Identification and characterization of human Polycomb-group proteins.
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Citation for published version (APA):

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CHAPTER 4

Interference with the expression of a novel human Polycomb protein, HPC2, results in cellular transformation and apoptosis.


4. Human Polycomb protein and cellular transformation

Interference with the Expression of a Novel Human Polycomb Protein, hPc2, Results in Cellular Transformation and Apoptosis

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Received 19 March 1997/Returned for modification 3 May 1997/Accepted 1 July 1997

Polycomb (Pc) is involved in the stable and heritable repression of homeotic gene activity during Drosophila development. Here, we report the identification of a novel human Pc homolog, hPc2. This gene is more closely related to a Xenopus Pc homolog, XPc, than to a previously described human Pc homolog, CBX2 (hPc1). However, the hPc2 and CBX2/hPc1 proteins colocalize in interphase nuclei of human U-2 OS osteosarcoma cells, suggesting that the proteins are part of a common protein complex. To study the functions of the novel human Pc homolog, we generated a mutant protein, ΔhPc2, which lacks an evolutionarily conserved C-terminal domain. This C-terminal domain is important for hPc2 function, since the ΔhPc2 mutant protein which lacks the C-terminal domain is unable to repress gene activity. Expression of the ΔhPc2 protein, but not of the wild-type hPc2 protein, results in cellular transformation of mammalian cell lines as judged by phenotypic changes, altered marker gene expression, and anchorage-independent growth. Specifically in ΔhPc2-transformed cells, the expression of the c-myc proto-oncogene is strongly enhanced and serum deprivation results in apoptosis. In contrast, overexpression of the wild-type hPc2 protein results in decreased c-myc expression. Our data suggest that hPc2 is a repressor of proto-oncogene activity and that interference with hPc2 function can lead to derepression of proto-oncogene transcription and subsequently to cellular transformation.

The Drosophila Polycomb (Pc) gene is a member of the Polycomb group (PcG) gene family. These genes are part of a cellular memory system that is responsible for the inheritance of gene activity by progeny cells (7, 19, 21, 28, 38). Stable and heritable transmission of gene activity is crucial for the maintenance of the differentiated identity of cells over many cell generations. 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, including Pc, bind in overlapping patterns on polytene chromosomes in Drosophila salivary gland cells (32, 48). In Drosophila, the PcG protein Polyhomeotic (Ph), but no other known protein, coimmunoprecipitates or cofractionates with Pc (12). Recently, we found that two human counterparts of Ph, HPH1 and HPH2, coimmunoprecipitate, cofractionate, and colocitize in nuclear domains with both the vertebrate PcG protein BMI1 and a human Pc homolog, indicating that these proteins associate in vivo (16). A similar in vivo interaction between Bmi1 and a Ph homolog has been found in mice (1). These findings indicate the existence of a vertebrate PcG multimeric protein complex.

The Pc protein binds to about 100 loci on polytene chromosomes in Drosophila salivary gland cells (32, 48). These loci include the homeotic gene loci and other PcG gene loci (48), indicating that homeotic genes and PcG genes are target genes of Pc. Direct evidence for this idea stems from the observation that Pc is associated with chromatin of the silent part of the homeotic bithorax (Ubx) complex (27). Physical association of the Pc protein with chromatin of other target loci has not been reported so far.

Several vertebrate homologs of Pc have been identified (15, 30, 33), suggesting that repression of gene activity, mediated by Pc, is evolutionarily conserved. This idea is supported by the finding that a mouse Pc homolog, M33, is able to rescue the Drosophila Pc phenotype (25). Recently, it has become evident that M33 displays functions in mice similar to those of Pc in Drosophila. M33-deficient mice show homeotic transformations of the axial skeleton, along with sternal and limb malformations (6).

Many aspects of the molecular mechanism underlying the role of PcG proteins in the stable transmission of gene activity are enigmatic. An important clue about the molecular mechanism underlying Pc action is the observation that the Pc protein shares a homologous domain with the Drosophila heterochromatin-binding protein HP1 (29, 39). The shared motif between Pc and HP1 has been termed the chromodomain (chromatin organization modifier) (29). This discovery provides an important, direct link between the 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. The chromodomain is essential for binding of the Pc protein to chromatin. When the chromodomain is either mutated or deleted, it no longer binds to chromatin (24). Also, a conserved domain located in the C terminus of the Pc protein (30) is crucial for Pc function. In Drosophila, several naturally occurring Pc mutants either are mutated in the C-terminal domain or lack this...
domain entirely (13). A mutant Pc gene lacking the C-terminal domain is unable to repress gene activity (4, 24).

In the present study, we identified a novel human Pc homolog, hPc2. We showed that the hPc2 protein and a previously characterized human Pc protein, CBX2 (15), colocalize in nuclear domains of human U-2 OS osteosarcoma cells. To study the functions of hPc2, we designed an hPc2 mutant protein that lacks the C-terminal domain, which is crucial for the ability of hPc2 to repress gene activity. Expression of this hPc2 mutant in mammalian cell lines results in (i) cellular transformation, (ii) enhanced expression of the c-myc proto-oncogene, and (iii) apoptosis upon serum deprivation. Our data suggest that hPc2 is a repressor of proto-oncogene activity and that interference with hPc2 function can lead to derepression of proto-oncogene transcription and subsequently to cellular transformation.

