The EMBO Journal vol.13 no.11 pp.2553-2564, 1994

A *Polycomb* response element in the *Ubx* gene that determines an epigenetically inherited state of repression

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Communicated by V.Pirrotta

Segmentation genes provide the signals for the activation and regulation of homeotic genes in *Drosophila* but cannot maintain the resulting pattern of expression because their activity ceases halfway through embryogenesis. Maintenance of the pattern is due to the *Polycomb* group of genes (*Pc*-G) and the *trithorax* group of genes (*trx*-G), responsible for the persistence of the active or repressed state of homeotic genes. We have identified a regulatory element in the *Ubx* gene that responds to *Pc*-G and *trx*-G genes. Transposons carrying this element create new binding sites for *Pc*-G products in the polytene chromosomes. This *Pc*-G maintenance element (PRE), establishes a repressive complex that keeps enhancers repressed in cells in which they were originally repressed and maintains this state through many cell divisions. The *trx*-G products stimulate the expression of enhancers in cells in which they were originally active. This mechanism is responsible for the correct regulation of imaginal disc enhancers, which lack themselves antero-posterior positional information. The PRE also causes severe variegation of the mini-*white* gene present in the transposon, a phenomenon very similar to heterochromatic position-effect variegation. The significance of this mechanism for homeotic gene regulation is discussed.

Key words: chromatin state/epigenetic maintenance/homeotic gene regulation/*Pc*-G response/variegation

Introduction

In *Drosophila* embryos, homeotic genes like *Ultrabithorax* (*Ubx*), *Antennapedia*, *abdominal-A*, etc., determine the developmental identity of the segments in which they are expressed. The specification of the expression domain of each of these genes is therefore critical to the correct development of the different parts of the body along the anterior-posterior axis. The expression domain of the *Ubx* gene is limited to the region including parasegments (PS) 5-13. Genetic and molecular evidence have shown that the boundaries of this domain are set by the segmentation gap genes *hunchback* (*hb*) and *tailless* (*tll*), acting as repressors to prevent expression in more anterior and more posterior regions of the embryo (White and Lehmann, 1986; Reinitz and Levine, 1990; Qian *et al.*, 1993). Gap genes very likely set the expression domains of the other homeotic genes. Within the domain of expression, other segmentation genes function as activators to stimulate expression in distinct patterns. However, the expression of gap genes in their early domains ceases shortly after gastrulation, and expression of the activator segmentation genes in their segmental domains ceases during germ band extension. These early positional cues cannot suffice, therefore, to determine the correct pattern of expression of the homeotic genes through the rest of embryonic and larval development. To maintain expression, which continues until the adult stage, two other sets of genes are necessary: the *Polycomb* group (*Pc*-G), which maintains the repressed state in those cells in which the gene was originally repressed, and the *trithorax* group (*trx*-G), which stimulates continued expression in those cells in which the gene was originally active [reviewed by Duncan (1987) and McGinnis and Krumlauf (1992)].

In the *Ubx* gene, a set of enhancer elements has been identified that can act independently of one another to activate expression of a reporter gene in parasegmentally specific patterns (Simon *et al.*, 1990; Müller and Bienz, 1991; Qian *et al.*, 1991, 1993; V.Pirrotta, C.-S.Chan, D.McCabe and S.Qian, manuscript in preparation). These elements are the targets of both activator and repressor segmentation gene products and generate different patterns of expression; some in odd-numbered, some in even-numbered parasegments, and in distinct patterns within a parasegment. Negative factors such as *hb* and *tll* act locally by binding the enhancers at sites that overlap with the binding sites of positive factors and functioning as competitive inhibitors (Müller and Bienz, 1991; Qian *et al.*, 1993). When analysed individually, all the enhancers have correct initial expression domains but all, sooner or later, fail to maintain the correct boundaries and begin to express ectopically in more anterior parts of the embryo (V.Pirrotta, C.-S.Chan, D.McCabe and S.Qian, manuscript in preparation). Their different affinity for Hb repressor apparently determines whether the failure occurs earlier or later. After germ band extension, most of these enhancers also cease to be active and cannot alone account for the continued expression of the *Ubx* gene. The different enhancer elements vary in their ability to maintain the correct domain boundaries; some break down the restriction very early, during germ band extension, some maintain the correct domain boundaries until much later, when the activity of the enhancer has already greatly declined and the ectopic expression is consequently much less extensive. In some cases, their ability to maintain correct expression is weakly affected by *Pc* mutations (Qian *et al.*, 1993). However, the fact that all fail sooner or later, even in the presence of a full complement of *Pc* group genes, indicates that they do not contain effective target sites for *Pc*-G response. Their inability to continue functioning after germ band extension also indicates that they do not contain targets for the *trx*-G

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positive maintenance. These results imply that response to maintenance functions requires sequences that are distinct from those of the enhancers and promoters and that must therefore act at a distance to regulate enhancers or promoters.

The *Pc*-G genes have common phenotypes indicative of a general derepression not only of *Ubx* but of the other major homeotic genes, as well as certain other genes such as *engrailed* or *even-skipped* whose complex patterns of expression need to be maintained through later development (Dura and Ingham, 1988; Heemskerk *et al.*, 1991). Several lines of evidence show that many of the members of the group act together to form complexes involving large numbers of molecules. Many of the genes are haplo-insufficient, producing homeotic phenotypes when present in a single dose. These phenotypes are strongly enhanced by heterozygous combinations of different *Pc*-G mutations (Jürgens, 1985). In some cases, haplo-insufficiency in one gene can be compensated for by duplications of the chromosomal region containing another member of the *Pc*-G (Kennison and Russell, 1987). Several members of the *Pc*-G have now been cloned and their products have been shown to be localized at some 100 chromosomal sites on polytene chromosomes, many of which correspond to known targets of *Pc*-G regulation (Franke *et al.*, 1992; Martin and Adler, 1993; Rastelli *et al.*, 1993). The chromosomal distribution of the products of *Pc*, *polyhomeotic* (*ph*), *Posterior sex combs* (*Psc*) and *Suppressor of zeste(2)* [*Su(z)2*], frequently, but not always, coincides, suggesting that they often act together. None of these proteins by itself has sequence-specific DNA binding activity *in vitro*, suggesting that the interaction with chromatin involves the formation of higher order complexes. Some of the features of *Pc*-G genes are strongly reminiscent of the behaviour of suppressor of variegation [*Su(var)*] genes, which are also thought to act in the form of large multiprotein complexes responsible for the compact and transcriptionally inert properties of heterochromatin (Locke *et al.*, 1988). A more specific link between *Pc*-G products and heterochromatin proteins is provided by the discovery of a protein motif in the *Pc* product that is shared by the HP1 protein, the product of the *Su(var)205* gene (Eissenberg *et al.*, 1990; Paro and Hogness, 1991). More recently, some *Pc*-G mutations have been found to have variegation suppressing phenotypes like *Su(var)* mutations, suggesting that some proteins may participate in both kinds of complexes (DeCamillis *et al.*, 1992).

In the search for a sequence element from the *Ubx* regulatory region able to confer long-term maintenance of the correct domain of expression to the small enhancer elements, we began to look for such properties in larger fragments or combinations of fragments of the *Ubx* gene. Irvine *et al.* (1991) found that a reporter gene containing 35 kb of the upstream region of *Ubx* had maintenance properties, but that these were lost when the construct included only the promoter-proximal 20 kb. In addition, we observed that reporter genes containing larger fragments from the region −10 to −20 or −30 to −40 kb upstream of the *Ubx* promoter included enhancers, but failed to maintain their initial domain of expression. We therefore concentrated our efforts on the region −20 to −30 kb upstream of the promoter. In this work we show that, in addition to parasegmental and imaginal enhancers, this region also contains an element that (i) responds to *Pc*-G genes, (ii) forms a complex with products of this group detectable in polytene chromosomes and (iii) is able to maintain the repressed state of enhancers present in its vicinity throughout development. Following the definition of Simon *et al.* (1993), we will refer to this regulatory element as the *Pc*-G response element or PRE. The PRE has a powerful effect in inactivating not only the *Ubx* promoter but also other nearby genes, particularly the mini-*white* gene present in the transposon construct. This results in striking patterns of variegation of eye pigmentation, highly reminiscent of position-effect variegation (PEV) phenomena that are seen when chromosomal rearrangements place the *white* gene in the vicinity of heterochromatin.

