Identification and characterization of human Polycomb-group proteins.
Satijn, D.P.E.

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CHAPTER 2

Identification and characterization of interactions between the vertebrate Polycomb-group protein BMI1 and human homologs of Polyhomeotic.


Identification and Characterization of Interactions between the Vertebrate Polycomb-Group Protein BMI1 and Human Homologs of Polyhomeotic

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In Drosophila melanogaster, the Polycomb-group (PcG) genes have been identified as repressors of gene activity. They are part of a cellular memory system that is responsible for the stable transmission of gene activity to progeny cells. PcG proteins form a large multimeric, chromatin-associated protein complex, but the identity of its components is largely unknown. Here, we identify two human proteins, HP1H and HP2H, that are associated with the vertebrate PcG protein BMI1. HP1H and HP2H coimmunoprecipitate and cofractionate with each other and with BMI1. They also colocalize with BMI1 in interphase nuclei of U-2 OS human osteosarcoma and SW480 human colorectal adenocarcinoma cells. HP1H and HP2H have little sequence homology with each other, except in two highly conserved domains, designated homology domains I and II. They share these homology domains I and II with the Drosophila PcG protein Polyhomeotic (Ph), and we, therefore, have named the novel proteins HP1H1 and HP1H2. HP1H, HP2H, and BMI1 show distinct, although overlapping expression patterns in different tissues and cell lines. Two-hybrid analysis shows that homology domain II of HP1H interacts with both homology domains I and II of HP2H. In contrast, homology domain I of HP1H interacts only with homology domain II of HP2H, but not with homology domain I of HP2H. Furthermore, BMI1 does not interact with the individual homology domains. Instead, both intact homology domains I and II need to be present for interactions with BMI1. These data demonstrate the involvement of homology domains I and II in protein-protein interactions and indicate that HP1H and HP2H are able to heterodimerize.

In Drosophila melanogaster, the genes of the Polycomb group (PcG) and trithorax group (trxG) are part of a cellular memory system that is responsible for the stable inheritance of gene activity. The PcG and trxG genes have been identified in Drosophila as repressors (PcG) (17, 22, 30) and activators (trxG) (16) of homeotic gene activity. In PcG mutants, the expression patterns of homeotic genes are initially normal, but in later embryonic phases, homeotic genes become expressed in regions of the embryo where they normally are repressed (22, 29). Besides Polycomb (Pc), an estimated 30 to 40 mutants exhibit similar, characteristic posterior homeotic transformations. These are collectively referred to as Polycomb group (PcG) mutants (15). The Pc protein binds to about 100 loci on polytene chromosomes in the Drosophila salivary gland (37). Also the PcG proteins Polyhomeotic (Ph), Polycomblike (Pcl) and Posterior sex combs (Psc) share many, but not all of these binding sites with Pc (10, 13, 18, 25). This is consistent with the idea that PcG proteins work together in a multimeric protein complex. Direct evidence that Pc is part of a multimeric complex comes from experiments which showed that the PcG protein Ph coimmunoprecipitates with Pc (13).

An important clue about the molecular mechanism underlying PcG action is the observation that the Pc protein shares a homologous domain with the Drosophila heterochromatin-binding protein HP1 (23). 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. This is further suggested by the finding that Pc is associated with chromatin over stretches of many kilobases. Pc specifically covers the silent part of the homeotic bithorax complex, which indicates that Pc represses gene activity by heterochromatinization (21).

PcG genes have originally been identified in Drosophila, but mammalian homologs of PcG genes have been identified recently. A mouse Pc homolog, M33 (24), and a Xenopus Pc homolog, XPC (26), have been characterized. Another PcG gene, Posterior sex combs (Psc), possesses significant sequence homology to the murine oncogene Bmi1 (6, 34, 35). Targeted deletion of Bmi1 results in a posterior transformation of the embryo and an anterior shift in the expression of homeotic genes (32, 33). Vice versa, overexpression of Bmi1 results in an anterior transformation and a posterior shift in the expression pattern of the homeotic gene Hoxc-5 (3). Also, targeted deletion of mel-I8, a closely related Bmi1 homolog, results in a posterior transformation of the embryo and an anterior shift in the expression of homeotic genes (1). Collectively, these data show that, also in vertebrates, PcG proteins are involved in the regulation of homeotic genes.

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 the genetic two-hybrid system (7, 11, 12, 14). We screened a human skeletal muscle cDNA library for proteins that interact with the vertebrate PcG protein Bmi1. We report the identification of two novel human proteins that are associated with Bmi1. The proteins have little sequence homology with each other, except in two conserved homology domains, I and II, which they share with the Drosophila PcG protein Ph. Based on the sequence homologies with Ph, we named these proteins HP1H and HP1H2. Two-hybrid analysis indicates that the homology domains are involved in protein-protein interactions and that HP1H1 and HP1H2 can be able to heterodimerize through the homology domains.

