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Direct interaction of the *Polycomb* protein with *Antennapedia* regulatory sequences in polytene chromosomes of *Drosophila melanogaster*

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The *Polycomb* (*Pc*) gene is responsible for the elaboration and maintenance of the expression pattern of the homeotic genes during development of *Drosophila*. In mutant Pc^- embryos, homeotic transcripts are ectopically expressed, leading to abdominal transformations in all segments. From this it was suggested that Pc^+ acts as a repressor of homeotic gene transcription. We have mapped the *cis*-acting control sequences of the homeotic *Antennapedia (Antp)* gene regulated by *Pc*. Using *Antp* P1 and P2 promoter fragments linked to the *E.coli lacZ* reporter gene we show different expression patterns of β-galactosidase (β-gal) in transformed Pc^+ and Pc^- embryos. In addition we are able to visualize by immunocytochemical techniques on polytene chromosomes the direct binding of the *Pc* protein to the transposed *cis*-regulatory promoter fragments. However, short *Antp* P1 promoter constructs which are—due to position effects—ectopically activated in salivary glands, do not reveal a *Pc* binding signal.

Key words: Antennapedia/Drosophila/homeotic gene regulation/*Polycomb*/polytene chromosomes

Introduction

The homeotic genes of *Drosophila* determine the diverse developmental pathways by which each segment acquires a distinct larval and adult phenotype. Diversification of the head, thoracic and the abdominal segments results from the position-specific gene expression along the antero-posterior axis of the embryo of two clusters of homeotic genes called the bithorax complex (BX-C) and the Antennapedia complex (ANT-C) (for a review see Duncan, 1987; Gehring and Hiromi, 1986). The *Antennapedia (Antp)* gene is a member of the ANT-C. From genetic analyses it is known to be required for the correct development of the thoracic segments (Wakimoto and Kaufman, 1981; Martinez-Arias, 1986). The structural organization of the *Antp* gene is complex; eight relatively small exons span ~100 kb of DNA (Garber *et al.*, 1983; Scott *et al.*, 1983). Transcription initiates at either of two promoters P1 and P2 which are separated by 67 kb (Laughon *et al.*, 1986; Schneuwly *et al.*, 1986; Stroeher *et al.*, 1986). Genetic as well as molecular data indicate that the two promoters reflect individual functional units within the *Antp* gene that are differentially regulated (Abbott and Kaufman, 1986; Ingham and Martinez-Arias, 1986; Jorgensen and Garber, 1987; Irish *et al.*, 1989; Bermingham *et al.*, 1990). The two overlapping transcription units produce four different primary transcripts, by utilizing either of the two promoters and either of the two polyadenylation sites. Alternative splicing of the primary transcripts results in slightly different forms of the *Antp* protein, however, without any preference in splice site employment depending on the promoter and polyA site usage (Bermingham and Scott, 1988; Stroeher *et al.*, 1988). *Antp* transcripts and proteins are largely confined to the thorax of the embryo, the region which was found to be affected in $Antp^-$ homozygotes (Levine *et al.*, 1983; Carroll *et al.*, 1986; Martinez-Arias, 1986; Wirz *et al.*, 1986).

The differential expression of homeotic genes is under multigenic control. Maternal genes and segmentation genes delimit the expression of the homeotic genes to specific spatial domains early in development (Ingham and Martinez-Arias, 1986; Harding and Levine, 1988; Irish *et al.*, 1989). The initial pattern of expression of homeotic genes is then modulated and refined by cross-regulatory interactions among the ANT-C and BX-C genes themselves (Hafen *et al.*, 1984; Harding *et al.*, 1985). The correct spatial expression of the ANT-C and BX-C is furthermore dependent on a class of genes called the Polycomb group (Pc-G). These genes seem to be required for the establishment of a cellular memory, since their role is to maintain the homeotic genes in a repressed state in cells where the homeotic gene expression was not initiated by the early-acting maternal and segmentation genes. The function of Pc-G genes is most evident in mutants of the gene *Polycomb (Pc)*. *Pc* homozygous mutant embryos display posteriorly directed transformations of most body segments, a phenotype which was interpreted as a result of ectopic BX-C expression due to the lack of *Pc* repressor function (Lewis, 1978; Duncan and Lewis, 1982). The involvement of *Pc* in the control of ANT-C and BX-C gene expression has also been proposed from the adult *Pc* mutant phenotype. *Pc* heterozygote adults exhibit transformations that correspond to those described for gain-of-function mutations in the ANT-C (antenna-to-leg; extra sex combs on the second and third thoracic leg) and the BX-C (haltere-to-wing; fourth abdominal segment into the fifth) (Duncan and Lewis, 1982; Sato and Denell, 1985; Sato *et al.*, 1985; Capdevila *et al.*, 1986). Molecular analyses confirmed that the morphological transformations seen in *Pc* mutants result from ectopic distribution of homeotic gene products. For example, *Antp* transcripts and proteins are no longer predominantly distributed in the thoracic ganglia of the embryo, but are detected throughout the CNS at equal levels in the absence of *Pc* products (Carroll *et al.*, 1986; Wedeen *et al.*, 1986).

To determine whether *Pc* represents a *trans*-acting factor

interacting with *Antp* regulatory sequences, we analyze the expression of β-gal (β-galactosidase) from different *Antp* promoter−*lacZ* fusion genes in Pc^+ embryos and in embryos lacking a functional *Pc* gene. Comparing the β-gal expression pattern in Pc^+ and Pc^- mutant embryos, we find that the *Pc* gene is necessary for normal regulation of *Antp* P1 and P2 expression. By applying anti-*Pc* antibodies to polytene chromosomes, we had previously demonstrated that the *Pc* protein binds to chromatin encompassing the ANT-C, the BX-C and most of the Pc-G genes in order to exert its regulatory function (Zink and Paro, 1989). To visualize the interaction of the *Pc* protein with a single gene of the homeotic cluster at a higher resolution, we make use of various transformed fly strains carrying different *Antp* promoter and regulatory sequences fused to the *lacZ* gene. Immunostaining of polytene chromosomes reveals a new *Pc* binding site at the integration site of the transgene. Furthermore, the binding is dependent on the site of integration of the transgene, which implies that *Pc* binding is sensitive to the different structural chromatin configurations acquired by the *Antp* sequences at the various integration sites. In addition, we are able to show a functional correlation between binding of *Pc* protein to a specific promoter fragment and its repressor activity.

Results

Cis- and trans-acting elements regulating Antp gene expression

As a first step in analyzing the mechanisms regulating the expression of the *Antp* gene during development, an approach was taken to define *cis*-acting regulatory elements which control transcription from the two *Antp* promoters P1 and P2. We have constructed fusion genes that contain various amounts of 5′ flanking sequences, corresponding exons, and portions of the introns, of either P1 or P2, fused to the coding region of the *E.coli lacZ* reporter gene. After P-element mediated germ line transformation, the expression pattern of β-gal was analyzed in whole mount embryos. In order to identify *trans*-acting genes, we analyzed the expression of β-gal from different *Antp* promoter−*lacZ* fusion gene constructs in embryos homozygous for mutations in a number of developmentally regulated genes (Y.Engström and W.J.Gehring, in preparation). Here we describe the *Pc*-responsive *cis*-acting elements regulating the two *Antp* promoters.

