows that the presence of $E(z)^{son3}$, or of a *hb* mutation in the maternal background increases the number of abdominal segments produced by both of these *kni* mutant genotypes. As with the son phenotype, the strongest effect is produced in a background that carries both $E(z)^{son3}$ and *hb* mutations.

Thus, maternal genotypes that in *nos* and *bcd* mutant embryos cause derepression of the abdomen-promoting gap genes *kni* and *gt*, also increase the number of abdominal segments produced by embryos mutant for at least one weak *kni* mutation. Given the known role of E(z) and Hb in transcriptional repression, the increase in abdominal segments produced by kni^{14B} in $E(z)^{son}$ and *hb* mutant backgrounds can be interpreted as

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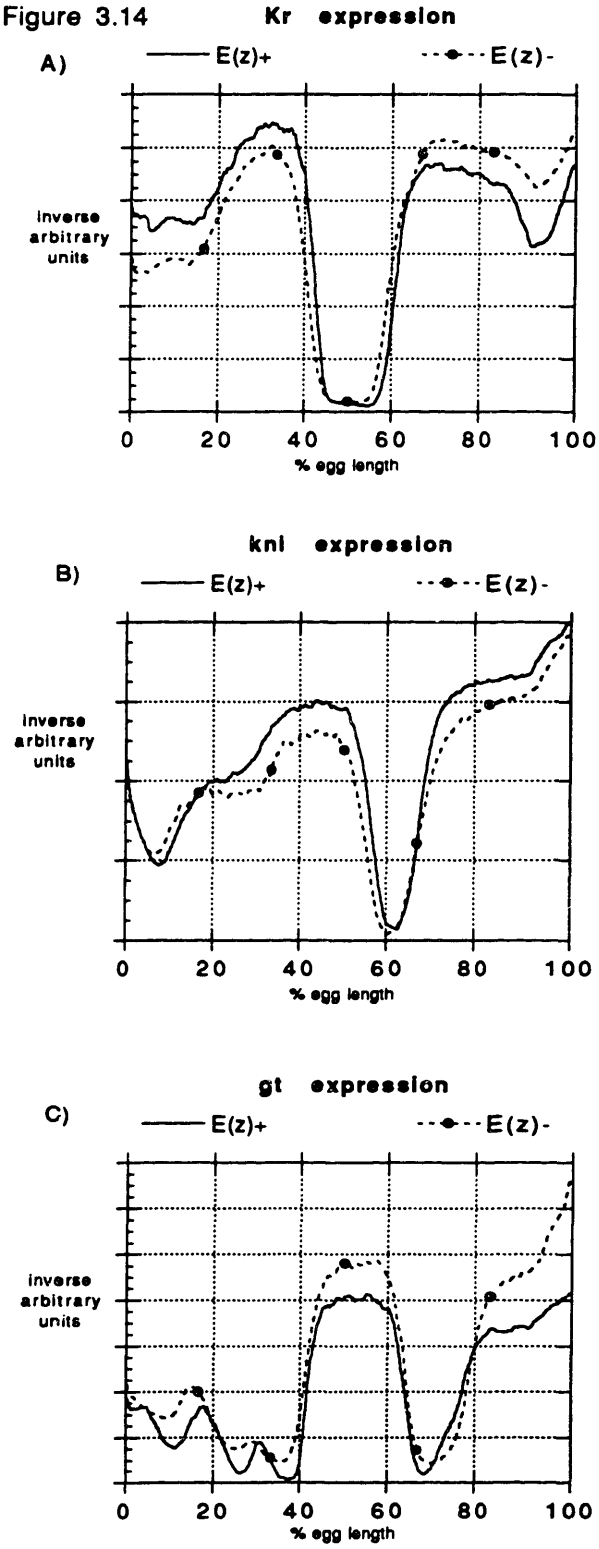
Figure 3.14. Expression profiles for *Kr*, *kni* and *gt* RNA expression in wild-type and $E(z)^{son2}/E(z)^{61}$ embryos at the late cellular blastoderm stage (s.14b).

Figure 3.14


Kr expression
A)
E(z)+
E(z)-
inverse arbitrary units
0
20
40
60
80
100
% egg length
kni expression
B)
E(z)+
E(z)-
inverse arbitrary units
0
20
40
60
80
100
% egg length
gt expression
C)
E(z)+
E(z)-
inverse arbitrary units
0
20
40
60
80
100
% egg length

being caused by an increase in transcription of these gene, and thus in the concentration of its partially functional product. The defect confered by the *kni*^{IIV} allele, on the other hand, may not be rescuable by an increase in the *kni*^{IIV} product.

The suppression of the abdominal phenotype caused by *kni*^{14B} mutations by *hb* and *E(z)*^{son} mutations suggests that in the wild-type embryo Hb^{mat} and E(z) are involved in establishing a certain level of *kni* transcription, perhaps being involved in an equilibrium between activating and repressing factors.

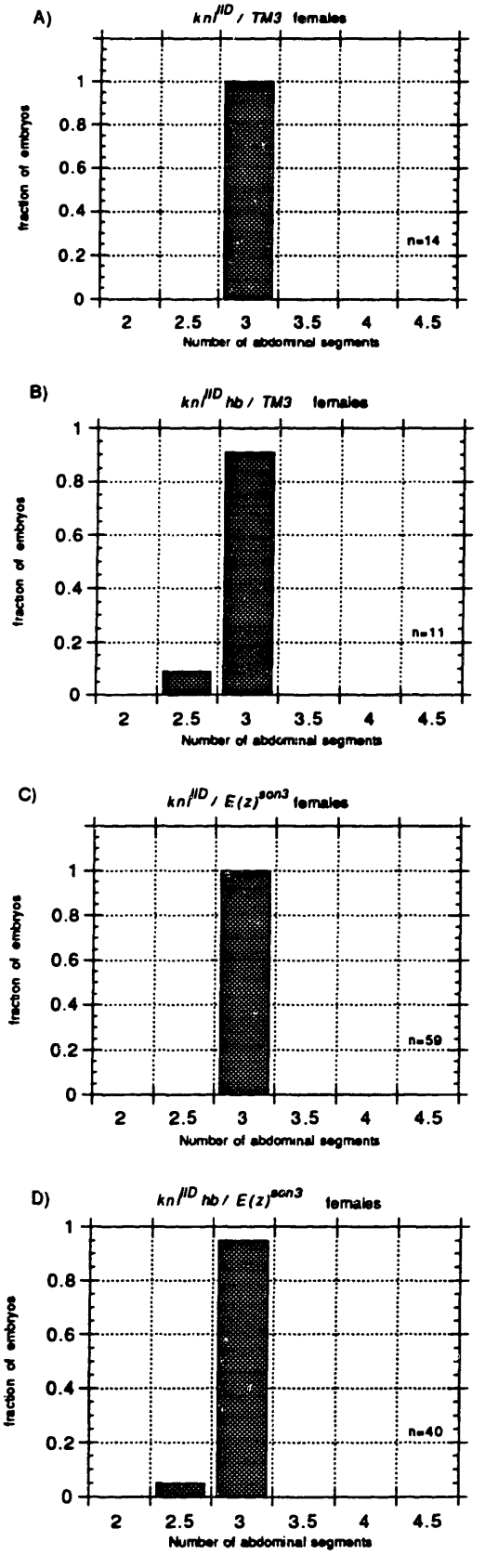
In conclusion, these two studies suggest that in the wild-type embryo, *E(z)* has a role in regulating *kni* expression. First, the anterior boundary of *kni* expression in *E(z)* mutant embryos exhibits a small but statistically significant anterior shift. Second, phenotypic studies of the effect of *E(z)* mutations on weak *kni* alleles are consistent with *E(z)* mutations causing an increased transcription of *kni* in an otherwise wild-type background. These data support a role of *E(z)* in the normal regulation of *kni*. Nevertheless, it should be kept in mind that in both of these studies the mutant *E(z)* background involved gain-of-function (antimorphic) *E(z)* alleles. Thus, it remains unknown whether a complete loss of *E(z)* function would have an effect on *kni* expression.

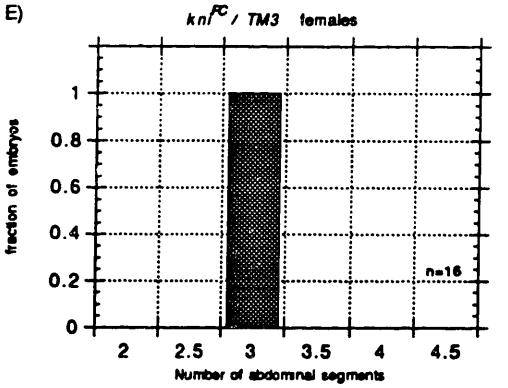
The lack of statistically significant effects on the anterior boundaries of *Kr* and *gt* expression in *E(z)* mutant embryos might reflect the fact that there are, for both of these boundaries, other strong interactions that do not depend on Hb^{mat}. In particular, the anterior *Kr* boundary is determined by Hb^{zyg}, and the anterior *gt* boundary is strongly repressed by Kr.

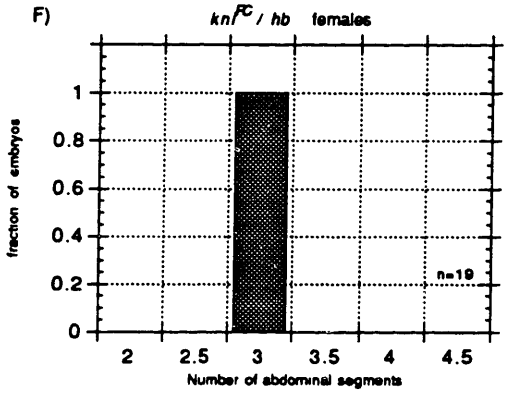
Figure 3.15. Mutations in *hb* and *E(z)*^{son3} do not affect the abdominal phenotype produced by the weak *kni*^{IIV} allele. Embryos mutant for the weak *kni*^{IIV} allele in trans to strong *kni* alleles (*kni*^{IID}, *kni*^{FC} and *kni*^{IL}), were tested for their degree of abdomen formation in four maternal backgrounds: wild type ((A), (E) , (I)), *hb*/+ ((B), (F), (J)), *E(z)*^{son3}/+ ((C), (G), (K)), and *E(z)*^{son3} +/*hb* + ((D), (H), (L)). *TM3* is a balancer chromosome that is wild-type for *kni*.

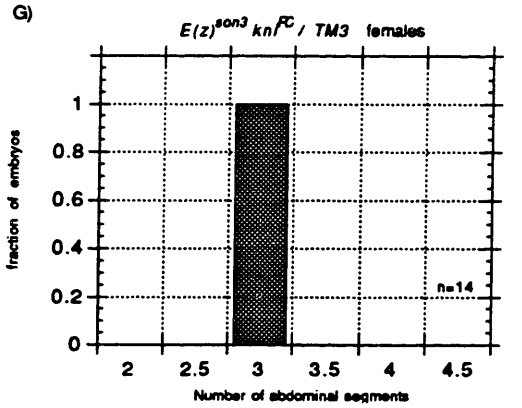
Figure 3.16. Mutations in *hb* and *E(z)*^{son3} alleviate the abdominal phenotype produced by the weak *kni*^{14B} allele. Embryos mutant for the weak *kni*^{14B} allele in trans to strong *kni* alleles (*kni*^{FC} and *kni*^{IL}), were tested for their degree of abdomen formation in four maternal backgrounds: wild type ((A), (E)), hb/+ ((B), (F)), *E(z)*^{son3}/+ ((C), (G)), and *E(z)*^{son3} +/*hb* + ((D), (H)). *TM3* is a balancer chromosome that is wild-type for *kni*.

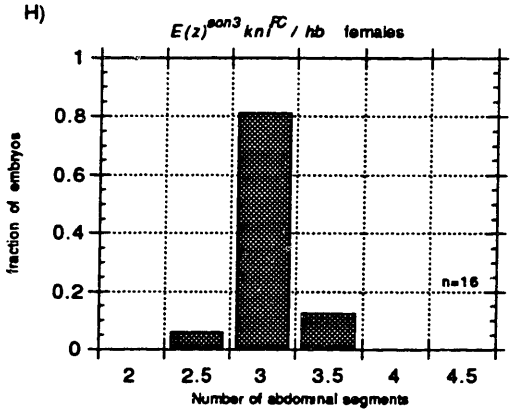
Figure 3.15 Paternal Genotype: kni^{IIV} / *TM3*

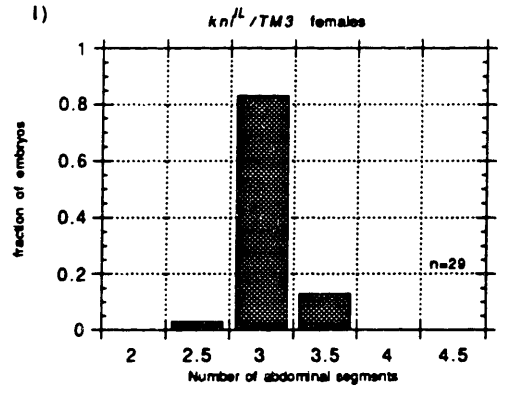


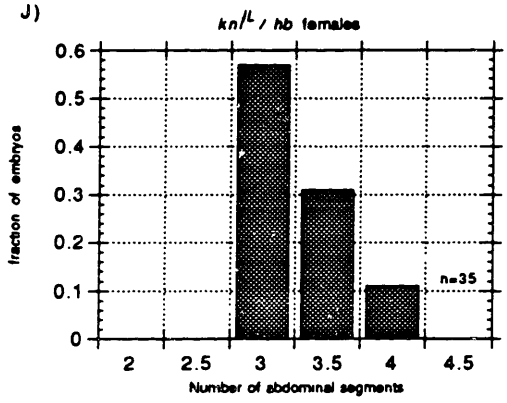

E)
kn^FC / TM3 females
fraction of embryos
1
0.8
0.6
0.4
0.2
0
n=16
2
2.5
3
3.5
4
4.5
Number of abdominal segments

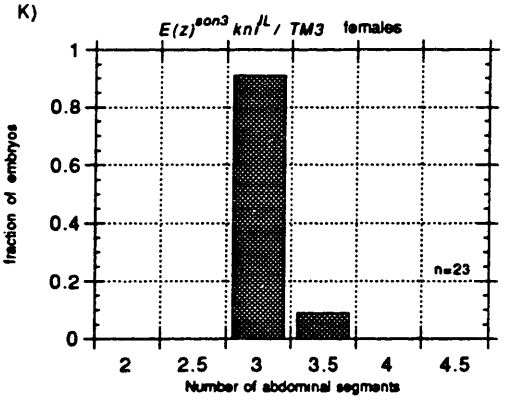

F)
kn^FC / hb females
fraction of embryos
1
0.8
0.6
0.4
0.2
0
n=19
2
2.5
3
3.5
4
4.5
Number of abdominal segments


G)
E(z)^son3 kn^FC / TM3 females
fraction of embryos
1
0.8
0.6
0.4
0.2
0
n=14
2
2.5
3
3.5
4
4.5
Number of abdominal segments


H)
E(z)^son3 kn^FC / hb females
fraction of embryos
1
0.8
0.6
0.4
0.2
0
n=16
2
2.5
3
3.5
4
4.5
Number of abdominal segments


I)
kn^L / TM3 females
fraction of embryos
1
0.8
0.6
0.4
0.2
0
n=29
2
2.5
3
3.5
4
4.5
Number of abdominal segments


J)
kn^L / hb females
fraction of embryos
0.6
0.5
0.4
0.3
0.2
0.1
0
n=35
2
2.5
3
3.5
4
4.5
Number of abdominal segments


K)
E(z)^son3 kn^L / TM3 females
fraction of embryos
1
0.8
0.6
0.4
0.2
0
n=23
2
2.5
3
3.5
4
4.5
Number of abdominal segments

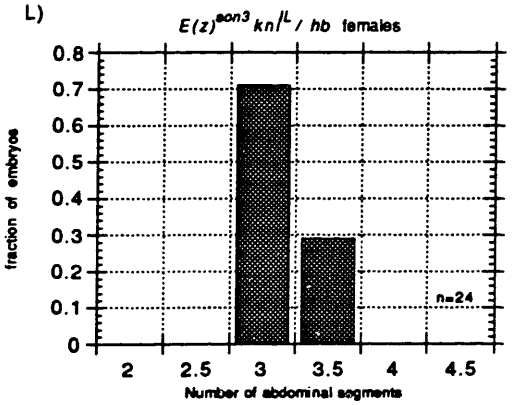
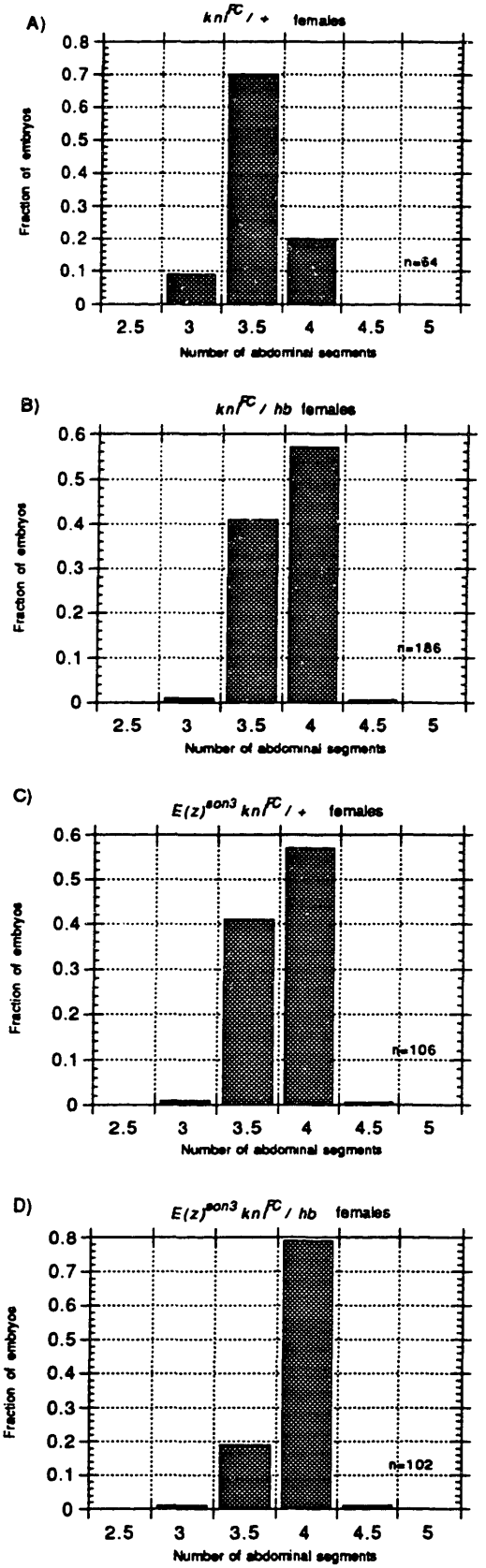
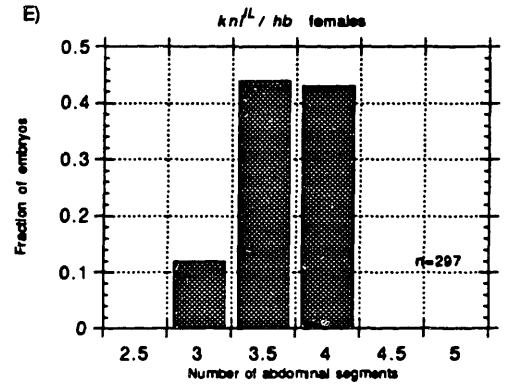
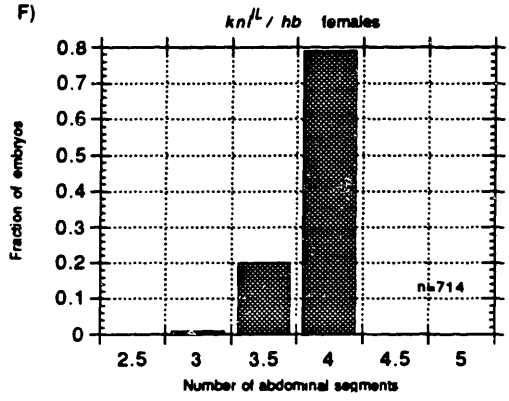
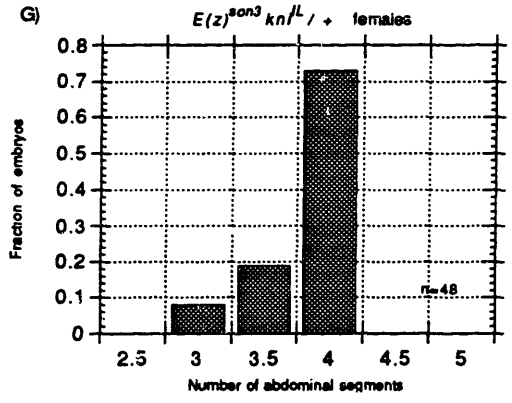

L)
E(z)^son3 kn^L / hb females
fraction of embryos
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
n=24
2
2.5
3
3.5
4
4.5
Number of abdominal segments

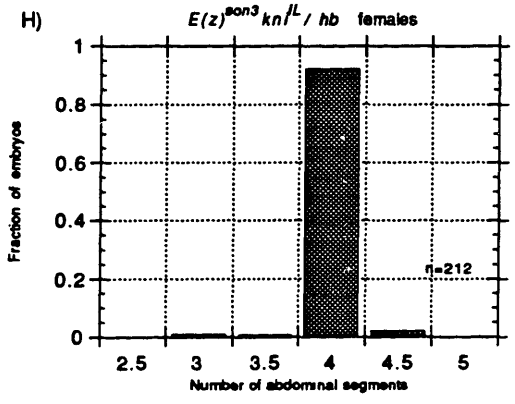
Figure 3.16 Paternal genotype: kni^{14B} / TM3




E)
kn^L / hb females
Fraction of embryos
0.5
0.4
0.3
0.2
0.1
0
n=297
2.5
3
3.5
4
4.5
5
Number of abdominal segments


F)
kn^L / hb females
Fraction of embryos
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
n=714
2.5
3
3.5
4
4.5
5
Number of abdominal segments


G)
E(z)^son3 kn^L / +
females
Fraction of embryos
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
n=48
2.5
3
3.5
4
4.5
5
Number of abdominal segments


H)
E(z)^son3 kn^L / hb
females
Fraction of embryos
1
0.8
0.6
0.4
0.2
0
n=212
2.5
3
3.5
4
4.5
5
Number of abdominal segments

In *bcd* embryos, mutations in *E(z)* do not exactly mimick lack of *hb* activity

During the studies on the role of *E(z)* mutations on the Hb^{mat} gradient I observed that the phenotypes conferred by *E(z)* mutations were not entirely equivalent to the lack of hb^{mat} function. In most embryos from *E(z) bcd* females the posterior abdomen was longer than the anterior (Figure 3.17A), while embryos that lack both Bcd and Hb^{mat} have short, symmetric double abdomens (Hülskamp et al. 1990). Similarly, most embryos from *E(z) bcd tsl* females form a polar abdomen with four or five segments (Figure 3.18A), while segments (Struhl et al. 1992). In order to better understand the effect of *E(z)* on these embryos, I further investigated possible cause(s) for this difference.
embryos from *tor bcd hb* germ line clones (mutations in *tor* and tsl, two genes required for the terminal pathway, should be roughly equivalent) form a polarized field of at most two

One likely cause is the fact that the *E(z)* mutant combination is not completely penetrant, and thus there is likely residual *E(z)* function in these embryos. Because of *E(z)* function is required for adult viability and oogenesis, the production of germ line clones heteroallelic for $E(z)^{son}$ mutations in a *bcd* mutant background would circumvent this problem.

