Function of the Polycomb protein is conserved in mice and flies

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SUMMARY

A key aspect of determination — the acquisition and propagation of cell fates — is the initiation of patterns of selector gene expression and their maintenance in groups of cells as they divide and develop. In *Drosophila*, in those groups of cells where particular selector genes must remain inactive, it is the *Polycomb-Group* of genes that keep them silenced. Here we show that M33, a mouse homologue of the *Drosophila* Polycomb protein, can substitute for Polycomb in transgenic flies. Polycomb protein is thought to join with other Polycomb-Group proteins to build a complex that silences selector genes. Since members of this group of proteins have their homologues in mice, our results suggest that the molecular mechanism of cell determination is widely conserved.

Key words: Polycomb, M33, determination, *Drosophila*, mouse

INTRODUCTION

The design of animals depends on a special class of homeotic selector genes (Lewis, 1963, 1978; Garcia-Bellido, 1975; Kaufman et al., 1980). Even between creatures as different as mice and flies, these genes are conserved in their arrangement on the chromosome, in their homeobox sequences, in some aspects of function and in their order of deployment from head to tail (e.g. Duboule and Dollé, 1989; Graham et al., 1989; McGinnis and Krumlauf, 1992). Early in development, a unique set of selector genes becomes activated in each group of primordial cells, the particular combination of active genes in a group determining which developmental pathway it will follow (Hadorn, 1965; Garcia-Bellido et al., 1979). The descendants of each primordial cell then maintain their determined state by keeping the same set of selector genes continuously active throughout development (Morata and Garcia-Bellido, 1976; Morata and Lawrence, 1977). Since ectopic expression of selector gene products, even at a late developmental stage, can change the determined state of a cell (e.g. Lewis, 1963; Schneuwly et al., 1987), it is equally important to keep selector genes stably inactive where their products are not wanted. This is achieved by repression: selector genes contain enhancers that are potentially active in many primordia, but whose activity is blocked or "silenced" in some (Bieberich et al., 1990; Müller and Bienz, 1991; Püschel et al., 1991). In *Drosophila*, heritable silencing of homeotic genes is achieved by a two-step process (Garcia-Bellido and Capdevila, 1978). The first step, initiation, provides the spatial specificity: gap gene products that are localised in the early embryo repress homeotic genes in sets of primordia (White and Lehmann, 1986; Harding and Levine, 1988; Irish et al., 1989; Qian et al., 1991; Zhang et al., 1991; Shimell et al., 1994). The second step, maintenance, keeps the homeotic genes silenced even though the gap proteins have gone and even though the cells divide. This heritable silencing depends on a set of more than 10 genes (Lewis, 1978; Struhl, 1981; Jürgens, 1985), called the *Polycomb-group* (*Pc-G*), which includes the *Polycomb* (*Pc*) gene itself. The *Pc-G* gene products are necessary for the proper regulation of homeotic genes in many parts of the body and are almost uniformly distributed in the embryo (Paro and Hogness, 1991; Franke et al., 1992; Martin and Adler, 1993); they therefore do not seem to carry any intrinsic positional information. The *Pc-G* proteins maintain cell determination, they function as a molecular memory.

Contemporary studies on *bmi-1*, a murine homologue of the *Pc-G* gene *Posterior sex combs* (*Psc*), provide evidence that it acts as a regulator of Hox gene expression in mice (van der Lugt et al., 1994; Alkema et al., 1995). Here we show that the M33 protein, a mouse homologue of Polycomb (Pearce et al., 1992, Paro and Hogness, 1991), can substitute functionally for the Pc protein in transformed flies. These two complementary lines of evidence suggest that at least the maintenance mechanism of cell determination has been widely conserved.

MATERIALS AND METHODS

Drosophila strains

A *cn;ry42* strain was used as host for transformation.

