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

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Manuscript received October 7, 1993
Accepted for publication December 9, 1993

ABSTRACT

Anteroposterior polarity of the Drosophila embryo is initiated by the localized activities of the maternal genes, bicoi'd 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\textsuperscript{mat}) to a gradient of zygotic Hb protein (Hb\textsuperscript{zg}) 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\textsuperscript{zg} 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 NÜSSLLEIN-VOLHARD 1986; BERLETH et al. 1988). Bcd RNA translation results in an anterior to posterior concentration gradient of Bcd protein (DRIEVER and NÜSSLLEIN-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 NÜSSLLEIN-VOLHARD 1989; DRIEVER et al. 1989; STRUHL et al. 1992).

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...
A gradient of either Hbmut or Hb78 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 Hb78 protein attains the high levels necessary for Kr repression) (Huls Graham 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 Hbmut is translated throughout the embryo. Uniformly high levels of Hbmut repress transcription of the abdomen-specific gap genes kni and gt and therefore these embryos lack abdomen. Since Hb is the major repressor of gap gene expression in nos mutants (Hüs Graham et al. 1989; Ikish et al. 1989a; Struhl 1989), we reasoned that additional genes required for the production or the activity of the Hbmut 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 Hbmut 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:** hB78 is a protein null (Lehmann and Nüsslein-Volhard 1987; Tautz 1988), nos−/− 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). hB78/nos+/?TM3 females were crossed to nos+/? homozygous males mutagenized with either 35 mm or 45 mm ethyl methanesulfonate (EMS). F1 hB78/nos+/?nos+/? females were tested in groups for producing hatching embryos. Single females were retested and lines were established from the F2 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 from the 35 mm batch and 3,482 from the 45 mm batch. To determine the efficiency of mutagenesis we also screened from the 35 mm batch and 3,482 from the 45 mm batch.

**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°C (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°C) 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)−/− mutations are alleles of Enhancer of zeste (synonym: polycombetic).

**Map position:** son1 and son2 map within 5 map units of E(z) (3-34.0), son1 to the 3-26.5 to 3-43.2 interval.

**Associated phenotypes:** E(z)+/nos alleles, like other E(z) alleles are larval lethal when trans-heterozygous. The alleles E(z)−/− and E(z)−/nos are lethal in trans to E(z) null alleles or deficiencies, E(z)−/nos 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)+/nos alleles (zygotic genotypes tested: E(z)+/E(z)+, E(z)+/E(z)−, and E(z)+/E(z)+nos), and reduces their suppressor-of-nos effect (maternal genotypes tested: in a hB78 heterozygous background, E(z)+/+ and E(z)+/nos+; in a wild-type hB78 background, E(z)+/E(z)+ and E(z)+/E(z)+nos). 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)E6, E(z)+, E(z)+ (deficiencies, E(z)+ partial deletion, weakly antimorphic, Jones and Gelbart 1993), E(z)−, E(z)+/E(z)+ (nulls, E(z)+ slightly antimorphic); E(z)+ (temperature sensitive, see legend of Table 1). E(z)+, E(z)+ (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 Brink et al. 1991)]. Su(z)2/TM3 Df(2R)vg-B [deletions of Psc, Su(z)2 and Su(z)2-Distal (Su(z)2-2/D)]; Su(z)2/TM3 Df(2)3.1 Df(2)3.1 (null), Su(z)2/TM3 Df(2)3.1 (null); [deletions of Psc, Su(z)2 and Su(z)2-Distal (Su(z)2-2/D)]; Su(z)2/TM3 Df(2)3.1 (null), Su(z)2/TM3 Df(2)3.1 (null); [deletions of Psc, Su(z)2 and Su(z)2-Distal (Su(z)2-2/D)]; Su(z)2/TM3 Df(2)3.1 (null), Su(z)2/TM3 Df(2)3.1 (null); [deletions of Psc, Su(z)2 and Su(z)2-Distal (Su(z)2-2/D)]; Su(z)2/TM3 Df(2)3.1 (null), Su(z)2/TM3 Df(2)3.1 (null); [deletions of Psc, Su(z)2 and Su(z)2-Distal (Su(z)2-2/D)].

**Pole cell transplants:** OvoD females were used as hosts for pole cell transplants as described in Lehmann and Nüsslein-Volhard (1987). OvoD females were used as hosts for pole cell transplants as described in Lehmann and Nüsslein-Volhard (1987).
Phenotype and/or other associated defects. At 25°C, 8/16 fertile females (presumably E(z)\textsuperscript{um1} nos/E(z)\textsuperscript{um2} nos) produced very few, abnormal eggs, most of which did not form cuticles. At 18°C, 3/4 fertile adult females (presumably E(z)\textsuperscript{um1} nos/E(z)\textsuperscript{um2} 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 reached adulthood. Many (84%, n = 32) showed head involution defects, similar to those observed in embryos with mild homeotic transformations (see, for example, JURGENS 1985).

