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Polycomb and *polyhomeotic* are constituents of a multimeric protein complex in chromatin of *Drosophila melanogaster*

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The polycomb group (Pc-G) genes are responsible for maintaining the repressed state of homeotic genes during development. It has been suggested that the Pc-G exerts its transcriptional control by regulating higher order chromatin structure. In particular, the finding of genetic and molecular similarities to components involved in heterochromatin formation, led to the proposal that homeotic genes are permanently repressed by mechanisms similar to those responsible for heterochromatin compaction. Because of synergistic effects, Pc-G gene products are thought to act in a multimeric complex. Using immunoprecipitation we show that two members of the Pc-G, *Polycomb* and *polyhomeotic*, are constituents of a soluble multimeric protein complex. Size fractionation indicates that a large portion of the two proteins are found in a distinct complex of molecular weight 2–5 × 10^6 Da. During embryogenesis the two proteins show the same spatial distribution. In addition, by double-immunofluorescence labelling we can demonstrate that *Polycomb* and *polyhomeotic* have exactly the same binding patterns on polytene chromosomes of larval salivary glands. We propose that some Pc-G proteins act in multimeric complexes to compact the chromatin of stably repressed genes like the homeotic regulators.

Key words: chromatin regulation/*Drosophila*/homeotic gene regulation/polycomb group/polytene chromosomes

Introduction

Regulatory mechanisms that maintain the determined state of a cell are necessary for the proper development of an organized body plan. In *Drosophila melanogaster*, the differential activity of the homeotic genes of the *Antennapedia*- (ANT-C) and *bithorax*-complex (BX-C) determine the various body structures along the anterior–posterior axis (Lewis, 1978; Kaufman *et al.*, 1980). Since their function is required throughout development, they have been used as a model system to identify and characterize the elements required for the transmission of the determined states which they define. Genetic analysis has revealed a distinct class of genes involved in part of this process. The genes of the Polycomb group (Pc-G) were found to be responsible for maintaining the repressed state of homeotic genes (reviewed in Paro, 1990). Mutations in members of the Pc-G show an ectopic expression of homeotic regulators (Struhl and Akam, 1985; Weeden *et al.*, 1986; Dura and Ingham, 1988; McKeon and Brock, 1991; Simon *et al.*, 1992), which can result in dramatic transformations of body structures (Struhl, 1981; Duncan, 1982; Duncan and Lewis, 1982; Ingham, 1984; Dura *et al.*, 1985; Jürgens, 1985; Breen and Duncan, 1986; Adler *et al.*, 1989; Jones and Gelbart, 1990). Pc-G genes are not only involved in maintaining homeotic gene expression patterns, but have been found to perform similar roles on other developmental regulators (Ingham, 1984; Breen and Duncan, 1986; Dura and Ingham, 1988; Smouse *et al.*, 1988; Busturia and Morata, 1988; Wu *et al.*, 1989).

Two genes of the Pc-G group have been extensively characterized at the genetic and molecular level. Homozygous mutant embryos of the *Polycomb* (*Pc*) gene display posterior transformations of all segments, characteristic for a deregulation of all the homeotic genes of the ANT-C and BX-C (Lewis, 1978; Denell and Frederick, 1983; Sato and Denell, 1985; Tiong and Russell, 1990). *Pc* was found to exert its regulatory role at the transcriptional level (Weeden *et al.*, 1986; Zink *et al.*, 1991). The *Pc* protein is a nuclear protein associated with ~100 different sites on polytene chromosomes of larval salivary glands (Zink and Paro, 1989; Zink, 1990). Reporter genes linked to homeotic *cis*-regulatory sequences become ectopically expressed when tested in a background lacking Pc^+ function (Müller and Bienz, 1991; Zink *et al.*, 1991). Additionally, Zink *et al.* (1991) showed that the *Pc* protein specifically binds to regulatory sequences of the *Antennapedia* gene.

Genetic analysis of the other well characterized gene, *polyhomeotic* (*ph*), revealed a high degree of pleiotropy of the mutant phenotype. Although the homeotic transformations observed classify it as a Pc-G gene, additional defects like cell death in the ventral epidermis (Dura *et al.*, 1987) and misrouting of central nervous system axons (Smouse *et al.*, 1988; Smouse and Perrimon, 1990) can be identified. *ph* shows a complex gene structure and is arranged as a tandemly duplicated unit. Only lesions in both units result in null or strong hypomorphic alleles, whereas single lesion alleles display only weak hypomorphic phenotypes. The two genetic units contained within a stretch of 25 kb of genomic DNA encode similar proteins that have certain characteristics of proteins interacting with DNA (Deatrick *et al.*, 1991; DeCamillis *et al.*, 1992). In addition to four blocks of glutamine repeats and serine/threonine-rich sequences, the *ph* protein displays a region with partial homology to the helix–loop–helix motif and a single C4 zinc finger. Like *Pc*, *ph* binds to ~100 polytene chromosome sites and *ph* specifically recognizes regulatory sequences from the *bithoraxoid* region (DeCamillis *et al.*, 1992).

The Pc-G genes display some common features with another distinct class of *Drosophila* genes, the modifiers of position effect variegation (PEV). These genes are thought to encode structural and regulatory constituents of chromatin (reviewed in Eissenberg, 1989; Henikoff, 1990). Some Pc-G genes show effects on PEV (D.Sinclair, N.Clegg, T.Grigliatti and H.W.Brock, submitted) and reciprocally, some modifiers of PEV display homeotic transformations (Reuter *et al.*, 1990; G.Reuter, personal communication). Paro and Hogness (1991) have found a molecular relationship between the *Pc* protein and the heterochromatin-associated HP1 protein, which is encoded by *Su(var)205* (Eissenberg *et al.*, 1990). Both proteins share the chromo domain, a 48 amino acid motif found to be responsible for specific chromatin binding (Messmer *et al.*, 1992). Based on these similarities, it has been proposed that the Pc-G genes repress homeotic genes by regionally compacting the chromatin (heterochromatinization) and thus eliminating the accessibility of DNA to diffusible transcription factors. This mechanism could imprint the higher order structure of chromatin with the determined state of the cell as defined by the differential expression pattern of developmental regulators like homeotic genes (Locke *et al.*, 1988; Gaunt and Singh, 1990; Paro, 1990; Reuter *et al.*, 1990).

Because modifiers of the PEV show a very high gene dose sensitivity, Locke *et al.* (1988) proposed that heterochromatin is packaged by reiterated multimeric protein complexes composed of the products of these genes. Heterochromatin apparently can spread along the chromosome in a cooperative fashion and thus transcriptionally inactivate large chromosomal regions. Jürgens (1985) has found that the double and triple mutant combination of four genes of the Pc-G, *Additional sex combs* (*Asx*), *Polycomb-like* (*Pcl*), *Posterior sex combs* (*Psc*) and *Sex combs on midleg* (*Scm*) showed a marked enhancement of the homeotic transformation compared with the single mutations. This synergistic effect of the Pc-G could be explained by a participation of the different Pc-G gene products in a common regulatory structure, i.e. a multimeric protein complex. Pc-G proteins could form multimeric protein units that are functionally and mechanistically equivalent to heterchromatic complexes, except that particular Pc-G proteins would target the complexes to euchromatic genes.