MATERIALS AND METHODS

Isolation of the hPc2 gene. For screening a ag101 human fetal brain cDNA library (Clontech, Palo Alto, Calif.), a probe encompassing the entire coding region of the XPc (33) except for the chromodomian (range from amino acids [aa] 60 in hPc2 to 556 in M33) was prepared by PCR. The filters were hybridized overnight at 0.5 X SSC (1 X SSC = 0.15 M NaCl/0.015 M sodium citrate)–100% Denhardt’s solution–10% dextran sulfate–0.1% sodium dodecyl sulfate (SDS)–100 ng/ml of cDNA probe and washed at 0.1 X SSC–0.1% SDS (ATP-labelleled DNA per ml of 100,000 cpnm). After being washed twice for 60 min at 55°C in 0.1 X SSC–0.1% SDS, the filters were exposed to X-ray films with intensifying screens for 2 days at –70°C. Positive plaques were isolated and purified. The complete nucleotide sequence of the longest cDNA was determined by sequence analysis.

Analysis of hPc2 transcripts. Multispecies Northern blots containing approximately 2 μg of poly(A)* RNA per lane from different human tissues or human cell lines were obtained by PCR. The filters were hybridized overnight at NfCT (6X SSC (0.9 M NaCl/0.09 M sodium citrate)–100% Denhardt’s solution–10% dextran sulfate–0.1% sodium dodecyl sulfate (SDS))–100 ng/ml of cDNA probe and washed at 0.1 X SSC–0.1% SDS (ATP-labelleled DNA per ml of 100,000 cpnm). After being washed twice for 60 min at 55°C in 0.1 X SSC–0.1% SDS, the filters were exposed to X-ray films with intensifying screens for 2 days at –70°C. The Lex A fusion repressor gene targeted repression assay. The LexA fusion reporter gene targeted repression assay was performed as described previously (4, 35). The chloramphenicol acetyltransferase (CAT) reporter gene has been replaced by the luciferase gene (LUC). U-2 OS cells were cultured in selective media and transfected with the heat shock factor (HSF)-inducible LUC reporter plasmid, 4 μg of the LexA fusion constructs, and 2 μg of the gspβ-Gal construct (Promega) by the calcium phosphate precipitation method. The HSF-inducible LUC reporter plasmid was activated by exposing the cells to 43°C for 1 h, followed by a 6 h recovery at 37°C, as described previously (4, 35). LUC activity was normalized to β-galactosidase activity. The absolute values of LUC activity varied between independent experiments. The LUC activity in cells transfected with the LexA reporter plasmid only was set at 100%, and LUC activities in cells which were cotransfected with indicated plasmids were expressed as a percentage of this control value. The degree of repression is expressed as the mean ± the standard error of the mean (SEM).

Establishment and characterization of stable C57MG and U-2 OS cell lines. Indicated cDNAs were cloned into the pCDNA3 vector (Invitrogen, San Diego, Calif.), which carries the neomycin resistance gene and in which the indicated cDNAs are under control of the enhancer from the immediate-early gene of human cytomegalovirus (CMV). C57MG, U-2 OS, or Rat1a cells were transfected with Lipofectin (Gibco BRL) as specified by the manufacturer, and stable transfected lines were selected by culturing the cells for 2 weeks in medium containing 900 μg of Geneticin (G418) per ml. The surviving cells were cloned by dilution for 2 to 4 weeks in medium containing 250 μg of G418 per ml. Individual C57MG and U-2 OS cell clones were selected and cultured in individual dishes. After five passages, the cell lines were characterized for [3H]thymidine incorporation and gene expression. [3H]Thymidine incorporation was measured as described previously (26). The numbers were normalized to the protein content. From C57MG cells, expressing low levels of c-myc, poly(A)* RNA was isolated. From U-2 OS cells, expressing high levels of c-myc, total RNA was isolated. isolation of RNA and Northern analysis were performed as standard procedures. The blots were hybridized with [α-32P]ATP-labelled DNA probes, and the bands were autoradiographed with intensifying screens at –70°C with preflushed X-ray films.

Western blot analysis of hPc2 and c-myc proteins. The expression levels of the hPc2 and c-myc proteins were analyzed by using cell lysates of the stably transfected C57MG and U-2 OS clones (35). For hPc2 detection, the blots were incubated with a 1:25,000 dilution of c-myc-specific antibodies. The monoclonal 9E10 antibody was used for the detection of c-myc (8). Equal amounts of proteins were loaded, as measured by the bicinchoninic acid method (41) and visualized by Coomassie blue staining of a gel.

Soft agar growth assays. Cell lines were analyzed for anchorage-independent growth as described previously (34, 40, 44). Rat1a cells were transfected by the calcium phosphate transfection procedure with full-length hPc2 (hPc2WT), the C-terminal deletion mutant (hPc2C), and a mutant lacking the chromodomian (hPc2Δchromo), which were all cloned in the pCDNA vector. As a positive control, the c-myc cDNA which was cloned in the pRC-CMV vector was transfected. The cells were subjected to selection with G418 (1 mg/ml) for 14 days, at which time the G418-resistant clones were counted. The clones were triplicated, and cells were counted. A total of 5 × 105 cells in 5 ml of Dulbecco’s modified Eagle’s medium containing 0.4% (w/v) glucose were seeded in 5-cm petri dishes containing 1% (w/v) agarose. The plates were inspected 14 to 21 days after seeding of the cells, and the colonies were counted. The entire procedure, including the transfection of the cDNAs, was performed in triplicate.