\(E(z)\textsuperscript{um1} nos/E(z)\textsuperscript{um2} nos\) germ line clones: 8/25 fertile females exhibited phenotypes similar to those observed in females carrying \(E(z)\textsuperscript{um1} nos/E(z)\textsuperscript{um2} nos\) 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 \(E(z)\textsuperscript{son}\) 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 (\(E(z)\textsuperscript{um1} nos^+\)). A more detailed description of these germ line clone results will appear elsewhere.

Analysis of expression patterns: \(E(z)\textsuperscript{um2}/E(z)\textsuperscript{um1}\) females were grown at 25°C and eggs were collected from young females. Mutant backgrounds: nos\textsuperscript{L7} homozygotes, bcd\textsuperscript{11} homozygotes, gt\textsuperscript{18} hemizygotes, ts\textsuperscript{F50}/ts\textsuperscript{18} trans-heterozygotes.

Whole-mount in situ hybridization with digoxigenin-labeled RNA probes for kni, gt, 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. JACKE (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\textsuperscript{ts} 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 \(hb\) \(nos^+/+\) females (Table 1) (HÜLSKAMP et al. 1989; IRISH et al. 1989; STRUHL 1989). We screened for EMS-induced mutations that allowed embryos from \(hb\) \(nos^+/+\) females to develop into adults (Figure 1). After screening approximately 13,000 F\textsubscript{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 \(E(z)\)\textsuperscript{um1}, \(E(z)\)\textsuperscript{um2} and \(E(z)\)\textsuperscript{um3}. 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 discs 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.

\(E(z)\)\textsuperscript{um3} 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 \(E(z)\)\textsuperscript{um} 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).

\(E(z)\)\textsuperscript{um} mutations are semidominant, and suppression of the \(nos\) phenotype is much stronger in embryos from \(nos\) females homozygous for \(E(z)\)\textsuperscript{um} (see Table 1). Table 1 shows the dominant maternal effect of \(E(z)\)\textsuperscript{um} 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 \(E(z)\)\textsuperscript{um} alleles. Thus the \(E(z)\)\textsuperscript{um} 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 \(E(z)\)\textsuperscript{um} 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 \(E(z)\)\textsuperscript{61} which has significantly reduced \(E(z)\) activity at the restrictive temperature (JONES and GELBART 1990).
We found that homozygosity or hemizygosity for $E(z)^{61}$ at semipermissive (25°C) and restrictive (29°C) 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 $E(z)^{nos}$ mutations are specific for abdomen formation. This specificity of the $E(z)^{nos}$ alleles is demonstrated most clearly by the phenotype of embryos derived from germ line cells mutant for the two strongest $E(z)^{nos}$ alleles ($E(z)^{ven}$ and $E(z)^{nos3}$). 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 $E(z)^{nos}$ 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
propose that the $E(z)$ protein is a complex molecule and that the $E(z)^{nos}$ mutations affect a specific function of this protein.

**$E(z)$ is required for maintenance of gap gene repression:** To understand how $E(z)^{nos}$ 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 $E(z)^{nos2}/E(z)^{y}$, 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) (Rotth et al. 1989; Eldon and Pirrotta 1991; Krut 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 $wasa$ 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-
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 \(Hb^{ma}\) 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 \(Hb^{ma}\) 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 \(Hb^{ma}\) protein. At the syncytial blastoderm stage, when the maternal \(Hb^{ma}\) protein is no longer detectable, \(hb^{ inefficient}\) is transcribed in the anterior half of the embryo under the control of \(bcd\). The concentration of either \(Hb^{ma}\) or \(Hb^{ inefficient}\) along the anteroposterior axis establishes the anterior boundaries of \(kni\) and \(gt\) expression (Hulskamp 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 \(Hb^{ma}\) but is dispensable if anteriorly expressed genes, such as \(Hb^{ efficiency}\), 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 \(Hb^{ma}\) gradient, we tested the effect of \(E(z)\) in \(bcd\) mutant embryos. In these embryos, the only source of anteroposterior polarity is the \(Hb^{ma}\) 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 \(Hb^{ma}\) 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
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\textsuperscript{mut} protein initiates the restrictions of kni and gt expression. At the cellular blastoderm stage, when Hb\textsuperscript{mut} 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\textsuperscript{mut} and E(z) mediated regulation. A 1.8-kbp fragment of the kni promoter fused to the *lar 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\textsuperscript{mut} we first examined the expression of the transgene in the progeny of nos and bcd mutant females. Like the endogenous kni expression,
This embryo shows an antl pattern in a bryos from pressed females (Figure 4A). 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) bed tsl embryos. (B) The anterior boundary of kni expression expands anteriorly in E(z) bed 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, bedc 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 bedc 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) bedc 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

