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Ring1A is a transcriptional repressor that interacts with the Polycomb-M33 protein and is expressed at rhombomere boundaries in the mouse hindbrain

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In *Drosophila*, the products of the Polycomb group (*Pc-G*) of genes act as chromatin-associated multimeric protein complexes that repress expression of homeotic genes. Vertebrate *Pc-G* homologues have been identified, but the nature of the complexes they form and the mechanisms of their action are largely unknown. The Polycomb homologue M33 is implicated in mesoderm patterning in the mouse and here we show that it acts as a transcriptional repressor in transiently transfected cells. Furthermore, we have identified two murine proteins, Ring1A and Ring1B, that interact directly with the repressor domain of M33. Ring1A and Ring1B display blocks of similarity throughout their sequences, including an N-terminal RING finger domain. However, the interaction with M33 occurs through a region at the C-terminus. Ring1A represses transcription through sequences not involved in M33 binding. Ring1A protein co-localizes in nuclear domains with M33 and other *Pc-G* homologues, such as Bmi1. The expression of Ring1A at early stages of development is restricted to the neural tube, whereas M33 is expressed ubiquitously. Within the neural tube, Ring1A RNA is located at the rhombomere boundaries of the hindbrain. Taken together, these data suggest that Ring1A may contribute to a tissue-specific function of *Pc-G*-protein complexes during mammalian development.

Keywords: M33/Polycomb/Ring1A/rhombomere boundaries/transcriptional repression

Introduction

Appropriate development of multicellular organisms requires the stable inheritance of the determined state of a wide variety of cell types. Genetic analysis in *Drosophila* has identified two antagonistic groups of genes which are

essential for the maintenance of the expression patterns of developmentally relevant genes through cell divisions. Hence, the products of the *Polycomb* group (*Pc-G*) of genes are required to maintain repressed transcriptional states, while the products of the *trithorax* group of genes (*trx-G*) are responsible for sustaining transcriptionally active states (reviewed in Kennison, 1995). In *Drosophila* *Pc-G* mutant embryos, patterns of homeotic gene expression are established correctly, but later in development, expression outside their normal boundaries occurs and, as a consequence, homeotic transformations are seen (Jürgens, 1985; Struhl and Akam, 1985; Simon *et al.*, 1992; Soto *et al.*, 1995). The *Pc-G* genes encode a structurally diverse group of proteins (reviewed in Simon, 1995), which appear to act in large complexes (Kennison and Tamkun, 1988; Franke *et al.*, 1992). Although there is no evidence for direct binding of individual *Pc-G* proteins to DNA, they bind to chromatin in a DNA-dependent manner (Zink *et al.*, 1991; DeCamillis *et al.*, 1992). *Pc-G* gene products have been shown to act on the two clusters of homeotic genes in *Drosophila*, the Antennapedia and Bithorax complexes, through specific *cis*-regulatory DNA sequences termed *Pc-G* response elements (PRE) (Simon *et al.*, 1993; Chan *et al.*, 1994; Christen and Bienz, 1994; Chiang *et al.*, 1995). Gene transfer experiments show that these PREs are required for appropriate expression patterns of homeotic transgenes. The silencing function of PREs results in expression patterns of reporter genes reminiscent of heterochromatin-related silencing effects seen in position-effect variegation (Fauvarque and Dura, 1993; Chan *et al.*, 1994; Zink and Paro, 1995). A current hypothesis proposes that *Pc-G* proteins are targeted to PREs, thus nucleating a compacted chromatin structure that can spread over neighbouring sequences and prevent activators from interacting with regulatory regions in the gene to be silenced (Orlando and Paro, 1993; Paro, 1993; Pirrotta, 1995).

Vertebrate homologues of *Pc-G* genes have been identified recently, and although very little is known about their regulatory properties, it is clear that they are also involved in the control of homeotic genes. Hence, targeted deletions of the *Bmi1* and *Mel18* genes, which encode proteins with homology to the product of the *Drosophila* *Posterior sex combs* (*Psc*) gene, show posterior transformation of the axial skeleton and deregulated expression of some *Hox* genes (Van der Lugt *et al.*, 1994, 1996; Akasaka *et al.*, 1996). In addition, overexpression of *Bmi1* in transgenic mice results in anterior transformation of the axial skeleton together with alteration of the anterior boundaries of *Hox* gene expression (Alkema *et al.*, 1995; Van der Lugt *et al.*, 1996).

Another *Drosophila* *Pc-G* protein for which vertebrate homologues have been found is Polycomb (*Pc*). Thus, the murine M33 protein and the *Xenopus* *Pc* (*XPc*) protein, both share an N-terminal chromo domain and a C-terminal

region with *Drosophila* Pc (Pearce *et al.*, 1992; Reijnen *et al.*, 1995). The chromo domain, a protein motif present in the heterochromatin-associated protein HP1 (Paro and Hogness, 1991), is required in *Drosophila* Pc for binding to chromosomal targets (Messmer *et al.*, 1992; Platero *et al.*, 1995), while the C-terminal domain is thought to recruit further Pc-G members to form a silencing complex (Franke *et al.*, 1995; Muller, 1995). Consistent with the presence of these homologous domains, M33 has been found to be a constituent of multimeric protein complexes in mouse embryos (Alkema *et al.*, 1997).

We are interested in the molecular mechanisms underlying the function of Pc-G proteins in mammals. Here, we have investigated the effect of M33 on transcriptional regulation and searched for M33 interactors. We show that the conserved C-terminal domain of M33 is necessary and sufficient for generalized repression when tethered to a promoter and report the identification of two murine RING finger proteins that interact directly with this domain. One of these, Ring1A, is identical to the human protein RING1 which is so far of unknown function. The human RING1 protein has been shown to interact with XPc (Satijn *et al.*, in preparation). Comparison of the expression patterns of Ring1A and M33 during mouse development, together with the finding that Ring1A acts as a repressor through a region that it is not involved in M33 binding, indicates that it may be a relevant component of the silencing function of Pc-G complexes in mammals.

Results

M33 is a transcriptional repressor

When recruited to a promoter, Pc and other *Drosophila* Pc-G proteins act as repressors in transiently transfected mammalian cells (Bunker and Kingston, 1994). The murine M33 gene was isolated by its homology to sequences encoding the chromo domain of the *Drosophila* Pc gene (Pearce *et al.*, 1992). However, apart from this domain and the C-terminal stretch of 30 amino acids, the rest of the sequences of the two proteins show no significant homology (Figure 1A). Therefore, we asked whether M33 would also have a general negative effect on transcription. The coding sequence of M33 was fused to amino acids 1–147 of the GAL4 DNA binding domain (pGAL4–M33), and the transcriptional activity of the fusion protein was assessed on the pG5tkCAT reporter plasmid, in which chloramphenicol acetyltransferase (CAT) expression is directed by a herpes simplex virus thymidine kinase (tk) promoter containing five GAL4 binding sites located 120 bp upstream of the TATA box. Co-transfection of pGAL–M33 and pG5tkCAT into NIH-3T3 cells resulted in a marked repression of the expression of CAT in a dose-dependent manner (Figure 1C). Repression was dependent on the presence of the GAL4 binding sites since CAT expression was minimally affected when a promoter–reporter construct lacking the GAL4 binding sites (ptkCAT) was used (Figure 1C). To test whether M33 can repress from a distance we used pG5-1.6-tkCAT, a plasmid in which five GAL4 binding sites are located 1.6 kb upstream of the TATA box. CAT expression was still repressed in the presence of GAL4–M33, although to a slightly lesser extent than seen with pG5tkCAT (Figure 1D). For comparison, we also tested a GAL4–Bmi1

fusion protein, which has previously been shown to have repression activity in transiently transfected cells (Bunker and Kingston, 1994; Cohen *et al.*, 1996). The transcriptional repression elicited by GAL–M33 and GAL4–Bmi1 was found to be similar on both reporter constructs (Figure 1D).

To define the region(s) in M33 responsible for the transcriptional repression, a number of N- and C-terminal deletions were cloned into the GAL4 expression vector and assayed for their ability to repress transcription from pG5tkCAT in NIH-3T3 cells. Previous studies with Pc showed that a C-terminal truncation of 86 amino acids reduced its repression activity, whereas the deletion of the C-terminal 118 amino acids had little effect (Bunker and Kingston, 1994). Here we show that a GAL4–M33 fusion protein containing a C-terminal truncation of 202 amino acids [GAL4–M33 (1–317)] showed little ability to repress CAT expression. In fact, the C-terminal deletion of only the conserved stretch of 30 amino acids [GAL4–M33(1–488)] results in a fusion protein with a very diminished repression activity (Figure 1E). To test whether the deletions could have their effect indirectly, for instance by disrupting the conformation of other parts of the protein, we compared repression mediated by GAL4–M33 with the N-terminal deletions [GAL4–M33 (318–519) and GAL4–M33(489–519)]; both appeared to be as efficient, or even more effective, as repressors than the full-length M33 (Figure 1E). Both C-terminally truncated GAL4–M33 fusion proteins were expressed at levels similar to those of the intact M33 fusion (Figure 1F). We conclude that, like Pc and other Pc-G proteins, M33 functions as a repressor when fused to a heterologous DNA binding domain and that the conserved C-terminal stretch of 30 amino acids of M33 is necessary and sufficient for transcriptional repression.

