Analysis of the functional role of the Polycomb chromo domain in *Drosophila melanogaster*

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The chromo domain was identified as a homologous protein motif between Polycomb (Pc)—a member of the Pc-group genes encoding transcriptional repressors of the homeotic genes—and HP1—a heterochromatin-associated protein encoded by the suppressor of position effect variegation gene Su(var)205. Together with previous genetic studies, this molecular similarity supports the suggestion of a common mechanism used for generating heterochromatin and for repressing homeotic genes. The evolutionary conservation of the chromo domain throughout the animal and plant kingdoms implies an important functional role for this protein motif. We have used transgenic lines as well as transient expression assays employing *Drosophila* tissue culture cells to study the functional role of the Pc chromo domain. Wild-type Pc protein is endogenously expressed in SL2 cells and is found in large immunologically visible complexes. Mutated Pc proteins were expressed as Pc-β-galactosidase fusion proteins, and their nuclear distribution was examined by indirect immunofluorescence in tissue culture cells and on polytene chromosomes of transgenic larvae. We show that carboxy-terminal truncations of the Pc protein do not affect chromosomal binding of the fusion protein. However, mutations affecting only the chromo domain including in vitro generated deletions, as well as point mutations, abolish chromosomal binding. Our results demonstrate for the first time that the chromo domain is important for the function of Pc and that it is absolutely required for binding of Pc protein to chromatin. Some of the nuclear patterns generated by the mutated forms of the fusion proteins suggest, furthermore, that the chromo domain could be involved in a packaging mechanism, essential for compacting chromosomal proteins within heterochromatin or heterochromatin-like complexes.

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The Polycomb-group (Pc-G) genes play a major role in the process of maintenance of a determined cellular state. In *Drosophila* development, this is particularly evident for homeotic gene regulation. Appropriate differential expression of the homeotic genes of the Antennapedia complex (ANT-C) and the bithorax complex (BX-C) is required for proper segmental determination [Lewis 1978; Kaufman et al. 1980]. In the early phase, homeotic genes are activated in their correct spatial domains by a complex interplay of transcriptional regulators, encoded by the early-acting maternal and segmentation genes [Harding and Levine 1988; Irish et al. 1989]. This early regulatory hierarchy decays after the establishment of the segmented pattern. However, homeotic genes need to be active throughout development to ensure proper segmental identity [Morata et al. 1983]. The Pc-G is part of the mechanism that maintains the homeotic expression boundaries after their setting up in early embryogenesis. In domains where particular homeotic genes are kept silent by the early initiation process, the Pc-G is subsequently responsible for maintaining the repressed state through the rest of development. Mutations in the Polycomb (Pc) gene show a normal distribution of homeotic genes early in embryogenesis [Kuziora and McGinnis 1988; Franke 1991], indicating that the initiation process is not disturbed. At later embryonic stages when the maintenance process should take over, in Pc− mutants homeotic transcripts and proteins become ectopically expressed [Beachy et al. 1985; Carroll et al. 1986; Wedeen et al. 1986; Kuziora and McGinnis 1988; Celniker et al. 1990], resulting in a completely posteriorly transformed embryo [Lewis 1978; Duncan and Lewis 1982; Denell and Frederick 1983; Sato and Denell 1985; Sato et al. 1985; Capdevila et al. 1986]. Similar effects on homeotic expression have been found for other members of the Pc-G, although the degree of transformations observed in mutants is highly variable depending on the gene analyzed [Dura et al. 1985; Jürgens 1985; Struhl and Akam 1985; Dura and Ingham 1988; Jones and Gelbart 1990; McKeon and Brock 1991; Simon et al. 1992]. Evidence that the Pc-G acts at the level of transcrip-
tional regulation has been well documented for the \( \text{Pc} \) gene. \( \text{Pc} \) encodes a nuclear protein found to be associated with \( \sim 100 \) loci on polytene chromosomes of larval salivary glands [Zink and Paro 1989]. Among the genes bound by \( \text{Pc} \) protein are the two homeotic complexes as well as other members of the \( \text{Pc-G} \). Reporter genes linked to \( \text{cis} \)-regulatory regions of the homeotic \( \text{Antennapedia} \) gene [Zink et al. 1991] or the \( \text{Ultrabithorax} \) gene [Müller and Bienz 1991] were found to become derepressed in \( \text{Pc} \) mutants. In addition, Zink et al. [1991] could show that on polytene chromosomes the \( \text{Pc} \) protein directly binds the \( \text{Antennapedia} \) regulatory fragment driving the reporter gene. However, no direct DNA binding of the \( \text{Pc} \) protein to its homeotic targets could be found in vitro [M. Oed and R. Paro, unpubl.]. This suggests that \( \text{Pc} \) is not a DNA sequence-specific binding protein. It probably achieves its specificity by interacting with more segment- or tissue-specific factors. This results in differential repression of homeotic genes along the anterior–posterior axis of the embryo.

Earlier genetic experiments have suggested a similarity at the mechanistic level between the \( \text{Pc-G} \) genes and the modifiers of position effect variegation (PEV) in \( \text{Drosophila} \). PEV describes the phenomenon that is observed when euchromatic genes are translocated into the vicinity of heterochromatin. This heterochromatin differentially represses the genes, resulting in a clonally inherited variegated pattern. This effect can spread over large chromosomal distances and was suggested to be the consequence of cooperative heterochromatin packaging (for review, see Eissenberg 1989; Henikoff 1990). Modifier genes are thought to encode structural proteins of heterochromatin [James and Elgin 1986; Locke et al. 1988; Eissenberg 1989; Hayashi et al. 1990, Reuter et al. 1990] or factors that modify chromosomal proteins [Dorn et al. 1986]. Interestingly, some members of the \( \text{Pc-G} \) have effects on PEV [H. Brock, pers. comm.]. Reciprocally, some modifiers of PEV show homeotic phenotypes reminiscent of ectopic homeotic expression patterns [Reuter et al. 1990]. Both classes show a strong dependence on gene doses. In the case of heterochromatin formation by the modifiers of PEV, Tartof and colleagues explained the phenomenon by the law of mass action [Locke et al. 1988]. They proposed that heterochromatin is composed of reiterated multimeric protein units that form large, compact chromosomal complexes.

