A Polycomb and GAGA Dependent Silencer Adjoins the Fab-7 Boundary in the Drosophila Bithorax Complex

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ABSTRACT

The homeotic genes of the Drosophila bithorax complex are controlled by a large cis-regulatory region that ensures their segmentally restricted pattern of expression. A deletion that removes the Frontal-7 cis-regulatory region (Fab-7) dominantly transforms parasegment 11 into parasegment 12. Previous studies suggested that removal of a domain boundary element on the proximal side of Fab-7 is responsible for this gain-of-function phenotype. In this article we demonstrate that the Fab-7 deletion also removes a silencer element, the iab-7 PRE, which maps to a different DNA segment and plays a different role in regulating parasegment-specific expression patterns of the Abd-B gene. The iab-7 PRE mediates pairing-sensitive silencing of mini-white, and can maintain the segmentally restricted expression pattern of a BXD, Ubx/lacZ reporter transgene. Both silencing activities depend upon Polycomb Group proteins. Pairing-sensitive silencing is relieved by removing the transvection protein Zeste, but is enhanced in a novel pairing-independent manner by the estate allele. The iab-7 PRE silencer is contained within a 0.8-kb fragment that spans a nuclease hypersensitive site, and silencing appears to depend on the chromatin remodeling protein, the GAGA factor.

DISRUPTIONS in development arise not only by failing to appropriately activate genes but also by failing to maintain their inactivity. A variety of different mechanisms are used to negatively regulate gene activity. In the best understood cases, a negative regulator blocks gene expression either by competing with a positive regulator for the same target sequence or by inactivating a positive regulator through direct protein-protein interactions (Jackson 1991; Johnson 1995). Less understood are the negative cis-regulatory elements called silencers. Like enhancers, silencers are able to regulate transcriptional activity from a distance, are often orientation independent and can act on different promoters (Brand et al. 1985). Silencers may repress gene activity by binding silencing proteins that inhibit the function of activator proteins or that directly interfere with the transcriptional machinery (Gray and Levine 1996). Alternatively, proteins bound to the silencer may repress gene activity by altering the chromatin structure (Laurenson and Rine 1992; Moehle and Paro 1994).

For most of the well-characterized silencers a single copy of the element is sufficient to repress a reporter gene in cis. However, recent studies in Drosophila have uncovered a novel class of regulatory elements whose silencing activity depends upon the presence of two copies of the element that are brought together by chromosomal pairing. This "pairing-sensitive silencing" is an example of a phenomenon in Drosophila known as "transvection," in which regulatory interactions occur in trans between elements on different chromosomes (Lewis 1954; reviewed in Tartof and Henikoff 1991; Wu 1993). The first example of such a "pairing-sensitive silencer" was discovered upstream of the en (en) gene (Kassis et al. 1991; Kassis 1994). When this regulatory element is included in a mini-white transgene, it represses or even eliminates mini-white expression when the animals are homozygous for the transgene insert. Like classical transvection, pairing-sensitive silencing seems to depend upon whether these elements can pair. Silencing is observed in animals homozygous for the same engrafted, mini-white insertion, but is usually not found in animals that have the transposon inserted at two different locations (Kassis et al. 1991). Regulatory elements that induce pairing-sensitive silencing have also been found at other developmental loci, including polyhomeotic (ph) (Fauvarque and Dura 1993), the homeotic genes Sex combs reduced (Scr) and proboscipedia (pb) of the Antennapedia (ANT-C) complex (Gindhart and Kaufman 1995; Kapoun and Kaufman 1995), and Ultrabithorax (Ubx) of the bithorax complex (BX-C) (Chan et al. 1994).

The pairing-sensitive silencers from the ANT-C and BX-C appear to be components of the elaborate cis-regulatory regions associated with each complex. These cis-regulatory regions are crucial for generating the proper parasegment-specific patterns of expression of
the ANT-C and BX-C homeotic genes. The cis-regulatory region of the BX-C spans more than 300 kb and is subdivided into nine domains (abx/bx, bxd/pbx, iab-2, iab-3, iab-4, iab-5, iab-6, iab-7, and iab-8) that direct the appropriate parasegmental transcription of the three homeotic genes: Ubx, abdominal-A (abd-A), and Abdominal-B ( Abd-B) (Duncan 1987). For example, the expression of Abd-B in the posterior parasegments (PS) PS10, PS11, PS12, and PS13 is directed by four cis-regulatory units, iab-5, iab-6, iab-7, and iab-8 (see Figure 1A). Loss-of-function mutations in one of these Abd-B cis-regulatory units transforms the corresponding parasegment into a copy of the parasegment immediately anterior (Karch et al. 1985).

The expression of the BX-C (and also the ANT-C) homeotic genes is regulated in two phases: initiation and maintenance. Early in development, gap and pair-rule gene products interact with target sequences in the nine cis-regulatory domains to initiate the parasegment-specific expression of the BX-C homeotic genes (e.g., Ingham and Martinez Arias 1986; White and Lehmann 1986; Shimell et al. 1994; Casares and Sanchez Herrero 1995). However, since the products of these early patterning genes are present only transiently, regulation switches to a maintenance mode that recognizes and propagates the initial pattern through the remainder of development. Maintenance requires the Polycomb-Group ( Pc-G) and trithorax-Group (trx-G) genes (reviewed in Paro 1990; Kennison 1993; Simon 1995). The products of the Pc-G genes function as negative regulators to maintain the inactive state of the homeotic genes. Experiments with homeotic reporter constructs have identified elements in several of the BX-C cis-regulatory domains that appear to be targets for Pc-G action. When these elements (called Polycomb response elements or PREs) are combined with parasegment-specific initiation elements, they are able to maintain segmentally restricted patterns of expression conferred on the reporter by the initiation elements (Simon et al. 1990, 1993; Muller and Bienz 1991; Busturia and Bienz 1993; Chan et al. 1994; Chiang et al. 1995; Gindhart and Kaufman 1995). Interestingly, some of the DNA sequences that function as PRE maintenance elements have also been found to cause Pc-G-dependent pairing-sensitive repression of mini-white (Chan et al. 1994; Gindhart and Kaufman 1995).  

In this article we describe a pairing-sensitive silencer associated with the Frontal abdominal-7 (Fab-7) region of the BX-C. Fab-7 has been defined by three small deletions, Fab-7', Fab-7', and Fab-7', in the DNA segment between the iab-6 and iab-7 cis-regulatory domains (Gyurkovics et al. 1990; Galloni et al. 1995). The original Fab-7 allele removes a 4kb DNA segment that contains a cluster of three major (HS1, HS2, and HS3) and several minor chromatin-specific nucleosome hypersensitive sites (see Figure 1B). The other two deletions are smaller than Fab-7' and are derived from the imprecise excision of a transposon, called Bluetail, inserted between HS2 and HS3 (Figure 1B). Both extend proximally from the Bluetail insertion site and remove HS1 and HS2, but leave HS3 intact. The three Fab-7 mutations have two unusual features that distinguish them from most other BX-C mutations. While mutations in a BX-C cis-regulatory domain such as iab-6 or iab-7 are typically recessive and have a loss-of-function phenotype, the Fab-7 mutations are dominant and have a gain-of-function phenotype. Thus, instead of the posterior to anterior transformation normally seen in BX-C mutants, the opposite phenotype is observed for the Fab-7 mutants: PS11 is transformed into a copy of PS12 (Gyurkovics et al. 1990). Consistent with this phenotypic transformation, Abd-B is expressed in a PS12-like pattern in PS11 (Galloni et al. 1993). The two smaller deletions Fab-7' and Fab-7' (but not Fab-7) also occasionally exhibit a second unusual phenotype in PS11: the clonal transformation of a small group of cells from PS11 to PS10 identity (Galloni et al. 1993; Mihaly et al. 1997).