Here we present evidence that two members of the Pc-G are part of a large multimeric protein complex. DeCamillis *et al.* (1992) have shown that the *ph* and *Pc* proteins have many overlapping binding sites on polytene chromosomes. We show that this *in vivo* correlation is based on a molecular association of the two proteins in a soluble nuclear complex. Using immunoprecipitations we can demonstrate that *Pc* and *ph* are found in a multimeric complex. We have furthermore refined the correlation of the *Pc* and *ph* binding patterns on polytene chromosomes by using double-immunofluorescence techniques. We propose that the Pc-G proteins exert their repressory function on the chromatin of homeotic genes as multimeric protein units.

Results

Immunoprecipitation of Pc and associated proteins from nuclear extracts

We used immunoprecipitations to enrich for proteins that may associate with the *Pc* protein. In an initial experiment we found that our previously described rabbit polyclonal anti-*Pc* antibodies (Zink and Paro, 1989) could be used to immunoprecipitate *in vitro* translated *Pc* protein as well as endogenous *Pc* protein from embryonic nuclear extracts, or from nuclear extracts of Schneider L-2 tissue culture cells. To perform the immunoprecipitations, we essentially followed the protocol of Gay *et al.* (1988), who were able to identify the association of the *engrailed* protein with other nuclear factors. In this method the nuclei are lysed by a hypertonic shock and the released soluble nuclear proteins partially purified and concentrated by a 30% ammonium sulfate precipitation. The redissolved soluble nuclear proteins were immunoprecipitated with anti-*Pc* antibodies and Protein A–Sepharose beads (see Materials and methods).

To determine if *Pc* is associated with other nuclear proteins in the soluble fraction, we separated the immunoprecipitate by SDS–PAGE. However, these initial experiments did not yield enough coprecipitated proteins to give a visible pattern in silver stained gels. To circumvent the sensitivity problem we tried to immunoprecipitate *in vivo* labelled nuclear proteins from tissue culture cells. *Pc* is endogenously expressed in these cells (Messmer *et al.*, 1992). Cells were labelled for 3 h with [^{35}S]methionine and [^{35}S]cysteine, and nuclear proteins were immunoprecipitated with anti-*Pc* antibodies and separated on gels. Disappointingly, only very weak signals could be observed after prolonged exposures (data not shown). Because the immunoprecipitated *Pc* protein seemed to have incorporated only a small amount of radioactivity, we attributed the negative result to the relatively high stability of *Pc* and the other associated proteins. Pc-G proteins might have a low turnover, such that a 3 h labelling period is too short to incorporate sufficient radioactivity. Indeed, we have evidence from the developmental profile of *Pc* that the protein is very stable during embryogenesis (B.Zink and R.Paro, unpublished results).

However, by radioactively labelling the proteins after immunoprecipitation, we were able to visualize the *Pc*-associated proteins. For this purpose we used a modified Bolton–Hunter reagent to couple ^{35}S to the precipitated proteins (Assoian *et al.*, 1980; see also Materials and methods). The disadvantage of this method is that the antibodies in the precipitate are also labelled and result in a strong signal on the autoradiogram that can obscure the signals of other proteins in the same molecular weight range. Figure 1 shows the result of this experiment. In lane 3, 10–15 new *Pc*-associated proteins are seen to coprecipitate with *Pc*, compared with the control lanes 4, 5 and 6 (see legend to Figure 1). In lane 3, no major band corresponding to the *Pc*-protein, shown in Lane 1 as an *in vitro* produced ^{35}S-labelled protein, is visible. However, this is not surprising, considering that the labelling of proteins with the ^{35}S-labelling reagent ^{35}SRL, can substantially change the mobility of proteins in SDS–PAGE (Assoian *et al.*, 1980). The results shown in Figure 1 clearly indicate that *Pc* is associated with several other proteins in the soluble nuclear fraction. The multimeric protein complex is stable enough to be immunoprecipitated with anti-*Pc* antibodies, providing a possible molecular means of characterizing the various components.

Polycomb and polyhomeotic are colocalized in the same multimeric protein complex

Biochemical identification of the coprecipitated proteins would be very difficult, because only small amounts of

material could be recovered. However, we were able to take advantage of the considerable amount of genetic and molecular data available on the Pc-G system. Obvious candidates for proteins interacting at the molecular level with *Pc* are other members of the Pc-G. Indeed, we have shown previously that *Pc* and *ph* have similar binding patterns on

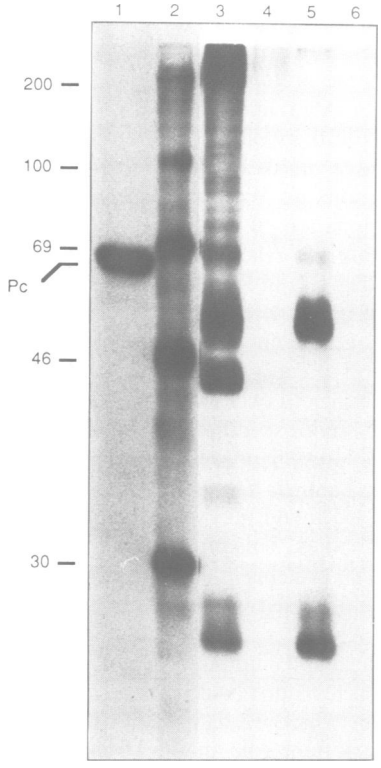

Fig. 1. Immunoprecipitation of *Pc*-associated proteins from embryonic nuclear extracts. 25 μl (protein concentration 15 mg/ml) of an embryonic nuclear extract was immunoprecipitated in a total volume of 500 μl using anti-*Pc* antibodies. The precipitate was labelled with ^{35}SLR (see Materials and methods) and fractionated on a 8% SDS−PAGE gel. The gel was dried on a piece of Whatman paper and exposed for 24 h. Lane 1, *in vitro* translated ^{35}S-labelled *Pc* protein as a size reference; lane 2, ^{14}C protein molecular mass standards with the corresponding sizes given on the left, lane 3, immunoprecipitations with anti-*Pc* antibodies and protein A−Sepharose; lane 4, the same immunoprecipitation as in lane 3 but omitting the anti-*Pc* antibodies; lane 5, mock-immunoprecipitation without nuclear extracts. Note the strong signal from the immunoglobulin chains; lane 6, mock-immunoprecipitation without nuclear extract and without antibodies.