Apoptosis assays. Exponentially growing cells were washed with phosphate-buffered saline, and culture medium containing 10% serum was exchanged for culture medium containing 0.1% serum. After 3 or 6 h, the cells were washed with cold phosphate-buffered saline and incubated with FITC-conjugated annexin V (Nexin Research, Hoeven, The Netherlands) for 15 min at 4°C. The binding conditions were as recommended by the manufacturer. After being washed, the cells were fixed in 2% paraformaldehyde for 15 min. The cells were then viable, since they excluded propidium iodide (36). The assay was repeated five times with similar results. Beside annexin V staining, C57MG/G2c cells showed hallmarksof apoptosis, such as membrane blebbing, cell shrinkage, and chromatin condensation after 24 to 48 h. As an independent assay to detect apoptosis, we monitored apoptosis-induced DNA strand breaks by the TUNEL (Terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling) assay (Boehringer), in which the terminal deoxynucleotidyltransferase (TdT) enzyme activity is measured. Also using this assay, we observed apoptosis, specifically in serum-deprived ΔhPc2-transfected cells (36).

Nucleotide sequence accession number. The complete hPc2 sequence has been deposited with GenBank under accession no. AF013956.

RESULTS

Isolation and characterization of a novel human Pc homolog, hPc2. To isolate human Pc homologs, we screened a human fetal brain cDNA library with a probe that encompasses the coding region of a Xenopus Pc homolog, XPc; except for the chromodomian (33). We isolated an 1,867-bp cDNA clone (Fig. 1). This clone contains a 1,674-bp open reading frame (Fig. 2A). The predicted 558 aa protein possesses a conserved region, the chromodomian, which is 96% identical to the Xpc chromodomian, 77% identical to the chromodomian of the mouse Pc homolog, M33, and 55% identical to the Drosophila Pc chromodomian (Fig. 2A) (29, 30, 33). A conserved C-terminal domain is 100% identical to Xpc at the protein level, 73% identical to M33, and 67% identical to Pc (Fig. 2). Overall, the human protein is 48% identical to Xpc.
When conservative changes are taken into account, the human protein is 80% similar to the Xpc protein. Also, at the nucleotide level the human cDNA has a striking 52% identity to the Xpc cDNA in the entire coding region. We conclude, therefore, that we have isolated the human homolog of the Xpc protein.

In contrast, the overall homology between the human Pc protein and the mouse Pc homolog M33 is a mere 24% identity. At the nucleotide level, the novel human Pc homolog has 29% identity to the M33 cDNA. This degree of homology is considerably lower than that between the human Pc homolog and the Xpc cDNA. However, when conservative changes are taken into account, the novel human Pc homolog is 68% similar to M33 at the protein level. This probably implies that the overall three-dimensional shapes of the two proteins are very similar. This may in turn indicate that the two proteins are functionally equivalent.

A partially characterized human homolog of M33, CBX2 (15), has 86% identity and 100% similarity to M33 at the protein level (Fig. 2B). Significant homology between CBX2 and our novel human Pc protein is limited to the conserved C-terminal domain (Fig. 2B). The CBX2 protein has a mere 25% overall identity but a significantly higher 80% similarity to the novel human Pc homolog (Fig. 2B).

We conclude that there are at least two human Pc homologs. They encode proteins that are homologous not only in the conserved C-terminal domain and presumably also in the chromodomains. The Pc gene that we have isolated is highly homologous to the Xpc homolog, whereas the other human Pc homolog, CBX2, is more homologous to the murine Pc homolog, M33. For convenience, we named the CBX2 gene hPc1 and the gene that we isolated hPc2.

**Distribution of hPc2 transcripts in human tissues and cancer cell lines.** The hPc2 cDNA clone that we have identified is 1,867 bp in length, of which 1,674 bp (encoding aa 1 to 558) encompasses the open reading frame. To study the distribution of hPc2 mRNA, we selected a probe which has little homology to the other vertebrate Pc genes, that is, between the conserved chromodomains and C-terminal domains ranging from aa 60 to 530. On a Northern blot, a single transcript of approximately 2.8 kb was detected in all the tissues and cell lines tested (Fig. 3). In normal human tissues, the highest level of hPc2 expression is found in the thymus (Fig. 3A, lane 2) and in peripheral blood leukocytes (lane 8). Expression levels are still pronounced in the spleen (lane 1), prostate (lane 3), testis (lane 4), ovary (lane 5) and small intestine (lane 6) but are very low in the colon (lane 7). This expression pattern of hPc2 is very similar to the expression pattern of the vertebrate PcG genes BMI1 and HP1H2. In human tissues, all three PcG genes are expressed at a high level, except in the colon (16). The expression levels of hPc2 in several human cancer cell lines vary more than in normal human tissues (Fig. 3B). The hPc2 transcripts are hardly detectable in the Burkitt's lymphoma Raji (Fig. 3B, lane 5) and lung carcinoma (lane 7) human cell lines. In contrast, the expression level of hPc2 is high in all other human cell lines.