Isolation of cDNAs coding for proteins that interact with the repressing domain of M33

To begin to understand the repression mediated by M33, the yeast two-hybrid system was used to isolate cDNAs encoding proteins that interact with M33. A fusion between the LexA protein (amino acids 1–202) and the C-terminal half of M33 (amino acids 318–519) was used as a bait to screen a library of mouse embryo cDNAs fused to the DNA sequence encoding the activation domain of GAL4. Approximately 2×10^6 yeast transformants were screened, out of which 200 were scored as positive. Sequence analysis of 30 of these revealed three classes of cDNA. The shortest one was a 800 bp cDNA containing an open reading frame (ORF) for a protein which is 96% identical to amino acids 201–377 of the human RING1 protein (Figure 2A). *RING1* is a gene of unknown function, which had been previously identified in association with a CpG island at the centromeric end of the human major histocompatibility complex (Lovering *et al.*, 1993). In order to isolate a full-length cDNA we performed 5'RACE on mouse embryo RNA. Two types of cDNA were obtained for which the deduced amino acid sequence further supported a close identity with human RING1. The shortest cDNA encodes an ORF of 383 amino acids, in which the first ATG is preceded by an upstream in-frame amber termination codon; however, another in-frame ATG in a better context for initiation translation is found down-

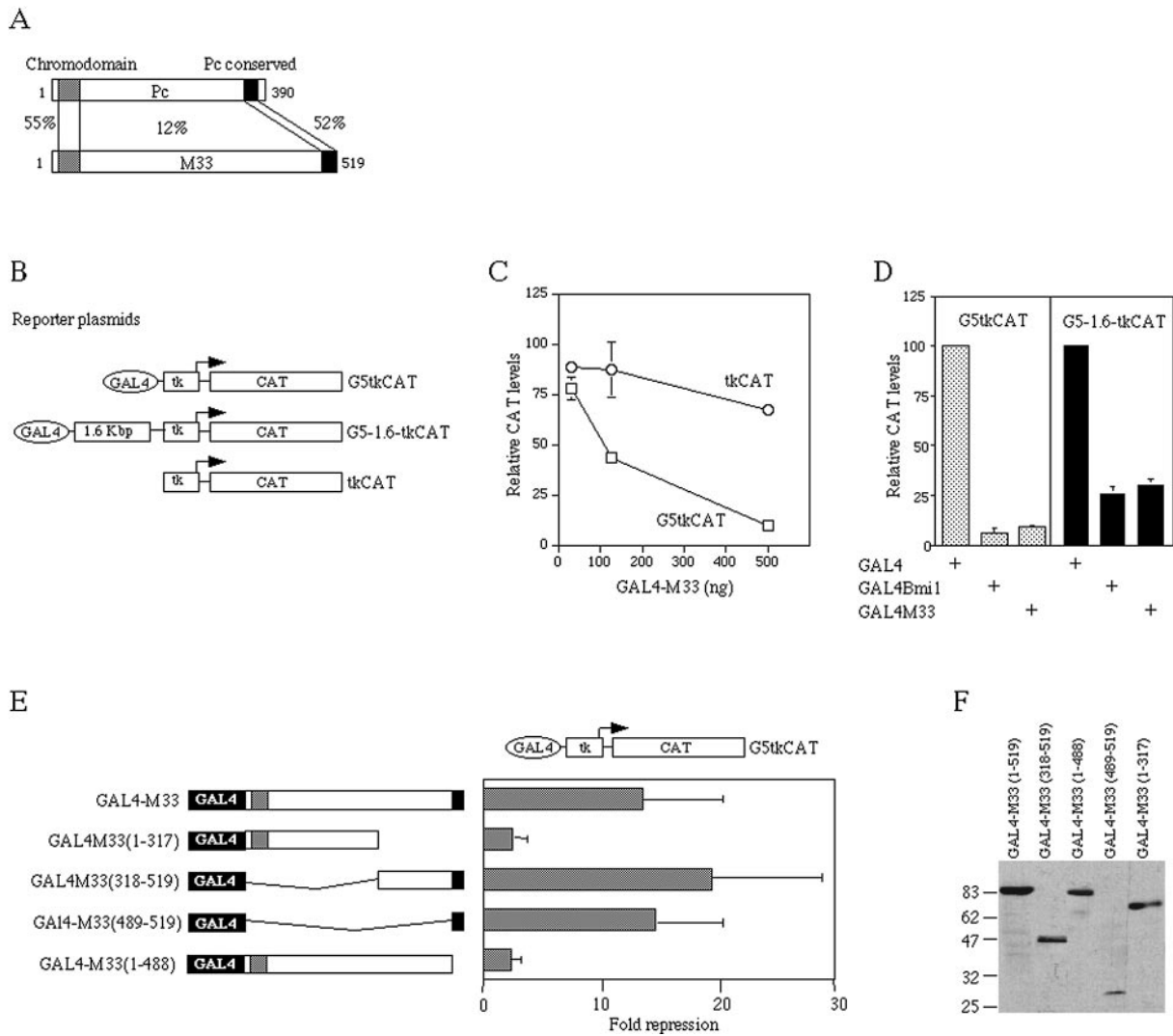


Fig. 1. Fusions to the GAL4 DNA binding domain reveal a repression function in the C-terminal region of M33. **(A)** Structural homologies between *Drosophila* Pc and murine M33. The chromo domain and the C-terminal Pc conserved region are represented, with the percentage of amino acid identity noted below each domain. **(B)** Schematic representation of the reporter constructs used. pG5tkCAT contains five GAL4 binding sites immediately upstream of the (-105 to +51) HSVtk promoter in plasmid pBLCAT2 (here termed tkCAT). pG5-1.6-tkCAT contains five GAL4 binding sites placed 1.6 kb upstream of the same HSVtk promoter. **(C)** GAL4-M33 fusion protein represses transcription in a dose-dependent manner. NIH-3T3 cells received 1.5 µg of pG5tkCAT or tkCAT, together with 50 ng of pCMVlacZ and increasing amounts of pGAL4-M33. The total amount of effector plasmid (0.5 µg), was kept constant by addition of the plasmid expressing only the GAL4 DNA binding domain. CAT protein levels were determined 40 h after transfection and normalized to β-galactosidase protein levels. Results are expressed as normalized CAT levels relative to those obtained in the presence of 0.5 µg of the GAL4 expressing vector. The results shown are an average of three experiments with the standard deviation indicated. **(D)** Repression at a distance by GAL4-M33 and GAL4-Bmi1. NIH-3T3 cells were co-transfected with pG5tkCAT or pG5-1.6-tkCAT (1.5 µg) and pCMVlacZ (50 ng) together with GAL4-M33 or GAL4-Bmi1 expression vectors (0.5 µg). Normalized CAT levels are expressed relative to those obtained in the presence of the GAL4 DNA binding domain alone. **(E)** Mapping the transcriptional repression domain of M33. NIH-3T3 cells were transfected with 1.5 µg of pG5tkCAT and 50 ng of pCMVlacZ together with plasmids expressing GAL4 DNA binding domain alone or fused to various regions of M33 (0.5 µg) as indicated. Fold repression is expressed as the ratio of normalized CAT protein values in the presence of GAL4 DNA binding domain expression plasmid over normalized CAT protein values in the presence of a given effector. Values represent the averages of three experiments with standard deviation indicated. **(F)** Immunoblots of extracts from COS-7 cells transiently transfected with plasmids expressing the indicated GAL4-M33 fusion proteins and probed with a monoclonal antibody against the DNA binding domain of GAL4. The positions of the molecular size markers (kDa) are indicated on the right.

stream. If the latter ATG were used as the initiation codon, a 377 amino acid protein would be expressed, as has been reported for the human RING1 protein (Lovering *et al.*, 1993). This conceptual mouse protein differs from human RING1 at only 11 positions and we have therefore termed it Ring1A. The longest RACE cDNA would encode for an ORF in which the first ATG was also preceded by in-frame stop codons and that would add 26 extra amino acids to the N-terminus of the Ring1A ORF (J.Schoorlemmer, data not shown).