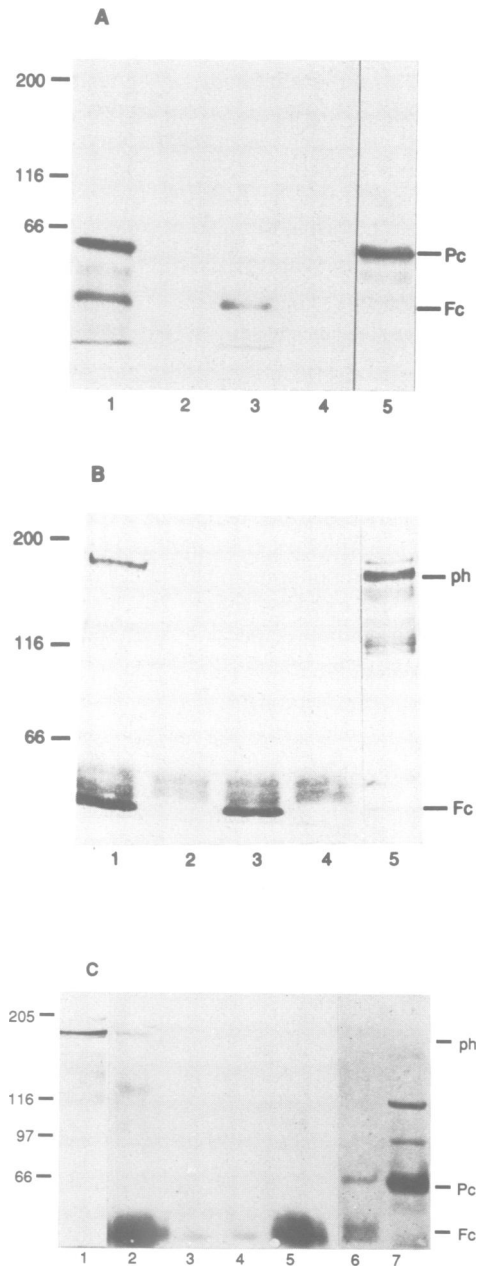

Fig. 2. The *Pc* and *ph* protein are coimmunoprecipitated in the same multimeric protein complex. Panels A and B show an immunoprecipitation of 100 μl embryonic nuclear extract (protein concentration 15 mg/ml) with anti-*Pc* antibodies in a total volume of 1.5 ml. One-third of the precipitate in A and two-thirds of the precipitate in B were separated on a 8% (**A**) and 6% (**B**) SDS−PAGE and transferred to nitrocellulose. Blot A was probed with anti-*Pc* antibodies and shows the specific precipitation of the *Pc* protein. Blot B was probed with anti-*ph* antibodies to show the co-purification of the *ph* protein. Lane 1, immunoprecipitation of nuclear extract with anti-*Pc* antibodies and Protein A−Sepharose; lane 2, mock-immunoprecipitation without primary antibodies; lane 3, mock-immunoprecipitation without nuclear extract; lane 4, mock-immunoprecipitation without nuclear extract and primary antibody; lane 5, 5 μl nuclear extract to show the mobility of the *Pc* and *ph* proteins, respectively. The *ph* gene encodes multiple protein forms. At least four different *ph* proteins can be detected in unstaged embryos (M.DeCamillis and W.H.Brock, in preparation). Due to the instability of the *ph* protein, some degradation seems to have occurred. Both Westerns were incubated with alkaline phosphatase coupled anti-rabbit antibody as secondary antibodies. Thus, the new bands in lane 1 are not due to cross-reactivity with the secondary antibody. The bands denoted with Fc in lanes 1 and 3 are caused by the cross-reactivity of the secondary antibodies with the anti-*Pc* antibodies. (**C**) Coprecipitation of *Pc* with anti-*ph* antibody. Embryonic nuclear extracts were immunoprecipitated with anti-*ph* antibody. The precipitates were separated by SDS−PAGE, transferred to nitrocellulose and examined using Western blotting for the presence of *ph* and *Pc*. Lanes 1−5 show Western blots probed with antibodies to *ph* and lanes 6−7 show western blots probed with antibodies to *Pc*. Lane 1, Western blot of an embryo nuclear extract before immunoprecipitation to show mobility of *ph* products; lane 2, immunoprecipitation of the same extract with *ph* antibody. Some degradation of *ph* occurs. Lane 3, mock-immunoprecipitation lacking Protein A−Sepharose; lane 4, mock-immunoprecipitation lacking antibody; lane 5, mock-immunoprecipitation lacking nuclear extract; lane 6, *Pc* antibody reacted with the same immunoprecipitate shown in lane 2 to show that *Pc* coprecipitates with anti-*ph* antibody; lane 7, Western blot of an embryo nuclear extract before immunoprecipitation to show mobility of *Pc*. The higher molecular weight bands above the *Pc* protein are due to cross-reactivity with the secondary antibody used in this particular experiment.

polytene chromosomes of salivary glands (DeCamillis *et al.*, 1992). We therefore tested if *ph* copurifies in the immunoprecipitation with anti-*Pc* antibodies. The results are shown in Figure 2A and 2B. Immunoprecipitations of embryonic nuclear extracts were performed, separated on SDS−PAGE and blotted onto nitrocellulose filters. In Figure 2A, the Western blot was probed with anti-*Pc* antibodies. Lane 1, which contains the normal immunoprecipitate clearly shows the enrichment of the *Pc* protein, when compared with the mock-immunoprecipitations in the control lanes 2, 3 and 4 (see legend to Figure 2A). The same set of immunoprecipitations were tested for the presence of the *ph* protein in Figure 2B. Lane 1 identifies *ph* as one of the proteins that can be copurified with *Pc,* indicating that *Pc* and *ph* are part of the same multimeric protein complex.

To prove the specificity of the interaction and to demonstrate the reproducibility of the immunoprecipitation protocol, we performed the reciprocal experiment. Using specific anti-*ph* antibodies (DeCamillis *et al.*, 1992), we were able to coimmunoprecipitate the *Pc* protein from embryonic nuclear extracts. Figure 2C, lane 2, shows the result of the immunoprecipitation analysed first for the presence of the *ph* protein. Although some degradation seems to have occurred, most of the *ph* products are found in the precipitate. In lane 6, the same immunoprecipitate is probed for the presence of the *Pc* protein. The comparison with the nuclear extract in lane 7 shows that *Pc* can also be precipitated by using anti-*ph* antibodies. We find that in general immunoprecipitation with anti-*ph* antibodies is less efficient than with the anti-*Pc* antibodies. This could reflect a reduced accessibility of the *ph* protein in the complex, or a difference in the protein composition of the complex for the two proteins. Alternatively, the discrepancy could be due to a different specificity of the two antibodies used, although this seems quite unlikely as the two antibodies gave comparable results on Western blots.

The coprecipitation of the two proteins could also be explained by a cross-reactivity of the anti-*Pc* and anti-*ph* antibodies. However, in Western blots probed with anti-*Pc* antibodies, we never detected a cross-reacting band at the expected size of the *ph* protein. This is also the case for the reciprocal experiment with anti-*ph* antibodies (see also Figure 2). In order to exclude completely this possibility, we have used a more specific approach. In vitro translated ^{35}S-labelled *Pc* and *ph* proteins were immunoprecipitated with anti-*ph* and anti-*Pc* antibodies, respectively (Figure 3). In both cases we found no evidence for a cross-reactivity of the two antibodies. This proves that the copurification of the *Pc* and the *ph* proteins in the immunoprecipitates are due to the presence of these two proteins in the same multimeric complex.