Antp P1−lacZ expression in Pc^+ and Pc^- embryos

The expression of β-gal in embryos carrying the P1 constructs pAPT1.8, pAPT1.4, pAPT1.1 and pAPT1.0 (see Figure 1B) was analyzed as described under Materials and methods. The pAPT1.8 construct reproduces essentially the expression pattern as seen for the wild-type distribution of P1 driven transcripts. In contrast, the shorter constructs reveal subpatterns of the wild-type pattern, including both lack of expression and ectopic expression in different regions of the embryo. A detailed description of the expression patterns from the different constructs during embryogenesis will be presented elsewhere (Y.Engström and W.J.Gehring, in preparation). In this context we only describe the patterns generated at and after the germ band extension stage, since this is the earliest time when *Antp*-transcripts as well as

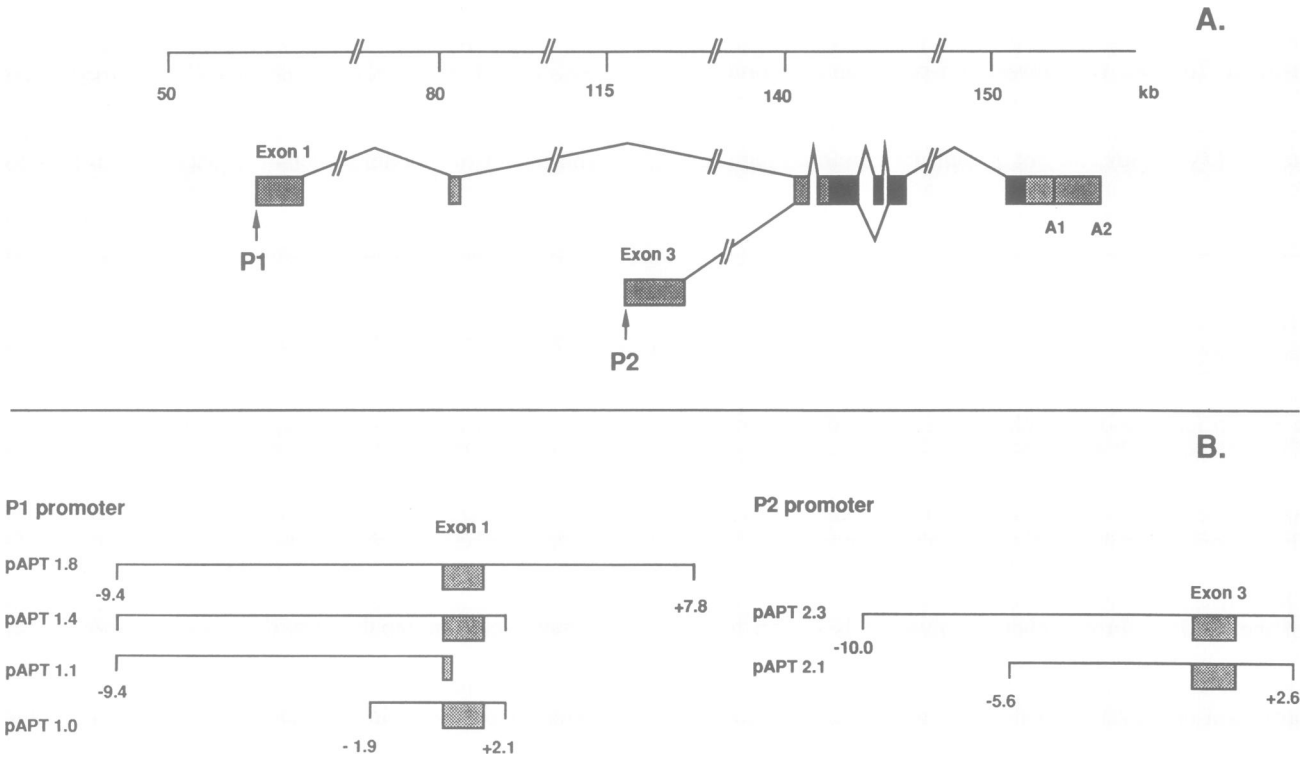

Fig. 1. Molecular map of the *Antennapedia* gene and of the P1 and P2 promoter fragments. (**A**) Schematic diagram showing the structure of the *Antennapedia* gene. The diagram is a composite from figures in Schneuwly *et al.* (1986) and Bermingham and Scott (1988). P1, P2: promoters. A1, A2: polyadenylation sites. Filled boxes: open reading frame. (**B**) *Antp* promoter sequences which were fused to the actin−*lacZ* reporter gene of the transformation vector pPT 12/2 (see Materials and methods). 5′- and 3′-flanking sequences of the *Antp* P1 and *Antp* P2 cap-site, respectively, are indicated in kb. The different pAPT constructs were introduced into the fly genome by P-element mediated transformation.

β-gal expression patterns show anteriorly directed ectopic expression in Pc^- embryos. An embryo carrying the pAPT1.8 construct (line pAPT1.8-20B), is shown in Figure 2A. At this retracted germ band stage, the P1 driven β-gal staining in the epidermis is confined to a broad region in the thorax, extending from the posterior compartment of T1 (pT1) to T3. Expression is also evident in mesodermal derivatives and in the ventral cord exhibiting high levels in the thoracic neuromeres pT1 to T3 and lower levels in the abdominal neuromeres A1 to anterior A7.

The line pAPT1.8-20B was analyzed in Pc^- mutant background, and as seen in Figure 2B the expression of β-gal is dramatically changed in the absence of the *Pc* gene product. β-gal staining is present in all neuromeres of the central nervous system (CNS), including the supraoesophageal ganglion (spg), the labial (lb), maxillary

