HPC3 Is a New Human Polycomb Orthologue That Interacts and Associates with RING1 and Bmi1 and Has Transcriptional Repression Properties*

Polycomb (PcG) proteins were first described in *Drosophila* as factors responsible for maintaining the transcriptionally repressed state of Hox/homeotic genes in a stable and heritable manner throughout development. A growing number of vertebrate genes related to the *Drosophila* PcG proteins have recently been identified, including two Polycomb orthologues, Pc2 and M33. PcG proteins form multiprotein complexes, termed PcG bodies, that are thought to repress transcription by altering chromatin structure. Here we report the identification and characterization of HPC3 (human Polycomb 3), a novel PcG protein isolated in a yeast two-hybrid screen using human RING1 as bait. HPC3 shows strong sequence similarity to *Drosophila* Pc and also to vertebrate Pc2 and M33, particularly within the chromo-domain and C-box. Previous studies indicate that M33 and human Pc2 (HPC2) can interact with RING1, and we show here that HPC3 also binds to RING1. This interaction is dependent upon the HPC3 C-box but, only partially, on the RING finger of RING1. In contrast to HPC2, HPC3 interactions with RING1 are only observed in *vivo* with covalently modified forms of RING1. HPC3 also colocalizes with other PcG proteins in human PcG bodies. Consistent with its role as a PcG member, HPC3 is able to act as a long range transcriptional silencer when targeted to a reporter gene by a heterologous DNA-binding domain. Taken together, these data suggest that HPC3 is part of a large multiprotein complex that also contains other PcG proteins and is involved in repression of transcriptional activity.

Genetic analyses in *Drosophila* have revealed two classes of genes that are involved in maintaining the long term expression patterns of Hox/homeotic genes (1–3), after they have been established by other mechanisms. The Polycomb group (PcG)1 gene products are transcriptional repressors that maintain the silenced state of Hox genes in a stable and heritable manner, while the members of the trithorax group act to maintain the state of transcriptional activation (reviewed in Refs. 4–10). It has been postulated that the PcG family comprises at least 30–40 genes (11, 12), which cooperate to maintain the repressed state of Hox genes, but at present less than half of these loci have been characterized at the molecular level. Loss of PcG function in *Drosophila* does not directly alter the initial Hox gene expression patterns, but instead leads to ectopic expression at later developmental stages that often results in transformations of segmental identity. One hypothesis is that PcG-mediated repression is achieved by altering higher order chromatin structure, but exactly how the PcG proteins achieve this heritable silenced state is still a matter of debate (discussed in Refs. 4 and 13–17). Recent data indicate that PcG-mediated repression of gene activity might be achieved by interaction with nucleosomes (18) and histone deacetylation (19) and suggest that PcG proteins may function antagonistically to the human SWI/SNF family of chromatin remodeling complexes (20).

A steadily increasing number of mammalian genes have been identified that share structural and functional similarity to members of the *Drosophila* PcG (reviewed in Refs. 21–23), providing opportunities for comparing the functions of these genes between mammals and flies. Early evidence for conservation of the PcG came from the work on the murine proto-oncogene Bmi1, which was originally identified as a collaborator of *Eμ-myc* in lymphomagenesis (24, 25). Recently, it has been shown that Bmi1 regulates cell proliferation and senescence as well as apoptosis via the *ink4a/arf* gene locus (26, 27). Both Bmi1 and the highly related Mel18 gene products share regions of high homology with the *Drosophila* PcG proteins Psc and Su(z)2 (28–30). Knockout mice for these genes show posterior transformations of the axial skeleton that often correlate with anterior shifts of *Hox* gene expression boundaries during development, similar to those seen in *Drosophila* PcG mutants (31, 32). Typically, mammalian PcG phenotypes are not as extensive as the more prominent *Drosophila* loss-of-function mutations, an observation that may be explained by the increased potential for overlapping PcG gene functions in vertebrate embryos (33).