Results

The 2212H6.5 region confers boundary maintenance

To identify a regulatory element dependent on the *Pc*-G genes and able to confer long-term maintenance, we constructed transposons containing fragments larger than enhancers or combinations of fragments from the region −20 to −30 kb upstream of the *Ubx* promoter [corresponding to positions −10 to 0 in the map of Bender *et al.* (1983)]. Many such constructs show in fact a partial maintenance in the sense that a substantial number of cells, but not all, show correct maintenance. The 6.5 kb 2212 *Hin*dIII fragment (see map in Figure 1), containing embryonic enhancers 2212S1 and S2, gives a considerable degree of maintenance in six of the seven lines obtained (Figure 2) and complete maintenance to the end of embryonic development in the seventh. In the partially maintaining lines, the boundary of the domain of expression is still visible throughout embryonic development, but scattered groups of cells express in the anterior region, as if the repressed state could be frequently but not always established in anterior cells and their descendants. In contrast, the separate 2212S1 and S2 enhancers express initially in PS6, 8, 10 and 12, but towards the end of germ band extension expression appears also in

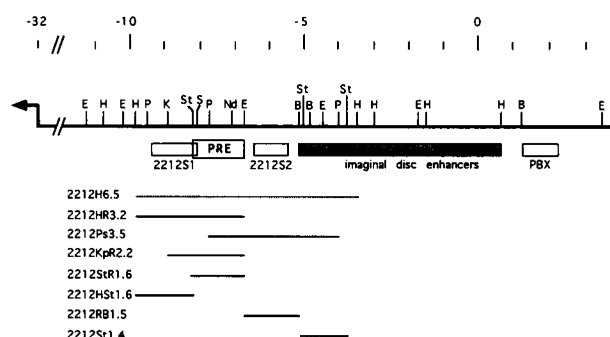

Fig. 1. Map of the upstream region of *Ubx* containing the maintenance region. The region shown begins 20 kb upstream of the transcription start site (indicated on the left). The scale in kb follows the numbering system of Bender *et al.* (1983) with the promoter at position −32. The parasegmental enhancer fragments 2212S1, 2212S2 and PBX are shown as white boxes. The region containing imaginal disc enhancers is indicated by a stippled box. The fragments used for various transposon constructs are indicated below by a dashed bar if they produce maintenance or eye variegation, and by a black bar if they do not. Restriction enzyme sites important for the constructs discussed are: E, *Eco*RI; H, *Hin*dIII; P, *Pst*; K, *Kpn*I; St, *Sty*I; S, *Sau*3A; Nd, *Nde*I; B, *Bam*HI.

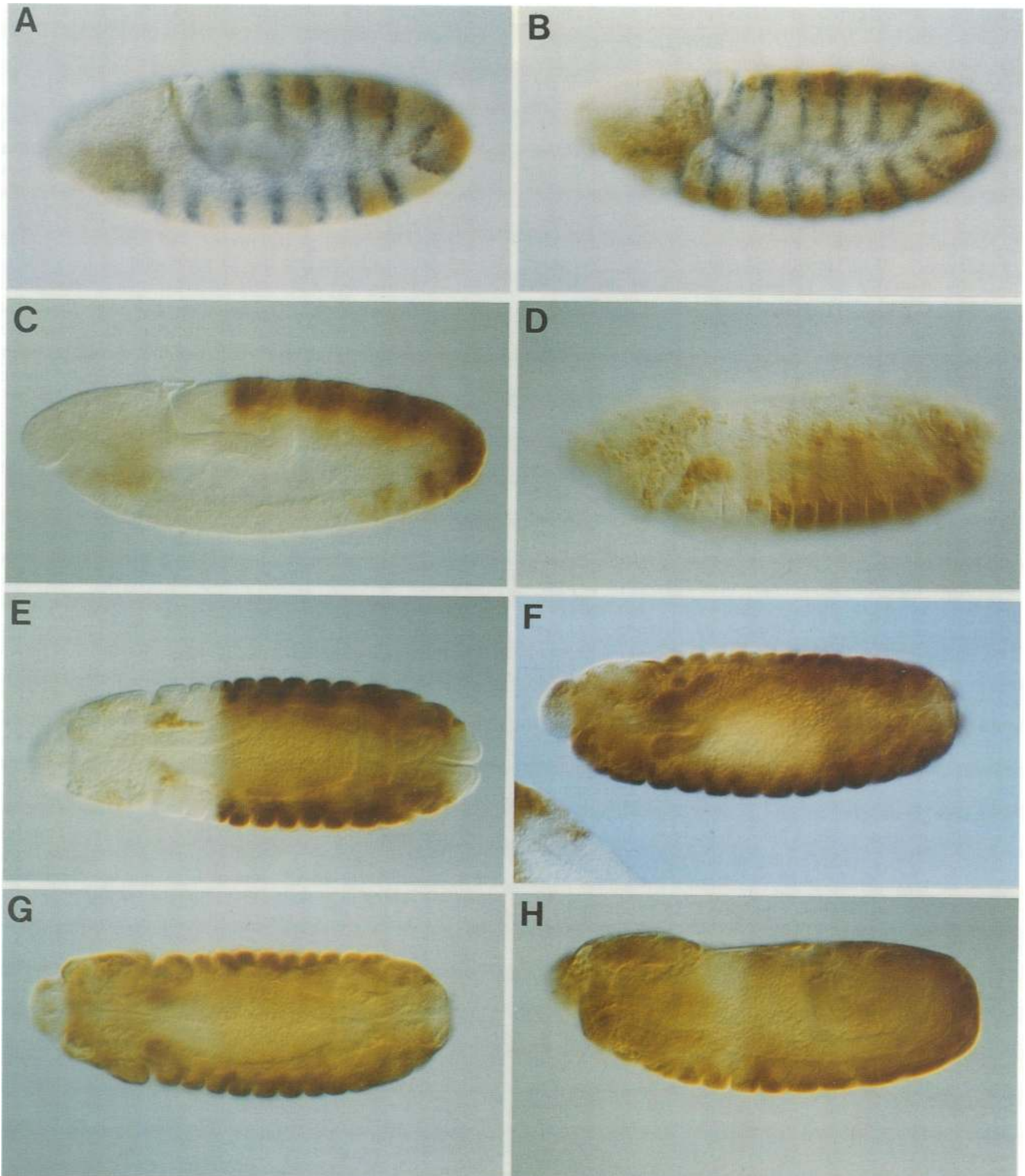

Fig. 2. Expression pattern of the 2212 region enhancers. Embryos of flies carrying the transposons were stained with anti-β-galactosidase antibody (brown colour). In some cases the embryos were also stained with anti-engrailed antibody to visualize the parasegmental borders (blue colour). Embryos carrying the 2212S1 transposon exhibit four stripes in the central and ventral region of PS6, 8, 10 and 12 during germ band extension (**A**). At the end of germ band extension, expression appears in all parasegments, including the thoracic region showing that repression cannot be maintained (**B**). Embryos containing the H6.5 transposon, which includes both 2212S1 and 2212S2, give a similar pattern but with varying degrees of maintenance. (**C** and **D**) A line with intermediate maintenance. (C) End of germ band extension, comparable with (B). (D) After germ band retraction. Some spotty ectopic expression is present in the head and thorax regions, but the correct pattern is substantially maintained. Embryos carrying the 2212H6.5 + PBX construct give even more complete maintenance at a comparable stage (**E**). Maintenance is dependent on *Pc*-G gene function: anterior repression is lifted in homozygous Pc^3 mutant embryos resulting in near ubiquitous expression of the 2212H6.5 transposon (**F**) and the H6.5 + PBX transposon (**G**). Complete derepression is also obtained in embryos homozygous for the *Suvar(3)7* mutation (**H**).

the odd-numbered parasegments and in the thoracic region. During germ band retraction, expression becomes nearly ubiquitous, indicating total inability to maintain the initial boundaries. Although we have not shown it in detail, the initial boundary of expression of the S1 and S2 enhancers is most likely set by Hb protein acting as a repressor, as in the BX and PBX enhancers (Müller and Bienz, 1991; Qian *et al.*, 1991). Hb binding sites are found in S1 and S2 but their affinity for Hb protein is substantially weaker than that of either BX or PBX elements (V.Pirrotta, C.-S.Chan, D.McCabe and S.Qian, manuscript in preparation). The loss of maintenance of the anterior boundary of S1 and S2 is most probably explained by the gradual release of Hb protein bound to the enhancers after the expression of *hb* in its gap gene domain ceases, at the beginning of germ band extension. These two enhancers, therefore, become derepressed in the anterior part of the embryo.