MATERIALS AND METHODS

Isolation of the HPFH1 and HPFH2 genes. The full-length coding region of Xbmi2 (7) was cloned into the pAS2 vector (7, 11, 14) (Clontech, Palo Alto, Calif.) and used as "bait" to screen for interacting proteins in a two-hybrid system (12). The pAS2.Xbmi2 plasmid was cotransfected with a human leukocyte MatchMak human genomic DNA library. Two-hybrid transformants were plated on selective medium lacking the leucine, tryptophan, and histidine amino acids, but containing 30 mM 3-aminotriazole (11, 14) Approximately 1.6 x 10^8 independent clones were obtained. Three hundred growing colonies were obtained from which two clones (HPH1 and HPH2) based on homologous domains with Drosophila Ph and Ras2 (20). A total of 1.2 kb of this clone was the 3' noncoding region. The coding region of HPHF2 as well as the first 5'-1,000 bp of the coding region of Ras2 were used to screen a 10g1 human fetal brain cDNA library (Clontech). Filters were hybridized overnight at 40°C in 0.5 x SSC (1 X SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 10 X Denhardt's solution, 1% dextran sulfate, 0.1% sodium dodecyl sulfate, (SDS), 100 μg of denatured hering sperm DNA per ml, and an α-32P-α-β-TAP labeled probe (5 x 10^6 cpm/ml). After being washed twice for 60 min at 55°C in 0.2 X SSC and 0.1% SDS, the filters were autoradiographed with intensifying screens for 2 days at -70°C. This led to the isolation of the HPFH2 cDNA.

RNA analysis. Multitissue Northern blots containing approximately 1 μg of poly(A)^+ RNA per lane from different human tissues or human cell lines were obtained commercially (Clontech). The U-2 OS cell line was not present on the commercial Northern blot. Poly(A)^+ RNA of U-2 OS was isolated and blotted, and the expression patterns of HPFH1, HPFH2, and Bmi1 were analyzed. To allow a comparison with the commercial Northern blot, we blotted poly(A)^+ RNA of SW480. The blot was reprobed on the commercial blot, and all the U-2 OS cDNA bands were strongly expressed in this cell line. The bands were hybridized with [α-32P]-α-β-TAP labeled DNA probes and the filters were autoradiographed with intensifying screens at -70°C with X-ray films.

Analysis of interacting protein domains with the two-hybrid system. Indicated fragments of the cDNAs encoding HPFH1 and HPFH2 were derived by PCR (Expand, Boehringer). The fragments were subcloned into the pAS2 (GALA-A-Domaining domain [GALA-BD]) or GALD10 (GALA-activation domain [GALA-AD]) vector. The fragments were sequenced over their entire length. The resulting plasmids were cotransformed into the yeast Y190 strain. The transformants were plated on medium lacking the leucine, tryptophan, and histidine amino acids, with or without 30 mM 3-aminotriazole. Interactions that were scored negative failed to grow in the presence of 30 mM 3-aminotriazole. Because of residual HIS3 promoter activity, they are able, however, to grow on medium that does not contain 3-aminotriazole (11, 14). Under these nonselective conditions, negative interactions were β-galactosidase negative and the colony color was indicated as white (Table 1). Positive interactions met the two criteria of growth in the presence of 30 mM 3-aminotriazole, and they were β-galactosidase positive. To quantify 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 the leucine, tryptophan, and histidine amino acids. No 3-aminotriazole was added in the case of negative interactors to allow them to grow. Cells were permeabilized, and β-galactosidase activity was measured as described previously (11, 14; technical instructions from Clontech). Measurements were performed in triplicate, starting with three independent cultures. To exclude the possibility that the negative interactors did not produce either one of the fusion proteins, we Western blotted equal amounts of protein and incubated the blots with monoclonal antibodies that specifically recognize the GALA-BD or the GALA-AD protein (Chotek). All positive and negative interactors expressed both GALA-BD fusions and the GALA AD fusions at approximately the same levels (data not shown).