The other two classes of cDNA isolated from the two-hybrid screening were overlapping clones of 1100 and 2300 bp, whose longest ORF encoded for a protein structurally related to Ring1A, which we have termed Ring1B (Figure 2B). Ring1A and Ring1B cDNAs are not products of a differentially spliced mRNA, but are encoded by two different genes (M.Vidal, data not shown). The Ring1A and Ring1B proteins have an N-terminal RING finger motif and display blocks of similarity throughout their sequences. The region of highest similarity comprises

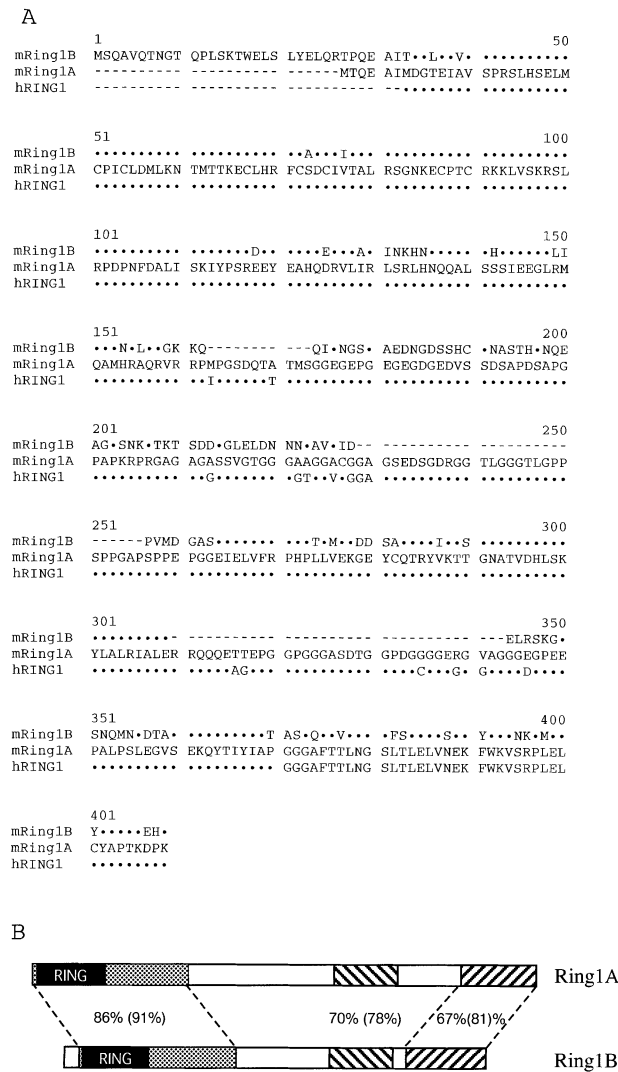


Fig. 2. Amino acid sequences for the Ring1 proteins that interact with M33. **(A)** The deduced amino acid sequences encoded by mouse Ring1A (mRing1A) and mouse Ring1B (mRingB) cDNAs are shown aligned to that encoded by human RING1 (hRING1). Sequences were aligned using the PILEUP algorithms (Genetics Computer Group, University of Wisconsin, Madison). The residues that are conserved between mRing1A and hRING1 or mRing1A and mRing1B are marked with a dot, while dashes represent gaps introduced to maximize the alignment. **(B)** Schematic representation of Ring1A and Ring1B proteins. Black boxes represent the RING finger domains, shaded and stippled boxes represent conserved sequence motifs and open boxes represent non-conserved regions. The approximate percentage of sequence identity (similarity in parenthesis) is given.

the RING finger and a contiguous domain, and is 86% identical between the two Ring1 proteins. Two other blocks of homology (70% and 67% identity respectively) are located in the C-terminal region, and are separated from each other and the conserved N-terminal region by non-conserved sequences (Figure 2B). In this study we have focused on Ring1A and the various constructs used are based on the ORF that encodes for the protein of 377 amino acids.

The C-terminus of M33 interacts with a domain in the carboxyl domain of Ring1A

To define the M33–Ring1A interaction domains we used the yeast two-hybrid system. As shown in Figure 3A, the

LexA–M33 protein containing amino acids 489–519 was the smallest fusion protein that is able to interact with a GAL4–Ring1A fusion protein. This region of M33 is precisely the C-terminal stretch of 30 amino acids that is also conserved with Pc and that is important for transcriptional repression in transfected mammalian cells. Removal of this C-terminal region generates an M33 protein that is unable to interact with Ring1A, indicating that the M33 chromo domain is not required for the M33–Ring1A interaction. On the other hand, the truncated Ring1A derivative containing amino acids 201–377 (originally isolated from the M33 two-hybrid screening) was the smallest GAL4–Ring1A protein that interacted with LexA–M33. This portion of Ring1A and its homologous Ring1B contain two conserved regions (Figure 2B). Deletion of either of the two blocks resulted in fusion proteins [GAL4–Ring1A(1–213) and GAL4–Ring1A(214–377)] which did not interact with M33 (Figure 3A). These results indicate that an extended domain in the carboxyl half of Ring1A is necessary for binding to M33 but that the RING finger is not required.

To determine whether the Ring1A protein can interact directly with M33 we used an *in vitro* protein binding assay. Sequences encoding the C-terminus of M33 (amino acids 333–519) were fused to the glutathione *S*-transferase (GST) gene, and the resulting hybrid protein was expressed in *Escherichia coli*. As a source of Ring1A protein, the entire Ring1A coding sequence (amino acids 1–377) or sequences encoding a C-terminal portion (amino acids 201–377) were transcribed and translated *in vitro* in the presence of ³⁵S-labelled methionine. The GST–M33 protein, immobilized on glutathione–Sepharose, was incubated with labelled Ring1A proteins. After the beads were washed, bound proteins were analysed by SDS–PAGE electrophoresis. As shown in Figure 3B, both the full-length and the N-terminal-deleted Ring1A proteins were able to bind to GST–M33, but did not exhibit appreciable binding to GST–Sepharose alone. Binding was not affected by high salt washes (500 mM NaCl). These results indicate that the C-terminal half of Ring1A is able to interact directly with the C-terminus of M33.

In vivo interaction of Ring1A and M33 and its co-localization with other Pc-G proteins

To obtain evidence for the association *in vivo* of the M33 and Ring1A proteins in mammalian cells, we performed immunoprecipitation studies using extracts from transfected cells. We used affinity purified polyclonal antibodies raised against purified GST–M33 and GST–Ring1A proteins. COS-7 cells were transiently transfected with expression vectors encoding M33 and Ring1A fused to the MYC epitope at its N-terminus. Figure 4A shows the results of immunoprecipitations of extracts from cells transfected with either M33 or Ring1A separately or both together, followed by Western blot analysis with a monoclonal antibody against the MYC tag. It can be seen that M33 and Ring1A can be immunoprecipitated specifically by their cognate antibodies. MYC-tagged M33 was detected in both non-immunoprecipitated extracts and in anti-M33 immunoprecipitated material as a 74 kDa protein doublet, whereas MYC-tagged Ring1A was detected as a 58 kDa band. Anti-M33 antibodies co-immunoprecipitated Ring1A from cell extracts containing both M33 and

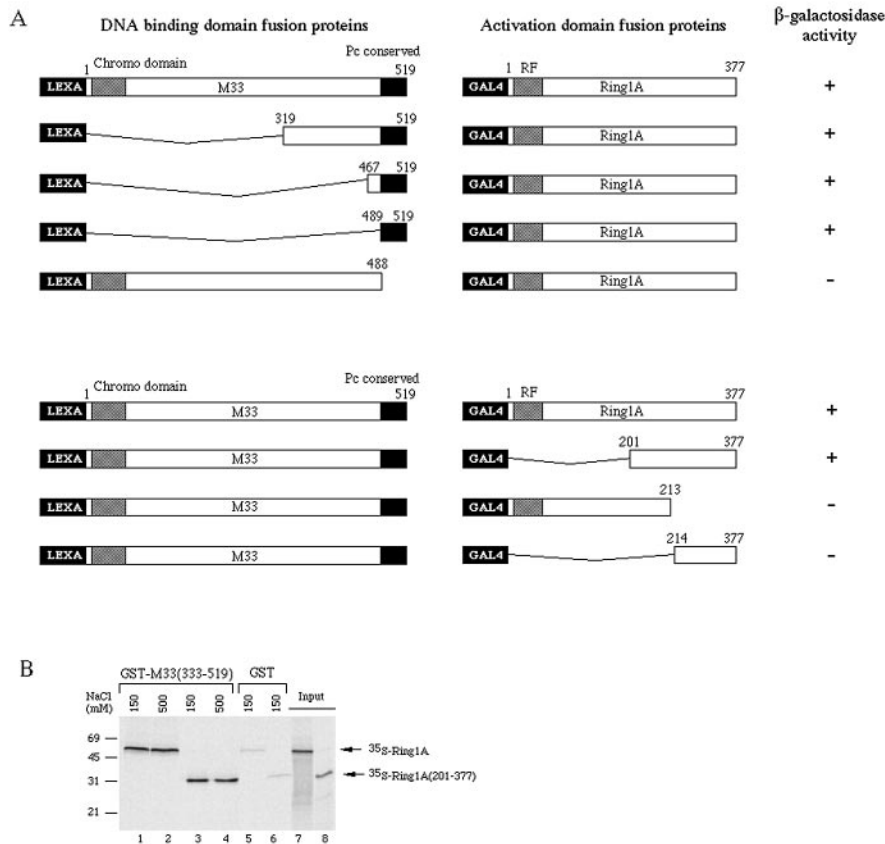


Fig. 3. M33/Ring1A interaction domains. **(A)** Schematic representation of plasmids used to determine the binding domains in yeast. Plasmids expressing DNA binding domain–M33 fusion proteins were constructed by fusing intact or truncated cDNAs in frame to the LexA cDNA. M33 protein motifs are indicated. Plasmids expressing transactivation domain–Ring1A fusion proteins were constructed by joining intact or truncated Ring1A cDNAs to the GAL4 activation domain cDNA encoding amino acids 768–881. The dark-shaded boxes denoted by RF represent the RING finger motif. Yeast strain L40 was co-transformed with the indicated pairs of plasmids and colonies scored for β -galactosidase activity using a colony lift assay. **(B)** *In vitro* binding of Ring1A to the C-terminal region of M33. Bacterially produced GST (30 μ g) or GST–M33(333–519) (5 μ g) immobilized on glutathione–Sephacrose were incubated with *in vitro* translated [35 S]Ring1A (lanes 1, 2 and 5) or [35 S]Ring1A(201–377) (lanes 3, 4 and 6). After incubation and washes with buffer containing the indicated amounts of NaCl, the bound proteins were separated by SDS–PAGE (10% gel). Input lanes (7 and 8) were 1/10 of the amount of the *in vitro* translation reaction used in the incubations. Phosphorimager analysis showed that protein bound to GST–M33(333–519)–Sephacrose was 25–30% of input, whereas protein bound to GST beads was ~3%. Sizes of molecular weight markers (in kDa) are indicated at the left.