The following mutant alleles were used to test for rescue activity of the transgenes: *Pc23937.4A*, *Pc26865.4*, *PcXT109* are three alleles that lack antigen for the Pc protein (R. Paro, personal communication). For some experiments, we used *Pc3*, an allele with an antimorphic effect (Duncan and Lewis, 1982) which we chose to minimise any rescue of the larval phenotype due to *Pc+* product given by the mother (Denell, 1982; Lawrence et al., 1983). In some experiments, an *Abd-BM1* allele was used for marking purposes.
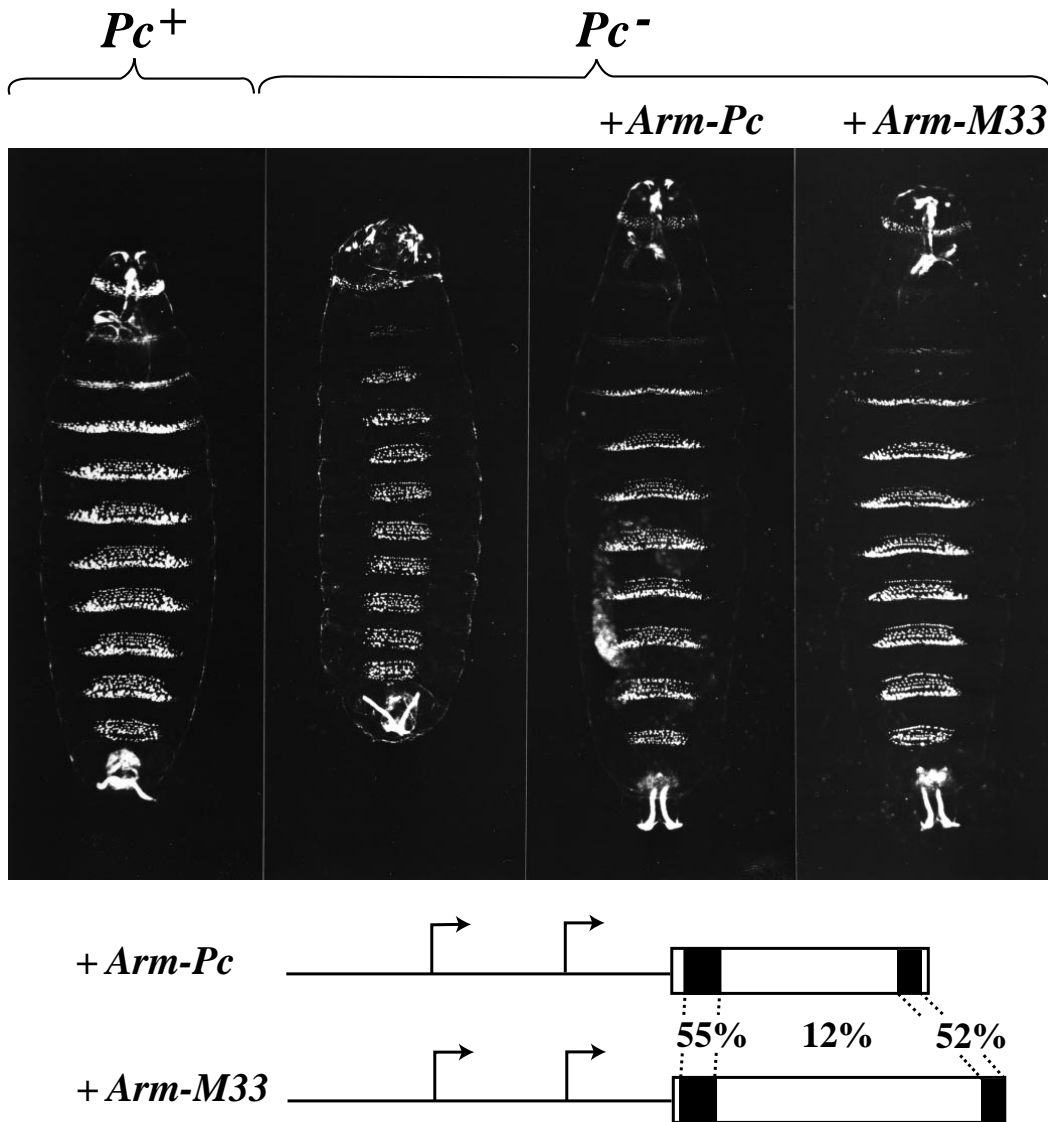
Fig. 1. Ventral views of first stage larval cuticles. Dark field. The wild-type larva (Pc^+) shows a sequence of 3 thoracic and 8 abdominal segments. In the Pc^- larva, the segments are transformed and mostly resemble the wild-type abdominal segment A8, they show severe head defects. In the presence of either *Arm-Pc* or *Arm-M33*, the wild-type phenotype is restored. Note the rescued larvae come from Pc^{XT109}/*TM2* stocks that also carry one or two copies of the transgenes. Each larva falls into one of three distinct classes consisting of either apparent wild types, or *TM2* homozygotes or *Pc* mutants; the proportions of the larvae in these classes is consistent with a complete rescue of the Pc^- cuticle phenotypes by either *Arm-Pc* or *Arm-M33*. Below, scale diagrams of the rescue constructs (thin line, *armadillo* promoter; boxes, fly Polycomb and mouse M33 coding regions). Black boxes show the conserved chromo- and C-terminal domains; a comparison of amino acid sequence gave the identities shown in percentages.