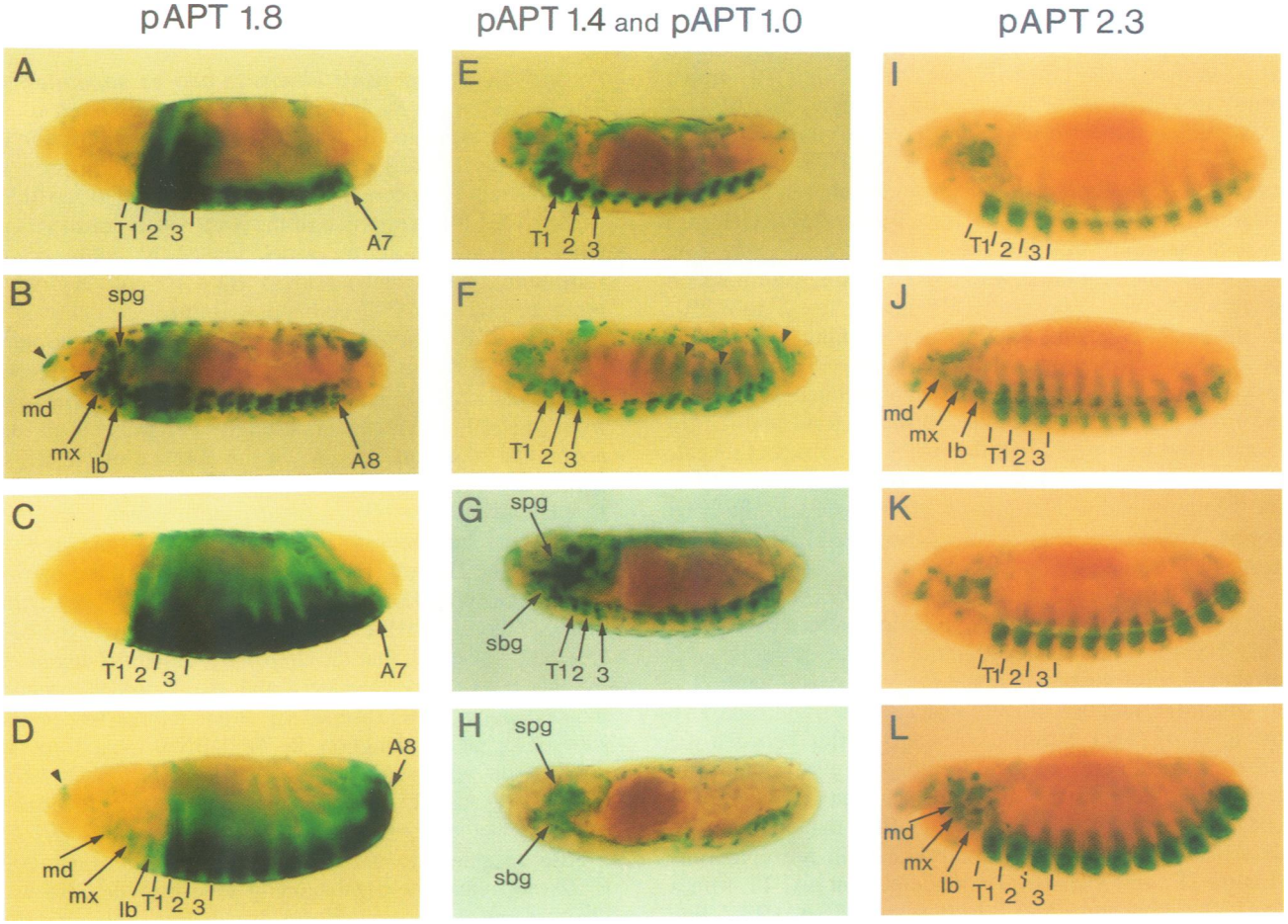

Fig. 2. β-gal expression directed from *Antp* P1 and P2 promoter sequences in wild-type and in homozygous Pc^- mutant embryos. Indicated on top of each panel is the *Antp−lacZ* construct, which is carried by the transgenic embryos (see Figure 1B). All embryos are shown in a lateral view except when indicated, and anterior is to the left. Arrows point to the respective neuromeres, while bars indicate segmental boundaries. For a description of the developmental stages, see Campos-Ortega and Hartenstein (1985). **(A)−(D)** Transformant embryos from the line pAPT1.8-20B. (A) Wild-type at stage 13. Expression is evident in the epidermis between pT1 and T3, and between pT1 and aA7 (parasegment (PS) 4−12) in the CNS. (B) Pc^- at stage 14 in a ventrolateral view. Note the ectopic expression in the supraoesophageal ganglion (spg), in the mandibular (md), maxillary (mx) and labial (lb) ganglia, as well as in the A8 neuromere and in the clypeolabrum (arrowhead). (C) BX-C^- at stage 14. Strong reporter gene expression extends from pT1 to aA7 (PS 4-12) both in the epidermis and in the CNS. (D) Pc^- BX-C^- at late stage 12. β-gal expression is detected in all neuromeres of the ventral cord and in the suboesophageal ganglia. Strong derepression is seen in the epidermis from pT1 to A8 and in the clypeolabrum (arrowhead). The relatively weak staining in the suboesophageal neuromeres in (D) in comparison with (B) is explained by the accumulation of β-gal in the VNS of the older embryo (B), and that the embryo in (B) but not in (D) is homozygous for the *Antp−lacZ* fusion gene. **(E)−(F)** Embryos from the transformant line pAPT1.4-49A at stage 15 to 16. (E) Wild-type, revealing β-gal expression in the CNS extending from the gnathal ganglia to the abdominal ganglion of A8. Observe the difference in staining intensity between the thoracic and the abdominal neuromeres. (F) Pc^-. The level of β-gal in the CNS is reduced and almost uniform comparing the different neuromeres, including A9. The arrowheads indicate stripes of ectopic β-gal staining in the epidermis. **(G)** and **(H)** are embryos transformed with the construct pAPT1.0-5C, stages 15 to 16. (G) Wild-type. β-gal staining is evident in all neuromeres of the CNS except A9, with high levels in the supraoesophageal (spg) and in the suboesophageal (sbg) ganglia, while low levels are seen in the A1 and A2 neuromeres. (H) Pc^-. The β-gal staining in the CNS is drastically reduced, except in the supraoesophageal and suboesophagial ganglia revealing moderate levels of expression. **(I)−(L)** Transformant embryos of the line pAPT2.3-30D, at stages 13 to 14. (I) Wild-type, revealing β-gal staining in the ventral cord. T1, T2 and T3 reveal equally strong expression while the nine abdominal neuromeres display lower levels. No expression is detected in the gnathal neuromeres and in the supraoesophageal ganglion. The staining in the ventrolateral epidermis and in the head region is out of focus in this micrograph. (J) Pc^- in a slightly ventrolateral view. In this micrograph, the ventrolateral stripes of epidermal staining are better exposed. The staining in the thorax and abdomen is similar in comparison with the wild-type embryo in (I), but in addition the mandibular (md), maxillary (mx) and labial (lb) neuromeres express β-gal. (K) BX-C^-. In comparison with wild-type (I) the β-gal staining in the VNS is intensified and equally distributed in the thoracic and abdominal neuromeres. The level of expression in the ventrolateral epidermis is increased in the abdominal segments, leading to equally strong staining in the thoracic and abdominal segments. (L) Pc^- BX-C^-. Strong derepression of the β-gal expression is seen in the thoracic and abdominal segments, both in the VNS and in the ventrolateral epidermis. Ectopic staining is also evident in the mandibular (md), maxillary (mx) and labial (lb) ganglia.

(mx) and mandibular (md) ganglia, as well as extending further posterior than in wild-type. The level of P1 expression in the ventral nerve cord was, however, lower than in wild-type embryos. Ectopic expression was also evident in the clypeolabrum (arrowhead). The expression in the thoracic epidermis was not spatially disturbed but significantly weaker than in pAPT1.8 Pc^+ embryos.

In order to delimit the *cis*-elements regulated by *Pc*, we studied the effect of the *Pc* gene on the shorter P1 constructs (see Figure 1B). The pAPT1.4 and pAPT1.1 embryos do not reproduce the same pattern of fusion gene expression as seen for pAPT1.8 and for normal *Antp* P1 driven transcript distribution. At germ band extension, the pAPT1.4 expression is ectodermal, forming a band around the embryo in pT1. The lack of expression in T2 and T3, indicate the existence of positive *cis* regulatory elements in the intron between +2.1 to +7.8 kb (Y.Engström and W.J.Gehring, in preparation). When the germ band retracts, expression becomes evident in the developing CNS in a pattern which partly resembles the expected P1 expression (Figure 2E). In the ventral ganglia the strongest staining was always observed in the thoracic neuromeres (T1−T3), moderate levels in the gnathal neuromeres and abdominal neuromeres A3−A8, and low levels in the neuromeres A1 and A2. In comparison with pAPT1.8, the pAPT1.4 construct reveals ectopic expression anterior to pT1 and posterior to A7 in the ventral ganglia, and also in other tissues in the head region. This ectopic expression was observed from extended germ band stage on.