Ring1A proteins. In the reciprocal experiment, anti-Ring1A antibodies also co-immunoprecipitated M33 protein. From these results we conclude that M33 and Ring1A can form complexes in mammalian cells.

Additional evidence for the *in vivo* association of Ring1A with M33 and with other Pc-G proteins was obtained from their intracellular localization in U2-OS cells as detected by indirect immunofluorescence (Figure 4B). The close similarity of mouse and human M33 and Ring1A proteins (Lovering *et al.*, 1993; Gecz *et al.*, 1995) allowed us to utilize the antibodies described above to show that endogenous Ring1A is located in the nucleus, where it showed a speckled pattern (Figure 4B, panel IV), similar to that seen for M33 (Figure 4B, panel I). Anti-Ring1A and anti-M33 antibodies were used together with chicken anti-Bmi1 antibodies (D.P.E.Satijn *et al.*, in preparation) in double labelling experiments. We found that Ring1A co-localized in large speckles with Bmi1 (Figure 4B, panels I–III). Likewise, we found that M33 and Bmi1 also co-localized in speckled structures (Figure 4B, panels IV–VI), similar to those of Ring1A and to those described previously (Alkema *et al.*, 1997). We

believe this result implies that Ring1A and M33 also co-localize in such nuclear structures, although direct evidence could not be obtained because both anti-Ring1A and anti-M33 antibodies were raised in rabbits. Recently it has been shown that these nuclear domains also contain HPH1 and HPH2, two human protein homologues of the *Drosophila* polyhomeotic protein (Gunster *et al.*, 1997). We conclude that the overlap in the localization of Ring1A, M33, Bmi1, HPH1 and HPH2 proteins is consistent with the idea that they all interact in a multiprotein complex.

Expression of Ring1A and M33 during embryogenesis

To further assess the functional relationship between Ring1A and M33 we investigated the expression pattern of their transcripts in the mouse embryo between E8.5 and E15.5 by non-radioactive *in situ* hybridization (Figure 5). From E8.5 to E11.5, expression of Ring1A mRNA was restricted to cells of the developing central nervous system (CNS; Figure 5A and B) while M33 transcripts were detected in most embryonic tissues including the CNS, the main exception being the heart (Figure 5D and

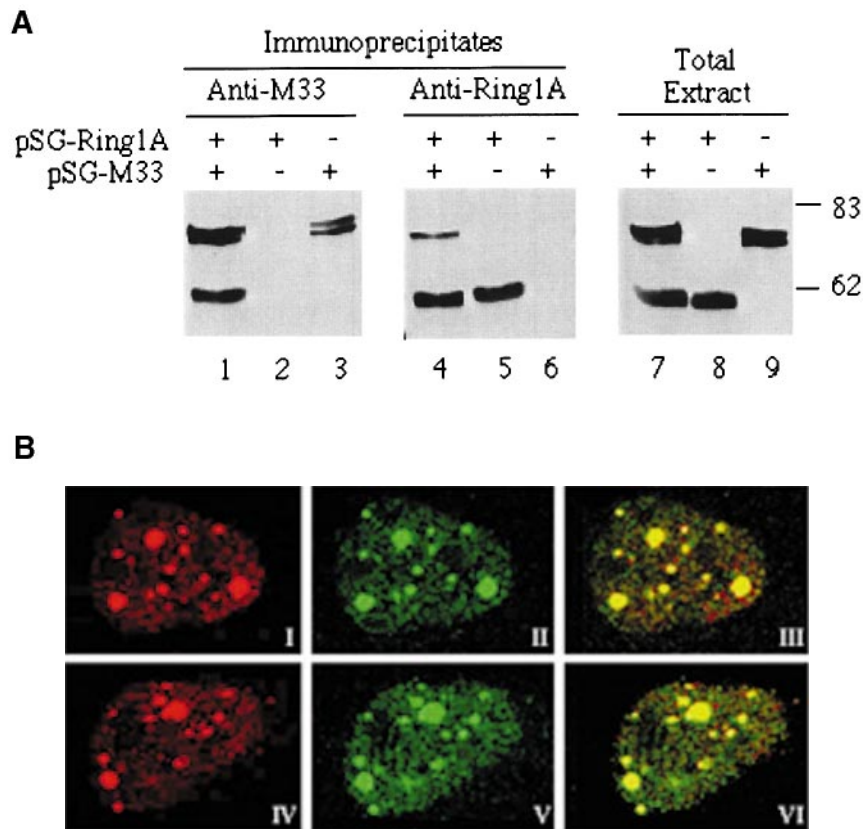


Fig. 4. *In vivo* association of Ring1A and Pc-G proteins. **(A)** Co-immunoprecipitation of Ring1A and M33 in COS-7 cells. Cells were transfected with plasmids expressing MYC-tagged Ring1A alone (lanes 2, 5 and 8) or MYC-tagged M33 alone (lanes 3, 6 and 9) or both (lanes 1, 4 and 7). Immunoprecipitation was with polyclonal antibodies against M33 or against Ring1A. Immunoprecipitated proteins (lanes 1–6) or total extracts from transfected cells (lanes 7–9) were immunoblotted with monoclonal anti-MYC antibody 9E10. Molecular weights (in kDa) are indicated at the right. **(B)** Co-localization of endogenous M33 and Ring1A with Bmi1 in the nucleus of U2-OS cells using indirect immunofluorescence and confocal microscopy. (I–III) Double labelling with affinity purified rabbit anti-M33 antibodies (I, red) and chicken anti-Bmi1 antibodies (II, green). The merge of both pictures (III) shows complete overlapping of distribution patterns. (IV–VI) Double labelling with affinity purified rabbit anti-Ring1A (IV, red) and chicken anti-Bmi1 antibodies (V, green). The merge of the two images (VI) shows that Ring1A and Bmi1 co-localize in most of the large labelled domains.

E). As embryogenesis progressed, the patterns of Ring1A and M33 expression became more similar. At E13.5, Ring1A and M33 transcripts were detected in both central and peripheral components of the nervous system (Figure 5C and F). In the CNS, expression of both genes was mostly localized to the ventricular zone of the brain and spinal cord. In the peripheral nervous system (PNS), transcripts for both molecules were mainly observed in sensory cranial and spinal ganglia (Figure 5C and F: Vg and sg). Other sites of Ring1A and M33 co-expression included the olfactory and tongue epithelia. Additionally, M33 mRNA was detected in the lung, gastrointestinal duct and urogenital system (Figure 5F). By E15.5, the pattern of Ring1A expression was most similar to that of M33. Transcripts for both genes were mostly observed in the developing CNS, the thymus and in various epithelial cell types including the olfactory, tooth and tongue epithelia (not shown).

Most strikingly, Ring1A expression within the hindbrain region of E8.5–E11.5 mouse embryos was restricted to stripes located between each rhombomere. To determine whether this signal represented the cells comprising rhombomere boundaries, we examined the distribution of Ring1A transcripts in relation to the neuroanatomical

pattern of the hindbrain. The distribution of developing fibre tracts has been shown to outline the organization of the hindbrain (McKay *et al.*, 1994). At E10.5, rhombomere boundaries are easily recognized by the alignment of axons along them. At this developmental stage, Ring1A transcripts were detected at the same location as the axons concentrated along rhombomere boundaries (Figure 5J and K). To confirm Ring1A expression in rhombomere boundaries, we compared the expression pattern of Ring1A to that of an established marker for rhombomere boundaries, PLZF (Cook *et al.*, 1995). As shown in Figure 5I, Ring1A expression mimics that of PLZF in rhombomere boundaries at E9.5.