Two models have been proposed to account for the genetic properties of the Fab-7 mutants. The first is that Fab-7 is a parasegment-specific silencer that is required to repress iab-7 in PS11 (cf. Busturia and Bienz 1993; Zink and Paro 1995). The second is that Fab-7 functions as a domain boundary that insulates iab-7 from cross-talk with regulatory elements in iab-6 (Gyurkovics et al. 1990; Galloni et al. 1993). Without the intervening boundary, the adjacent iab-6 and iab-7 regulatory domains “fuse,” leading either to the ectopic activation of iab-7 in PS11 (changing the identity of PS11 cells to PS12) or the ectopic silencing of iab-6 in PS11 (changing the identity of PS11 cells to PS10).

Using a white enhancer-mini-white reporter assay system we have found that the DNA sequence deleted in the larger Fab-7' mutation contains two distinct elements: an element that can function as a domain boundary in enhancer blocking assays (Hagstrom et al. 1996; see also Zhou et al. 1996), and an element that can function as a silencer (without parasegmental specificity). The studies reported here focus on the characterization of the silencer. In our mini-white assay system, this silencer induces pairing-sensitive repression of mini-white expression. We show that the silencing activity maps to a 0.8-kb DNA fragment that contains HS3. By contrast, the sequences required for Fab-7 boundary function either in the context of bithorax complex itself (Galloni et al. 1993; Mihaly et al. 1997) or in enhancer blocking assays using transgenes (Hagstrom et al. 1996) map to HS1 and HS2. The silencing activity of the HS3 fragment depends upon Pc-G proteins, the transsection protein Zeste and the chromatin remodeling protein, GAGA. Finally we present evidence that the HS3 fragment is likely to correspond to an iab-7 PRE that func-
tions to maintain the 

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MATERIALS AND METHODS

Generation of P element constructs and transgenic lines: The basic construct is a P element derived from the "pRW" vector described in Vazquez and Schedel (1994). It was modified to contain a white enhancer fragment extending only from BamHI to HindIII (bp 4433 to bp 5540 in O'Hare and Schedel 1985; Pirrotta et al. 1985) as well as a number of Zeste protein binding sites (Benson and Pirrotta 1988).

The various Fab-7 restriction fragments described in Figure 1 were isolated from 3.35-kb HincII to XhoI Fab-7 fragment inserted in Bluescript. This Fab-7 region was originally isolated from phage N8053 (Karch et al. 1985) and spans bp 163-3517 as indicated in (Karch et al. 1994).

Each construct (0.5 mg/ml) was injected along with 0.1 mg/ml of P-turbo helper plasmid (pUCHspA2-3wc) into w6 embryos, and transfectants were selected by rescue of the w6 eye color. Homozygous balanced transfectants were established. Flies were raised and examined at 22°C unless otherwise stated.

Localization of inserts by in situ hybridization: Polytenic chromosome squashes and in situ hybridization with a biotinylated probe of pCasPER were performed as described in Ashburner (1989).

Crossovers to mutant strains: Extra Y: Males from the stock C(1) RM, Y to f/X Yw11180 (provided by D. Dorer and S. Henikoff) contain an attached X Y (Dorer and Henikoff 1994). These males were mated to homozygous transgenic females. The resulting female progeny are heterozygous for the P element and carry a Y chromosome, while male progeny lack a Y chromosome. These were crossed to homozygous transgensics generated by backcrossing to w+6. Attached X Y males were also crossed to the variegating rearrangement of w6 (P+c17) to confirm their interaction with this P element.

Pc-G, trz-G, GAGA factor: Crosses of autosomal transgenic inserts to mutant alleles that were on a different chromosome and had a visible phenotype as heterozygotes (e.g., extra sex combs for Pclom, Se6, Pcl3, and Su(z)26) and halters to wing transformation for trz(2) were performed as follows. Homozygous transgenic females (P/P) were crossed to mutant/balancer (mut/bal) males. In the next generation, P/+; mut/+ males were compared to P/+ generated by a backcross to w6. These flies were then crossed to females from the double balancer stock w+151; Goy TM3 Sb/Xa. Progeny carrying P/bal; mut/bal were selected and crossed back to the homozygous transgenic stock. In the final generation, P/F; mut/+ flies were compared to P/F stock.

The mutant alleles with visible phenotypes were also tested for interactions with some transgenes on the same chromosome by recombination. P/F females were crossed to mut/bal males. P/F mut female progeny were collected and crossed to a balanced stock. Balanced transfectants in the next generation were selected by eye color and the mutant phenotype, then crossed to w6 or P/F to inspect the effect of the mutation on P/+ or P/F, respectively.

If the mutant allele had no obvious phenotype (hmb2, kis2, kdt1, Tl(1)kk, Tp(1)kk), then both the mut stock and the transgenic stock were crossed to the double marked, double balancer w1118; SpCyO; Gcd/TM3 Sb. From the mutant cross, males that are mut/marked chromosome; +/bal are collected and mated to females from the transgene cross that are +/+; bal/other marked chromosome. In the next generation, P/F; bal/mut flies are selected. These are crossed to w6 or P/F to generate P/+; mut/+ or P/F; mut/+ flies for comparison to P+ or P/F.

For all comparisons, flies of the same sex and age were compared. In all cases the balancer chromosomes were not present in the final genotypes to avoid the potential effects of this difference in genetic background.

Zeste: Crosses to generate P/P; z/Y were performed using a double marked, double balancer line as described above and compared to P/F; z/Y. Homozygous transgenic males were crossed to z/Y; w1118 homzygous females (provided by J. Kassis). The resulting z/Y; w1118/Y; P/+ males and z/Y; w1118/Y; P/+ females were compared to P/+ males and females.

Pc cross to Fab-7 P RE, BND, Ubx/lacZ maintenance lines: Homozygous Fab-7 PRE, BXD, Ubx/lacZ lines on the second chromosome were crossed to Pc3/TM6 Sh. In the next generation, P/+; Pc+/+ males and females were selected and mated, and their embryos were stained. Pc+ mutant embryos were detected by the obvious ectopic expression pattern.

Immunological detection of β-galactosidase activity: Zero-to 15-hr embryos were collected and β-galactosidase activity was visualized using the antibody staining protocol outlined in Patel (1994). A mouse monoclonal antibody against β-galactosidase (Promega, used at 1:500) was followed by a goat anti-mouse IgG conjugated to AP (Jackson Immuno Research Labs, used at 1:300), then an alkaline phosphatase reaction as described.