Biochemical evidence in both *Drosophila* and vertebrates indicates that PcG proteins form large multiprotein complexes that are associated with DNA. Yeast two-hybrid and co-immunoprecipitation experiments reveal interactions between the following mouse and human PcG proteins: Mph1/Mph2, Hph1/Hph2, Bmi1, Mel18, M33, Mcp2, and Hpc2 (34–38). Recently, it has been shown that human as well as murine RING1 is also part of this complex (39–41), which may be large...
but heterogeneous in size (36). All of these proteins colocalize in human cell lines as large nuclear domains termed PcG bodies (34–37, 39, 41, 42). PcG bodies have been shown to be stably associated with large areas of pericentromeric heterochromatin in a cell cycle-independent manner (42).

It has previously been shown that PcG complexes in Drosophila are likely to vary in composition, depending on chromosomal localization (15). In support of this, the PRC1 complex of PcG proteins, although in excess of 2 million daltons in size, only contains a subset of PcG proteins (20). The existence of different complexes has also been observed in vertebrates. For example, the mammalian homologues of Drosophila E(z) and ESC, EZH1/EZH2 and EED, constitute part of a mammalian PcG complex that is biochemically and cytologically distinct from the PcG complex formed by human BMI1, RING1, HPC2, HPH1/HPH2, or their murine counterparts (43–45), and it has been suggested that the different PcG complexes function in an antagonistic manner (46).

To further characterize the mammalian PcG complex, we set out to identify additional RING1-interacting proteins by yeast two-hybrid screening. Here we describe the identification and characterization of a new RING1-interacting protein, Pc3. Pc3, Pc3 shows strong similarity within the conserved chromodomain and C-box to the known vertebrate Polycomb proteins Pc2 and M33 but, in addition, possesses an atypical RED domain containing a long stretch of alternating Arg-Asp and Arg-Glu repeats. We show that HPC3 (human Polycrom 3) can bind and associate specifically to two other PcG members, Bmi1 and RING1, but interestingly, in vivo interactions are only observed with covalently modified forms of RING1. In line with its role as a Polycrom protein, HPC3 is also able to function as a long range transcriptional repressor when recruited to DNA by a heterologous DNA-binding domain. Taken together, these data suggest that HPC3 is a member of the PcG complex of transcriptional repressors and may contribute to its gene silencing function.

MATERIALS AND METHODS

Yeast Two-hybrid Screens and Interaction Assays—Full-length RING1 (47) was cloned into the pAS2−vector (CLONTECH) and used as bait to screen a human lung (WI-38) Matchmaker two-hybrid library (Clontech). Instead of using the RING1 interacting clone, isolated from the yeast two-hybrid screens, cDNA library filters were hybridized in CHURCH buffer (0.5 M NaH2PO4, pH 7.5, 10 mM EDTA, 7% SDS, 100 µg/ml sonicated salmon sperm cDNA (Amersham Pharmacia Biotech), 15% (v/v) formamide overnight at 60 °C and then washed to a stringency of 0.2× standard sodium citrate, 0.1% SDS at 65 °C. Rapid amplification of cDNA ends were performed by centrifugation at 14,000 rpm for 5 min at 4 °C. Lysates were then loaded by using the Marathon kit (CLONTECH). A full-length HPC3 cDNA clone was subsequently obtained and subcloned into the mammalian expression vector pMLV2T (50).

Immunoprecipitations and Western Blotting—Human 293 (HT1080) fibrosarcoma cells were grown to approximately 70% confluence and transfected using the Qiagen SuperFect transfection reagent. Cells expresses the HPC3 and RING1 interaction in vivo were precleared for 1 h using normal mouse antibodies (10 µg; Sigma) and protein G-Sepharose (Amersham Pharmacia Biotech) to minimize nonspecific binding. Precleared lysates were then incubated with the 9E10 monoclonal antibody for 1 h at 4 °C with gentle agitation. Immunocomplexes were then incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody (1:1000; DAKO) and horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:2000; DAKO) used as secondary antibodies. All blots were developed using the ECL reagent system (Amersham Pharmacia Biotech). In vivo, 2C4 cells were washed twice with PBS and once in distilled water before mounting and analysis by confocal microscopy. All samples were visualized and recorded on a Zeiss LSM510 confocal microscope.