Plasmid constructions and transformants

The *Arm-M33* and *Arm-Pc* transgenes were assembled in bluescript from the following starting plasmids: Arm bs, containing a 1.8 kb fragment of the *armadillo* promoter (Vincent et al., 1994); M33bs, a subclone containing the *M33* coding region (Pearce et al., 1992); hb-GAL-Pc bs, a subclone that contains the *Pc* coding region, a fragment with the *hb* translation initiation codon and 5′ untranslated leader sequences (Müller, 1995). Convenient restriction sites were engineered by site-directed mutagenesis to link the different fragments isolated from these plasmids and obtain Arm-M33 bs [*Kpn*I-*Pvu*II-(*arm* promoter nucleotides 11-1792)-*Bam*HI-*ACTAGA*-(*hb* 5′ untranslated leader nucleotides 4675-4683)-*ATG*AAGCTT-(*M33* codons 2-519)-*TGAGACCAT*-*Sal*I] and Arm-Pc bs [*Kpn*I-*Pvu*II-(*arm* promoter nucleotides 11-1792)-*Bam*HI-*ACTAGA*-(*hb* 5′ untranslated leader nucleotides 4675-4683)-*ATG*AAGCTT-(*Pc* codons 3-390)-*TGAGC*-*Sal*I]. Each transgene was then subcloned as a *Kpn*I-*Sal*I fragment into the backbone of a modified transformation vector '*pry*' to obtain ARM-M33 pry and ARM-Pc pry, respectively; '*pry*' contains RNA trailer sequences from the *Drosophila hsp70* gene downstream of the *Sal*I site (Müller and Bienz, 1991). Detailed maps of the plasmids used are available on request.

Expression levels of the transgenes

Three (*Arm-Pc*) and three (*Arm-M33*) independent lines of transformed flies were examined and gave almost identical results.

All the three *Arm-Pc* lines that we used are viable when homozygous, but the homozygotes frequently show defects in A4 of adults (ventrally only in most cases). First instar larvae show a hierarchical series of defects. (1) A gap in the midline in the region of ps 9 of the CNS of developing embryos and in the cuticle where the A4 denticle band is split and displaced laterally. (2) Complete lack of A4. (3) Loss of A4 plus reduction of A2 and A8. Since these effects are not observed in zygotes carrying only one paternally derived copy of the transgene, we wondered if they might be a consequence of very high levels of Pc protein donated by the mother to young embryos (staining of *Arm-lacZ* constructs suggests that the *armadillo* promoter is very active during oogenesis) and whether an abnormally high level could affect the expression of genes that are not otherwise known to be controlled by Pc. However, the *armadillo* promoter is not always so active: our rescue data of adults suggest the *armadillo* promoter functions less well than the endogenous *Pc* promoter (see below).