The pAPT1.4 lines were analyzed in Pc^- background, and as seen in Figure 2F the expression pattern of β-gal in the CNS was clearly changed in the absence of the *Pc* gene product. Unlike the transformant Pc^+ embryos (Figure 2E), in which the number of cells that express β-gal, and also the level of β-gal expression, clearly differ between the different metameres, the Pc^- embryos reveal a uniform level of β-gal expression in all the segments of the ventral cord, including A9. Many of the Pc^- mutant embryos displayed stripes of ectopic expression in the epidermis (Figure 2F, arrowheads). Two independent pAPT1.4 lines (pAPT1.4-49A and pAPT1.4-20D) and one pAPT1.1 line were tested in Pc^- embryos, revealing in all three cases the same altered β-gal staining pattern.

To dissect the *cis* control regions further, we analyzed the effect of *Pc* on the shortest P1 construct (pAPT1.0, see Figure 1B). In analyzing the pAPT1.0 transformants in wild-type background, we observed conspicuous variations in the patterns of β-gal staining when different lines were compared. Only a few of the transformant lines (3 out of 10) revealed β-gal expression patterns in the CNS which were similar to the one found in the transformants carrying pAPT1.4, with additional ectopic expression in the supraoesophageal ganglion. As an example, the line pAPT1.0-5C is shown in Figure 2G. The levels of P1 expression between the different neuromeres correspond to the levels found in pAPT1.4, while the pattern of stained cells is more divergent. The same lines were analyzed in Pc^- background (Figure 2H). A striking reduction of *Antp* expression, except in the supra- and suboesophageal ganglia, and only subtle, ectopic activation in *Antp* expression can be observed. Thus, it becomes evident that the effect *Pc* has on *Antp* P1 regulatory sequences diminishes the shorter the constructs are. In contrast, the repressing effect of the BX-C genes is even more pronounced on the shorter constructs (see below). In addition, two of the pAPT1.0 lines, pAPT1.0-55C and pAPT1.0-79A, which diverged more from the expected pattern of *Antp* P1 expression, were analyzed in Pc^+ and Pc^- backgrounds. We could not detect any difference in the β-gal expression patterns when Pc^+ and Pc^- embryos were compared. This indicates that regulatory factors are inhibited, due to position effects, from controlling the spatial expression of the short P1 constructs in a wild-type fashion.

Pc and BX-C responsive elements can be separated

The overall lower β-gal expression in Pc^- embryos could possibly be explained by a repression of P1 transcription in *trans* by the BX-C genes, which are also derepressed in Pc^- mutants (Wedeen *et al.*, 1986). In order to test this, we analyzed the expression of the *Antp−lacZ* constructs in embryos homozygous for a deletion of the entire BX-C [Df(3R)P9, here referred to as BX-C^-] and in double mutant Pc^- BX-C^- embryos. Figure 2C shows a pAPT1.8-20B embryo lacking the BX-C genes, and Figure 2D a pAPT1.8-20B embryo lacking both the BX-C genes and a functional *Pc* gene. It is evident that the expression from the P1 promoter is strongly derepressed in the abdominal segments A1−A7 in the absence of the BX-C genes. In contrast, embryos that lack both the BX-C and the *Pc* gene products exhibit a β-gal pattern which resembles the sum of the aberrant patterns in BX-C^- and in Pc^- mutants (see Figure 2D). The ectopic expression anterior to pT1 and posterior to A7 in the Pc^- embryos (Figure 2B), can therefore be separated from the activity of the BX-C genes, indicating that the *Antp* P1 promoter is regulated by the BX-C gene products and the *Pc* gene product independently. The absence of β-gal expression in the supraoesophageal ganglion in Pc^- BX-C^- embryos in comparison with Pc^- embryos (cf. Figure 2B and 2D) cannot be easily explained, but might be due to effects induced by other ectopically expressed genes of the ANT-C.

After crossing the pAPT1.4 and pAPT1.0 lines into BX-C^- background, the β-gal staining pattern in the CNS was changed into a strong uniform distribution. Interestingly, pAPT1.0 was derepressed more effectively than pAPT1.4. A weak, general derepression was also observed in the inner layers of the embryo, not in the epidermis however, as seen for the pAPT1.8 construct. This shows that BX-C responsive elements, repressing *Antp* P1 transcription in the CNS are located within the −1.9 to +2.1 kb region, whereas epidermis-specific repressing elements are located in the intron region +2.1 to +7.8 kb. The increased CNS specific derepression of pAPT1.0 in comparison with pAPT1.4 possibly indicates that upstream sequences and/or factors present in pAPT1.4 but not in pAPT1.0, normally suppress the BX-C repression on the *Antp* sequences located between −1.9 and +2.1 kb. The level of β-gal expression in the CNS from pAPT1.0 was further increased in Pc^- BX-C^- double mutants, while the general staining in the inner layers was absent (data not shown). Thus also on the shortest construct, *Pc* responsive elements can be separated from the BX-C responsive elements.

Antp P2−lacZ expression in Pc^+ and Pc^- embryos

The expression from the *Antp* P2 promoter in wild-type background was examined in embryos carrying two different

Antp P2−*lacZ* constructs (see Figure 1B). The longer P2 construct, pAPT2.3, showed the best correspondence between the expressed pattern of the reporter gene and the expected P2 expression pattern (Boulet and Scott, 1988; Y.Engström and W.J.Gehring, in preparation). Shown in Figure 2I is an embryo during germ band retraction of the transformant line pATP2.3-30D. The stripes of β-gal expression in the epidermis are located on the ventro-lateral side of the embryo, but were not detected in the most ventral epidermal layer. The expression in the ventral nerve cord followed the same segmental pattern as in the epidermis, the three thoracic metameres revealed stronger β-gal staining than the nine abdominal ones. The same line was also investigated in the absence of a functional *Pc* gene product. The effect on the regulation of the *Antp* P2 promoter by the elimination of the *Pc* gene was not as easy to identify as in the P1 transformants. This is partly explained because the β-gal staining in embryos from the P2−*lacZ* constructs was generally weaker than the expression derived from the P1−*lacZ* constructs. At extended germ band stage, ectopic expression was detected in the head regions: in addition to the β-gal expression described above for the Pc^+ embryos, the Pc^- embryos also displayed β-gal staining in the epidermis of the labial lobe, the maxillary lobe and of the dorsal ridge (data not shown). After retraction of the germband we observed ectopic expression in anterior segments of the CNS (Figure 2J). Ectopic β-gal staining was found in the labial (lb), maxillary (mx) and in the mandibular neuromeres (md). The level of β-gal staining in the gnathal neuromeres was comparable with the one in the abdominal neuromeres. In contrast to the P1 transformants, however, the P2 *lacZ* expression in the thoracic part of the nerve cord remained stronger than in the abdominal part. This result was confirmed using another transformant line, pAPT2.3-9B. Many of the examined pAPT2.3 Pc^- embryos exhibited in addition a general derepression of P2 transcription in most cells of the embryo, leading to an overall blue staining. These observations indicate that the *Antp* P2 promoter, including the flanking sequences present in pAPT2.3, was deregulated in the head segments as well as weakly derepressed in most cells of the embryo, as a consequence of the missing *Pc* gene product.

We extended our analysis of the regulation of the *Antp* P2 promoter by the *Pc* gene, to also include a shorter construct. Two transformant lines carrying the construct pAPT2.1 were analyzed in Pc^- mutant background (pAPT2.1-13B and pAPT2.1-44E/55B). The results from the pAPT2.1 transformants were more difficult to interpret than from the pAPT2.3 lines described above, because the pAPT2.1 fusion gene reveals a weak but significant expression of β-gal in the labial and maxillary neuromeres already in the Pc^+ embryos. This suggests that some *cis*-acting elements in the pAPT2.1 construct are missing in order to repress P2 expression completely in these anterior segments. The pattern of β-gal expression in the CNS of pAPT2.1 transformed wild-type embryo is almost identical to the one described for the pAPT2.3 constructs in Pc^- embryos. Thus, we cannot determine whether the ectopic expression in the gnathal segments of pAPT2.1 Pc^- embryos is due to the lack of *cis*-acting elements in the pAPT2.1 construct or due to the absence of the *trans*-acting gene product in the Pc^- embryos.