The relationship between the \( \text{Pc-G} \) and the modifiers of PEV was substantiated recently by the finding of an extended homology between the \( \text{Pc} \) protein and the heterochromatin-associated protein HP1 of \( \text{Drosophila melanogaster} \) [Paro and Hogness 1991]. The \( \text{Pc} \) and HP1 proteins share a region of extended similarity [24 of 37 amino acids are identical] at the amino terminus of both proteins defining the chromatin organization modifier, or chromo domain. HP1 is encoded by the gene \( \text{Suppressor of variegation 205} \) [\( \text{Su(var)205} \)] [Eissenberg et al. 1990]. In polytene chromosomes, HP1 was shown to be associated with \( \beta \)-heterochromatin, localizing HP1 to chromosomal regions connecting the chromocenter to the chromosome arms, throughout the fourth chromosomal and on most telomeric regions [James and Elgin 1986; James et al. 1989]. Mutations in the gene, including deficiencies and missplicing of the transcript, lead to dominant suppression of PEV, whereas duplication of this locus enhances the variegated phenotype [Locke et al. 1988; Wustmann et al. 1989; Eissenberg et al. 1990].

The genetic and molecular similarities described above strongly point to a common mechanism of gene regulation that is used for generating heterochromatin and for keeping developmental regulators [i.e., homeotic genes] repressed. In the current model it is suggested that \( \text{Pc} \) together with other products of the \( \text{Pc-G} \) form multimeric protein complexes compacting the chromatin into a heterochromatin-like conformation and thereby keeping the expression of the homeotic genes repressed. Imprinted into the higher-order chromatin structure, the repressed state would then be stably and clonally inherited through development like heterochromatic domains [Gaunt and Singh 1990, Paro 1990]. At the mechanistic level, the chromo domain most probably plays an important role in the formation of such regulatory chromosomal domains. Interestingly, the chromo domain was found to be conserved across the animal and plant kingdoms [Singh et al. 1991; Epstein et al. 1992, Pearce et al. 1992].

In this work we investigate the functional role of the \( \text{Pc} \) chromo domain. We show that two-thirds of the \( \text{Pc} \) protein, containing the chromo domain, is sufficient to mediate specific binding to the same loci on polytene chromosomes that are recognized by the endogenous \( \text{Pc} \) protein. We find that in \( \text{Drosophila} \) tissue culture cells \( \text{Pc} \) is endogenously expressed and is distributed in the nuclei in a punctated pattern. By expressing mutant \( \text{Pc} \) proteins as \( \beta \)-galactosidase fusion proteins [\( \text{Pc-} \beta \text{gal} \)], we determined their nuclear localization by indirect immunofluorescence staining. We demonstrate that alterations in the chromo domain of the \( \text{Pc-} \beta \text{gal} \) fusion proteins affect the distribution in tissue culture cell nuclei and disrupt the binding to polytene chromosomes.

Results

Some mutant \( \text{Pc} \) alleles have point mutations in the chromo domain

To identify functional domains of the \( \text{Pc} \) protein, we have taken advantage of the large number of different \( \text{Pc} \) alleles isolated. We have collected homozygous embryos from 19 different \( \text{Pc} \) alleles [see Materials and methods]. Using our rabbit anti-\( \text{Pc} \) antibodies on Western blot analysis, we have identified 10 alleles that still express the \( \text{Pc} \) protein and thus potentially have mutations in the protein-coding part of the gene. The genomic sequence of these alleles was isolated by the Polymerase chain reaction (PCR) technique, and the DNA alterations were identified by sequencing. A detailed description of the various mutations identified will be reported elsewhere [A. Franke and R. Paro, in prep.]. Here, we concentrate on the two mutant alleles in which we have identified a point mutation in the chromo domain. The allele \( \text{Pc}^{196} \)
allele show a very strong haploinsufficient Pc phenotype. This latter deletion, however, falls outside the originally defined chromo domain (Paro and Hogness 1991). The results of Singh et al. (1991), comparing chromo domains from different species, show that carboxy-terminally additional amino acids are conserved. Taken together with

Figure 1. (A) Part of the chromo box sequence comparing the wild-type Pc sequence (WT) with the sequence of the mutant Pc alleles (Pc1°6 and PcXL5). (Left) The point mutation in the chromo box is shown leading to an amino acid exchange from Ile to Phe at position 31 resulting in the mutant Pc allele Pc1°6; (right) the 6 nucleotides encompassing the deletion are shown, leading to a 2-amino-acid deletion in the chromo domain resulting in mutant allele PcXL5 and designated as Pca69-70 in the fusion protein. (B) Schematic drawing of wild-type Pc protein (for the Pc sequence, see Paro and Hogness 1991). The chromo domain is indicated by a hatched box, each of the histidine repeats by a stippled box, and the four presumptive nuclear localization signals by asterisks (*). The lines designated as ab 1 and ab 2 indicate the carboxy-terminal 199 amino acids [192–390] and 114 amino acids [277–390] of the Pc protein used as antigen to produce polyclonal antibodies in rabbits and mice, respectively (see Materials and methods). (C) Pc/bgal fusion proteins carrying either carboxy-terminal truncations or chromo domain mutations of the Pc protein. Pcl-266 indicates the structure of the basic Pc/bgal fusion construct. (Above) The three different truncated Pc proteins, carrying 190, 117, or 65 amino acids of the amino-terminal Pc protein part fused to bgal. (Below) The basic construct of the fusion proteins with the various chromo domain mutations. The amino acid sequence of the wild-type Pc chromo domain is shown, extending over 48 amino acids between amino acids 26 and 73. Taking into account the results of Singh et al. (1991) and Pearce et al. (1992) and our analysis of the Pc mutant alleles, we have extended the size of the presumptive chromo domain compared with that reported previously (Paro and Hogness 1991). The amino acid substitution in Pc(Ile-31→Phe) and the 2-amino-acid deletion in Pc[Δ69–70] are both lethal Pc alleles (see Fig. 1A). The chromo domain deletions Pc[Δ26–36], Pc[Δ42–65], and Pc[Δ46–52] were constructed by site-directed mutagenesis. A dash represents a deleted amino acid. (Hatched box) Chromo domain; (stippled box) histidine repeat; [asterisks] presumptive nuclear localization signal.
our mutant analysis, this indicates that the extent of a functional chromo domain is larger than we suggested previously. In Figure 1C, we have increased the size of the chromo domain, now extending from position 26 to 73, to take these findings into account. Below the wild-type sequence, the amino acid alterations in the two mutants are shown. The strong phylogenetic conservation of the chromo domain suggested an important function for this protein motif. Our results are a direct proof that mutations in the chromo domain disrupt Pc function. Because the physiological role of the chromo domain was unknown, we have tried to devise means to identify its function.