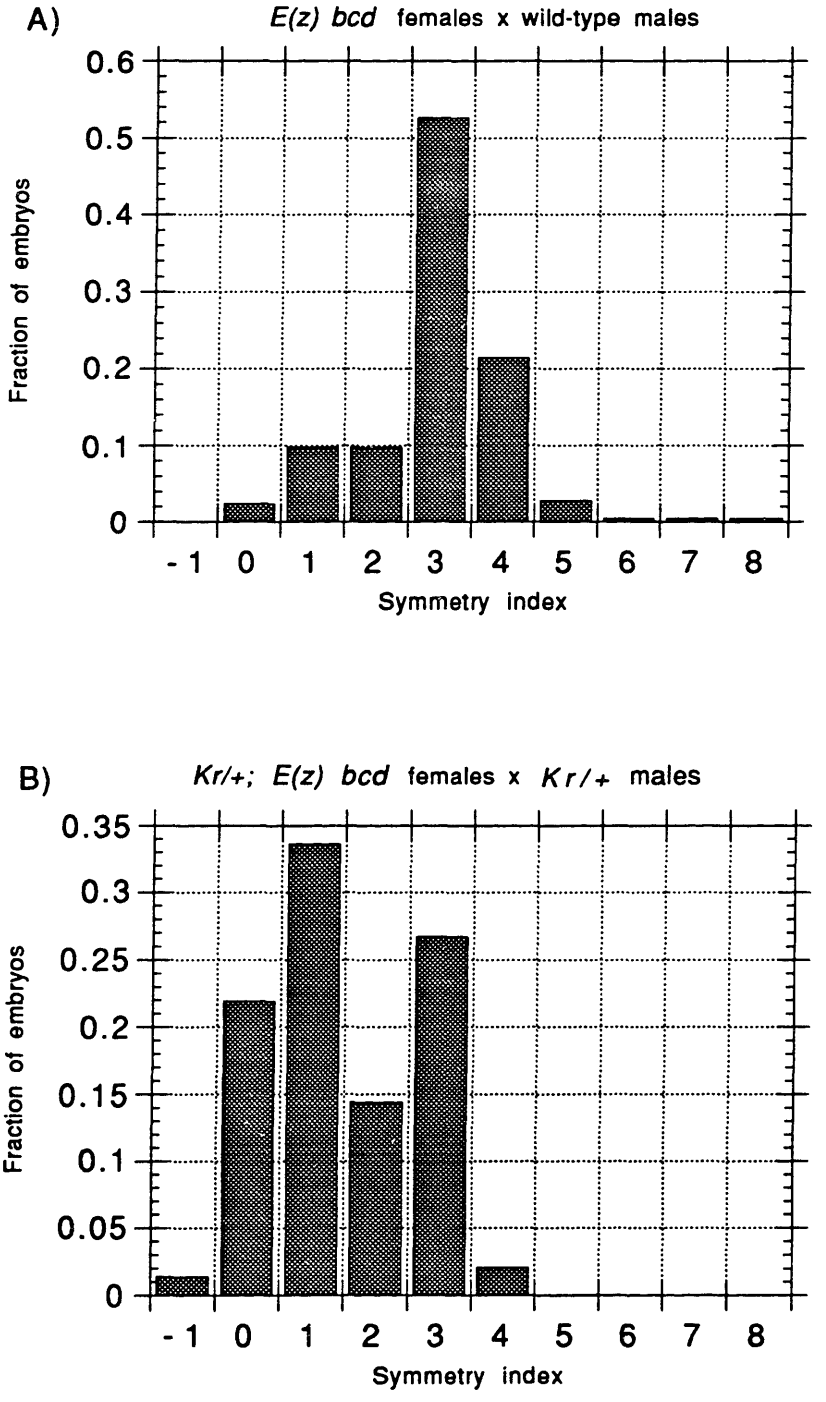
Another likely cause for the non-equivalence between *E(z)* and hb^{mat} mutations is that, while *E(z)* mutations cause Hb^{mat} to be effectively mutant for its repressive function on *kni* and *gt*, the Hb^{mat} protein itself is still present and in principle capable of functioning as an activator of *Kr*. The Kr protein is in turn a repressor of both *kni* and *gt* and thus would tend to prevent their expression in the anterior half of the embryo. To test whether the activation of *Kr* by Hb^{mat} is involved in these differences, embryos from *E(z) bcd* and *E(z)bcd tsl* females with reduced *Kr* levels were compared to similar embryos with the wild-type *Kr* dosage. In these experiments the "*Kr* mutant" collection consisted of one quarter homozygous *Kr* mutant embryos, one half *Kr* heterozygotes embryos and one quarter embryos with the wild-type *Kr* dosage.

Indeed, activation of *Kr* contributes to the differences between *E(z)* and *hb* mutations. A reduction in *Kr* dosage in *E(z) bcd* embryos leads to an increase in the degree of symmetry of these embryos (compare Figures 3.17A and B), so that a greater fraction of

---

Figure 3.17. A reduction in *Kr* dosage increases the symmetry of embryos from *E(z) bcd* females. A) $E(z)^{son2}$ *bcd*/$E(z)^{61}$ *bcd* females crossed to wild type males. B) Kr^{1}/+; $E(z)^{son2}$ *bcd*/$E(z)^{61}$ bcd females crossed to Kr^{1}/+ males. One-quarter and one-half of this progeny are expected to have no and half the normal *Kr* dosage, respectively. Symmetry index is defined as (# of abdominal segments in the posterior abdomen - # of abdominal segments in the anterior abdomen). Symmetrical embryos have a symmetry index of 0.

Figure 3.17


A)
E(z) bcd females x wild-type males
Fraction of embryos
0.6
0.5
0.4
0.3
0.2
0.1
0
-1 0 1 2 3 4 5 6 7 8
Symmetry index
B)
Kr/+; E(z) bcd females x Kr/+ males
Fraction of embryos
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-1 0 1 2 3 4 5 6 7 8
Symmetry index

embryos are similar to *bcd* hb^{mat} mutant embryos. Similarly, a reduction in *Kr* dosage causes a reduction in the number of abdominal segments produced by embryos from *E(z) bcd tsl* females (compare Figures 3.18A and B), so that they approach the morphology of *tor bcd* hb^{mat} mutant embryos.

Thus although mutations in *E(z)* alone are not equivalent to the complete absence of hb^{mat}, mutations in both *E(z)* and *Kr* approach the hb^{mat} mutant phenotype. These data suggests that mutations in *E(z)*, while inactivating the maintenance of the repressive function of the Hb^{mat} protein, do not affect the Hb^{mat} protein per se nor its activation of *Kr*.

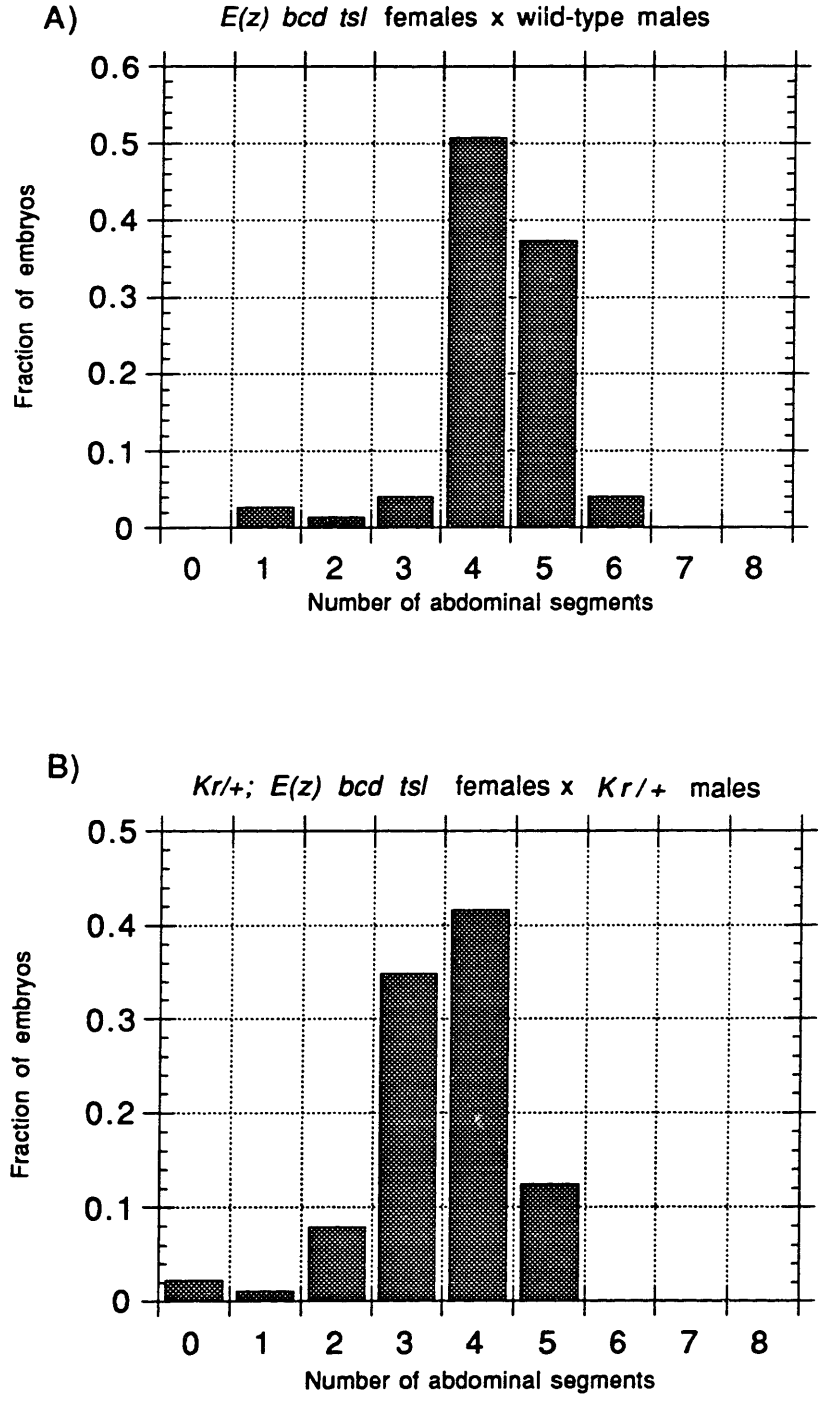
Interactions between *nanos*, *suppressors-of-nanos* and *zeste*

Some of the Pc-G genes involved in the regulation of gap genes, such as *E(z)* and the genes in the Su(z)2 complex, have also been reported to be involved in the repression of *white (w)* by the $Zeste^1$ (Z^1) product. The following two studies expand on this possible connection. First, I compare the son phenotype and the suppression of the zeste-white interaction confered by a number of *son*, *E(z)* and other Pc-G mutations. Second, I ask whether the wild-type Zeste product, which has been proposed to facilitate transcription of genes, is required for the expression of *kni* and *gt*.

Effect of mutations in *E(z)*, other *son* genes and other Pc-G genes on the zeste-white interaction. Mutations in some Pc-G gene, such as *E(z)*, *Sex comb on midleg* (*Scm*) and the Su(z)2 complex, are known to affect the repression of *white* by the $Zeste^1$ (Z^1) product (Jones and Gelbart 1990; Wu et al. 1989). The wild-type Zeste protein is a weak transcriptional activator of *white*. Flies homozygous for null *zeste* mutations (such as the allele z^a) have a mild reduction in eye pigmentation (Kaufman et al. 1973). The z^1 allele codes for an aberrant Zeste protein that produces a yellow eye phenotype because it represses *white* transcription when two or more copies of the 5' region of *white* are in close proximity (see Chen and Pirrotta (1993) and references therein). Null mutations in some Pc-G genes, such as *E(z)*, *Scm* and *Su(z)2*, suppress this phenotype, leading to a darker eye. Thus, these Pc-G gene products may be required for the transcriptional repression of *white* by Z^1.

Figure 3.18. A reduction in *Kr* dosage decreases the number of abdominal segments produced by embryos from *E(z) bcd tsl* females. A) $E(z)^{son2}$ *bcd tsl*/$E(z)^{61}$ *bcd tsl* females crossed to wild type males. B) Kr^1/+; $E(z)^{son2}$ *bcd tsl*/$E(z)^{61}$ *bcd tsl* females crossed to Kr^1/+ males. One-quarter and one-half of this progeny are expected to have no and half the normal *Kr* dosage, respectively.

Figure 3.18


A)
E(z) bcd tsl females x wild-type males
Fraction of embryos
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8
Number of abdominal segments
B)
Kr/+; E(z) bcd tsl females x Kr/+ males
Fraction of embryos
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8
Number of abdominal segments

Since the effect of mutations in these Pc-G genes can only be observed when *white* is repressed by Z^1, it is unclear what role, if any, these Pc-G genes normally have in the transcription of white. Nevertheless, the repression of white by Z^1 is a simple assay to determine whether genes are involved in the repressive process that Z^1 promotes. This process may involve multimeric structures containing Pc-G products similar to those that have been proposed to act on homeotic genes (see Chapter I).

The effect of the $E(z)^{son}$ mutations, as well as that of other *son* mutations, on the zeste-white interaction is shown in Table 3.20. In an otherwise wild-type background, z^1 w^{is} / Y males exhibit an orange eye color. As previously reported (Jones and Gelbart 1990), control $E(z)^{null}$ mutations have a mild suppression effect on the repression of w^{is} by Z^1, leading to a dark orange color. The $E(z)^{son}$ mutations have a stronger suppression effect of the zeste-white interaction, which shows that they are gain-of-function alleles which interfere with the wild-type *E(z)* function (this had also been inferred by their son phenotypes-see Chapter II). This effect is comparable to that of the previously known gain-of-function $E(z)^{60}$ allele. The *son* mutations son^{29}, son^{59} and son^{126} did not have a detectable effect on the repression of w^{is} by Z^1.

Table 3.21 compares the dominant son phenotype and suppression of the zeste-white interaction by $E(z)^{son}$ and other *son* mutations. In some cases *E(z)* mutations affect both processes with corresponding strength. For example, $E(z)^{null}$ alleles and deficiencies cause a mild son phenotype and a mild suppression of the z-w interaction, while $E(z)^{son}$ alleles have a strong effect in both processes.

Nevertheless, there is not an absolute correspondence of the effect of mutations on these two processes. The gain-of-function allele $E(z)^{60}$, for example, is a strong suppressor of the z-w interaction but does not appreciably suppress the *nos* phenotype. The opposite case, when mutations affect the *nos* phenotype but not the z-w interaction is also observed, as in this case with the *son* mutations son^{29} and son^{126}.

I have also tested a number of Pc-G mutations for their effect on the z-w interaction. Table 3.22 shows the effects of mutations in Pc-G genes on the z-w interaction. I found that in addition to mutations in *E(z)* and the Su(z)2 complex, a *Polycomb like* allele, Pcl^{D5}, also causes a dominant suppression of the z-w interaction. Mutations in the genes *extra sex combs* (*esc*), *pleiohomeotic* (*pho*) and *Super sex combs* (*Sxc*) did not appreciably modify the z-w effect.

Table 3.23 compares the son phenotype and suppression of z-w effect for a number of Pc-G genes. Again, there seems to be no absolute correlation between son effects and the suppression of the z-w interaction. Mutations in some genes, such as *Pcl*, act as strong suppressors of the repression of *w* by Z^1 but do not have a detectable dominant son

Table 3.20. Suppression of the zeste-white interaction by *E(z)* alleles and other *son* mutations.

Genotype	Eye color in z^1 w^{is} / Y males
+/+	orange
Df(3L)Ez6 / +	dark orange
$E(z)^{65}$ / +	dark orange
$E(z)^{63}$ / +	dark orange
$E(z)^{64}$ / +	dark orange
$E(z)^{1}$ / +	yellow
$E(z)^{60}$ / +	reddish (1)
$E(z)^{son1}$ / +	reddish (1)
$E(z)^{son2}$ / +	reddish (1)
$E(z)^{son3}$ / +	reddish (1)
$E(z)^{son2}$ / *Df(3L)Ez6*	red (wild-type)
son^{29} / +	orange
son^{59} / +	orange
son^{126} / +	orange

(1) Careful observations in two separate experiments, one of them with an independent observer, establish the order of strength of suppression of the z-w interaction as: (lighter eye color) $E(z)^{60} = E(z)^{son2} < E(z)^{son1} = E(z)^{son3}$ (darker eye color).

Table 3.21. Comparison of the suppression of *nos* and suppression of the zeste-white interaction phenotypes by *E(z)* and other *son* mutations.

E(z) genotype	Suppression of the *nos* phenotype (1)	Suppression of the z-w interaction (2)
+/+	0	0
Df(3L)Ez6 / +	+	+
$E(z)^{65}$ / +	0	+
$E(z)^{63}$ / +	+	+
$E(z)^{64}$ / +	+	+
$E(z)^{1}$ / +	0 (3)	-
$E(z)^{60}$ / +	0	++
$E(z)^{son1}$ / +	+++	++
$E(z)^{son2}$ / +	++	++
$E(z)^{son3}$ / +	+++	++
$E(z)^{son2}$ / *Df(3L)Ez6*	+++(+) (4)	+++
son^{29} / +	+++	0
son^{59} / +	0	0
son^{126} / +	++	0

(1) Legend of symbols for the suppression of the *nos* phenotype. symbol (% of embryos with ≥ 3 abdominal segments in a *hb nos/ nos* background): 0 (0-5%), + (6-15%), ++ (16-30%), +++ (51-100%).

(2) Legend of symbols for the suppression of the z-w interaction. symbol (eye color of z^{1} w^{is} / Y males): - (yellow 0 - enhancement), 0 (orange - no suppression), + (dark orange), ++ (reddish), +++ (red (wild-type) color).

(3) It is possible that $E(z)^{1}$ enhances the *hb nos / nos* phenotype in the same way that it enhances the z-w effect. This enhancement would be difficult to detect in this particular background because the background level of abdominal segment formation is already very low.

(4) Data for son phenotype is from the genotype $E(z)^{son2}$/ $E(z)^{65}$ in a *nos*, hb^{+} background (see Table 3.10, $E(z)^{65}$ is a null). A (+) has been added for comparison according to the expected enhancement of the son phenotype in a *hb* heterozygous background.

Table 3.22. Suppression of the zeste-white interaction by mutations in Pc-G genes.

Genotype	Eye color in z^1 w^{is} / Y males
+ / +	orange
E(z)^{null} / +	dark orange
E(z)^{son} / +	reddish
esc / +	orange
Pcl / +	reddish
pho / +	orange
Su(z)2-C / +	reddish
Sxc / +	orange

Table 3.23. Comparison of the suppression of *nos* and the suppression of the zeste-white interaction phenotypes caused by mutations in Pc-G genes.

Pc-G gene	Suppression of the nos phenotype (1)		Suppression of the z-w interaction (2)
	dominant	recessive (3)	
Asx	0	n. d.	n. d.
E(z)^{null}	0 or +	(+)++	+
E(z)^{son}	+++	(+)+++	++
esc	0	0	0
Pc	0	n. d.	n. d.
Pcl	0	n. d.	++
ph	0	n. d.	n. d.
pho	0 or +	(+)+++	0
Scm	0	n.d.	n.d.
Su(z)2-C	++	n.d.	++
Sxc	0	n.d.	0

(1) Legend as in Figure 2.21, (1).

(2) Legend as in Figure 2.22, (2).

(3) The degree of suppression for this column is based on values in a background with full hb^{mat} dose. To facilitate a comparison with the dominant effect column, a (+) has been added to account for the expected enhancement in a hb^{mat} heterozygous background.

phenotype. On the other hand, mutations in *pho* have dominant and recessive effects on the *nos* phenotype but do not act as suppressors of the z-w effect.

In conclusion, I have found that there are both allele and gene specific effects for both the suppression of the nos phenotype and the suppression of the repression of *w* by Z^1. The allele specificity suggests that *E(z)* is a complex protein which functions in the repression of gap genes and *white* by using different functional domains. Further evidence of the complexity of *E(z)* is presented in Chapter III, where we show that $E(z)^{son}$ mutations strongly interfere with gap gene repression but retain other *E(z)*-dependent functions.

The gene specificity of Pc-G genes in their effects on the *nos* phenotype and the z-w interaction suggests that different combinations of Pc-G genes are used for the repression of different genes. Nevertheless, in most cases the recessive effect of null mutations in both of these processes has not been determined, which leaves open the possibility that the same set of Pc-G products may be required for the repression of gap genes and *w*, but that these two processes are differentially sensitive to reductions in Pc-G gene function.

***zeste* is not absolutely required for the son effect.** The wild-type *zeste* gene has been shown to be required for pairing-dependent effects at a number of loci, including the Bithorax complex (reviewed in Wu and Goldberg, 1989). The Zeste protein has been proposed to facilitate transcriptional activation and has been shown to activate genes containing Zeste binding sites in vitro and in vivo (Pirrotta et al. 1987; Biggin et al. 1988; Laney and Biggin 1992). Nevertheless, the Zeste protein must be part of a redundant system of transcriptional activation since flies homozygous for null *zeste* alleles exhibit as their only phenotype a mild reduction in eye pigmentation (Kaufman et al. 1973; Goldberg et al. 1989).

Given the involvement of Zeste in in vivo transcription from *white* and *Ubx* promoters (Kaufman et al. 1973; Laney and Biggin 1992), and the effect of mutations in the *E(z)* and Su(z)2-C genes in both gap genes and white regulation, it seemed possible that the wild-type Zeste product may be involved in the activation of *kni* and *gt*. Clearly, Zeste is not required for the activation of these genes in an otherwise wild-type background, since females homozygous for *zeste* null alleles are viable and fertile. Nevertheless, perhaps the derepression of *kni* and *gt* by *E(z)* mutations in a *nos* background is dependent on Zeste activity, or the *E(z)* mutations rendered the *kni* and *gt* promoters more sensitive to the lack of Zeste activity. Thus I tested for the effect of *zeste* mutations on the son phenotype.

Table 3.24 shows that the son phenotype still occurs in the absence of maternal wild-type Zeste product (z^a/z^a). Thus, the Zeste product is not essential for the son phenotype.

A maternal genotype homozygous for the z^1 allele seems to produce a reduction in the son phenotype caused by $E(z)^{son3}$, both in the full dose and heterozygous hb^{mat} backgrounds. This effect appears to be dependent on two copies of the Z^1 product, since it is not observed in embryos from z^a/z^1 females. It is possible that the gain-of-function Z^1 product interferes with expression of *kni* and/or *gt* in a manner similar to its repression of *w* transcription, although in the latter case z^a/z^1 individuals also exhibit the a phenotype. Nevertheless, these results should be considered preliminary since these experiments have not been repeated, and it is also possible that the reduction of the son phenotype in z^1 homozygotes is caused by an additional mutation present on the z^1 chromosome.

In summary, these data show that *zeste* is not essential for the son phenotype. If *zeste* is involved in the activation of *kni* and *gt*, there is sufficient redundant activators in the early embryo for the normal transcription of these gap genes. Evidence for the presence of activators which can substitute for *zeste* function in the in vivo activation of the *Ubx* promoter has been recently obtained (Laney and Biggin 1992).

Table 3.24. *zeste* function is not required for the son phenotype.

Maternal *zeste* genotype	Maternal Genotype			
	E(z)son3 nos / nos		*E(z)son3 nos/ hb nos*	
	% rescue (1)	n	% rescue (1)	n
z^a / z^a	10	48	67	63
z^a / z^1	10	147	93	293
z^1 / z^1	1	268	38	215

(1) % of embryos with ≥ 3 abdominal segments.

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CHAPTER IV. A possible role of trithorax group genes in the regulation of gap genes

ABSTRACT

Previous experiments have shown that Polycomb group (Pc-G) genes are required very early in embryogenesis for the patterning of abdominal gap genes. Specifically, some Pc-G genes are required for the maintenance of the repression of *knirps* and *giant* initiated by the maternal Hunchback (Hb^{mat}) protein. Here, I ask whether trithorax group (trx-G) genes, which have been shown to antagonize Pc-G genes in the regulation of homeotic genes, also have a role in gap gene regulation. In genetic backgrounds that depend on repression by the Hb^{mat} protein trx-G gene mutations exert an effect on abdominal segmentation that is synergistic and not antagonistic to that of Pc-G gene mutations. A strong effect of *trx* mutations depends on an alleviation of the repression of *knirps* and *giant* by Hb^{mat} and Pc-G genes. These results are consistent with trx-G genes acting through a pathway that is independent of, but secondary to, that of repression by Hb^{mat} and Pc-G genes. Genetic data show that the effect of *trx* mutations is not mediated indirectly by Kr, another factor that can repress *knirps* and *giant* expression. Possible models for a role of trx-G genes in the regulation of abdominal gap genes are discussed.