RESULTS

Fab-7 can mediate the pairing-sensitive repression of white. In experiments initially aimed at determining whether Fab-7 has boundary activity, we tested Fab-7 fragments in an enhancer blocking assay consisting of the eye and testes enhancers from the white locus and the mini-white reporter gene. A 3.5-kb Fab-7 fragment that includes the three strong and several weak nuclease hypersensitive sites deleted by the Fab-7 mutation (Figure 1B; Galloni et al. 1993) was introduced into this vector, either between the enhancer and the promoter (Figure 1C, 1), or upstream of the enhancer (Figure 1C, 2). Although the 3.3-kb Fab-7 fragment functioned as a boundary element in this mini-white assay system (blocking enhancer-promoter communication when placed between the enhancer and the promoter: Hagstrom et al. 1996; Zhou et al. 1996), it also had silencing activity. For both constructs 1 and 2, we observed a silencing activity in ~10% of the transformants. Normally animals homozygous for the white enhancer:mini-white transgene have darker eyes than their heterozygous siblings (Figure 2A). However, when Fab-7 DNA is included in the transgene, the homozygotes of these unusual lines have a lighter, not darker, eye color than siblings who carry only a single copy of the transgene (Figure 2, B-D). As mentioned in the
**FIGURE 1.**—Schematic of the *Abd-B* cis-regulatory domains and the *Fab-7* region; summary of the *P* element constructs and silencing results. (A) Expression of the *Abd-B* gene (arrow) in parasegments (PS) 10 through PS13 is controlled by the parasegment-specific cis-regulatory domains *iab-5* through *iab-8*. Boundary elements (boxes) are thought to define and insulate the domains. The *Fab-7* region lies between *iab-6* and *iab-7*, and appears to contain both a boundary element and a silencer (circle). (B) A 3.35-kb *HindIII* to *XbaI* fragment from the *Fab-7* region includes one minor and three major nuclease hypersensitive sites (HS1, HS2, and HS3) whose extent is indicated by boxes (KARCH et al. 1994). Half-ovals represent the presumed position of nucleosomes. The dotted lines above indicate the extent of three *Fab-7* deletions: *Fab-7<sup>3</sup>* is the original deletion and transforms PS11 to PS12 (GVRKOVICS et al. 1990). The PS11 to PS12 transformation is often incomplete in *Fab-7<sup>1</sup>* and *Fab-7<sup>2</sup>*; and in these mutants small clones of cells are sometimes observed in PS11 that have a PS10 (rather than a PS12) identity (GALLONI et al. 1993; MIHALY et al. 1997). The insertion site of the *BlueTail* transposon, which is subject to control by *iab-7* but not *iab-6*, is also shown (GALLONI et al. 1993). Brackets below indicate the extent of the *Fab-7* boundary (HAGSTROM et al. 1996) and the element defined in this article as the *iab-7 PRE*. *H*, *HindIII*; *N*, *NsiI*; *A*, *ApaI*; *E*, *EcoRI*; *X*, *XbaI*. (C) The *P* element constructs include a white enhancer fragment that drives expression in the eye and testes, a restriction fragment of *Fab-7* (restriction sites correspond to those in B), the *mini-white* eye color gene, and *ses* to minimize position effects from this side (KELLUM and SCHEDL 1991). On the right are shown the number of independent transformant lines out of the total generated that show reduced *mini-white* expression when the lines are made homozygous (pairing-sensitive lines/total).
**Figure 2**—*Fab-7* mediates pairing-sensitive repression of *mini-white*. Eye colors of flies transformed with *white* enhancer: *mini-white* constructs are shown, with heterozygotes on the left and homozygotes on the right. (A) A line transformed with the control *white* enhancer: *mini-white* construct (6) shows the typical uniform orange/red eye color as a heterozygote, and bright red color as a homozygote. (B–D) Examples of transformant lines that include *Fab-7* fragments in the *white* enhancer: *mini-white* construct (1 or 5), and that induce pairing-sensitive repression of *mini-white* in homozygotes. (Line names as shown in Table 1: B, 8.56.5; C, 8.51.7; D, 18.8.6)

**Figure 3**—*Fab-7* can also mediate pairing-sensitive repression of *mini-white* in the testes. This example shows testes of a 5-day-old heterozygous (left) or homozygous (right) male from a line transformed with construct 5 (line 18.59.3). As is also observed in the eyes, *mini-white* expression in the testes of this line is variegated in both heterozygotes and homozygotes, and reduced *mini-white* expression is observed in homozygotes.

introduction, this phenomenon has been observed for regulatory elements from other loci and is referred to as “pairing-sensitive” repression since it seems to depend on the homologous pairing of the transgenes.

Figure 2 shows the eye color in animals heterozygous and homozygous for the transgene insert in representative pairing-sensitive lines. As can be seen by comparing the different panels, the extent of *mini-white* pairing-sensitive repression varies from line to line; however, all homozygotes within a line have the same eye color. In some cases *Fab-7* mediated repression results in only a slight, uniform reduction of *mini-white* expression in homozygotes (Figure 2B). In other cases, the silencing is nearly complete and the eye color of the homozygotes is close to that of *white*- animals (Figure 2D). Silencing can also be associated with variegated expression (Figure 2C).

Since the *white* enhancer also directs the expression of *mini-white* in the testes, we examined *mini-white* expression in testes from the pairing-sensitive transgenic lines. We found that all lines that show pairing-sensitive repression of *mini-white* in the eyes also show pairing-sensitive repression of *mini-white* in the testes. This pairing-sensitive silencing of *mini-white* in the testes is illustrated for one of the transgenic lines in Figure 3. The fact that *mini-white* is repressed not only in the eyes, but also in the testes suggests that the silencing activity of *Fab-7* is not restricted to a particular parasegment or tissue.

A 0.8-kb DNA segment on the distal side of the *Fab-7* boundary is responsible for pairing-sensitive repression: The observations described in the previous sec-
tion (together with the studies of BUSTURIA and BIENZ 1993 and ZINK and PARO 1995) indicate that DNA sequences within the Fab-7' deletion are capable of functioning as a silencer. Since previous studies indicated that Fab-7 acts as a boundary to insulate the iab-6 and iab-7 cis-regulatory domains (GYUKOVICS et al. 1990; GALLONI et al. 1993; HAGSTROM et al. 1996; ZHOU et al. 1996), an obvious question is the relationship between this boundary activity and the silencing we observe in our mini-white assay system. Is silencing an aspect of boundary function, or are these two activities distinct?

To investigate this question, we attempted to localize the sequences within the 3.3-kb Fab-7 fragment that are responsible for inducing pairing-sensitive repression. As shown in Figure 1C, the 3.3-kb fragment was subdivided into three smaller fragments, and these were tested in the white enhancer:mini-white construct (Figure 1C, 3–5). The 1.4-kb HindIII-Nsid fragment in construct 3 is from the proximal side of Fab-7 and includes one of the minor DNase hypersensitive sites (see diagram in Figure 1B). The 1.1-kb Nsd-EaeI covers the central region and spans HS1 and HS2. As indicated in Figure 1B, deletion of this central region (Fab-7') or of this central region plus the more of the proximal HindIII-Nsid fragment (Fab-7') disrupts Fab-7 boundary function (GALLONI et al. 1993; KARCH et al. 1994). Finally, construct 5 has a 0.8-kb Apal-XbaI fragment that contains Hs3. Previous studies on the expression pattern of the Bluetail transposon, a Ubx/lacZ reporter inserted just proximal to Hs3 (Figure 1), suggest that this region is not important for boundary function (see GALLONI et al. 1993).