The Pc(1-266)-β-galactosidase fusion protein mimics chromosomal binding of endogenous Pc protein

As part of an ongoing dissection of the Pc regulatory region, we have constructed transgenic fly lines expressing β-galactosidase (βgal) under the control of the Pc promoter and various portions of the Pc-coding regions. Our goal was to construct a Pc/lacZ fusion gene that directs expression of βgal in a pattern resembling the endogenous Pc protein pattern observed in wild-type embryonal and larval tissue. The analysis of βgal distribution patterns of various independent Pc/lacZ transgenic lines indicates, that only a construct encoding a fusion protein with 266 of 390 amino acids of the wild-type Pc protein fused to βgal fulfilled this criterion. In addition to the 725 bp of the Pc promoter, the fusion construct includes 1229 bp of the transcribed sequences from the Pc gene encompassing 118 nucleotides of untranslated leader and the amino-terminal 266 amino acids spliced in-frame to the lacZ gene.

The wild-type Pc protein and the Pc(1-266)-βgal fusion protein are schematically drawn in Figure 1, B and C, respectively. The Pc portion of the fusion is designated Pc[1-266], as it contains 266 amino acids of the 390 amino acids of the wild-type Pc protein. This Pc protein portion includes all important domains identified by primary amino acid sequence analysis (Paro and Hogness 1991). Included are the amino-terminally localized chromo domain, the two homopolymeric stretches of 10 and 8 histidines, and four potential nuclear localization signals identified to be similar to the nuclear targeting sequence of SV40 large T antigen (Kalderon et al. 1984). The fourth nuclear entry signal (from amino acid 170 to 173) downstream of the second histidine repeat was not published previously, but was identified to be essential for complete nuclear localization of carboxy-terminally truncated Pc/βgal fusion proteins in Drosophila cells (see below).

To identify the functional domains of the Pc protein required for specific recognition of its target genes, we have asked whether the 266 amino acids of the Pc protein are sufficient to mediate the same binding pattern on polytene chromosomes as the full-length Pc protein. Salivary glands of transgenic third-instar larvae expressing the Pc(1-266)-βgal fusion protein were dissected, and the βgal pattern was determined by indirect immunofluorescence. As the endogenous wild-type Pc protein (Fig. 2A) the Pc[1-266]-βgal fusion protein (Fig. 2B) becomes localized completely in the nuclei of salivary glands and is distributed in a bright-banded pattern. Performing double immunofluorescence staining on spread chromosomes of transgenic lines expressing Pc(1-266)-βgal, we found that the fusion protein (Fig. 2D) also binds to the same target genes as the endogenous Pc protein (Fig. 2C). This result demonstrates that two-thirds of the Pc protein contain all relevant domains needed for specific binding to distinct chromosomal loci. Thus, the Pc(1-266)-βgal fusion protein could be used as a powerful tool to analyze in vivo the functional domains of the Pc protein.

Expression of Pc protein in Drosophila tissue culture cells

Transient expression assays using Drosophila Schneider line 2 (SL2) cells (Schneider 1972) have been shown to be useful to rapidly analyze the function and subcellular distribution of in vitro manipulated proteins (Fehon et al. 1990; Rebay et al. 1991). To take advantage of this system we have first established that the Pc protein is expressed in SL2 cells. By immunofluorescence staining with polyclonal rabbit anti-Pc antibodies (ab 1 in Fig. 1B), we have found Pc protein localized in the nuclei of SL2 cells accumulated mainly in bright spots (Fig. 3A). This distribution is reminiscent of the bands visible in whole nuclei of salivary glands (Fig. 2A), where we have shown that they are caused by the binding of the Pc protein to specific chromosomal loci (Fig. 2C). Thus, the speckled pattern in SL2 cell nuclei might also suggest a binding of the Pc protein to specific chromosomal locations. Immunostaining with anti-βgal antibodies of SL2 cells transiently expressing the Pc(1-266)-βgal fusion protein revealed a speckled nuclear pattern similar to that of the endogenous Pc protein, implying a similar association of the fusion protein with interphase chromatin (Fig. 3B). Note that A and B are two different nuclei. Neither the endogenous Pc protein nor the transiently expressed Pc[1-266]-βgal fusion protein are localized in the nucleolus.