We tested the anti-*Pc* immunoprecipitate for the presence of additional proteins that could play a part in the regulatory system, and for which we had specific antibodies. Histone H1 has an important role in the compaction of inactive chromatin (reviewed in Grunstein, 1990). Using antibodies against *Drosophila* Histone H1 (kindly provided by S.Elgin, St. Louis) we have found that H1 does not coprecipitate with *Pc* and thus is not a major constituent of the multimeric protein complex (data not shown). A similar negative result

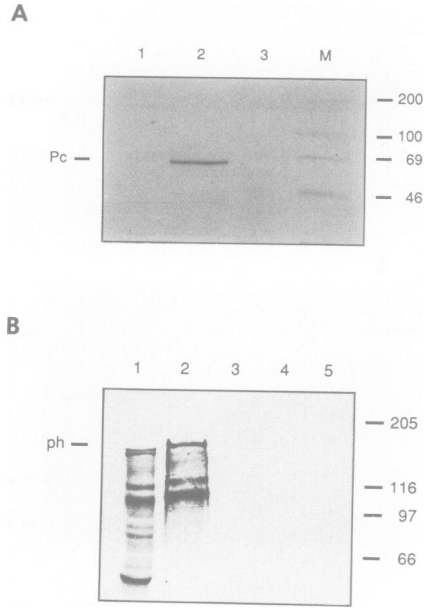

Fig. 3. Control of cross-reactivity of the anti-*ph* antibody with *Pc* and of the anti-*Pc* antibody with *ph.* (**A**) Immunoprecipitation of ^{35}S-labelled *Pc* protein produced in a reticulocyte lysate. 5 μg of the translation mixture was incubated in a total volume of 500 μl. The precipitate was fractionated on an 8% SDS−PAGE gel and exposed overnight. Lane 1, immunoprecipitation with anti-*ph* antibodies and Protein A−Sepharose; lane 2, immunoprecipitation with anti-*Pc* antibodies and Protein A−Sepharose; lane 3, mock-immunoprecipitation without antibody; lane M, ^{14}C protein marker as size standards that are given on the right. (**B**) Control for cross-reactivity of anti-*Pc* antibody with *ph* protein. *ph* proteins that had been translated in a reticulocyte lysate were immunoprecipitated with antibodies to *ph* and *Pc.* The immunoprecipitates were separated by SDS−PAGE and exposed to autoradiography. Lane 1, *ph* mRNA translated in a reticulocyte lysate containing [^{35}S]methionine; lane 2, immunoprecipitation of *ph* translation products with anti-*ph* antibody; lane 3, immunoprecipitation of *ph* translation products with anti-*Pc* antibodies. Note the absence of immunoprecipitation. Lane 4, immunoprecipitation using *ph* antibody of a lysate that did not contain *ph* message; lane 5, mock-immunoprecipitation of lysate containing *ph* translation products and protein A, but lacking *ph* antibody.

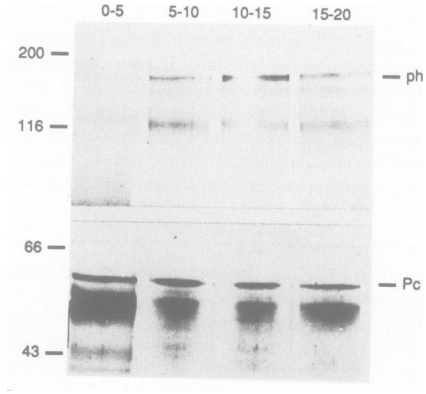

Fig. 4. Immunoprecipitations of nuclear extracts from staged embryos with anti-*Pc* antibodies. The precipitates were separated by SDS−PAGE and transferred to nitrocellulose. The upper filter was incubated with anti-*ph* antibodies and the lower filter with anti-*Pc* antibodies. At every stage analysed the protein concentration was normalized to have a constant amount of immunoprecipitated *Pc* protein. On top of the lanes the individual embryonic stages are denoted by the time (h) from egg laying. Both filters were incubated with alkaline phosphatase coupled anti-rabbit antibodies as secondary antibodies. The strong band below the *Pc* signal is due to the cross-reactivity with the heavy chains of the primary antibodies. Molecular weight standards are indicated on the left.

was obtained when the immunoprecipitate was probed with antibodies against the homeotic proteins *Antennapedia* (kindly provided by W.Gehring, Basel) and *Ultrabithorax* (kindly provided by R.White, Cambridge). This indicates that Pc-G proteins do not directly interact with homeotic proteins to exert their negative regulatory role. This conclusion had also been suggested by genetic experiments (Castelli-Gair and Garcia-Bellido, 1990). We found no evidence of a coprecipitation of the *engrailed* protein, indicating that the *engrailed*-associated multimeric protein complexes identified by Gay *et al.* (1988) are distinct from the *Pc–ph* complexes identified here.

Developmental profile of the Pc–pH interaction

The activity of the Pc-G is needed after germ band elongation, to take over and maintain the homeotic expression pattern initiated by the early maternal and segmentation genes (Struhl and Akam, 1985; Weeden *et al.*, 1986; Kuziora and McGinnis, 1988; Celniker *et al.*, 1990; McKeon and Brock, 1991; Simcox *et al.*, 1991; Simon *et al.*, 1992). We wanted to test whether this activity is regulated at the level of complex formation. In particular, we have tried to identify at what embryonic stages *Pc* is associated with *ph*. We collected nuclear extracts from staged embryos, performed immunoprecipitations with anti-*Pc* antibodies and analysed for the copurification of *ph*. We normalized the mass of immunoprecipitated proteins to give a constant amount of *Pc* protein in each lane (Figure 4). Our data suggest that *Pc* and *ph* can be coprecipitated over the entire period of embryogenesis. Although the ratio between the coprecipitated *ph* and *Pc* proteins clearly increases during embryogenesis, there is no stage at which the two proteins are not associated. This shows that the *Pc–ph* complexes form early in development during the initiation stage of homeotic gene regulation, suggesting that Pc-G repression of homeotic expression is most probably controlled by a different means than formation of the Pc-G complex.

Size measurements of the multimeric protein complex

The data we have presented suggest that *Pc* is associated with 10–15 other nuclear proteins and show that the Pc-G member *ph* is one of the constituents of this soluble multimeric complex. Using gel filtration and density gradient centrifugation methods, we have determined the approximate molecular weight of the complex. As starting material we used the same embryonic nuclear extracts and buffers as for the immunoprecipitations, since under these conditions the complex was shown to be stable. The best separation of nuclear extract was achieved by gel filtration chromatography on a Sephacryl S-400 HR column (see Materials and methods). Different fractions were collected, separated by SDS–PAGE, blotted onto nitrocellulose and tested for the presence of *Pc* and *ph* protein, respectively. Figure 5 shows that significant amounts of *Pc* and *ph* proteins copurify as a high molecular weight complex. Most of the *Pc–ph* protein elutes over a narrow molecular weight range. Extrapolating from the molecular weight markers indicated (see legend to Figure 5), we have estimated that the weight of the *Pc–ph* complex is $\sim 2-5 \times 10^3$ kDa. A more accurate weight could not be obtained because of the absence of a suitable molecular weight marker. There is a clear upper band corresponding to the size of the complex (Figure 5, lane 8), but there is a smear of smaller complexes decreasing in molecular weight to the size of the monomers. Molecular weights of these smaller complexes were estimated using a Sephacryl S-300 HR column that separates molecules in the range of 10–1500 kDa (data not shown). It is not clear whether the smear is due to partial dissociation of the complexes during preparation, or whether the two proteins are associated with other proteins, resulting in multimeric complexes of different composition and thus of different sizes.