INTRODUCTION

The initiation of gene expression patterns in the Drosophila embryo depends on inputs from transient regional factors, such as the products of maternal, gap and segmentation genes (for reviews, see Hülskamp and Tautz (1991); Hoch and Jäckle (1993); Kornberg and Tabata (1993) and Chapter I). The stabilization of those patterns depends on maintenance mechanisms such as cross-regulatory interactions, autoregulation and regulation by general factors such as the products of the trithorax (trx-) and Polycomb group (Pc-G) genes (for reviews, see Bienz (1992); and Chapter I).

A number of lines of evidence suggests that regulation by the trithorax group (trx-G) and Polycomb group (Pc-G) of genes occurs at the level of chromatin (for reviews, see Paro (1990); Kennison and Tamkun (1992); Kennison (1993); Chapter I). In particular, trx-G and Pc-G genes are thought to promote the formation of,

respectively, open and closed chromatin domains thereby stabilizing gene expression patterns.

Both of these groups of genes are believed to affect a number of targets, including the homeotic genes. However, mutations in trx-G genes cause homeotic phenotypes characteristic of loss of homeotic gene function, while mutations in Pc-G genes lead to phenotypes resulting from ectopic homeotic gene expression. In both cases, the effect has been shown to occur at the level of homeotic gene transcription (for trx-G gene, see Ingham (1985); Tamkun et al. (1992); Breen and Harte (1991); Breen and Harte (1993); for Pc-G genes, see Struhl and Akam (1985); Glicksman and Brower (1990); Jones and Gelbart (1990); McKeon and Brock (1991); Simon et al. (1992); Zhang and Bienz (1992)). Consistent with these opposite phenotypes, mutations in trx-G genes suppress the homeotic phenotypes caused by Pc-G gene mutations (Ingham 1985; Mazo et al. 1990; Breen and Harte 1991; Tamkun et al. 1992; Breen and Harte 1993).

We have previously shown that the Pc-G gene *Enhancer of zeste* (*E(z)*) and other Pc-G genes are involved in the repression of gap genes (Chapter III). In particular, mutations in *E(z)* affect the maintenance of the repression of the abdominal gap genes *knirps* (*kni*) and *giant* (*gt*) that is initiated by the maternal Hunchback (Hb^{mat}) protein gradient. This maintenance function has been shown to stabilize the anterior boundaries of expression of *kni* and *gt*.

Here, I ask whether the trx-G genes, like the Pc-G, may be similarly involved in the regulation of gap gene expression. This question is particularly interesting given the recent finding that the E(z) and Trx proteins have a common C-terminal domain, which has been proposed to interact with a common target (Jones and Gelbart 1993). Interestingly, the role of *E(z)* as a gap gene regulator is dispensable in the wild-type embryo (Chapter III). The role of *E(z)* in the maintenance of gap gene repression is normally obscured by the overlying network of redundant gap gene zygotic interactions, such as repression by Bicoid-activated genes. It is possible that similar redundant interactions might be obscuring a role for trx-G gene function in the regulation of gap genes.

In the hope of circumventing interactions that could substitute for a role of trx function in gap gene regulation, I have tested the effect of trx-G mutations in mutant backgrounds where the phenotype is dependent on the transient function of the Hb^{mat} morphogenetic gradient. These backgrounds are the same that previously allowed us to observe the effect of Pc-G genes on gap gene regulation.

The first mutant background lacks *nanos* (*nos*) function and leads to the ectopic translation of Hb^{mat} protein in the prospective abdominal region (Tautz 1988; Wang and Lehmann 1991). Hb^{mat} protein represses *kni* and *gt* transcription and leads to embryos that completely lack abdominal structures. This mutant background allows testing a possible role of *trx* mutations in the activation and/or repression of the abdominal gap genes.

The second background in which *trx* mutations were tested was a *bicoid* (*bcd*) mutant background. The Bcd protein activates transcription of a number of anteriorly expressed genes, including the zygotic *hunchback* (hb^{zyg}) gene (Schröder et al. 1988; Tautz 1988; Driever and Nüsslein-Volhard 1989; Driever et al. 1989; Struhl et al. 1989). Thus, in *bcd* mutant embryos, many possibly redundant interactions are not present. Moreover, in these embryos, the only source of anteroposterior polarity is the Hb^{mat} protein gradient (Hülskamp et al. 1990; Struhl et al. 1992). This mutant background allows testing for a possible role of trx-G gene mutations in patterning by the Hb^{mat} morphogen.

We find that trx-G mutations have an effect in both mutant backgrounds. To our surprise, and in contrast to the case of homeotic gene expression, the effect of *trx* and Pc-G gene mutations on abdominal gap gene expression is synergistic. The effect of trx-G mutations does not occur by affecting the levels of the abdominal gap gene repressor Kr. Thus trx-G mutations must be acting on the expression of gap genes either indirectly through an unknown pathway, or directly in an unprecedented manner.

MATERIALS AND METHODS

Genetic analysis and strains: All experiments were conducted at a constant temperature of 25°C. Embryos were allowed to develop cuticle structures (24 hrs at 25°C). In the *nos* mutant background, the number of abdominal segments was scored directly under a dissecting scope (the embryos being cleared by a film of mineral oil). In the *bcd* mutant background, the embryos were scored as cuticle preparations embedded in Hoyer's medium (Wieschaus and Nüsslein-Volhard 1986).

The background mutations used were nos^{L7}, which lacks the function required for abdomen formation (Lehmann and Nüsslein-Volhard 1991), bcd^{E1}, a strong allele (Struhl et al. 1989) and hb^{7M}, a protein null (Lehmann and Nüsslein-Volhard 1987b; Tautz 1988). The $E(z)^{son}$ mutations are gain-of-function dominant suppressor-of nos- (*son*) mutations. $E(z)^{65}$ deletes the *E(z)* locus (Jones and Gelbart, 1993) and $E(z)^{63}$ is a null (Jones and Gelbart, 1990). The *son* mutations son^{29}, son^{59}, son^{126} and

Pson^{82} are described in Chapter II and Appendix A. The trx-G mutations tested were (unless otherwise stated, see Lindsley and Zimm 1992 for references): *trithorax*, *Df(3R)red-P52* (=*Df(trx)* in the text), a deficiency that deletes the *trx* locus), *trx*^{1}(a temperature-sensitive loss-of-function allele) *trx*^{E2} (a strong suppressor of Pc); *brahma*, *brm*^{2} (loss-of-function, (Tamkun et al. 1992, J. Kennison, personal communication), *brm*^{20} (partial loss-of-function, an additional trx-G-like mutation is also present in this chromosome, J. Kennison, personnal communication); *kohtalo*: *kto*^{1}; *moira*: *mor*^{1} (loss-of-function); *osa*: *osa*^{2}. The *Krüppel* allele used, *Kr*^{1}, is a null.

Antibody staining: Embryos were fixed with paraformaldehyde and stained according to Gavis and Lehmann, 1993. Anti-Kr rabbit antibody, a gift from M. Levine, was used at a 1:2000 dilution. Anti-Hb rat antibody, a gift from G. Struhl, was used at a 1:5000 dilution.

A note on nomenclature

The "nos phenotype" refers to the lack of abdominal segmentation exhibited by embryos females mutant for the *nanos* (*nos*) gene.

Mutations isolated in screens for suppressors of the nos phenotype (i.e. those that allow the formation of abdominal segments in the absence of *nos* function) are called "*son* (suppressor-of-*nos*) mutations". The "son phenotype" refers to the partial or complete rescue of the nos phenotype by these *son* mutations, by mutations in some Pc-G genes, and by maternal heterozygosity for *hb*.

Mutations in *trx* enhance the tendency of son mutations to suppress the nos phenotype. This effect is referred to as the "*trx*-dependent enhancement of the son phenotype".

The effect of *nos*, *hb* and *son* mutations on the expression of *kni* and *gt* has been shown to depend solely on the maternal genotype (Lehmann and Nüsslein-Volhard 1991; Chapter III; see below). For this reason, I refer to embryos according to their maternal genotype, and not their zygotic genotype (e.g. embryos from *nos* or *E(z)* mutant females as *nos* or *E(z)* mutant embryos. A similar nomenclature is sometimes used for trx-G mutations. In this case an exclusively maternal effect seems likely, but has not been rigorously tested (see below).

RESULTS

Trithorax mutations enhance the son phenotype

In the case of homeotic genes, the effects produced by mutations in Pc-G genes are counteracted by mutations in trx-G genes. I tested whether a similar genetic interaction occurs in the case of gap gene regulation, i.e. whether mutations in trx-G genes suppress (revert) the son phenotype caused by *E(z)* mutations to a more "nanos-like" phenotype.

Initially, I tested for the dominant effect of a deficiency that includes the *trx* locus on the *E(z)*-dependent son phenotype. This experiment involved comparing the percent of embryos that formed abdominal segments in the progenies of + *Df(trx)* *nos/E(z)*$^{-}$ + *nos* and + + *nos/E(z)*$^{-}$ + *nos* females. Surprisingly, the *trx* deficiency enhanced the son phenotype associated with *E(z)* mutations (Table 4.1). This effect is most apparent with *E(z)*^{son} alleles, which are gain-of-function alleles that have a strong dominant son phenotype (Chapter III).

In a similar experiment, I also tested whether other mutations that had been isolated in screens for *son* mutations (see Chapter II) were enhanced by the *trx* deficiency. Table 4.1 shows that the *trx* deficiency enhanced the son phenotype conferred by *Pson*^{82}, but not that conferred by *son*^{29} or by mutations in the *son*^{59,126} complementation group. This suggests that *Pson*^{82} may have a role similar to that of *E(z)* in the repression of gap genes. (The implications of the different behavior in this assay of *son*^{29} are discussed in Appendix A.). The dominant son phenotype of the *son*^{59} and *son*^{126} mutations may be too weak to be observable, even if enhanced by *trx* mutations. Thus, a deficiency of *trx* enhanced the son phenotype conferred by some, but not all, dominant *son* mutations.

A mutation in *Polycomb*, which does not have on its own a dominant son phenotype, also did not exhibit any *trx*-dependent enhancement of the son phenotype (Table 4.1).

The dominant enhancement of the son phenotype was also induced by other *trx* alleles, such as *trx*^{E2} and the temperature-sensitive *trx*^{1} allele (Table 4.2). Interestingly, this enhancement was in turn reduced in embryos from females doubly heterozygous for *trx*^{E2} and the *brahma* allele *brm*^{2}. A similar effect has been observed in a *bcd* mutant background (data from experiments in *bcd* mutant embryos suggests that the effect of *trx* alleles on the son phenotype is more representative of the effect

Table 4.1. Enhancement of the son phenotype by *Df(trx)*.

Maternal genotype	Maternal genotype			
	nos / nos		*Df(trx) nos/+ nos*	
	% rescue (1)	n	% rescue (1)	n
+/+	0	625	0.3	666
$E(z)^{son1}/+$	2	246	13	373
$E(z)^{son2}/+$	0.5	440	9	691
$E(z)^{son3}/+$	1	299	47 (2)	234
$E(z)^{65}/+$	0	409	0.5	220
$E(z)^{63}/+$	0	403	1	519
$son^{29}/+$	37	596	32	820
$son^{59}/+$	0.2	847	0	637
$son^{126}/+$	0	605	0	254
$Pson^{82}/+$	0	807	12	378
$Pc_5/+$	0	732	0	84

(1) % of embryos with ≥ 3 abdominal segments.

(2) This value is taken from a previous experiment where $E(z)^{son3}$ + / + *Df(trx)* transheterozygotes were viable ($E(z)^{son3}$ + / + + control: 2%, n=166). For this reason it is not directly comparable to other values in this table. On this and subsequent tests $E(z)^{son3}$ + / + *Df(trx)* transheterozygotes were lethal. The lethality may be caused by the accumulation of a genetic modifier and is not with the maternal genotype.

Table 4.2. Effect of *trx* mutations on the suppression of *nos* by *hb* heterozygosity and *E(z)*^{son} alleles @

		nos^{L7}		hb^{7m} nos^{L7}		$E(z)^{son1}$ nos^{L7}		$E(z)^{son3}$ nos^{L7}	
		% ≥ 3 seg	n	% ≥ 3 seg	n	% ≥ 3 seg	n	% ≥ 3 seg	n
nos^{L7}		0	314	0	258	0	43	0.9	241
$Df(trx)nos^{L7}$	(1)	0	726	12	614	53	237	LETHAL (47)	(234)
	(2)	0	372	14	301	34	581	LETHAL (46)	(189)
trx^{1} nos^{L7}	(1)	0.1	673	0.1	1440	18	179	35	463
	(2)	0	632	0	273	6	226	18	402
trx^{E2} nos^{L7}		0	767	6	525	11	55	62	263
$brm^{2}trx^{E2}nos^{L7}$	(1)	0	538	0.5	436	N.D.		8	374
	(2)	0	797	0	618	8 - 10	261	4	166

@ Rows and columns indicate different sets of gametes. Their intersection in the table corresponds to results for the appropiate combined genotype . (1) and (2) refer to different recombinant lines.

caused by mutations in the trx-G as a whole rather than that caused by the *brm*^{2} mutation, see below).

Because in all tests mutant females are crossed to wild-type males, it seems likely that the effect of *trx* mutations is, as in the case of *E(z)* (Chapter III), solely due to maternal *trx* contribution. This would be consistent with the fact that the abdominal gap genes are part of the first tier of zygotic gene expression, which relies solely on regulation by maternal products. Nevertheless, proper tests addressing the possibility of zygotic trx-G gene contribution to the son phenotype have not been performed.

These data suggest that trx and Pc-G genes do not have antagonistic roles on the expression of the abdominal gap genes, as in the case of homeotic genes. Rather, the phenotypic effects suggest that the wild-type products of both trx and Pc-G genes may contribute, directly or indirectly, to the repression of *kni* and *gt* in *nos* mutant embryos.

The enhancement of the son phenotype by *trx* mutations depends on mutations that weaken the repression of abdominal gap genes

In order to better characterize the effect of *trx* mutations in the repression of gap genes, I asked whether *trx* mutations can cause a son phenotype in a *nos* (otherwise wild-type) background. *trx* mutations did not cause a son phenotype, even in combinations such as *trx*^{1}*/Df(trx)*, which were expected to have severely reduced *trx* function (Table 4.3). The lack of a recessive enhancement of the nos phenotype by *trx* mutations contrasts with their strong dominant enhancement in backgrounds that contain one copy of *son*, *E(z)*^{son} or *hb* mutations (Tables 4.1 and 4.2). Thus, *trx* mutations enhanced the son phenotype induced by other mutations that affect *hb* function, but could not on their own induce a son phenotype. This suggests that *trx* mutations act through a pathway that is independent of, but secondary to, that of repression of *kni* and *gt* by Hb^{mat}.

The enhancement of the son phenotype by *trx* mutations is independent of *Kr* function

The known role of trx-G genes as activators of homeotic genes is in apparent contradiction with their negative effect on abdomen formation, and presumably *kni* and *gt* expression. These two facts may be reconciled if trx-G genes are required for the expression of a second factor that in turn represses *kni* and *gt*. One such factor might be the product of the gene *Kr*. In nos mutant embryos, ectopic Hb^{mat} protein present

Table 4.3. *trx* mutations do not affect the nos phenotype in a wild-type *E(z)* background

Maternal genotype (*nos* background)	% rescue (1)	n
+ / +	0	476
trx^1 / +	0	821
trx^{E2} / +	0.2	870
Df (trx) / +	0	710
trx^1 / Df (trx)	0	639

(1) % of embryos with ≥3 abdominal segments.

in the prospective abdominal leads to the activation of *Kr* in this same domain (Gaul and Jäckle 1987; Gaul and Jäckle 1989; Struhl et al. 1992). Kr in turn can act as a repressor of *kni* and *gt* transcription (Hülskamp et al. 1989; Eldon and Pirrotta 1991). Thus, both Hb^{mat} and Kr likely contribute to the repression of *kni* and *gt* in *nos* mutant embryos.

To test whether the effect of *trx* mutations is mediated through Kr, I tested whether a reduction in the zygotic dose of *Kr* further enhanced the son phenotype. Females of the genotype + $trx^{(+or-)}$ *nos* / $E(z)^{son3}$ + *nos* were mated to *Kr* heterozygous males. Half of the embryos from this cross were expected to have one dose of *Kr*. The son phenotype was mildly enhanced in embryos that have a reduced *Kr* dosage when their mothers carry the wild-type *trx* or trx^{E2} alleles (Table 4.4). This effect was not observed in embryos from females that carry the trx^{1} allele. Thus, these experiments were consistent with a mild, although variable, enhancement of the son phenotype by reduced Kr levels.

In order to conclusively test whether *trx* mutations act through Kr we examined whether the *trx*-dependent enhancement of the son phenotype depends on *Kr* function. The effect of combinations of mutant $E(z)^{son3}$ and trx^{1} alleles on a *nos* mutant background was tested in embryos that were homozygous mutant for a *Kr* null allele (Table 4.5). The results of this experiment showed that even in the absence of *Kr* function, *trx* mutations caused an increase in the number of abdominal segments produced by *nos* mutant embryos. This shows that the effect of *trx* on the nos phenotype is likely through target(s) other than *Kr*.

In *nos* embryos, abolishing repression by Kr does not lead to the formation of abdominal structures (Kraut and Levine 1991a; Table 4.5). However, repression by ectopic Kr in *nos* embryos likely contributes to the nos phenotype. This is suggested by the fact that, while in *nos* embryos the posterior spiracles (Fizkörper) are never stretched, *Kr nos* double mutant embryos often exhibit stretched posterior spiracles (68% with one or more stretched spiracles). This mild contribution of Kr to the nos phenotype may explain the mild enhancement of the son phenotype by a reduced *Kr* dosage described above.

Effect of trithorax group mutations in *bcd* mutant embryos

In order to test whether other trx-G genes have a role in the anteroposterior patterning organized by the Hb^{mat} protein, I tested the effect of mutations in several trx-G genes, such as *brahma* (*brm*) *kohtalo* (*kto*), *moira* (*mor*) and *osa*, in *bcd* mutant

Table 4.4. A decrease in *Kr* dosage may enhance the effect of *trx* mutations on the son phenotype.

Maternal Genotype ($E(z)^{son3}$ *nos* / $E(z)^{+}$ *nos* background)	Paternal Genotype			
	+/+		Kr^{1} / +	
	% rescue (1)	n	% rescue (1)	n
+/+	0.2	462	1.1	285
trx^{1} / +	41	582	36	734
trx^{E2} / +	15	668	25	590

(1) % of embryos with ≥ 3 abdominal segments.

Table 4.5. *trx* mutations enhance the son phenotype in the absence of *Kr* function.

Maternal genotype	Maternal genotype: *nos* / *nos*			
	% with ≥ 1 segment formed (1)	n	% with ≥ 3 segments formed (1)	n
+ +/+ +	0	36	0.5	201
$E(z)^{son3}$ +/ + +	47	37	12	106
+ +/+ trx^{1}	17	42	0	85
$E(z)^{son3}$ +/ + trx^{1}	76	70	48	171

(1) Note that percentage values refer to different numbers of abdominal segments.

embryos. In a *bcd* mutant background, anterior zygotic genes, including hb^{zyg}, are not activated, and the only source of anteroposterior polarity is the Hb^{mat} protein gradient.

Embryos that are mutant for *bcd* have a duplicated telson at the anterior end of the embryo which has unstretched Filzkörper material (Figure 4.1A). Nevertheless, these embryos have a unique anteroposterior polarity that span their entire axis.

Females mutant for *bcd* which in addition carry mutations in trx-G genes, such as *kto*, *mor*, *osa* and *trx*, produce embryos that have at their anterior end a field of denticles that is often in opposite polarity to the normal axis (Table 4.6, Figure 4.1B) In addition, the anterior Filzkörper material is often stretched. This is likely an indication that some abdominal segmentation has occurred. These effects are also observed in *bcd* mutant embryos that have only one dose of Hb^{mat} product (Table 4.6, Figure 4.1C).

Table 4.6 shows the dominant effects of mutations in trx-G genes in *bcd* mutant background with either the normal Hb^{mat} complement, or half the Hb^{mat} dosage, using Filzkörper stretching as a quantitative measure. Although results are somewhat variable, some trends in this data can be observed. Mutations in *kto*, *mor*, *osa*, and *trx* appear to have a significant dominant effect on the *bcd* phenotype, while *brm* mutations do not appear to produce a significant effect. The same general trends, at a higher penetrance, are observed in a background with half a dose of Hb^{mat}.

The effect of combinations of two trx-G mutations was, in general, stronger than that caused by single mutations. An exception was that a mutation in brm^2 reverses the effect of a *trx* mutation. This effect is similar to that conferred by this *brm* allele in *nos* mutant embryos (see above). (The chromosome carrying the hypomorphic brm^{20} allele contains a second mutation that is a strong suppressor of *Polycomb*, J. Kennison, personal communication. Thus the effects of this chromosome may not represent solely the effect produced by the brm^{20} allele). This phenomenon could be explained if the balance between Trx and Brm gene products, and not their absolute amounts, is important for the proper function of a trx-G gene complex.

The effect of trx-G mutations in the *bcd* background was similar to that in *nos*

Figure 4.1. Cuticle preparations of *bcd*, *bcd trx-G* and *bcd, hb* heterozygous embryos. A) *bcd* embryo. Anterior Filzkörper (arrowhead) are not stretched and the anterior denticle bands have the normal polarity. B) *bcd trx-G* (actual genotype: *bcd* trx^1 *osa / bcd mor*) mutant embryo. C) *bcd, hb* heterozygous embryo. In B) and C), there is a small reversal of denticle polarity at the anterior of the embryo and the Filzkörper (arrowheads) are stretched. Arrow indicates abdominal polarity, from anterior to posterior. Dark-field optics. Anterior is left, dorsal is up.