Transcriptional repression by Ring1 in transfected mammalian cells does not require the M33 binding domain

In the light of its interaction with M33 and its presence in mammalian Pc-G complexes, we investigated whether Ring1A could also repress the transcription of a reporter gene in transiently transfected mammalian cells. Figure 6A shows that co-transfection of a plasmid expressing a GAL4–Ring1A fusion protein and pG5tkCAT resulted in a significant decrease in the transcriptional activity of the

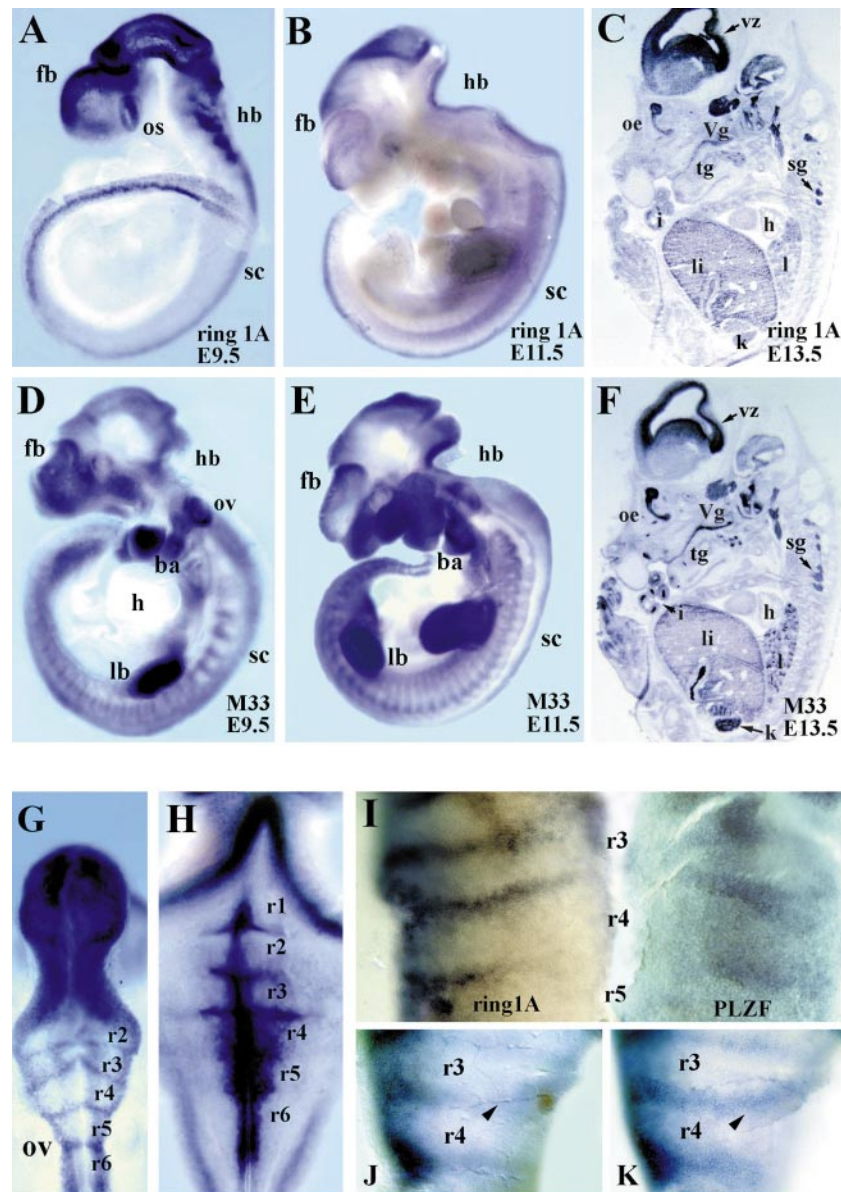


Fig. 5. (A–F) *In situ* hybridization analysis of Ring1A and M33 mRNA expression during mouse embryogenesis. (A and B) Lateral views of whole-mount preparations of E9.5 (A) and E11.5 (B) mouse embryos hybridized with a Ring1A cRNA probe. Expression of Ring1A mRNA is restricted to the developing CNS, including the forebrain (fb), hindbrain (hb) and dorsal spinal cord (sc). (C) Sagittal section of an E13.5 mouse embryo hybridized with a Ring1A cRNA probe. In the developing CNS, transcripts for Ring1A are detected in the ventricular zone (vz). In the PNS, expression is observed in the spinal (sg) and trigeminal (Vg) ganglia. (D and E) Whole-mount *in situ* hybridization histochemistry of E9.5 (D) and E11.5 (E) mouse embryos using a M33 riboprobe. M33 signal is detected in most embryonic tissues with the main exception of the heart (h). As is the case for Ring1A, M33 expression is detected throughout the CNS. High levels of M33 expression are also detected in the optic vesicle (ov), branchial arches (ba) and the developing limb buds (lb). (F) Sagittal section adjacent to that in (C) of an E13.5 mouse embryo hybridized with a M33 cRNA probe. Similarly to Ring1A, M33 transcripts are observed in the ventricular zone, and spinal and trigeminal ganglia. Outside the CNS, M33 signal is present in the olfactory (oe) and tongue (tg) epithelia, lung (l), kidney (k) and intestine (i). (G–K) Expression of Ring1A mRNA in rhombomere boundaries. (G and H) Dorsal views of the developing hindbrain of E9.5 (G) and E11.5 (H) mouse embryos hybridized with a Ring1A riboprobe. Transcripts for Ring1A are detected in the boundaries dividing each rhombomere (r1–r6). (I) Flat-mount preparation of the left and right halves of an E9.5 hindbrain hybridized with a Ring1A and a PLZF riboprobe respectively. Expression of Ring1A mimics that of PLZF in the rhombomere boundaries. (J and K) Two different planes of focus of an E10.5 flat-mounted hindbrain hybridized with a Ring1A cRNA probe. Ring1A signal (K) coincides with the location of rhombomere boundaries which are outlined here by the axons concentrated along them (J). Other abbreviations: optic stalk (os), liver (li).

reporter gene. The extent of GAL4–Ring1A repression was dependent on the amount of transfected effector plasmid. Although some repression of ptkCAT was also observed, GAL4–Ring1 repression was more efficient on the reporter construct with GAL4 binding sites. We also studied the activity of the Ring1A cDNA that encodes the Ring1A protein variant with 26 extra amino acids at its

N-terminus, and found no differences with that of the shorter Ring1A protein used throughout this study (data not shown). Like GAL4–M33 and GAL4–Bmi1, the GAL4–Ring1A fusion protein also repressed transcription from pG5-1.6-tkCAT (not shown), indicating that it was able to repress transcription at a distance.

To define the region(s) in Ring1A that is responsible

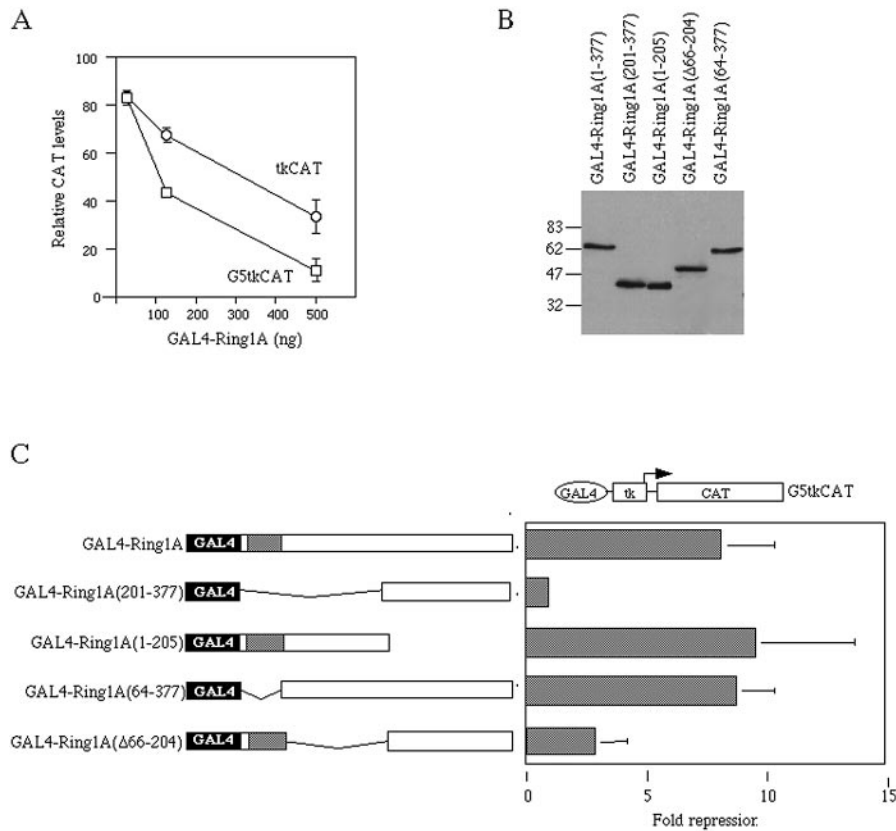


Fig. 6. Ring1A represses transcription in transiently transfected mammalian cells. (A) Dose-dependent repression by GAL4–Ring1A fusion protein. NIH-3T3 cells were co-transfected with 1.5 μ g of pG5tkCAT or tkCAT, together with 50 ng of pCMVlacZ and increasing amounts of pGAL4–Ring1A, which expresses the Ring1A cDNA as a fusion with the GAL4 DNA binding domain. The total amount of effector plasmid (0.5 μ g), was kept constant by addition of the plasmid expressing only the GAL4 DNA binding domain. Relative CAT levels are expressed as described in Figure 1C. (B) Immunoblots of extracts from COS-7 cells transiently transfected with plasmids expressing the indicated GAL4–Ring1A fusion proteins and probed with a monoclonal antibody against the DNA binding domain of GAL4. The position of molecular size markers (kDa) are indicated on the left. (C) Ring1A repression activity locates to a domain in its N-terminal region. NIH-3T3 cells were co-transfected with 1.5 μ g of pG5tkCAT and 50 ng of pCMVlacZ together with 0.5 μ g of the indicated plasmids expressing various GAL4–Ring1A fusion proteins. Fold repression is expressed as in Figure 1E.