We have obtained comparable results by separating the nuclear complexes on sucrose gradients (data not shown). After centrifugation, most of the *Pc* and *ph* is found in the

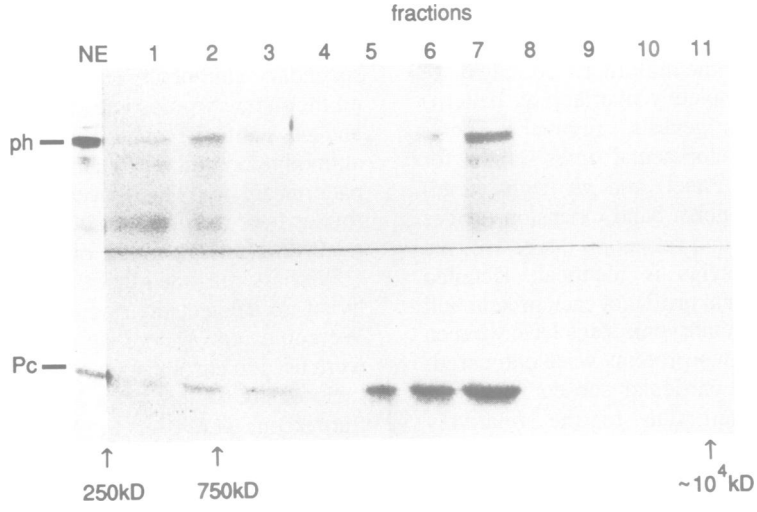

Fig. 5. Cofractionation of *Pc* and *ph* in multimeric protein complexes separated by gel filtration. Nuclear protein extracts of wild type embryos (200 μl, 15 mg/ml) was applied to a column of Sephacryl S-400 HR (90 cm long, 2 cm wide). Fractions were collected after the expected elution of catalase (240 kDa, see Materials and methods). 12 × 10 ml fractions were collected until the exclusion volume was reached. Fractions were concentrated and applied to a 7.5% SDS–PAGE gel. The electrophoretically separated proteins were transferred to nitrocellulose. The upper half of the filter was probed with anti-*ph* antibodies and the lower half with anti-*Pc* antibodies. The Western blot was developed using alkaline phosphatase coupled anti-rabbit antibodies as secondary antibodies. Lane NE, 5 μl (15 mg/ml) embryonic nuclear extract to indicate the expected size of *Pc* and *ph;* lanes 1–11, different fractions analysed. The column was equilibrated separately with marker proteins indicated at the bottom. 240 kDa, catalase; 750 kDa, mouse proteasome. The exclusion volume was determined by specifications given by the supplier (Pharmacia). Dextran blue 2000 (2×10^6 Da) could not be used as a size standard since it seemed to interact with the matrix, resulting in a broad elution peak.

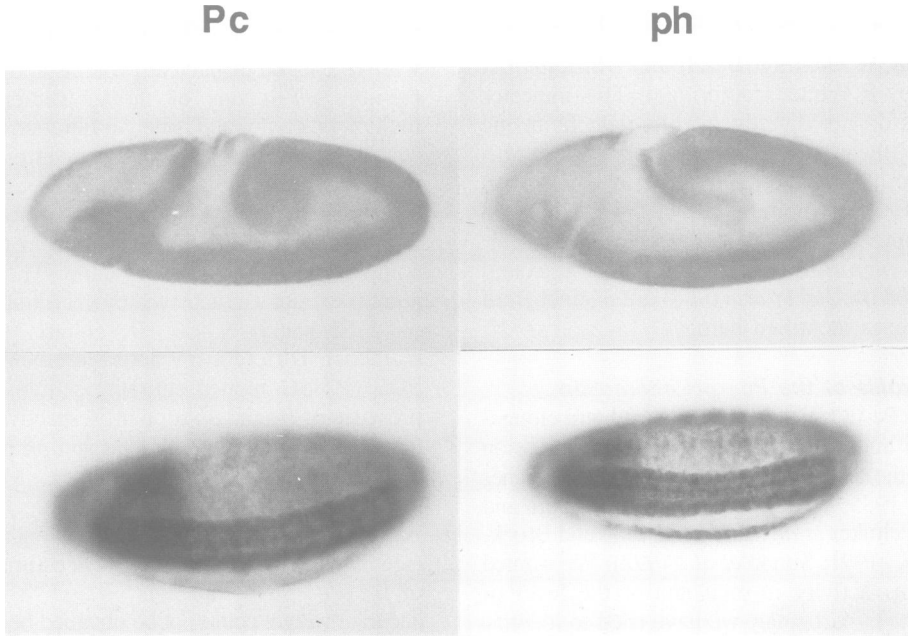

Fig. 6. Distribution of the *Pc* and *ph* protein in embryos. The spatial distribution of the two proteins were the same at all embryonic stages. As an example embryos at germ band elongation (top) and after germ band retraction at stage 16 are depicted. Note the intense labelling of the CNS in both latter cases.

pellet, suggesting that the weight of the complex exceeds the separation capacity of this method. The elution profiles of *Pc* and *ph* were the same using gel exclusion or centrifugation in sucrose gradients. These results suggest that independent of the techniques used, *Pc* and *ph* participate in the same high molecular weight complexes. We estimate that ~25% of all *Pc* protein found in the different fractions is localized in the large distinct complex seen in lanes 5–7 in Figure 5.

Pc and ph show the same distribution in embryogenesis and bind to the same target genes on polytene chromosomes

If *Pc* and *ph* associate in the same multimeric complex, we would expect equivalent, completely overlapping patterns of expression during embryogenesis. Figure 6 shows embryos at two different developmental times stained for the presence of the *Pc* (left Panel) and *ph* (right panel) proteins. At the stage of the germ band extension (stages 7–9), as well as after germ band retraction (stage 16), the distribution of the two proteins is identical. Detailed descriptions of the developmental profile of each protein will be presented elsewhere. At no embryonic stage have we seen a major difference when the two proteins were compared, although small differences in particular subsets of tissues might have gone undetected due to the relatively homogeneous distribution of the two proteins.