Immunochemical staining of tissue culture cells. U-2 OS and SW480 cells were cultured and labelled as described recently (29). Labelling has been ana-

<table>
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<tr>
<th>DNA-binding domain interaction (aa)</th>
<th>Activation domain fusion (aa)</th>
<th>Colony color</th>
<th>% Relative β-galactosidase activity</th>
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<td>HPH2 (137-432)</td>
<td>Blue</td>
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</tr>
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<td>HPH2 (137-375)</td>
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* White colonies were obtained on medium lacking both histidine and 3-aminotriazole. Blue colonies were obtained on medium lacking histidine, but in the presence of 3-aminotriazole.
Isolation and characterization of the human *HPH1* and *HPH2* genes. To identify genes encoding proteins that interact with or are part of the vertebrate PeG multimeric protein complex, we performed a genetic two-hybrid screen (7, 11, 12, 14). As target protein, we chose the *Xenopus* homolog of the vertebrate PeG protein Bmi1 (2, 3, 26, 35). At the protein level, Xbmi1 is 90% identical and 95% similar to mouse and human Bmi1 (26). We screened a human two-hybrid cDNA library. The full-length coding region of Xbmi1 (26) was cloned into the pAS2 vector (11, 14). The pAS2-Xbmi1 plasmid was co-transformed with a human leukocyte Matchmaker two-hybrid library (Clontech) into the yeast Y190 strain. The transformants were plated on selective medium lacking the histidine, leucine, and tryptophan amino acids and in the presence of 3 mM 3-aminotriazole (11, 14). Of approximately 1.6 x 10^6 independent clones, 300 colonies were His^+^, of which 16 were also β-galactosidase positive. After DNA isolation and retransformation, six colonies remained His^+^ β-Gal^+. These clones did not grow on His^-^ plates with 3-aminotriazole when cotransformed with either the empty pAS2 vector or with fusion proteins unrelated to the target protein.

Two of these clones were identical. They were 2.1 kb in length and contained a 900-bp coding region and a 1,200-bp 3' noncoding region. The predicted protein coincides with two highly homologous domains of the Rae28 protein (Fig. 1 and 2). Rae28 (retinoic acid-activated Early-28) has been identified in a differential screen for genes which respond to retinoic acid (20). Employing a two-hybrid screen, with Bmi1 as the target protein, Rae28 has been identified as interacting with Bmi1 (4). Rae28 shares considerable homology with the *Drosophila* PeG protein Ph, particularly in two domains, designated homology domains I and II, that are, respectively, 29 and 67 amino acids long (9, 10, 20 [compare Fig. 2]). Sequence analysis indicated that the clone we isolated encompasses these two homology domains (Fig. 1 and 2). Homology domain I is 90 and 52% identical with homology domain I of Rae28 and Ph, respectively. Homology domain II is 79 and 56% identical with homology domain II of Rae28 and Ph, respectively (Fig. 2). Also, a Cys2-Cys2 zinc finger motif is conserved between the protein we isolated and the Rae28 and Ph proteins. Not only the structure of this zinc finger motif, but also its location between homology domains I and II is conserved (Fig. 2). Based on these homologies, we named the clone isolated *HPH2*. Following the same reasoning, Alkema et al. recently renamed Rae28 Mph1 (4).

The HPH2 clone starts at homology domain I, suggesting that we did not isolate the full-length clone, but only the 3' region of the gene. To isolate full-length cDNAs of *HPH2*, we screened a human fetal brain library. Initially we used the 1,300-bp 3' noncoding region of *HPH2* as probe, but isolated...
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no cDNAs which contained additional 5' sequences. We then used the coding sequences of HPH2 as probe, as well as the most upstream 5' 1,000 bp of the coding region of Rac28/Mph1. We isolated a cDNA clone that overlapped with the HPH2 two-hybrid clone, extending HPH2 with 140 aa (Fig. 1) and the HPH2 cDNA to 2,554 bp. The length of the HPH2 transcript and the molecular mass of HPH2 (see below) suggest that we isolated the full-length HPH2 cDNA.

Besides isolation of the extended HPH2 clone, we also isolated two overlapping cDNA clones which are, in contrast with HPH2, highly homologous with Rac28/Mph1 (Fig. 2). The predicted 1,012-aa long protein is 95% identical with Rae28/Mph1 and is only homologous with HPH2 and Ph in homology domains I and II and the Cys2-Cys2 zinc finger (Fig. 2). Besides these sequence homologies, the protein shares a glutamine-rich stretch (aa 400 to 450) with Ph (Fig. 2 [9, 10, 20]). Given the high degree of homology with Ph, but also with the earlier characterized Rae28/Mph1, we named this clone HPH1.

Homology domain II is also shared with other proteins. The products of the recently identified Pgc1 gene, Sex comb on midleg (Scr) and of the Drosophila gene lethal(3)malignant brain tumor (l(3)mbl) share homology domain II (referred to as the SPM domain) with Ph, HPH1, and HPH2 (5, 36). The SPM domain in the Scr protein is essential for its function (5). The occurrence of this domain in these diverse proteins suggests the existence of a family of proteins containing these homology domains.