Immunofluorescence Microscopy—Cells were grown to near confluence on glass coverslips and washed with PBS before fixing with 4% formaldehyde, followed by permeabilization with 0.5% Triton in PBS for 10 min. After washing with PBS, cells were incubated with block solution (10% fetal calf serum in PBS with 0.1% Tween 20) for at least 10 min at room temperature. Incubation with antibodies was in block solution for 1 h at room temperature, followed by washing once with PBS and once with PBS containing 0.1% Tween 20 and 0.1% BSA. Coverslips were washed twice with secondary fluorochrome-conjugated antibodies (swine anti-rabbit fluorescein isothiocyanate conjugate, DAKO, and sheep anti-mouse Texas Red; Amersham Pharmacia Biotech) diluted 1:200 in block solution for 1 h at room temperature. Coverslips were then washed once in PBS plus 0.1% Tween 20, twice in PBS, and once in distilled water before mounting and analysis by confocal microscopy. All samples were visualized and recorded on a Zeiss LSM510 confocal microscope.

Regression Assays—Full-length HPC3 and the indicated HPC3 fragments were subcloned into the mammalian expression vector, pMLV-GAL4, as N-terminal fusions with the GAL4 DNA-binding domain. The CAT reporter plasmids used were those described by Schoorlemmer et al. (54). pG5tkCAT contains five GAL4-binding sites upstream of a HSVtk promoter; pG5–1.6tkCAT contains an additional 1.6-kb fragment inserted between the GAL4-binding sites and the promoter; ptkCAT contains the same HSVtk promoter but lacks the GAL4-binding sites; pG3-7 and HT1080 cells were grown in DME plus 10% fetal calf serum and split 1:8–1:10 the day before transfection into 3-mm culture plates at 37 °C. Total cDNA transfected per well of cells was 5 µg: 2.5 µg of CAT reporter plasmid, 1.67 µg of effector plasmid, and 0.83 µg of pSV-β-Gal (Promega) reference plasmid. Cells were harvested 40–48 h after transfection, and reporter gene expression was determined in the same cell extract using CAT and β-galactosidase enzyme-linked immunosorbent assay kits with substrate enhancer (Roche Molecular Biochemicals). Expression values are standardized against β-galactosidase expression from the pSV-β-Gal reference plasmid. CAT expression values obtained with the reporter plasmid in the presence of empty pMLV-GAL4 effector plasmid were set to 100 arbitrary units, and CAT levels in cells transfected with HPC3 or RING1 were expressed relative to those obtained with the empty GAL4 vector. The results represent the mean values of five independent experiments.
RESULTS

Identification and Isolation of a RING1-interacting cDNA Clone—As RING1 has been previously identified as a mammalian PcG protein (39), we wanted to identify novel RING1-interacting proteins, which, by inference, could be part of the PcG complex. We therefore performed a yeast two-hybrid screen of a nontransformed human lung cDNA library, where RING1 is known to be expressed (data not shown), using full-length RING1 as bait. After cDNA isolation and subsequent retransformation to test for false positives, 57 colonies remained of potential interest and were sequenced. Twelve of these clones, representing overlapping cDNA fragments from the same gene, could all be translated in an identical reading frame (data not shown) with high sequence identity to the previously described “C-box” interaction domain present in other PcG proteins (see Fig. 1; Ref. 53). The interaction of other clones with RING1 is currently under investigation.