By analysing the distribution of *Pc* and *ph* on the polytene chromosomes of salivary glands, we could demonstrate that the biochemically identified *Pc–ph* complexes are also present *in vivo* on their site of action. DeCamillis *et al.* (1992) have presented evidence that *Pc* and *ph* have overlapping patterns on polytene chromosomes, but these comparisons were difficult to interpret because only rabbit polyclonal antibodies were available for each protein. Thus the cytological locations of *Pc* and *ph* had to be compared from different glands and different chromosomes, and our experience has shown that a comparison of so many binding sites, in the case of *Pc* and *ph* ~100, can be very difficult. Furthermore, we have found that different preparations can show different signal intensities due to the complexity of the fixation/immunostaining procedure. To avoid these problems we have isolated mouse polyclonal anti-*Pc* antibodies. These have allowed us to superimpose the binding patterns of *Pc* and *ph* on the same preparation by using double-immunofluorescence techniques. In Figure 7, the two binding patterns are compared. Panel A shows the distribution of the *Pc* protein visualized by using specific mouse anti-*Pc* antibodies and DTAF-conjugated anti-mouse antibodies as secondary antibodies. In panel B, *ph* protein is visualized on the same chromosome set by using the rabbit polyclonal anti-*ph* antibodies and rhodamine-conjugated anti-rabbit antibodies as secondary antibodies. It is clear that the two patterns are completely overlapping. We found no evidence for separate binding sites of *Pc* or *ph* in many preparations from different glands of different developmental stages.

We have excluded that the overlapping pattern is caused by an insufficient filtering of the two fluorescence signals. We could not observe signals when filters for rhodamine were used in chromosomes stained for *Pc* protein alone and reciprocally we could not observe a signal when filters for fluorescein were used in chromosomes stained for the *ph* protein alone (see Materials and methods for filter specifications). Additionally, we have compared the pattern of *Pc* with that of the nuclear protein *modulo* (Krejci *et al.*, 1989; antibodies kindly provided by J.Pradel, Marseille) in double-immunofluorescence staining. *Modulo* shows a distinct pattern from *Pc*. The two patterns could clearly be restrained to the individual fluorescence signal by using the appropriate filters (data not shown). Our results show that *Pc* and *ph* target the same set of genes on polytene chromosomes. Taken together, the signals visible on the chromosomes most

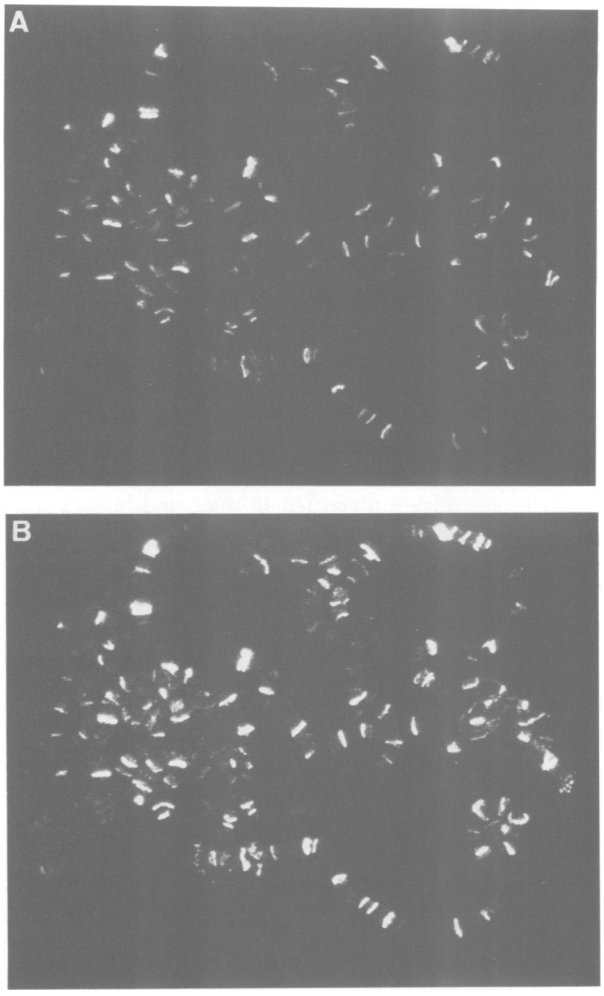

Fig. 7. Double-immunofluorescence of polytene chromosomes to visualize the distribution of the *Pc* and *ph* proteins. (**A**) Staining of chromosomes with polyclonal mouse anti-*Pc* antibodies and DTAF-conjugated anti-mouse antibodies. (**B**) Staining of chromosomes with polyclonal rabbit anti-*ph* antibodies and rhodamine-conjugated anti-rabbit antibodies. The distribution of the two proteins is identical. Chromosomes were photographed with an epifluorescence equipped Zeiss Axiophot using the appropriate filters (see Materials and methods).

probably represent the binding of the *Pc−ph* multimeric complexes to their respective target sequences.

Discussion

Pc-G genes were grouped together because of their similar homeotic phenotype. This suggests that they repress the homeotic genes using a common mechanism. Here we show that two members of the Pc-G, *Polycomb* and *polyhomeotic*, are constituents of a large multimeric nuclear protein complex. The soluble complex was stable enough to be purified by immunoprecipitation. Formally however, we have only proven that the *Pc* and *ph* proteins are part of the complex. The coprecipitation of 10−15 additional proteins depicted in Figure 1 is suggestive evidence that other proteins are potentially associated with these two Pc-G gene proteins. What could be the identity of these other proteins? Obviously other members of the Pc-G will be good candidates for interacting in the same molecular structure. Based on genetic grounds, Jürgens (1985) estimated that there should be around 30−40 different Pc-G genes. Most probably only a subset of them will directly take part in the multimeric complex. Others might be involved in modifying functions or in indirectly controlling the Pc-G/homeotic regulatory system. The complex might also contain proteins that fulfil a more general, structural function and thus may not be readily identified as Pc-G genes. Modifiers of PEV, which were also found to exhibit some homeotic phenotypes, could be such elements. The recently characterized protein BJ1 (Frasch, 1991), which shows homology to the vertebrate gene RCC1 (Regulator of Chromosome Condensation; Ohtsubo *et al.*, 1991), could be another constituent. Both BJ1 and the *Pc−ph* complexes are localized in the condensed bands in polytene chromosomes and have partially overlapping patterns. Most probably, the multimeric complex is structured by such basic building units that are used to compact many other chromatin domains. The addition of particular Pc-G gene products could subsequently render the complexes more specific for chromatin of homeotic genes or other developmental regulators.

Our data from the gel filtration and velocity gradient ultracentrifugation experiments suggest that a large portion of *Pc* and *ph* proteins are colocalized in a discrete complex of a molecular mass of $2-5 \times 10^6$ Da. However, some of these two proteins are also found in lower molecular weight complexes. Though this could be explained by a partial dissociation of the complex during the preparation, it could also indicate a much more heterogeneous composition of the multimeric units. Tartof and colleagues (Locke *et al.*, 1988; Tartof and Bremer, 1990) have suggested a combinatorial model for the formation of heterochromatin. They interpreted the dose sensitivity of the modifiers of PEV with the law of mass action. Multimeric protein complexes, sensitive to the dose of each individual component, could compact heterochromatin in a cooperative way. Each individual domain of heterochromatin would be composed of different combinations of proteins some of which may be commonly shared. Here we present for the first time molecular evidence that the Pc-G proteins could use similar multimeric complexes to perform their regulatory role.