for transcriptional repression we assayed the ability of a number of truncated Ring1A proteins fused to the GAL4 DNA binding domain to repress transcription from pG5tkCAT. The GAL4–Ring1A(201–377) derivative, which lacks the N-terminal half, showed no activity (Figure 6C), whereas the complementary truncated protein, GAL4–Ring1A(1–205), repressed expression of pG5tkCAT with an efficiency similar to that of the GAL4–Ring1A fusion protein. The deletion of the RING finger domain in GAL4–Ring1A(64–377) resulted in a truncated fusion protein with full repression activity, indicating that either the RING finger is not required for Ring1A repression or that Ring1A has redundant repressor domains. To distinguish between these two possibilities we used the GAL4–Ring1A(Δ 66–204) derivative, which eliminates the central region of Ring1A but leaves the RING finger. This truncated protein showed some repression activity, although far lower than that of the full-length Ring1A or that of the RING finger derivative, suggesting that Ring1A transcriptional repression is mostly mediated by a central domain of the Ring1A protein. The differences in repression activity could not be related to differences in protein stability or expression levels, as shown by the detection of the various fusion proteins in cell extracts (Figure 6D). Thus, repression by Ring1A is

located in a domain in its N-terminus region that can be separated from the region required for binding to M33.

Discussion

The murine M33 protein is a homologue of Pc and it can substitute, at least in part, for Pc function in transgenic flies (Muller *et al.*, 1995). M33-deficient mice show homeotic transformations of the axial skeleton, as well as sternal and limb malformations (Coré *et al.*, 1997). The mechanisms by which M33 exerts its function(s) are not known. In this study we have shown that M33 functions as a repressor of transcription in transiently transfected cells. In addition, using a yeast two-hybrid screen, we have identified two structurally related M33-interacting proteins. One of these (Ring1A) is the murine homologue of the human protein of unknown function, RING1. Biochemical assays indicate that the M33 and Ring1A proteins interact directly and that Ring1A can also behave as a transcriptional repressor.

The repression domain of M33 interacts with the RING finger proteins Ring1A and Ring1B

We have shown that a GAL4–M33 protein containing only the conserved stretch of 30 C-terminal amino acids

represses promoter-driven reporter expression. In a two-hybrid screen of embryonic cDNAs encoding proteins that interact with the C-terminal portion of M33 we found two different but related cDNAs. One encoded Ring1A, the mouse counterpart of human RING1, a protein of unknown function (Lovering *et al.*, 1993). The other, which shares large stretches of conserved sequences with Ring1A, encodes a new RING finger protein, which we termed Ring1B because of its relatedness to Ring1A. It is worth noting that the RING finger motif is also found in some Pc-G proteins, such as Psc, Bmi1 and Mel18 (van Lohuizen *et al.*, 1991b; Ishida *et al.*, 1993). Interaction assays in yeast and *in vitro* showed that M33 and Ring1A use neither the chromo domain nor the RING finger domains (two protein motifs believed to mediate protein-protein interaction) for their mutual interaction, but rather make use of domains located at their respective C-terminal regions. Ring1A interacts directly with M33 through a large C-terminal domain which contains two of the blocks of sequences conserved between Ring1A and Ring1B. Truncated Ring1A proteins lacking either of these two domains fail to bind M33, which suggests the conserved sequences at the C-terminus of Ring1A are relevant to M33 binding. In contrast, the M33 domain that interacts with Ring1A was shown to be within the stretch of 30 amino acids at the C-terminus conserved with other Pc proteins. Except for the RING finger, database searches showed no similarity of any of the conserved regions of Ring1A and B to any previously characterized protein motifs. Interestingly, the RING finger domain of Ring1A and Ring1B, and to a lesser extent the homology block adjacent to it, showed homology with a gene of unknown function that was identified as a *Drosophila* expressed-sequence tag (dbEST Id 841906) (D.Harvey, L.Hong, M.Evans-Holm, J.Pendleton, C.Su, P.Brokstein, S.Lewis and G.M.Rubin, unpublished). We are trying to map its cytological location to see whether it corresponds to any of the *Drosophila* Pc-G mutations mapped but not yet cloned. Until genetic evidence is available, such a piece of information might help in indicating whether Ring1 proteins are new members of the Pc group of proteins. Indeed, this might be expected since all of the putative mammalian Pc-G proteins identified to date have homologous counterparts among *Drosophila* Pc-G proteins (van Lohuizen *et al.*, 1991a; Pearce *et al.*, 1992; Hobert *et al.*, 1996; Motaleb *et al.*, 1996; Schumacher *et al.*, 1996).

Ring1A and M33 repressor function

A number of Pc alleles consist of chromo domain or C-terminal truncated Pc proteins (Messmer *et al.*, 1992; Franke *et al.*, 1995). This indicates that chromatin binding, which depends on the chromo domain (Messmer *et al.*, 1992) is not sufficient for Pc function. Besides, the chromo domain of Pc is sufficient to bind Pc and Pc-bound proteins (Platero *et al.*, 1996). Therefore, in addition to the ability to make protein complexes, it seems that the transcriptional activity associated with the conserved C-terminal region of Pc (Muller, 1995) and M33 is relevant to their function.

Here we have shown that the Ring1A protein, which interacts with the repressing domain of M33, is itself a repressor when tethered to promoters by means of a DNA binding domain. The repression activity of Ring1A is mediated by the N-terminal half of the protein, particularly

through sequences C-terminal to the RING finger. A similar analysis showed that the RING finger of Bmi1 was dispensable for repression of a GAL4tk-luciferase gene by a GAL4-Bmi1 fusion protein (Cohen *et al.*, 1996). Instead, the RING finger of Bmi1 has been associated with the nuclear localization of the protein (Cohen *et al.*, 1996; Alkema *et al.*, 1997). Early studies have speculated on the DNA binding properties of the RING finger of the RING1 protein, although more recently such an activity is considered to be more artifactual than of true *in vivo* relevance (Borden and Freemont, 1996). It is intriguing, however, that the Ring1A derivative lacking amino acids 66–204 shows a low but substantial repression activity, which suggests that the RING finger may contribute in some way, to the full repressing activity of the intact Ring1A protein. A more detailed deletion analysis of the transcriptional activity of Ring1A is currently underway to explore this possibility.

The region of Ring1A responsible for repression is distinct from the domain which interacts with M33, whereas the repression domain of M33 coincides exactly with the region of M33 that binds directly to Ring1A. It might be expected that in cells co-expressing the two proteins, M33 repression could be mediated by the Ring1A protein. However, overexpression of Ring1A did not significantly increase repression of a GAL4-tkCAT gene mediated by the GAL4-M33 fusion protein (M.Vidal, unpublished observations) even though these cells may have been expected to have limiting amounts of Ring1A. It is possible that M33 and Ring1A associate with each other functionally only as a large complex which requires the participation of additional proteins. Previous studies showed that different promoters responded differently to the various Pc-G proteins (Bunker and Kingston, 1994). Thus, it could be that co-operation among M33 and Ring1A proteins is observed with promoters other than the HSVtk promoter used in our constructs. Nevertheless, the more restricted expression of Ring1A in early stages of development indicates that if M33 acts in every cell type as a repressor, then it can do so without Ring1A. The participation of additional, as yet unknown proteins in M33 repression cannot be ruled out. It is possible, for instance, that Ring1B substitutes for Ring1A. We have yet to determine the expression pattern of Ring1B during development, and work on the transcriptional properties of Ring1B is ongoing.