Note that larvae and adults carrying two copies of the *Arm-Pc* transgene plus two copies of the endogenous Pc^+ gene do not show

Pc^+B^- Pc^-B^-

$+Arm\text{-}Pc$ $+Arm\text{-}M33$

◄A9
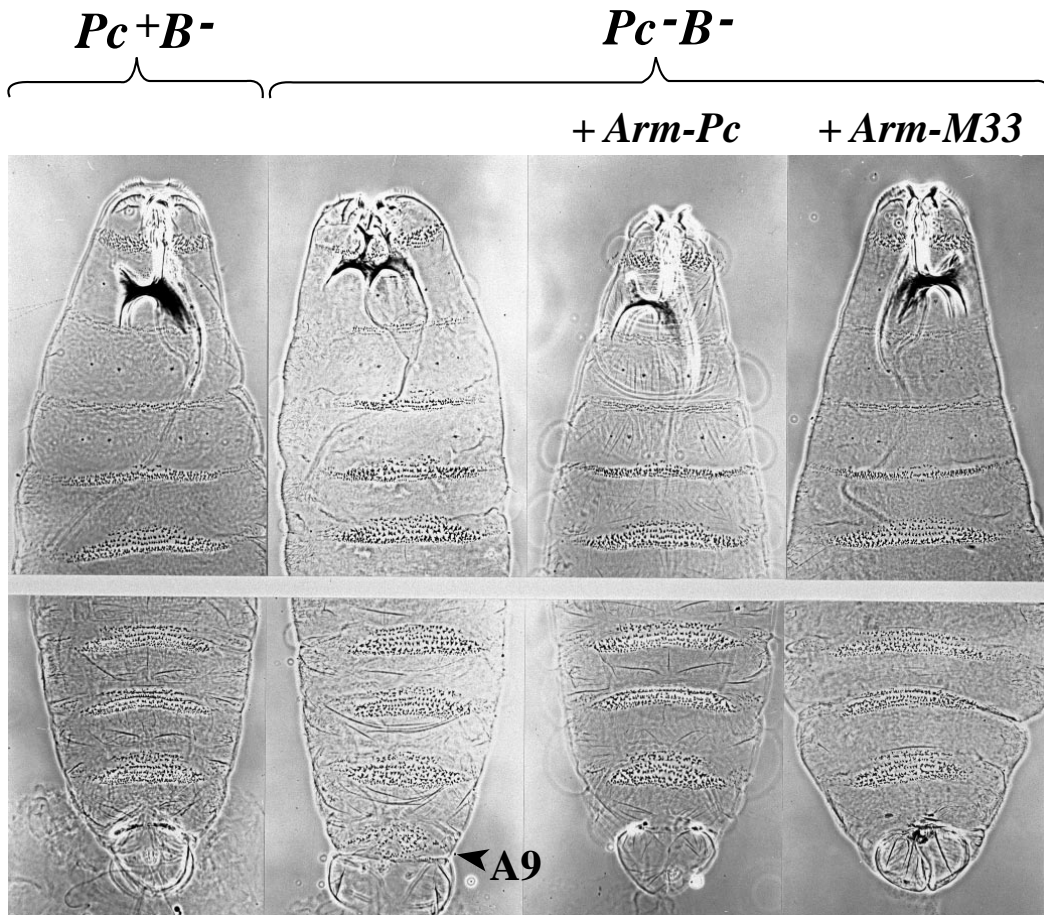
Fig. 2. Ventral views of anterior (above) and posterior (below) portions of larval cuticles. Phase contrast. The purpose of this figure is to compare the action of the transgenes on embryos that are unequivocally marked. All these larvae show the effects of the $Abd\text{-}B^{M1}$ marker mutation. The leftmost larva ($Pc^+ B^-$) carries no other mutations, all the other larvae are homozygous for a $Abd\text{-}B^{M1} Pc^3$ chromosome ($Pc^- B^-$). These Pc^- larvae are either unrescued, or carry one dose of a paternally derived transgene as indicated. In the absence of Pc (Pc^-B^-) the head is defective, a new denticle band appears at A9 (arrowhead) and the larvae fail to hatch (for details see Casanova et al., 1986). As shown, one paternal dose of either of the two transgenes ($Arm\text{-}Pc$ or $Arm\text{-}M33$) is sufficient to correct all those aspects of the phenotype due to Pc^- (compare the rescued larvae with the $Pc^- B^-$ and $Pc^+ B^-$ larvae) and the rescued larvae hatch.