We have found that the Pc protein becomes evenly distributed throughout the cells during mitosis. In immunofluorescence stainings we have failed to detect any Pc protein associated with mitotic chromosomes (Fig. 3C-E). In contrast, we have found that the Pc[1-266]-βgal fusion protein is bound to the chromosomes throughout mitosis (Fig. 3F-H). Most probably in this case the large βgal moiety acts as an extended arm easily recognized by the anti-βgal antibodies. The Pc[1-266]-βgal protein does not show a punctuated pattern but a uniform staining of the mitotic chromosomes. This distribution most likely reflects the compact nature of the chromatin. Taken together, these data suggest that some Pc protein is left on the chromosomes during cell division, though not visible in immunostainings, owing to inaccessibility by the anti-Pc antibodies. This result would be in agreement with the notion that Pc is needed...
Pc chromo domain required for chromatin binding

Figure 2. Subcellular localization of wild-type Pc protein (A, C) and Pc(1–266)–βgal fusion protein (B, D) in larval salivary glands. (A) The distribution of wild-type Pc protein in the nuclei of salivary glands from wild-type third-instar larvae detected by polyclonal rabbit anti-Pc antibodies (ab 1 in Fig. 1B) and DTAF-coupled secondary antibodies. (B) The distribution of Pc(1–266)–βgal fusion protein in the nuclei of salivary glands from transgenic third-instar larvae visualized by monoclonal mouse anti-βgal antibodies and secondary antibodies conjugated to DTAF. The brightly staining bands in the nuclei represent the binding of the respective proteins to the giant chromosomes. (C, D) Double-label immunostaining of the squashed salivary gland chromosomes prepared from a transgenic line expressing the Pc(1–266)–βgal fusion protein. In C, the binding pattern of the endogenous Pc protein is shown detected by polyclonal mouse anti-Pc antibodies (ab 2 in Fig. 1B) and secondary anti-mouse antibodies conjugated to DTAF. The mouse polyclonal antibodies also stain unspecifically the nucleolus of the salivary gland nuclei. However, in polytene chromosome preparations, often the nucleolus is broken away, as is the case in C, allowing an analysis of the binding pattern. In D, the binding pattern of the fusion protein is shown visualized by polyclonal rabbit anti-βgal antibodies and secondary anti-rabbit antibodies conjugated to rhodamine. The two patterns are completely overlapping.

to stably repress homeotic genes throughout development and, thus, through many cell divisions.

Unfortunately, we were not able to perform double-labeling experiments with tissue culture cells. The polyclonal mouse anti-Pc antibodies (ab 2 in Fig. 1B), which recognize only the endogenous Pc protein and not the fusion protein, cross-reacted unspecifically with the nucleolus. The strong immunofluorescent signal of the nucleolus obscured the speckled pattern of the Pc protein in the extranucleolar space preventing a direct comparison of the endogenous Pc and the Pc/βgal fusion protein patterns in the same cell.

Carboxy-terminal truncations of the Pc protein do not affect complex formation

In the first series of experiments, we have generated a set of truncated Pc/lacZ fusion constructs by successive carboxy-terminal deletion of the two-thirds of the Pc protein-coding sequence using exonuclease III digestion. After this enzymatic reaction, the Pc protein-coding portion was fused in-frame to lacZ again [see Material and methods]. The carboxy-terminal-truncated Pc proteins analyzed are shown schematically in Figure 1C. By reference to the remaining amino acids in the Pc protein portion, they were designated Pc1-190, Pc1-117, and Pc1-65, respectively. SL2 cells were transfected with the fusion protein encoding constructs, and after 2 days the subcellular distribution of the truncated fusion proteins was visualized with anti-βgal antibodies. For comparison, the punctated distribution pattern of Pc(1–266)–βgal fusion protein in SL2 cell nuclei is shown in Figure 4A. Analyzing the distribution pattern of Pc(1–190)–βgal fusion protein, we found that the removal of 76 amino acids affects neither nuclear localization nor the punctated pattern (Fig. 4B). Carboxy-terminal deletions encompassing either one [data not shown] or both histidine repeats lead in the latter case to the Pc(1–117)–βgal fusion protein whose punctuated nuclear pattern in SL2 cells is similar to the basic construct (Fig. 4C). However, the deletion of two presumptive nuclear targeting sequences (NTS) also leads to a weak cytoplasmic retention of this fusion protein. It seems that the deletion of the fourth NTS [starting with the first NTS at the amino terminus] is responsible for this faint cytoplasmic localization, because a carboxy-terminal truncated fusion protein missing only this NTS shows the same nuclear and cytoplasmic distribution pattern in SL2 cells as Pc(1–117)–βgal fusion protein [data not shown]. Carboxy-terminal truncations extending into the chromo domain alter the distribution of the Pc(1–65)–βgal fusion protein dramatically. Figure 4D shows that this fusion protein is concentrated normally in the nucleus of SL2 cells but is distributed homogeneously throughout the
extranucleolar nucleoplasm. A weak cytoplasmic staining like that in cells expressing Pc[1-117]βgal (Fig. 4C) is also visible. Taken together, these results demonstrate that as soon as the chromo domain is affected by the carboxy-terminal truncations, the punctated pattern in SL2 cell nuclei is lost.

Single mutations in the chromo domain disrupt the punctated Pc protein pattern in SL2 cell nuclei and prevent binding to polytene chromosomes

The previous experiments have suggested that for the formation of specific protein complexes, the Pc/βgal fusion proteins need the entire chromo domain. To prove this, we have restricted the extent of the mutated protein parts by constructing a second set of altered Pc/βgal fusion proteins affecting only the chromo domain (Fig. 1C). We have replaced the wild-type chromo domain by the chromo domain of the two mutant Pc alleles Pc10c and PcXL5, described above, giving rise to Pc[Ile-31→Phe]βgal and to Pc[Δ69-70]βgal fusion proteins, respectively. Furthermore, we used specific oligonucleotide primers to generate several chromo box deletions (see Material and methods). In the Pc[Δ26-36]βgal fusion protein, the amino-terminal part of the chromo domain, including the first presumptive NTS, is missing. Fusion protein Pc[Δ46-52]βgal carries an internal deletion in the chromo domain, whereas the deletion in Pc[Δ42-65]βgal fusion protein extends into the carboxyl terminus of the chromo domain. The constructs coding for these chromo domain mutant Pc/βgal fusion proteins were transfected into SL2 cells, and the subcellular distributions of the encoded fusion proteins were visualized by immunofluorescence staining. The results are shown in Figure 5. Starting with the far left panels [Fig. 5A], the distribution of the Pc[1-266]βgal fusion protein is shown for comparison (top panel). The amino acid substitution from isoleucine to phenylalanine results in the fusion protein Pc[Ile-31→Phe]βgal, which becomes concentrated in the nucleus but is distributed homogeneously throughout the extranucleolar nucleoplasm (Fig. 5B). The 2-amino-acid deletion in the chromo domain leads to the fusion protein Pc[Δ69-70]βgal, which is also homogeneously distributed in the nucleus (Fig. 5C). In both cases, no punctated pattern could be seen. Deletion of the amino-terminal amino acids of the chromo domain, thereby destroying the first potential nuclear targeting sequence, results in a fusion protein that is still localized exclusively and distributed homogeneously in the nucleus (Fig. 5D). This distribution pattern is indistinguishable from the homogeneous nuclear distribution of the former two mutant Pc/βgal fusion proteins. No cytoplasmic staining could be detected. The internal deletion of amino acids 46–52 in the chromo domain leads to a fusion protein that is localized in the nucleus, but in
Pc chromo domain required for chromatin binding