Tissue-specific expression of Ring1A and different Pc-G complexes

Whereas Ring1A expression in E8.5–10.5 embryos was restricted to the CNS, M33 transcripts were expressed almost ubiquitously (our data; Pearce *et al.*, 1992). Here, we show that transfected Ring1A and M33 were able to interact *in vivo* and also that they co-localize with other Pc-G proteins to speckled structures in the cell nucleus. Taken together, these data support the idea that Pc-G complexes containing M33 can be heterogenous, depending upon the tissue-specific expression of the various proteins which are able to form complexes with it. Such a heterogeneity may provide the basis for tissue specificity of Pc-G function, for which evidence already exists in *Drosophila* (Soto *et al.*, 1995). Thus, early in development, the activity of M33-containing complexes

in the CNS, where Ring1A transcripts are present, could differ in some way from that of M33 complexes in the rest of the embryo, where Ring1A is absent. Within the CNS, *Ring1A* transcripts in the hindbrain were restricted to cells surrounding the rhombomeric boundaries. Together with other genes such as *FGF-3* and *PLZF* (Cook *et al.*, 1995; Mahmood *et al.*, 1995), *Ring1A* provides an intracellular marker that supports the distinctive identity of this group of cells. The segmented distribution of *Ring1A* in the hindbrain, however, makes it difficult to envisage a direct relationship between *Ring1A* and the regulation of *Hox* genes, which are thought to be among the targets of Pc-G regulation and are expressed evenly through the hindbrain in domains whose anterior boundaries coincide with boundaries between rhombomeres (Wilkinson *et al.*, 1989). In vertebrates, the *Hox* complexes are progressively transcribed during development, starting with the genes at the extreme 3' end of the clusters, and extending along the complex in a 5' direction (Duboule and Dollé, 1989; Graham *et al.*, 1989). It has been proposed that this occurs concomitantly with a re-organization of chromatin from a 'closed to open' structure, in opposition to an 'open to closed' transition in *Drosophila* (Van der Hoeven *et al.*, 1996). It should be pointed out that, as yet, there has been no demonstration of regulation of *Hox* gene expression by Pc-G genes through chromatin modification. In fact, it has been found that Pc-G silencing in *Drosophila* is not related to chromatin accessibility to restriction enzymes (Schlossherr *et al.*, 1994). So far, no PRE elements have been identified in vertebrate *Hox* gene clusters, although a number of *cis*-acting elements are used to control expression of vertebrate *Hox* genes in discrete regions (Whiting *et al.*, 1991; Vesque *et al.*, 1996). Thus, in addition to their general role as repressors, it is possible that Pc-G complexes also contribute to the fine tuning of *Hox* gene expression.

Later in development, Ring1A expression expands to a variety of tissues, most of which also express M33. Some of these cell types, for example those in the neuroectoderm, the epidermis and the thymus, are among those with the highest proliferation rates in the embryo. Interestingly, the cell proliferation defects observed for a number of cell types in M33 null mice (Coré *et al.*, 1997) suggests that the M33 protein may play a role, although perhaps indirectly, in the control of proliferation. It follows, therefore, that Ring1A may also participate in such a function.

In summary, we have shown that the M33 protein can act as a transcriptional repressor, and that its conserved C-terminal domain is sufficient for this repression. In addition, we have found that the Ring1A protein interacts with M33, thus providing a clue about its unknown function, and that Ring1A itself shows a repression ability, which together with its expression pattern most likely suggests that it is a developmentally relevant protein in the context of Pc-G function.

Materials and methods

RT-PCR and RACE cDNA cloning

Full-length M33 was obtained by PCR from random primed reverse transcribed total C57Bl/6 mouse embryo RNA. The PCR primers used were 5'-CTGAATTCGAGGAGCTGAGCAGCGT-3' (forward) and

5'-GGCATCAAAGCAAACCAGAGACT-3' (reverse). M33 cDNA encoding amino acids 319–519 was obtained by PCR from C57Bl/6 mouse genomic DNA using primers 5'-CAGAGCAGCAGAGAGGG-AACCATT-3' (forward) and 5'-GGCATCAAAGCAAACCAGAGACT-3' (reverse). The protein sequence deduced from our M33 cDNAs has a valine at position 85 instead of alanine, as previously published. This difference was also observed when a phage cDNA library made from P19 cell RNA was used as a template for PCR. RACE cDNA cloning was performed using the Marathon kit (Clontech) using poly(A)⁺ RNA from day E11.5 mouse embryos, following manufacturer's instructions.

Plasmids

Plasmid manipulations were performed according to established procedures and when PCR fragments were involved their sequences were verified by sequencing. DNA binding domain and activation domain fusion proteins were expressed in yeast from the plasmids pBTM116 (a gift of P.Bartel and S.Fields) and pGAD10 (Clontech) respectively. Plasmids for transfection studies in mammalian cells were CsCl purified or isolated using Qiagen columns. The CAT reporter plasmids include the following: pG5tkCAT contains five GAL4 binding sites upstream of the -110 to +56 (relative to the transcription initiation site) herpes simplex virus thymidine kinase promoter (Shi *et al.*, 1991); ptkCAT, also called pBLCAT2 (Luckow and Schütz, 1987), uses the same minimal HSVtk promoter but lacks GAL4 binding sites; pCMVlacZ contains the enhancer and promoter of the immediate early promoter of cytomegalovirus in front of the *lacZ* gene of *E.coli*. The GAL4 DNA binding domain chimeras were constructed by subcloning of the indicated cDNAs into pSG424 (Sadowski and Ptashne, 1989; a gift of M.Ptashne). For epitope-tagging of M33 and Ring1A, the 5' ends of their coding sequences were fused to sequences encoding the Myc epitope recognized by the monoclonal antibody 9E10 (Evan *et al.*, 1985) in the expression vector pSG5 (Green *et al.*, 1988). Murine Bmi1 cDNA (Van Lohuizen *et al.*, 1991b) was obtained from M.Alkema. Details of plasmid constructions are available upon request.

Yeast two hybrid screen and interaction assays

A GAL4 activation domain-tagged cDNA library from day E11.5 mouse embryo RNA constructed in the leucine-selectable plasmid pGAD10 (Clontech) was introduced by LiAc transformation into the *Saccharomyces cerevisiae* L40 strain [MATa trp1-901 leu 23, 112 his3-Δ200 ade2 LYS2::*(LexAop)4-HIS3 URA3::*(LexAop)8-lacZ**] (Hollenberg *et al.*, 1995) expressing the fusion protein LexA-M33(319–519) from the tryptophan-selectable expression vector pBTM116 (Vojtek *et al.*, 1993). After overnight recovery in yeast complete medium (Trp⁻Leu⁻Ura⁻), the transformants were plated on selective medium for histidine prototrophy (Trp⁻Leu⁻Ura⁻His⁺Lys⁻). His⁺ clones exhibiting β-galactosidase activity on filters were isolated and further analysed. Library plasmids were rescued on *E.coli* strain HB101 (leuB⁻) and selected for leucine prototrophy on minimal plates. These plasmids were then retransformed into L40 along with pBTM116-M33(319–519) or plasmids expressing irrelevant LexA fusion proteins, such as LexA-lamin and LexA-daughterless (gifts from S.Hollenberg). To map the interaction domains of M33 and Ring1A, cDNA fragments were subcloned in plasmids pBTM116 and or pGAD10 and co-transformed into L40. The resulting colonies were assayed for β-galactosidase activity using a colony lift assay.

Cell lines, transient transfection and repression assays

The NIH-3T3 and COS-7 cells were obtained from P.Rodríguez-Viciano (ICRF, London); U2-OS cells were obtained from ATCC. COS-7 and U2-OS cells were propagated in DMEM-10% fetal calf serum, whereas NIH-3T3 cells were grown in DMEM-10% newborn calf serum. All transfections were done using Lipofectamine (Gibco-BRL), according to the manufacturer's instructions. COS-7 cells (1×10⁵ per 3 cm dish, 2×10⁵ per 6 cm dish) received 1 μg of plasmid per 1×10⁵ cells and were harvested 40 h after transfection. For repression assays, NIH-3T3 cells were plated the day before transfection at 1.5×10⁵ cells per 3 cm dish. Transfection mixtures contained 2 μg of plasmid per dish; 0.5 μg of effector plasmid, 1.5 μg of CAT reporter plasmids and 50 ng of a CMVlacZ reference plasmid. Cells were harvested 48 h after transfection. Reporter gene activities were determined in the same cell extract using commercial CAT and β-galactosidase ELISA kits (Boehringer Mannheim). The reporter gene activity was standardized against the reference gene activity. Normalized CAT protein values obtained with the reporter plasmid in the presence of empty expression plasmids were set to 100. Fold repression is expressed as the ratio of normalized CAT protein values in the presence of empty expression plasmids over

normalized CAT protein values in the presence of a given effector. In each case, the results shown represent the mean values of at least three independent experiments.