These three constructs were introduced into flies and tested for pairing-sensitive repression of mini-white. We examined 40 independent lines for construct 3, and 31 independent lines for construct 4. In all cases eye color in the homozygotes was darker than eye color in the heterozygotes and there was no indication of pairing-sensitive repression. A different result was obtained for construct 5. In this case we observed pairing-sensitive repression in eight out of the 25 independent lines. (It should be noted that none of these three fragments have boundary activity. However, boundary activity was observed when the Fab-7 fragment in 4 was combined with the distal part of the fragment in 3: HAGSTROM et al. 1996). These findings map the silencer element to the distal side of the 3.3-kb Fab-7 fragment within the iab-7 cis-regulatory domain and suggest that silencing is disctinct from the Fab-7 boundary function. To distinguish this silencer from the Fab-7 boundary we have renamed this iab-7 silencing element the "iab-7 PRE." (The PRE designation is explained below.)

Silencing of mini-white depends on pairing: To provide further evidence that the silencing of mini-white by the Fab-7 sequence requires pairing of the transgene inserts, we generated flies trans-heterozygous for five independent insertions on the third chromosome. Of the 10 possible pairwise combinations, only one pair showed mini-white silencing in trans. In this exceptional case, the eye color of the trans-heterozygote was intermediate between the eye color of homozygotes for each transgene (data not shown). In situ hybridization to polytene chromosomes revealed that these two interacting transgenes are inserted in relatively close proximity; one is at 82B and the other is at 84E.

The iab-7 PRE induces variegation distinct from classic position effect variegation: We were interested in identifying trans-acting factors that might contribute to iab-7 PRE induced silencing of mini-white. We observed that most of the iab-7 PRE lines that show pairing-sensitive repression of mini-white have a variegated or nonuniform eye color (i.e., Figure 2C). Of the 10 pairing-sensitive lines obtained with constructs 1 and 5, five show a variegated or nonuniform expression of mini-white when either heterozygous or homozygous for the transgene. Two additional lines show nonuniform mini-white expression as homozygotes. A similar variegated eye color is observed when rearrangements place the endogenous white gene near heterochromatin or when white transgenes are inserted near heterochromatin. This phenomenon is called position effect variegation (PEV) and appears to be due to the spread of a repressive chromatin structure from the heterochromatin into the euchromatic gene (reviewed in HENIKOFF 1990; REUTER and SPIERER 1992). These observations raised the possibility that the iab-7 PRE might silence mini-white by a mechanism similar to heterochromatic silencing or PEV.

We used two different tests to determine whether the iab-7 PRE silencing of mini-white involves a mechanism related to classical PEV. The first was to test whether the eye color of the variegating lines is affected by the introduction of an extra Y. While heterochromatin induced silencing can be suppressed by an extra copy of the Y (DIMITRI and PSANO 1989), we found that an extra Y produced no change in the eye color of the variegating pairing-sensitive lines (data not shown). The second test was the effect of temperature. Classical PEV shows a temperature dependence: it is suppressed by growth at elevated temperatures and enhanced by growth at lower temperatures (GOWEN and GAY 1933; SPOFFORD 1976). In contrast, we found that iab-7 PRE mediated repression shows the opposite response to temperature; high temperature enhances repression in all pairing-sensitive lines (data not shown). Temperature has no effect on the eye color of control mini-white lines lacking the iab-7 PRE. We also tested whether temperature alters the eye color in 11 of the iab-7 PRE mini-white lines that are not pairing-sensitive. In nine of the 11 lines, temperature had no effect. The two exceptional iab-7 PRE lines showed a modest reduction in the eye color of homozygotes at the elevated temperature and hence could actually be classified as weakly
pairing-sensitive. (These two lines are also unusual with respect to their interactions with z': see below.) Thus, iab-7 PRE induced variegation appears to be distinct from PEV.

Pairing-sensitive repression is altered in Polycomb Group gene mutant backgrounds: The pairing-sensitive elements from other loci can be divided into two classes based on their response to mutations in the Pc-G. The repression of mini-white by pairing-sensitive elements from Sce, ph, and Ubx can be alleviated by loss-of-function mutations in the Pc-G genes (FAUVARQUE and DURA 1993; CHAN et al. 1994; GINDHART and KAUFMAN 1995). In contrast, the pairing-sensitive element of pb is reported to be insensitive to Pc-G mutations (KAPOUN and KAUFMAN 1995). Hence it was of interest to determine whether the silencing activity of the iab-7 PRE is altered by mutations in the Pc-G genes. We introduced mutations in several of the Pc-G genes into the iab-7 PRE transgenic lines and examined the eye color phenotype. The results of this analysis are summarized in Table 1.

Of the eight iab-7 PRE pairing-sensitive lines tested for interactions with the Polycomb allele Pc^{106}, we found that pairing-sensitive repression was alleviated in five lines while no effect was observed in the other three (Table 1). The enhanced mini-white expression in animals heterozygous for the Pc^{106} allele is illustrated for one of the pairing-sensitive lines in Figure 4A. These findings suggest that the Polycomb protein contributes to the iab-7 PRE pairing-sensitive repression of mini-white. The fact that a few of the lines fail to respond to the Pc^{106} mutation is most likely explained by chromosomal position effects. This is not unusual as silencers from other loci have also been reported to display position dependent differences in their response to Polycomb mutations (FAUVARQUE and DURA 1993; KASSIS 1994). The lack of response to the Polycomb mutation could simply indicate that a twofold reduction in the Polycomb dose is not sufficient to disrupt silencing at these particular insertion sites. Alternatively, it is possible that nearby elements enable other Pc-G proteins (see below) to substitute for Polycomb. Interestingly, in two of our interacting lines, a reduction in the dose of Polycomb enhanced mini-white expression not only in animals homozygous for the transgene, but also in animals heterozygous for the transgene (Table 1). Thus, the mini-white expression in animals heterozygous for the transgene to a heterozygous Polycomb allele, Pc^{106}.+ increased mini-white expression; 0, no change in expression.

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The response of eight iab-7 PRE pairing-sensitive lines heterozygous (het) or homozygous (hom) for the transgene to a heterozygous Polycomb allele, Pc^{106}.+ increased mini-white expression; 0, no change in expression.
gene in these particular lines is apparently repressed by a Polycomb dependent mechanism even when there is only a single copy of the transgene. For these transgenes, it is possible that elements in the surrounding chromosomal environment are able to partially substitute for the PRE activity contributed by the second copy of the transgene.

Table 2 shows the results of examining the interaction of five of our iab-7 PRE pairing-sensitive lines with mutations in several other Pe-G genes, Sex-combs extra (Sc), Polycomb-like (Pcl), and Sex-combs on midleg (Scm). In general, those lines that respond to a reduction in the dose of Pe also respond to a reduction in the dose of the four other Pe-G genes and show enhanced mini-white expression. This is illustrated for Pcl in Figure 4B. We also find that one of the lines, 8.56.5, which is apparently insensitive to the Pcl mutation, does respond to mutations in both Pcl and Scm. These results provide further support for the suggestion that the silencing activity of the iab-7 PRE is dependent upon proteins from the Pe-G. They also indicate that chromosomal position effects are important in determining whether the silencing activity of the iab-7 PRE will be sensitive to reductions in the dose of a particular Pe-G protein.