Thirteen additional cDNA clones for this RING1-interacting gene were obtained by high stringency screening of a human breast carcinoma cDNA library, and sequence analysis revealed that all clones overlapped with the original yeast clone but contained additional 5′ sequence. Further 5′ sequence was obtained by 5′ rapid amplification of cDNA ends. Conceptual translation of the assembled cDNA sequence indicates the presence of a 1167-base pair open reading frame encoding a previously unidentified 389-amino acid protein (Fig. 1A).

HPC3 Has High Sequence Homology with Other Polycomb Genes—Database searches resulted in a number of significant matches to known Polycomb-related genes, including Drosophila Pc, murine M33 and human Pc2 (Table I). We thus call this new gene Polycomb 3 or Pc3 (Fig. 1A). The level of sequence identity is much higher within the N- and C-terminal regions of the predicted protein (Table I; Fig. 1, B and C), which include two previously identified Pc subdomains. The N-terminal region contains a chromatin organization modifier domain or chromodomain (Fig. 1B), which is found in over 40 proteins, including a subset of PcG proteins (54). Multiple sequence alignment of the HPC3 chromodomain with other chromodoms shows a high degree of sequence identity ranging from 60 to 65%, with the highest level of identity found within the core of the domain (Table I; Fig. 1B). The C-terminal region of HPC3 contains a C-box protein-protein interaction domain found in a number of Pc homologues (55). Compared with other vertebrate PcG proteins, Pc3 is most similar in sequence to the mammalian Pc3, a New Mammalian Polycomb Protein

PcG—A diversity of interacting proteins, which, by inference, could be part of the PcG complex (39), have been reported. Using the yeast two-hybrid system, we have identified a novel protein encoded by the Pc3 gene that interacts specifically with RING1. The interaction is shown to be specific for RING1, since it is not observed with other RING1 interacting proteins. The interaction of RING1 with Pc3 is also shown to be specific for the C-terminal region of Pc3 and the C-box domain of RING1.

Mapping the Interaction Domains between RING1 and HPC3—The C-box domain of HPC3 was isolated as the RING1-interacting sequence in the yeast two-hybrid screen. This conserved domain has previously been shown to be necessary and sufficient for the interactions of both HPC3 and XPC with RING1 (39, 40). To determine whether this also applies to HPC3 and to test for the specificity of the HPC3-RING1 interaction, we used the yeast two-hybrid system. Constructs were made that express the GAL4 DNA-binding domain (GAL4 DBD) fused to either full-length RING1, RING1 lacking the RING finger, or the RING finger alone (Fig. 2A). Constructs were also made for HPC3 with the GAL4 activation domain (GAL4 AD) fused to full-length HPC3, the HPC3 C-box, or HPC3 lacking the C-box (Fig. 2A). The particular yeast strain (PJ69–4A) used in these assays shows significant endogenous β-galactosidase activity, and thus several negative controls were also included (Fig. 2B).

To quantitate relative interaction strengths between the various RING1 and HPC3 fusion proteins, a β-galactosidase liquid assay was used (Fig. 2B). The interaction between full-length RING1 and the HPC3 C-box alone is comparable with that seen
GAL4 fusion constructs were assayed for domain.

with full-length HPC3 (Fig. 2B). Consistent with this, removal of the C-box results in a substantial reduction in the strength of the interaction (Fig. 2B). This demonstrates that the C-box is necessary and sufficient for the interaction of HPC3 with RING1. Our observations are similar to those for the HPC2 and Xp-RING1 interaction (39, 40). The domains within RING1 that are necessary for an interaction with HPC3 are less clear, and although the RING finger alone is not sufficient, in the context of full-length RING1, it does appear to contribute to the overall interaction strength (Fig. 2B).

