RING1 Is Associated with the Polycomb Group Protein Complex and Acts as a Transcriptional Repressor

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The Polycomb (Pc) protein is a component of a multimeric, chromatin-associated Polycomb group (PcG) protein complex, which is involved in stable repression of gene activity. The identities of components of the PcG protein complex are largely unknown. In a two-hybrid screen with a vertebrate Pc homolog as a target, we identify the human RING1 protein as interacting with Pc. RING1 is a protein that contains the RING finger motif, a specific zinc-binding domain, which is found in many regulatory proteins. So far, the function of the RING1 protein has remained enigmatic. Here, we show that RING1 coimmunoprecipitates with a human Pc homolog, the vertebrate PcG protein BMI1, and HPH1, a human homolog of the PcG protein Polyhomeotic (Ph). Also, RING1 colocalizes with these vertebrate PcG proteins in nuclear domains of SW480 human colorectal adenocarcinoma and Saos-2 human osteosarcoma cells. Finally, we show that RING1, like Pc, is able to repress gene activity when targeted to a reporter gene. Our findings indicate that RING1 is associated with the human PcG protein complex and that RING1, like PcG proteins, can act as a transcriptional repressor.

The *Drosophila Polycomb* (*Pc*) gene is a member of the *Polycomb* group (*PcG*) gene family which is part of a cellular memory system responsible for the stable inheritance of gene activity. The *PcG* genes have been identified in *Drosophila* as repressors (18–20, 23, 30, 38, 39) of homeotic gene activity. An important clue as to the molecular mechanism underlying *Pc* action is the observation that the Pc protein has a domain homologous with the *Drosophila* heterochromatin-binding protein HP1 (31, 33, 40). This domain has been designated the chromodomain. This discovery provides an important, direct link between regulation of gene activity and chromatin structure. It suggests that Pc and HP1 operate through common mechanisms, which may involve the formation of heterochromatin-like structures (29). The chromodomain has been found to be essential for binding of Pc to chromatin. When the chromodomain is either mutated or deleted, it no longer binds to chromatin (27). Also, a conserved domain located in the C terminus of the Pc protein (32) is crucial for Pc function. A mutant *Pc* gene lacking this COOH box is unable to repress gene activity (5, 28).

The Pc protein binds to about 100 loci on polytene chromosomes in *Drosophila* salivary gland cells (44). The PcG proteins Polyhomeotic (Ph), Polycomblike (Pcl), and Posterior sex combs (Psc) share many, but not all, of these binding sites with Pc (10, 25, 34). This is consistent with the idea that PcG proteins act together in a multimeric complex. Direct evidence that supports this idea comes from immunoprecipitation (IP) experiments which showed that the PcG protein Ph coimmunoprecipitated with Pc (10).

Considering the many novel features of regulation of gene activity by changes in chromatin structure, amazingly little is known about the molecular nature of chromatin structure. In particular, the composition of the chromatin-associated PcG complex is largely unknown. In order to characterize the molecular nature of the vertebrate PcG protein complex, we employed a genetic two-hybrid system (7, 9, 16). We screened a human leukocyte two-hybrid cDNA library for proteins that interact with a vertebrate homolog of Pc, *Xenopus* Pc (XPc) (35). Here, we report the identification of a previously characterized protein, RING1, that interacts with XPc. We show that RING1 coimmunoprecipitates with a human Pc homolog, the vertebrate PcG protein BMI1 (1, 2, 4, 17, 42, 43), and HPH1, a human homolog of the PcG protein Ph (3, 15). In addition, RING1 colocalizes with these three PcG proteins in nuclear domains of SW480 human colorectal adenocarcinoma and Saos-2 human osteosarcoma cells. PcG proteins have been found not to bind directly to DNA, but it has been shown that when they are targeted as LexA or GAL4 fusion proteins, PcG proteins and HP1 can mediate transcriptional repression of reporter genes in mammalian cells and *Drosophila* embryos (5, 22, 28). Similarly, we find that when targeted to reporter genes, RING1 represses their activity. Our findings indicate that RING1 is associated with the human multimeric PcG protein complex and that RING1, like PcG proteins, can act as a repressor of gene activity.

MATERIALS AND METHODS

Yeast two-hybrid screen. The full-length coding region of XPc (35) was cloned into the pAS2 vector (7, 16) (Clontech, Palo Alto, Calif.) and used as a target to screen for interacting proteins in a two-hybrid screen (9). The other RING1 and *XPc* hybrids were derived by PCR (Expand; Boehringer) and were sequenced entirely. The pAS2-*XPc* plasmid was cotransformed with a human leukocyte Matchmaker two-hybrid library (Clontech) into the Y190 strain of *Saccharomyces cerevisiae*. The transformants were plated on selective medium lacking leucine, tryptophan, and histidine but containing 30 mM 3-amino-1,2,4-triazole (3-AT) (7, 16). From approximately 1.6×10^6 independent clones, 125 growing colonies were obtained, of which 20 were β-galactosidase positive. After DNA isolation and rescreening, two colonies remained histidine and β-galactosidase positive. These clones were further characterized by sequencing and analyzed for gene homology by using the BLAST database. The transformants were plated on medium lacking leucine, tryptophan, and histidine, with or without 30 mM 3-AT. Cells with interactions that were scored as negative failed to grow in the presence

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of 30 mM 3-AT. Due to residual HIS3 promoter activity, however, they are able to grow on medium that does not contain 3-AT (7, 16). Under these nonselective conditions, cells with negative interactions were β-galactosidase negative, and the colony color was indicated as white (see Table 1). Positive interactions meet the two criteria of growth in the presence of 30 mM 3-AT and β-galactosidase positivity. To quantitate the β-galactosidase activity, cultures of 2.5 ml were grown to an optical density at 600 nm of 1.0 to 1.2 in medium lacking leucine, tryptophan, and histidine. No 3-AT was added in the case of negative interactors to allow them to grow. The cells were permeabilized, and β-galactosidase activity was measured as described elsewhere (16; technical instructions of Clontech). Measurements were performed in triplicate, starting with three independent colonies.

Production of polyclonal rabbit and chicken antibodies. Fusion proteins were made with the C-terminal region of RING1, which was recovered from the two-hybrid screen (amino acids [aa] 214 to 375), hPc2 (aa 60 to 558), BMI1 (the entire coding region), and HPH1 (aa 165 to 700) (15). cDNAs were cloned into pET-23 expression vectors (Novagen, Madison, Wis.). Fusion proteins were produced in *Escherichia coli* BL21(DE), and the purified fusion proteins were injected into a rabbit (RING1, HPH1, hPc2 and BMI1) or a chicken (hPc2 and BMI1). Serum was affinity purified over an antigen-coupled CNBr-Sepharose column (Pharmacia, Uppsala, Sweden).