In both the BX-C and ANT-C, the trx-G genes have an opposite regulatory role from that of the Pe-G, functioning to activate, not repress, expression (Kennison 1993). The pairing-dependent silencers from Ser, Ubx, en and Ph have been tested for their response to mutations intrx-G genes. Repression of mini-white by the Ser and Ubx silencers was enhanced in a heterozygous trx-G mutant background, while the trx-G mutations had no apparent effect on the Ph silencer and had a variable effect on the en silencer (Chan et al. 1994; Kassis 1994; Gindhart and Kaufman 1995; Kapoun and Kaufman 1995). As indicated in Table 2, most iab-7 PRE pairing-sensitive lines are insensitive to mutations in several different trx-G genes. One exception is line 18.73.1 in which mini-white expression is further reduced in a trxP2 background.

**The GAGA factor may influence the accessibility of the iab-7 PRE to silencing proteins:** As indicated in Figure 1B, the 0.8-kb Apal-XhoI fragment containing the iab-7 PRE includes the chromatin-specific nuclease hypersensitive site HS3 (Galloni et al. 1993). We examined the DNA sequence of HS3 for sites that match the specificity of known Drosophila DNA binding proteins and found two consensus binding sites for the GAGA factor (Soeller et al. 1993). The GAGA factor is encoded by the Trithorax-like (Trl) gene that is a member of the trithorax Group. Trl mutations can reduce the expression of hemostatic loci and cause posterior to anterior segmental transformations (Farkas et al. 1994). Consistent with these phenotypic effects, GAGA binding sites are found in the Ubx promoter and the GAGA factor has been shown to stimulate its activity in vitro transcription assays (Biggin and Tjian 1988; see also Soeller et al. 1993; Granok et al. 1995).

The presence of GAGA binding sites in HS3 suggested that the GAGA factor might influence the functioning of the iab-7 PRE. Since the GAGA gene is a member of the trithorax Group, one possibility is that these binding sites are part of a Trithorax response element (TRE) that overlaps the iab-7 PRE (see Chinwalla et al. 1995). If this is the case, Trl mutations might be expected to enhance the silencing of mini-white by the iab-7 PRE. An alternative hypothesis is suggested by the recent finding that the GAGA factor func-
tions in the establishment and/or maintenance of nucleosome-free regions of chromatin (Tsukiyama et al. 1994). It is conceivable that the GAGA factor is required to generate HS3 so that target sequences for Pc-G proteins within this nucleosome-free region are accessible for interactions in vivo. In this case, Trl mutations, like mutations in Pc-G genes, might be expected to reduce the silencing activity of the iab-7 PRE.

To test these two hypotheses, we introduced a mutation in the gene encoding the GAGA factor (the hypomorphic Trl<sup>3C</sup> allele) into four pairing-sensitive lines on the second chromosome. We found that the Trl<sup>3C</sup> mutation suppressed the silencing of mini-white in three out of the four lines, giving a darker eye color. The suppression of pairing-sensitive silencing in heterozygous Trl<sup>3C</sup> flies is illustrated for one line in Figure 4C. Suppression of silencing was even stronger when the Trl<sup>3C</sup> mutation was made homozygous (not shown). Pairing-sensitive silencing could also be relieved in animals heterozygous for a null allele, Trl<sup>895</sup>. The one line, 18.8.6, which was not suppressed by either Trl<sup>3C</sup> or Trl<sup>895</sup>, shows the most extreme pairing-sensitive silencing (see Figure 2D) and did not respond to mutations in several different Pc-G genes (Table 1 and data not shown).

While these findings indicate that wild-type GAGA activity is required for silencing by the iab-7 PRE, it is possible that this involves a mechanism that is not specific for this particular PRE. For example, Trl mutations might reduce silencing activity by causing a general decrease in the expression of Pc-G genes. In this case, Trl mutations should also suppress the silencing activity of other PREs. To address this question, we tested whether mutations in Trl would suppress the pairing-sensitive silencing of mini-white by a PRE from the Mep region of the BX-C (Vazquez et al. 1993). The Mep PRE resembles the iab-7 PRE in that it is associated with a chromatin-specific nucleosome hypersensitive region; however, the Mep hypersensitive region contains only a single consensus GAGA binding site (Karch et al. 1994). We found that Trl mutations had no apparent effect on the silencing of mini-white by the Mep PRE (data not shown). These findings show the iab-7 PRE, but not the Mep PRE, requires the GAGA factor for silencing activity.

The transgene protein Zeste is required for pairing-dependent silencing: The nucleosome hypersensitive site, HS3, in the iab-7 PRE fragment also contains three sequences that match the consensus binding site for the Zeste protein (Pirrotta 1991). Zeste has been implicated previously in pairing-dependent regulatory interactions (a phenomenon called transvection) at the BX-C, white, and other loci (for review see Pirrotta 1991; Wu 1993). Hence, it could potentially promote mini-white silencing by facilitating pairing between the iab-7 PREs in the transgenes on each homologue. In this case, loss-of-function z mutations should suppress pairing dependent silencing. On the other hand, Zeste also appears to function as a transcriptional activator for genes such as Ubx and white (Biggins and Tjian 1988; Lany and Biggins 1992). Since the white enhancer in our construct also has z binding sites, it is possible that z mutations might interfere with mini-white expression. In fact, both of these effects were observed when we examined mini-white expression in the loss-of-function z mutation, z'.

In animals carrying a single copy of the iab-7 PRE mini-white transgene the z' mutation reduces white expression, and in all cases the eye color was lighter than in the z' control. This reduction in white expression was observed not only for the pairing-sensitive lines but also for the nonpairing-sensitive lines, and for control lines which have mini-white transgenes lacking the PRE (data not shown). We presume that the lighter eye color of these different mini-white transgenic lines in the z' mutant background reflects a requirement for the Zeste gene product in mini-white expression.

z' also reduced mini-white expression in animals homozygous for nonpairing-sensitive iab-7 PRE mini-white transgenes and for control transgenes that lack the PRE (Figure 5A). However, a different result was obtained for animals carrying two copies of the pairing-sensitive iab-7 PRE mini-white transgenes. As illustrated in Figure 5B, the eye color of the pairing-sensitive lines becomes darker, not lighter, in a z' mutant background. We presume that the observed suppression of iab-7 PRE silencing by the z' mutation would be even stronger except for the fact that the loss of zeste activity is simultaneously reducing the expression of the mini-white reporter by interfering with white enhancer or promoter function. These findings suggest that the Zeste protein is required for efficient silencing by the iab-7 PRE.

The neomorphic Zeste<sup>+</sup> protein causes pairing-independent enhancement of iab-7 PRE silencing: We also examined the effects of the neomorphic z allele, z', on mini-white expression. The z' mutation induces an unusual pairing-dependent repression of the endogenous white (w) gene. Since this "zeste-white effect" has many features in common with the pairing-dependent repression of mini-white by the iab-7 PRE (Jack and Judd 1979; Pirrotta and Rastelli 1994), we tested whether the z' mutation influences mini-white repression by the iab-7 PRE.