In summary, the hPc2 gene is expressed in normal human tissues with almost equal abundance. The hPc2 mRNA levels are more variable in the different human cell lines.

**Characterization of antibodies against hPc2 and hPc1/M33.** To further strengthen our conclusion that we have isolated a novel human Pc homolog that is different from the previously isolated human M33 homolog, CBX2 (hPc1) (15), we raised polyclonal antibodies against XPc2/M33 and hPc2/M33. We raised antibodies against M33 since the full-length cDNA of CBX2 has not been reported. The published CBX2 sequence is virtually identical to M33 at the protein level, and we reasoned that polyclonal antibodies raised against M33 will recognize the

![FIG. 1. Nucleotide sequence of hPc2 and its predicted amino acid sequence. The chromodomains in the N terminus of the protein and the conserved C-terminal domain of the protein are shaded. A putative nuclear localization signal is underlined. The stop codon of the hPc2 gene is indicated by an asterisk.](image-url)
closely related human CBX2 protein as well. The predicted molecular masses of the 558-aa hPc2 and the 519-aa M33 are 61 and 55 kDa, respectively. The fusion proteins include a 3-kDa T7 tag. A mouse monoclonal antibody against T7 recognized an 85-kDa T7-hPc2 fusion protein and a 79-kDa T7-M33 fusion protein in extracts of the E. coli strain in which the fusion proteins were produced (Fig. 4, lanes 1 and 2, respectively). Both proteins have an aberrant mobility on SDS-PAGE. The predicted size difference of 6 kDa between hPc2 and M33, however, remains visible on SDS-PAGE.

We raised polyclonal chicken antibodies against T7-tagged hPc2 and polyclonal rabbit antibodies against T7-tagged M33. The chicken anti-hPc2 antibody recognizes the T7-hPc2 fusion protein but not the T7-M33 fusion protein (Fig. 4, lanes 3 and 4, respectively). The rabbit anti-M33 antibody recognizes the T7-M33 fusion protein but not the T7-hPc2 fusion protein (lane 6 and 5, respectively). In extracts of U-2 OS human osteosarcoma cells, the hPc2 antibody detected an 82-kDa protein (lane 7) and the M33 antibody detected a 76-kDa protein (lane 8). The detected molecular masses in extracts of human cells are consistent with the molecular masses of the hPc2 and M33 fusion proteins. We will refer to the human protein that is detected by the anti-M33 antibody as hPc1/M33. Although M33 is a murine protein, the human CBX2 (hPc1) protein is virtually identical to M33 (Fig. 2B), and it is therefore likely that our anti-M33 antibody detects the hPc1 protein in extracts of human U-2 OS cells. In support of this notion, we find that the hPc2 and M33 antibodies recognize proteins with identical molecular masses of, respectively, 82 and 76 kDa in extracts of the murine P19 embryonic carcinoma cell line (36) and the murine mammary epithelial cell line C57MG (see also Fig. 7). We conclude that the antibodies specifically recognize hPc2 and hPc1/M33 in extracts of human and murine cells.

hPc2 and hPc1/M33 colocalize in nuclei of U-2 OS cells. We next analyzed the subcellular localization of the hPc2 protein in relation to the hPc1/M33 protein by performing immunofluorescence labelling experiments. The use of chicken anti-hPc2 and rabbit anti-M33 allowed double-labelling experiments. We used human U-2 OS osteosarcoma cells in which several human PcG genes are expressed at a high level (Fig. 3B, lane 10) (1, 16). Both the hPc2 and hPc1/M33 proteins were detected in the nuclei of U-2 OS cells, throughout the nucleoplasm. They are detected in a fine granular pattern as well as in large, brightly labelled domains (Fig. 5). hPc2 and hPc1/M33 colocalize in these large domains. Using these hPc2- and M33-directed antibodies, we have shown previously that hPc2 coimmunoprecipitates and colocalizes with the vertebrate PcG proteins BMI1 and HPFI1 (35). Also, M33 protein coimmunoprecipitates and colocalizes with BMI1 (1). We therefore conclude that hPc2 and hPc1/M33 colocalize in interphase nuclei of U-2 OS human osteosarcoma cells.

An hPc2 mutant lacking the conserved C-terminal region is not able to repress gene activity. The PcG complex proteins are known to be involved in repressing homeotic gene activity