any homeotic transformations. Note also that in the three *Arm-M33* lines, homozygous larvae and flies show no defects.

Rescue tests

We used different *trans*-combinations ($Pc^{26865.4}/Pc^{XT109}$; Pc^{XT109}/Pc^{XT109}; $Pc^{23937.4A}/Pc^{XT109}$; $Pc^{26865.4}/Pc^{26865.4}$) and either one or two copies of the transgene to test for adult rescue and all gave survivors with *Arm-Pc* but not with *Arm-M33*. The survivors fell into two classes: flies carrying two doses of the transgene were either wild type in phenotype or showed slight defects in the wings but had no other *Pc* phenotype; flies carrying one dose showed sexcombs on all three legs, antennae partially transformed to legs, abnormal wings, abdominal cuticle and bristles showing slight transformation towards analia, that is fewer cell hairs and longer finer bristles. These flies are sick and sterile. These results suggest that, in later development, the *armadillo* promoter is somewhat less active than the wild-type *Pc* promoter, which in one dose gives more *Pc* function (although the flies are still not wild type).

To analyse *Ubx* expression in putatively rescued embryos, a cross was made in which the second chromosome was heterozygous for the transgene and the third chromosome carried $Abd\text{-}B^{M1} Pc^3/TM2$. As expected one quarter of the embryos were $Abd\text{-}B^{M1} Pc^3/Abd\text{-}B^{M1} Pc^3$ and of these only one quarter showed the complete Pc^- phenotype, as would be predicted if one dose of either transgene substantially rescued the Pc^- phenotype. The same result was obtained in a similar cross using the Pc^{XT109} allele; as the chromosome in this case was not marked, we counted all the embryos and found that approximately 1 in 16 showed the complete Pc^- phenotype.

Antibody stainings and cuticle preparations

Embryos were fixed and stained with a monoclonal antibody against

Ubx protein (White and Wilcox, 1984), as previously described (Lawrence and Johnston, 1989). Preparations of larval and adult cuticles were by standard methods (Roberts, 1986).

Clonal analysis

To make clones in the adult abdomen, *y; Arm-Pc/+; Dp(1;3)sc^{J4}, y$^+$ M(3)67C/Pc^{XT109}* **or** *y; Arm-M33/+; Dp(1;3)sc^{J4}, y$^+$ M(3)67C/Pc^{XT109}* males were generated and irradiated as larvae to induce somatic recombination (1000 R of X rays). Clones from these and their *Sternopleura (Sp)* control siblings (*y; Sp/+; Dp(1;3)sc^{J4}, y$^+$ M(3)67C/Pc^{XT109}* males) were compared. Because of the positions of the *yellow* marker gene and the *Pc* mutation on the third chromosome, only about half of the *yellow* clones are Pc^-/Pc^-, the other half are Pc^-/Pc^+ (Busturia and Morata, 1988) and due to recombination distal to the *Pc* locus. In those flies carrying no transgene, 37% of the abdominal clones (n=38) showed a complete *Pc* mutant phenotype. In flies carrying one copy of *Arm-Pc* all of the abdominal clones (n=27) were wild type in phenotype. In flies carrying one copy of *Arm-M33*, 51% of the clones (n=47) were rescued to an intermediate phenotype with both wild type and Pc^- character but some gave an apparent Pc^- phenotype. As expected, the remaining half of the clones (49%) were wild type.