We anticipated that, as is the case for the endogenous white gene, we would only observe z' effects in flies homozygous for the transgenes. However, to our surprise, we found that all of the pairing-sensitive transgenic lines show strong interactions with z' when the transgene insert is heterozygous (see Figure 6, C and D). The strong effect of z' on mini-white expression can be seen by comparing the eye color of males heterozygous for one of the iab-7 PRE transgene inserts and hemizygous for either z' (Figure 6A) or z' (Figure 6C). By contrast, z'
shows no apparent effect on heterozygous control *mini-white* lines that lack the *iab-7 PRE*, or on nine of 11 lines that contain the *iab-7 PRE* but are not pairing-sensitive (compare Figure 6, E and G). (The two exceptional lines were the ones that showed evidence of silencing when raised at 25°; see above.)

*z¹* silencing of the endogenous *white* gene in females requires not only the pairing of two *white* genes, but also the absence of a wild-type copy of *zeste*. Again the repression of *mini-white* by the *iab-7 PRE* is unusual in that it is dominantly enhanced by *z¹*. As can be seen by comparing Figure 6, D and B, the eye color of *z¹/z¹* females heterozygous for the *iab-7 PRE* transgene is lighter than the eye color of their *z¹/z¹* sisters. This dominant enhancement of *iab-7 PRE* repression was observed in females of all the pairing-sensitive lines, but there was no apparent effect of *z¹* on *mini-white* expression in transgenic lines carrying the control constructs or in nonpairing-sensitive *iab-7 PRE* lines (Figure 6H).

The 0.8-kb *iab-7 PRE* is sufficient to confer Pc-G dependent maintenance: One question raised by the findings described in the previous sections is whether the silencing activity of the *iab-7 PRE* on *mini-white* reflects the function of this element in the regulation of the BX-C homeotic genes. Studies on transgenes containing regulatory regions from BX-C have identified two classes of *cis*-acting regulatory elements. The first are the initiation elements that can program parasegmentally restricted patterns of expression. The initiation elements are, however, unable to maintain these expression patterns later in development unless they
are combined with the second class of elements, the maintenance elements or PREs. Since Pe-G proteins appear to be involved in the repression of mini-white by the iab-7 PRE DNA fragment, an obvious possibility is that the iab-7 PRE is a cis-component of the Pe-G dependent maintenance system. This possibility is also suggested by the earlier study of BUSTURIA and BIENZ (1993). They showed that when a large 4.0-kb fragment from the Fab-7 region was introduced into a Ubx/lacZ reporter construct that has a BXD initiation element (PS6), the Fab-7 fragment was able to confer Pe dependent maintenance in three out of five transgenic lines.

We used the assay system of BUSTURIA and BIENZ to test whether the 0.8-kb iab-7 PRE fragment has maintenance activity. The iab-7 PRE was introduced into the reporter construct and transgenic flies were isolated. Staining of transgenic embryos for β-galactosidase activity revealed that the iab-7 PRE can function as a maintenance element. β-galactosidase activity spreads anteriorly from PS6 in transgenic embryos carrying the starting BXD,Ubx/lacZ reporter (Figure 7A), but this anterior activation is not observed in seven out of 11 independent iab-7 PRE lines and expression remains predominately restricted to PS6 and more posterior parasegments (Figure 7B). As illustrated in Figure 7C, the introduction of a Pe mutation, Pe<sup>c</sup>, (LEWIS 1978) into an iab-7 PRE containing line abolishes this maintenance activity, and the Ubx/lacZ reporter is activated anterior to PS6.

**DISCUSSION**

The Fab-7 regulatory region contains a boundary and a silencer: Segmental identity in the posterior of the fly depends on the precise expression patterns of the bithorax complex genes. A large cis-regulatory region, organized into parasegment-specific domains, controls the BX-C homeotic genes and ensures that they are expressed in the appropriate pattern in each parasegment. Mutations that disrupt one of these parasegment-specific cis-regulatory domains are typically recessive and have a loss-of-function phenotype, transforming the corresponding parasegment into a copy of the parasegment immediately anterior. Fab-7 mutations are unusual in that they are dominant and usually have an opposite gain-of-function phenotype, transforming parasegment 11 into parasegment 12. Only this gain-of-function phenotype is observed in the original 4.0-kb Fab-7 deletion mutant, Fab-7<sup>*</sup>. While the same PS11 to PS12 transformation is also observed in the two smaller Fab-7 deletions, Fab-7<sup>2</sup> and Fab-7<sup>3</sup>, these mutants infrequently have small clones of cells in PS11 that have a PS10 identity (MIHÁLY et al. 1997; GALLONI et al. 1993). Thus, within the same parasegment (PS11) it is possible to observe both a gain- and loss-of-function phenotype.

The results presented in this article provide a plausible explanation for why the phenotypic effects of these Fab-7 alleles differ. As indicated in Figure 1B, all three deletions remove nuclease hypersensitive sites HS1 and HS2 (KARCH et al. 1994). Previous studies have shown that sequences spanning these two nuclease hypersensitive sites can function as a domain boundary in transgenic enhancer blocking assays (HAGSTROM et al. 1996). Thus, all of the deletions apparently lack the boundary sequences critical for ensuring the functional autonomy of the iab-6 and iab-7 cis-regulatory domains, and this would result in the formation of a fused iab-6:iab-7 cis-regulatory domain.

The mutants, however, differ in that HS3 is additionally deleted in Fab-7<sup>2</sup> while it still present in both Fab-7<sup>2</sup> and Fab-7<sup>3</sup> (KARCH et al. 1994). We have shown here that sequences spanning HS3 function as a Pe-G depen-
dent silencing element. This silencing element mediates pairing-sensitive repression of a min-cut reporter gene in the eye and testes, and can maintain the parasegmentally restricted pattern of expression of a BXD, Ubx/lacZ reporter construct. We show that both silencing activities depend upon Pc-G proteins. These results, together with the studies of BUSTURIA and BIENZ (1993) and ZINK and PARO (1995), argue that the HS3 silencer provides a nucleation site for the assembly of a Pc-G silencing complex, and functions as a PRE for the iab-7 cis-regulatory domain. cis-acting elements with similar properties have been found in other BX-C cis-regulatory domains including abx/bx, bxd/bx, iab-2, and iab-3 (SIMON et al. 1990; MÜLLER and BIENZ 1991; SIMON et al. 1993; CHAN et al. 1994; CHIANG et al. 1995), as well as iab-5, iab-6 and iab-8 (K. HAGSTROM, M. MULLER, P. SCHEDL, J. MIHALY, F. KARCH and H. GYURKOVICS, unpublished data).

When this iab-7 PRE is deleted along with the Fab-7 boundary, as in the Fab-7* mutant, a critical component for establishing and/or maintaining iab-7 in the inactive state in PS11 is missing. Consequently, the fusion of the iab-6 and iab-7 cis-regulatory domains leads in every case to the ectopic activation of iab-7 in PS11, transforming the cells in this parasegment to a PS12 identity. When the Fab-7 boundary is deleted but the iab-7 PRE is intact, as in Fab-7* and Fab-7', the balance between positive and negative regulatory elements in the fused domain still favors the ectopic activation of iab-7 in PS11 cells and their transformation to PS12 identity. However, in some cells, ectopic activation of iab-7 is apparently prevented, and instead the iab-7 PRE is able to nucleate a silencing complex that ectopically represses iab-6 in PS11. This would account for the infrequent PS11 to PS10 transformation in these two Fab-7 alleles.