Figure 4. Immunolocalization of carboxy-terminal truncated Pc/βgal fusion proteins in SL2 cells. Representative cells for each construct are shown. The cells were transfected with plasmids encoding various forms of the Pc protein fused to βgal (indicated at top; the corresponding constructs are shown in Fig. 1C), and the subcellular distribution of each transiently expressed Pc/βgal fusion protein was visualized by mouse monoclonal anti-βgal antibodies and by secondary DTAF-coupled anti-mouse antibodies (anti-βgal, top panel). DNA of the same cell stained with DAPI (middle panels). Phase-contrast view (lower panels) showing the morphology of the same cell. In A, (anti-βgal), the punctated distribution pattern of the Pc(1-266)-βgal fusion protein in the SL2 cell nuclei is shown. Compared with this speckled pattern, carboxy-terminal deletions of the 266-amino-acid-long Pc protein portion neither prevent the Pc(1-190)-βgal (B) nor the Pc(1-117)-βgal (C) fusion protein from being concentrated in a punctated pattern in the nuclei. Further truncations into the chromo domain abolish the punctated pattern, leading to a homogeneous distribution of the Pc(1-65)-βgal fusion protein in the cell nucleus (D). The faint cytoplasmic staining in cells expressing Pc(1-117)-βgal and Pc(1-65)-βgal fusion protein is probably caused by the deletion of the fourth nuclear targeting sequence (for details, see text).

two different ways. In most cells we have found a complete homogeneous nuclear distribution, as reported for the three previous cases (Fig. 5E). In about one-third of the cells we have seen additional accumulations in globular structures varying in their numbers and sizes [Fig. 5F shows a big single heavily stained ball-shaped structure]. The fusion protein Pc[Δ42-65]-βgal with the large carboxy-terminal deletion of the chromo domain shows an aberrant distribution in all cells. Although a faint homogeneous background is visible in the nucleus, most of the fusion protein is localized along large, thick fibers [Fig. 5G]. By changing the plane of focus, one realizes that the threads are linked to one another, forming a three-dimensional meshwork.

The homogeneous nuclear distribution of the Pc[ile-31 → Phe]-βgal mutant fusion protein in tissue culture cells is reproduced also in transgenic lines expressing this construct. Figure 6A shows the distribution of the fusion protein in nuclei of third-instar larval salivary glands. The nuclear staining is clearly much more homogeneous compared with the basic construct in Figure 2B. Immunostaining of the polytene chromosomes shows that the single-amino-acid exchange in the chromo domain completely abolishes chromosomal binding [Fig. 6C]. In comparison, no effect is seen for the distribution of the endogenous Pc protein [Fig. 6B]. Similar results were obtained for the Pc[Δ69-70]-βgal fusion protein that carries the mutant chromo domain of the PcX2.5 allele [data not shown].

In summary, we find that alterations in the chromo domain do not affect nuclear accumulation of the Pc/βgal fusion proteins but do dramatically change the nuclear distribution in diploid cells as well as the binding to polytene chromosomes.
Figure 5. Transient expression and immunolocalization of the chromo domain mutant Pc/βgal fusion proteins in SL2 cells. The distribution of the fusion proteins was visualized as in Fig. 4 (anti-βgal, top panel). For comparison, the punctated pattern of the Pc[1–266]-βgal fusion protein in the nucleus of SL2 cells is shown in [A]. All mutations in the chromo domain disrupt the punctated appearance of the fusion protein and result mostly in a homogeneous staining of the nucleus. In addition, two mutations show a peculiar distribution of the fusion protein. While most of the PcA46–52 transfected cells show a homogeneous staining of the nucleus (E), some (30%) of the cells contain one or several large aggregates of the fusion protein [F]. In these cells the homogeneous background staining is reduced accordingly. These aggregates do not seem to correlate with cells in particular cell cycles. The mutant PcA42–65 displays a peculiar network of long, thick fiber-like aggregates. Double stainings with DAPI of early mitotic cells indicate that the fibers do not follow the paths of the chromosomes.
Discussion

Pc protein forms large complexes in cell nuclei

The normal Pc protein, as well as the Pc[1-266]-βgal fusion protein, must be localized in multiple copies in large structures to be seen as such bright spots in the immunofluorescence stainings reported here. By analogy to the situation on salivary gland polytene chromosomes, the structures visible on tissue culture cell nuclei are most probably large Pc–chromatin complexes bound to the respective target genes. We are aware, however, that the dots [Fig. 3A,B] cannot be compared directly to the bands seen in whole nuclei preparations of salivary glands [Fig. 2A,B], as the latter are more a consequence of the high degree of polyteny of these nuclei. They seem to coincide with condensed chromatin, however. In Figure 5A, the DAPI[4', 6'-diamidino-2-phenylindole]-stained chromatin most likely shows a nucleus in early prophase. Some of the speckles of the Pc[1-266]-βgal protein overlap with the DAPI-stained spots. Figure 3C clearly demonstrates that this fusion protein is associated with chromosomes. Similar punctated distributions have been reported for proteins involved in the splicing metabolism [Carmo-Fonseca et al. 1991a, b; Li and Bingham 1991]. The subnuclear structures formed by the complex splicing apparatus are comparable in size and distribution to the Pc-formed nuclear foci. This suggests that Pc might participate in distinct multimeric protein structures comparable to the subnuclear compartments where splicing factors are concentrated.