RESULTS AND DISCUSSION

The mouse *M33* gene (Pearce et al., 1992) was isolated by its homology to the *Pc* chromobox (Paro and Hogness, 1991), a sequence motif of about 40 amino acids also found in a variety of other genes (James and Elgin, 1986; Delmas et al., 1993; Tschiersch et al., 1994). The chromodomain of Pc is thought to

Pc^+B^+ Pc^-B^-
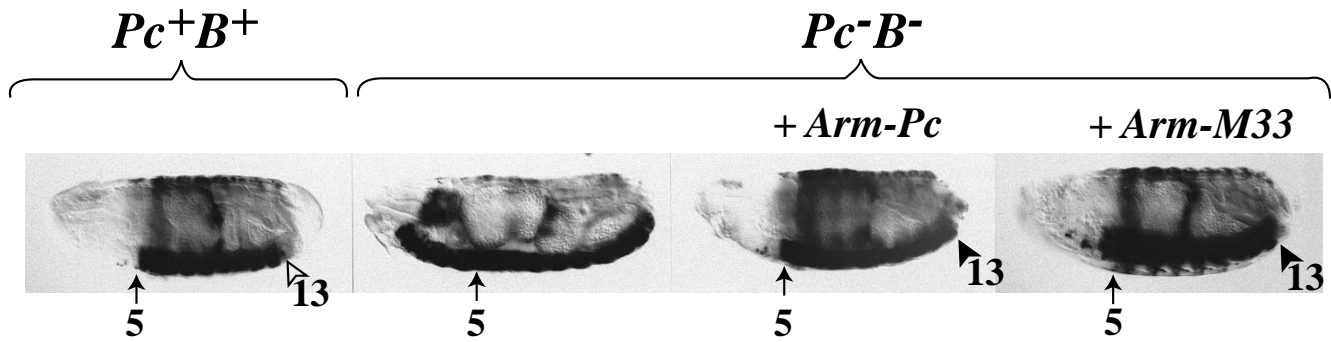
+ Arm-Pc   + Arm-M33

Fig. 3. Function of Arm-M33 and Arm-Pc transgenes in *Pc*⁻ mutant embryos. Lateral views of 14-16 hours old embryos stained with an antibody against Ubx protein, the product of one of the *Drosophila* homeotic genes. We use the same marking method as in Fig. 2, the *Abd-B^{M1}* phenotype being shown by the ectopic expression of *Ubx* in parasegment 13 (Struhl and White, 1985), as indicated. The leftmost embryo is wild type (*Pc^+B^+*), the others are mutant for *Pc* as well as for *Abd-B* (*Pc^-B^-*) and carry either the *Arm-Pc* or *Arm-M33* transgene as indicated. In wild-type embryos (*Pc^+B^+*), *Ubx* is strongly expressed in four midline cells in parasegment (ps) 4 and then from ps 5 (anterior margin marked with closed arrow) to ps 12; only a few cells express in ps 13 (open arrowhead, cf Struhl and White, 1985). *Pc* mutant embryos show *Ubx* expression from head to tail (ps 1 to ps 14). Note the strong staining anterior to ps 5. In *Pc*⁻ embryos carrying one or two copies of *Arm-Pc*, the pattern of *Ubx* expression is restored to the normal. This embryo is identified as a *Abd-B^{M1} Pc^3* homozygote by the strong expression in ps13 (closed arrowhead, compare with the *Pc^+ B^+* embryo) which is due to the absence of *Abd-B* function (Struhl and White, 1985). *Arm-M33* can also rescue the epidermis of *Pc*⁻embryos completely, giving a wild-type pattern of staining for Ubx; however, the CNS shows some ectopic expression. The embryo illustrated has only a few stained cells anterior to ps 5.

be responsible for recognising specific targets on the chromosome (Messmer et al., 1992), probably via one or more other proteins — the Pc protein does not seem to bind to DNA on its own (Franke et al., 1992). In addition to the chromodomain, M33 contains a C-terminal stretch of about 30 aminoacids that is well conserved in Pc but identified so far in no other proteins (Pearce et al., 1992); however, over the remaining 85% of their sequence the two proteins share no significant homology (Fig. 1). Both the chromodomain and the C-terminal domain are essential for *Pc* function in flies (Messmer et al., 1992; Franke et al., 1995; Müller, 1995); the conservation of these two regions between the *Pc* and *M33* genes suggests that the two proteins might be functionally homologous.