Figure 4.1

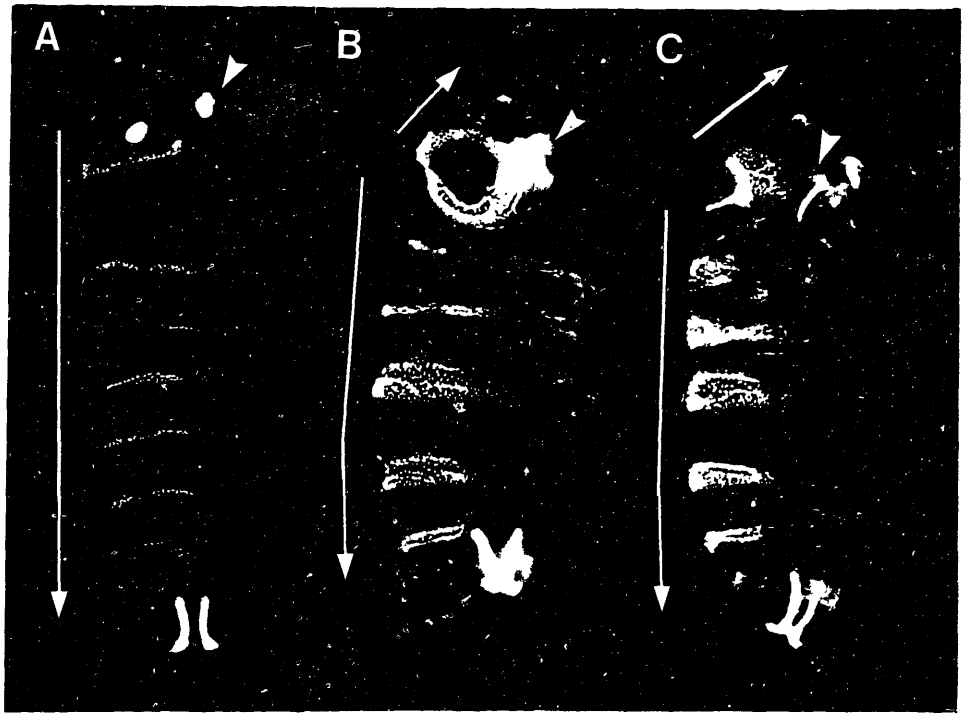

Table 4.6. Effect of trx-group mutations on the *bcd* phenotype.

background genotype	*bcd / bcd*		*bcd / hb bcd*	
	% ≥ 1 FK^{s} (1)	n	% ≥ 1 FK^{s} (1)	n
+ / +	0	117	31	29
brm^{2} / +	0.8	244	16	123
brm^{20} / +	0	70	43	73
kto^{1} / +	4.2	119	73	150
mor^{1} / +	19	26	55	74
osa^{2} / +	1.7	116	10	153
trx^{1} / +	6.6	152	85	89
Df(trx) / +	2.6	154	24	95
brm^{2} trx^{1} / +	2.2	326	57	184
brm^{2} Df(trx) / +	0.3	312	5.6	36
brm^{20} trx^{1} / + (1)	2.3	172	63	91
(2)	6.9	131	79	167
brm^{20} Df(trx) / + (1)	1.8	223	37	19
(2)	1.2	167	20	213
trx^{1} mor^{1} / +	5.1	198	64	126
trx^{1} osa^{2} / +	14	189	89	92

(1) % of embryos with more than one stretched anterior Filzkörper.

mutant embryos, i.e. enhancing the formation of an "abdomen", although this time at the anterior end of the embryo. The tendency to form abdominal structures is presumably caused by leaky expression of abdominal gap genes at anterior regions, where they normally are repressed.

The effect of *trx* mutations in a *bcd* background with the normal hb^{mat} complement was further enhanced when multiple trx-G gene mutations were combined (Figure 4.2) Again, in spite of the somewhat variable background, it was apparent that the additive effects of mutations in different trx-G genes, such as in the genetic combinations trx^1 + osa^2 /+ mor^1 + and trx^1+ osa^2 + /trx^1 mor^1 +, led to a high proportion of *bcd* embryos with rudimentary anterior abdomens.

Expression of Hb^{mat} and Kr in trx-G mutant embryos

Embryos from *bcd* mutant females which in addition carried the trx-G mutant combination trx^1 + osa^2 /+ mor^1 + exhibited phenotypic effects at a penetrance of about 50% (Figures 4.1 and 4.2). Embryos from these females will be referred to as *bcd*, trx-G mutant embryos.

Using the *bcd*, trx-G mutant embryos, I attempted to obtain more evidence on the possible mechanism of action of trx on gap gene regulation. Previously I had shown, in a *nos* background, that the effect of *trx* mutations is mediated through a target different than *Kr*. I attempted to directly confirm this result by testing whether Kr expression is affected by trx-G mutations. In addition, I tested whether the effect of trx on abdomen formation is mediated through the Hb^{mat} protein (for example, if trx-G gene function was required for activation of hbmat expression in the nurse cells).

Kr and Hb^{mat} protein were detected in *bcd*, trx-G mutant embryos using antibody staining (not shown). For both proteins, the staining patterns and intensities were similar to those observed in parallel stainings of *bcd* (wild type for *trx*) embryos.

Figure 4.2. Effect of multiple mutations in trx-G genes on a *bcd* mutant background. Rows and columns represent different gametes, boxes represent the genotype of this combination. The background genotype is homozygous for *bcd*. In each box, the value at the top left corner represents the percent of embryos that produce stretched Filzkörper, and the value in the bottom right corner is the sample size. Two different lines carrying the brm^{20} mutation were used, to increase the possibilities that at least one of them also carried the associated trx-G-like mutation (see Materials and Methods). Boxes and double boxes indicate genotypes that led to layings with 5-15% and > 16% embryos with stretched anterior Filzkörper, respectively. "L" indicates that the genetic combination is lethal.

Figure 4.2. Effect of trx-Group allelic combinations on the bcd phenotype

(*bcd / bcd* background)

	brm^{2}	*brm*^{20}	*kto*^{1}	*mor*^{1}	*osa*^{2}	*trx*^{1}	*Df trx*	*brm*^{2} *trx*^{1}	*brm*^{2} *Df trx*	*brm*^{20} *trx*^{1} *a*	*brm*^{20} *trx*^{1} *b*	*brm*^{20} *Dftrx*^{a}	*brm*^{20} *Dftrx*^{b}	*trx*^{1} *mor*^{1}	*trx*^{1} *osa*^{2}
brm^{2}		L	0 3	0 24	0 10	0 99	0 85	L	L	L	L	L	L	4.0 167	2.1 48
brm^{20}			L	1.7 60	SL	1.1 183	0 100	L	L	L	L	L	L	0.8 118	0.8 0.8
kto^{1}				1.9 104	0 105	7.9 126	0 138	0 9	0 1	0 9	7.7 13	0 5	n.d.	4.8 124	1.4 73
mor^{1}					0 9	13 48	0 22	10 97	1.9 53	3.0 33	19 31	1.4 71	n.d.	6.0 *84*	45 49
osa^{2}						1.1 90	L	2.5 40	L	3 43	4 25	L	L	3.8 105	0 2
trx^{1}						9.1 11	1.3 77	1 33	4.1 49	15 13	2.9 34	1.6 126	6.3 80	1.6 135	8.5 59
Df trx								1.0 104	L	2.8 72	0 27	L	L	3.0 53	2.8 36
brm^{2} *trx*^{1}									L	L	L	L	L	3.5 142	9.9 81
brm^{2} *Df trx*										L	L	L	L	n.d.	4.1 98
brm^{20} *trx*^{1} *a*											L	L	L	7.6 131	13 8
brm^{20} *trx*^{1} *b*												L	L	1.9 52	33 60
brm^{20} *Dftrx*^{a}													L	4.7 106	1.9 105
brm^{20} *Dftrx*^{b}														1.2 161	4.7 170
trx^{1} *mor*^{1}															25 102
trx^{1} *osa*^{2}															

In addition, the staining intensities for both proteins in these two mutant backgrounds were clearly higher than those in control embryos that carried half the dose of Hb^{mat} protein (i.e. from *bcd hb/bcd* + females).

Unfortunately, in this large scale experiment, the penetrance of the phenotypic effect observed in *bcd*, trx-G mutant embryos was significantly lower than that observed in *bcd hb/bcd* + embryos (9% compared to 37%, respectively). This leaves open the possibility that trx-G mutations affect the levels of Kr and/or Hb^{mat} protein, but the penetrance of this effect is too low to be observed.

DISCUSSION

Here, I report the effects of trx-G mutations in *nos* and *bcd* mutant backgrounds. In *nos* mutant embryos, *trx* mutations enhance the suppressor-of-*nos* phenotype produced by mutations that alleviate repression of *kni* and *gt* by Hb^{mat}. In *bcd* mutant embryos, *trx* mutations promote the formation of small mirror-image abdomens in anterior regions. Both of these phenotypes can be explained if mutations in trx-G genes increase the tendency of abdominal gap genes to be expressed in spite of high Hb^{mat} levels.

Possible models for the effects of *trx* mutations on abdominal gap genes

trx-G and Pc-G genes are genetic antagonists of each other. At the molecular level, they are thought to be involved in the activation and repression of homeotic genes (Figure 4.3A), perhaps by promoting stable chromatin structures (for reviews, see Paro (1990); Kennison and Tamkun (1992)). Previously, I had shown that Pc-G mutations repress the abdominal gap genes in a manner similar to their effect on homeotic genes (Chapter III). On the other hand, the synergistic enhancement of the son phenotype by *trx* and Pc-G mutations suggests that trx-G genes have an inhibitory, and not a positive, role on gap gene expression. Thus our data argue against a model for gap gene regulation similar to that in the regulation of homeotics (Figure 4.3B).

An alternative model that accommodates the phenotypic effect of trx-G mutations and the fact that trx-G genes are thought to be involved in gene activation is presented in Figure 4.3C. In this model, trx-G genes are required for the expression of a second factor (X), which in turn is a repressor of *kni* and *gt*. An obvious factor with the properties of X could be the Kr protein. However, this study shows that the effect

of *trx* mutations can be observed in the absence of *Kr* function. Thus, *trx* must act in the pathway of abdomen regulation through a target other than Kr.

Another candidate for a factor with the properties of X in model C could be the Tll protein. In *tll* mutant embryos, *kni* expression expands posteriorly, showing that Tll is normally a repressor of *kni* (Pankratz et al. 1990; Pankratz et al. 1992). In *nos* and *bcd* mutant embryos, Tll could be contributing significantly to the repression of abdominal gap genes in posterior and anterior regions, respectively. This repression would be alleviated in *trx* mutant background if *tll* expression depends on trx-G activity. This model is particularly interesting, since the identity of the terminal genes activator is unknown. The possibility that the effect of *trx* mutations on the son phenotype is mediated through Tll is currently being tested.

In another model, trx-G genes are required for hb^{mat} expression (Figure 4.3D). No apparent differences in the levels of Hb^{mat} protein were observed trx-G *bcd* mutant embryos when compared to *bcd* mutant embryos. Nevertheless, because in this experiment the penetrance of the rudimentary anterior abdomen phenotype in trx-G *bcd* embryos was significantly lower than that in control *bcd* embryos with half the dose of Hb^{mat}, it is not possible to conclude with certainty that mutations in trx-G genes do not affect Hb^{mat} levels. Future experiments should try to achieve a more penetrant background, perhaps by using genetic backgrounds homozygous mutant for strong trx-G mutations. Because trx-G genes are required for adult viability, this will require the creation of germ line clone chimeras.

Additional models can be proposed in which the integrity of trx-G gene complexes is required for the stability of Pc-G complexes, or of a common target required for the function of trx- and Pc-G genes in the early embryo (Figure 4.3E). Indeed, genetic and molecular studies in Drosophila and homologous systems in yeast provide some precedents for these possibilities (Franke et al. 1992; Peterson and Herskowitz 1992; Jones and Gelbart 1993).

Another, although unprecedented, possibility is that some trx-G gene products can act as direct repressors of gap genes (Figure 4.3F).

Synergistic interactions between *trx* and *son* mutations

In this report, I show that the that enhancement of the son phenotype by *trx* mutations depends in turn on mutations that weaken the repression of *kni* and *gt* in *nos* mutants. In a *nos* (but otherwise wild-type) background, *trx* mutations, even when homozygosed, do not induce a strong son phenotype. On the other hand, a strong

effect of even one mutant copy of trx can be observed when additional genetic mutations (*son*, Pc-G genes or hb^{mat}) weaken the repression of *kni* and *gt* in the prospective abdominal region. The genetic behavior of *trx* is different from that of hb^{mat} or Pc-G gene mutations, which when homozygosed cause a strong suppression of the nos phenotype (Hülskamp et al. 1989; Irish et al. 1989a; Struhl 1989; Chapter III).

In a *bcd* mutant background, one can observe mild effects caused by *trx* mutations in the absence of additional son-type mutations. Given the results in a nos background, however, it seems likely that this effect is small compared to that in synergism with son mutations.

The fact that the effect of *trx* mutations is greatly enhanced when repression by Hb^{mat} is compromised has implications for models of *trx* function. In particular, this phenomenon seems most consistent with a model in which trx acts through a pathway which is independent of, but secondary to, the input from Hb^{mat}/Pc-G genes (as in Figure 4.3C).

A redundant role for trx-G genes in embryogenesis

In the absence of both maternal and zygotic Trx product, embryonic development can proceed normally to a large extent, with embryos exhibiting only minor homeotic and segmentation defects (Ingham 1983). Nevertheless, early embryos

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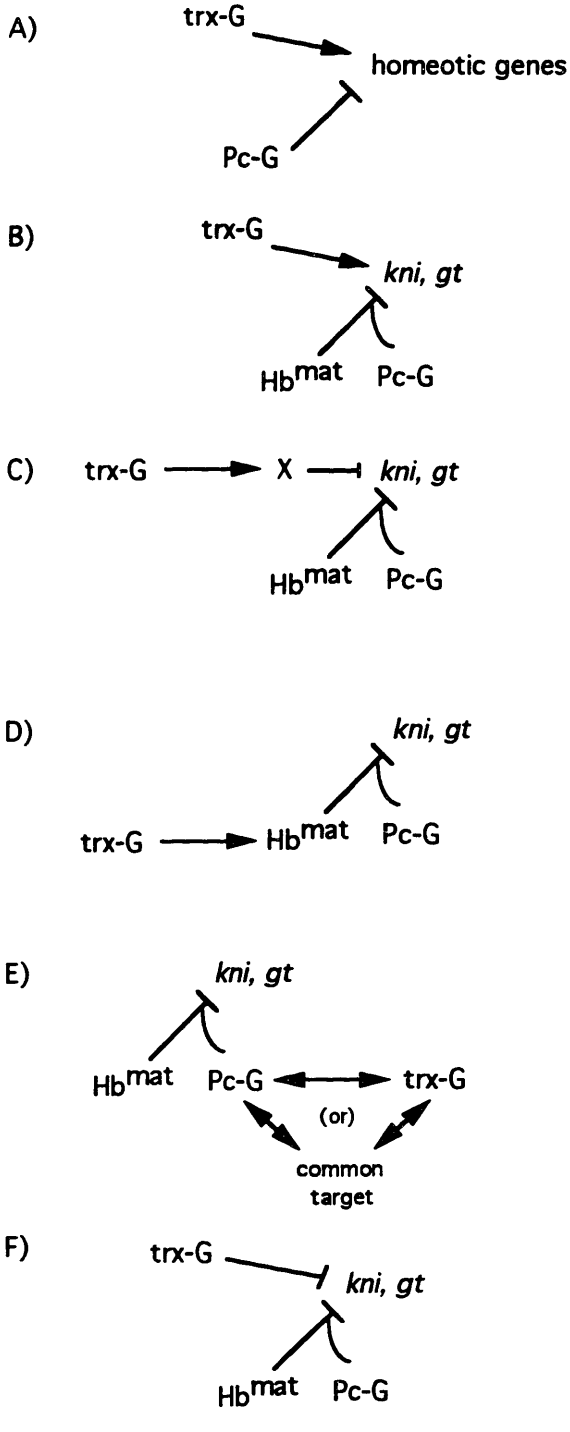
Figure 4.3. Possible models for a role for trx-G and Pc-G genes in the regulation of the abdominal gap genes *kni* and *gt*. A) trx-G and Pc-G products are involved in the maintenance of activated and repressed states, respectively, of homeotic genes. The initiators of homeotic gene expression (not shown) are regions specific transcription factors such as maternal, gap and pair rule gene products. B-F) Hypothetically models for the regulation of gap genes by trx- and Pc-G genes. Pc-G genes are involved in the maintenance of the repression initiated by the Hb^{mat} protein (Chapter III). The activators, whose identity is still unknown, may be general "default" factors. B) trx-G genes are required for the activation of *kni* and *gt*. The enhancement of the son phenotype by *trx* mutations suggests that this model is incorrect. C) trx-G genes are required to activate a repressor (X) of *kni* and *gt*. Candidates for this repressor could be Kr and Tll, although expression and genetic studies show that Kr does not mediate the effect of *trx*. D) trx-G genes are required for the expression of the Hb^{mat} protein. Antibody staining studies show that this model is incorrect. E) In genetic backgrounds with mutations in trx-G genes, trx-G gene products form complexes that are unstable. The unstability of these complexes leads in turn to the degradation of Pc-G gene product complexes, or, alternatively, a common target required for the function of both of these groups of genes. F) In the early embryo, trx-G genes have an unprecedented, direct repressive role on gap gene regulation.

Figure 4.3. **Models for trx-G gene function**



Comments

established model

does not fit genetic data

possible,
X not = Kr
X= Tll, other ?

Hb^{mat} unaffected by *trx* mutations, but could be a detection problem

Possible

Unprecedented role for trx-G genes

contain abundant trx RNA (Mozer and Dawid 1989), and *trx* is known to influence the expression of homeotic genes in the embryo (Ingham 1983; Breen and Harte 1991; Breen and Harte 1993). It is possible that in the embryo trx-G genes substitute for one another. Alternatively, as it is the case of *E(z)* in abdominal gap gene regulation, additional activators present in the embryo provide redundant functions that obviate the requirement for trx-G function.

I have attempted to observe a role for *trx* and other trx-G genes in the regulation of gap genes by testing mutations of these genes in genetic backgrounds whose phenotypes rely upon the function of the Hb^{mat} protein. In particular, a possibly redundant role of *trx* in the regulation of abdominal gap genes by the Hb^{mat} gradient can be tested in *bcd* mutant embryos, since in these embryos other sources of anteroposterior polarity have been removed. My results are suggestive of such a role for trx-G gene products. Nevertheless, additional experiments are required to clarify the exact nature of this role.

Our data argue that, in contrast to the case of homeotic regulation, trx-G genes are not required to activate abdominal gap genes. The difference between homeotic and gap gene activation may depend on the type of activators involved in these two processes. While for homeotic genes the activators are specific transcription factors (Duncan 1986; Ingham and Martinez-Arias 1986; Jack and McGinnis 1990; Reinitz and Levine 1990; Irish et al. 1989b; Harding and Levine 1988; Qian et al. 1991; Müller and Bienz 1992; Busturia and Bienz 1993), in the case of the abdominal gap genes, the activators are not known, and may be more general factors (Pankratz et al. 1992). It is possible that this second class of factors act independently of trx-G genes function.

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CONCLUSIONS AND FUTURE DIRECTIONS

A screen for suppressors of *nanos* identifies a number of functionally related genes involved in the regulation of gap gene patterning

Embryos that lack the *nanos* (*nos*) product do not form abdominal structures because the abdominal gap genes, *knirps* (*kni*) and *giant* (*gt*) are not expressed. The requirement of Nos to activate abdominal gap genes is indirect, where Nos is required to repress in posterior regions of the embryo translation of the maternal Hunchback (Hb^{mat}) protein, which would otherwise repress transcription of *kni* and *gt*.

This indirect pathway suggested that mutations in genes required for the production or activity of the Hb^{mat} protein would suppress the *nos* phenotype. Therefore, we carried out several screens for dominant suppressor-of-*nos* (son) mutations. Mutations in at least 5 complementation groups were isolated. One of these complementation groups corresponds to the gene *Enhancer of zeste* (*E(z)*).

Genetic analysis shows that the $E(z)^{son}$ alleles isolated in our screen are gain-of-function (antimorphic) alleles. Interestingly, they specifically affect the function required for the regulation of abdominal gap genes, while other functions, including regulation of homeotic genes, are comparatively less affected. The molecular characterization of the lesions present in these mutations may identify a domain that is specifically involved in gap gene regulation. The sequencing of the $E(z)^{son}$ alleles is currently under way in the laboratory of Rick Jones (SMU, Texas), who cloned the *E(z)* gene.

Unlike the $E(z)^{son}$ alleles, loss-of-function mutations of *E(z)* do not behave as strong dominant suppressors of *nos*. We were able to show, using a temperature sensitive *E(z)* allele, that loss-of-function mutations in *E(z)* act as recessive suppressors of *nos*. This proved that the wild-type *E(z)* product is required for the *nos* phenotype.

Since *E(z)* is a member of the Polycomb group (Pc-G) of genes, it seems possible that other Pc-G genes are also involved in abdominal development. Unfortunately, the fact that loss-of-function mutations in genes that are involved in the regulation of gap genes may not show a dominant son phenotype makes it difficult to prove whether other Pc-G genes also regulate gap gene expression. This is because most Pc-G genes are required zygotically, and thus testing their recessive effect involves the creation of homozygous mutant germ line clones in a wild-type soma. Nevertheless, determining which Pc-G genes are involved in abdominal gap gene regulation will require the systematic creation of such germ line chimeras. Similar tests could also be applied to mutations in genes from families related to the Pc-G family, such as the modifiers of position effect variegation.

Our analysis suggests that other Pc-G genes are also involved in the regulation of abdominal patterning. A number of other mutations in Pc-G genes, such as in *pleiohomeotic* (*pho*) and the Su(z)-2 complex showed dominant suppression of *nos*. In the case of *pho*, homozygous adults for a weak mutation are viable and show a strong son phenotype. The case of the Su(z)2 complex is interesting, since individual loss-of-function mutations in genes of the complex exhibit weak or no dominant son phenotype, while the simultaneous reduction of several products using deficiencies encompassing the region produces a stronger effect. This suggests that combining several Pc-G mutations may lead to an observable dominant effect that would implicate the tested genes in abdominal gap gene regulation.

On the other hand, we have been able to show that embryos that completely lack *extra sex combs* (*esc*) product do not show a son phenotype. Thus, at least one Pc-G gene is not involved in the regulation of abdominal gap genes.

Mutations in many of the other genes isolated as suppressors of *nos* exhibit genetic interactions that suggest that these genes form part of a functionally related family of genes. For example, transheterozygous combinations of the son^{59} or son^{126} mutations and an $E(z)^{son}$ allele lead to wing and bristle defects, and similarly, transheterozygous combinations of $E(z)^{son}$ mutations and the son^{29} mutation produce a strong female sterile phenotype. A screen for revertants of this female sterile interaction has led to the isolation of mutations in another interacting gene, *Sufsi-1*.

It is possible that the functional family defined by the *son* mutations may overlap the Pc-G family in more than the common *E(z)* gene. Indeed, phenotypes exhibited by certain *son* mutations are reminiscent of phenotypes produced by Pc-G mutations. For example, females with mutations in $Pson^{49}$, son^{29}, and *Sufsi-1* produce embryos that exhibit head involution defects and, in the case of *Sufsi-1*, pair-rule like phenotypes, both of which have been associated with Pc-G gene mutations. It would be interesting to test whether double mutant combinations of these mutations among themselves, or in combination with Pc-G gene mutations, lead to an enhanced homeotic phenotype.

The role of Pc-G genes in the regulation of abdominal gap genes

We have studied the basis for the son phenotype conferred by Pc-g gene mutations in genetic backgrounds mutant for *E(z)*. Mutations in this gene lead to the derepression of *kni* and *gt* in *nos* mutant embryos in spite of uniformly high levels of Hb^{mat} protein.

Thus, *E(z)* is required to act in conjunction with the Hb^{mat} protein to repress transcription of the abdominal gap genes *kni* and *gt*.

Because the Hb protein gradient determines by repression the anterior boundaries of expression of *kni* and *gt*, we hypothesized that *E(z)* might be also required for the determination of these boundaries. Nevertheless, the gap gene expression patterns in *E(z)* mutant embryos that are otherwise wild type are largely normal. This lack of phenotype is perhaps not surprising, since previous studies showed that proper development can occur in the absence of Hb^{mat} protein. Clearly other redundant zygotic products, including the zygotic Hunchback (Hb^{zyg}) protein gradient, can substitute for the function of the Hb^{mat} and E(z) proteins in the early embryo.

In order to better test for an role of *E(z)* function in the determination of anteroposterior patterning by the Hb^{mat} protein gradient, we tested the effect of *E(z)* mutations in embryos that are mutant for the gene *bicoid* (*bcd*). In *bcd* mutant embryos many anteriorly expressed zygotic genes, including hb^{zyg}, are not expressed, and thus the only source of anteroposterior polarity in the embryo is the Hb^{mat} protein gradient. We find that, in *bcd* embryos, *E(z)* is required for the proper determination of anterior boundaries of *kni* and *gt*.