Even more complete maintenance was obtained with a transposon containing both the 2212H6.5 fragment and a 0.6 kb PBX fragment (Figure 2). In this case repression is complete in the anterior region throughout embryonic development. Transposons containing the 2212H6.5 fragment together with a 1.7 kb BX enhancer give partial maintenance, similar to, though better than, that usually obtained with 2212H6.5 alone. Although the PBX or the BX fragments alone maintain longer than 2212S1 or S2, they also begin to express anteriorly during germ band retraction. Their better maintenance is probably due to the fact that they have much greater affinity for the Hb repressor (V.Pirrotta, C.-S.Chan, D.McCabe and S.Qian, manuscript in preparation). Other combinations of enhancer fragments, such as PBX + 2212S2, PBX + 2212S1 and 2218R6 + 2212S1, showed no change in the degree of maintenance obtained with the separate fragments. These results indicate that some activity in the 2212H6.5 fragment can maintain repression in the anterior region, even when Hb protein is no longer present, and that the repression operates not only on the 2212H6.5 enhancers but on other enhancers present in the same construct. In all cases, some lines gave much better maintenance than others, suggesting that the chromosomal environment influences the ability to establish the repressed state.

Maintenance properties are Pc group-dependent

To determine if the maintenance element present in 2212H6.5 is dependent on *Pc*-G genes, we introduced the various transposons into a *Pc* mutant background. A cross between flies heterozygous for the Pc^3 mutation gave embryos in which maintenance at late stages was lost either partially or completely. We interpret embryos showing partial loss as heterozygous and the embryos showing total breakdown as homozygous for the *Pc* mutation (Figure 2F and G). Embryos produced by parents heterozygous for mutations in the *Psc* gene, another member of the *Pc*-G, showed only weaker loss of maintenance effects visible only at later stages. This is not unexpected since *Psc* has an important maternal component that is detectable throughout embryonic development (Martin and Adler, 1993). Another *Pc*-G gene, *E(z)*, also has a major maternal contribution, but in this case a temperature-sensitive allele is available (Jones and Gelbart, 1990). Embryos from parents homozygous for the $E(z)^{S2}$ temperature-sensitive mutation, kept at non-permissive temperature, gave loss of maintenance. For reasons that will become apparent below and because of the similarity between the action of *Pc*-G genes and *Su(var)* genes, we also tested some *Su(var)* mutations for their effect on the maintenance property. To our surprise, *Suvar(3)7* also affects maintenance in a dramatic way in homozygous embryos, resulting in uniform expression of the transposon, entirely analogous to the effect of the *Pc* mutation (Figure 2), although the transposon is inserted at 14C, far from heterochromatin. These results indicate that the maintenance element present in 2212H6.5 is dependent on *Pc* group function and is therefore a true PRE. They also suggest that at least some of the *Su(var)* genes that affect heterochromatin condensation can also affect the establishment of *Pc*-G complexes. However, the effect of *Suvar(3)7* is probably not directly on the PRE but on the sequences flanking the transposon insertion site because other lines are not affected by the *Suvar(3)7* mutation nor is the expression of the endogenous *Ubx* gene.

Posterior shift of the anterior boundary

A surprising feature of the maintenance observed with the 2212H6.5 construct is that the boundary is set not at PS6, like the initial boundaries of the S1 and S2 enhancers contained within the 2212H6.5 fragment, but at PS8 (Figure 3A and B). In addition, the onset of expression is greatly delayed or at least so greatly reduced in level that no staining is observed at the blastoderm stage, when expression of the separate S1 and S2 enhancers usually starts. This is not due to an overall weakening of the expression because, after staining appears at the beginning of germ band extension, it rapidly becomes as intense or more intense than in most of the lines containing the separate S1 or S2 enhancers. Furthermore, the pattern differs from that of S1 or S2 from the early stages. At blastoderm, the separate enhancers have a PS6 band followed by stronger bands at PS8 and PS10. Instead, for 2212H6.5, the earliest visible pattern at the end of gastrulation has a weak PS6 band which then becomes weaker and disappears. The PS8 band is strong, while the PS10 and PS12 bands appear later during germ band extension, followed by the odd-numbered bands at PS9, 11 and 13. The posterior shift of the boundary occurs to a lesser and more variable extent in the lines of 2212H6.5 that maintain less well (see Figure 2A). We see a weaker effect in the same direction with the 2212HR3.2 transposon (Figure 3E and F) which contains only the S1 enhancer but includes the *Pc*-G response region (see below). In the majority of embryos at the germ band elongation stage the PS6 stripe is absent and the pattern begins at PS7. Frequently the PS7 stripe is weaker than the others and in a few cases it is entirely absent and the pattern begins at PS8 like that of the 2212H6.5 construct.

Neither maintenance nor the posterior shift of the boundary is seen with the 2212HS1.6 construct, which contains the 2212S1 enhancer but not the *Pc*-G response element PRE. In constructs that include the PRE, the extent of the shift parallels the degree of maintenance of the line, suggesting that it is related to the *Pc*-G-dependent maintenance. To confirm this, we looked at the onset of expression and the position of the anterior boundary in embryos homozygous for the loss of function Pc^3 mutation. In the mutant background, expression of a strongly maintaining 2212H6.5 line is now no longer delayed but starts before the cellular blastoderm stage in the form of stripes. During germ band