Pc and ph regulate chromatin by being part of a protein complex

Genetic analysis suggests that some Pc-G genes can interact synergistically (Jürgens, 1985; Kennison and Russel, 1987). The finding that *Pc* and *ph* participate in a multimeric complex could explain some or all of this synergism. Compared with the individual wild-type protein distributions, we have found no difference of *ph* protein distribution in Pc^- embryos, or of *Pc* protein distribution in ph^- embryos (Franke, 1991; M.DeCamillis and H.W.Brock, unpublished results). This suggests that the synergistic interactions between these two proteins (Dura *et al.*, 1985; R.Campbell and H.W.Brock, unpublished) are not at the transcriptional level, but are more likely to be protein−protein interactions.

Pc-G repression of homeotic genes can be first detected at the stage of germ band elongation, even when the maternal Pc-G contribution is eliminated (Struhl and Akam, 1985; Jones and Gelbart, 1990). *Pc* and *ph* can be coprecipitated at all stages in embryogenesis, including 0−5 h post-fertilization, when the homeotic genes are not repressed in Pc-G mutant embryos. Thus, it appears that *Pc−ph* complex

formation is not sufficient for homeotic gene derepression. There could be several explanations for this finding. It may be that the complex is active early in development, but it does not bind to the homeotic genes in early embryogenesis, perhaps because the chromatin of the homeotic genes is inaccessible to the complex. Mutations in *Additional sex combs* permit ectopic expression of *even-skipped* at the blastoderm stage (Sinclair *et al.*, 1992), supporting the suggestion that a complex containing Pc-G genes could be active in early development. Alternatively, the complex may lack components or modifications that allow recognition of homeotic genes, which in turn implies that the complex could have different compositions or properties at different developmental stages.

Pc and ph have the same distribution during embryogenesis and a completely overlapping binding pattern on polytene chromosomes

DeCamillis *et al.* (1992) suggested that most *ph* binding sites on polytene chromosomes overlapped with *Pc* binding sites, but that ~10% of the sites appeared to differ. However, it is difficult to compare sites on separate polytene chromosome preparations that may differ in age, puffing stage, amount of squashing, or intensity of the immunostaining. Using double-label immunofluorescence, we now demonstrate that all of the target sites detectable using these reagents are the same for *Pc* and *ph*. We analysed glands of different ages and the ~100 sites always overlapped completely, making it unlikely that there are developmental differences of binding specificity of the two proteins in this tissue.

The completely overlapping pattern of *Pc* and *ph* binding to polytene chromosomes poses a paradox, because mutations in each gene have different phenotypes. Three explanations cannot be ruled out. First, minor polytene binding sites that have not been detected in our procedure might differ for each protein and account for the differences in phenotype. Secondly, binding of *Pc* and *ph* to polytene chromosomes might differ from binding to chromosomes of diploid tissues or in other polytene tissues. Thirdly, complexes containing *Pc* and *ph* may bind in the same regions at cytological resolution, but have different binding sites at the DNA level. Notwithstanding the arguments above, the simplest interpretation of our results is that binding to a particular site on polytene chromosomes might not necessarily mean that the complex is functional. It may be that the *Pc–ph* complexes are always associated with all their potential target genes and that additional components or modifications of the complex determine it gene-specific activity. Constitutive binding is a commonly used mechanism for gene regulation. The recruitment of particular accessory proteins or a specific modification of the regulatory structure seems to be necessary to invoke the transcriptional activity. Examples for this are the c-*fos* induction by the serum response factor (Herrera *et al.*, 1989; Dalton and Treisman, 1992) in mammalian cells or the phosphorylation of the heat shock factor in yeast (Sorger and Pelham, 1988). In this view, *Pc* or *ph* mutations have different phenotypes because complexes lacking each protein are differentially susceptible to modification or activation.

We show that two Pc-G genes are constituents of a large multimeric protein complex. Additional experiments will be needed to determine what defines the binding site of the complex. Zink *et al.* (1991) and DeCamillis *et al.* (1992) have shown that binding of *Pc* and *ph* to polytene chromosomes is DNA sequence-dependent. However, neither *Pc* nor *ph* protein appears to bind DNA *in vitro* (M.Oed and R.Paro, N.Cheng and H.W.Brock, unpublished results). One candidate for the DNA binding is the *Posterior sex combs* (*Psc*) protein which contains presumptive DNA binding motifs (Brunk *et al.*, 1991; van Lohuizen *et al.*, 1991a). Another question is whether the multimeric complex binds a limited number of sites at target genes and acts to regulate the formation of chromatin without being a structural component itself, or whether the complex is a constituent of chromatin that is itself directly required to alter chromatin structure through cooperative effects over long chromosomal distances.

Potential homologues of Pc-G genes have been found in mammals. Using the *Pc* chromo domain as a probe, similar proteins have been identified in mice and humans (Singh *et al.*, 1991; Pearce *et al.*, 1992). Similarly, the *Psc* protein is highly conserved. A related murine protein, *bmi-1* was found to increase the incidence of B-cell lymphomagenesis when overexpressed together with the *myc* protein (van Lohuizen *et al.*, 1991b). It will also be interesting to see whether vertebrates use similar multimeric complexes to stably repress developmental regulators by regulating higher order chromatin structure.

Materials and methods

Preparation of nuclear extracts

Nuclear proteins were prepared by the method of Gay *et al.* (1988), with some minor modifications. All steps were performed at 4°C. As starting material, 3–6 g dechorionated wild type embryos from an overnight egg lay were suspended in buffer B (15 mM HEPES pH 7.6, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 1 mM DTT, 2 μg/ml Leupeptin, 2 μg/ml Pepstatin and 2.5 μg/ml Aprotinin; 4 ml/g embryos). Embryos were homogenized with 10–15 strokes in a Kontes glass homogenizer using a type A pestle. The homogenate was pre-cleared by a passage through two layers of Miracloth (Calbiochem) and subsequently centrifuged at 2000 *g* for 10 min. The pellet was resuspended in buffer B (2 ml/g embryos). The solution was overlayed on a cushion of the same volume of buffer B plus 0.8 M sucrose in a centrifuge tube and spun in a swing-out rotor at 1000 *g* for 10 min. The nuclear pellet was resuspended in buffer B–150 mM KCl (0.7 ml/g embryos). Nuclei were lysed by the addition of 4M $(NH_4)_2SO_4$ to a final concentration of 0.4 M and further incubation for 15 min on ice. The lysate was cleared by centrifugation of 120 000 *g* for 1 h. The soluble nuclear proteins in the supernatant were precipitated by slow addition of $(NH_4)_2SO_4$ to a final concentration of 30% (w/v), centrifuged at 30 000 *g* for 15 min and carefully resuspended in buffer C (25 mM HEPES pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 2 μg/ml Leupeptin, 2 μg/ml Pepstatin, 2.5 μg/ml Aprotinin and 10% glycerol; 100 μl/g embryos). 50–100 μl aliquots were frozen in liquid nitrogen and stored for later use at −80°C. Protein concentration was measured by the Bradford method (Bradford, 1976).