In conclusion, a two-hybrid screen with the Xenopus homolog of the vertebrate Pgc protein Bmi1 as the target resulted in the isolation of a human cDNA which encodes a protein that shares two homology domains with Rac28/Mph1 and the Pgc protein Ph. Subsequent screening of a cDNA library to isolate full-length clones of this cDNA led to the isolation of a second cDNA which is the human homolog of Rac28/Mph1. We named the proteins HPH1 and HPH2. The longer HPH1 protein is the virtually identical human homolog of Rac28/Mph1. The shorter HPH2 protein which was isolated in the two-hybrid screen shares the homology domains, but further it has only limited homology with HPH1.

Distribution of HPH1 and HPH2 transcripts in human tissues and cancer cell lines. Besides limited sequence homology outside the homology domains, the HPH2 cDNA clone is considerably shorter (2.55 kb) than the HPH1 and Rac28/Mph1 cDNA clones (3.2 and 3.5 kb, respectively [20]). To verify this difference in length at the level of mRNA transcripts, we characterized the expression levels of the HPH1 and HPH2 genes.

We analyzed multiple tissue Northern blots containing poly(A)+ mRNA from different human tissues or human cell lines (Clontech). As probes, we selected the 5' coding region from the HPH1 cDNA, since this region is not present in the HPH2 cDNA (Fig. 2) and the 3' noncoding region from the HPH2 cDNA.

We detected approximately 4.4- and 6-kb transcripts for the HPH1 gene and a single approximately 2.5-kb transcript for the HPH2 gene (Fig. 3 and 4). The length of the transcripts confirms that HPH1 and HPH2 are different genes. The highest level of expression of HPH1 is found in thymus (lane 2), testsis (lane 4), and ovary (lane 5) (Fig. 3). Low expression levels were detected in spleen (lane 1), prostate (lane 3), small intestine (lane 6), colon (lane 7), and peripheral blood leukocytes (lane 8). In contrast, HPH2, like Bmi1, was expressed ubiquitously (Fig. 3).

The differences in the abundance of HPH1, HPH2, and Bmi1 transcripts are more pronounced in human cell lines than in normal human tissues (Fig. 4). The HPH1 gene was strongly expressed in the Burkitt's lymphoma Raji (lane 5), colorectal adenocarcinoma SW480 (lane 6), melanoma G381 (lane 8), and U-2 OS osteosarcoma (lane 10) cell lines and at a much lower level in the other cell lines (Fig. 4). In contrast, both HPH2 and Bmi1 were expressed at a low level in Burkitt's lymphoma Raji cells. As in normal human tissues, no major differences in expression levels of HPH2 were observed, except in Burkitt's lymphoma Raji cells (lane 5). An aberrant HPH2 transcript was detected, specifically in the colorectal adenocarcinoma SW480 (lane 6). This approximately 5.5-kb transcript is expressed at a much lower level than the 2.5-kb transcript. No aberrant transcripts were detected in the mRNA from the other cell lines, not even after longer exposures. Bmi1 expression is more varied than HPH2 expression. Bmi1 being most strongly expressed in the K-562 (lane 3), SW480 (lanes 6 and 9), and U-2 OS (lane 10) cell lines.

In summary, the HPH1, HPH2, and Bmi1 genes are differentially expressed in normal human tissues and cancer cell lines. The length of the HPH1 and HPH2 transcripts confirm the existence of two different genes and also verifies that the HPH2 cDNA is considerably shorter than the HPH1 cDNA. HPH1 and HPH2 coimmunoprecipitate with Bmi1. In order to test whether HPH1, HPH2, and Bmi1 exist in vivo as part of a common protein complex, we performed immunoprecipitation experiments with antibodies raised against the Rac28/Mph1, HPH2, and Bmi1 proteins. The predicted molecular mass of Rac28/Mph1, which is virtually identical to that of

![FIG. 3. Expression patterns of HPH1, HPH2, and Bmi1 in human tissues.](image-url)

Shown are expression levels in spleen (lane 1), thymus (lane 2), prostate (lane 3), testis (lane 4), ovary (lane 5), small intestine (lane 6), colon (lane 7), and peripheral blood leukocytes (lane 8). As probes, we selected the full-length Bmi1 cDNA, the 5' coding region from the HPH1 cDNA, since this region is not present in the HPH2 cDNA, and the 3' noncoding region from the HPH2 cDNA. The filter was rehybridized with a probe for GAPDH to verify the loading of RNA in each lane.
HPh1, is 108 kDa. On Western blots, the affinity-purified antibody recognizes a protein with a size of approximately 120 kDa (Fig. 5A). However, when a lower-percentage gel is used, it becomes apparent that HPh1 runs as a doublet protein (Fig. 5D). It is possible that the HPh1 protein doublet is a reflection of the two, 4.4- and 6-kb HPH1 transcripts (Fig. 3 and 4). It is, however, also possible that the HPh1 protein doublet arises from posttransational modifications.