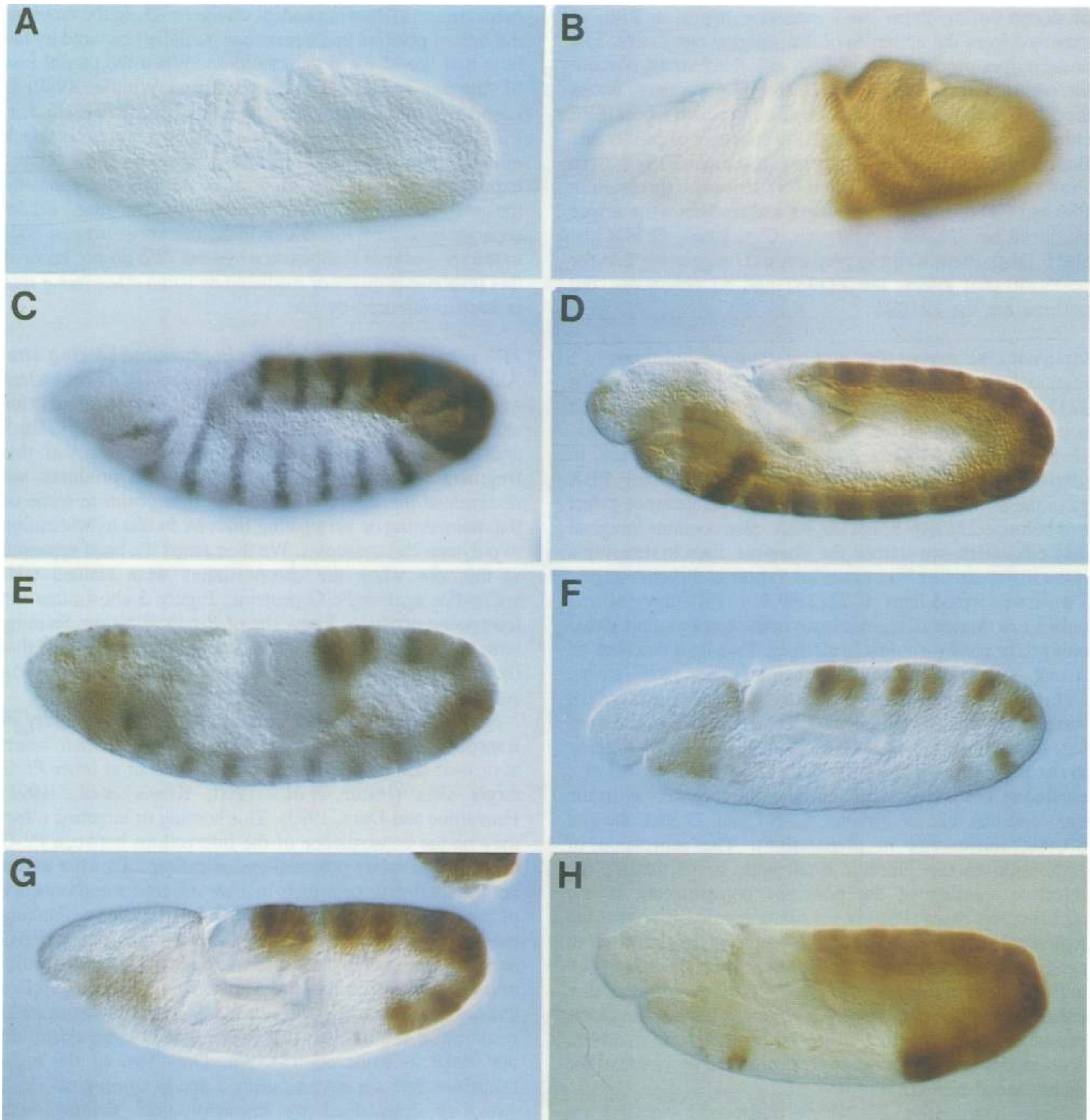

Fig. 3. Posterior shift of the expression boundary. The presence of the PRE causes a weaker and later onset of expression of the 2212H6.5 transposon: (**A**) H6.5 in a wild-type embryo at the beginning of germ band extension; (**B**) H6.5 in a homozygous Pc^- embryo at the same stage. At the fully extended germ band stage, a strongly maintaining line of H6.5 shows that the anterior boundary is pushed back to PS8 (**C**), but the same line in a Pc^- background gives complete derepression (**D**). A similar but less complete posterior shift is observed with the 2212HR3.2 transposon which contains only 2212S1 and the PRE during (**E**) or at the end of germ band extension (**F**). In contrast, no posterior shift is seen in embryos carrying the 2212HS1.6 + HB which contains 2212S1 plus synthetic Hb binding sites but no PRE (**G**). A posterior shift is also not visible in the case of the 2212H6.5 + PBX transposon (**H**), indicating that the PBX enhancer, at least, is not affected.

extension, these give rise to the PS6, 8, 10 and 12 stripes. Expression in the odd-numbered parasegments begins towards the end of germ band extension followed by the thoracic segments and complete breakdown leading to uniform expression in the late embryo and, later, to expression in all imaginal discs. We conclude that the posterior shift is due to the *Pc*-G genes, whose products have three apparently different effects on these transposons: (i) delay of initiation of expression at blastoderm; (ii) extension of repression two parasegments more posteriorly; and (iii) maintenance of the resulting pattern throughout embryogenesis (and, later, in the imaginal discs). In combinations with other enhancers, this posterior shift seems to affect only the S1 and S2 enhancers present in 2212H6.5. Expression of the 2212H6.5 + PBX or 2212H6.5 + BX constructs is easily detected by the blastoderm stage and the pattern

produced during germ band extension begins at PS6, as expected from the activities of the separate enhancers. The broad stripes produced by the PBX or BX enhancers obscure the contribution made by the S1 and S2 enhancers, but in the 2212H6.5 + 2218R6 combination, in which the 2218R6 enhancer [corresponding to the BXD enhancer of Müller and Bienz (1991)] is initially weakly expressed, the early pattern shows weak or missing PS6 and PS7 stripes. Expression in PS6 and PS7 appears at later stages and resembles the stripes produced by 2218R6 (V.Pirrotta, C.-S.Chan, D.McCabe and S.Qian, manuscript in preparation), suggesting that the posterior shift affects the 2212S1 and S2 enhancers, but perhaps not the 2218R6.

Imaginal disc expression and positive maintenance

Imaginal disc enhancers are located in the region between 2212S2 and PBX and are partially included in the 2212H6.5 construct but not in the PBX element alone (V.Pirrotta, C.-S.Chan, D.McCabe and S.Qian, manuscript in preparation). Because the combination 2212H6.5 + PBX gave the strongest and most consistent maintenance effect and because 2212H6.5, but not PBX, also contains imaginal disc enhancers, we stained the imaginal discs to determine if the maintenance effect extended to post-embryonic stages. Two transformed lines of 2212H6.5 + PBX that showed only a low degree of maintenance in the embryo gave either uniform or patchy staining in all discs. Two lines that showed strong eye variegation (see below) and gave strong embryonic maintenance produced a distinct pattern of staining in the posterior compartment of the haltere and third leg, but no staining or only occasional patches of staining in the other discs (Figure 4). Separately, the imaginal disc enhancers are active in all thoracic discs as well as in the eye–antenna disc (V.Pirrotta, C.-S.Chan, D.McCabe and S.Qian, manuscript in preparation). The repression of expression in the anterior discs and, in particular, the selective staining of the posterior compartment in the metathoracic discs (PS6) is a striking confirmation that the maintenance of the boundary established in the embryo persists during later development after several rounds of cell division. Even more striking is the fact that the imaginal disc enhancers, which contain no Hb binding sites and therefore, presumably, no anterior–posterior positional information, acquire this correct boundary of expression by virtue of the maintenance element.

Once again, the 2212H6.5 transposon presents an interesting case. Although the 2212H6.5 construct is potentially active in the imaginal discs, strongly maintaining lines carrying this fragment alone give no expression at all in imaginal discs. In weakly maintaining lines, however, disc expression is found in all discs, including the eye–antenna disc. We conclude that the lack of imaginal disc expression when maintenance is established is a consequence of the posterior shift observed with this construct. If expression is repressed up to PS8 this would eliminate activity in the third leg and haltere disc as well. These results, together with the eye variegation phenomenon described below, indicate that the repressed state conferred by the maintenance element is established in the embryo and is epigenetically inherited by the progeny of embryonic cells that give rise to imaginal structures.

Expression in the imaginal discs allows us also to determine whether the construct responds to the positive maintenance system dependent on the *trx*-G. In the embryo, the lack of positive maintenance is partially obscured by the long half life of the β-galactosidase. When the partial loss of function *trx*[1] mutation (Ingham and Whittle, 1980) is introduced into a strongly maintaining line of 2212H6.5 + PBX, homozygous larvae show a great decrease or total lack of expression in the imaginal discs (Figure 4). Even ectopic expression, in lines that show it, is strongly decreased by the *trx*[1] mutation. These results indicate that, unlike separate enhancers like BX or PBX, this construct responds to the *trx*-positive maintenance system. We do not know if this response is direct or mediated by some other factor that is itself influenced by *trx*.

PRE creates a new polytene chromosome binding site

Although the 2212H6.5 region confers *Pc*-G-dependent maintenance, this could be an indirect effect mediated, for example, by some other factor that interacts with 2212H6.5 and is itself regulated by the *Pc*-G. To show that this fragment contains a direct target for *Pc*-G products, we determined the insertion site of the transposon in some of the maintaining or variegating lines by *in situ* hybridization to polytene chromosomes. We then asked if a band appeared at this site when the chromosomes were stained with antibodies against *Pc*-G proteins. Figure 5 shows that the transposon produces a new site of *Psc* localization. Staining with antibodies against *Pc* and *Su(z)2* products reveals that the transposon insertion site also produces a new binding site for these two proteins (not shown).