IPs and Western blotting (immunoblotting). SW480 human colorectal adenocarcinoma cells, which were grown to confluence, were lysed in ELB lysis buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES [pH 7.0], 5 mM EDTA) containing 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors, including leupeptin, benzamidine, pepstatin, and aprotinin. The cell lysate was sonicated three times with bursts of 15 s. The lysate was centrifuged at 14,000 × g at 4°C for 10 min, and the supernatant (500 μl) was aliquoted and stored at −70°C. A 25-μl volume of the supernatant was subsequently incubated with the antibodies indicated for 2 h at 4°C. Two hours of incubation gave better results than incubation for 4 h to overnight (15), since we noted that longer incubation times resulted in considerable breakdown of the antigens (data not shown). Goat anti-rabbit or goat anti-chicken immunoglobulin G (IgG) antibodies (Jackson ImmunoResearch Laboratories) were added to the mixture, and the mixture was incubated for 1 h at 4°C. Protein A-Sepharose CL-4B (Pharmacia) and ELB buffer were added to increase the volume of the mixture to 300 μl. The mixture was incubated for 1 h at 4°C, with continuous mixing. The mixture was centrifuged at 1,500 × g at 4°C for 1 min, washed with 1 ml of ice-cold ELB buffer without protease inhibitors, and centrifuged at 1,500 × g at 4°C for 1 min. This washing procedure was repeated five times. After heating and centrifugation to remove the protein A-Sepharose beads, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The blots were probed with a 1:1,000 to 1:5,000 dilution of affinity-purified RING1, hPc2, BMI1, and HPH1 antibodies. When the IP was performed with rabbit antibodies, the blot was incubated with chicken antibodies to prevent detection of the heavy chains (Fc) of the IP antibodies that remain present in the blotted immunoprecipitates. Vice versa, when chicken antibodies were used in the IP, the blots were incubated with rabbit antibodies. The secondary alkaline phosphatase-conjugated goat anti-rabbit or donkey anti-chicken IgG (heavy plus light chain) antibodies (Jackson) were diluted 1:10,000, and nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT-BCIP) (Boehringer) was used as a substrate for detection. We titrated the amount of the RING1, hPc2, BMI1, or HPH1 antibodies that was required to achieve optimal recovery of the antigen from the lysates. By optimizing the time of incubation and the amount of antibody, the majority of the RING1, hPc2, BMI1, or HPH1 proteins were immunoprecipitated or coimmunoprecipitated, indicating that the proteins indeed coimmunoprecipitated. Only residual amounts of RING1, hPc2, BMI1, or HPH1 protein were detected in the supernatants after IP (data not shown).

Immunofluorescence labelling of tissue culture cells. SW480 and Saos-2 cells were cultured and labelled as described recently (15, 37). The labelling has been analyzed by confocal laser scanning microscopy, of which single optical sections are shown below (see Fig. 4 through 6). The first two pictures of each row represent the two different scanned channels for imaging the double labelling, whereas the last picture represents the reconstituted image. For labelling, donkey anti-rabbit IgG coupled to Cy3 (Jackson ImmunoResearch Laboratories) and donkey anti-chicken or anti-mouse IgG-coupled 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF; Jackson) were used.

LexA fusion reporter gene-targeted repression assay. A LexA fusion reporter gene-targeted repression assay was performed as described previously (5). NIH 3T3 or P19 embryonic carcinoma (EC) cells were cultured in a 25-cm² flask and cotransfected with 4 μg of the HEB expression vector, 2 μg of the HEB- or heat shock factor (HSF)-inducible chloramphenicol acetyltransferase (CAT) reporter plasmid (5), 4 μg of the LexA fusion constructs, and 2 μg of the pSV/β-Gal construct (Promega) by the calcium phosphate precipitation method. The HSF-inducible CAT reporter plasmid was activated by exposure of the cells at 43°C for 1 h followed by a 6-h recovery at 37°C, as described previously (5). CAT activity (14) was normalized to β-galactosidase activity. The absolute values of CAT activity varied between independent experiments. The CAT activity in cells transfected with the CAT reporter plasmid only was therefore set at 100%, and CAT activities in cells which were cotransfected with other plasmids were expressed as percentages of this control value. The degrees of repression by LexA-
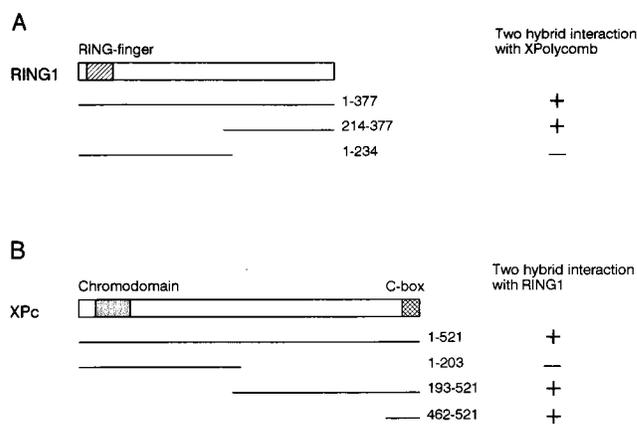

FIG. 1. RING1 and XPc interact in the yeast two-hybrid system. (A) Various regions of RING1 fused to the GAL4 activation domain were tested against full-length XPc, fused to the GAL4 DNA-binding domain. The interaction is considered positive (+) when both the *His3* (growth) and the *LacZ* (blue coloring) reporter genes are activated. Absence of detectable color or cell growth is considered negative (−). The C-terminal domain of RING1 (aa 214 to 377) was recovered from the two-hybrid screen. The domain which contains the RING finger (aa 1 to 234) does not interact with XPc. (B) Various regions of XPc fused to the GAL4 DNA-binding domain were tested against full-length RING1 fused to the GAL4 activation domain. RING1 interacts with the C-terminal region of XPc (aa 193 to 521 and 462 to 521) and not with the region that contains the chromodomain (aa 1 to 203).

RING1 or LexA-XPc are expressed as means ± standard errors of the means (SEM).

Nucleotide sequence accession number. The hPC2 sequence has been deposited with GenBank under accession no. U94344.

RESULTS

Identification of interactions between RING1 and XPc or hPc2 in the two-hybrid system. To identify genes encoding proteins that interact with or are part of the PcG multimeric protein complex, we performed a genetic two-hybrid screen (7, 9, 16). As a target protein, we chose the highly conserved *Xenopus* homolog of the PcG protein Pc (35). We screened a human two-hybrid cDNA library. The full-length coding region of *XPc* (35) was cloned into pAS2 vector (7, 16). The pAS2-*XPc* plasmid was cotransformed with a human leukocyte Matchmaker two-hybrid library (Clontech) into the yeast Y190 strain. The transformants were plated on selective medium lacking histidine, tryptophan, and leucine (7, 16). Of approximately 1.6 × 10⁶ independent clones, 125 colonies were His⁺, of which 20 were also β-galactosidase positive. After DNA isolation and retransformation, two colonies remained His⁺ and β-galactosidase positive. These clones did not grow on His⁻ plates when cotransformed with either the empty pAS2 vector or fusion proteins unrelated to the target protein.