FIG. 2. Comparison of the hPc2 protein with other Pc homologs. (A) The sequence is aligned to Xenopus Pc, M33, and the CBX2 (hPc1) protein sequences. Identical amino acids are shown, and nonidentical amino acids are indicated by dashes. The conserved chromodomains and C-terminal domain (COOH box) are boxed. (B) Alignment of the identified amino acids of CBX2 (hPc1) with corresponding regions of the hPc2 and M33 proteins. Double dots indicate identical amino acids, and single dots indicate conservative changes.
in *Drosophila* (21, 28). So far, no PcG protein has been found to bind directly to DNA (28). To investigate the ability of PcG proteins to repress gene activity, they have been targeted to reporter genes as LexA or Gal4 fusion proteins (4, 35). Previously, we have analyzed the ability of LexA-XPC and LexA-hPc2 fusion proteins to repress gene activity in different cell lines by using CAT reporter constructs (35). We observed that LexA-XPC and LexA-hPc2 repress HSF-induced CAT expression to approximately 20% (35). In *Drosophila*, several naturally occurring Pc mutants either are mutated in the conserved C-terminal domain or lack this domain entirely (13). A mutant hPc2 protein that lacks the C-terminal domain has lost its ability to repress gene activity (Fig. 6). On the other hand, the chromodomain is essential for binding of the Pc protein to chromatin (23). Based on the different functions of these two conserved domains, we reasoned that a mutant hPc2 protein that possesses a functional intact chromodomain but does not contain the C-terminal domain might still be able to bind to the chromatin but be unable to repress gene activity. Competition of the mutant hPc2 protein with the endogenous wild-type hPc2 protein might interfere with wild-type hPc2 function.

To test this idea, we expressed wild-type hPc2 (hPc2WT) and the C-terminal deletion mutant (ΔhPc2) in the murine mammary epithelial C57MG (2, 3, 26) and NIH 3T3 cell lines and in the human U-2 OS osteosarcoma cell line. To test whether the proteins are indeed properly overexpressed, we analyzed the hPc2 protein levels of the different, individual clones by Western blotting (Fig. 7). We found higher levels of the hPc2 protein in hPc2WT- (Fig. 7, lane 2) and in ΔhPc2 (lane 3)-transfected C57MG cells than in the untransfected C57MG cells (lane 1). The 82-kDa protein which is detected in the untransfected C57MG cells is presumably the murine homolog of hPc2. Similarly, we found higher levels of hPc2 protein in hPc2WT- (lane 5) and ΔhPc2 (lane 6)-transfected U-2 OS cells than in the untransfected U-2 OS cells (lane 4).

**Expression of ΔhPc2 results in cellular transformation.** To study potential functions of hPc2, we expressed the protein in several different mammalian cell lines. In *Drosophila*, several naturally occurring Pc mutants either are mutated in the conserved C-terminal domain or lack this domain entirely (13). A mutant hPc2 protein that lacks the C-terminal domain has lost its ability to repress gene activity (Fig. 6). On the other hand, the chromodomain is essential for binding of the Pc protein to chromatin (23). Based on the different functions of these two conserved domains, we reasoned that a mutant hPc2 protein that possesses a functional intact chromodomain but does not contain the C-terminal domain might still be able to bind to the chromatin but be unable to repress gene activity. Competition of the mutant hPc2 protein with the endogenous wild-type hPc2 protein might interfere with wild-type hPc2 function.

To test this idea, we expressed wild-type hPc2 (hPc2WT) and the C-terminal deletion mutant (ΔhPc2) in the murine mammary epithelial C57MG (2, 3, 26) and NIH 3T3 cell lines and in the human U-2 OS osteosarcoma cell line. To test whether the proteins are indeed properly overexpressed, we analyzed the hPc2 protein levels of the different, individual clones by Western blotting (Fig. 7). We found higher levels of the hPc2 protein in hPc2WT- (Fig. 7, lane 2) and in ΔhPc2 (lane 3)-transfected C57MG cells than in the untransfected C57MG cells (lane 1). The 82-kDa protein which is detected in the untransfected C57MG cells is presumably the murine homolog of hPc2. Similarly, we found higher levels of hPc2 protein in hPc2WT- (lane 5) and ΔhPc2 (lane 6)-transfected U-2 OS cells than in the untransfected U-2 OS cells (lane 4). These
results show that the transfected constructs are expressed in the selected cell clones.

When cultured to confluence, ΔhPc2-transfected C57MG cells showed striking changes in morphology compared to the parental cell line. Whereas untransfected C57MG cells exhibit a "cuboidal" morphology (Fig. 8), C57MG/ΔhPc2 cells exhibit a "streaming" phenotype, characterized by highly elongated cells (Fig. 8). This latter phenotype is commonly observed in transformed cells and is very similar to the phenotype obtained when the wnt1 oncogene is transfected into C57MG cells (Fig. 8) (2, 3, 26). No phenotypic changes were observed in cells transfected with hPc2WT (Fig. 8). To test whether the phenotypic changes induced by the ΔhPc2 mutant were specific for the deletion of the C-terminal domain, we also transfected a construct containing a mutant lacking the chromodomain (Δchromo) but retaining the C-terminal domain. This did not result in phenotypic changes (36). We also transfected the ΔhPc2-transformed cells with additional hPc2WT. This resulted in reversal of the transformed phenotype (36), suggesting that the cellular transformation caused by the ΔhPc2 mutant is due to a dominant-negative effect.