Some evidence has been accumulating for the ability of transposons containing targets sites for *Pc*-G genes to insert at or near homologous chromosomal sites or at other *Pc*-G target sites (Hama *et al.*, 1990; Kassis *et al.*, 1991; Fauvarque and Dura, 1993). This homing or targeting effect may be the consequence of the interactions between *Pc*-G proteins and raises potential complications. To what extent are the maintenance effects that we observe a consequence of possible *Pc*-G complexes formed at sequences flanking the insertion site and to what extent are they due to *Pc*-G response elements in the transposon? Although the variability of the different lines obtained with a given transposon implies that the chromosomal environment affects the degree of *Pc*-G response, it is also clear that in our case homing effects do not make a major contribution. Only two of the eight insertions that we have examined are in cytological *Pc*-G sites, as determined by immunological staining with antibodies against *Pc*-G gene products in salivary gland chromosomes (Rastelli *et al.*, 1993). Given the number of *Pc*-G sites in the genome and the cytological resolution, this may not be a significant indication of homing. Many of the stronger maintaining or variegating lines do not correspond to previously known sites of *Pc*-G product localization, but we cannot exclude the possibility that some chromosomal sites form *Pc*-G complexes that are not visible in salivary gland cells but exist at other stages or in other tissues. We conclude that the PRE present in these transposons is most likely itself directly responsible for the maintenance and variegation phenomena.

Variegation

A striking property of flies carrying transposons that include the 2212H6.5 fragment is that in a majority of the lines the mini-*white* gene contained in the CaSpeR transposon vector

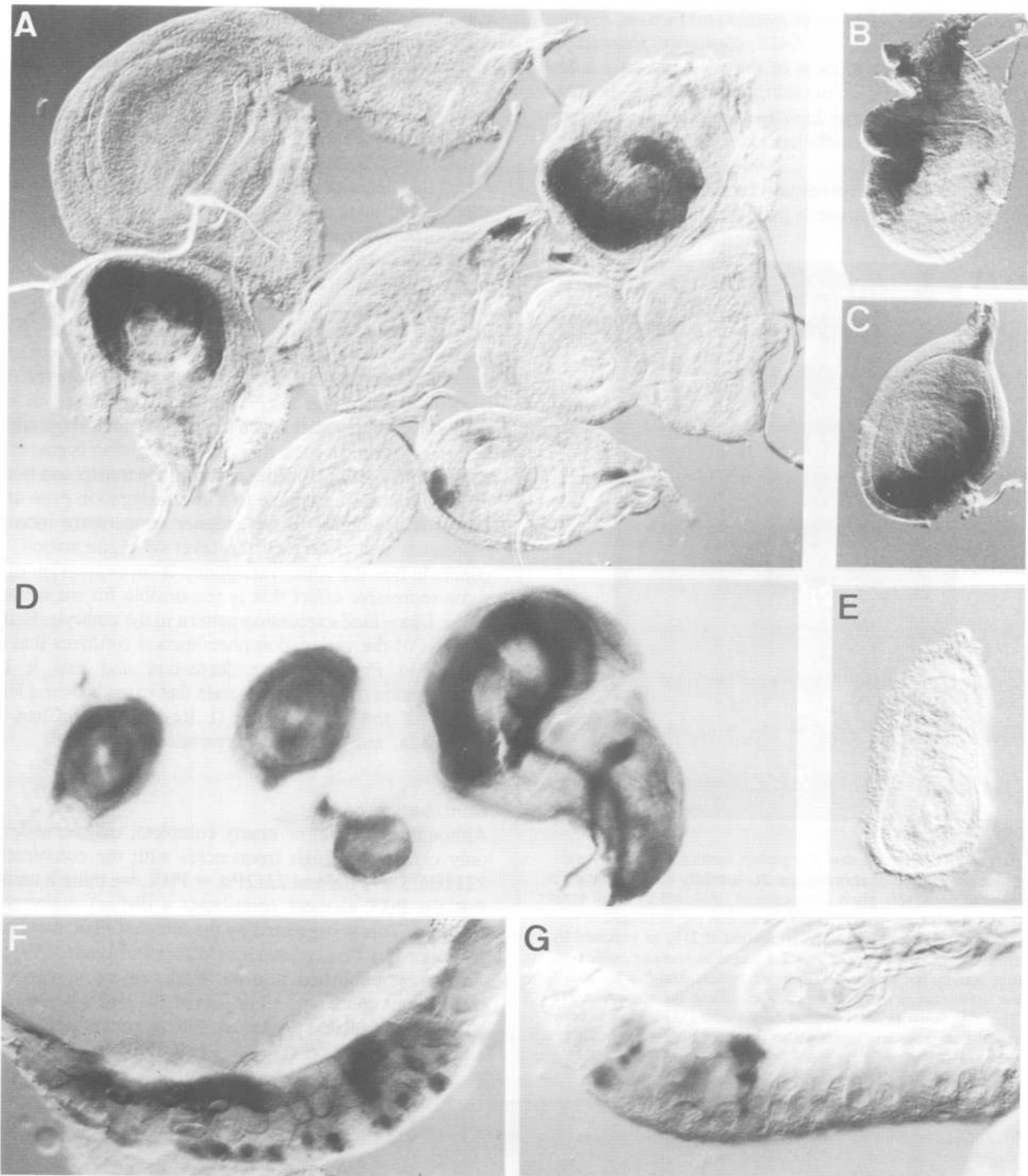

Fig. 4. Maintenance of imaginal disc expression. Imaginal discs of larvae carrying the 2212H6.5 + PBX transposon (A) show expression only in the haltere (B) and third leg (C) in a pattern corresponding to the posterior compartment of the disc (PS6). No expression is found in discs of larvae carrying the H6.5 transposon (not shown) in a strongly maintaining line, consistent with the posterior shift of the boundary to PS8. A weakly maintaining line of 2212H6.5 gives expression in all discs (D). Imaginal disc expression from either transposon is dependent on *trx*. In a trx^1 background, expression of H6.5 + PBX is totally absent in haltere discs (E), and even the ectopic expression seen in salivary glands when repression is incomplete (F) is greatly decreased in the mutant (G).

is partially but non-uniformly repressed, giving rise to a variegated pigmentation in the eye (Figure 6A). Such a variegated appearance has been seen with *white* transposons in rare cases in which the insertion has occurred in the vicinity of β-heterochromatin [see, for example, Steller and Pirrotta (1985b)]. In our case, a large proportion of the lines shows variegated pigmentation to a greater or lesser degree. Some lines show extreme effects, giving only a few pigmented ommatidia in a *white* background. The low frequency of transformation obtained with this transposon suggests, in fact, that many transformed lines may go undetected because of extreme variegation effects. This was confirmed in the case of some transposons used in this study which were constructed using a vector that contained both

the mini-*white* and *hs-neo* genes (Steller and Pirrotta, 1985a). Some lines selected using G418 resistance showed no detectable eye colour in most of the flies and only a few pigmented ommatidia in occasional individuals. In these cases we suppose that the *hsp70−neo* gene was active in at least some tissues to a sufficient extent to confer resistance to G418.