The effect of *E(z)* mutations in both *nos* and *bcd* mutant backgrounds is observable only at late stages. This timing roughly coincides with the disappearance of the Hb^{mat} protein. Thus we propose that *E(z)* is required for the maintenance of the repression of *kni* and *gt* that is initiated by the Hb^{mat} protein. This mechanism of gap gene regulation by *E(z)* agrees well with the previously described roles of *E(z)* in the maintenance of repression of homeotic genes and segment polarity genes.

The fact that Pc-G genes are thought to regulate expression through chromatin structure has led us to speculate that abdominal gap gene regulation involves regulation at the chromatin level. This model predicts that the early Hb^{mat} gradient promotes, together with Pc-G gene products, spatial differences in chromatin accessibility at the *kni* and *gt* promoters. Thus, in the anterior of the embryo, where Hb^{mat} levels are high, the promoters of *kni* and *gt* would acquire through Pc-G products a more condensed form that is less accessible to transcription factors. In posterior regions, on the other hand, these promoters would remain in open, accessible conformations. At later stages, this "chromatin infrastructure" would be further refined by the network of appearing gap gene products.

Clearly, this model is likely an oversimplification, since both *kni* and *gt* have anterior domains of expression and therefore in those regions they must be accessible to activation by transcription factors. Perhaps the presence of the Bcd protein in these regions

counteracts the repressive role of Hb^{mat} and Pc-G products. The fact that the Bcd protein has been shown to cooperate in yeast cells with yeast trithorax group-like products suggests that Bcd may be interacting with trithorax-group genes in the Drosophila embryo to establish open chromatin domains.

We have found that a 1.8 Kb region of the *kni* promoter is sufficient to confer regulation by Hb^{mat} and *E(z)*. Thus this region may contain Pc-G responsive elements which may interact with Pc-G genes to promote a closed chromatin structure. This region is, to my knowledge, the smallest fragment of DNA which is sensitive to Pc-G genes. The simplicity of the *kni* promoter region, in comparison to those of homeotic genes, may be an advantage in future molecular studies involving Pc-G regulation.

Using this *kni* promoter region, I am currently attempting to prove the model that Hb^{mat} and Pc-G products confer chromatin accessibility differences along the anteroposterior axis of the embryo. These experiments rely on transcription from a bacteriophage T7 promoter inserted within the *E(z)*-responsive *kni* promoter fragment as an in vivo measure of chromatin accessibility (an idea borrowed from Kim McCall and Welcome Bender in one of our joint group meetings).

I am also using this *E(z)*-responsive *kni* promoter region in a different experiment. The Posterior sex combs protein, which genetic experiments predict is involved in the repression of *kni* (*Psc* is part of the Su(z)2 complex), binds to a band in salivary gland chromosomes that corresponds to the location of the endogenous *kni* locus. Thus, it is possible that Psc protein could be found associated with *kni* promoter regions inserted at other locations in the genome. This association would provide evidence for a physical association between Pc-G genes and *kni* regulatory regions.

The Nos/Hb^{mat}/Pc-G pathway: a selected patterning system?

It has been proposed that redundant systems of positional information may be favored through evolutionary time, since independent overlapping functions would make the patterning process more resilient to internal or external fluctuations (Tautz, 1992). The redundant Nos/Hb^{mat}/Pc-G patterning system could provide such redundancy at the level of chromatin imprinting. Here, I would like to propose that one reason for the presence of the "dispensable" Nos/Hb^{mat}/Pc-G patterning system in the Drosophila embryo, is the natural selection for enhanced reliability in patterning.

I have attempted to determine whether the nos/Hb^{mat}/Pc-G patterning system contributes to the reliability of gap gene patterning by carefully examining gap gene expression patterns in embryos that are mutant for *E(z)* but are otherwise wild type. These

experiments have not shown a major contribution on the determination of gap gene boundaries by this patterning system. It is likely, however, that the challenges of natural selection are more discriminatory than laboratory conditions.

Do trithorax group genes have a role in the regulation of gap genes?

trithorax group (trx-G) genes are thought to be required for the establishment of active or open chromatin domains. Because trithorax group genes antagonize Pc-G genes in the regulation of homeotic genes, and because Pc-G genes are required for the repression of gap genes by the Hb^{mat} protein, I have tested the effect of trx-G mutations in two backgrounds whose phenotypes depend on Hb^{mat} function. These were the same genetic backgrounds, mutant for *nos* or *bcd* functions, that allowed us to observe an effect of Pc-G mutations on gap gene regulation.

Mutations in trx-group genes do exhibit a phenotype in these mutant backgrounds. As inferred from their phenotypic effects, mutations in these genes seem to enhance the expression of abdominal gap genes. Thus, trx-G products appear to have an inhibitory role on gap gene expression. This effect is unexpected considering the role of trx-G genes in the activation of homeotic gene expression. Currently, the exact cause of this effect is unknown, although indirect effects mediated by the gap gene *Kr* have been ruled out. Nevertheless, these results support a role for trx-G function in the regulation of very early patterning genes. Additional experiments to understand the way in which mutations in trx-G genes affect abdominal development are under way.

APPENDIXES TO CHAPTERS

APPENDIX A: Genetic analysis of *son^{29}* and the isolation of a second site modifier locus, *Sufsi-1*

ABSTRACT

The suppressor-of-*nos* mutation *son^{29}* has genetic properties that are slightly different than those exhibited by the *E(z)* mutations. It has a maternal effect son phenotype that is less dependent on maternal hunchback and trithorax dosage. This suggests that it may act at a different step of the pathway of abdominal regulation. *son^{29}* mutations interact with gain-of-function *E(z)* suppressor-of-*nos* alleles to produce a strong female sterile interaction. I have isolated mutations that suppress this sterile interaction. Three mutations are allelic and define the *Suppressor of the female sterile interaction-1* (*Sufsi-1*) gene. A viable allele of *Sufsi-1* produces maternal effect phenotypes that are similar to those caused by the female sterile interaction itself. Thus, *son^{29}* and *Sufsi-1* are interacting genes that may regulate a number of target genes, including the gap genes *knirps* and *giant.*

INTRODUCTION

son^{29} is the strongest dominant suppressor-of-*nos* (*son*) mutation isolated that is not an *Enhancer of zeste (E(z))* allele. The following studies further characterize the function and identity of *son^{29}*.

In addition I describe here the isolation of second site modifiers of *son^{29}* and *E(z)*. The *son^{29}* allele and the *E(z)* alleles that are strong dominant *son* mutations (*E(z)^{son}* alleles) interact with each other, so that transheterozygous females are largely sterile. I carried out a screen for suppressors of this female sterility to possibly isolate revertants of *son^{29}*, whose nature is unknown, or additional second site modifier mutations that may interact with *son^{29}* and *E(z)*. The results of the screen led to the isolation of mutations in the gene *Suppressor of the female sterile interaction-1 (Sufsi-1)*. Surprisingly, maternal homozygosity for at least one allele of *Sufsi-1* leads to embryos that exhibit phenotypes similar to those that are produced by the son^{29}/E(z)^{son} female sterile interaction.

I discuss possible models for the action of the *son^{29}* and *Sufsi-1* genes.

MATERIALS AND METHODS

Genetic strains: Genetic strains are as in Chapter II, III and IV.

Genetic analysis of *son^{29}*: The genetic mapping of *son^{29}* is described in Chapter I.

Screen for *Su(fsi)*: The screen for suppressors of the son^{29}/E(z)^{son} female sterile interaction (*Su(fsi)*) was carried out as shown in Figure A.2. The *son^{29}*-carrying chromosome is a recombinant derivative of the *nos^{L7}, st e* chromosome in which the son mutations where induced (Chapter II). In this recombinant the left and right arm of the second chromosome have been exchanged with the "neuple" (*th st ri p^{p} sr e*) chromosome (see new set of markers in Figure A.2). Males homozygous for an isogenic *son^{29}* chromosome were mutagenized with 35mM Ethyl Methyl Sulfonate (EMS), a point mutagen, and crossed to *E(z)^{son3} nos / TM3* females. The *E(z)^{son3}*-containing chromosome used carried a *nos* mutation since the presence of *nos* mutations appears to enhance the female sterile interaction (fsi) phenotype caused by the + *son^{29} / E(z)^{son}* + genotypic background (see Table A.5). This chromosome also provided appropriate visible markers that allowed it to be distinguished from the *son^{29}*-containing chromosome. For simplicity, this screen only attempted to isolate *Su(fsi)* mutations on the third chromosome.

An estimate of the mutagenesis frequency was obtained by scoring for newly induced *p^{p}* (in * *son^{29}/TM3(p^{p-})* males) and *cu* (in *E(z)^{son3} (cu^{-})/* son^{29}* females, see Figure A.2). Of the approximately 3,000 individuals screened in each case, 5 *p^{p}* and 3 *cu* newly induced mutations were observed. This suggests a frequency of mutagenesis of 1.3 hits/ locus/1,000 chromosomes.

Genetic analysis of *Sufsi-1* alleles: Genetic mapping of *Sufsi-1*: The lethality associated with *Sufsi-1^{63}* and *Sufsi-1^{82}* was mapped to within the *ru - h* interval (3-0.0 to 3-26.5), at approximately positions 3-20 and 3-15, respectively (number of lines used: 19 and 45, respectively). Thus, *Sufsi-1* is inferred to map at position near 3-17.5. Recombinant chromosomes carrying only the *ru-h* region from the original *Su(fsi)^{4}*-carrying chromosome also showed the semilethality and embryonic phenotypes associated with the original chromosome, and the embryonic phenotypes associated with *Sufsi-1^{4}* are enhanced in transheterozygous combinations of *Sufsi-1^{4}* and *Sufsi-1^{63}* and *Sufsi-1^{82}* (see Tables A.7-9). Therefore *Sufsi-1^{4}* is regarded here as another *Sufsi-1* allele, although because of its rough mapping, it is formally possible that *Sufsi-1^{4}* is a mutation in a different, but interacting, gene in this chromosomal region.

The screen for *Su(fsi)* mutations allowed recombination in the F1 females. In order to insure that the phenotypes associated with the *Sufsi-1* mutations were not related to a *E(z)^{son3}* allele recombined onto the *son^{29}*-carrying chromosome, the chromosomes carrying *Sufsi-1^{4}* , *Sufsi-1^{63}* and *Sufsi-1^{82}* were backcrossed to chromosomes carrying

the *E(z)* alleles $E(z)^{65}$ and $E(z)^{64}$. In all instances, transheterozygote combinations were viable, which indicates that the *Su(fsi)*-carrying chromosomes did not contain the $E(z)^{son3}$ allele.

Tests involving the *Sufsi-1* alleles involved recombinant chromosomes where the only region of the originally mutagenized son^{29} chromosome is the *ru-h* interval.

RESULTS

Genetic properties of son^{29}

Effect on the suppression of *nos*.

The suppression of *nos* by son^{29} is strictly maternal. The son effect only occurs when son^{29} is introduced through the mother and not when introduced through the father (Table A.1). Thus, like in the case of *E(z)*, it is the maternal product that is involved in abdominal regulation.

In contrast to the son phenotype conferred by *E(z)* mutations, homozygosity for son^{29} does not have a much higher effect than heterozygosity over a wild-type copy (Table A.1, see also Chapter II). In addition, higher temperatures do not induce a drastic increase in the degree of suppression of the nos phenotype (Table A.1)

In addition, the suppression of *nos* by son^{29} is much less dependent on the number of wild-type hb^{mat} and *trithorax* copies than suppression by *E(z)* mutations (Tables A.2 and A.3).

B) Additional phenotypes caused by son^{29} mutations

Adults homozygous for the son^{29} mutation frequently (21% of the flies, n=62) exhibit thoracic bristle duplications. son^{29}/*TM3* balanced adults also exhibit this phenotype, to an extent similar to that caused by son^{29}/son^{29} homozygotes (21%, n=91). Thus it is possible that this effect is associated with mutations in the stock different than son^{29}. Nevertheless, it seems likely that the bristle duplication phenotype is indeed associated with son^{29}, since mutations in a second site suppressors of son^{29}, *Sufsi-1*, also exhibit the recessive (but not the dominant) phenotype, and this phenotype is enhanced by additional son^{29} mutations (see below).

The son^{29} mutation also exhibits, at a low penetrance, a maternal effect zygotic phenotype. A small fraction (about 6%) of the embryos from son^{29} homozygous mothers show head involution defects which are similar to those observed by us with the $E(z)^{son}$ germ line clones (Chapter III) and others by other mutations that induce weak homeotic phenotypes (see, for example, Jürgens (1985)).

Table A.1. Maternal-effect suppression of the nos phenotype by *son^{29}*.

genetic cross (**females** x males)	% rescue (1)	n
son^{29} nos / nos x OR	46	109
son^{29} nos / son^{29} nos x OR (25°C)	42	868
son^{29} nos / son^{29} nos x OR (29°C) (2)	44	758
nos / nos x *nos / nos*	0	292
nos / nos x *son^{29} nos/ son^{29} nos*	0	271

(1) % of embryos with ≥ 3 abdominal segments.

(2) Females were grown at 25°C, then shifted to 29°C

Table A.2. Suppression of the nos phenotype by son^{29} is less hb^{mat}-dependent than that by $E(z)^{son}$ alleles

Maternal genotype	hb^+ / hb^+		hb^- / hb^+		*hb* -dependence
	% rescue(1)	n	% rescue(1)	n	(2)
experiment 1					
$son^{29} / +$	46	109	63	172	1.4
$E(z)^{son1} / +$	11	156	76	186	6.9
$E(z)^{son2} / +$	6	160	42	145	7
$E(z)^{son3} / +$	13	895	88	179	6.8
experiment 2					
$son^{29} / +$	36	439	68	212	1.9
$E(z)^{son2} / +$	1	372	14	482	14
$E(z)^{son3} / +$	2	259	86	478	43

(1) % of embryos with ≥ 3 segments.

(2) Value shown is the ratio (% rescue in hb^- / hb^+ over % rescue in hb^+ / hb^+. This ratio represents a measure of the enhancement of the son phenotype produced by *hb* heterozygosity.

Table A.3. Suppression of the nos phenotype by son^{29} is less *trx*-dependent than that by $E(z)^{son}$ alleles

maternal genotype	trx^+ / trx^+		$Df(trx) / trx^+$		*trx* -dependence
	% rescue(1)	n	% rescue(1)	n	(2)
$son^{29} / +$	37	596	32	820	0.86
$E(z)^{son1} / +$	2	246	13	373	6.5
$E(z)^{son2} / +$	0.5	440	4	691	8
$E(z)^{son3} / +$	1	299	47 (3)	234	47 (3)

(1) % of embryos with ≥ 3 abdominal segments.

(2) Value shown is the ratio (% rescue in $Df(trx) / trx^+$ over % rescue in trx^+ / trx^+). This ratio represents a measure of the enhancement of the son phenotype produced by *trx* heterozygosity.

(3) Value is from a different experiment and may not be directly comparable to other values in this table. See footnote (2) in Table 4.1.

Female sterile interaction of the *son*^{29} mutation in trans to *E(z)*^{son} alleles

Transheterozygous females of *son*^{29} and *E(z)*^{son3} exhibit a strong sterile interaction where most of the eggs do not show any development, probably because of a defect in fertilization (as inferred by the lack of appearance of brown pigment in the embryos of the embryos). A smaller percentage of eggs do develop into embryos that form a cuticle. Most of these embryos do not hatch due to various defects, such as apparent head involution defects and segmentation defects (Figure A.1A) These phenotypes had been previously observed in embryos from females homozygous for *son*^{29} and for *E(z)*^{son} mutations.

This sterile phenotype can not be induced paternally, since wild type females are fertile when crossed to *son*^{29}, *E(z)*^{son3} transheterozygous males (Table A.4). This indicates that the sterility is not due to chromosomal aberrations, but is likely caused by maternal-effect mutations.

This female sterile interaction is not dependent on the *nos* mutations present in the original chromosome in which these *son* mutations were induced, since it can still be observed in a wild-type *nos* background (Table A.5). Nevertheless, the presence of one or two mutant *nos* copies appears to increase the frequency of unfertilized eggs in layings from *son*^{29}, *E(z)*^{son} transheterozygotes. This may be due to additive effects of separate mutations, since *nos* also has a function during oogenesis.

The E(z)^{son}/son^{29} interaction is strictly dependent on the gain-of-function nature of the *E(z)*^{son} alleles, as it is not observed in progeny from *son*^{29}/*E(z)*^{null} females (Table A.5). This suggests that the female sterility depends on the ability of the *E(z)*^{son} product, or an E(z)^{son}/Son^{29} complex, to act as a poison in processes required during both oogenesis and embryogenesis.

A genetic screen for revertants/suppressors of the son^{29}/E(z)^{son} maternal sterile interaction leads to mutations in a second site modifier locus

It is possible that *son*^{29} is a gain-of-function mutation and that the fsi depends on that gain of function character. This argument has some support in the fact that in the screen for suppressors of nos, only one allele of *son*^{29} was recovered, a frequency which was lower than that for the appearance of the gain-of-function *E(z)*^{son} alleles. Also, the sterility of *E(z)*^{son} +/+ *son*^{29} females is dependent on the gain-of-function nature of the

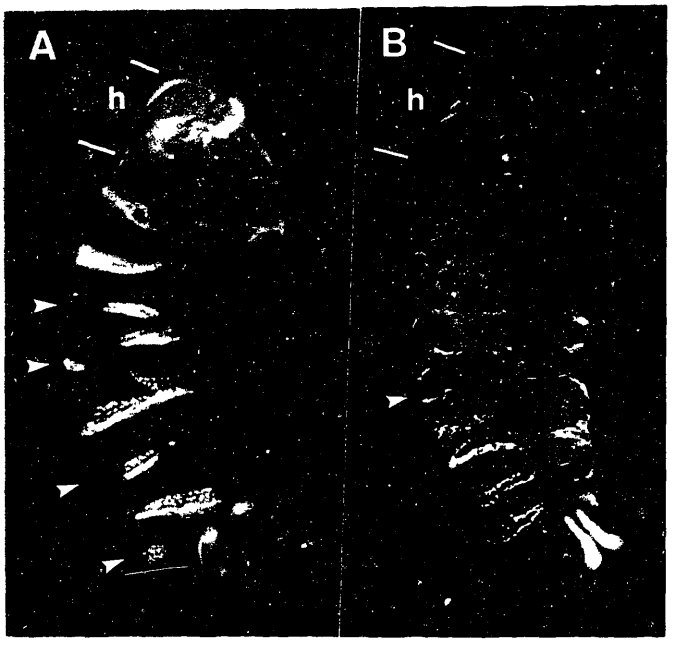

Figure A.1. Embryonic phenotypes exhibited by embryos from $+son^{29}/E(z)^{son}$ + transheterozygous females and from *Sufsi-1*^{4} homozygous females. A) Embryo from $+son^{29}/E(z)^{son3}$ + transheterozygous females, which show the female sterile interaction between son^{29} and $E(z)^{son}$ alleles. Embryos exhibit various defects, such as head involution and segmentation defects. 3) Embryo from from *Sufsi-1*^{4} homozygous females. *Sufsi-1*^{4} is a mutation isolated as a dominant suppressor of the $son^{29}/E(z)^{son}$ sterile interaction (see below). Homozygosity for *Sufsi-1*^{4} causes phenotypes similar to those it suppresses in one copy. Dark field optics. h: Head parts that have not involuted properly; arrowheads: defective abdominal segments (compare with the phenotypically wild-type embryo shown in Figure 3.2B). Anterior (head) top and ventral left.

Table A.4. The *son*^{29} / *E(z)*^{son} interaction is not paternally induced.

genotype of males (1)	% affected eggs (2)	n
son^{29} / *E(z)*^{son3}	4	23
son^{29} / *TM3*	0 (3)	41

(1) Females used in the cross were wild type for *son*^{29} and *E(z)*. Their particular genotype was *hb*^{7m} *nos* / *TM3*.

(2) Includes mostly apparently unfertilized eggs and embryos which formed a cuticle but did not hatch due to visible cuticular defects.

(3) 10 of 41 embryos did not hatch but had no cuticular defects. This phenotype is due to the expected 1/4 *TM3* homozygous embryos.

Table A.5. The dominant sterile interaction *son*^{29} / *E(z)*^{son3} depends on the gain of function nature of the *E(z)*^{son} allele.

maternal genotype	% unfertilized eggs	% non-hatchers	% hatchers	n
son^{29} / *son*^{29}	28	5	67	1138
son^{29} / *E(z)*^{son1}	68	29	3	147
son^{29} *nos* / *E(z)*^{son1} *nos*	92	8	0	153
son^{29} / *E(z)*^{son2}	39	26	35	151
son^{29} / *E(z)*^{son3}	35	38	27	2281
son^{29} / *E(z)*^{son3} *nos*	87	8	5	3897
son^{29} / *DfE(z)6*	9	0.3	91	1504
son^{29} *nos* / *DfE(z)6*	4	6	90	289

E(z)^{son} alleles. Nevertheless, the possibility that *E(z)^{son}, son29^{null}* transheterozygous females do not exhibit the female sterile phenotype can not be properly tested due to the absence of *son^{29}* null alleles or deficiencies that clearly remove this locus (see Chapter II).

If the female sterile interaction depends on the hypothetical gain-of-function nature of the *son^{29}* allele, a screen for suppressors of this female sterile interaction (*Su(fsi)*) could lead to the isolation of *son^{29}* null alleles. Regardless of this assumption, a screen for suppression of the son^{29}/E(z)^{son} interaction could in addition lead to the isolation of mutations in second site modifier genes which code for products that interact with the Son^{29} and/or E(z)^{son} products.

A screen for suppressors of the son^{29}/E(z)^{son} female sterile interaction was carried out as shown in Figure A.2. Approximately 3,000 F1 *son^{29} / E(z)^{son3} nos* females were individually tested in laying blocks and their progeny observed for three consecutive days. Typically, more than 90% of the eggs from females of this genotype are affected (see above). Seventy-three F1 females (2.4% of the total) whose progeny exhibited a markedly lower fraction of affected eggs were selected and placed into individual vials. The mutagenized chromosomes were recovered from each of these females and backcrossed to the *E(z)^{son3} nos* chromosome in order to retest for their Su(fsi) phenotype.

From the results of the retest lines were classified in four groups (number of lines in each group are in parenthesis): 0 (30), I (28), II (10) and III (3), which correspond to retest frequencies of >60%, 31-60%, 11-30% and ≤10% affected eggs, respectively. Table A.6 presents the Su(fsi) phenotype for lines in groups II and III.

Chromosomes in groups II and III (13 lines, 0.04% of the total number of mutagenized chromosomes) were tested in a single inter se matrix for possible interactions such as effects on viability, visible adult phenotypes and fertility. Only one set of three mutations, *Su(fsi)^{4}*, *Su(fsi)^{63}* and *Su(fsi)^{82}*, did not complement each other. These three mutations appear to be alleles of a single gene essential for viability, which will be referred to as *Sufsi-1* (see below).

The rest of the lines in Groups II and III were kept as balanced stocks. The mutations in Group I were tested for effects on viability in trans to *Sufsi-1^{63}* and *Sufsi-1^{82}*. None of the transheterozygous combinations exhibited visible adult phenotypes or an obvious effect on viability, and Group I lines were discarded thereafter.

The *Sufsi-1* gene: *Sufsi-1* maps at approximately 3-17.5 and is represented by three alleles, *Sufsi-1^{4}*, *Sufsi-1^{63}* and *Sufsi-1^{82}* (see Methods). This map position is

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Figure A.2. Screen for revertants and/or second site suppressors of the *son^{29}/E(z)^{son}* female sterile interaction. See Materials and Methods for details.