Here we ask whether the M33 protein functions in flies by testing its ability to substitute for the fly Pc protein. As the promoter of the fly gene is ubiquitously active but has not been defined, we used the *armadillo* promoter which functions in the ovary, the embryo and imaginal discs (Vincent et al., 1994). The staining of *Arm-LacZ* (Vincent et al., 1994) and our own results suggest that this promoter is stronger than the Pc promoter in oogenesis but weaker in the imaginal discs (see Materials and Methods). Using a construct in which the *armadillo* promoter directs expression of the fly Pc protein (*Arm-Pc*), we find that two doses can rescue *Pc*⁻ mutants and give flies that are wild type in appearance. We then turned to the mouse Polycomb protein, and asked whether, in transformed flies, the *Arm-M33* transgene would also be able to rescue *Pc*⁻ mutants.

First, we looked at the cuticle pattern of larvae. In the wild type, each metamere bears a characteristic belt of denticles, the pattern being specified by the combination of homeotic genes that are active at each locale (Figs 1, 2). In *Pc* mutants, silencing fails and all homeotic genes tend to become universally expressed. As a consequence, and because of a functional hierarchy amongst homeotic proteins (Lewis, 1978; Struhl, 1981; Struhl and White, 1985; González-Reyes and Morata, 1990), all denticle belts become transformed towards that of

the eighth abdominal segment (Fig.1; see legends for details). Both the control *Arm-Pc* and the experimental *Arm-M33* transgenes rescue the *Pc*⁻ cuticle phenotype completely (Figs 1, 2) and the rescued larvae hatch. However, unlike the situation with *Arm-Pc*, we saw no rescue to the adult by *Arm-M33*.

Next, we studied expression of a homeotic gene in embryos (Fig. 3). In *Pc* mutant embryos carrying either the *Arm-Pc* or the *Arm-M33* transgenes, homeotic gene expression is restricted – that is, silenced – until the end of embryogenesis (Fig. 3). No ectopic expression is seen in the epidermis or mesoderm, but, in the central nervous system of mutant embryos carrying *Arm-M33*, a few cells begin to show ectopic expression at late stages (Fig. 3). Thus it appears that the M33 protein can substitute for fly Pc although it is somewhat less efficient.

The function of *Pc* is necessary throughout development (Struhl, 1981; Duncan and Lewis, 1982; Busturia and Morata, 1988) and we therefore asked if the transgenes can rescue clones of *Pc*⁻ adult cells. We found that one dose of the *Arm-Pc* transgene rescues *Pc*⁻ cells and gave clones with a completely wild-type phenotype, while one dose of *Arm-M33* also rescues but gave clones with an intermediate phenotype (Fig. 4). Thus the M33 protein can emulate Pc both early and late in development.