There is an excellent correlation between variegation and the ability of the transposon to maintain anterior repression as if *white* variegation were a particularly sensitive indicator of *Pc*-G activity. Although not all lines obtained with a PRE-containing transposon variegate, maintenance of the *Ubx−lacZ* reporter gene is only observed in lines that variegate (Table I). Furthermore, lines that variegate strongly also maintain strongly while lines that variegate weakly maintain only partially. In fact, it is possible to interpret the partial maintenance observed in many lines as a variegation effect such that, while many cells are able to repress transposon expression in the anterior part of the embryo, some cells escape repression and their progeny continue to express. In contrast to embryonic expression of the *Ubx−lacZ* reporter gene, variegation is only slightly affected in flies heterozygous for the Pc^3 mutation. Since homozygous *Pc* flies die as embryos, we could not study the effect of complete loss of *Pc* function. However, flies homozygous for the $E(z)^{s2}$ mutation and raised at non-permissive temperature reach the pharate adult stage and die before eclosing. In such flies, eye variegation is essentially abolished (Figure 6B). Flies carrying the transposon but not the *E(z)* mutation continue to show variegation even at the higher temperature. In fact, higher temperature increases repression and decreases the level of pigmentation. We conclude that the *white* variegation is an expression of the same repressive effect that is responsible for maintenance of the *Ubx−lacZ* expression pattern in the embryo. Further analysis of the variegation phenomenon confirms that it is related to *Pc*-G complex formation and that it is a manifestation of a chromatin state that extends over a larger region of the chromosome (L.Rastelli, C.-S.Chan and V.Pirrotta, manuscript in preparation).

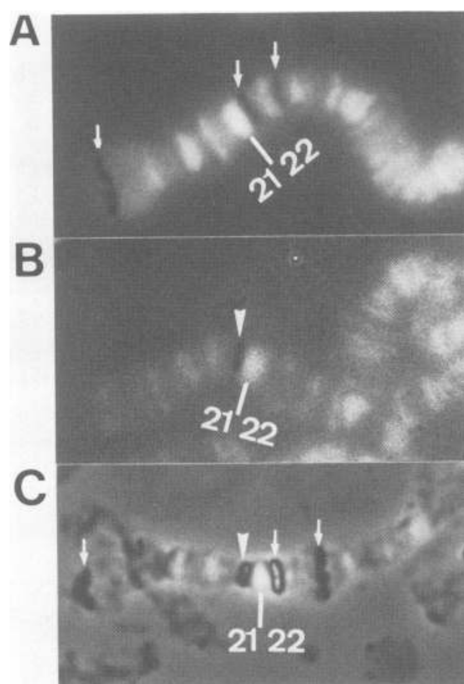

Fig. 5. The PRE creates a new *Psc* protein binding site in polytene chromosomes. The tip of chromosome 2L normally contains three *Psc* binding sites in salivary gland chromosomes, indicated by small arrows in (**A**) and (**C**). *In situ* hybridization shows that the 2212KpR2.2 transposon in this transformed line is inserted at 21F, as indicated by the arrowhead in (**B**). The 2212KpR2.2 fragment contains neither enhancer activity nor detectable Hb binding sites. Anti-*Psc* antibody staining shows that in this line a new *Psc* binding site appears at 21F (C). The chromosomes were counterstained with DAPI so as to better visualize the chromosome bands which therefore appear brightly fluorescing.

Contribution of Hb

Although complete, or nearly complete, maintenance was only obtained at high frequencies with the combinations 2212H6.5 + PBX and 2212Pst + PBX, we think it unlikely that the PBX element contributes a distinct maintenance function. This is suggested by the fact that PBX itself is not influenced by *Pc*-G mutations (Müller and Bienz, 1991; C.-S.Chan, unpublished results). Furthermore, a variety of transposons containing all or part of 2212H6.5 but not PBX show a set of related properties with respect to maintenance and eye variegation (see also L.Rastelli, C.-S.Chan and

Fig. 6. The PRE causes variegation of *white* gene expression. (**A**) Female (above) and male (below) with one copy of the 2212H6.5 + PBX transposon on the X chromosome. (**B**) Same line in a homozygous $E(z)^{S2}$ background, transferred to 29°C after embryonic development. Lethality occurs throughout larval growth, but some individuals reach the pharate adult stage shown here, extracted from the pupal case, and have strong and uniform pigmentation.

V.Pirrotta, manuscript in preparation) that are dependent of *Pc*-G genes and differ only in the degree and frequency with which ectopic expression is repressed. Neither PBX nor any other fragment of the *Ubx* upstream region produced eye variegation or late embryonic pattern maintenance. Of all the enhancers of the *Ubx* gene that we have examined, the isolated PBX enhancer maintains its correct pattern of expression longest, even though it lacks a PRE. We have shown that early breakdown of the Hb-mediated repression in the anterior part of the embryo is closely correlated with the affinity for Hb protein shown by each enhancer element and that PBX contains the strongest binding sites (V.Pirrotta, C.-S.Chan, D.McCabe and S.Qian, manuscript in preparation). We suppose therefore that if the Hb-mediated repression is beginning to break down at the time the *Pc*-G repression is established, the resulting *Pc*-G repression will be patchy and incomplete, while if strong Hb binding sites guarantee a longer-lasting repression, the *Pc*-G complex will be much more likely to form and to establish complete and long-lasting repression.

This interpretation raises the question whether the mere presence of PBX or other strong Hb target can confer better Hb response to an enhancer that, by itself, loses Hb-mediated repression early in development. To test whether the presence of high-affinity Hb binding sites was responsible for more complete maintenance, we added the PBX element to the 2212RB fragment which contains little more than the 2212S2 enhancer. Embryos containing the resulting transposon continue to show loss of maintenance during germ band retraction. We also added a set of seven synthetic Hb binding sites to the 2212HSt1.6 fragment, which contains little more than the 2212S1 enhancer. The 2212HSt1.6 + Hb construct shows longer-lasting maintenance than 2212S1, but this is not affected by *Pc* mutations and still breaks down during germ band retraction. Addition of Hb sites to 2212HR3.2, placed at a distance of several hundred base pairs from the S1 enhancer to mimic the configuration of the 2212H6.5 + PBX construct, gave ambiguous results. Although maintenance is somewhat more complete in some lines of 2212HR3.2 + HB, other lines showed no detectable difference compared with lines of 2212HR3.2.

Table I. Maintenance properties and eye variegation

Construct	Maintenance	Variegation
2212H6.5	1 complete/6 partial	–
2212H6.5	–	5/8
2212H6.5 + PBX1.3	complete	2/4
2212H6.5 + PBX0.6	complete	3/3
2212H6.5 + BX1.5 + PBX1.3	complete	2/3
2212H6.5 + 2218R6	complete	3/5
2212H6.5 + BX1.5	partial	3/4
2212HR3.2	partial	6/9
2212HR3.2 + PBX1.3	complete	1/4
2212Ps3.5	partial	5/8
2212Ps3.5 + PBX1.3	complete	2/3
2212HR3.2 + HB	partial	5/10
2212hst1.6 + HB	none	0/8
2212StR1.6	no expression	23/43
2212StR1.6 + 2212S	partial	2/3
2212StR1.6 + 2218R6	partial	7/13
2212RB1.5 + PBX1.3	none	0/10
2212St1.4	no expression	0/7
2212KpR2.2	–	7/7

In all cases maintenance is observed only in lines that show eye variegation and the extent of maintenance is closely related to the degree of variegation. The degree of maintenance indicated in the second column refers only to the lines that show detectable maintenance (which are also the lines that variegate). Lines that do not maintain begin to show ectopic expression at stage 10 and become completely stained after germ band retraction. The degree of maintenance could be determined only for transposons constructed using the Casper *Ubx–lacZ* vector to reveal embryonic enhancer activity. Constructs containing no *Ubx–lacZ* reporter gene are indicated by a dash in the maintenance column. The 2212H6.5 construct was cloned in a pUChsneo *Ubx–lacZ* vector and used G418 resistance instead of eye pigmentation as a selective marker for transformation. Some of the constructs contain no enhancers and give no *lacZ* expression. Another set of 2212H6.5 constructs was made in pUChsneo containing a mini-*white* gene but lacking the *Ubx–lacZ* reporter gene. The remaining constructs were assembled in the CaSpeR4 vector.