The two isolated clones were identical. They were 0.9 kb in length and are identical with the human *RING1* gene (11, 26). The fusion of the GAL4 activation domain began at aa 214 of the RING1 protein and ended 400 bp after the stop codon. To determine which regions of RING1 are involved in the XPc-RING1 protein-protein interaction, different RING1 hybrids were constructed and tested in the two-hybrid system. Only the C-terminal portion of the RING1 protein (aa 214 to 377) interacted with XPc (Fig. 1A). The N-terminal region of RING1 (aa 1 to 234), containing the RING finger motif, did not interact with XPc (Fig. 1A).

We further determined which regions of XPc are involved in the XPc-RING1 protein-protein interaction. RING1 interacted with the small, conserved C-terminal COOH box (aa 462

TABLE 1. β-Galactosidase activities of XPc, hPc2, and RING1 interactions in the two-hybrid system

DNA-binding domain fusion protein (aa)	Activation domain fusion protein (aa)	Colony color[a]	% Relative β-galactosidase activity
XPc (1–521)	RING1 (1–377)	Blue	100[b]
	RING1 (1–234)	White	<1
	RING1 (214–377)	Blue	59
	pGAD10	White	<1
XPc (1–203)	RING1 (1–377)	White	<1
XPc (193–521)	RING1 (1–377)	Blue	95
XPc (462–521)	RING1 (1–377)	Blue	91
pAS2	RING1 (1–377)	White	<1
hPc2 (1–558)	RING1 (1–377)	Blue	110
	RING1 (1–234)	White	<1
	RING1 (214–377)	Blue	65

[a] White colonies were obtained on medium lacking both histidine and 3-AT. Blue colonies were obtained on medium lacking histidine but containing 3-AT.
[b] The average β-galactosidase activity in a triplicate experiment was 35 U. This activity was set at 100%.

to 521), which is important for the ability of Pc to repress gene activity (5, 28). In contrast, RING1 did not interact with the N-terminal domain, containing the conserved chromodomain (aa 1 to 203) (Fig. 1B).

To quantify the strengths of the interactions between the different portions of the RING1 and XPc proteins, we prepared lysates of the transformants and measured the β-galactosidase activity. The interaction between full-length XPc (aa 1 to 521) and full-length RING1 (aa 1 to 377) was found to be the strongest. The relative strengths of the other interactions are given as percentages of this interaction (Table 1). The interactions between XPc (aa 193 to 521) or XPc (aa 462 to 521) and full-length RING1 (aa 1 to 377) were both about 90% of the interaction between full-length XPc and full-length RING1. The interaction which was identified in our original two-hybrid screen, between XPc (aa 1 to 521) and RING1 (aa 214 to 377), was about 60% of the maximum strength (Table 1).

The original two-hybrid screen was performed with XPc. Recently, we isolated a novel human Pc homolog, hPc2 (35a). hPc2 shows an overall identity of 70% at the protein level with XPc. In contrast, the overall identity between hPc2 and the mouse Pc homolog, M33, is a mere 24%. Also in the C-terminal region, which we found to interact with RING1, the identity between XPc and hPc2 is almost 70% (Fig. 2). hPc2 is a human Pc homolog different from a previously described, partially characterized human Pc homolog, CBX2, or hPc1 (13). Homology between CBX2 and our novel human Pc protein is restricted to the highly conserved COOH box (Fig. 2). In contrast, hPc1 shows 86% identity and 100% similarity at the protein level with the mouse Pc homolog, M33 (Fig. 2). We conclude that there are at least two human Pc homologs. The

hPc2 protein is highly homologous to the XPc protein, whereas the other human Pc homolog, CBX2, or hPc1, is more homologous to the murine Pc homolog M33.

We tested whether full-length RING1 and the C-terminal region of RING1 also interacted with hPc2. Both interactions were positive, and the strength of the interaction between full-length hPc2 (aa 1 to 558) and RING1 (1 to 377) is slightly stronger than the interaction between full-length XPc and full-length RING1 (Table 1). Like with XPc, the full-length hPc2 does not interact with the N-terminal domain of RING1 (aa 1 to 234) which contains the RING finger motif but interacts only with the C-terminal domain of RING1 (aa 214 to 377) (Table 1). We conclude that by using a vertebrate homolog of Pc, XPc, as a target protein in the two-hybrid system, we identified the RING1 protein as interacting with both XPc and a human Pc homolog, hPc2.

RING1, hPc2, BMI1, and HPH1 coimmunoprecipitate from extracts of SW480 cells. In order to test whether the interaction between RING1 and hPc2 in the two-hybrid assay signifies an in vivo interaction, we performed IP experiments. We used extracts from SW480 human colorectal adenocarcinoma cells in which PcG proteins are expressed at a high level (15). With these extracts we found that the vertebrate PcG protein BMI1 (1, 2, 42, 43) and HPH1 and HPH2, human homologs of the PcG protein Ph, coimmunoprecipitate (15). We used polyclonal rabbit and/or chicken antibodies against RING1 and hPc2. We further used antibodies against BMI1 and HPH1 to test whether RING1 also coimmunoprecipitates with these other vertebrate PcG proteins. We found that RING1 (Fig. 3A, Input lane) was present in the immunoprecipitates with hPc2 and BMI1 antibodies (Fig. 3A, hPc2 IP and BMI1 IP lanes, respectively). We also tried to immunoprecipitate RING1 by using HPH1 antibodies. Since both RING1 and HPH1 antibodies are rabbit derived, the heavy chains (Fc) (~50 kDa) of the rabbit anti-HPH1 antibodies are detected by the goat anti-rabbit alkaline phosphatase-conjugated antibody. Unfortunately, the 54-kDa RING1 molecule migrates at the same position as these Fc fragments, thus preventing detection of RING1 in immunoprecipitates with the HPH1 antibodies.

Using chicken antibodies to detect hPc2, we found that hPc2 (Fig. 3B, Input lane) coimmunoprecipitates when rabbit antibodies against RING1, BMI1, or HPH1 are used for IP (Fig. 3B). Furthermore, using chicken antibodies against BMI1, we found that BMI1 (Fig. 3C, Input lane) coimmunoprecipitates with RING1, hPc2, and HPH1 (Fig. 3C). Finally, HPH1 (Fig. 3D, Input lane) coimmunoprecipitates with RING1, hPc2, and BMI1 (Fig. 3D). None of the antigens being investigated were detected when the specific IP antibodies were replaced by preimmune sera (Fig. 3, Mock IP lanes) or unrelated antibodies or when the first antibody was merely omitted from the IPs (data not shown). This underlines the specificity of the IPs.

In conclusion, using specific antibodies, we show that RING1, hPc2, BMI1, and HPH1 coimmunoprecipitate from extracts of SW480 human colorectal adenocarcinoma cells.