Antp P2 repression by the BX-C genes

Transformant lines carrying the two constructs pAPT2.3 and pAPT2.1 (lines pAPT2.3-5C, pAPT2.3-30D and

Table I. *Pc* protein binding to *Antp* P1 and P2 promoters

Transformed lines	Chromosomal site of integration	Extent of promoter fragment	Pc-binding	β-gal expression in salivary glands
Antp-P1				
pAPT 1.8-20B	20B	− 9.4 to +7.8	+	−
-22D	22D	− 9.4 to +7.8	+	−
-99F	99F	− 9.4 to +7.8	+	−
pAPT 1.4-20D	20D	− 9.4 to +2.1	+	−
-49A	49A	− 9.4 to +2.1	+	−
-79B	79B	− 9.4 to +2.1	+	−
pAPT 1.0-5C	5C	− 1.9 to +2.1	+	−
-33A	33A	− 1.9 to +2.1	+	−
-97F	97F	− 1.9 to +2.1	+	−
-55C	55C	− 1.9 to +2.1	−	+
-79A	79A	− 1.9 to +2.1	−	++
Antp-P2				
pAPT 2.3-99B	99B 7-11	−10.0 to +2.6	+	−
-9B	9B	−10.0 to +2.6	−	−
-30D	30D	−10.0 to +2.6	−	−
pAPT 2.1-44 E/55 B	44 E/55 B	− 5.6 to +2.6	+(44E)	−

Antp P1− and *Antp* P2−*lacZ* transformant lines analyzed for binding of the *Pc* protein to the inserted promoter sequences, as well as for ectopic β-gal expression in salivary glands. *Antp* sequences present within the pAPT1.8, 1.4, 1.0, 2.3 and 2.1 transformant lines are outlined in Figure 1B. The sites of integration of the *Antp*−*lacZ* fusion genes were mapped by *in situ* hybridization with pPT 12/2 vector DNA. *Pc*-binding to the *Antp* regulatory sequences is reflected by an additional band appearing at the insertion site (see Figure 3) and is indicated with (+). The accurate quantification of the *Pc* binding signal by comparing labeling intensities of different promoter fragments is limited in this assay for reasons explained in the discussion. In panel 5 the results are compiled from the experiment, in which ectopic β-galactosidase expression from the respective *Antp*−*lacZ* constructs was determined in salivary glands of third instar larvae. The relative value of β-gal activity is indicated with (+).

pAPT2.1-13B) were also analyzed in BX-C$^-$ and in *Pc*$^-$ BX-C$^-$ background. As shown in Figure 2K, the β-gal expression from pAPT2.3 was derepressed in the nervous system and the ventro-lateral epidermis of the abdominal segments of BX-C$^-$ embryos. Thereby, all the thoracic and abdominal segments revealed an identical staining pattern. A similar derepression in BX-C$^-$ background was seen for the shorter construct pAPT2.1 (data not shown). After crossing the same pAPT2.3 lines into the double mutant *Pc*$^-$ BX-C$^-$, the reporter gene expression was further increased as shown in Figure 2L. The staining was more intense and covered a larger area in each segment. Furthermore, ectopic expression was evident in the mandibular, maxillary and labial ganglia. These data show that within −10.0 and +2.6 kb, the P2 promoter is also repressed by the BX-C genes and the *Pc* gene independently. Transformants carrying the shorter pAPT2.1 construct were also analyzed in the double mutant. However, we were not able to demonstrate a reproducible difference in the staining pattern between BX-C$^-$ and *Pc*$^-$ BX-C$^-$ embryos. Thus we cannot disclose whether the pAPT2.1 construct contains *Pc* responsive elements, necessary for proper embryonic regulation.

Pc protein is associated with chromatin of the Antp P1 and P2 promoters

Knowing that the *Pc* gene was necessary for the normal regulation of *Antp* transcription via *Antp cis*-acting elements it was important to analyze the mechanism of this interaction. Does *Pc* directly interact with the *Antp cis*-acting elements at the chromosomal level or does it exert its function through other *trans*-regulators of *Antp*? We have previously shown that *Pc* encodes a nuclear protein which is associated with discrete locations on salivary gland chromosomes (Zink and Paro, 1989). We found that the ANT-C is among the most strongly labeled *Pc* binding sites and that the staining signal spans the whole complex, though with different intensities from proximal to distal. Thus *Pc* potentially binds and regulates several of the homeotic genes within the ANT-C. Performing immunostaining of polytene chromosomes of transgenic flies having an *Antp* promoter−*lacZ* construct inserted into the genome, we now had the tools to visualize the interaction of *Pc* with promoter sequences of just one of the homeotic genes of the ANT-C, the *Antennapedia* gene. For our experiments we chose transformants which had the *Antp*−*lacZ* construct inserted in a region of the genome which lacked *Pc* binding sites in wild-type chromosomes. The site of integration of the various fusion gene constructs in the transformant lines was determined by *in situ* hybridization using the pPT 12/2 vector as a probe. The insertion sites of the different lines used in this study are listed in Table I. Of the *Antp* P1 promoter constructs tested previously for the embryonic β-gal pattern we analyzed the 17.2 kb long pAPT1.8, the 11.5 kb long pAPT1.4, and the 4.0 kb long pAPT1.0 constructs for *Pc* binding (Table I). In all transformant lines containing one of the two larger constructs we detected a new *Pc* binding site at the mapped integration site of the construct. Figure 3A shows an example of an additional binding site to the pAPT1.4 construct at the location 49A on the second chromosome. This data clearly demonstrate that the *Pc* protein interacts with the chromatin encompassing *Antp* P1 promoter sequences and implies that *Pc* regulates *Antp* P1 transcription directly at the chromosomal level.

During the investigation of five independent lines carrying the shorter (pAPT1.0) construct we made some interesting observations. Though we found that the *Pc* protein was also associated with this smaller *Antp* P1 fragment, this was not true for all the different lines tested. Interestingly, binding of the *Pc* protein to the smaller 4.0 kb P1 promoter region is dependent on the chromosomal site of integration of the target sequences. *Pc* binds to this sequence only at three out of five chromosomal sites we analyzed, implying that binding of *Pc* to promoter sequences is also influenced by sequences and/or factors in the vicinity of the actual binding site(s). We found *Pc* binding to pAPT1.0 when it is inserted at 5C, 33A and 97F (see Table I). This result correlates with the embryonal β-gal expression pattern in the different pAPT1.0 lines: At the analyzed insertion sites 5C and 33A we find that the pAPT1.0 sequences are able to mediate an almost *Antp* P1 like expression of the reporter gene, which in turn is altered in the absence of *Pc*$^+$ function. However, at the integration sites 55C and 79A, where we did not find any *Pc* signal on the polytene chromosomes, the β-gal expression in the embryo seems no longer to be directed by the pAPT1.0 *Antp* sequences, but largely to be controlled by the neighboring control elements. In addition, this ectopic β-gal expression pattern is not changed when *Pc* is absent.