Figure A.2

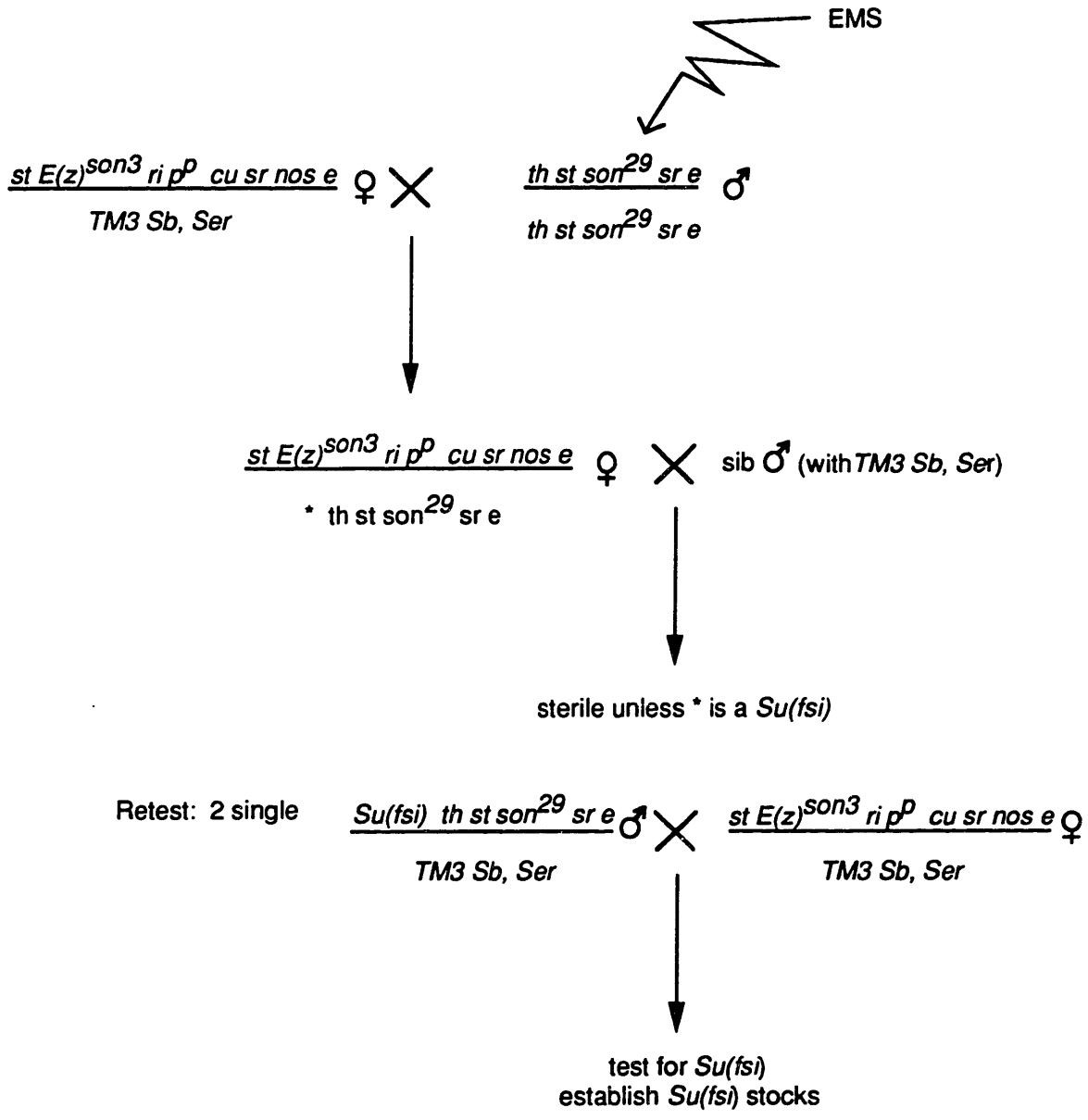

EMS
st E(z)son3 ri pp cu sr nos e
TM3 Sb, Ser
th st son29 sr e
th st son29 sr e
st E(z)son3 ri pp cu sr nos e
* th st son29 sr e
sib ♂ (with TM3 Sb, Ser)
sterile unless * is a Su(fsi)
Retest: 2 single
Su(fsi) th st son29 sr e
TM3 Sb, Ser
st E(z)son3 ri pp cu sr nos e
TM3 Sb, Ser
test for Su(fsi)
establish Su(fsi) stocks

Table A.6. Dominant reversion of the son^{29} / $E(z)^{son}$ female sterile interaction by *Su(fsi)* mutations.

Su(fsi) mutation (maternal background genotype: son^{29} / $E(z)^{son}$ *nos*)	% affected eggs (1)	n
none	95	3897
Type II *Su(fsi)*		
$Su(fsi)^{20}$ / +	20	154
$Su(fsi)^{21}$ / +	30	158
$Su(fsi)^{40}$ / +	28	158
$Su(fsi)^{51}$ / +	27	173
$Su(fsi)^{62}$ / +	24	66
$Su(fsi)^{63}$ / +	30	125
$Su(fsi)^{67}$ / +	29	165
$Su(fsi)^{75}$ / +	24	139
$Su(fsi)^{76}$ / +	29	238
$Su(fsi)^{82}$ / +	17	211
Type III *Su(fsi)*		
$Su(fsi)^{4}$ / +	9	115
$Su(fsi)^{19}$ / +	10	232
$Su(fsi)^{34}$ / +	5	134

(1) % of eggs that do not hatch (i.e. unfertilized + unhatched)

different from that of the *son*^{29} mutation (3-46.6) so that these mutations are clearly not revertants of *son*^{29} but rather second site suppressors of the son^{29}/E(z)^{son} female sterile interaction.

Sufsi-1^{63}/*Sufsi-1*^{82} individuals are lethal, and *Sufsi-1*^{4}/*Sufsi-1*^{63} and *Sufsi-1*^{4}/*Sufsi-1*^{82} have a reduced viability (Table A.7). This deleterious effect on viability is enhanced in the presence of *son*^{29} mutations, suggesting that both *Sufsi-1* and *son*^{29} functions are important for viability.

In addition, *Sufsi-1* mutations in heteroallelic combinations cause extra bristles in the thoracic region (Tables A.7 and A.8). This phenotype is similar to that caused by *son*^{29} mutations, although *Sufsi-1* mutations, in contrast to *son*^{29} mutations, have only a very weak dominant effect. The penetrance of the extra bristles phenotype caused by *Sufsi-1* mutations is enhanced by the presence of *son*^{29} mutations. Thus the *son*^{29} and *Sufsi-1* genes may both be involved in a process related to thoracic bristle determination.

In addition, *Sufsi-1* mutations have dominant and recessive maternal-effect embryonic phenotypes. A fraction of the progeny from *Sufsi-1*^{4} (but not *Sufsi-1*^{63} and *Sufsi-1*^{82}) heterozygous females exhibit pair rule-like phenotypes, even when crossed to wild type males (Table A.9). Progeny from *Sufsi-1*^{4} homozygous females exhibit this effect at a higher penetrance and in addition exhibit head involution and pair rule-like defects (Figure A.1B). These phenotypes are enhanced in the progeny of *Sufsi-1*^{4}/*Sufsi-1*^{63} and *Sufsi-1*^{4}/*Sufsi-1*^{82} females (Table A.9). In general, the penetrance of these maternal effect phenotypes is enhanced at higher temperatures (Table A.9). These results suggest that the *Su(fsi)-1* maternal product is involved in embryonic development.

This maternal-effect embryonic phenotype is enhanced when females of the same genotype are crossed to males carrying Sufsi-1 mutations (Table A.9). Thus, zygotic *Sufsi-1* product is also likely involved in embryogenesis. Nevertheless, the maternal *Sufsi-1* product appears to be sufficient for embryogenesis even in the absence of zygotic products, since stocks carrying the presumed loss-of-function alleles *Sufsi-1*^{63} and *Sufsi-1*^{82} (see below) do not exhibit these embryonic defects.

As observed for the adult phenotypes described above, the embryonic phenotype of *Sufsi-1* mutations is enhanced by the presence of additional mutations in *son*^{29} (Table A.9). Thus the Son^{29} and Sufsi-1 products appear to interact in processes required for embryonic patterning.

In the absence of known *Sufsi-1* null alleles or deficiencies that uncover this gene, the nature of the *Sufsi-1* alleles is subject to speculation. The alleles *Sufsi-1*^{63} and *Sufsi-1*^{82} may be loss of function mutations. This is suggested by their lethal phenotype, their

Table A.7. *Su(fsi)*^{4,63,82} mutations are associated with a reduction in viability and an extra bristle phenotype.

Genetic cross (**females** x males)	# of mutations (1)	Standarized viability of trans-heterozygous(2)	n	Standarized fraction of extra bristles (3)	n
son*^{29}*/TM3 x *son*^{29}*/TM3*	2	1	153	1	62
Su(fsl)*^{4}*/TM3 x *son*^{29}*/TM3*	2	0.98	208	1.6	83
Su(fsl)*^{63}*/TM3 x *son*^{29}*/TM3*	2	1.07	116	0.95	51
Su(fsl)*^{82}*/TM3 x *son*^{29}*/TM3*	2	1.02	131	0.71	55
Su(fsl)*^{63}*/TM3 x *Su(fsi)*^{4}*/TM3*	2	0.76	172	1.4	54
Su(fsl)*^{82}*/TM3 x *Su(fsi)*^{4}*/TM3*	2	0.76	137	2.7	42
Su(fsl)*^{63}*/TM3 x *Su(fsi)*^{82}*/TM3*	2	0	96	n. a.	-
Su(fsl)*^{4} *son*^{29}*/TM3 x *son*^{29}*/TM3*	3	0.71	137	2.8	40
Su(fsl)*^{63}*/TM3 x *Su(fsi)*^{4} *son*^{29}*/TM3*	3	0.44	148	3	27
Su(fsl)*^{82}*/TM3 x *Su(fsi)*^{4} *son*^{29}*/TM3*	3	0.68	154	2.3	43

(1) # of mutations include each mutation in *son*^{29} or *Su(fsi)*^{4,63,82}.

(2) Fraction of progeny as counted 20 days of setting up the cross. The value shown is standarized against the viability of *son*^{29} homozygotes, which was 0.41 of the total progeny (expected was 0.33; the difference in these two values is likely due to semi-lethal effects associated with the *TM3* balancer).

(3) Fraction of scored adult progeny with extra thoracic bristles. The value shown is standarized against the fraction of *son*^{29} homozygote adult found with extra bristles, which was 0.21.

Table A.8. Dominant and recessive bristle duplication phenotype conferred by *son*^{29} and *Sufsi-1* mutations.

Mutation	recessive effect (1)		dominant effect (2)	
	%	n	%	n
son29	21	62	21	91
Sufsi-1^{4}	n.d.		6	108
Sufsi-1^{63}	n.a. (lethal)		3	116
Sufsi-1^{82}	n.a. (lethal)		4	102
trans-heterozygous combinations	%	n		
Sufsi-1^{4}/*Sufsi-1*^{63}	30	54		
Sufsi1^{4}/*Sufsi-1*^{82}	57	42		
Sufsi-1^{63}/*Sufsi-1*^{82}	n.a. (lethal)			

(1) Effect of homozygosity for the mutation

(2) Effect of heterozygosity for the mutation in trans to a wild-type allele

Table A.9. Embryonic phenotypes produced by *son*^{29} and *Sufsi-1* mutations.

Maternal genotype		x wild-type males		x sib males (of same genotype as females)	
		% embryos with defects	n	% embryos with defects	n
son^{29}/*son*^{29}	25°C	3	29	0	21
Sufsi-1^{4}/*Sufsi-1*^{4}	25°C	13	191	7	300
Sufsi-1^{4}/*Sufsi1*^{63}	25°-29°C	n.d.		21	33
Sufsi-1^{4}/*Sufsi-1*^{82}	25-29°C	n.d.		26	137
Sufsi-1^{4} *son*^{29}/ *Sufsi-1*^{63} *son*^{29}	18°C	15	48	39	64
	25°C	8	156	19	149
	25-29°C	25	155	58	142
	29°C	n.d.		78	73
Sufsi-1^{4} *son*^{29}/ *Sufsi-1*^{82} *son*^{29}	18°C	25	146	51	136
	25°C	47	163	70	87
	25-29°C	56	141	75	113
	29°C	n.d		97	87
Sufsi-1^{4} *son*^{29}/*TM3*	25°C			15	131
Sufsi-1^{63} *son*^{29}/*TM3*	25°C			1	167
Sufsi-1^{82} *son*^{29}/*TM3*	25°C			1	206
Sufsi-1^{4} *son*^{29}/*TM3* (1)	25°C	8, 4	25, 24	4	46

(1) Results from a different experiment as in row 13. The two different values represent results when females were mated to Oregon R and *nos*^{L7}, *st e/TM3* males, respectively.

lack of a dominant phenotype and the fact that the frequency in which these alleles were obtained is similar to that of induced loss-of-function mutations during mutagenesis (approximately 0.7 and 1.3 hits/locus/1,000 chromosomes, respectively, see Methods). The allele *Sufsi-1*^{4} retains some wild-type *Sufsi-1* function, as indicated by its ability to support development into adulthood. *Sufsi-1*^{4} appears to have in addition a gain of function character, since it causes dominant maternal effect embryonic defects.

DISCUSSION

The role of *son*^{29} in gap gene regulation

son^{29} homozygotes are mostly viable and fertile. Homozygous adults often exhibit extra thoracic bristles and, at a low frequency, females produce embryos with embryonic defects such as head involution defects. Its most obvious phenotype is its strong suppression-of-nos phenotype.

Deficiencies near the candidate *son*^{29} region do not exhibit any strong interaction with *son*^{29} (see Chapter II). Nevertheless, the limits of this deficiencies with respect to the position of *son*^{29} are not well defined, and thus it is not known with certainty whether *son*^{29} maps within or outside of the region covered by these deficiencies. It is also possible that one copy of the *son*^{29} mutant product retains enough wild type function to support development, or that *son*^{29} function is largely dispensable.

The son phenotype conferred by *son*^{29} mutations differs from that conferred by the better studied *son*^{29} mutations in several aspects. Although *son*^{29} has a relatively strong dominant son phenotype, homozygosity for *son*^{29} does not have a much greater son phenotype. This is in contrast to *E(z)* mutations, which cause a weak dominant son phenotype and a much greater son phenotype when homozygosed (see Chapter III). It is possible that a single *son*^{29} copy is adequate for the son phenotype and a greater dosage is above a threshold and has no further effects. It is also possible that the *son*^{29} mutations requires a wild-type allele to exert its son effect, and that in *son*^{29} homozygotes the addition of an extra *son*^{29} mutant copy is offset by the absence of wild-type *son*^{29} function.

Heterozygosity for maternal *hb* and *trx* enhances the son phenotype conferred by *son*^{29} to a relatively small extent, whereas the son phenotype conferred by *E(z)* mutations is extremely sensitive to the dosages of these two genes. These results could be explained if the effect of *son*^{29} in the pathway that leads to *knirps* and *giant* transcriptional regulation occurs at a step that is downstream from the step where *E(z)*, *hb* and *trx* act, and thus is less sensitive to variations in the dosages of these genes.

Because of the uncertain cytological location of *son*^{29}, and the absence of known *son*^{29} null alleles, the nature of the *son*^{29} mutation itself remains unclear. This in turn causes uncertainty on the function of the wild-type *son*^{29} function. At present one can only speculate on the possible functions of the wild-type *son*^{29} product.

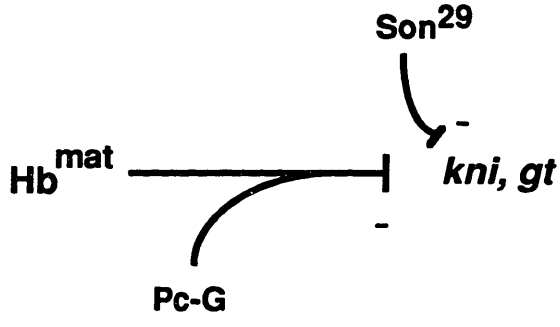
One possibility is that the function of the wild-type *son*^{29} gene is similar to that of Polycomb-group genes, so that it would be required for the repression of a number of genes, including the gap genes *kni* and *gt* (Figure A.3A). In this scenario, the *son*^{29} allele could code for a loss of function or a dominant negative gain-of-function. This explanation is consistent with the head involution phenotype observed in a small fraction of embryos from *son*^{29}/*son*^{29} females. Head involution defects have been observed in embryos with weak homeotic transformations caused by mutations in Pc-G genes, including E(z) mutations (see, for example, Jürgens (1985),; see also Chapter III).

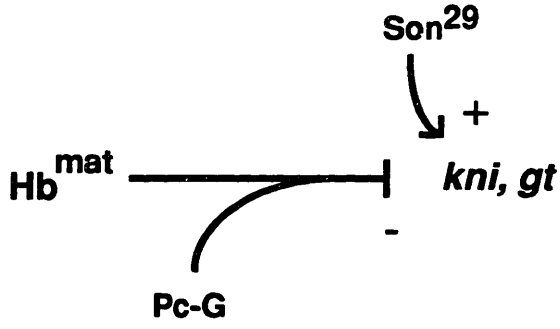
This explanation is also consistent with the strong female sterile interaction of *son*^{29} with the mutation in the Pc-G gene *E(z)* (*E(z)*^{son} mutations). + *son*^{29}/*E(z)*^{son3} + females lay many unfertilized eggs, perhaps due to misregulation of unknown genes during oogenesis. Progeny embryos from these females that do develop a cuticle exhibit a high frequency of head involution and pair-rule like defects. Again, the head involution defects observed in these embryos could be the result of weak posterior transformations. Similarly, pair-rule-like defects can be caused by mutations in certain Pc-G genes (Breen and Duncan 1986; Sinclair et al. 1992).

An alternative possibility is that the wild-type *son*^{29} gene is involved in the activation of *kni* and/or *gt* (Figure A.3B). In this scenario, the *son*^{29} allele would code for an overactive, gain-of-function allele. This possibility is particularly interesting since thus far the activators of kni and gt in the embryo are unknown. The phenotypes associated with *son*^{29} by itself and in trans to *E(z)*^{son} mutations could also be explained in this case if the *son*^{29} mutation, when combined with an *E(z)*^{son} mutation, leads to the unregulated expression of a number of genes involved in oogenesis and embryogenesis.

Figure A.3. Models for the interaction of the Son^{29} wild-type product with gap genes. A) If the *son*^{29} mutation is a loss-of-function or antimorphic allele, then the wild-type *son*^{29} product may be involved in gap gene repression. B) If the *son*^{29} mutation is a gain-of-function (overactive) allele, then the wild-type Son^{29} product may be involved in gap gene regulation.

Figure A.3.





A screen for suppressors of the son^{29}/E(z)^{son} female sterile interaction: identification of the interacting gene *Sufsi-1*

I carried out a screen for suppressors of the female sterility exhibited by son^{29}, $E(z)^{son}$ transheterozygous females. The original purpose of this experiment was the isolation of new son^{29} alleles. This idea was based on two assumptions: first, that son^{29} is a gain-of-function mutation, and second, that the sterile interaction is dependent on this gain-of-function character, and would not be caused by son^{29} loss-of-function mutations.

A number of suppressor mutations were isolated. An inter se complementation matrix revealed interactions between three alleles which map genetically at a position that is different from that of son^{29}. Thus these mutations are not son^{29} revertants. It is unclear whether any of the other isolated suppressor mutations are son^{29} revertants. The absence of additional complementation groups in the inter se matrix suggests that no son^{29} revertant was isolated. This could indicate that the starting assumption of the screen is incorrect, i.e. that son^{29} does not have a gain-of-function character that is required for the female sterile interaction. Alternatively, only one son^{29} revertant allele was isolated and thus would show no interactions in an inter se matrix, or more than one son^{29} allele was isolated but the wild-type son^{29} function is dispensable.

The three suppressor mutations isolated appear to be alleles of a single gene, which has been named *Sufsi-1*. Interestingly, *Sufsi-1* mutations cause on their own phenotypes also observed in son^{29} homozygotes and in son^{29}, $E(z)^{son3}$ transheterozygotes, such as extra thoracic bristles in the adult and head involution defects in embryos. *Sufsi-1* mutations have in addition a maternal effect pair rule-like phenotype in embryos. These phenotypes are in general enhanced by additional son^{29} mutations.

son^{29} and *Sufsi-1* mutations cause on their own similar phenotypes, and Sufsi and son^{29} mutations mutually enhance the bristle and embryonic phenotypes. Thus, it is somewhat surprising that *Sufsi-1* mutations suppress, rather than enhance, the son^{29}/E(z)^{son} female sterile interaction. It is possible that the son^{29} and *Sufsi-1* products act together in a complex, perhaps related to that formed by Polycomb group products. This complex would be generally required for viability and in particular in processes such as adult bristle determination, and embryonic head involution and segmentation.

In this model, the reduction of *Sufsi-1* product could suppress the E(z)^{son}/son^{29} dominant negative interaction in two ways. It may eliminate the function of the complex altogether, and perhaps the aberrant interaction initiated by the gain-of-function $E(z)^{son}$ (note that $E(z)^{nulls}$ do not cause the female sterile phenotype). Alternatively, the balance between Sufsi-1 and Son29 products may be important and the reduction in Sufsi-1 product may counteract the son^{29} mutation in $E(z)^{son}$, son^{29} transheterozygotes.

It is not known what misregulated target genes are responsible for the sterility of *E(z)^{son}, son^{29}* heterozygous females, and therefore, what targets may be affected by *Sufsi-1*. The head involution and pair-rule embryonic phenotypes caused by *Sufsi-1* mutations suggest that this gene is involved in the regulation of, respectively, homeotic and pair rule genes. Currently, it is also unknown whether *Sufsi-1* affects the regulation of abdominal gap genes *knirps* and *giant.*

In summary, the combination of the suppressor-of-*nos* and female sterile interaction screens has led to the isolation of at least two new genes which may be involved in the regulation of a number of targets during development, including the abdominal gap genes *knirps* and *giant.*

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Appendix B. Genetic and preliminary molecular characterization of *Pson^{49}*, a P-element induced suppressor-of-*nanos* mutation

ABSTRACT

Pson^{49} was isolated as a suppressor-of-*nos* (*son*) mutation induced by P-element mutagenesis. In addition, homozygosity for *Pson^{49}* leads to a maternal effect embryonic phenotype. Here, I show that the son phenotype and embryonic phenotypes are associated with the presence of the P-element insertion. These experiments suggest that *Pson^{49}* is involved in the regulation of gap genes and perhaps other genes such as homeotic and pair rule genes. The son and embryonic phenotypes caused by *Pson^{49}* mutations are similar to those produced by mutations in members of the Polycomb group of genes. Thus, *Pson^{49}* may be a new member of this family of genes.

I have cloned about 50 Kb of the *Pson^{49}* genomic region. Northern analysis has revealed a set of structurally related transcripts produced by genomic sequences within 4 Kb from the P-element insertion. This set of transcripts consists of one major 7 Kb transcript and several, less abundant larger transcripts. No other transcripts are detected using probes spanning this 50 Kb region. Thus, these large transcripts are good candidates to be mRNAs produced by the *Pson^{49}* gene.

INTRODUCTION

Chapter II describes a genetic screen for supressors of *nos* (*son*) using P-element mutagenesis. *Pson^{49}* is the only induced son mutation that is conclusively associated with a P-element insertion. Here, I describe genetic results that show this association, as well as the son and other phenotypes associated with this mutation. I also describe preliminary molecular analysis of the *Pson^{49}* genomic region.

MATERIALS AND METHODS

Genetic analysis and strains: Genetic tests and strains were performed as in Chapters II and III. Genetic mapping of *Pson^{49}* by virtue of the w^+ marker of the P-element is described in Chapter I.

P-element construct: The P-element used for mutagenesis (Chapter II) is described in detail in (Bier et al. 1989). It contains a mini-white gene, a *lacZ* gene that acts

as an enhancer trap construct in the flies, and a an ampicillin resistance and bacterial origin of replication for easy cloning of flanking DNA.

Molecular cloning of the *Pson^{49}* region: Genomic DNA from flies of a *Pson^{49}/Cyo* stock was digested with either XbaI or SacII and used to transform competent bacteria (Pirrotta 1986). Fragments from each of these clones (see Figure B.4) were used to screen a NotBamNot-CaSpeR cosmid genomic library (Tamkun et al. 1992). DNA fragments were hybridized to a Northern blot containing 10 μg of poly-A+ selected mRNA (a gift from A. Williamson). The blots were stripped of hybridized signal in between experiments. Cloning and hybridization techniques are according to standard procedures (Maniatis et al. 1982).