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Xenopus Pc   LQEPDIILLDSDLDEPIDLRCVKSRCDSDQVDKPIQVETQNPQSVDVDV----------------------CESQFKPFFGNIVITDVTANCLTVTFKEYITV
             :..:.:..:::::::::::::: ::::: ....    :: . : :..:.:               . :.:::::::.:::::::::::::::::::.::
Human Pc2    LRQPEVILLDSDLDEPIDLRSVKSRSEAGEPPSSLQVKPETPASAAVAVAAAAAPTTTAEKPPAEAQDEPAESLSEFKPFFGNIIITDVTANCLTVTFKEYVTV
                                                                                ::::::::::::::::::::::::::::
                             :::: :: . :.. . . ...  :: . :...                 .. ...:::::::.::: ::. :
Human Pc1          LPEMSAGEESSSSDSDPDSASPPSTGQNPSVSVQTSQDWKPTRS-------------LIEHVFVTDVTANLITVTVKESPTSVGFFNLRHY
                  :.:.:.::::.:::::::::.:.::.:.::.:::::::::      :::::::::::::::::::::::::::::::::::::::
M33          SVAASGGQEGHTAPGEGRKPPALSELSTGEENSSSDSDPDSTSLPSAAQNLSVAIQTSQDWKPTRS-------------LIEHVFVTDVTANLITVTVKESPTSVGFFNLRHY
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FIG. 2. Partial sequence of the hPc2 protein. The predicted amino acid sequence of hPc2 is aligned with the XPc, human Pc1 (CBX2), and M33 protein sequences. The region corresponds to the partially characterized and published sequence of CBX2 (hPc1). Identical amino acids (double dots), conservative substitutions (single dots), and the conserved COOH box (shaded region) are indicated.
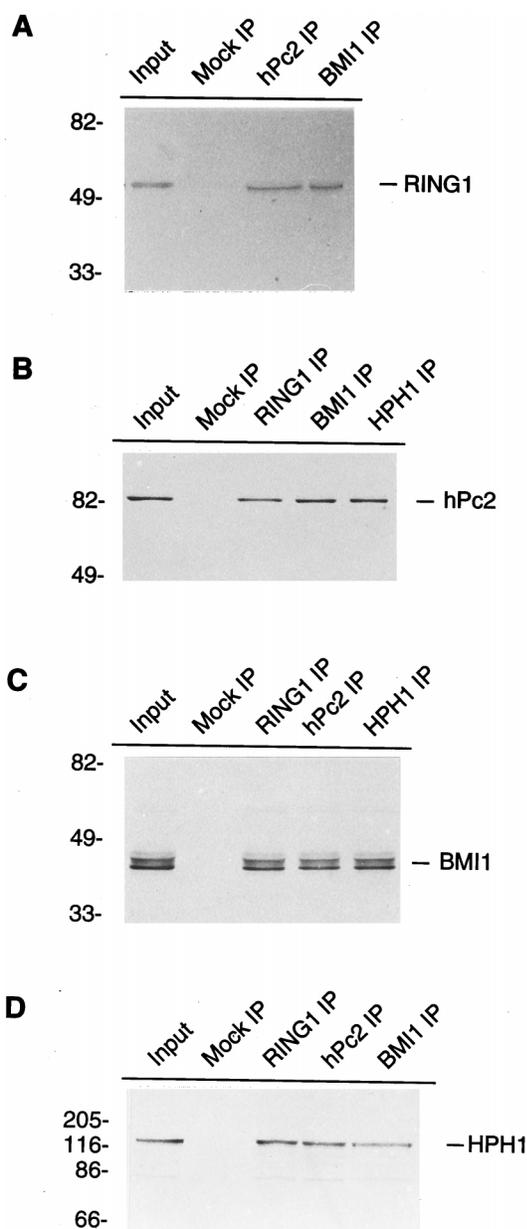
FIG. 3. RING1, hPc2, BMI1, and HPH1 coimmunoprecipitate from extracts of SW480 cells. IP experiments were performed with extracts of SW480 human colorectal adenocarcinoma cells. (A) IP was performed with polyclonal chicken antibody against hPc (hPc2 IP) or BMI1 (BMI1 IP) or preimmune serum (Mock IP). The resulting immunoprecipitates were Western blotted and incubated with rabbit anti-RING1 antibody. The approximately 54-kDa RING1 protein was detected in the SW480 cell extract (Input) and in the immunoprecipitates. (B) IP was performed with polyclonal rabbit antibody against RING1 (RING1 IP), BMI1 (BMI1 IP), or HPH1 (HPH1 IP) or preimmune serum (Mock IP). The resulting immunoprecipitates were Western blotted and incubated with chicken anti-hPc2 antibody. The approximately 82-kDa hPc2 protein was detected in the SW480 cell extract (Input) and in the immunoprecipitates. (C) IP was performed with polyclonal rabbit antibody against RING1 (RING1 IP), hPc2 (hPc2 IP), or HPH1 (HPH1 IP) or preimmune serum (Mock IP). The resulting immunoprecipitates were Western blotted and incubated with chicken anti-BMI1 antibody. The approximately 44- to 47-kDa BMI1 protein was detected in the SW480 cell extract (Input) and in the immunoprecipitates. (D) IP was performed with polyclonal rabbit antibody against RING1 (RING1 IP), hPc2 (hPc2 IP), or BMI1 (BMI1 IP) or preimmune serum (Mock IP). The resulting immunoprecipitates were Western blotted and incubated with rabbit anti-HPH1 antibody. The approximately 120- to 124-kDa HPH1 protein (15) was detected in the SW480 cell extract (Input) and in the immunoprecipitates. Molecular masses (in kilodaltons) are indicated on the left.

This indicates an in vivo association between RING1 and the PcG proteins hPc2, BMI1, and HPH1.

RING1, hPc2, BMI1, and HPH1 colocalize in nuclei of human SW480 and Saos-2 cells. We next analyzed the subcellular localization of RING1 in relation to that of the PcG proteins hPc2, BMI1, and HPH1 by performing immunofluorescence labelling experiments. We used SW480 cells, in which we found that RING1, hPc2, BMI1, and HPH1 coimmunoprecipitate (Fig. 3). The use of rabbit anti-RING1 and chicken anti-hPc2 and anti-BMI1 antibodies allowed double-labelling experiments. Both the RING1 and hPc2 proteins were found in the nuclei of SW480 cells, throughout the nucleoplasm. They completely colocalize in large, brightly labelled domains (Fig. 4A to C). Colocalization is not obvious for the more homogeneous distribution pattern. The fine granular pattern is too complex to allow analysis of any systematic colocalization. RING1 and BMI1 also colocalize in the same brightly labelled domains (Fig. 4D to F). Furthermore, hPc2 and BMI1 colocalize in the large domains (Fig. 4G to I), which is in agreement with our earlier finding that the XPc and Xbmi1 proteins interact with each other in vitro (35). Finally, hPc2 and HPH1 colocalize in the same brightly labelled domains (Fig. 4J to L). Also, the related human homolog of Ph HPH2 (15) colocalizes with hPc2 in these domains (data not shown). RING1 colocalizes with hPc2 and BMI1 (Fig. 4A to F). HPH1 colocalizes with hPc2 (Fig. 4J to K) and with BMI1 (15) in the large domains. This implies that RING1 and HPH1 colocalize in these domains, although we could not directly test this, due to the fact that both the RING1 and HPH1 antibodies are rabbit derived.