The highest expression levels of the HPH1, HPH2, and BMI1 genes are found in the SW480 and U-2 OS cell lines (Fig. 4). We used extracts of U-2 OS human osteosarcoma cells to perform coimmunoprecipitation experiments. Extracts of U-2 OS cells were incubated with a mouse anti-Bmi1 antibody and subsequently with agarose-coupled goat anti-mouse IgG antibodies. The resulting IP was Western blotted and incubated with rabbit anti-Rae28/Mph1 antibody. The approximately 30-kDa HPH1 protein (Input) was detected in the BMI1 BMI1 IP. In this particular experiment, HPH1 runs as a single protein, but when a lower percentage of polyacrylamide is used, the HPH1 protein runs as a doublet (compare with panel D). When the Bmi1 antibody was omitted, no HPH1 protein was detected (Mock IP). (B) The mouse anti-Bmi1 antibody immunoprecipitated the approximately 54-kDa HPH2 protein (Input) from extracts of U-2 OS cells (BMI1 IP). No HPH2 protein was detected when the Bmi1 antibody was omitted from the immunoprecipitation (Mock IP). (C) The HPH1 (HPH1, IP) and HPH2 (HPH2 IP) antibodies immunoprecipitated the approximately 44- and 46-kDa BMI1 proteins (Input) from extracts of U-2 OS cells. No BMI1 protein was detected when the HPH antibodies were omitted from the immunoprecipitation (Mock IP). (D) The HPH2 antibody immunoprecipitated the approximately 120-kDa HPH1 protein (Input) from extracts of U-2 OS cells (HPH2 IP). No HPH1 protein was detected when another, unrelated antibody was used in the immunoprecipitation (Mock IP). Note the rabbit heavy chain (Fc) in the HPH2 and mock IP.

The predicted molecular mass of human BMI1, based on amino acid composition, is 38 kDa (2). On Western blots, the antibody recognizes a doublet of 44- and 46-kDa proteins (Fig. 5C, Input). Both the Rae28/Mph1 and HPH2 antibodies were able to immunoprecipitate the BMI1 protein from extracts of U-2 OS cells (Fig. 5C, HPH1 IP and HPH2 IP, respectively). No BMI1 was detected when the antibodies were omitted (Fig. 5C, Mock IP) or when an unrelated antibody was added to the immunoprecipitations.

These results suggest that in vivo, HPH1 and HPH2 are part of a protein complex which contains BMI1. We further strengthened this notion by showing that the HPH2 antibody was able to immunoprecipitate the HPH1 protein from ex-
tracts of U-2 OS cells (Fig. 5D, HPH2 IP). The HPH2 immunoprecipitations were specific, since no HPH1 proteins were detected when an unrelated antibody was added to the immunoprecipitations (Fig. 5D, Mock IP). We also tried to immunoprecipitate HPH2 by using the Rae28/Mph1 antibodies. Since both Rae28/Mph1 and HPH2 antibodies are rabbit-derived, the heavy chains (Fc) of the rabbit anti-HPH2 antibodies (Fig. 5D, Mock IP and HPH2 IP) or the rabbit anti-Rae28/Mph1 antibody are detected by the goat-anti rabbit alkaline-phosphatase-conjugated antibody. Unfortunately, HPH2 migrates at the same position of these Fc heavy chains (compare Fig. 5B and D), thus preventing detection of HPH2 in IPs by the Rae28/Mph1 antibodies.

The major part of the immunoprecipitated or coimmunoprecipitated HPH1, HPH2, and BMI1 proteins was present in the pellet, indicating that the proteins indeed coimmunoprecipitated. We could, however, still detect residual amounts of BMI1, HPH1, or HPH2 proteins in the supernatants after immunoprecipitation with the BMI1, Rae-28/Mph1, or HPH2 antibodies (data not shown). Therefore, neither the immunoprecipitation nor the coimmunoprecipitation can be considered quantitative. However, since the majority of the proteins were removed from the supernatants and present in the pellets, this indicates that HPH1, HPH2, and BMI1 proteins coimmunoprecipitate from extracts of U-2 OS cells. This suggests that in vivo, the proteins are part of a common protein complex.