Immunological reagents

To generate antibodies against M33 and Ring1A, GST-M33, GST-Ring1A and MBP-Ring1A fusion proteins were produced in *E. coli*. BL21(DE3) pGST-M33 was constructed by cloning a cDNA fragment encoding amino acids 333–519 in pGEX-KG (a gift of A.Hall). pGST-Ring1A and pMALc-Ring1A were constructed by cloning a cDNA fragment obtained by PCR corresponding to amino acids 200–300 in pGEX-4T1 (Pharmacia) or pMALc2 (New England Biolabs) respectively. Expression of the fusion proteins was induced for 2 h at 37°C with 0.4 mM isopropyl- β -D-thiogalactopyranoside. The cells were resuspended in 0.05 M Tris-HCl, pH 8.0, buffer containing 0.15 M NaCl, 1 mM DTT, 0.1% Triton X-100, 10% glycerol and protease inhibitors (Complete®, Boehringer Mannheim) and sonicated. The cell lysate was centrifuged at 14 000 g for 10 min at 4°C and the supernatant collected. GST fusion proteins were isolated from bacterial extracts by affinity chromatography using glutathione-Sepharose (Pharmacia) and further purified by SDS-PAGE for use in immunization of rabbits. MBP-Ring1A protein was isolated from extracts by affinity chromatography on an amylose column (New England Biolabs). Affinity chromatography columns were prepared by coupling purified bacterially expressed proteins to CNBr-activated Sepharose (Sigma Chemical Co.). The GST-M33 antiserum was absorbed with GST-Sepharose and the anti-M33 antibodies isolated by affinity chromatography on GST-M33-Sepharose. Chicken antibodies against human Bmi1 were raised against a recombinant protein and affinity purified as described (Satijn *et al.*, 1997).

Immunoprecipitations

For immunoprecipitations, total protein extracts were made from transfected COS-7 cells. Cells were scraped in lysis buffer containing 0.05 M HEPES, pH 7.9, 0.25 M NaCl, 5 mM EDTA, 1.0 mM DTT, 0.1% NP-40 and protease inhibitors (Complete®, Boehringer Mannheim) using 0.3 ml per 6 cm dish. Cell lysates were sonicated and spun in an Eppendorf centrifuge at 12 000 r.p.m. for 15 min at 4°C. For use in immunoprecipitations, affinity purified antibodies were covalently coupled to protein A-Sepharose (Pharmacia) using dimethylpelimide (Sigma Chemical Co.). The supernatant of the lysates was precleared for 1 h with non-immune rabbit IgG-protein A-Sepharose. The precleared lysates were then incubated with anti-M33 or anti-Ring1A-Sepharose beads (15 μ l) for 1 h at 4°C with continuous rotation. After 1 h at 4°C, the beads were washed in lysis buffer two times, and transferred to fresh tubes for a final wash. Immunoprecipitated proteins were eluted in Laemmli's buffer, separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose for Western blot analysis.

Western blot analysis

Total cell extracts were prepared by adding 0.3 ml of boiling 0.01 M Tris-HCl, pH 8.0, buffer containing 1% SDS per 3 cm dish and boiling the lysates for 5 min. The viscosity of the lysates was reduced by passage through a syringe. Proteins in 10–20 μ l of extract were separated by SDS-PAGE and transferred to either nitrocellulose (Schleicher & Schüll) or Immobilon-P (Millipore) membranes. After overnight incubation in TBST (Tris-buffered saline, 0.1% Tween 20) containing 5% non-fat dried milk at 4°C, membranes were subsequently incubated with the indicated antibodies diluted in TBST for 1 h at room temperature. After washing, membranes were incubated with horseradish peroxidase-coupled goat anti-mouse IgG antibodies (Nordic) in TBST for 1 h at room temperature. Bound antibodies were detected by chemiluminescence (ECL, Amersham).

Immunofluorescence

Cells growing on glass coverslips were washed three times in PBS and fixed in freshly prepared 4% paraformaldehyde for 10 min at room temperature. The cells were washed twice for 5 min in PBS and permeabilized with PBS containing 0.5% Triton X-100 for 5 min at room temperature. After two 5 min PBS washes, the cells were incubated for 10 min in 0.1 M glycine in PBS. The cells were washed in PBS and incubated in blocking solution (PBS containing 1% non-fat dried milk, 5% horse serum, 2% bovine serum albumin and 0.1% Tween 20) for 30 min at room temperature. The fixed cells were then transferred to blocking solution without milk containing rabbit or chicken antibodies for 1 h at room temperature. Coverslips were washed three times for 5 min in PBS/0.1% Tween 20. The cells were then incubated with donkey anti-rabbit IgG coupled to Cy3 and donkey anti-chicken IgG

coupled to DTAF (both from Jackson ImmunoResearch Laboratories) diluted 1:100 in blocking solution for 1 h at room temperature. After washing four times for 5 min in PBS-Tween, cells were mounted and analysed by confocal microscopy. Fluorescent signals were then processed using image analysis software.

In vitro transcription-translation and GST protein binding assay

Full-length Ring1A cDNA (amino acids 1–377) or truncated Ring1A cDNA (amino acids 200–377) were subcloned in the pCITE4-1 vector (Novagen). RNA was synthesized with 500 ng of supercoiled plasmids and translated in the presence of 40 μ Ci of [³⁵S]Met (10 mCi/ml, 800 Ci/mmol, Amersham) using a rabbit reticulocyte lysate (Single Tube Protein System 2, Novagen). For the GST pull-down assay, 20 μ l of glutathione-agarose (Pharmacia) and bacterial protein extracts containing either GST or GST-M33 (amino acids 333–519) were mixed and rotated at 4°C for 30 min. Agarose beads were washed three times with 0.05 M Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM DTT and protease inhibitors. Immobilized GST proteins were then resuspended in 200 μ l of the same buffer containing 2 μ l of the *in vitro* translation mixtures and incubated for 1 h at 4°C with rotation. The beads were washed twice with 1 ml of buffer, transferred to fresh tubes and washed one more time. After adding 20 μ l of loading buffer, bound proteins were separated in a 10% SDS-polyacrylamide gel. Dried gels were analysed using a Phosphor-Imager (Molecular Dynamics).

In situ hybridization

Sense and antisense probes were obtained from pBluescript plasmids (Stratagene) containing full-length Ring1A, M33 or PLZF (a gift of A.Zelent) cDNAs. After linearization, *in vitro* transcription was performed using T3 or T7 RNA polymerase and digoxigenin-labelled rUTP (Boehringer Mannheim). Whole mount *in situ* hybridization was performed on day E8.5–10.5 embryos as previously described (Wilkinson, 1992). Briefly, dissected embryos were fixed overnight in 4% paraformaldehyde in PBS, dehydrated in 100% methanol, rehydrated, washed in PTW (0.1% Tween 20 in PBS), bleached with 6% H₂O₂ in PTW for 1 h, proteinase treated for 15 min (10 μ g/ml proteinase K in PTW), washed with 2 mg/ml glycine in PTW for 10 min, fixed with 0.2% glutaraldehyde/4% paraformaldehyde in PTW and prehybridized for 2 h at 70°C in hybridization buffer (50% formamide, 5 \times SSC, 50 μ g/ml yeast RNA, 1% SDS, 50 μ g/ml heparin). Hybridization was carried out for 12–16 h at 70°C in hybridization mix containing 1 μ g/ml digoxigenin-labelled riboprobe. Post-hybridization washes were as follows: 2 \times 30 min at 70°C in sol 1 (50% formamide, 5 \times SSC, 1% SDS), 10 min at 70°C in 1:1 sol 1/sol 2, 3 \times 10 min at room temperature in sol 2 (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween 20), 2 \times 30 min at 37°C in sol 2 containing 100 μ g/ml RNase A, a rapid washing at room temperature with solution 2 and then solution 3 (50% formamide, 2 \times SSC) and 2 \times 30 min at 60°C in sol 3. After three 5 min PTW washes, the embryos were blocked for 2 h in PTW containing 10% sheep serum. Detection was performed by overnight incubation at 4°C using an anti-digoxigenin Fab fragment conjugated to alkaline phosphatase (Boehringer Mannheim). After extensive PTW washes, the embryos were incubated in BCL buffer (0.1M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM MgCl₂) and the signal detected by incubation in BCL buffer containing 4.5 μ l/ml NBT and 3.5 μ l/ml BCIP.

Non-radioactive *in situ* hybridization on sections from day E11.5–17.5 mouse embryos was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993). Cryosections were fixed for 10 min in 4% paraformaldehyde, washed three times for 3 min with PBS and then incubated for 10 min in 0.1M triethanolamine (pH 8.0) containing 0.25% acetic anhydride. After three 5 min washes with PBS, the sections were prehybridized for 6 h at ambient temperature with 200–300 μ l of hybridization buffer (50% formamide, 5 \times SSC, 5 \times Denhardt's, 250 μ g/ml yeast RNA, 500 μ g/ml salmon sperm DNA) in a 5 \times SSC humidified chamber. The slides were then placed in a 50% formamide/5 \times SSC humidified chamber and incubated overnight at 65°C with 100 μ l hybridization buffer containing 200–400 ng/ml digoxigenin-labelled riboprobe. Post-hybridization washes were as follows: rapid submersion in 5 \times SSC, 1 \times 1 h in 0.2 \times SSC at 65°C and 1 \times 5 min in buffer B1 (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) at ambient temperature. Detection was carried out in B1 buffer as described above. Signals were detected by the NBT/BCIP substrate reaction in BCL buffer.

Accession numbers

The murine Ring1A (accession number Y12881) and Ring1B (accession numbers Y12880 and Y12783) cDNA sequences have been deposited in the DDBJ/EMBL/GenBank database.

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