The fly Pc protein has two functional domains: the chromodomain and the C-terminal region. The chromodomain is found in at least one other protein that binds to chromosomes with a characteristic distribution. Whereas the Pc protein can be detected at about 100 sites on the polytene chromosomes (Zink and Paro, 1989), the HP1 chromodomain protein is mainly located in the centromeric heterochromatin (James and Elgin, 1986). Pc protein with a defective chromodomain can still work if artificially tethered to the DNA (Müller, 1995) — suggesting that the normal function of the chromodomain is to bind to a chromosomal target. This binding is presumably via *Polycomb-group* proteins, which themselves recognise particular base sequences of DNA (Chan et al., 1994; Christen and Bienz, 1994; Müller, 1995). The other functional part of the
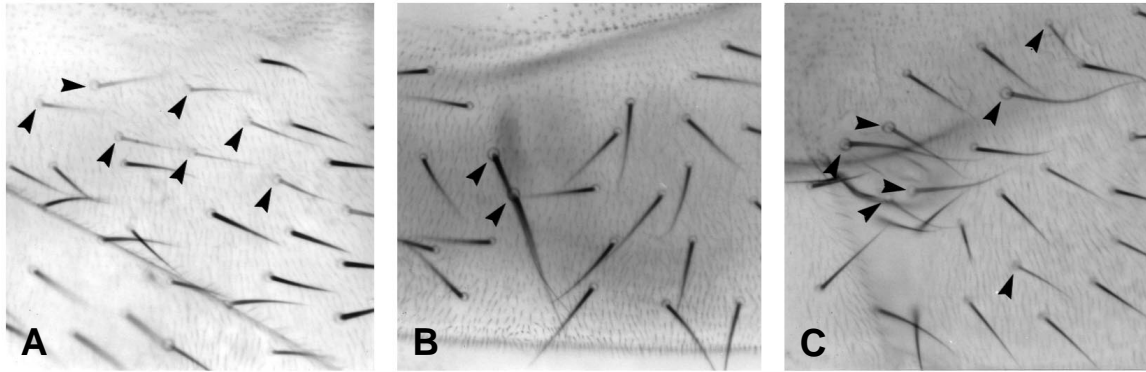
Fig. 4. Rescue of Pc⁻ in adult cells. The pictures show portions of the first abdominal segment of adults. Shown are clones of *Pc⁻/Pc⁻* cells induced in *Pc⁻/Pc⁺* individuals; *Pc* mutant cells are genetically marked with *yellow*. Flies carry *Arm-Pc* (A), no transgene (B) or *Arm-M33* (C). (B) In the absence of a transgene, the clones are completely transformed and resemble structures found more posteriorly (Busturia and Morata, 1988), note that the mutant bristles (arrowheads) are much longer and finer than their wild-type neighbours. (A) In the presence of *Arm-Pc* all clones are wild type in phenotype; note the wild-type shape and length of the rescued bristles (arrowheads). (C) In the presence of *Arm-M33*, the clones are rescued to an intermediate phenotype; note that all bristles in the clone are shorter than unrescued *Pc⁻/Pc⁻* bristles found in B and, in some cases, have nearly wild-type length and shape.

protein, the C-terminal domain, most likely acts to recruit further members of the *Polycomb-group* of proteins to form a silencing complex (Müller, 1995, Franke et al., 1995). Note that complexes containing the Pc protein have been isolated from *Drosophila* embryos and contain at least one other *Polycomb-group* protein (Franke et al., 1992).

Our finding that the M33 protein, having these two domains but no other homology with Pc, can rescue *Pc* mutants makes the following points: First, it is striking that even though the two conserved domains constitute a small fraction of the protein, the mouse protein is effective in flies. Second, if the fly Pc chromodomain does recognise proteins on the DNA, it would follow that the mouse homologue can bind to these fly proteins, suggesting that they have been conserved between mice and flies. Third, if the C-terminal domain is indeed there to recruit one or more proteins to form a multimeric complex, then the proteins that are recruited may also be conserved. This universalist view has other support: mice lacking the *bmi-1* gene, a homologue of the *Drosophila Polycomb-group* gene *Posterior sex combs*, show "homeotic" transformations along the whole body axis (van der Lugt, 1994). As in *Drosophila*, these transformations change patterns characteristic of anterior segments towards those characteristic of more posterior ones.

Determination results in "a reproducible cell state propagated ...... by cell heredity" (Hadorn, 1965). This requires the transmission of the silenced state of homeotic selector genes through many cell divisions. In flies this heritable silencing depends on the *Polycomb-group* of genes. Our results, taken together with studies of the *bmi-1* gene (van der Lugt et al. 1994; Alkema et al., 1995) suggest that cell determination in vertebrates depends on a group of genes that is homologous to the *Polycomb-group* of flies. Thus, although mice and flies might use different molecules and mechanisms to initiate determination, the genetic and molecular mechanisms that maintain the silenced state of homeotic genes through cell divisions may be the same.

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