In situ hybridization to salivary chromosomes: Performed essentially as in Laverty (1990). The probe used was a biotinylated pC4bgal plasmid (Thummel et al. 1988), which contains *LacZ* sequences.

RESULTS

Association of *P-son^{49}* with a P-element insertion

The suppressor-of-*nos* phenotype present in the *Pson^{49}* line cosegregated with the *white^{+}* marker (Table B.1). Maternal homozygosity for the *w^{+}*-carrying *Pson^{49}* chromosome led to the production of abdominal segments in nos embryos (Figure B.1A). Thus *Pson^{49}* is a suppressor-of-*nos* mutation associated with the p[*w^{+}*] element.

Aside from the nos phenotype, mutant females homozygous for the *Pson^{49}* chromosome also exhibit on their own a number of phenotypes. *Pson^{49}/Pson^{49}* homozygous flies are less viable and fertile than wild-type flies (not shown). In addition, maternal homozygosity for *Pson^{49}* leads to embryonic lethality in a fraction of the embryos. Embryos exhibit a range of phenotypes, such as segment deletions and fusions, often in a pair-rule-like fashion, external heads indicative of head involution defects and, in some cases, absence of head and cuticular structures (Figure B.2). This suggests that the Pson49 product is required for a number of developmental processes.

To test conclusively whether the P-element insertion is responsible for the son and other phenotypes associated with *Pson^{49}*, I carried out a scheme to isolate excision derivatives of the putative *Pson^{49}* insertion mutation (Figure B.3). Balanced stocks of w^{-} excision derivatives were created. Lines were classified according to their viability when homozygosed (Table B.2). A number of lines were significantly more healthy as homozygous stocks than the original *Pson^{49}* chromosome. In addition, embryos from females homozygous for these lines did not show the high degree of inviability

Table B.1. The son phenotype in the *Pson*^{49} line is associated with the *w+* marker.

white phenotype (1)	Maternal Genotype			
	nos/nos		*hb nos/nos*	
	% rescue (2)	n	% rescue (2)	n
w+	5	348	35	277
w-	0	215	17	69

(1) Females tested were obtained from the following cross: *yw / Y; Pson*^{49} */ +; nos st e / +* males x *yw / yw; + / +; (hb) nos st e / +* females. Females were tested individually to distinguish those that had lost the *nos* mutation through recombination.

(2) % of embryos with ≥ 3 abdominal embryos.

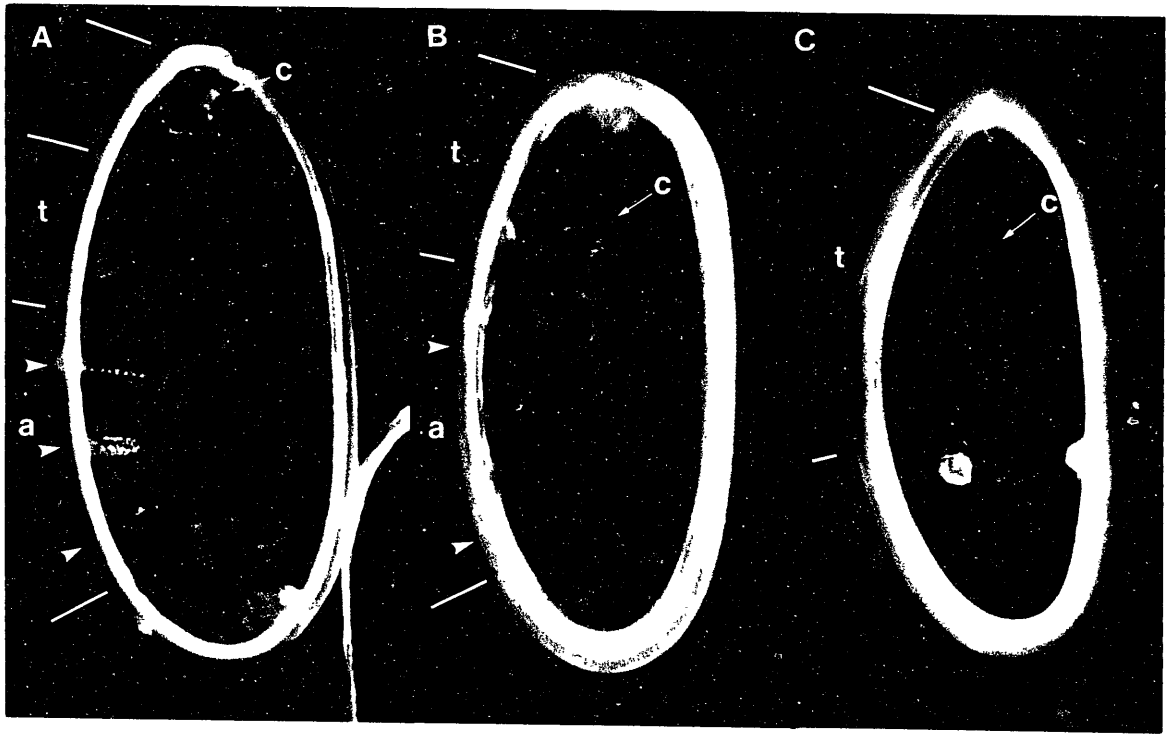

Figure B.1. Suppressor-of-*nos* phenotype exhibited by $Pson^{49}$ and P-element excision derivatives. A) Embryos from $Pson^{49}$ *nos* / $Pson^{49}$ *nos* females form several abdominal segments. These embryos also exhibit head involution defects. B) Embryos from nos mutant females that in addition are homozygous for the $Pson^{49}$-derivative R^{24} (R^{24} *nos* / R^{24} *nos*) exhibit a weaker son phenotype. C) Embryos from R^{22} *nos* / R^{22} *nos* females do not exhibit a suppression-of-*nos* phenotype. A similar result has been obtained with the derivative R^{16}. Embryos in (B) and (C) do not exhibit head involution defects. Arrowheads point to abdominal segments. t: thoracic region; a: abdominal region; c: cephalopharyngeal skeleton, which is external in (A, see also Figure B.2) and internal in (B, C). Darkfield optics. Anterior top, ventral left.

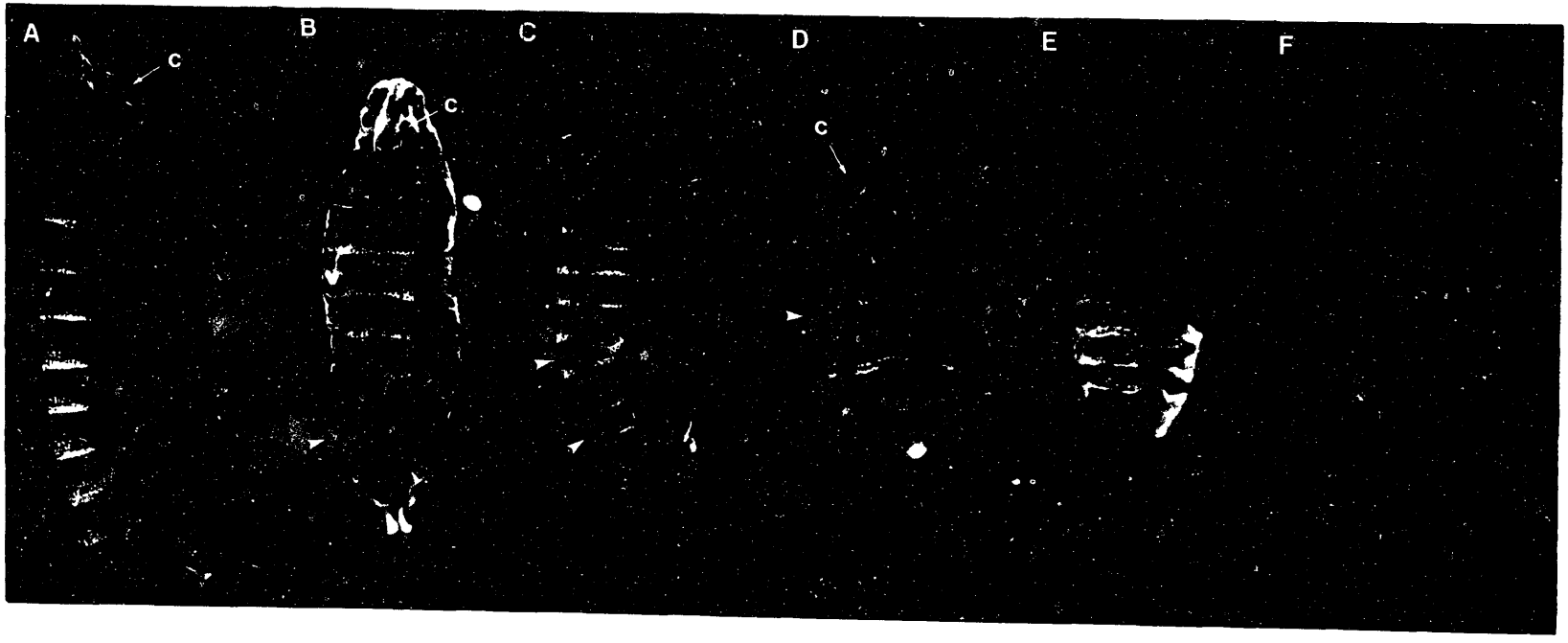

Figure B.2. Embryonic phenotypes associated with *Pson*^{49} mutations. A) Phenotypically wild-type embryo. B-F) Embryos from *Pson*^{49} homozygous females exhibiting progressively stronger defects. Embryos exhibit segment deletions and pair-rule-like fusions (arrowheads in (B-D)), head involution defects (C, D), and, in the stronger cases, absence of head (E) and other cuticular structures (F). c: cephalopharyngeal skeleton, which is internal in (A, B), external in (C, D) and absent in (E, F).

characteristic of embryos from *Pson*^{49} homozygous females (Table B.3). Therefore, both the viability of adults and embryos appears to be associated with the presence of the P-element.

Three viable revertant lines were tested for their recessive suppression-of-*nos* phenotype. One of them, *R24*, has a reduced suppression-of-*nos* phenotype in comparison to *Pson*^{49} (Figure B.1B), while two other lines completely lack the ability to suppress the nos phenotype. Thus, the excision of the P-element present in the *Pson*^{49} chromosome as assayed by the w^+ marker gene is associated with reversion to a wild-type *Pson*^{49} function. This indicates that the *Pson*^{49} mutations are likely produced by the insertion of the p[w^+] element.

Preliminary molecular analysis of the *Pson*^{49} genomic region

The P-element associated with the *Pson*^{49} mutation carries a bacterial drug resistance gene and an origin of replication that allow direct cloning from genomic mutant DNA (Bier et al. 1989). Thus I used these features to directly clone genomic regions that flank the *Pson*^{49}-associated P-element (see Materials and Methods). The structures of the isolated clones are shown in Figure B.4. Fragments from each flanking region were used to probe a genomic cosmid library (Tamkun et al. 1992), which led to the isolation of about 50 Kb of genomic region that flanks the P-element insertion (Figure B.5A). The correct identity of these clones was corroborated by in situ hybridization to salivary gland polytene chromosomes using one of the genomic cosmids (Figure B.6).

A Northern blot carrying 0 -2.5 hr poly+ RNA was sequentially probed using different fragments of these genomic walk (Figure B.5B). These results show that only

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Figure B.3. Scheme for the isolation of P-element excision derivatives of *Pson*^{49} ([w$^+$]). *Delta 2,3* is a stable source of transposase used to mobilize the P-element (Robertson et al 1988). *Sb, Sp* are dominant visible markers; *y* and *white (w)* are recessive visible markers. *CyO* and *TM6* are second and third chromosome balancers, respectively. * represents an excision event as indicated by a *white* mutant phenotype that is not associated with the *CyO* chromosome in the F_2 males.

Figure B.4. Structure of the P-lacW plasmid rescue construct and the flanking fragments isolated from *Pson*^{49} genomic DNA. P-lacW contains a bacterial origin of replication and an ampicillin resistance marker as indicated. X and S genomic clones were isolated by transfecting competent bacteria with *Pson*^{49} genomic DNA that had been digested with either XbaI or SacII, respectively. Probes L and R indicate left and right probes used to isolate the genomic cosmid clones represented in Figure B.5. (H) Hind III; (E) EcoRI; (S) SacII; (X) XbaI; (G) BglII; (P) PstI; (B) BamHI.

Figure B.3

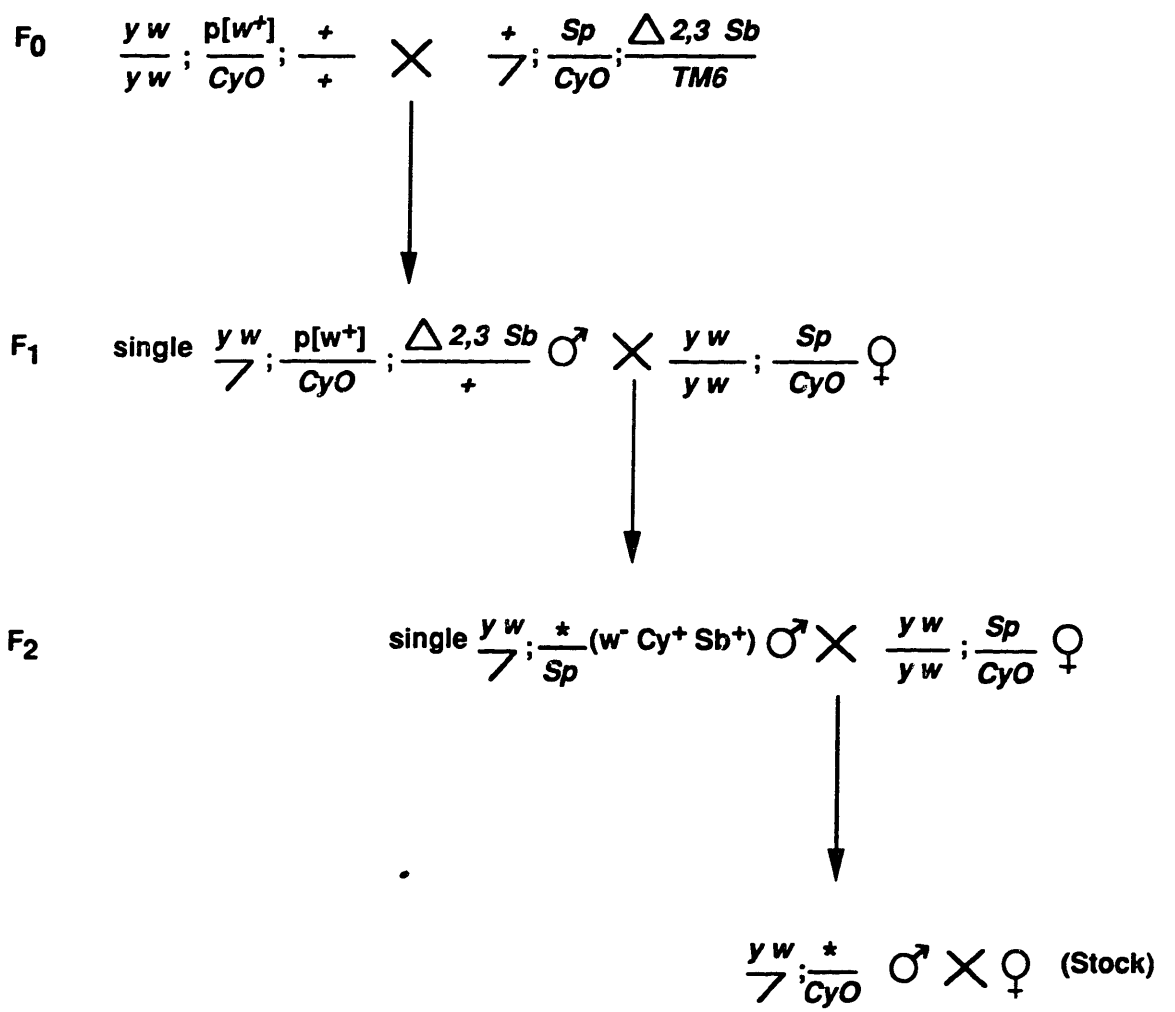

F0
y w / y w ; p[w+] / CyO ; + / +
+ / ; Sp / CyO ; Δ 2,3 Sb / TM6
F1
single y w / ; p[w+] / CyO ; Δ 2,3 Sb / + ♂
y w / y w ; Sp / CyO ♀
F2
single y w / ; * / Sp (w- Cy+ Sb+) ♂
y w / y w ; Sp / CyO ♀
y w / ; * / CyO ♂ ♀ (Stock)

Table B.2. Types of P-element excision *Pson*^{49} derivatives.

Chromosomal line (1)	Effect of homozygosity
Pson^{49}	sick
R3	viable
R9a	viable
R9b	viable
R14b	viable
R22	viable
R24	viable
R1 (2)	viable?
R14a (2)	viable?
R26b (2)	viable?
R2	inviable
R5a	inviable
R5b	inviable
R5c	inviable
R6a	inviable
R6b	inviable
R8	inviable
R12	inviable
R17a	inviable
R17b	inviable
R17c	inviable
R23a	inviable
R23b	inviable
R23c	inviable
R23d	inviable
R26a	inviable

(1) Lines denoted by the same number but different letters originate from the same F1 male in the excision crosses (Figure B.3). Therefore it is possible that these lines are derived from an identical excision event.

(2) These three lines exhibit a moderate to high frequency of embryonic lethality in sibling crosses, which is characteristic of the inviable lines (not shown). In the latter case, the embryonic lethality appears to be associated with the *CyO* balancer. It is possible that these lines are homozygous inviable and the "homozygous" tested females are in actuality heterozygous females that do not exhibit the Cy phenotype (*CyO* is not completely penetrant). Two other lines initially classified as homozygous viable by these tests were found upon further testing to be homozygous lethal.

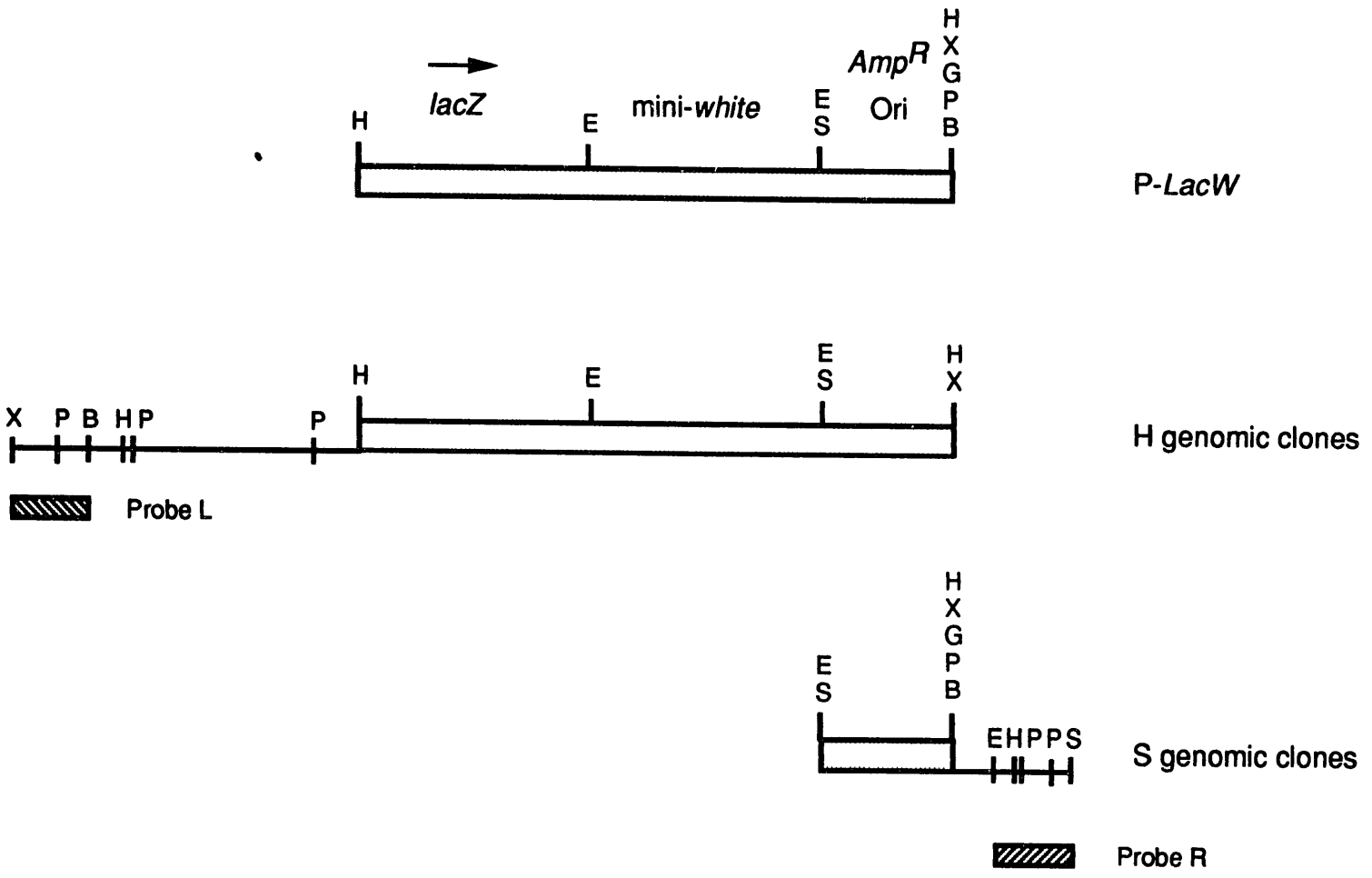
Table B.3. Viable P-element excision derivatives of *Pson*^{49} exhibit a reduced embryonic phenotype.

Maternal genotype (x to sib *CyO* males)	% embryonic lethality (1)	n
Control: *Pson*^{49} recombinant line *Pson*^{49}, *al*$^{+}$*dp*$^{+}$*b*$^{-}$*pr*$^{-}$*cu*$^{-}$*px*$^{-}$*sp*$^{-}$/ *Pson*^{49}, *al*$^{+}$*dp*$^{+}$*b*$^{-}$*pr*$^{-}$*cu*$^{-}$*px*$^{-}$*sp*$^{-}$	32	114
Control: *w*$^{+}$ chromosomes isolated after excision scheme		
C1/C1 (2)	(< 14 unf. eggs) (2)	129
C2/C2	17	
C5/C5 (2)	(40 unf. eggs) (2)	
Excision lines (*w*$^{-}$) that are viable as homozygotes and have low embryonic mortality		
R3/R3	6	118
R9a/R9a	6	112
R9b/R9b	3	196
R14b/R14b	6	126
R22/R22	2	192
R24/R24	5	85

(1) % of embryos that formed a cuticle that did not hatch.

(2) unf.= unfertilized. These chromosomes may have acquired unrelated mutations that affect oogenesis during the P-element mobilization crosses.

Figure B.4


lacZ
mini-white
Amp^R
Ori
H
E
E S
H X G P B
P-LacW
X P B H P
P
H
E
E S
H X
H genomic clones
Probe L
E S
H X G P B
E H P P S
S genomic clones
Probe R

one detectable set of likely structurally related transcripts are encoded by this genomic region. Thus, these transcripts are likely candidates to be the *Pson*^{49} mRNAs.

Discussion

Here I show that the P-element associated *Pson*^{49} mutation leads to a variety of phenotypes, including the suppression-of-*nos* phenotype, head involution defects, and pair-rule-like segmentation defects.