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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. 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. 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, STAIRNS 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 and TAMMINEN 1988), activators and repressors of homeotic genes (KARRISON AND TAMKIN 1988), and products involved in signal transduction during photoreceptor cell determination (SIMON AND BISHOP 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 abdome-specific gap genes knirps (kni) and giant (gt). Our genetic analysis indicates that E(z) maintains the proper
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. 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 hh<sup>mat</sup> RNA is deregulated resulting in high levels of Hb<sup>mat</sup> protein throughout the prospective abdominal region (Tautz 1988; Wang and Lehmann 1991). Hb<sup>mat</sup> 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<sup>mat</sup>. In bcd embryos, hh<sup>p</sup> is not expressed, and the Hb<sup>mat</sup> 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<sup>mat</sup> protein. We were unable to detect any significant reduction in the levels of Hb<sup>mat</sup> in embryos from E(z) females. Thus, E(z) does not seem to affect the production of Hb<sup>mat</sup> protein. In addition, the effects of E(z) mutations in embryos are only apparent when the Hb<sup>mat</sup> 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)bed embryos than in Kr;bed double mutant embryos [this report and Struhl et al. (1992)]. We favor the idea that E(z), like hh<sup>mat</sup>, 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 hh<sup>mat</sup> (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-
The determination of anterior boundaries of gap gene expression by Hb<sup>mat</sup> 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 PeG-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<sup>mat</sup>) obviate the absolute requirement for the PeG-function in the determination of gap gene boundaries. Regulators like Hb<sup>mat</sup> may not require a PeG-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 PeG genes.

**Polycomb group genes and the chromatin link:** The PeG 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 PeG 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 PeG genes are involved in the negative regulation of gap genes by Hb<sup>mat</sup>. At least one PeG 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 PeG 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 PeG 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 the

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

<sup>a</sup>A "+" refers to the wild-type copy of the particular PeG gene tested.

<sup>b</sup>Percent of embryos with ≥3 abdominal segments.

<sup>c</sup>Data shown is for the deficiency Su(z)2<sup>1/67</sup>. A similar result was obtained using the deficiency Df(2R)vg-B.

<sup>d</sup>Test performed at 18°.

**Figure 7.—Suppression of the nos phenotype by other Polycomb group genes.** Cuticle preparations of larvae derived from females mutant for nos and various PeG 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.
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1.751

**FIGURE 8.**—Polycomb group genes are required for two anteroposterior patterning pathways during embryogenesis. (Left side) Model for gap gene regulation by Hb<sup>mat</sup> and Pc-G genes as proposed in this article (top, syncytial stages; bottom, cellular blastoderm stage). The situation depicted is that in hbd mutant embryos, where the only source of anteroposterior polarity is the Hb<sup>mat</sup> gradient. During the syncytial stages Hb<sup>mat</sup> establishes the boundaries of gap genes such as gt (shown) and kni. By the end of the blastoderm stage, when Hb<sup>mat</sup> 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<sup>mat</sup> and Pc-G genes as previously proposed (top, beginning of gastrulation stage; bottom, germ band extended stage). Gap gene products (e.g., Hb<sup>mat</sup>) 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).

Redundant gradients in AP axis determination: The discovery that nos function is dispensable for abdomen formation in the absence of functional Hb<sup>mat</sup> product raises the question about the importance of the nos-dependent patterning system. In particular, Hb<sup>mat</sup>, 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<sup>mat</sup> (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<sup>mat</sup> 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 maternal 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.

We are grateful to Anne Ephrussi for her collaboration in the screen for suppressors of nos. We also thank all members of the Lehmann laboratory, and specially Doug Barker and Liz Gavis, for help and advice at different stages of this project. We are indebted to Rick Jones and Michael Pankratz for sending us fly stocks carrying the E(s) transcription unit and the kni promoter/lac Z fusion constructs prior to publication. We also thank Margaret Fuller, Rick Jones, Pedro Santamaria, Jeff Simon and C.-Ting Wu for sending us fly stocks. Finally, we thank Steve Burden and Dan Curtis for criticisms on the manuscript. This work was supported by a David and Lucile Packard fellowship. R.L. is an associate investigator of the Howard Hughes Medical Institute.

LITERATURE CITED