**HPH1, HPH2, and BMI1 cotraffocate in a sucrose gradient.** The coimmunoprecipitation experiments suggest that HPH1, HPH2, and BMI1 may be part of a high-molecular-mass protein complex. To test this idea, we performed velocity gradient ultracentrifugation experiments. We loaded extracts of U-2 OS cells, which had been prepared as in the immunoprecipitation experiments, on a 5 to 30% sucrose gradient (5 ml). After centrifugation 14 350-μl fractions were collected. We calibrated the lower-molecular-mass range of the gradient with catalase (232 kDa) and thyroglobulin (669 kDa), which were detected in fractions 1 and 3, respectively (Fig. 6). Lack of suitable markers prevented calibration of the higher-molecular-mass range of the gradient. The proteins in the 14 fractions of the gradient on which the U-2 OS cell extract was loaded were separated by SDS-PAGE, blotted on nitrocellulose, and probed with antibodies against HPH1, HPH2, and BMI1. We found that the largest portion of the HPH1, HPH2, and BMI1 proteins co-traffocate in fractions 7 through 11 (Fig. 6). This result indicates that the HPH1, HPH2, and BMI1 proteins are present in a complex with a molecular mass higher than 669 kDa.

These results are in agreement with earlier results that demonstrated that the *Drosophila* Polycomb and Polyhomerotic proteins cotraffocate by gel filtration and velocity gradient ultracentrifugation (13). We used a gradient ranging from 5 to 30% sucrose, which we found to result in a better separation than when the reported 5 to 20% gradient was used (13). Similar to what has been reported previously (13), we found a portion of the HPH1 and BMI1 proteins to be present in the pellet of the gradient (Fig. 6, fraction 14). We also found a substantial portion of the HPH1 and BMI1 proteins to be present in fraction 1 (Fig. 6). In contrast, no HPH2 protein was detected in either fraction 1 or fraction 14. The meaning of these observations is unclear. It has been speculated that the presence of the proteins in the lower-molecular-mass range of the gradient can be explained by partial dissociation of the complex during the preparation (13). Alternatively, it could indicate a heterogeneous composition of the multimeric complex (13). At present, it is not clear which is the right interpretation. The presence of the HPH1, HPH2, and BMI1 proteins in fractions 7 through 11, however, supports the idea that the proteins are part of a common, multimeric protein complex.

**HPH1 and HPH2 colocalize with BMI1 in nuclei of U-2 OS and SW480 cells.** We next analyzed the subcellular localization of the HPH1 and HPH2 proteins in relation to the PcG protein BMI1 by performing immunofluorescence labelling experiments. The use of mouse anti-Bmi1 and rabbit anti-Rae28/Mph1 and HPH2 allows double-labelling experiments. We used U-2 OS human osteosarcoma cells and SW480 human colorectal adenocarcinoma cells, in which we found that HPH1, HPH2, and BMI1 are all expressed at a high level (Fig. 4). In extracts of U-2 OS cells, we found that HPH1 and HPH2 coimmunoprecipitate and co-traffocate with each other and with BMI1 (Fig. 5 and 6). In U-2 OS cells, both HPH1 and BMI1 are found in the nucleus and throughout the nucleoplasm, and they colocalize in large, brightly labelled domains (Fig. 7A to C). Also HPH2 and BMI1 colocalize in the same brightly labelled domains (Fig. 7D to F). HPH1 and HPH2 colocalize with BMI1 in the large domains, but as yet, this is not clear for the more homogeneously distributed pattern. The fine granular pattern is too complex to allow analysis of any systematic colocalization.

The observed labelling patterns of HPH1, HPH2, and BMI1 are in contrast with recently published labelling patterns of the Bmi1 protein (8). It should be pointed out, however, that these workers detected overexpressed Bmi1 protein in Rat1 fibroblasts or COS cells in which no endogenous Bmi1 could be detected (8). We, on the other hand, detected endogenous levels of BMI1 in cell lines which express the Bmi1 gene at a high level (Fig. 4). This is a likely explanation for the observed differences in labelling patterns. The significance of the do-
mains in which HPH1, HPH2, and BMI1 colocalize is further underlined by our recent finding that a human homolog of the Polycomb protein also colocalizes with BMI1 in these domains (27). This human Pc homolog also coimmunoprecipitates with BMI1 (27).

We further analyzed the subcellular localization of HPH1 and HPH2 in relation to BMI1 in SW480 cells. As in U-2 OS cells, we found complete colocalization between HPH1 (Fig. 8A to C) and HPH2 (Fig. 8D to F) and BMI1 in large, brightly labelled domains. These results demonstrate that the colocalization of HPH1 and HPH2 with the PcG protein BMI1 is not restricted to one cell type.

We conclude that HPH1 and HPH2 colocalize with BMI1 in large domains in the nuclei of U-2 OS and SW480 cells. These results further underline the validity of the identified two-hybrid interaction between HPH2 and BMI1. They strengthen the notion that HPH1, HPH2, and BMI1 are part of a multimeric protein complex.