The role of the chromo domain in forming large Pc-chromatin complexes

The chromo domain is a phylogenetically highly conserved protein motif found in multiple copies in species ranging from plants to man [Singh et al. 1991; Epstein et al. 1992; Pearce et al. 1992]. Elucidation of its function would certainly shed light on an important and ubiquitously used biological mechanism. Here, we have shown that two Pc alleles have mutations in the chromo domain. This clearly proves the functional importance of this protein segment for wild-type Pc activity. The data presented here, as well as the comparative analysis of Singh et al. (1991) and Pearce et al. (1992), have allowed us to extend the boundaries of a presumptive functional chromo domain [see Fig. 1C]. A precise delimitation will obviously need additional functional analyzes.

Figure 6. Mutations in the chromo domain prevent binding to the polytene chromosomes. Transgenic lines expressing Pc[Ile-31 → Phe] [Pc106], the chromo domain mutation with the amino acid exchange, were analyzed for the distribution of the fusion protein. (A) The fusion protein is detected on whole salivary glands using anti-βgal antibodies. Note the homogeneous distribution in the nucleus in comparison with the banded pattern of the wild-type or Pc[1-266] protein in Fig. 2, A and B. (B) The polytene chromosomes of these transgenic lines were immunostained for the presence of the wild-type Pc protein using mouse anti-Pc antibodies. The Pc protein shows the normal distribution. The same chromosomes were also immunostained for the presence of the Pc[Ile-31 → Phe] [Pc106] fusion protein with rabbit anti-βgal antibodies [C]. The chromo domain-mutated fusion protein is clearly not associated with the chromosomes anymore. They are found in both preparations [stars in B,C]. Note that at the most intense wild-type Pc-labeled site at 49EF in B [arrow] no fusion protein can be found at the same site in C.
Using a tagged Pc protein we have begun to gain insight into the physiological role of the chromo domain. The basic Pc[1-266]-βgal fusion protein containing a complete chromo domain retains its binding specificity on polytene chromosomes. In the case of the tissue culture system, all Pc/βgal fusion proteins with a complete chromo domain retained the ability to form large complexes in the nuclei, reflecting the same behavior as the normal Pc protein. Although we have evidence that the carboxy-terminal third of the Pc protein contains additional important sequences for Pc function, it seems not to be necessary for the binding specificity. The mutant Pc[1-117]-βgal protein still retained the ability to form complexes and, thus, probably the capacity of the fusion protein to bind specifically to the appropriate target genes (Fig. 4C). This capability was completely eliminated either in the Pc[1-65]-βgal fusion protein, leaving only a truncated chromo domain fused to the βgal tag (Fig. 4D), or in the case of the different chromo domain mutations analyzed from Figure 5, B–G. Expression of the Pc[Ile-31→Phen]-βgal mutant fusion protein in transgenic lines shows even more convincingly that the chromo domain is responsible for the chromatin-binding specificity. The exchange of isoleucine to phenylalanine in the chromo domain completely abolishes the association of the fusion protein to its specific target genes (Fig. 6C). Considering that the isolated Pc protein does not bind DNA directly, these results suggest that the Pc protein could achieve its target gene specificity through protein–protein interactions via the chromo domain. Intriguing in this respect is the fact that on polytene chromosomes the HP1 protein and the Pc protein have completely different binding patterns (see introductory section; James et al. 1989; Zink and Paro 1989; S. Elgin, pers. comm.). Therefore, the binding specificity must either be determined by the slightly different amino acid composition of the Pc and HP1 chromo domains or by additional domains in the two proteins.

The chromo domain could be part of a chromatin packaging mechanism

The presence of the chromo domain in the heterochromatin-associated protein HP1 suggests an interesting additional function for this protein motif. As mentioned in the introductory section, heterochromatin is thought to be condensed by reiterated multimeric protein units (Locke et al. 1988). Heterochromatin seems to be capable of spreading in a cooperative way along the chromosome. The punctated pattern of the Pc protein in tissue culture cell nuclei resembles large multimeric protein complexes. Because the dots are disrupted in chromo domain mutants, an attractive additional function of the chromo domain could be an involvement in a “packaging” mechanism. The Pc chromo domain might cooperate with other Pc-G proteins in condensing the chromatin of repressed genes (i.e., homeotic genes). Similarly, the HP1 chromo domain might be involved in condensing heterochromatin in cooperation with other modifiers of PEV.

A support for this novel function comes from the analysis of the patterns of the two chromo domain mutants Pc[Δ46–52]-βgal and Pc[Δ42–65]-βgal. The fusion protein Pc[Δ46–52]-βgal shows in some cells very large globular aggregates (Fig. 5F). The Pc[Δ42–65]-βgal mutant shows an even more dramatic distribution, being localized in thick, long fibers arranged in a three-dimensional mesh work (Fig. 5C). Both patterns could be interpreted as being the result of an uncontrolled polymerization/packaging effect of the chromo domain. Because the deletions in the two mutants overlap (Fig. 1C), they might disrupt a part of the chromo domain involved in controlling the extent of the packaging.

A simpler explanation for the odd distribution of the two mutant fusion proteins could be a solubility problem. The mutations in the chromo domain might have rendered the fusion protein insoluble [in the nucleus], resulting in large precipitates. Similar thread-like structures are found in erythrocytes of sickle-cell anemia patients. An amino acid replacement in the β-globin chain renders the deoxyhemoglobin insoluble and causes it to aggregate into long straight fibers extending through the whole length of the sickled cell [Finch et al. 1973]. The threads could also be caused by an unspecific association of the mutant Pc[Δ42–65]-βgal protein with a yet unknown nuclear matrix component. In these latter cases, the aberrant pattern might not give any clues toward the real function of the chromo domain. Additional experiments, including the biochemical analysis of these aggregates, will obviously be needed to discriminate between these different possibilities.