We next analyzed the subcellular localization of RING1 in relation to that of hPc2 and BMI1 in Saos-2 human osteosarcoma cells. We found that RING1, hPc2, and BMI1 are expressed at a high level in these cells (35a). As for SW480 cells, we found colocalization of RING1 and hPc2 in large, brightly labelled domains (Fig. 5A to C). RING1 and BMI1 also colocalize in the same brightly labelled domains (Fig. 5D to F). These results demonstrate that the colocalization of RING1 with PcG proteins hPc2 and BMI1 is not restricted to one cell type.

The labelling pattern of the large PcG domains in SW480 cells is reminiscent of the distribution of the PML protein (21). The PML protein is a RING finger-containing protein with growth suppressor properties (24) and which is disturbed in acute promyelocytic leukemia (8). The PML protein is concentrated in 10 to 20 nuclear bodies per nucleus (21). Double-labelling experiments utilizing rabbit anti-RING1 and mouse anti-PML antibody 5E10 (41) showed two distinct labelling patterns (Fig. 6A to C). The RING1 and PML proteins apparently do not colocalize despite the presence of the RING finger motif in both proteins. We also tested the possibility that the PcG domains colocalize with other, well-characterized nuclear domains. Distinct, speckled nuclear domains highly enriched in splicing factors were detected when a mouse monoclonal antibody against the SC35 splicing factor was used (12). The RING1 protein (Fig. 6D) and the speckles, recognized by the anti-SC35 antibody (Fig. 6E), do not colocalize (Fig. 6F). Finally, we compared the distribution patterns of RING1 (Fig. 6G) with that of kinetochores, which are centromere-associated protein structures. Kinetochores are recognized by the human autoimmune serum H33 (Fig. 6H) (41). Although some brightly labelled domains appear to be in close contact, the majority clearly do not colocalize (Fig. 6I). The results underline the specificity of the domains in which RING1 and PcG proteins colocalize.

We conclude that RING1 colocalizes with the vertebrate PcG proteins hPc2, BMI1, and HPH1 in large nuclear domains
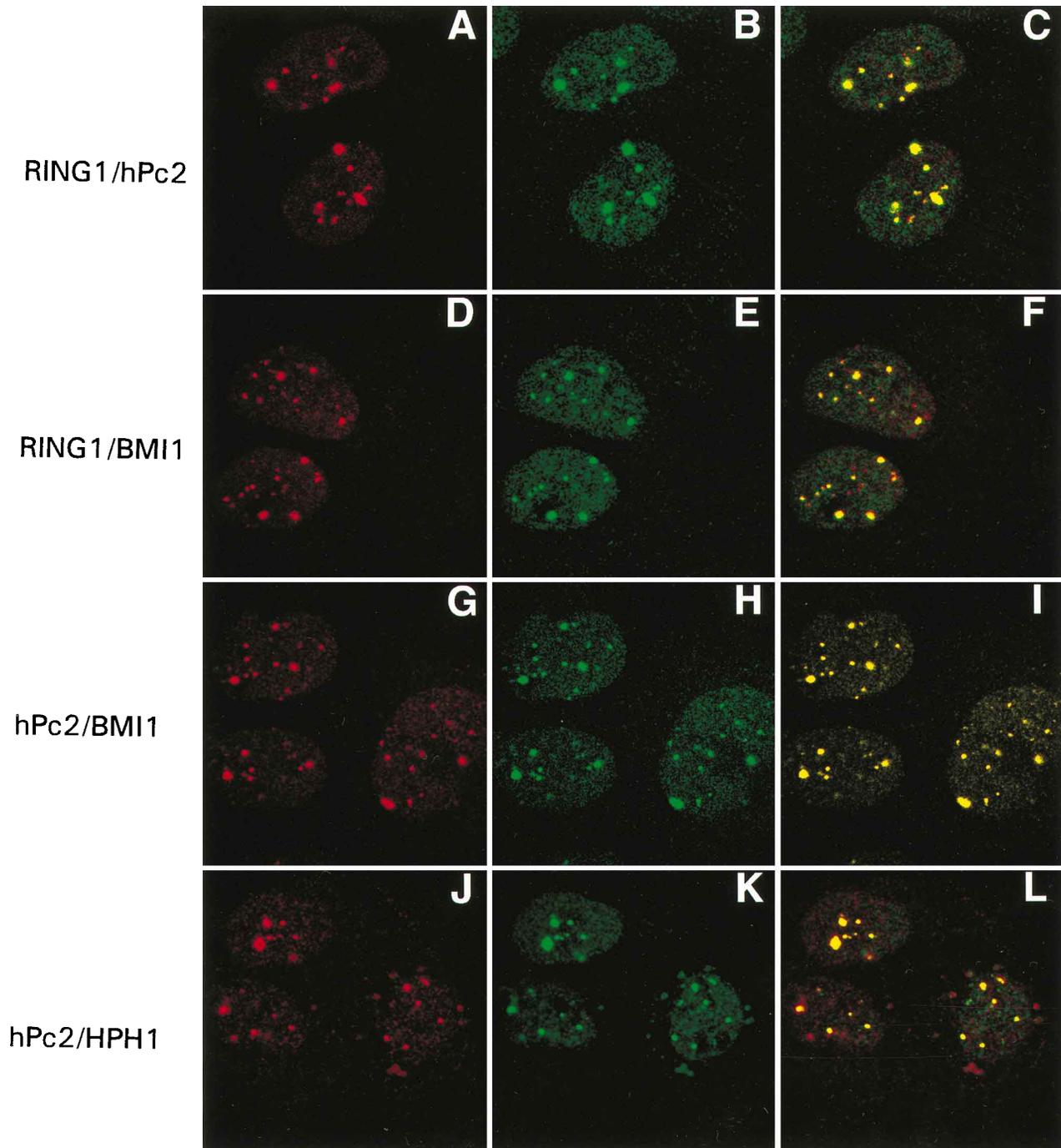
FIG. 4. RING1 colocalizes with PcG proteins in nuclear domains of SW480 cells. Rabbit anti-RING1 (A) and chicken anti-hPc2 (B) double labelling demonstrates that RING1 and hPc2 are homogeneously distributed in the nucleus but are also concentrated in large, brightly labelled domains. RING1 and hPc2 colocalize in these large domains (C) (yellow). Rabbit anti-RING1 (D) and chicken anti-BMI1 (E) double labelling demonstrates that the staining patterns of RING1 and BMI1 are very similar, again with a homogeneous staining throughout the nucleus and bright labelling in numerous large domains. RING1 and BMI1 are found to colocalize in the large, bright domains (F). Chicken anti-hPc2 (G) and rabbit anti-BMI1 (H) double labelling demonstrates colocalization of hPc2 and BMI1 in the large, bright domains (I). Chicken anti-hPc2 (J) and rabbit anti-HPH1 (K) double labelling demonstrates colocalization of hPc2 and HPH1 in the large, bright domains (L).

of SW480 human colorectal adenocarcinoma cells and Saos-2 human osteosarcoma cells. These results further underline the identified two-hybrid interaction between RING1 and XPc or hPc2. They strengthen the notion that RING1, hPc2, BMI1, and HPH1 are part of one multimeric protein complex.