**Interactions between homology domains I and II of HPH1 and HPH2.** We found that HPH2 is able to immunoprecipitate HPH1 (Fig. 5D), that HPH1 and HPH2 cofractionate in a sucrose gradient (Fig. 6), and that HPH1 and HPH2 colocalize with BMI1 in the nuclei of interphase cells (Fig. 7 and 8). These results suggest that HPH1 and HPH2 are part of a common protein complex, but they do not address the question of whether HPH1 and HPH2 interact with each other. Homology domains I and II are potentially involved in protein-protein interactions. Significant homology has been found between the Rac28/Mph1 and Ph homology domain II and the recently characterized protein encoded by the PcG gene Sex comb on midleg (Scm) (5). This domain has been named SPM domain and it has been suggested that this domain is involved in protein-protein interactions (5). By using the two-hybrid assay, we therefore tested whether the homology domains of HPH1 and HPH2 can mediate protein-protein interactions. The region encompassing homology domains I and II from HPH1 (aa 722 to 1013 [Fig. 9A]) was fused to the GAL4 DNA binding domain. The plasmid was cotransformed with the HPH2 two-hybrid clone, encompassing homology domains I and II (aa 137 to 432 [Fig. 9A]) (see Fig. 1). The transformants were able to grow on selective medium lacking histidine, in the presence of 3-aminotriazole, and were also β-galactosidase positive. This result indicates an interaction between homology domains I and II of HPH1 and HPH2, and we subsequently tested potential interactions between the individual homology domains. We found that homology domain I of HPH1 interacts with homology domain II, but not with homology domain I of HPH2. Also, homology domain II of HPH1 interacts with both homology domains I and II of HPH2 (Fig. 9A). In contrast, BMI1 does not interact with individual homology domains I and II. For an interaction with BMI1, both intact homology domains I and II need to be present in the fusion protein (Fig. 9B).

To quantify the strength of the interactions between the different portions of the HPH1 and HPH2 proteins, we prepared lysates of the transformants and measured the β-galactosidase activity. The interaction between Xbmi1 and HPH2 (aa 137 to 375), which was identified in our original two-hybrid screen (Fig. 1), was found to be the strongest. The relative strengths of the other interactions are given as percentage of the Xbmi1-HPH2 (aa 137 to 375) interaction (Table 1). The second strongest interaction was found between HPH1 (aa 722

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FIG. 7. Colocalization of HPH1 and HPH2 with BMI1 in nuclei of U-2 OS cells. (A to C) Rabbit anti-Rae28/Mph1 (A) and mouse anti-BMI1 (B) labeling is shown in single confocal optical sections. Panels A and B represent the two different detection channels of the dual image, whereas panel C represents the false color overlay. HPH1 and BMI1 are homogeneously distributed in the nucleus, but are also concentrated in large, brightly labeled domains. HPH1 and BMI1 colocalize in these large domains (C) (indicated by yellow). With rabbit anti-HPH2 (D) and mouse anti-BMI1 (E) antibodies, similar distribution patterns and colocalizations (F) were observed.
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FIG. 8. Colocalization of HPH1 and HPH2 with BMI1 in nuclei of SW480 cells. (A to C) Rabbit anti-Rae28/Mph1 (A) and mouse anti-BMI1 (B) labeling and the false color overlay (C) are shown. Similar to in U-2 OS cells (Fig. 7), HPH1 and BMI1 colocalize in large, brightly labeled domains (C) (indicated by yellow). With rabbit anti-HPH2 (D) and mouse anti-BMI1 (E) antibodies, similar distribution patterns and colocalizations (F) were observed.

to 1013 and HPH2 (aa 137 to 432), in which both intact homology domains of HPH1 and HPH2 were present. When single homology domains were tested against each other, a decrease in the strength of the interactions was observed (Table 1).

Taken together, these results demonstrate the importance of the homology domains in protein-protein interactions. They further suggest that HPH1 and HPH2 can heterodimerize in vivo through these homology domains.

DISCUSSION

Are HPH1, HPH2, and the PcG protein BMI1 part of a multimeric protein complex? It has been proposed that PcG proteins repress homeotic gene expression via the formation of multimeric complexes. This model is based on the observation that different PcG proteins bind in overlapping patterns to about 100 loci on polytene chromosomes in the Drosophila salivary gland (10, 13, 18, 25, 37). However, so far, only the PcG protein Polyhomeotic, but no other known proteins, has been shown to coimmunoprecipitate or cofractionate with Pc (13).