We also analyzed whether *Pc* was associated with *Antp* P2 promoter sequences. Chromosomes of three independent pAPT2.3 lines (see Table I), carrying 12.6 kb from −10.0 to +2.6 kb of the P2 transcription start site were immunostained. These relatively long P2 constructs showed to be similarly affected by the site of integration as was observed for the short P1 (pAPT1.0) promoter constructs. Only one out of three transgenic lines, namely pAPT2.3-99B, displayed a new band at the insertion site (see Figure 3C). No additional binding site could be detected at the insertion sites of pAPT2.3-30D and pAPT2.3-9B. The strain pAPT2.1-44E/55B contains two insertions of the pAPT2.1 construct on the second chromosome at 44E and 55B. This shorter construct extends from −5.6 to +2.6 kb. The insertion at 44E also showed binding by *Pc* (see Figure 3B). The insertion at 55B could not be analyzed since it is co-localized with a *Pc* binding site present in wild-type chromosomes. These results demonstrate that *Pc* also regulates P2 directly at the chromosomal level.

To ascertain that the observed *Pc* binding signal was specific for the *Antp* promoter sequences and not due to unspecific binding of *Pc* protein to the P-element vector sequences, we chose P[1ArB]-transformed lines as controls (Bellen *et al.*, 1989). The P[1ArB] vector contains all the elements present in the pPT 12/2 vector (P-element, *ry*, *E.coli lacZ*, *hsp70*), with the exception of the *actin* 88F sequences. Since the endogenous 88F *actin* locus was found to lack a *Pc* signal, P[1ArB] is a suitable control. We analyzed seven independent P[1ArB] lines for *Pc* binding at the cytological map positions of the insertions and found no evidence for *Pc* binding.

Pc protein is not bound to Antp P1 fragments expressing β-gal ectopically in salivary glands

In order to establish the physiological function of the various *Antp* promoter constructs in the same tissue as analyzed for binding, we stained salivary glands for β-gal activity. The endogenous *Antp* gene is transcriptionally inactive in salivary glands of third instar larvae (W.Gehring, unpublished data). Ectopic expression of β-gal in this tissue would indicate a

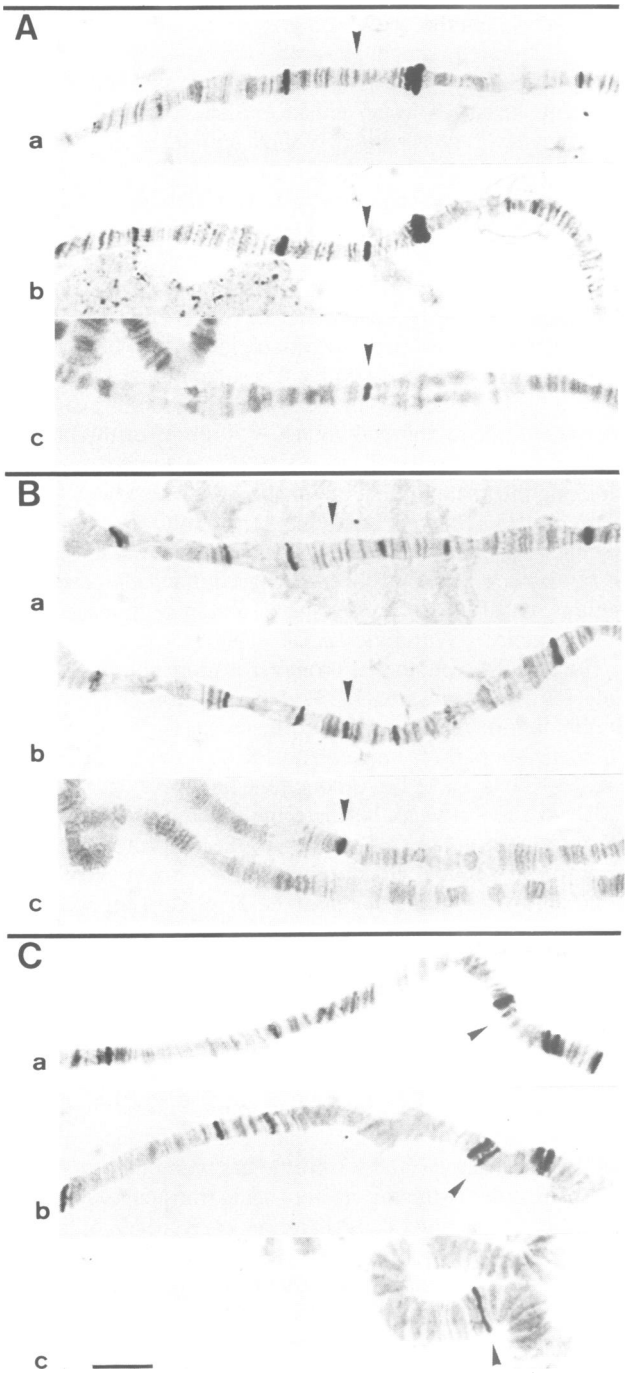

Fig. 3. Immunohistochemical localization of the *Pc* antigen on polytene chromosomes of salivary glands of third instar larvae carrying an *Antp* promoter−*lacZ* construct. Immunohistochemical comparison between sections of homologous chromosomes of wild-type (**a**) and transformed (**b**) strains shows that the binding pattern of *Pc* protein remains very constant, except at the site of integration of the *Antp*−*lacZ* constructs where a new *Pc* protein band can be localized (arrow). (**c**) The insertion sites of the fusion constructs were mapped by *in situ* hybridization to polytene chromosomes of transgenic flies, using pPT 12/2 vector DNA as a probe. The proximal−distal orientation of all chromosomes is from left to right. (**A**) Binding of *Pc* protein to a 11.5 kb (−9.4 to +2.1) *Antp* P1 promoter fragment (pAPT1.4-49A). (a) Section of a wild-type 2nd chromosome. (**b**) Corresponding section of the 2nd chromosome of a transgenic animal having the pAPT1.4 construct inserted at the cytological position 49A. Compared with the wild-type chromosome in (a) an additional *Pc* binding site appears at 49A. (c) *In situ* hybridization with vector DNA. (**B**) Binding of *Pc* protein to 8.2 kb (−5.6 to +2.6) *Antp* P2 promoter sequences (pAPT2.1-44E/55B). (a) Section of a wild-type 2nd chromosome. (b) Corresponding chromosomal section of the transformant line which shows the insertion of the pAPT2.1 construct at 44E. (c) *In situ* hybridization with vector DNA. (**C**) Association of the *Pc* protein with a 12.6 kb (−10.0 to +2.6) *Antp* P2 promoter fragment (pAPT2.3-99B). (a) Tip of the right arm of a wild-type 3rd chromosome. (b) Section equivalent to that in (a) from a transformant line which carries the pAPT2.3 construct inserted at 99B. At 99B there is already a faint *Pc* band evident in wild-type chromosomes which becomes significantly enhanced upon insertion of the pAPT2.3 construct, however. In addition, the chromosome seems to be at a different developmental stage than the wild-type chromosome in (a), as evidenced by the puffed structure of the telomere. No *Pc* signal is visible in this puffed telomere. This could indicate that in this instance we are observing a displacement of the repressor *Pc* by transcriptional activating mechanisms. Conceivably could the loss of the *Pc* signal also be caused by the dilution effect of the stain due to the puffed organization of the chromosome. (c) *In situ* hybridization with vector DNA. The bar represents 10 μm.

misregulation of the *cis*-acting elements analyzed. The long P1 constructs (pAPT1.8 and pAPT1.4) did not express β-gal in salivary glands as expected for a proper wild-type regulation. When we analyzed the five short pAPT1.0 lines for β-gal expression, however, we found that two lines, pAPT1.0-55C and pAPT1.0-79A, express β-gal ectopically in their salivary glands (data not shown). This result complements the binding data and indicates, that *Pc* binding and transcription from the *Antp* P1 promoter are mutually exclusive in this particular tissue and in these cases tested (Table I). The β-gal enzyme activity in salivary glands is different in the two lines analyzed. In pAPT1.0-79A the level of β-gal expression is many fold higher than in pAPT1.0-55C, reflecting a modulating effect on transcriptional activation of the chromosomal environment, irrespective of *Pc*.