Similar phenotypic changes, characterized by elongated cells, were observed in NIH 3T3 and U-2 OS cells after transfection with ΔhPc2 but not after transfection with hPc2WT. However, transformed C57MG cells are well characterized in terms of morphology and changes in the expression of marker genes, and we therefore further characterized the C57MG cells. Transformed C57MG cells that are transfected with either the neuT, wnt1, or wnt2 gene have decreased expression of the wnt4 gene (2, 3, 14, 26). This indicates that wnt4 expression can be used as a marker for cellular transformation of C57MG cells resulting from the overexpression of proto-oncogenes. Considering the striking similarities between ΔhPc2-induced and wnt1-induced phenotypic changes, we tested whether ΔPc-induced phenotypic changes are accompanied by changes in wnt4 expression level. We found that wnt4 is strongly down-regulated in both C57MG/ΔhPc2 and C57MG/wnt1 cells whereas no changes in wnt4 levels were observed in C57MG/hPc2WT cells (Fig. 9A). In contrast, overexpression of wild-type hPc2 did not have such an effect.

It has further been shown that after reaching confluence, C57MG/wnt1 cells continue to divide, as measured by [%H]thymidine incorporation (2, 3, 26). Similarly, we found that C57MG/ΔhPc2 but not C57MG/hPc2WT cells continued to proliferate at the same rate as C57MG/wnt1 cells (Fig. 9B).

We conclude that expression of the ΔhPc2 mutant protein results in a partial transformation of C57MG, NIH 3T3 and U-2 OS cell lines. A likely mechanism is that this transformation is due to interference of the ΔhPc2 mutant protein with the PcG protein complex.

Enhanced, deregulated expression of c-myc in ΔhPc2-transformed cells. The PcG protein complex is involved in repression of gene activity, and our results suggest that interference with hPc2 protein function results in derepression of at least one oncogene. Deregulated expression of this oncogene will subsequently lead to cellular transformation. To test this idea, we probed a Northern blot containing poly(A)+ mRNA of C57MG, C57MG/wnt1, C57MG/hPc2WT, and C57MG/ΔhPc2.

![FIG. 5. Colocalization of the hPc2 and hPc1/M33 proteins in nuclei of U-2 OS cells. Rabbit anti-M33 (A) and chicken anti-hPc2 (B) labelling is shown in single confocal optical sections. (A and B) Two different detection channels of the dual image; (C) false-color overlay. hPc2 and hPc1/M33 are homogeneously distributed in the nucleus but are also concentrated in large, brightly labelled domains. hPc2 and hPc1/M33 colocalize in these large domains (C) (yellow).](image)

![FIG. 6. ΔhPc2, lacking the C-terminal domain, is not able to repress HSF-induced LUC gene activity. LUC reporter expression is maximally induced by HSF in the absence of any LexA fusion protein (Control). Cotransfection of LexA alone has hardly any influence on HSF-induced LUC expression, which is still about 97% of the total (LexA). Cotransfection of LexA and hPc2 represses LUC activity fivefold. Cotransfection of LexA and ΔhPc2 has no significant repression effect on LUC activity, which is still 90% of the total. The bars represent the average degree of repression by LexA-hPc2 or LexA-ΔhPc2 in four independent experiments (mean ± SEM). The actual values are indicated in the text.](image)
FIG. 7. Western analysis of transfected hPc2 proteins from cell extracts of C57MG and U-2 OS cells. Equal amounts of C57MG and U-2 OS cell extracts were Western blotted. The blots were incubated with a rabbit anti-hPc2 antibody. Endogenous hPc2 levels were detected in the untransfected U-2 OS cells (lane 4). Endogenous levels of a murine hPc2 homolog were detected in untransfected C57MG cells (lane 1). Elevated levels of the hPc2 protein were detected in the hPc2WT- (lanes 2 and 5) and in the ΔhPc2 (lanes 3 and 6)-transfected cell lines. Analysis was performed with a 10% polyacrylamide gel. Molecular masses of the proteins are indicated (in kilodaltons). The C-terminal 30-aa deletion was too small to be detected on these Western blots.

cells with cDNA fragments of several oncogenes. We found that in C57MG/ΔhPc2 cells the expression of the c-myc proto-oncogene is strikingly enhanced, concomitant with a decrease in the wnt4 expression level (Fig. 10). In C57MG/wnt1 cells, expression of c-myc was also increased, but this increase was sixfold lower than that in C57MG/ΔhPc2 cells. No significant changes in the expression levels of the bmi1, bcl-2, c-fos, and c-jun oncogenes were observed (36). This underlines the specificity of the effect of ΔhPc2 expression on c-myc expression in C57MG/ΔhPc2 cells.

The U-2 OS osteosarcoma cell line shows phenotypic characteristics of cellular transformation similar to those seen in C57MG after the transfection with ΔhPc2. We therefore also examined the RNA levels of c-myc in the different U-2 OS clones. We found that in U-2 OS/ΔhPc2 cells the expression of c-myc is enhanced (Fig. 11A, lane 3) compared to the control U-2 OS cells (lane 1). Surprisingly, we detected reduced c-myc RNA levels in U-2 OS/hPc2WT cells (lane 2). This effect was specific for c-myc expression, since no reduced c-fos and c-jun expression in U-2 OS/hPc2WT cells was observed (36). We also analyzed the expression of the 67-kDa c-myc protein, using the 9E10 monoclonal antibody, which specifically detects the human c-myc protein (8). We found that also the c-myc protein levels are elevated in U-2 OS/ΔhPc2 (Fig. 11B, lane 3) cells in comparison with control U-2 OS cells (lane 1). As with c-myc RNA levels, a lower level of c-myc protein was found in U-2 OS/hPc2WT cells (lane 2).