In addition to explaining the phenotypic differences between the Fab-7 alleles, the subdivision of the Fab-7 region into a boundary and a PRE resolves the apparently contradictory lines of evidence arguing in favor of either a silencer or boundary model for Fab-7 function. Although we have confirmed previous reports that the Fab-7 region constrains a silencer (BUSTURIA and BIENZ 1993; ZINK and PARO 1995), we show this silencer is distinct both in its location and in its function from the Fab-7 boundary. Moreover, it is the deletion of the boundary element and not the silencer that is responsible for the dominant gain-of-function phenotypes of the Fab-7 mutations. This has been demonstrated directly by MIHALY et al. (1997) who generated small BX-C deletions that remove either the Fab-7 boundary or the iab-7 PRE. They found that deletions that remove only the iab-7 PRE generally have no phenotypic consequences either as heterozygotes or homozygotes (however see below). This result would also suggest that the iab-7 cis-regulatory domain must contain other PRE-like elements that can substitute for the PRE near the Fab-7 boundary.

**PREs may cooperate in cis and trans, and boundaries may limit PRE-PRE cooperation:** One striking feature of the iab-7 PRE mediated silencing of the min-cut reporter is that it typically requires a pair of transgenes, one on each homologue. Is silencing in pairs a phenomenon that is peculiar to our min-cut assay system or does it instead reflect some intrinsic property of the iab-7 PRE that normally restricts its activity? Arguing that it may be a peculiarity of the min-cut assay system is the ability of the iab-7 PRE to maintain a parasegmentally restricted pattern of lacZ expression in animals carrying only a single copy of the BXD, Ubx/lacZ reporter. However, this apparent difference between the min-cut and BXD, Ubx/lacZ reporters may be somewhat misleading as recent studies by POUX et al. (1997) have shown that the BXD fragment itself has a weak PRE activity. In this case, the maintenance activity exhibited by the iab-7 PRE in the BXD, Ubx/lacZ reporter may simply reflect its ability to cooperate with the weak BXD PRE in cis rather than an ability to establish and maintain a silencing complex as a single autonomous unit.

The idea that two or more PREs may cooperate to form a functional silencing complex is further supported by the studies of MIHALY et al. (1997) on the genetic properties of small deletions that remove the iab-7 PRE. While these iab-7 PRE deletions generally have no phenotypic consequences as homozygotes, a small number (~2%) of mutant animals had a weak Fab-7-like phenotype in which a small group of cells in PS11 were misspecified as PS12. Significantly the penetrance of this weak Fab-7-like phenotype could be enhanced (to 10% of the animals) when the iab-7 PRE deletion mutant was trans to larger deficiencies that remove the iab-7 PRE. While these iab-7 PRE deletions generally have no phenotypic consequences as homozygotes, a small number (~2%) of mutant animals had a weak Fab-7-like phenotype in which a small group of cells in PS11 were misspecified as PS12. Significantly the penetrance of this weak Fab-7-like phenotype could be enhanced (to 10% of the animals) when the iab-7 PRE deletion mutant was trans to larger deficiencies that removed either the entire iab-7 cis-regulatory domain or all of BX-C. Two conclusions are suggested by these observations. First, the iab-7 PRE next to the Fab-7 boundary plays an important, but partially dispensable, role in the formation and/or maintenance of an inactive iab-7 cis-regulatory domain in PS11. Second, PRE cooperation in trans helps the other PRE-like elements in the iab-7 cis-regulatory domain compensate for the deletion of the iab-7 PRE. Hence, it is reasonable to suppose that PRE cooperation either in trans or in cis may be actually be an important feature of the silencing process within the BX-C rather than simply an aberration of the min-cut assay system.

Of course, PRE cooperation in the context of the BX-C requires a mechanism to prevent cis or trans interactions between PREs in adjacent cis-regulatory domains that could promote ectopic silencing. From the phenotypic properties of the Fab-7* and Fab-7' deletions it seems likely that one of the functions of BX-C boundary elements like Fab-7 is to prevent inappropriate cooperation between PREs in nearby cis-regulatory domains. A prediction of this model is that boundary deletions
elsewhere in the complex need not always have a gain-of-function phenotype. They might instead cause ectopic silencing.

Chromatin structure of the iab-7 PRE and the roles of Zeste and GAGA: The small fragment that contains the iab-7 PRE spans a landmark of chromatin structure: a prominent chromatin-specific nuclease hypersensitive site, HS3. While we have not yet pinpointed the sequences required for iab-7 PRE activity, there are reasons to believe that some of the critical elements for PRE function are located within this 215-bp nuclease hypersensitive site. As described in KARCH et al. (1994), HS3 has a 40-bp sequence that is 70% identical to a sequence from another cis-regulatory region of the BX-C, Mcp. Fragments containing this corresponding Mcp sequence, like the 0.8-kb iab-7 PRE, behave as pairing-sensitive silencers (VAZQUEZ et al. 1993). Within the conserved 40-bp sequence, there is a shorter region of homology that is also present in pairing-sensitive elements from the en gene (KASSIS 1994) and in other PREs from the BX-C (K. HAGSTROM, M. MULLER, P. SCHEDI, J. MIHALY, F. KARCH and H. GYURKOVICS, unpublished data). We have found that an oligo spanning the core homology region is capable of silencing mini-white, although the frequency of pairing-sensitive lines is rather low (K. HAGSTROM, unpublished data). Finally, HS3 contains consensus binding sequences for two proteins, Zeste and the GAGA factor, which seem to be play an important role in the functioning of the iab-7 PRE.

Zeste and pairing-dependent regulatory interactions: There are three consensus binding sites for the Zeste protein within HS3, and a fourth just beyond the proximal edge of the hypersensitive region. Zeste has a well-documented role in pairing-dependent regulatory interactions at many different loci (see PIRROTTA 1991; WU 1994) and hence would be an excellent candidate for a protein that could facilitate cooperative interactions between iab-7 PREs on sister chromosomes. A prediction of this hypothesis is that loss-of-function z mutations such as z1 should suppress iab-7 PRE mediated silencing of mini-white. However, testing this hypothesis is complicated by the fact that Zeste seems to do more than simply promote trans interactions. It also appears to stimulate the transcriptional activity of genes, such as white, which have Zeste binding sites in their promoters and/or upstream regulatory elements (BIGGIN and TJIAN 1988; LANEY and BIGGIN 1992). A model that accounts for Zeste activity in both transvection and transcription is that the self-aggregating Zeste protein facilitates long-distance interactions (looping in cis or trans) between regulatory elements (PIRROTTA 1991). Presumably because of Zeste's function in cis-regulatory interactions, we observe a reduction in eye pigmentation when the z1 mutation is introduced into transgenic lines carrying one copy of control mini-white constructs that lack the iab-7 PRE sequence. The z1 mutation has similar effects on mini-white expression in both pairing-sensitive and nonpairing-sensitive iab-7 PRE lines when this construct is also present in only a single copy. However, precisely the opposite effect is observed in homozygous pairing-sensitive lines. In these animals, the z1 mutation suppresses the iab-7 PRE mediated silencing of the mini-white reporter.