RING1 represses HEB- and HSF-induced CAT gene activity. The PcG complex proteins are known to be involved in repressing homeotic gene activity in *Drosophila* (23, 30). So far, no PcG protein has been found to bind directly to DNA (20, 30, 39). To investigate the ability of PcG proteins to repress
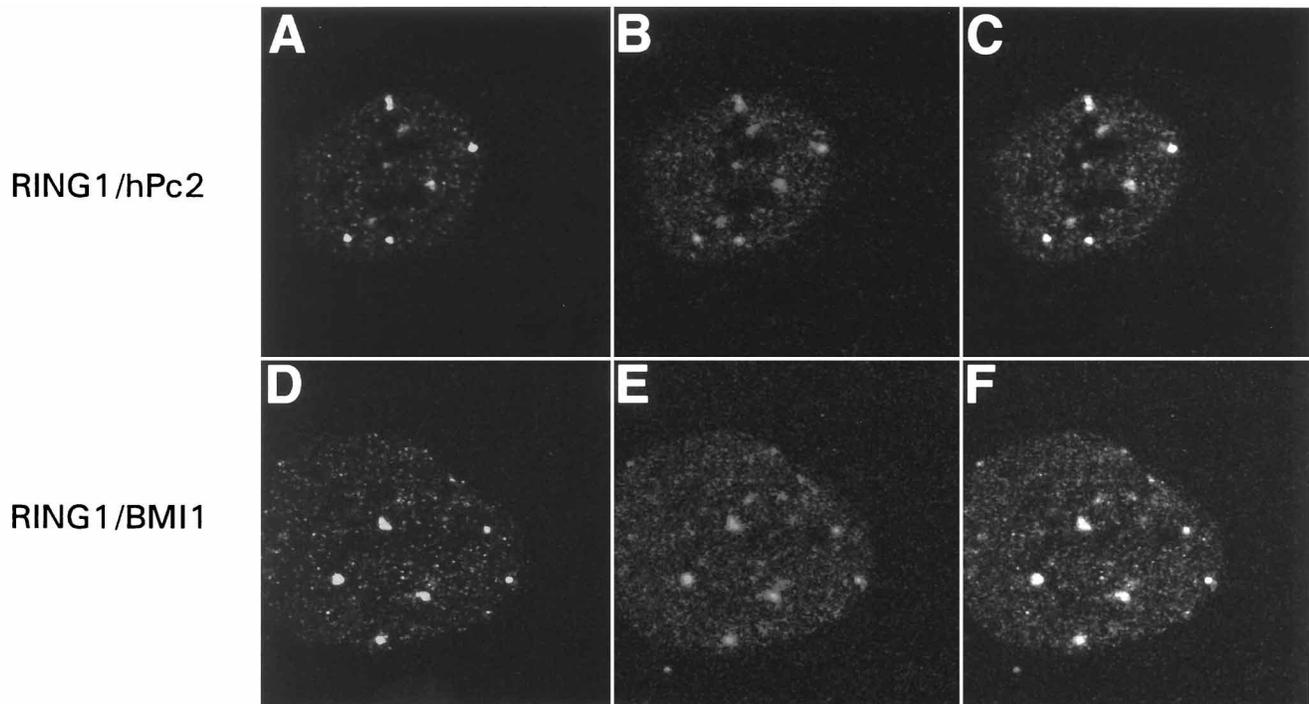
FIG. 5. RING1 colocalizes with PcG proteins in nuclear domains of Saos-2 cells. Rabbit anti-RING1 (A) and chicken anti-hPc2 (B) double labelling demonstrates that, as in SW480 cells (Fig. 4), RING1 and hPc2 colocalize in large, brightly labelled domains (C). With rabbit anti-RING1 (D) and chicken anti-BMI1 (E), similar distribution patterns and colocalizations (F) were observed.

gene activity, they have been targeted to reporter genes as LexA or GAL4 fusion proteins. In this manner, it has been shown that PcG proteins, as well as the chromodomain-containing heterochromatin-binding protein HP1, can mediate transcriptional silencing of reporter genes in mammalian cells and *Drosophila* embryos (5, 22, 28). Since RING1 interacts with and colocalizes with the vertebrate PcG proteins hPc2 and BMI1, we tested the possibility that RING1 also functions as a repressor when targeted to a promoter. We analyzed the abilities of LexA-RING1 and LexA-XPc fusion proteins to repress gene activity, using different CAT reporter constructs, as described previously (5).

NIH 3T3 or P19 EC cells were transfected with a construct containing a tandem of four LexA operators, binding sites for transcriptional activators (HEB or HSF), and the *hsp70* TATA promoter region, immediately upstream of the CAT reporter gene (5). As transcriptional activators, plasmids encoding HEB or the endogenous HSF were employed. In the absence of HEB or HSF, no CAT activity was observed (data not shown). Maximum CAT activity in the presence of HEB or HSF was set at 100% (control; Fig. 7). Cotransfection of LexA alone had no significant influence on HEB-induced (Fig. 7a; 96% ± 8% [mean ± SEM; $n = 7$]) or HSF-induced (Fig. 7b; 97% ± 7% [mean ± SEM; $n = 5$]) CAT activity. We found that LexA-RING1 repressed CAT expression to approximately 30%, with both HEB (Fig. 7a; 30% ± 7% [mean ± SEM; $n = 7$]) and HSF (Fig. 7b; 33% ± 10% [mean ± SEM; $n = 5$]). LexA-XPc repressed CAT expression to approximately 20%, with both HEB (Fig. 7a; 25% ± 8% [mean ± SEM; $n = 7$]) and HSF (Fig. 7b; 19% ± 9% [mean ± SEM; $n = 5$]). The degree of repression we observe for RING1 and XPc is very similar to the previously reported repression to about 20% by *Drosophila* Pc when targeted to a reporter gene in both mammalian and *Drosophila* cell lines (5, 6, 22). Also, targeting to a reporter

gene of HP1 leads to a three- to fourfold decrease in the transcriptional activity of the reporter gene (reference 22 and data not shown).

The interaction between RING1 and PcG proteins suggests that they might also collaborate in repressing gene activity. To test this, we cotransfected LexA-RING1 with hPc2. Vice versa, we cotransfected LexA-hPc2 (instead of LexA-XPc) with RING1. RING1 and hPc2 were cloned into the pcDNA3 vector (Invitrogen, San Diego, Calif.), in which the cDNAs are under control of the enhancer from the immediate early gene of human cytomegalovirus for high-level transcription. Four micrograms of these plasmids was cotransfected with various amounts of LexA-RING1 or LexA-hPc2 as indicated in Fig. 8. Repression of HSF-induced CAT expression was found to increase with the amount of plasmid encoding the LexA fusion protein (Fig. 8). Transfection of still larger amounts of LexA fusion proteins did not result in higher levels of repression (data not shown). It is also significant that the degrees of repression we observed for LexA-hPc2 and LexA-XPc are very similar (compare Fig. 7 and 8). We also found that cotransfection of the hPc2 (Fig. 8A) or RING1 (Fig. 8B) protein enhanced the repression of CAT expression by LexA-RING1 and LexA-hPc2, respectively. The maximum effect was observed with the smallest amount of LexA fusion proteins (0.5 μg). hPc2 was able to enhance LexA-RING1-mediated repression of CAT expression most efficiently (Fig. 8A). Taking these results together, we conclude that RING1, like PcG proteins, is able to repress gene activity when targeted to a reporter gene.