Our results show that the Pc protein is found in large immunologically visible complexes in diploid cells. Taken together, this supports the hypothesis that Pc-G proteins, like modifiers of PEV, are chromosomal proteins that have multiple contacts with other molecules to produce higher-order complexes for regulating gene expression. We were able to isolate by gel filtration from embryonic nuclear extract large multimeric protein complexes containing the Pc protein and at least one other member of the Pc-G (Franke et al. 1992). Here, we show that the chromo domain plays a major role in forming these complexes and thus in condensing chromatin of repressed genes. Additional experiments will be needed to define the exact molecular mechanisms and to identify the interacting partners by which this novel protein motif achieves its functional role.

Materials and methods

Cloning and sequencing of mutant Pc alleles

The homozygous mutant Pc embryos could be distinguished from the heterozygotes after ~20 hr of development because of their lack of head involution [Denell and Frederick 1983]. One hundred homozygous embryos were homogenized in protein sample buffer, and the proteins were separated on an 8% discontinuous SDS–protein gel [Laemmli 1970]. The proteins were transferred to nitrocellulose, and the Pc protein-expressing alleles were identified by staining the filter with polyclonal rabbit
anti-Pc antibodies and secondary alkaline phosphatase-conjugated antibodies. To identify the corresponding point mutations, 100 embryos were homogenized gently in 500 μl of lysis buffer (100 mM Tris-Cl at pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% Triton X-100), treated with proteinase K (100 μg/ml) for 2 hr at 37°C, phenol-extracted, treated with RNase (100 μg/ml) for 1 hr at 37°C, and phenol- and chloroform-extracted. The genomic DNA was ethanol-precipitated and resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA at pH 7.5). One-tenth of the genomic DNA (150 ng) was used for the PCR amplification of the Pc sequences, performing 30 cycles (Saiki et al. 1988). The oligonucleotides had the sequence 5′-GACACGGCTACT-TGCTAAAG-3′, complementary to a region 180 bp upstream of the Pc open reading frame (ORF), and 5′-GGCAGCAAAAGGGCTGTAA-3′, complementary to a region 16 bp downstream of the Pc ORF. The resulting 1711-bp fragments were subcloned in Bluescript (Stratagene) and sequenced with internal primers according to the double-stranded sequencing procedure of Chen and Seeburg (1988), by use of the T7 sequencing kit (Pharmacia). Of each allele, three independent clones were sequenced over the entire ORF.

**Recombinant plasmids**

The genomic 1954-bp PstI–PvuII PcDNA fragment containing the entire Pc promoter, the first exon, the intron, and two-thirds of the second exon (for Pc sequence, see Paro and Hogness 1991) was cloned into the pC4βgal vector (Thummel et al. 1988). For this purpose this vector was digested with BamHI, and the ends were filled in with Klenow enzyme and cleaved with PstI. The orientation-directed insertion of the 1954-bp Pc fragment generated an in-frame fusion to the lacZ gene. The encoded fusion protein contains 266 amino acids of Pc fused to the amino-terminal end of βgal. This subclone, pC4 Pc[1–266]–βgal, was partially digested with EcoRI (one EcoRI site resides within the Pc promoter, the other is 3′ to the SV40 polyadenylation signal) and then digested with PstI to completion. The isolated PstI–lacZ fusion gene was cloned into the PstI–EcoRI sites of the polylinker of the P-element transformation vector pW8 [Klemenz et al. 1987], resulting in a transcriptional orientation opposite the white gene. P-element transformation was performed according to Rubin and Spradling (1982), coinjecting 300 μg/ml of pW8 Pc[1–266]–βgal together with 50 μg/ml of ppc25.7wc[Kaess and Rubin 1984] P-factor helper plasmid into embryos of the white-eyed w mn fly strain.

For the carboxy-terminally truncated Pc/βgal fusion proteins, the transformation vector pW8 Pc[1–266]–βgal was cleaved at the single BamHI site, thereby separating the Pc sequence from the lacZ gene. A synthetic double-stranded oligonucleotide fragment was inserted carrying unique restriction sites for BamHI and KpnI. The correct orientation of this oligonucleotide [BamHI site 3′ to the Pc carboxyl terminus and KpnI site 5′ to the lacZ amino terminus] was verified by restriction enzyme analysis. Following the BamHI and KpnI digest, unidirectional truncations of the carboxy-terminal Pc sequence were performed with exonuclease III [Henikoff 1984] according to the manufacturer's protocol [Erase-a-base system, Promega]. For ligation, the ends were blunted by treatment with Klenow polymerase. The deletion breakpoint and the in-frame fusion to lacZ were verified by dideoxy sequencing by use of the T7 sequencing kit of Pharmacia.

The in vitro-generated internal chromo box deletions were introduced by oligonucleotide site-directed mutagenesis in M13 (Taylor et al. 1985), following the manufacturer's protocol (Amersham). A 1654-bp EcoRI–PvuII genomic Pc DNA fragment missing the first 300 bp of the 1954-bp PstI–PvuII Pc fragment was isolated and inserted between the EcoRI and blunt-ended BamHI polynucleotide sites of a M13mp19 vector. Ligation restored the BamHI site. The 50- to 56-bp-long synthetic oligonucleotide primers used for the in vitro mutagenesis were purified through a sequencing gel followed by a desalting Sep-Pak C18 cartridge [Waters Chromatography Division, Millipore]. Efficiencies of deletion mutagenesis were very high, ranging between 90% and 95%. Deletions were verified by restriction enzyme analysis, and DNA polymerase errors were excluded by dideoxy sequencing of the entire Pc DNA fragment. The cloning of the internally deleted Pc DNA fragments in-frame to the lacZ gene of the P-element transformation vector pCaSpeR-βgal (Thummel et al. 1988) was done in several steps. First, all Pc fragments carrying the desired deletion were isolated from the M13mp19 subclones by digestion with ClaI and BamHI and used to replace the respective wild-type Pc fragment Pc[1–1954] subcloned in pSP64. Although this subclone has no function in the final constructs, it facilitated the cloning by providing a single ClaI and BamHI site. Mutant Pc alleles subcloned in Bluescript (Stratagene) were isolated by ClaI and BclI digestions and inserted between ClaI and BclI sites of pSP64 Pc[1–1954] replacing the wild-type Pc sequence. Finally, the whole Pc DNA fragments carrying the desired internal chromo box deletions were isolated by treatment with PstI and BamHI and inserted into the PstI–BamHI polynucleotide sites of pCaSpeR-βgal transformation vector in-frame to the βgal coding region.