This finding indicates that Zeste is required for efficient silencing by the iab-7 PRE and would be consistent with a model in which the wild-type Zeste protein functions primarily as an ancillary factor, facilitating cooperative interactions between the iab-7 PREs on each homologue. An alternative, and we believe less likely hypothesis is that the Zeste protein plays an active role in silencing, perhaps functioning as a component of the Pc-G silencing complex (see, however, z1 below). Since at least three of the Zeste consensus sequences in the iab-7 PRE should be accessible in chromatin, we presume that the Zeste protein interacts with these particular sites to promote the trans-association of the PREs. Of course, we cannot exclude the possibility that Zeste protein associated with binding sites in the white enhancer also contributes to the trans-association of the iab-7 PRE.

The zeste1 allele causes pairing-independent enhancement of iab-7 PRE silencing: The zeste1 allele encodes a neomorphic protein that represses, instead of activates, white expression. The repression of w by z1 is similar in many respects to the repression of mini-white by the iab-7 PRE: both are pairing-dependent, enhanced by high temperature, and suppressed by mutations in some Pc-G genes (PIRROTTA and RASTELLI 1994). Given these similarities and the presence of Zeste binding sites in our transgene (in the white enhancer and the iab-7 PRE), we expected that z1 might enhance mini-white silencing by the iab-7 PRE. Indeed this is the case. In contrast to the loss-of-function z1 mutation that reduces mini-white silencing by the iab-7 PRE, the neomorphic z1 increases silencing.

However, we were surprised to discover that the z1 enhancement of mini-white silencing by the iab-7 PRE violates the rules that normally govern interactions between z1 and the endogenous w+ locus. First, pairing of the iab-7 PRE containing transgene is not required; z1 enhances the iab-7 PRE mediated repression in animals carrying only a single copy of the transgene. Second, in its interactions with the endogenous w+ locus, z1 is normally recessive to a z1 allele. However, z1 enhances the iab-7 PRE mediated repression of mini-white even in the presence of a wild-type z allele.

The unusual interaction between z1 and the iab-7 PRE is instructive. It appears that z1 proteins bound to target sites within the iab-7 PRE and/or the white enhancer are able to facilitate the establishment of a silencing complex, in effect substituting for the PRE activity nor-
would be interesting to know whether these transgenes 
else where could stimulate the 
formation or maintenance of nucleosome free re-
protein that could stimulate the 
Hex promoter in 
unpublished data). While z' enhances the silencing of all 
pairing-sensitive iab-7 PRE transgenes as heterozygotes, 
such interactions are not typically observed with non-
pairing-sensitive iab-7 PRE lines. In the two exceptional 
cases, the nonpairing-sensitive lines show evidence of 
pairing-dependent silencing at elevated temperatures. 
These findings would imply that chromosomal environ-
ments that are permissive for Pc-G protein mediated 
silencing of mini-white by iab-7 PREs in trans are also 
permissive for synergistic interactions with the Z' pro-
tein in cis. Although our control constructs also showed 
no interactions with z' in cis, there have been reports 
of unusual w transgene inserts that are repressed by z' as 
heterozygotes (Hazelrigg and Peterson 1992). It 
would be interesting to know whether these transgenes 
are inserted near PREs.

The role of the GAGA factor: The GAGA factor was 
first identified as a sequence specific DNA binding pro-
tein that could stimulate the en and Ubx promoters in 
in vitro transcription assays (Biggin and Tjian 1988; 
Soeller et al. 1988). GAGA binding sites were subse-
quently identified in the promoters and upstream regu-
latory regions of other patterning genes and also of 
housekeeping genes (Granok 1995). Although the 
GAGA factor appears to be required for the expression 
of many of these genes (Bhat et al. 1996), both in vitro 
and in vivo experiments indicate that the protein proba-
bly does not act as a classical transcription activator; 
rather it functions as an “anti-repressor” facilitating 
the formation or maintenance of nucleosome free re-
gions of chromatin (Glaser et al. 1990; Groston et al. 
1991; Lu et al. 1993; Tsukiyama et al. 1994).

The nucleosome hypersensitive site, HS3, associated with 
the iab-7 PRE has two consensus binding sequences for 
the GAGA protein, which are located in close proximity 
to the PRE homology region described above. The pres-
ence of these GAGA binding sites in a nucleosome-free 
region of chromatin suggested that the GAGA factor 
may play some role in the functioning of the iab-7 PRE. 
Consistent with this possibility, we have found that 
mutations in the GAGA gene, Trl, suppress the iab-7 PRE 
meditated silencing of mini-white.

How can we reconcile this result with the idea that 
the GAGA factor functions as an anti-repressor, alleviat-
ing the repressive effects of chromatin and/or chroma-
tin associated proteins? One possibility is that the sup-
pression of iab-7 PRE silencing activity by Trl mutations 
may be indirect; for example, the GAGA factor could 
be required for the transcription of Pc-G group genes. 
Although we cannot rule out this possibility, we believe 
it is unlikely. The phenotypic effects of Trl on the home-
otic gene complex are not like those associated with 
mutations in Pc-G genes, rather they most closely resemble 
those associated with mutations in the trx-G (Farkas 
et al. 1994). Moreover, we have found that Trl mutations 
do not suppress silencing by the Mep PRE. By contrast, 
a general defect in the expression of Pc-G genes would 
be expected to perturb the activity of all PREs. Of 
course, Trl could be required for the expression a spe-
cific Pc-G gene that is essential for iab-7 PRE silencing, 
but not for Mep silencing. Finally, the function of the 
GAGA factor is not restricted to promoting gene expres-
sion; it also seems to be involved in such processes as 
chromosome condensation and decatenation (Bhat et al. 
1996).

An alternative, and we believe more likely, hypothesis 

is that the GAGA factor is required to generate or main-
tain the iab-7 PRE nucleosome-free region HS3. This 
would facilitate silencing by making critical sequences 
in the iab-7 PRE (such as the homology region or the 
Zeste binding sites) accessible to Pc-G proteins. Al-
though largely circumstantial, several lines of evidence 
favor this view. First, the GAGA factor has already been 
implicated in PRE silencing by Laney and Biggin 
(1992). These workers showed that addition of reiter-
ated GAGA binding sites enhanced the Pc-G dependent 
maintenance of a homeotic reporter construct. Second, 
a function in chromatin remodeling is consistent with 
the known in vitro activities of the GAGA factor (Cronds-
ton et al. 1991; Tsukiyama et al. 1994). Third, a link 
between PRE activity and nucleosome-free regions of 
chromatin is not unique to the iab-7 PRE; at least three 
other BX-C PREs (the PRE from the Mcp region, plus 
PREs in iab-6 and iab-8) are also contained in small DNA 
segments that have prominent nucleosome hypersensitive 
sites (K. Hagstrom, M. Muller, P. Schedl, J. Mihaly, 
F. Karch and H. Henikoff, unpublished data). In 
each of these cases, the PRE homology region is 
contained within the nucleosome-free region. (Since the 
Mcp PRE does not respond to Trl mutations, we pre-
sume that some other GAGA-like factor is responsible 
for generation the Mcp nucleosome-free region. The 
other PREs have not been tested). Although a function 
in the formation or maintenance of HS3 would seem 
to be most consistent with the known properties of 
the GAGA factor, we cannot exclude the possibility that 
the factor has some more direct or active role in iab-7 PRE 
silencing (cf. Laney and Biggin 1992). Clearly, further 
experiments will be required to exclude this possibility.

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