Discussion

The Pc-G maintenance element

The PRE control region that we describe in this paper is a new kind of regulatory element that bears some similarity to the silencer described in yeast [see review by Alberts and Sternglanz (1990)]. Like the silencer, the PRE affects nearby genes over some distance and involves proteins that act upon other heterochromatinized regions. In our case, the element responds to members of the *Pc*-G but it can also be affected by a gene involved in heterochromatin and heterochromatin-related silencing, like *Suvar(3)7*. In the case of the *Pc*-G we have established that the interaction is direct because transposons containing this element create a new binding site for *Pc*-G products on polytene chromosomes. The chromosome staining results also implicate in the PRE complex the product of *Su(z)2*, a *Pc*-G related gene which was not known previously to be directly involved in the regulation of *Ubx* (Rastelli *et al.*, 1993; L.Rastelli, C.-S.Chan and V.Pirrotta, manuscript in preparation).

The PRE, located ~24 kb from the transcription start site, is most probably the only element in the *Ubx* gene able to respond directly to the *Pc*-G genes. We have found no other element in the upstream region that exhibits similar properties with respect to maintenance, interaction with *Pc* products or eye variegation. Furthermore, Simon *et al.* (1993) found only one region in the *Ubx* gene that mediates repression by *Pc*-G products. This region, which they localized in a 15 kb transposon from 12.5 to 28.0 kb upstream of the promoter, includes the site of the PRE we have identified. The separate *Ubx* enhancers do not respond to *Pc*-G mutations, although slight effects were reported for an 8.7 kb fragment containing the BX enhancer (Qian *et al.*, 1993). We cannot explain the results of Müller and Bienz (1991) and Zhang and Bienz (1992) who found that although the separate PBX and BXD (our 2218R6) enhancers do not respond to *Pc*-G genes, when combined in the same construct they appear coordinately to maintain the boundaries of expression in a *Pc*-G-dependent manner. The separate fragments do not seem to be targets of *Pc*-G activity in our experiments either separately or in combination with other enhancers, they do not generate new *Pc* binding sites on polytene chromosomes, do not induce variegation of the *white* gene and do not produce the plethora of distinct phenomena that we find associated with the PRE that we have identified (see also L.Rastelli, C.-S.Chan and V.Pirrotta, manuscript in preparation). We cannot exclude

the possibility that the *Ubx* regulatory region may contain other weaker target sites for the *Pc*-G complex that are normally unable to produce the maintenance and variegation effects associated with the PRE or to generate a new binding site on polytene chromosomes by themselves. Weaker sites might contribute to the spread of an expanding *Pc*-G complex on the chromatin. Recently, Orlando and Paro (1993) have shown that in cells in which the *Ubx* gene is repressed, Pc protein becomes associated with virtually the entire length of the gene, providing a physical proof of the spreading of the complex and the involvement of other DNA sequences, in addition to the PRE. The existence of accessory sites might explain, for example, the striking improvement in the maintenance properties of the 2212H6.5 fragment when the PBX region is present in the same construct. An alternative interpretation is that the seven exceptionally strong Hb binding sites present in the PBX enhancer may stabilize Hb binding also to the 2212H6.5 enhancers through cooperative interactions between Hb bound at different sites over the whole region. As a result, the direct repression exercised by Hb would last longer and give more time for a repressive *Pc*-G complex to be established. However, our attempts to demonstrate such an effect by the addition of synthetic Hb binding sites have not given clear cut results.

The dramatic posterior shift in the position of the expression boundary observed with the 2212H6.5 transposon or its derivatives raises more possibilities. We can see three possible interpretations for this shift. (i) An initial complex formed at the PRE increases the binding efficiency of Hb or other gap gene repressors, such as *Krüppel* product, retarding initiation of transcription and extending repression more posteriorly. *Pc*-G products might enhance *Krüppel* repression just as they enhance the repression of gap genes *giant* and *knirps* by Hb (Pelegri and Lehmann, 1994). (ii) Proximity of an enhancer to the PRE causes stronger repression that prevents expression even in the presence of lower concentrations of Hb. (iii) Repression, perhaps due to an initial complex formed at the PRE, interferes with the initiation of expression of some enhancers but not others that employ other initiating factors. These explanations are not mutually exclusive and are not entirely satisfactory, but in some combinations they might account for the fact that the 2212S1 and S2 enhancers are affected but the PBX and BX enhancers are not. Furthermore, the possibility that the distance from the PRE has an effect on the timing or degree of repression may have important consequences for the behaviour of the *Ubx* gene as a whole. These possibilities are currently being explored.

Model for establishment of maintenance

Our results strongly support the idea that the formation of a chromatin complex is the basis for the maintenance phenomenon. In vertebrates, such phenomena are generally associated with DNA methylation, but no methylation has been detected in *Drosophila* DNA although the existence of a few hundred methylated bases per genome could have escaped detection. More likely, as has been suggested frequently [see Paro (1990) and Bienz (1992) for recent reviews], the products of the *Pc*-G genes form a chromatin complex that can survive several rounds of cell division and is sufficient to maintain repression in *Drosophila* where the organism is relatively short-lived and only a few cell divisions intervene between the time the *Ubx* pattern is determined and the end of development.

Two basic models can be considered for the position-dependent establishment of the maintenance complex. Because Hb is responsible for establishing the anterior boundary of the expression domain of *Ubx* and because all the enhancers contain Hb binding sites, one possible model envisages that the PRE, in the vicinity of an enhancer or promoters, is the site of an initial complex which needs an additional signal to progress to an effective maintenance complex. This signal might be given by Hb protein bound to the enhancer which acts in concert with the PRE to recruit additional components of the *Pc*-G complex. Such a model, in which Hb plays a 'tethering' role for *Pc*-G proteins, has been proposed by Bienz (1992). If this model is correct, while the precise position of Hb binding sites, overlapping the activator binding sites is critical to prevent transcriptional activation, it would not be important for the establishment of the maintenance complex. Strong Hb binding in the general vicinity might be sufficient to recruit components for *Pc*-G complex formation. This model might help explain the strong enhancement of repression when the PBX element with its strong Hb binding sites is added to the 2212H6.5 fragment. It would be consistent with our finding of an early effect of the PRE in delaying initiation of expression of the 2212S1 and S2 enhancers. An interaction between Hb and *Pc*-G products would also fit well with the discovery by Pelegri and Lehmann (1994) that some members of the *Pc*-G, in particular *E(z)* but not *Pc* itself, are involved in stabilizing the ability of Hb to repress the gap genes *knirps* and *giant*.

An alternative model presupposes that an early complex formed at the PRE senses the state of activity of promoters or enhancers in the vicinity. If they are in the active state, e.g. devoid of nucleosomes or involved in an activation complex by the formation of an enhancer–promoter loop etc., repression is not established and the enhancer–promoter complex may even be locked in an active or potentially active configuration. If the region is not transcriptionally active, the maintenance complex proceeds to assemble, locking the chromatin region in a configuration that prevents expression. This is often conceived as a compaction of chromatin into an inactive heterochromatin-like state [see, for example, Paro (1990) or Fauvarque and Dura (1993)], although evidence for compaction is lacking. It could also be envisaged as a stabilization of nucleosomes that prevent the access of transcriptional activators, an interference with the looping that juxtaposes enhancer and promoter, prevention of localization in a nuclear compartment in which transcriptional activity takes place or a variety of other possible mechanisms that have been proposed to explain silencing [see also Wu (1993)]. Although we have not excluded the first model, we favour the alternative. The addition of Hb binding sites makes at best a small contribution to the ability of the PRE to establish maintenance. On the other hand, elements such as the imaginal disc enhancers, that are not active in the embryo at the time the repressive complex is established, are very efficiently repressed although they contain no Hb binding sites and, by themselves, would be expressed in all imaginal discs. The *white* gene, which contains no Hb binding sites, is efficiently repressed by fragments containing the PRE,

but no *Ubx* enhancer or promoter. Finally, it would be unreasonable to suppose that Hb mediates the ability of the *Pc*-G complex to repress a wide variety of genes, including the anterior homeotic genes, other *Pc*-G genes themselves, or a segment polarity gene like *engrailed* that are *Pc*-G targets but whose repression is not Hb-dependent. We do not know why some chromosomal insertion sites do not support the function of the PRE. According to the second model, sites in which the promoter has too high a basal level or in which it is activated by some nearby enhancer might result in locking the promoter in an active state. However, we have no evidence that this is the case in the lines that fail to maintain or to variegate.