In many cell lines, as is the case for C57MG, the expression levels of c-myc are very low. Therefore, we needed to isolate poly(A)⁺ RNA to be able to detect c-myc RNA in C57MG cells. On the other hand, c-myc levels in U-2 OS cells are known to be relatively high. In many osteosarcomas, the c-myc gene is amplified (17, 37). Therefore, we were able to detect

FIG. 8. Overexpression of a ΔhPc2 deletion mutant induces partial transformation of C57MG cells. The C57MG cells were stably transformed with the wnt1 oncogene (C57MG/wnt1), the full-length hPc2 gene (C57MG/hPc2WT), and the ΔhPc2 deletion mutant, which lacks the conserved C-terminal domain (C57MG/ΔhPc2). The cells were cultured to confluence before the photographs were taken.
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![Image](image-url)

**FIG. 9.** Changes in gene expression and growth characteristics in C57MG/AhPc2 cells. (A) Poly(A)⁺ mRNA of C57MG control cells (lane 1), C57MG cells transfected with only the pcDNA3 plasmid carrying the neomycin resistance gene (lane 2), C57MG/wnt1 (lane 3), C57MG/hPc2WT (lane 4), and C57MG/AhPc2 (lane 5) cells was Northern blotted and probed with a fragment of the wnt1 gene. To verify equal RNA loading, the filter was hybridized with a GAPDH probe. (B) Untransfected C57MG cells and C57MG/neo, C57MG/wnt1, C57MG/hPc2WT, and C57MG/AhPc2 cells were cultured to confluence, and [³H]thymidine was added to the culture medium. The cells were cultured for an additional 2 days before [³H]thymidine incorporation was determined. The counts were normalized to the protein content. The [³H]thymidine incorporation by control, untransfected C57MG cells is set at 100%. Representative results of four independent experiments are shown.

c-myc RNA expression in total RNA in U-2 OS cells. The difference in expression levels of c-myc in C57MG and U-2 OS cells is also a likely explanation for our detection of a reduction in c-myc expression levels in U-2 OS/hPc2WT cells but not in C57MG/hPc2WT cells. In the last cell line, the c-myc levels are already low.

In summary, we find that c-myc expression is elevated after expression of the ΔhPc2 protein in two different cell lines. We conclude that interference with the expression of hPc2 results in deregulated, enhanced c-myc transcription. At the same time, a reduction of c-myc RNA and c-myc protein levels is detected in U-2 OS cells by the overexpression of hPc2WT, providing extra evidence for a role of hPc2 in the regulation of c-myc expression.

**ΔhPc2 induces anchorage-independent growth.** Rat 1a cells, a fibroblast cell line, have been found to transform and display anchorage-independent growth in soft agarose by overexpression of c-myc alone (40, 44). Previous studies have shown that Bmi-1, a vertebrate PcG protein and oncprotein, is able to induce cellular transformation and anchorage-independent growth in Rat 1a cells (5). Since overexpression of ΔhPc2 enhances c-myc transcription, it is possible that ΔhPc2, like c-myc, is able to induce anchorage-independent growth of Rat 1a cells. We therefore used Rat 1a cells to test the transforming effect of ΔhPc2 as an independent assay for cellular transformation.

Rat 1a cells were transfected with c-myc (Rat 1a-myc), hPc2WT (Rat 1a-hPc2WT), ΔhPc2 (Rat 1a-ΔhPc2), and an hPc2 mutant lacking the chromodomain (Rat 1a-Δchromo). The results demonstrate that ΔhPc2 (Rat 1a-ΔhPc2) as well as c-myc (Rat1a-myc) overexpression alone is sufficient to transform cells (Table 1). The number of colonies induced by ΔhPc2 overexpression is comparable to the effect of c-myc overexpression alone. In contrast, overexpression of hPc2WT and Δchromo did not induce colonies of Rat1a cells (Table 1). These results demonstrate that ΔhPc2 is able to induce anchorage-independent growth when expressed in Rat 1a cells.