Employing the genetic two-hybrid system, we identified a protein that specifically interacts with the vertebrate PcG protein BMI1. Based on the occurrence of two conserved homology domains that are shared with Ph and the vertebrate Rae28/Mph1 protein, we name this protein HPH2 (see below). We also identified the human homolog of the protein expressed by the Rae28/Mph1 gene, which we name HPH1. Our results indicate that mammals have at least two proteins that bear similarity to the Drosophila Ph protein. The homology domains appear to be involved in mediating interactions between HPH1 and HPH2 as well as between Xbm1 and HPH2. We further show that HPH1 and HPH2 coimmunoprecipitate with BMI1 and with each other, indicating an in vivo association between these proteins. This notion is further supported by our finding that HPH1, HPH2, and BMI1 cofractionate in a sucrose gradient. Furthermore, HPH1, HPH2, and BMI1 colocalize in interphase nuclei of two different human lines. Together, these data provide evidence for an in vivo association and indicate that HPH1 and HPH2 are part of a human multimeric protein complex which also contains the vertebrate PcG protein BMI1.

In summary, four different approaches, namely (i) the two-hybrid assay, (ii) coimmunoprecipitation experiments, (iii) cofractionation experiments, and (iv) immunocolocalization experiments, all indicate that HPH1, HPH2, and BMI1 are part of a common, multimeric protein complex.

Are HPH1 and HPH2 homologs of the PcG protein Polyhomeotic? Two lines of evidence suggest that HPH1 and HPH2 are functional homologs of the Drosophila PcG protein Polyhomeotic (Ph). In the first place, based on four different approaches (see above) we find evidence for the notion that HPH1, HPH2, and BMI1 are part of a multimeric protein complex. BMI1 is a vertebrate PcG protein (3, 32, 33) and possibly a functional vertebrate homolog of the Drosophila PcG protein Posterior sex combs (6, 35). It is feasible that proteins that form a complex with PcG proteins are also PcG proteins. In the second place, the HPH1 and HPH2 proteins share extensive sequence and overall structural homology with the Drosophila PcG protein Ph. Both HPH1 and HPH2 share the conserved homology domains I and II with Ph. In addition, a particular Cys2-Cys2 zinc finger motif is conserved, both in terms of sequence homology and in terms of its position between the two homology domains. Also, a glutamine-rich re-
region is located in HPH1 in the same region of the protein as in Ph. In this context, a similar situation with the murine Ph homolog M33 is very relevant. M33 shares only the conserved chromodomain (52 aa long) and C terminus (17 aa long) with homolog M33 is very relevant. M33 shares only the conserved chromodomain (52 aa long) and C terminus (17 aa long) with Ph. Although the homology is restricted to small regions and the overall composition of the protein, M33 can be considered to be a functional homolog of Ph.

Both lines of evidence are supportive for the idea that HPH1 and HPH2 are human homologs of Ph. Although the sequence and structural homologies between Ph and Rae28 have been noted before (20), at that time it was unclear whether this implied that the Rae28 protein is a functional, mammalian homolog of the Ph protein Ph. We now show that the human Rae28 homolog, HPH1, as well as HPH2, interacts with and colocalizes with a noted member of the vertebrate PcG family, BMI1. We are, therefore, tempted to conclude that we have identified components of a human PcG protein complex. It will be of considerable interest to test whether loss-of-function mutations of the mouse Rae28/Mphi gene and the murine homolog of HPH1 result in homeotic transformations.

Possible functional significance of the existence of multiple HPH proteins. We have presented evidence that HPH1, HPH2, and BMI1 are part of a multimeric protein complex. Our data also indicate that homology domains I and II of HPH1 and HPH2 are involved in mediating protein-protein interactions. These data suggest that HPH1 and HPH2 are able to form heterodimers through their respective homology domains I and II. This may imply that their function is exerted via heterodimerization and that the human multimeric PcG complex requires the presence of both HPH1 and HPH2.

The occurrence of two proteins with homology to Ph may also point towards the interesting possibility of the existence of different mammalian multimeric PcG complexes. This idea is supported by the differential expression patterns of HPH1, HPH2, and BMI1 (Fig. 3 and 4). HPH2, like BMI1, is expressed ubiquitously in all tissues and cell lines tested, whereas HPH1 has a more limited expression pattern. Since we screened a peripheral blood leukocyte two-hybrid library, it is not amazing that we isolated HPH2 as a BMI1 binding protein. HPH2 is abundantly expressed in this tissue, and HPH1 is expressed only at a very low level. This might indicate that in leukocytes, HPH2 and BMI1 are components of the PcG complex and HPH1 is not.

The idea that PcG complexes exist with different compositions is reinforced by the observation that the PcG protein Psc shares many, but not all, homology domains with Psc proteins Ph, Ph, and Ph on salivary gland polytene chromosomes (10, 18, 25, 37). Distinct compositions of the PcG complexes could lead to specificity in the repression of different loci in different tissues. This provides flexibility, and it increases the possibilities of regulating a wide range of target genes with only a limited number of components.

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