Immunoprecipitation and radioactive labelling of nuclear proteins

Protein A–Sepharose CL-4B (PAS: Pharmacia LKB) was equilibrated in buffer CBX (buffer C plus 10 mg/ml BSA and 0.1% Triton X-100) for 30 min. Buffer CBX was added to give a 50% Protein A–Sepharose suspension. Embryonic nuclear extract (~25–50 μl) was mixed with 16 vol of buffer CBX. An appropriate amount of antiserum was added to the mixture and incubated by slow rotation for 30 min at 4°C. The optimal amount of antiserum needs to be determined for each antibody individually. Two volumes of the Protein A–Sepharose suspension were added and the incubation continued for 30 min at 4°C. The complexes were precipitated at 2000 *g* for 1 min in an Eppendorf centrifuge. The pellet was washed five times for 1 min in buffer C plus 0.1% Triton X-100 and 0.05% SDS and three times in buffer C only. Between each wash the complexes were precipitated as above. After the last wash the pellet was either resuspended

in 3–4 vol of SDS–PAGE loading buffer for direct analysis or resuspended in 100 μl of 0.1 M Na–borate buffer pH 8.5 for radioactive labelling of the proteins.

Immunoprecipitated proteins were labelled using the ^{35}SLR (Amersham International). 50 μCi ^{35}SLR (1 mCi/ml, specific activity >800 Ci/mmol) were dried in a speed vac. The resuspended immunoprecipitate was added, mixed and incubated for 30 min on ice. After the reaction unincorporated ^{35}SLR was neutralized by the addition of an equal volume of 0.2 M glycine in Na–borate buffer for 5 min. SDS and β-mercaptoethanol were added to a final concentration of 2 and 5%, respectively and the protein–Sepharose complex dissociated for 5 min at 60°C. Labelled proteins were separated from the ^{35}SLR–glycine by gel filtration over a small column (Bio-Gel P 10; Biorad). The pooled protein fractions were concentrated by precipitation with 2 vol of ethanol for 30 min on ice and a centrifugation for 15 min at 30 000 *g*. The protein pellet was resuspended in SDS–PAGE loading buffer.

Gel filtration chromatography and sucrose gradient ultracentrifugation

For gel filtration we used two media with different separation capacities. Sephacryl S-300 HR (Pharmacia) separates molecules in the range between 10–1500 kDa, Sephacryl S-400 HR in the range between 20–1 × 10^4 kDa. The material was suspended in running buffer (25 mM HEPES pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 10% glycerol) to reach a final ratio of 2/3 Sephacryl and 1/3 buffer. The suspension was loaded into a glass column that was 90 cm long and 2 cm wide. The column was calibrated separately using proteins of known molecular weight. For the Sephacryl S-300 HR column: cytochrome C (13.3 kDa, Boehringer Mannheim), BSA (66 kDa, Merck), catalase (240 kDa, Boehringer Mannheim) and for the Sephacryl 400-HR; catalase (240 kDa, Boehringer Mannheim) and mouse proteasome (750 kDa, Rivett,A.J., 1985, kindly provided by M.Gernold and P.Kloetzel). The exclusion volume of the Sephacryl 300-HR column was determined with Blue dextran 2000 (Pharmacia). 200 μl of embryonic nuclear extracts were mixed with 1.5 ml running buffer and loaded on the column. 5 ml fractions were collected. 2 μg of BSA were added as carrier and the fractions mixed with an equal volume of 20% TCA–50% acetone. The proteins were precipitated by incubation on ice for 30 min, centrifuged at 10 000 r.p.m. for 10 min and resuspended in SDS–PAGE loading buffer. The samples were neutralized by the addition of Na_2CO_3 crystals and stored at −20°C. For the sucrose gradient centrifugation a SW 60 Ti rotor (Beckman Instruments) was used. A 5–20% sucrose gradient in buffer C (25 mM HEPES pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 2 μg/ml Leupeptin, 2 μg/ml Pepstatin and 2.5 μg/ml Aprotinin) was prepared in a 4 ml centrifuge tube. 40 μl of embryonic nuclear extracts were mixed with 160 μl buffer C and loaded on top of the gradient. Centrifugation was at 50 000 r.p.m. for 12 h at 4°C. 20 × 200 μl fractions were collected with an ISCO Model 640. Proteins were precipitated with 2 vol ethanol and resuspended in SDS–PAGE loading buffer. The gradient was calibrated in separate tubes using different enzymes of known molecular weight (all obtained from Boehringer Mannheim): horse radish peroxidase (44 kDa, 1 mg/gradient), lactate dehydrogenase (140 kDa, 0.5 mg/gradient), fumarase (194 kDa, 0.2 mg/gradient), catalase (240 kDa, 0.4 mg/gradient). The localization of the proteins in the different fractions was determined by the corresponding enzyme reaction.

Immunolocalization of proteins on embryos and on polytene chromosomes spreads

Immunostaining of embryos was based on the procedure described by MacDonald and Struhl (1986). Fixation and spreading of the chromosomes essentially followed the protocol of Zink and Paro (1989) and Zink *et al.* (1991). The chromosome immunostaining protocol was modified slightly in order to reduce background staining. After washing slides in PBS, they were transferred to blocking solution (10% BSA, 10% dry milk, 0.2% NP-40 and 0.2% Tween 20-80 in PBS) and incubated for 1 h at 37°C. Although the blocking solution was very cloudy, it did not appear to interfere with the immunostaining reactions. Slides were briefly washed in PBS and then the chromosomes were incubated with the primary antibody at the appropriate concentration in blocking solution for 1 h at 37°C. We used the purified mouse polyclonal anti-*Pc* and rabbit polyclonal anti-*ph* antibodies at a 1:100 dilution. Slides were briefly washed in PBS and then vigorously shaken for 15 min in 300 mM NaCl, 0.2% NP-40 and 0.2% Tween 20-80 in PBS, and for additional 15 min in 400 mM NaCl, 0.2% NP-40 and 0.2% Tween 20-80 in PBS. Slides were again briefly washed in PBS and blocked in blocking solution as above. The incubation with the secondary antibody was for 1 h at 37°C in blocking solution. For the *Pc* protein, we used DTAF-conjugated anti-mouse antibodies (Jackson) diluted 1:100 and for the *ph* protein, rhodamine-conjugated anti-rabbit-antibodies (Jackson) diluted 1:40. All steps involving fluorochromes were performed under reduced light. Slides were washed as after the incubation with the primary antibody. The preparations were washed in PBS and embedded in Moviol–2.5% DABCO (1,4-diazobicyclo-(2,2,2)-octane). Chromosomes were photographed with an epifluorescence equipped Zeiss Axiophot using the appropriate filters (rhodamine, filter number 48 79 15; DTAF, filter number 48 79 09) using Kodak T-Max 400.

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