Neither with the long nor with the shorter P2 constructs was an ectopic expression of β-gal in the salivary glands found. In particular the two lines pAPT2.3-9B and pAPT2.3-30D, which lack *Pc* protein binding, were not expressing β-gal ectopically in salivary glands. The presence or absence of the *Pc* protein at the P2 promoter therefore does not correlate with transcriptional activity, indicating that additional factors are responsible for maintaining P2 in salivary glands in a repressed state.

Discussion

The large *Antp* regulatory region is under the influence of multiple *trans*-acting factors. Among these the *Polycomb* gene product has been suggested to act as a repressor of *Antp* gene transcription. We have linked *Antp* promoter fragments to a reporter gene in order to identify the *cis*-acting sequences regulated by *Pc*. Previous experiments designed to demonstrate sequence-specific DNA binding of *Pc* protein produced either *in vitro* or in *E.coli* by standard binding assays were unsuccessful (R.Paro, unpublished data). Therefore, we have introduced in this study a new tool which allowed us to show that the *Pc* protein exerts its regulatory function by interacting with *Antp cis*-regulatory sequences in polytene chromosomes. The experiments we describe above clearly demonstrate that *Pc* acts at the level of chromatin as a repressor of *Antp* transcription by specifically binding and regulating the *Antp* promoters. The lack of a DNA-binding activity of the *Pc* protein suggests that *Pc* achieves its specific association with the homeotic regulatory

regions through protein–protein interactions within a chromatin complex. *Pc* might be part of a higher order chromatin structure which is necessary to maintain homeotic transcription in a repressed state (Paro, 1990).

Pc regulates both the Antp P1 and P2 promoters

Both P1 and P2 constructs were ectopically expressed in Pc^- embryos, showing that *Pc* regulates both promoters. The comparison of expression patterns mediated by the shorter pAPT1.4 and the longer pAPT1.8 constructs showed that many *cis*-regulatory elements must be located downstream of the P1 transcription start site in the intronic region. In particular correct segment-specific epidermal expression seems to require positive as well as negative *cis*-regulatory regions between +2.1 kb and +7.8 kb downstream of the P1 start site. As with other *trans*-regulatory factors the repressor function of *Pc* also requires large *cis*-acting sequences. Larger constructs show a more pronounced effect, than smaller ones, when β-gal patterns in Pc^+ and Pc^- were compared. On the other hand, increased ectopic expression of β-gal could be observed the smaller the analyzed constructs were. Although the two effects might conceivably have different causes, the similarity of the derepressed pattern by deleting *cis*-regulatory DNA sequences or by eliminating the *trans*-acting factor *Pc* suggests that the two components are functionally interacting.

The P2 promoter seems to require an even larger *cis*-regulatory segment than the 12.6 kb fragment analyzed in this study, since this P2 construct is not sufficient to mediate complete repression of β-gal expression in anterior segments (see Figure 2I). Similar results for the P2 promoter were reported by Boulet and Scott (1988). Their results indicate that additional sequences located between 10 and 35 kb upstream of P2 are required to give appropriate repression in head segments. Together with our results this suggests that important regulatory elements of *Antp* transcription are located between the two transcriptional start sites. The possibility that the P2 promoter needs to be located downstream of the P1 promoter in order to be correctly regulated seems to be unlikely. The analysis of mutational breakpoints separating P1 from P2 by chromosomal aberrations suggests that the two promoters can be regulated independently (Laughon *et al.*, 1986; Boulet and Scott, 1988).

Some Antp promoter fragments are prone to strong position effects

The 4.0 kb *Antp* P1 constructs (pAPT1.0) showed pronounced position effects. This was observed when the embryonic β-gal patterns of the different lines, as well as the binding of the *Pc* protein to these *Antp* sequences was analyzed. While the three cases that reproduce an almost wild-type expression pattern in the embryo (pAPT1.0-5C,-33A and -97F), retain *Pc* protein bound to the P1 fragment, the aberrantly expressing transformants pAPT1.0-55C and -79A were found to be devoid of *Pc* protein at the respective sites. The absence of *Pc* protein at these constructs correlates well with the functional assay showing an ectopic expression of β-gal in the salivary glands (Table I). However, this correlation does not allow us to decide whether the lack of the *Pc* protein binding is the cause or the consequence of the ectopic activity of the short *Antp* P1 constructs in these two lines.

The higher susceptibility of the short constructs to influences from the neighboring sequences into which they have integrated, might indicate the lack of important buffering elements. We assume that this transposon functions in many instances as an enhancer detector (O'Kane and Gehring, 1987) consisting of a basal *Antp* P1 promoter. The need for large regulatory regions in homeotic genes might not only reflect the requirement for multiple *cis*-regulatory elements used by numerous *trans*-activators and repressors, but also point to a complex buffering mechanism which protects the sophisticated and sensitive interactions of the homeotic regulators from adjacent enhancers and other regulatory elements. Far upstream regions which buffer gene expression from influences by the surrounding chromatin have also been identified in mammalian cells. For example, such a control region was found ~50 kb 5′ of the human β-globin gene (Grosveld *et al.*, 1987). A similar element blocking the distant effect of enhancers has also been found bordering the chicken lysozyme gene (Stief *et al.*, 1989). Recently, boundary elements have been found in the BX-C of *Drosophila* and it has been proposed that such boundary regions may be the primary site of action of at least some Pc-like genes (Gyurkovics *et al.*, 1990).

The situation with the P2 promoter is more complex. Even long P2 constructs (pAPT2.3) show position effects when the binding of the *Pc* protein to these inserts is compared. Only one out of three long constructs analyzed binds *Pc* (see Figure 3C and Table I). Although the lines pAPT2.3-9B and -30D do show a weak but detectable derepression in Pc^- embryos, we do not find binding of the *Pc* protein at these insertion sites in the salivary glands. This could indicate that in these two cases the amount of the *Pc* protein bound might be below our detection level, but sufficient to show effects in embryonic tissues or that the regulation of the P2 promoter by *Pc* differs between the two types of tissue. In contrast to the P1 constructs, the absence of *Pc* binding to the P2 constructs is not accompanied by ectopically P2-driven β-gal expression in salivary glands. Taken together these results suggest that P2 differs from P1 in that it can be repressed by *trans*-acting factors other than *Pc*. Differences in the regulation of the P1 and P2 promoters were also found by analyzing other regulatory factors using transient expression assays in tissue culture cells (Krasnow *et al.*, 1989; Winslow *et al.*, 1989) and by determining transcript distributions in mutant embryos (Irish *et al.*, 1989).