RING1-GAL4 DNA binding domain fusion proteins

RING1 RING finger

HPC3-GAL4 Activation domain fusion proteins

HPC3 C-box

co-immunoprecipitations of transiently co-transfected Myc-tagged HPC3 and RING1 were performed. The high molecular mass isoforms of RING1 can be resolved into two main species (Fig. 3C, lane 3, single and double arrowheads) that correspond with the bands observed in Fig. 3, A and B. The presence of transfected Myc-HPC3 protein was confirmed by immunoblot analysis using the anti-Myc antibody 9E10 (Fig. 3B, lane 4, single arrowhead). No protein could be detected in cells that had been transfected with the empty expression vector, thus confirming the specificity of the anti-Myc antibody (Fig. 3C, lane 1). Co-expression of RING1 and HPC3 in 2C4 cells, followed by immunoprecipitation of Myc-tagged HPC3, did not produce the low molecular mass RING1 band (Fig. 3C, lane 5, absence of single arrowhead species). However, the higher molecular mass RING1-specific band is represented in co-precipitations with HPC3 (Fig. 3C, lane 5, double arrowheads), although we do observe an intermediate RING1 species (Fig. 3B, single arrowhead). Together these data suggest that RING1 can be post-translationally modified, although the nature of this modification is not known at present.

To investigate the in vivo interaction of HPC3 and RING1, co-immunoprecipitations of transiently co-transfected Myc-tagged HPC3 and RING1 were performed. The high molecular mass isoforms of RING1 can be resolved into two main species (Fig. 3C, lane 3, single and double arrowheads) that correspond with the bands observed in Fig. 3, A and B. The presence of transfected Myc-HPC3 protein was confirmed by immunoblot analysis using the anti-Myc antibody 9E10 (Fig. 3B, lane 4, single arrowhead). No protein could be detected in cells that had been transfected with the empty expression vector, thus confirming the specificity of the anti-Myc antibody (Fig. 3C, lane 1). Co-expression of RING1 and HPC3 in 2C4 cells, followed by immunoprecipitation of Myc-tagged HPC3, did not produce the low molecular mass RING1 band (Fig. 3C, lane 5, absence of single arrowhead species). However, the higher molecular mass RING1-specific band is represented in co-precipitations with HPC3 (Fig. 3C, lane 5, double arrowheads), although we do observe an intermediate RING1 species (Fig. 3C, lane 5, double asterisks), the nature of which is unclear. The anti-Myc-antibody was not able to precipitate any RING1 species from cells transfected only with the empty expression vector (Fig. 3C, lane 4). We conclude from these data that RING1 exists in a number of isoforms in vivo and that a covalently modified form is associated with HPC3.

HPC3 Localizes with RING1 and BMI1 in PcG Bodies—It has previously been shown that RING1 colocalizes with a number of other mammalian PcG proteins in discrete nuclear foci that have been termed PcG bodies (39, 42). To characterize the subcellular localization of HPC3, double immunolabeling experiments with RING1, HPC3 and the human PcG protein BMI1 were performed in a number of cell lines. An anti-HPC3 peptide antiserum ASA12 (raised against residues 201–215) was used to stain HPC3, while the anti-RING1 peptide antiserum ASA8 or a BMI1 monoclonal antibody was used to stain PcG bodies (42). The specificity of the anti-HPC3 antibody was confirmed by immunoblot analysis of transfected cells and in vitro translated HPC3 (data not shown). In the three cell lines tested, we found that endogenous HPC3 colocalizes with the same PcG bodies that contain RING1 and BMI1 (Fig. 4, A and B). Additionally, a homogeneous microparticulate labeling of HPC3 throughout the nucleus is also observed (Fig. 4B). The number and size of PcG bodies is known to vary in different cell

| Protein | No. of residues | Overall | Chromodomain | C-box | Excluding chromodomain/C-box |
|---------|----------------|---------|--------------|-------|------------------------------|
| Pc      | 390            | 24      | 48           | 91    | 13                           |
| XPc     | 471            | 29      | 66           | 54    | 16                           |
| M33     | 519            | 32      | 62           | 51    | 16                           |
| HPC2    | 58             | 32      | 72           | 21    | 22                           |