Interestingly, this set of phenotypes is similar to phenotypes exhibited by Pc-G genes. Mutations in these genes lead to the suppression of the nos phenotype (Chapter III), segmentation defects of a pair-rule character (Breen and Duncan 1986; Sinclair et al. 1983), and homeotic defects, which can result in head involution defects (see, for example, Jürgens (1985). Therefore it is tempting to speculate that the *Pson*^{49} Pson49 gene is another m ember of the Polycomb group family. This class of genes have been proposed to be involved in the trancriptional repression of target genes at the chromatin level (Paro 1990). This is, therefore, a candidate function for the *Pson*^{49} gene.

The phenotypes associated with the *Pson*^{49} mutation can be reverted by P-element excision, which shows that the *Pson*^{49} mutation is caused by the insertion of a transposable element. I have cloned the genomic region adjacent to this P-element. Prelimary analysis suggests that the *Pson*^{49} gene transcribes a set of structurally related large ($\geq$ 7 Kb) transcripts. Further work will be required to confirm this hypothesis and better understand the role of the *Pson*^{49} and other *son* genes.

Figure B.5. Genomic region of the *Pson*^{49} locus. A) Structure of the genomic cosmids isolated that encompass about 50 Kb flanking the *Pson*^{49} P-element insertion. Sizes of EcoRI fragments are indicated. B) DNA probes used as probes on a 0-2.5 hr embryonic polyA$^+$ RNA. All fragments tested which contained the region indicated with a filled box showed hybridization to a 7 Kb transcript and several less abundant transcripts of about 10 Kb and > 11 Kb. All tested fragments that did not contain this region (open boxes) did not hybridize to any detectable RNAs. Probes encompassing the entire 50 Kb genomic region did not detect any additional transcripts.

Figure B.6. In situ hybridization to salivary gland chromosomes. A, B) Hybridization of *Pson*^{49} chromosomes with a DNA fragment complementary to the *lacZ* portion of the P-element construct. C) Hybridization of wild-type chromosomes with the genomic clone 7.6 (see Figure B.5). In both cases a unique hybridization band is detected in chromosomal region 26A.

Figure B.5

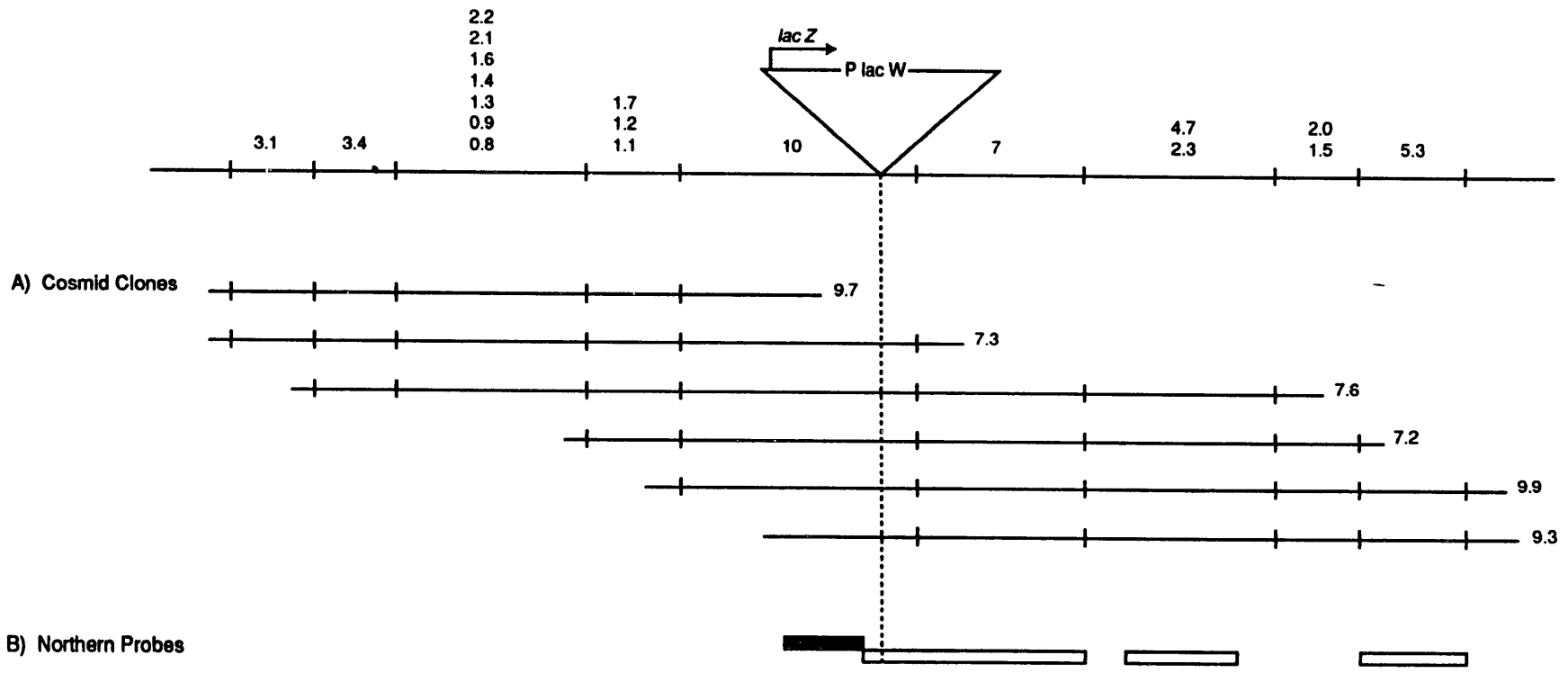
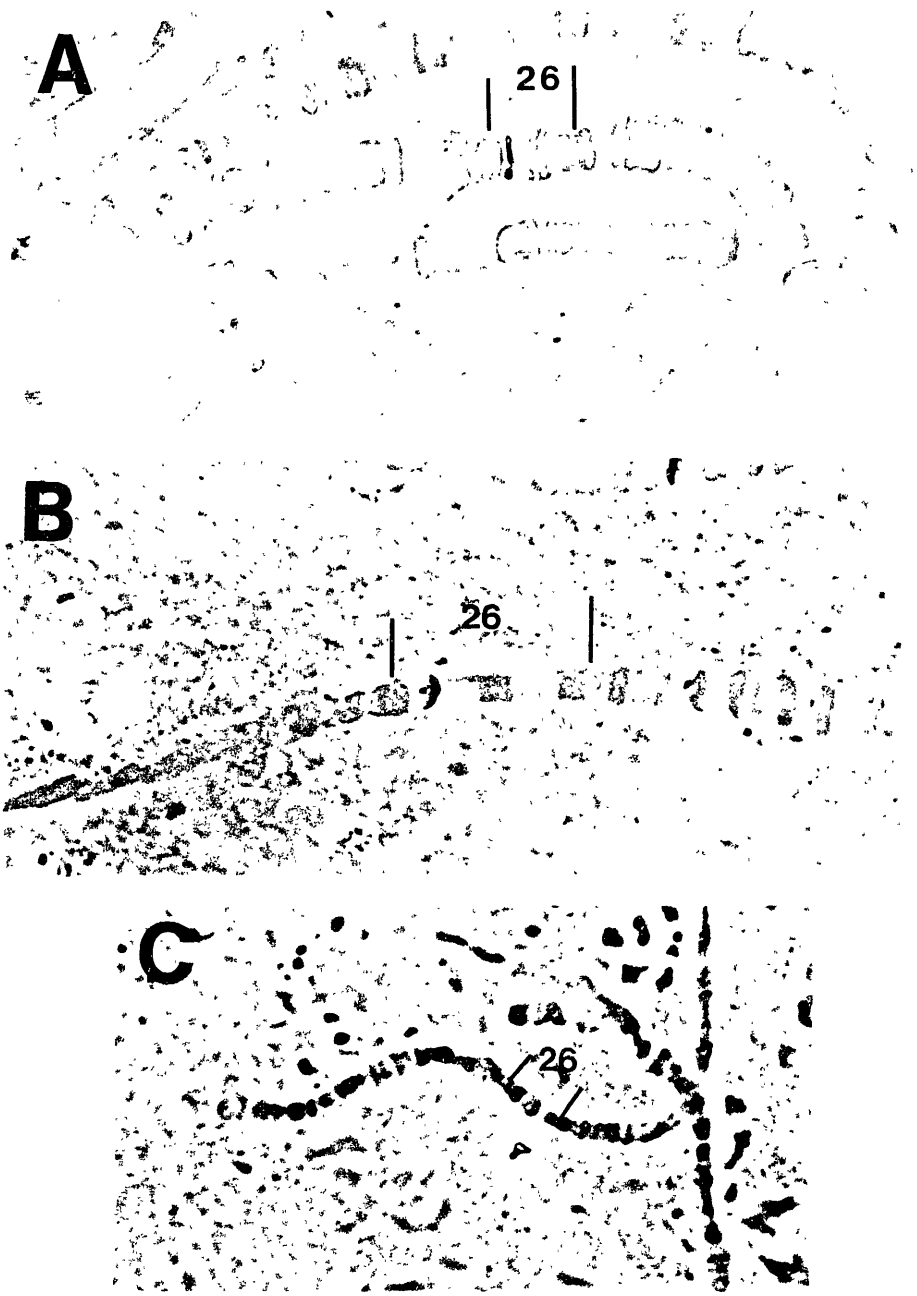

2.2
2.1
1.6
1.4
1.3
0.9
0.8
1.7
1.2
1.1
lac Z
P lac W
3.1
3.4
10
7
4.7
2.3
2.0
1.5
5.3
A) Cosmid Clones
9.7
7.3
7.6
7.2
9.9
9.3
B) Northern Probes

Figure B.6



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Appendix C. Isolation of suppressors of the *Bicaudal-D* mutation.

ABSTRACT

Embryos from females that are mutant for *Bicaudal-D^D (Bic-D^D)* mutations exhibit at their anterior ends mirror image duplications of the abdomen. Mutations in some genes involved in oocyte determination and pole plasm formation, such as *egalitarian* and *vasa*, act as dominant suppressors of the Bic-D^D phenotype. In order to isolate interacting components in this pathway, we carried out a screen for suppressors of *Bic-D^D*. Genetic characterization of the suppressor-of-*Bic-D^D* mutations shows that 4 new *egalitarian* and 3 new *vasa* alleles were isolated. In addition, a number of mutations that act as suppressors of *Bic-D^D* define at least two other complementation groups required for viability and oogenesis. Genetic interactions between suppressor-of-*Bic-D^D* mutations suggest that these genes form part of a functionally interacting gene network.

INTRODUCTION

Loss-of-function mutations in the *Bicaudal-D (Bic-D)* locus interfere with the differentiation of the oocyte during oogenesis (Suter et al. 1989; Suter and Steward 1991). *Bic-D* encodes a coiled-coil protein similar to the myosin tail (Wharton and Struhl 1989; Suter et al. 1989), and the Bic-D product is required for the accumulation in the pro-oocyte of its own and other RNAs (Suter and Steward 1991). Thus it is thought that the Bic-D protein is a component of the cytoskeletal network that is required for early transport of products into the oocyte.

Gain-of-function Bic-D mutations (*Bic-D^D*), on the other hand, lead to defects that are observed during embryogenesis, namely, the formation of anterior mirror image abdominal duplications (Nüsslein-Volhard et al. 1984; Wieschaus et al. 1984; Mohler and Wieschaus 1986, see Figure C.1). These defects are caused by the ectopic anterior localization of nos RNA by the Bic-D^D product. Bic-D protein is uniformly distributed in wild-type embryos but is enriched at the anterior of the oocyte in *Bic-D^D* mutant embryos. It is this abnormally distributed protein that is thought to gather at the anterior pole components of the pole plasm, including the nos RNA (Wharton and Struhl 1989).

The duplicated mirror image abdomens produced by *Bic-D^D* mutations can be modified by a reduction of in dosage of certain genes involved in the pathway of oocyte determination and posterior pattern formation (Mohler and Wieschaus 1986). Mutations in the gene *staufen* (*stau*), for example, act as dominant enhancers of the bicaudal phenotype.

This may reflect a requirement of *stau* for the transport of pole plasm components to the posterior of the embryo (St. Johnston et al. 1991). Mutations in the genes *egalitarian* (*egl*) and *vasa* (*vas*), on the other hand, act as dominant suppressors of the Bic-D^{D} phenotype. *egl* and *vas* may be required for the function of the wild-type and mutant gain-of-function Bic-D protein. Indeed, *egl* loss-of-function alleles have oogenesis phenotypes very similar to those of *Bic-D* loss-of-function mutations, suggesting that both products act in a common process.

The dominant suppression of the Bic-D^{D} phenotype by genes involved in the pathway of oocyte determination suggested that a screen for dominant suppressors of the Bic-D^{D} phenotype could be useful as a means of identifying further components in the pathway of oocyte development and/or pole plasm formation. Here, I present the results of such a screen. As expected, the screen for suppressors of *Bic-D^{D}* mutations led to the isolation of new *vas* and *egl* alleles. In addition, we isolated mutations in several complementation groups that appear to be required for viability and/ or oogenesis.

MATERIALS AND METHODS

Genetic analysis and strains: The dominant *Bic-D* mutation used in the genetic screen was *Bic-D$^{71.34}$* (Nüsslein-Volhard et al. 1984; Wieschaus et al. 1984; Mohler and Wieschaus 1986). The *staufen* mutation used was *stau^{D3}* (Lehmann and Nüsslein-Volhard 1991). See legend of Figure C.2 for a description of the screen. Embryos were examined under a dissecting microscope after clearing with a thin film of oil. Recombinational mapping was carried out using the multiply marked chromosome *S Sp Tft mam^{N2G} Pu^{2}*.

RESULTS

A screen for suppressors of the dominant Bicaudal-D phenotype was carried out as shown in Figure C.2. Heterozygosity for the *stau* mutation was used in the screened mutant background to increase the penetrance of the *Bic-D^{D}* mutation (Lehmann and Nüsslein-Volhard 1991). Our preliminary results suggested that the enhancement of the Bic-D^{D} phenotype by the *stau* mutation did not affect the strong dominant suppression phenotype exhibited by mutations in *egl* and *vas*.

About 7,600 *Bic-D^{D} stau* / + + females carrying newly induced mutations were screened for suppression of the Bic-D^{D} phenotype. Of these, 189 (2.5 %) females were

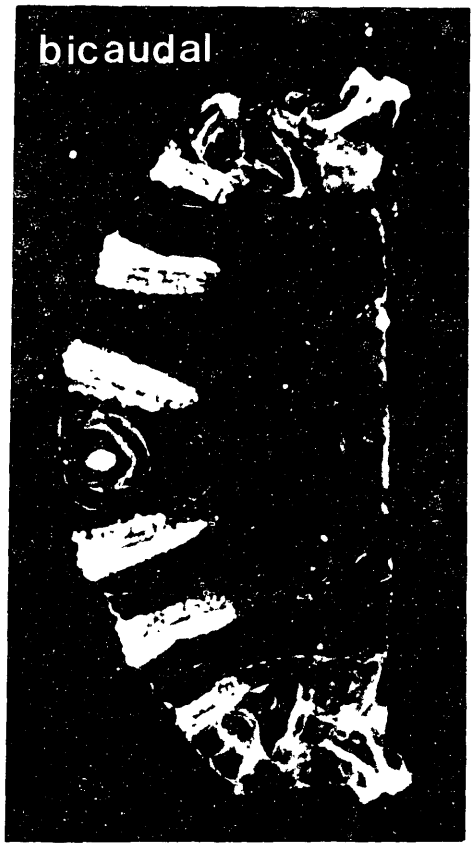


Figure C.1. The bicaudal phenotype. Embryo exhibiting two mirror image abdomens. (Photo courtesy of Jen Mach)

separated into vials and retested for suppression of *Bic-D^{D}*. Upon retest, 36 lines (0.4%) seemed to contain a second chromosomal suppressor of *Bic-D^{D}*. These chromosomes were saved as balanced second chromosome stocks.

The combination of an inter se matrix and genetic mapping has allowed the classification of 13 mutations into 4 or 5 complementation groups. The following is a brief description of these groups:

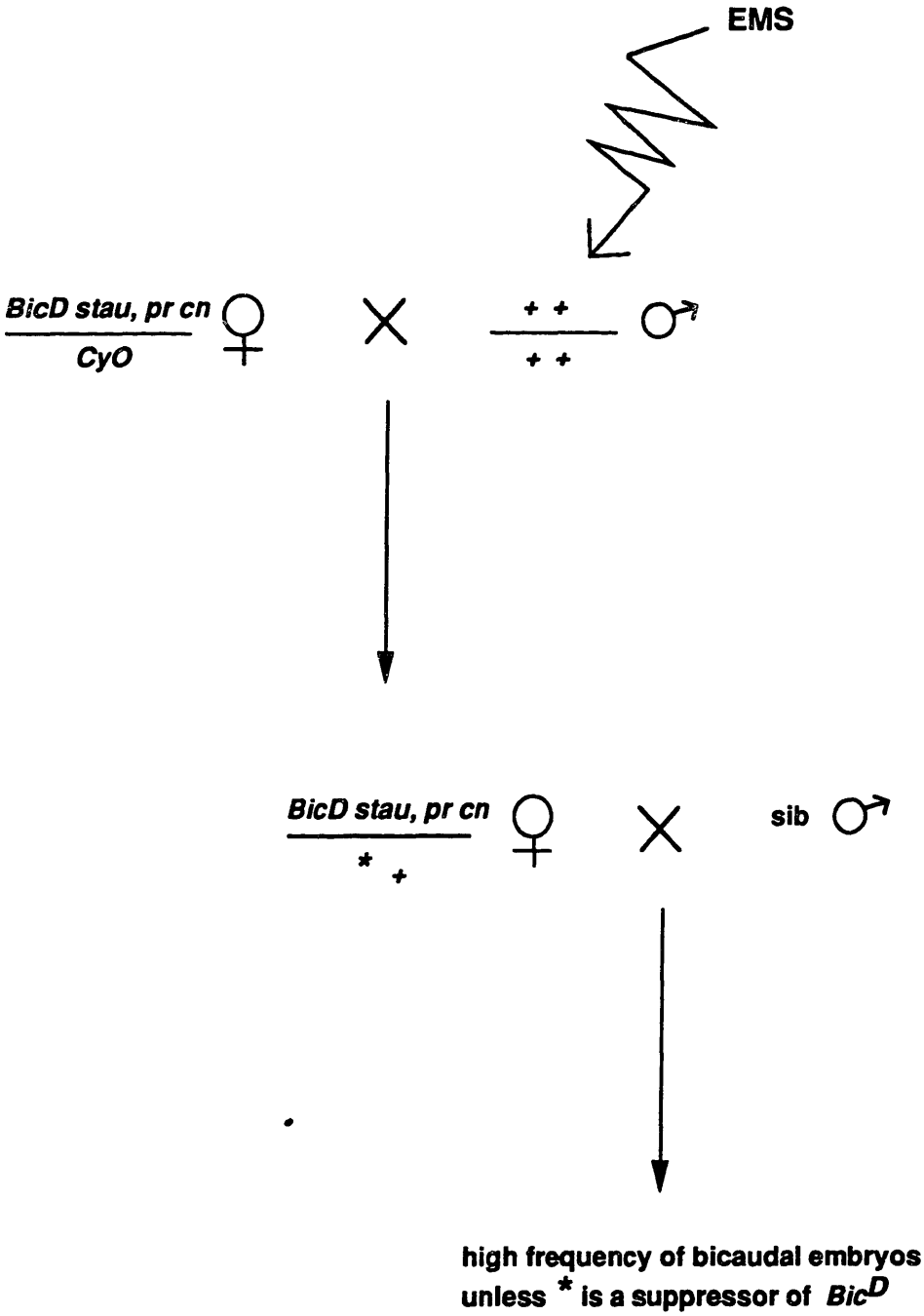
The *vasa* group: At least three new vasa alleles have been identified: *vas^{2c}*, *vas^{3f}*, and *vas^{4c}*. Inter se combinations of these mutations lead to egg and embryonic defects characteristic of *vas* mutations. Genetic mapping shows that these three alleles map near 2-50, a position very close to that of the *vas* locus (2-51). In addition, the mutation *Su(Bic-D^{D})^{6e}* interacts with these three alleles to produce unfertilized and apparently undifferentiated embryos and maps within the *Sp- Tft* interval, which includes the *vas* locus. Thus this mutation may be an additional *vas* allele.

The *egalitarian* group: Four mutations appear to be newly induced alleles of *egl*: *egl^{2b}*, *egl^{2e}*, *egl^{3e}* and *egl^{4e}*. Females transheterozygous for combinations of these alleles lay very few eggs, if any. The mutations *egl^{2b}*, *egl^{3e}* and *egl^{4e}* have been mapped to the right hand side of the *mam^{N-2G}* (map position: 2-70.3) mutation, a position consistent with them being alleles of *egl* (map position: 2-104). One of the alleles, *egl^{3e}*, is particularly interesting, since it produces only a few eggs. This is the only known *egl* leaky allele.

The *Su(Bic-D^{D})-Lethal* (*Su(Bic-D^{D})^{L}*) group: Three mutations: *Su(Bic-D^{D})L1e*, *Su(Bic-D^{D})L5e* and *Su(Bic-D^{D})L6b* have been mapped to the same interval, between the *Sp* and *Tft* markers (2-22 to 53.6). *Su(Bic-D^{D})L6b* and *Su(Bic-D^{D})L5e* are lethal in trans to each other. The combinations *Su(Bic-D^{D})L6b* and *Su(Bic-D^{D})L1e* , and *Su(Bic-D^{D})L5e* and *Su(Bic-D^{D})L1e* are viable in trans to each other, and females exhibit a very high frequency of infertility (> 95% of the eggs appear unfertilized).

The *Su(BicD)-Sterile* (*Su(Bic^{D})^{S}*) group: This group consists of two mutations *Su(Bic-D^{D})S3b* and *Su(Bic-D^{D})S5c*. Both of these mutations have been mapped to the *mam^{N-2G}-Pu^{2}* interval (2-70.3 to 97). Transheterozygous combinations of these two alleles are 100% sterile. In addition, *Su(Bic-D^{D})S5c* homozygotes are also 100% sterile. In both cases, the eggs appear to be unfertilized. *Su(Bic-D^{D})S3b* homozygotes are not viable. It is unknown whether this is related to a second mutation in the same chromosome, or whether *Su(Bic-D^{D})S*, like *Su(Bic-D^{D})L*, is also required for viability.

Figure C.2


EMS
BicD stau, pr cn
CyO
+ +
+ +
BicD stau, pr cn
* +
sib
high frequency of bicaudal embryos
unless * is a suppressor of Bic^D

It appears that these mutations form a set of interacting products. For example, *Su(Bic-D^{D})L1* and *Su(Bic-D^{D})L6b* are, respectively, 95% and 75% sterile in trans to *vas^{3f}*, *Su(Bic-D^{D})L5e* is 70% sterile in trans to *egl^{3e}*, and *Su(Bic-D^{D})S3b* is 90% sterile in trans to *vas^{6e}*.

Conclusion

Here, I present the results of a screen for suppressors of the dominant Bicaudal-D phenotype. As expected, new alleles of the genes *vas* and *egl* were isolated. In addition, two new complementation groups were identified. These genes, *Su(Bic-D^{D})L* and *Su(Bic-D^{D})S*, are required for viability and/or oogenesis. The isolated suppressor-of-*Bicaudal-D^{D}* mutations appear to interact with each other. Because *Bic-D* and *egl* are involved in oocyte determination and all the isolated genes interact with *Bic-D^{D}* and with one another, it is possible that they form part of a genetic network involved in oocyte differentiation.

Figure C.2. Screen for dominant suppressors of the dominant Bicaudal-D phenotype. Mutagenized wild-type chromosomes were introduced into a *Bic-D^{D} stau* mutant background. *Bic-D^{D} stau* doubly heterozygous females carrying newly induced mutations were tested in groups of six. Females from pools that produced a higher than background proportion of hatching embryos were separated into individual vials. Suppressor mutations were recovered from the progeny of the "suppressed" females. Retests and the creation of balanced stocks were carried out using appropriate balancer chromosomes. *pr* and *cn* are visible recessive markers.

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