DISCUSSION

RING1 is associated with a human PcG complex. PcG proteins are involved in the stable repression of gene activity during embryonic development. It has been proposed that PcG
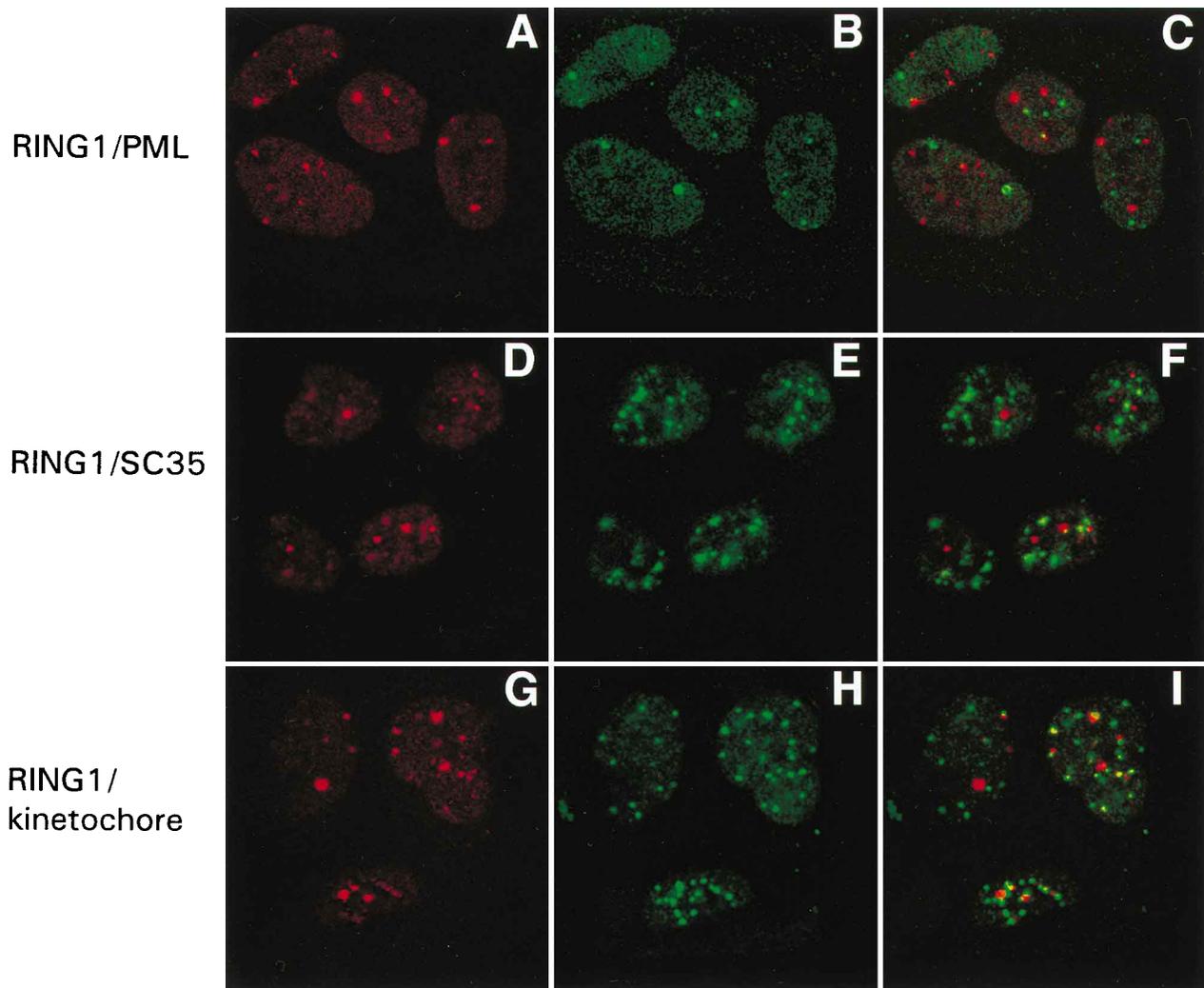
FIG. 6. RING1 does not colocalize with well-characterized nuclear factors. Double labelling of SW480 cells with rabbit anti-RING1 (A) and mouse anti-PML (B) shows that the distribution patterns of RING1 and PML bodies are distinct and no colocalization is detected (C). RING1 (D) also does not colocalize with speckles (E), nuclear domains which are highly enriched in splicing factors. Speckles are detected with a mouse monoclonal antibody against the SC35 splicing factor (E and F). The distribution pattern of RING1 (G) also differs from that of kinetochores (H), which are centromere-associated protein structures. Kinetochores are recognized by the human autoimmune serum H33 (H). Although some domains appear to be in close contact, the majority clearly do not colocalize (I).

proteins form multimeric complexes that bind to chromatin. This idea is based on the observations that different PcG proteins bind in overlapping patterns to about 100 loci on polytene chromosomes in *Drosophila* salivary gland cells (34, 44) and that the *Drosophila* PcG proteins Pc and Ph coimmunoprecipitate (10). Employing the two-hybrid system, we have now identified a protein, RING1, that specifically interacts with vertebrate homologs of *Drosophila* Pc, XPc and hPc2. Our data further indicate an in vivo association between RING1, hPc2, BMI1, and HPH1. RING1 coimmunoprecipitates with hPc2, BMI1, and HPH1, and these proteins colocalize in nuclear domains in human SW480 and Saos-2 cells. Our present data extend our previous finding that BMI1, and HPH1 and HPH2, two human homologs of the PcG protein Ph, are part of a human PcG protein complex (15). Together, our data indicate that RING1, hPc2, BMI1, HPH1, and HPH2 are part of a human PcG protein complex.

Functional significance of the interaction between RING1 and PcG proteins. The *RING1* gene was isolated and characterized several years ago. As yet, no function has been assigned to the RING1 protein (11, 26). The significance of RING1 is based on the presence of the RING finger, a particular zinc finger motif (36). The RING finger has been found in a wide variety of proteins, such as BMI1 and PML, the former being a PcG protein. Our results show that the interaction between RING1 and XPc does not involve the RING finger motif. However, this does not exclude the possibility that RING1 is associated with other proteins through the RING finger. Our data therefore indicate the existence of at least two functional domains in RING1, i.e., the RING finger motif and the C-terminal domain that is involved in the interaction with Pc.