**FIG. 2.** HPC3 interacts with RING1 via its conserved C-box domain. A, schematic representation of the constructs used in the yeast two-hybrid system; B, co-transformants of HPC3- and RING1-GAL4 fusion constructs were assayed for β-galactosidase activity (arbitrary units). The combinations of RING1 and HPC3 constructs are listed. Negative controls were co-transformations of the empty GAL4 DBD and AD vectors with RING1 and HPC3, respectively. Measurements were taken in triplicates, with the S.D. value indicated.
lines (42). In 2C4 (HT1080) fibrosarcoma cells, HPC3 is concentrated into two prominent nuclear foci (Fig. 4B, upper panels), whereas in U-2 OS and SAOS-2 osteosarcoma cells, colocalization in multiple PcG bodies can be observed (Fig. 4B, middle and lower panels). These results clearly demonstrate that HPC3 colocalizes with BMI1 and RING1 in PcG bodies, although in U-2 OS and SAOS-2 cells, HPC3 appears to form additional foci that do not contain BMI1 (Fig. 4B). The association of HPC3 and BMI1 was further analyzed by yeast interaction experiments, which indicate that these two proteins not only colocalize but that HPC3 also interacts with full-length BMI1 (data not shown).

HPC3 Is a Transcriptional Repressor—In order to further classify HPC3 as a mammalian PcG protein, we assessed its ability to silence transcription when targeted to a reporter gene in vivo (Fig. 5). Since RING1/RING1a has been shown to repress transcription of reporter genes (39, 41), we also tested this protein in parallel with HPC3. We transiently expressed HPC3-GAL4 and RING1-GAL4 fusion constructs and assayed for CAT expression from reporter plasmids containing five UAS binding sites for the GAL4 DBD either adjacent to a HSVtk promoter (pG5tkCAT; Fig. 5A) or 1.6 kb upstream from the promoter (pG5–1.6-tkCAT; Fig. 5A). Expression of increasing amounts of HPC3-GAL4 resulted in decreased CAT expression (Fig. 5B). The repression activity is dose-dependent, with half-maximal CAT expression obtained from transfections containing 25-fold less HPC3-GAL4 construct than CAT reporter. Maximal repression by HPC3 is dependent on the presence of the UAS sites, although we do observe some repression at high concentrations with the ptkCAT control vector (Fig. 5B). This may be due to squelching of the basal transcriptional machinery by HPC3-GAL4 overexpression. Compared with equivalent amounts of RING1 plasmid, HPC3 displays a 3-fold greater repression (Fig. 5C). This is true for both reporter plasmids...
where HPC3 appears to be better at repression from a distance than RING1 (Fig. 5C). Similar results were also obtained in HT1080 cells (data not shown).

Transcriptional repression by both M33 and HPC2 is dependent on the presence of the C-box domain (39, 41, 53). To assess the role of the HPC3 C-box in repression activity, constructs were made for the C-box domain alone and HPC3 lacking the C-box (compare Fig. 2A). Co-transfection of HPC3 \( \Delta \) C-box with the pG5tkCAT reporter plasmid results in maximal repression of CAT expression and is indistinguishable from full-length HPC3 (Fig. 5D). However, the HPC3 C-box alone retains some repression activity, since the GAL4-C-box fusion results in a 2-fold decrease in CAT expression (Fig. 5D). Thus, the C-box domain, which is critical for the HPC3-RING1 interaction, is not necessary for repression by HPC3 in this assay.