Implications for maintenance of imaginal expression

Our finding that imaginal disc enhancers are separate and distinct from the embryonic enhancers, that they have no Hb binding sites and receive therefore no direct positional information from the segmentation genes, raises the question of how they acquire their segmental specificity. The long-range action of the maintenance mechanism provides a plausible explanation: the imaginal disc enhancers receive the positional information from the embryonic enhancers through the mediation of the PRE. According to this scenario, the state of activity of an embryonic enhancer in a given cell determines whether that regulatory region will be open or maintained in a repressed state by the *Pc*-G complex and, therefore, whether the nearby imaginal enhancer will be open or repressed in that cell. Imaginal enhancers have been found in two regions of the *Ubx* gene: (i) in the vicinity of the ABX enhancer but expressing in all discs; and (ii) in the *pbx* region. We suppose therefore that they will be controlled primarily by the state of activity of the ABX and PBX enhancers, respectively. *abx* mutations will cause principal loss of activity of imaginal enhancers in the odd-numbered parasegments, the domain of activity of the ABX enhancer. The pbx^1 deletion, which removes both the PBX enhancer and the nearby imaginal enhancers, should cause loss of expression primarily in the even-numbered parasegments, the principal domain of activity of the PBX enhancer. A somewhat more complicated situation is presented by the pbx^2 deletion which removes not only the imaginal enhancers, but also the entire PRE region. This should result in the inability to establish repression and therefore in ectopic expression in the anterior part of the embryo. That this is not the case may be explained if the same region deleted by the pbx^2, but not by the pbx^1, mutation also deletes the positive maintenance element which responds to the *trithorax* product. Genetic experiments indicate that when both the *Pc*-G system and the *trx*-G system are inactivated, the effects cancel out, resulting in near normal development (Capdevila and Garcia-Bellido, 1981; Ingham, 1983). We might expect therefore that the simultaneous loss of the positive and negative maintenance would leave pbx^2 with a phenotype just due to the loss of the imaginal enhancers and very similar to that of pbx^1. However, we do not know whether there are other *trx* response elements elsewhere in the gene.

Variegation of the white gene

The presence of the PRE in a transposon containing the *white* minigene causes strong variegating effects and sometimes complete repression of eye pigmentation. The clonal nature of repression is evident in the patchwise distribution of pigmented or unpigmented cells and indicates that repression is established very early in the embryo when the primordia of the eye imaginal disc are limited to a small number of cells. The degree of variegation in any given line is correlated closely with the degree of maintenance of the *Ubx−lacZ* expression in the embryo and in imaginal discs. A similar variegation effect of the *white* gene has also been noted by Kassis *et al.* (1991) in the presence of a fragment from the *engrailed* regulatory region and by Fauvarque and Dura (1993) with the *ph* regulatory region, loci that are known to be regulated by *Pc*-G genes. In our case, the repressive effect occurs even when no other *Ubx* fragment, enhancer or promoter is included in the transposon, indicating that Hb-mediated repression is not itself essential for the establishment of the repressive complex. Fauvarque and Dura (1993) found that the eye variegation obtained with transposons containing the *ph* regulatory region is also affected by some of the suppressors of PEV. This, together with our observation that the maintenance function of the PRE can be influenced by *Suvar(3)7*, strengthens the conclusion that the complexes formed by *Su(var)* proteins are related to those formed by the *Pc*-G proteins.

The eye variegation phenomenon indicates that, as in the case of the *engrailed* and *ph* regulatory regions, the repressive effects of the PRE can operate on heterologous promoters and not just on the *Ubx* promoter and that they affect a broad region of DNA because the PRE can repress simultaneously the *Ubx* promoter and the *white* promoter contained in the same transposon. Evidence that the repressive complex affects an even broader chromatin region is presented by L.Rastelli *et al.* (manuscript in preparation), together with a more thorough analysis of the variegation phenomenon and of the factors affecting it.

Materials and methods

Transposon construction and germ line transformation

Most of the constructs were assembled in the CaSpeR4 vector for germ line transformation (Thummel and Pirrotta, 1991) where the fragments of the *Ubx* regulatory region were placed in front of the *Ubx−lacZ* reporter gene [see Qian *et al.* (1991) for details]. In some cases, when we expected that severe variegation would impede the identification of transformed flies through eye pigmentation, we made use of the pUChsneo vector (Steller and Pirrotta, 1985a) with the addition of the *Ubx−lacZ* reporter gene or the mini-*white* gene. When the *Ubx* fragment to be tested lacked an embryonic enhancer, we used the pUChsneo vector carrying the mini-*white* gene or the CaSpeR vector without the *Ubx−lacZ* reporter gene and monitored the effects on eye colour variegation. Selection of flies transformed with transposons containing the *hsp70−neo* gene was done by growing the larvae on food containing 0.5 mg/ml G418 (Geneticin, Sigma). All injections were made in embryos carrying the w^{67c2} deletion, a small homozygous viable deficiency removing the first exon and upstream sequence of the *white* gene. Synthetic Hb binding sites were added to some constructs. They were constructed from a monomer GATCCACATTTTTTATGGCGC, whose sequence contains Hb binding site Hb1 from the PBX enhancer (V.Pirrotta *et al.*, manuscript in preparation). The oligonucleotide was concatemerized to obtain a heptamer in random orientations whose structure was confirmed by direct sequencing.

Polytene chromosome staining

In situ hybridization to salivary gland chromosomes was carried out using a transposon probe digoxygenin-labelled with the Genius kit (Boehringer). The signal was detected using anti-digoxygenin antibody conjugated with alkaline phosphatase. Immunological staining of the chromosomes with anti-Pc, anti-Psc or anti-Su(z)2 antibodies was performed as described previously

(Rastelli *et al.*, 1993). Counterstaining the chromosomes with DAPI increased the sensitivity of detection and facilitated the identification of the chromosomal bands.

Staining of embryos and imaginal discs

Embryos were fixed, stained and mounted using the methods of Lawrence and Johnston (1989). The rabbit anti-β-galactosidase antibody (Cappel) was used as described by Qian *et al.* (1991). Monoclonal mouse anti-engrailed antibody, a gift from T.Kornberg, was used at 1:1000 dilution. The antibody was detected as a brown colour using the Vectastain ABC-HRP kit (Vector Labs), as instructed. For double staining, the embryos were washed after anti-β-galactosidase staining and incubated overnight with second antibody, then reacted with the Vectastain ABC-AP kit to produce a blue colour. In some cases, the second antibody was stained with the ABC-HRP kit but in the presence of nickel ion to give a black instead of brown colour. To stain imaginal discs and salivary glands, the tissues were fixed with glutaraldehyde and stained with X-gal as described by Bellen *et al.* (1989).

Eye pigmentation

Flies were raised at 22–24°C and photographed about 1 day after eclosion, except for the homozygous *E(z)* mutant in Figure 6B which was raised at 29°C and extracted from the pupal case. Temperature affects the degree of variegation in the opposite direction to the commonly observed effect in PEV. That is, higher temperatures result in increased variegation and decreased pigmentation.

Acknowledgements

We thank Su Qian for critical reading of the manuscript and Tom Kornberg and Phil Ingham for antibodies and mutant flies. We are grateful to Jackie Guiard and Marie Paule Barrillat for excellent technical support, and Elvyre Martinez and Micheline Vautravers for photographic services. C.-S.C. was on leave from the Baylor College of Medicine and was supported by a stipend from the University of Geneva. L.R. was the recipient of a fellowship from the CNR Progetto 'Ricerche Tecnologiche e Innovazione'. The Georges and Antoine Claraz Donation is gratefully acknowledged. This work was supported in part by a grant to V.P. from the NIH and in part by the University of Geneva.

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Received on January 17, 1994; revised on March 2, 1994