Quantification of the Pc binding signal

We have attempted to quantify the chromosomal *Pc* protein binding signal in comparison to the size of the different constructs tested. Although we clearly see an enhancement in signal intensity when constructs of increasing length were compared, we found that a precise quantification is very difficult due to several problems we have encountered: First, the amount of the cross-linked protein slightly varies between individual squashes, due to small uncontrollable variations in the handling of the chromosomes during the squashing procedure. Second, the intensity of the binding signal depends upon the chromatin structure near the insertion site (i.e. under-replication, heterochromatin, etc.). Third, the individual cells and glands might be at slightly different stages of differentiation and thus differ in the amount of *Pc* protein bound to the target sites. In order to control these effects and to determine relative numbers of binding sites,

a thorough statistical analysis of a particular construct from different insertion sites is needed. However, for a semi-quantitative analysis the technique of identifying protein binding to defined DNA segments on polytene chromosomes is very valuable. The major advantage being the possibility to analyze regulatory mechanisms of protein−protein or protein−DNA interactions *in situ*, in their normal chromosomal environment.

Materials and methods

Drosophila strains

The ry^{506} (ORM) strain served as recipient for the fusion gene constructs. The following balancer chromosomes were used to maintain transformant lines: (1st) FM6; ry^{506}, (2nd) CyO; ry^{506}, (3rd) TM3 ry^{rk}; ry^{506} fiz^{9H34} e^{s}. For a description of marker genes and balancer chromosomes, see Lindsley and Grell (1968). The Pc^3 allele was used for the analysis of *Antp−lacZ* expression in Pc^- background. The marked TM2 balancer chromosome (strain c40.1 S_3) was obtained from Hugo Bellen. For the analysis of reporter gene expression in embryos lacking the entire BX-C, the strains Df(3R)P9/TM1 (Lewis, 1978) and Pc^3 Df(3R)P9/TM1 (Duncan and Lewis, 1982) were used.

Vector and constructs

The vector pPT 12/2 (constructed by S.Schneuwly) is a derivative of the Carnegie 20 vector (Rubin and Spradling, 1983). It contains P-element borders that allow stable integration into the *Drosophila* genome; the $rosy^+(ry^+)$ gene as a marker for germ line transformation; a unique *Kpn*I cloning site 5′ of a 0.8 kb fragment of the *Drosophila actin*-88F gene (*act* 88F), which provides a splice acceptor site and the translational start site in frame with the *E.coli lacZ* gene; the termination codon and the polyadenylation signal is provided by the *Drosophila hsp70* gene. The five different *Antp−lacZ* constructs used in this study were constructed by inserting different fragments from the *Antp* gene, covering either the *Antp* exon 1 or exon 3 plus respective flanking sequences, into the unique *Kpn*I cloning site of the vector pPT 12/2. A more detailed description of the construction of the vector and the fusion gene constructs will be published elsewhere (Y.Engström and W.J.Gehring, in preparation).

Germ line transformation and analysis of β-gal expression

P-element mediated transformation was carried out essentially as described by Rubin and Spradling (1982). Embryos (ry^{506}) were injected with 100 μg/ml of helper plasmid (p25.7 wc) DNA and 300 μg/ml of *Antp−lacZ* DNA. Transformants were identified on the basis of their ry^+ phenotype and established as homozygous or as balanced heterozygous stocks. To localize the expression from the reporter gene *E.coli lacZ*, a β-galactosidase assay was performed on fixed whole embryos as well as on isolated salivary glands of third instar larvae using X-gal as a substrate for the enzyme. We essentially used the procedure described by Hiromi *et al.* (1985) except that the incubation in X-gal was performed at a lower pH (7.2) as described by Simon *et al.* (1985). In order to study the expression from the fusion gene constructs in Pc^- background, we chose transformant fly lines which had the P-element insert on the X or on the 2nd chromosome and crossed them to flies carrying the Pc^3 allele. The homozygous mutant embryos were identified by morphological changes and by altered β-gal staining patterns. In cases where these criteria could not be applied, lines carrying a marked balancer chromosome were used. The balancer chromosome has a *lacZ* gene insert which produces β-gal homogenously at a high level in the whole animal throughout development after the onset of germ band extension (TM2 c40.1 S_3) (Bellen *et al.*, 1989). Lines were established being homozygous for the *Antp−lacZ* constructs on the second chromosome and having the Pc^3 allele balanced over TM2 c40.1 S_3. Embryos heterozygous or homozygous for the balancer chromosome were identified by the overall staining, while embryos homozygous for the Pc^3 chromosome expressed β-gal exclusively from the *Antp−lacZ* gene insert on the second chromosome.

Preparation of chromosome spreads and immunolocalization of antigen on polytene chromosomes

Drosophila polytene chromosome spreads were prepared from salivary glands of third instar larvae. For higher polyteny, larvae were grown at 18°C on medium with live yeast. Salivary glands were dissected in PBS pH 7.5, 0.1% Triton X-100. For fixation, two pairs of glands were transferred into a droplet of sol 1 for 10−20 s (sol 1:3.7% formaldehyde, 1% Triton X-100, PBS pH 7.5) [37% formaldehyde stock solution: paraformaldehyde (Sigma) was dissolved in 15 mM KOH by boiling]. Glands were then immediately moved into a 35 μl droplet of sol 2 on a 22 mm × 22 mm coverslip (Corning) for 2−3 min (sol 2:3.7% formaldehyde, 50% acetic acid). Sols 1 and 2 were made fresh about every 2−3 h. The coverslip was picked up with a poly-L-lysine coated slide [coating of slides: clean slides were dipped into a 0.1% poly-L-lysine solution (Sigma) and air dried]. The coverslip was then gently, but extensively tapped and slightly moved with a pencil and the eraser end of a pencil, respectively. The slide was turned over onto blotting paper and a thumb pressure was applied to the preparation. The coverslip was flipped off with a razor blade after freezing in liquid nitrogen. Slides were washed in PBS (two times 15 min, agitating). Slides can be kept for up to one week in 50% ammonium sulfate at 4°C. For immunostaining slides were placed from PBS into PBT (two times 10 min, agitating; PBT:PBS pH 7.5, 0.1% BSA, 0.2% Tween 20). 40 μl affinity purified primary antibody (rabbit-anti-Polycomb; Zink and Paro, 1989) diluted in PBT were added per slide. For all incubation steps slides were kept in a humid atmosphere with a coverslip placed over the chromosomes. After a 1 h incubation at room temperature (rt), slides were washed in PBT (twice for 10 min, agitating). Biotinylated secondary antibody (goat anti-rabbit, Vector; preabsorbed to wild-type embryos) diluted in PBT, 2% normal goat serum was added. After 45 min incubation at rt, slides were washed as above. The avidin−biotin−HRP complex (Vector Vectastain ABC kit; preincubation in PBT according to manufacturer's protocol) was added for 45 min at rt. Slides were washed as above and then incubated with DAB solution (0.5 mg/ml in PBT) for 5 min. The DAB solution was replaced by DAB, 0.01% H_2O_2 solution. The staining reaction (1−10 min) was checked under the microscope and stopped with PBS. Chromosomes were counterstained with Giemsa (Merck, 1:130 dilution in 10 mM sodium phosphate buffer pH 6.8) for 20 s. After a brief rinse in dH_2O, chromosomes were rinsed with and mounted in 99.5% glycerol and immediately examined under the microscope. To get sufficient contrast for black and white photography the DAB precipitate was enhanced by applying a silver amplification system (Amersham). The enhancement was performed according to manufacturer's specifications, except that the silver amplification step was shortened to 1 min. Photos were taken on Ilford PANF film.

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