This study identifies, for the first time, interactions between RING1 and proteins with a known function. This finding potentially categorizes RING1 as a PcG protein (see below). This idea is reinforced by our observation that RING1 can act as a transcriptional repressor, indicating that RING1 is involved in transcriptional regulation. Transcriptional silencing by LexA-RING1 is enhanced by hPc2, and vice versa. Furthermore, it is important that the degrees of repression by RING1 and XPc or hPc2 in the transient-targeting assay are similar. In this con-
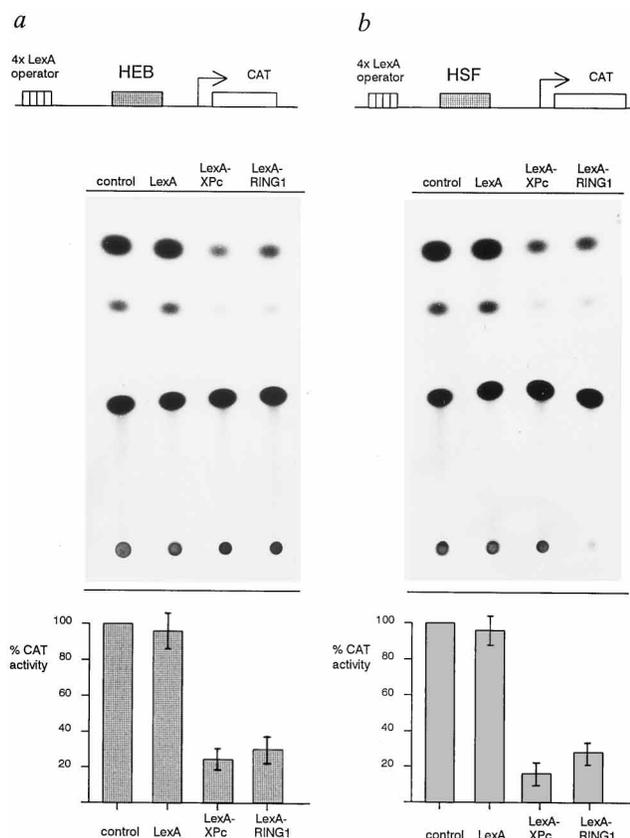
a

b

FIG. 7. RING1 represses HEB- and HSF-induced CAT gene activity. (a) Repression of activation by cotransfected HEB. CAT reporter expression is maximally induced by HEB in the absence of any LexA fusion protein (control). Cotransfection of LexA alone has hardly any influence on HEB-induced CAT expression, which is still about 97% of the total (LexA). Cotransfection of either LexA-RING1 or LexA-XPc represses CAT activity by three- or fourfold, respectively. (b) Repression of activation by endogenous HSF. Cotransfection of either LexA-RING1 or LexA-XPc represses CAT activity by three- or fivefold, respectively. The overall repression by the LexA fusion constructs is a bit higher when the HSF-inducible CAT expression vector is used. Visualization of CAT activity by means of thin-layer chromatography of a representative experiment is shown. The CAT activity in cells transfected with the CAT reporter plasmid only was set at 100%, and CAT activities in cells which were cotransfected with other plasmids were expressed as percentages of this control value. The bars represent the average degree of repression by LexA-RING1 or LexA-XPc in seven (a) or five (b) independent experiments (means ± SEM). The actual values are indicated in the text.
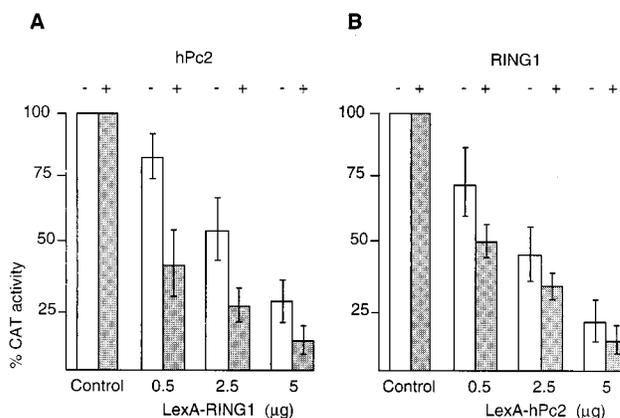

FIG. 8. RING1 and hPc2 collaborate in repression of HSF-induced CAT gene activity. The indicated amounts of LexA-RING1 (A) or LexA-hPc2 (B) were cotransfected with 4 μg of hPc2 (A) or with 4 μg of RING1 (B). Repression of HSF-induced CAT expression with (+) or without (−) hPc2 or RING1 is indicated. As in Fig. 7, CAT activity in cells transfected with the CAT reporter plasmid only was set at 100%, and CAT activities in cells which were cotransfected with other plasmids were expressed as percentages of this control value. The bars represent the average degree of repression by LexA-RING1 or LexA-hPc2 in three independent experiments (means ± SEM).

text, it may also be important that RING1 specifically interacts with the small, highly conserved domain in the C termini of Pc proteins (32). When this domain is deleted, the Pc protein loses its ability to repress gene activity (5, 28). The interaction of RING1 with specifically this C-terminal domain suggests that the involvement of this domain in transcriptional repression depends on or is in part mediated by its binding to RING1.

In conclusion, the association of RING1 with the PcG complex and its ability to repress gene activity suggest that RING1 is involved in the PcG-mediated repression of gene activity.

Is RING1 a PcG protein? RING1 is associated in vivo with PcG proteins, and, like PcG proteins, RING1 acts as a transcriptional repressor. Does this imply that RING1 is a novel PcG protein? About 15 *PcG* genes have been described genetically (18). At present, seven *Drosophila PcG* genes have actually been cloned and characterized (19, 39). It is therefore possible that RING1 is a vertebrate homolog of the product of

a *Drosophila PcG* gene that has not yet been characterized. No *Drosophila* RING1 homolog has been described. It is, however, also possible that RING1 is a typical vertebrate PcG protein. Although functions of PcG proteins appear to be conserved (for a review, see reference 39), this does not imply that the respective PcG complexes must have exactly the same compositions.

Another point to be considered is that in *Drosophila*, a gene is defined as a *PcG* gene when a mutation in this gene results in homeotic transformations. If RING1 is a *PcG* gene product in this strict sense, then mutations in a *Drosophila* RING1 homolog should result in homeotic transformations. It is, however, clear that PcG proteins bind to more target genes than homeotic genes (34, 44). RING1 could be involved in the repression of target genes other than homeotic genes. This can be achieved if RING1 is part of a subset of PcG complexes with a partially different composition or if RING1 targets PcG complexes with loci other than homeotic genes. In that case, no homeotic transformations are to be expected, while RING1 is still involved in the PcG-mediated repression of gene activity.

In summary, we are tempted to conclude that we provide novel clues about the molecular nature of the PcG complex by identifying RING1 as being associated with the PcG protein complex. The association with the PcG protein complex, together with the ability of RING1 to repress gene activity, is in agreement with similar roles for RING1 and PcG proteins. Most importantly, our data provide insight into possible roles of RING1, whose function has been enigmatic since its discovery.

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