**Antibodies**

Two different primary anti-Pc antibodies were generated either in rabbits or in mice. Antibodies raised in rabbits against a lacZ–Pc fusion protein containing the carboxy-terminal 199 amino acids of Pc protein [indicated by the ab 1 line in Fig. 1] have been described previously [Zink and Paro 1989]. These polyclonal antibodies were used for single immunofluorescence stainings. For double immunofluorescence staining, affinity-purified polyclonal rabbit anti-Pc antibodies were used. These were raised against a lacZ–Pc fusion protein containing only the carboxy-terminal 114 amino acids of Pc protein [indicated by the ab 2 line in Fig. 1]. Affinity purification protocols were as described for the rabbit antibodies. On Western blots of bacterial and embryonic lysates, we have shown that the polyclonal rabbit anti-Pc antibodies react with the antigenic fusion protein and with the Pc[1–266]–βgal fusion protein. The polyclonal mouse anti-Pc antibodies reacted only with the fusion protein. Both antibodies did not react with βgal alone (data not shown).

**Immunofluorescence staining of whole salivary glands and polytene chromosomes**

Glands were dissected in PBS, incubated in PBS/0.5% NP-40 for 20 min, and fixed for 10 min in PEM buffer (0.1 M PIPES at pH 6.5, 2 mM EGTA, 1 mM MgSO4) containing 2% formaldehyde. All subsequent incubation steps were performed in a 0.5-ml reaction tube under slow but continuous rotation at room temperature. Glands were rinsed in PBS and blocked for 2 hr in PBT [PBS at pH 7.5/0.5% Triton/10% BSA]. After blocking, glands were incubated for 2 hr in PBT containing either affinity-purified polyclonal rabbit anti-Pc antibodies diluted to the desired concentration or mouse monoclonal anti-βgal antibodies [Promega] diluted 1:500. After incubation, glands were washed three times for 10 min each in PBT and treated for 2 hr with DTAF [dichlorotriazinyl-amino-fluorescein]-conjugated goat anti-rabbit antibodies [Jackson Laboratories, preabsorbed 1:10 to 1- to 20-hr wild-type embryos and diluted to a final concentration of 1:200 in PBT containing 10% normal goat serum] to
detect the endogenous Pc protein. DTAF-coupled anti-mouse IgG (Jackson Laboratories, preabsorbed 1:10 to 1- to 20-hr Pc(1-266)-βgal transgenic embryos and diluted to a final concentration of 1:200 in PBT containing 10% normal goat serum) were used to visualize the Pc/βgal fusion protein.

Immunostaining of polytene chromosomes was done essentially as described by Zink et al. (1991). For double immunofluorescence staining polyclonal rabbit anti-βgal antibodies (U.S. Biochemical, diluted 1:100) and secondary rhodamine-conjugated goat anti-rabbit antibodies (Jackson Laboratories; diluted 1:50) were used in detecting the βgal fusion protein. The affinity-purified polyclonal mouse anti-Pc antibodies (diluted 1:5) and secondary DTAF-conjugated goat anti-mouse antibodies (Jackson Laboratories; diluted 1:50) were used to detect wild-type Pc protein. Incubation with primary and secondary antibodies was done for 2 hr each at room temperature. Stained glands and chromosomes were mounted in Mowiol (Osborn and Weber 1982) containing 2.5% DABCO [1,4-diazabicyclo[2.2.2]octan] to reduce fading. Photographs were taken at a magnification of 400× with Kodak Tmax 400 film using a Zeiss Axiofluar fluorescence microscope.

Drosophila cell culture, DNA transfection, and immunofluorescence

Drosophila SL2 cells (Schneider 1972) were grown and maintained as described (Di Nocera and Dawid 1983; Krasnow et al. 1989) in Schneider’s media (Serva or Gibco). One day before transfection, the cells were diluted to a density of 5 × 10^6 cells/ml, and 5-ml aliquots of this dilution were plated onto 6-cm-diam. tissue culture plates (Falcon). After 16 hr, the cells were transfected by the calcium–phosphate technique described by Di Nocera and Dawid (1983), except that the calcium–phosphate DNA precipitate was prepared by adding drop-wise a 250 μl Ca-DNA solution (0.25 M CaCl₂, 10 μg of CsCl-purified plasmid DNA) to 250 μl of 2× HeBS solution. The precipitation mixture was incubated at room temperature for 30 min and added to the cell culture. The cells were incubated with the calcium phosphate–DNA coprecipitate for 24 hr at 25°C. After this period the medium was removed, the cells were washed gently with PBS, and fresh medium was added. After an additional 24 hr at 25°C, aliquots of cultures [5 × 10⁶ cells] were transferred into one chamber of a two-chamber LAB-TEK tissue culture slide (Nunc). Slides were fixed onto a microtiter plate and simple method for sequencing plasmid DNA. 0.1 μg/ml and incubated for 1 min. The cells were then washed three times for 5 min each in PBS. Finally, the chambers were removed from the slides, and the cells were embedded in Mowiol. Photographs were taken at a magnification of 1200× on Agapan APX 100 using a Zeiss Axiofluar fluorescence microscope.

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