**DISCUSSION**

**HPC3 Is a Human Orthologue of Drosophila Polycomb**—The amino acid sequence of HPC3 comprises several domains that have been identified in proteins that show similarity to Drosophila Polycomb (56, 57). Drosophila Pc contains an N-terminal chromodomain, implicated in chromatin binding (57, 58), as well as a conserved C-terminal protein-protein interaction motif termed the C-box, which is required for transcriptional repression (59, 60). Like the other vertebrate homologues, HPC3 also contains an N-terminal chromodomain and C-terminal C-box (Fig. 1). The HPC3 chromodomain is more highly related to that of HPC2 than M33, XPc or Pc, whereas the C-box of HPC3 is more similar to XPc and Pc than to HPC2 (Table I). This may have implications for the specificity of the interactions of HPC1 and HPC2 with RING1, given that we have shown that HPC3 appears to only interact with higher molecular mass isoforms of RING1. A sequence alignment of HPC3 and Pc shows identities distributed throughout the length of both sequences, with the HPC3 RED/RD domain in a similar linear position to the polyhistidine sequence in Drosophila Pc (Fig. 1D). This domain is found in a wide range of different proteins such as the cytokine IK factor (61), Drosophila shuttle craft (62), atrophin-1 (63), RD protein (64), and the U1–70K snRNP protein (65), but its function is not clear at present.

**HPC3 Interaction with RING1**—Previously, it has been reported that RING1 interacts with the human PcG homologue HPC2 and is associated with, or is part of, the human PcG complex (39). Recently, it has been shown, that in addition to HPC2, RING1 can also interact with the well characterized mammalian PcG protein BMI1 (40). As BMI1 interacts in vivo with the human homologues of Polyhomeotic, HP1 and HP1H2 (34, 36), it would appear that RING1 is part of a large and distinct PcG complex (40). HPC3, HPC2, and mouse M33 all contain a C-box, which is required for binding to RING1, an interaction that is not dependent on the RING finger of RING1 or on the HPC3 chromodomain. However, as with HPC2, if the RING finger is not present, the RING1-HPC3 association is reduced (Fig. 2B), suggesting that the RING finger contributes to the full integrity of the interaction. The identification of HPC3 as a third Polycomb orthologue that can interact with RING1 raises the question as to whether all Pc proteins interact with RING1 in an equivalent manner or whether there might be specificity differences. Our results show that HPC3 is associated with a high molecular mass isoform of RING1 (Fig. 3). The Myc tag transfection experiments clearly demonstrate that both the high and low molecular mass isoforms of RING1...
can be generated from one cDNA species and are not produced by alternative RNA splicing. The nature of the covalent modification that produces these RING1 isoforms is unclear, but our data are consistent with the idea that this modification is required to facilitate the interaction with HPC3. These results might explain why both HPC2 and HPC3 utilize a similar RING1 interaction domain, yet each appears to interact with different RING1 isoforms. It is tempting to speculate that the different specificities of HPC2 and HPC3 for RING1 are dependent not on the presence of a functional interaction domain per se but on the post-translational modification state of RING1. Further studies are in progress to address these issues.

In contrast to the results reported here, Satijn et al. (39) did not observe modified forms of RING1 in SW480 cells by Western blot analysis. The reasons for these discrepancies are unclear, but they could relate to either the RING1 antisera used in both studies or the nature of the cell extracts. Further studies will be necessary to clarify these apparent discrepancies, although in this study, two different RING1-specific antisera gave consistent results, with modified RING1 isoforms observed in both in vitro translated RING1 (Fig. 3A) and Myc-tagged RING1 transfections (Fig. 3B).

**HPC3 Colocalizes to PcG Bodies**—Recent data suggest that PcG bodies are generated by a network of multiple interactions between the participating proteins and that subsets of complexes exist that differ in composition (36, 38, 40, 43–45). Murine Bmi1 corresponds to the vertebrate homologue of Drosophila Psc (28, 29) and is also involved in multiple protein-protein interactions (34, 36–38, 40). It has recently been suggested that Bmi1 and RING1 may serve as core proteins for the establishment of a multimeric PcG complex (40). Similar to HPC2, we also find that murine Bmi1 can interact with HPC3 in yeast (data not shown). Given that HPC3 is a new vertebrate Pc homologue, we would have predicted that HPC3 colocalizes with other PcG proteins in the same recently identified PcG bodies that are known to contain HPC2, BMI1 and RING1 (42). Our results clearly support this prediction, in that HPC3 colocalizes with PcG bodies in three different human cell lines (Fig. 4). Thus, it appears that RING1 can be associated with BMI1, HPC2, and HPC3 within the same PcG body, although the exact nature of these interactions in vivo remains to be determined. We also note that, in addition to the major HPC3 immunofluorescence foci, a more diffuse granular staining pattern is observed (Fig. 4B), similar to that reported for BMI1, HPC2, RING1, and HPH1 (36, 39), and it is possible that HPC2 and HPC3 are distributed differently within these smaller foci.

**HPC3 Is a Transcriptional Repressor**—PC proteins in both Drosophila and mice are involved in repressing Hox gene activity (for recent reviews, see Refs. 17 and 23). Several individual PcG proteins, including Pc, Bmi1, RING1, Ring1A, Mpc2, M33, and HPC2, have been demonstrated to act directly as transcriptional repressors in mammalian cells (35, 39, 41, 53,

![Fig. 5. HPC3 represses transcription of a CAT reporter gene.](http://www.jbc.org/)

**A** schematic representations of the reporter plasmids used in the repression assays. pG5tkCAT contains five GAL4 UAS immediately upstream of the HSVk promoter. pG5–1.6-tkCAT contains five GAL4 UAS placed 1.6 kb upstream of the same HSVk promoter. ptkCAT uses the same promoter but lacks the GAL4 UAS. **B** HPC3 represses transcription in a dose-dependent manner. COS-7 cells were transfected with either pG5tkCAT (open squares) or ptkCAT (open circles), together with pSV-β-Gal reference plasmid and increasing amounts of HPC3-GAL4. **C** transcriptional repression by HPC3 and RING1. COS-7 cells were transfected with pG5tkCAT (gray columns) or pG5–1.6-tkCAT (black columns) together with HPC3-GAL4, RING1-GAL4, or the empty pMLVGA4 plasmid. **D** mapping the repression domain of HPC3. COS-7 cells were transfected with pG5tkCAT and the indicated HPC3-GAL4 constructs. In all assays, CAT expression was determined 40–48 h after transfection and normalized to β-galactosidase levels from the pSV-β-Gal reference plasmid. Results are expressed as normalized CAT levels (arbitrary units) relative to those obtained in the presence of the pMLVGA4 plasmid. Values represent the averages of five independent experiments, with the S.D. values indicated.
59, 66). Here we extend these observations by showing that HPC3 can function as a repressor of reporter gene activity when bound either proximal or 1.6 kb away from a promoter (Fig. 5, B and C). This repression-at-a-distance is similar to that observed with M33 (41) and is consistent with other experiments showing that several mammalian and Drosophila PcG proteins can exert their repressive effects over several kilobases (4, 59, 67).

Previous studies have shown that repression by Pc, M33, and HPC2 requires the conserved C-box domain (41, 53, 59). In contrast to these studies, we show here that although the C-box alone does have some weak repressive function, it does not appear to be required for repression in the context of full-length HPC3 (Fig. 5D). This finding is not easily explained and could reflect the presence of an additional repression domain in HPC3 that is not found in the other Polycomb genes. However, we cannot exclude the possibility that this surprising observation is due to differences in the reporter systems, cell types, or construct deletions used. With regard to this last possibility, it is notable that studies with Drosophila Pc showed that a C-terminal 118 residue deletion was less deleterious for repression than an 86-residue deletion (59). A similar observation was made for M33, where an N-terminally extended GAL4 fusion of the M33 C-box was a more potent repressor than either full-length M33 protein or the C-box alone (41). Taken together, these data suggest that HPC3 is part of a large multigene complex that also contains other PcG proteins and is involved in repression of transcriptional activity.

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