**Induction of apoptosis, specifically in ΔhPc2-transformed cells.** We addressed the functional significance of the enhanced c-myc expression in C57MG/ΔhPc2 and U-2 OS/ΔhPc2 cells. It has been shown that overexpression of c-myc, in combination with exposure to culture medium containing a low serum concentration, leads to apoptosis (9-11, 47). To test whether the enhanced expression of c-myc in the two different cell lines also results in apoptosis, we cultured the cells in 0.1% serum. We observed extensive cell death of C57MG/ΔhPc2 cells 24 to 48 h after serum deprivation (Fig. 12C). In contrast, the numbers of C57MG cells and C57MG/hPc2WT cells had increased three- to fourfold 48 h after serum deprivation (Fig. 12A and B, respectively). One of the earliest hallmarks of apoptosis is the redistribution of phosphatidylinerine from the inner face of the plasma membrane to the cell surface (20, 22). This redistribution can be detected with an FITC conjugate of annexin V, a protein that has a high affinity for phosphatidylinerine (20, 22). After 4 to 6 h of serum deprivation, we observed a substantial number of C57MG/ΔhPc2 cells and cells that could be labelled with annexin V-FITC at the outside of the cell, 9% ± 3% in five independent experiments (Fig. 12F). In contrast, no annexin V-FITC-positive cells were observed in C57MG and C57MG/hPc2WT cells 6 h after serum deprivation (Fig. 12D and E). Similar results were detected with U-2 OS cells (36). We conclude that serum deprivation of C57MG/ΔhPc2 and U-2 OS/ΔhPc2 cells results in extensive cell death, which shows hallmarks of apoptosis.
**FIG. 11.** c-myc expression is enhanced in U-2 OS/hPc2 cells. (A) Total RNA of U-2 OS control (lane 1), U-2 OS/hPc2WT (lane 2), and U-2 OS/hPc2 (lane 3) cells was Northern blotted and probed with a fragment of the c-myc gene. To verify equal RNA loading, the filter was hybridized with a GAPDH probe. (B) Cell extracts of U-2 OS control (lane 1), U-2 OS/hPc2WT (lane 2), and U-2 OS/hPc2 (lane 3) cells were analyzed on a Western blot. The blots were incubated with the mouse monoclonal antibody 9E10, which specifically recognizes the human c-myc protein. Molecular masses of the proteins are indicated (in kilodaltons).

**DISCUSSION**

Two human Pc homologs exist. The Pc protein is involved in the stable and heritable repression of gene activity during *Drosophila* development. To study the functions of vertebrate Pc homologs, we have isolated and characterized a novel human Pc homolog. To isolate this gene, we used the *Xenopus* homolog of *Pc*, XPC, as a probe. Overall, the novel human Pc homolog has 48% identity and 80% similarity to the XPC protein. Based on sequence homologies with another, previously characterized human Pc homolog, CBX2 or hPc1, and the murine Pc homolog, M33, we conclude that we have isolated a novel human Pc homolog, hPc2, which is distinct from hPc1/M33. Whereas the hPc2 protein is most closely related to XPC, the hPc1 protein is most closely related to M33.

It appears to be a common feature among vertebrate PcG proteins that each of them exists as a pair of closely related proteins. For instance, the vertebrate PcG proteins Bmi1 (33) and mel-18 (45) have large identical regions (alignment shown in reference 33). Furthermore, we recently identified two human proteins, HPH1 and HPH2, that both have extensive sequence homology to the *Drosophila* PcG protein Polyhomeotic (Ph) in two conserved homology domains (16). Strikingly, homology between the HPH1 and HPH2 proteins themselves is restricted to these conserved homology domains; outside these domains, homologies are very limited. This is very similar to what we report here for hPc1/M33 and hPc2. Overall homologies between hPc2 and hPc1/M33 do not exceed the homologies between these vertebrate Pc homologs and *Drosophila* Pc. It is important, however, to note that when not only identical amino acids but also conservative changes are taken into consideration, the similarity between hPc2 and hPc1/M33 is 68%. This is significantly higher than the 29% identity. As already pointed out, this could indicate that the overall three-dimensional structures of hPc2 and hPc1/M33 are very similar, which may imply that the two proteins are functionally equivalent. It is also possible, however, that the strong conservation in the chromodomain and C-terminal domain is enough to provide functional equivalence to the proteins. Although homology between M33 and *Drosophila* Pc is restricted to the chromodomain and the C-terminal domain, M33 is able to partly rescue the *Drosophila* Pc phenotype when overexpressed in the Pc mutant (25). This indicates that hPc1/M33 can be considered a functional homolog of Pc, and it signifies the importance of the conserved regions. The potential functional relationship between the two human Pc homologs is further underlined by our finding that hPc1/M33 and hPc2 colocalize in nuclei of human U-2 OS cells, suggesting that hPc1 and hPc2 are part of a human PcG protein complex. In this context, it is significant that the human PcG protein BM1 and the human Polyhomeotic-related HPH1 and HPH2 proteins also colocalize with hPc2 and hPc1/M33 in the same nuclear domains of several human cell lines (1, 16).

We conclude that there are at least two human Pc homologs. It is not clear why these closely related human Pc proteins exist. Since other vertebrate PcG-related proteins have been found to exist as functional pairs as well, this may have functional significance. It is possible that small differences induce subtle changes in, for instance, their specificities for binding to target genes.

**c-myc is a potential target gene of hPc2.** In *Drosophila*, PcG proteins have been identified as repressors of gene expression. The only identified target genes of PcG proteins are homeotic genes and gap genes (28, 31, 38, 48). These genes are all involved in developmental decisions. In this study we found that expression of a mutant hPc2 cDNA in two different mammalian cell lines, U-2 OS and C57MG, results in deregulated, enhanced expression of a gene that controls a different process. This gene, c-myc, is involved in cell cycle and differentiation events. The mutant hPc2 protein lacks a conserved C-terminal domain that is crucial for the ability of the hPc2 protein to repress gene activity. Further, overexpression of the wild-type hPc2 cDNA results in decreased expression of c-myc. It is therefore likely that it is due to interference with hPc2 function that c-myc expression is deregulated and enhanced. From these data, however, it cannot be concluded whether the effect on c-myc expression is a direct or indirect effect. It is tempting to speculate that hPc2 interacts directly with the c-myc locus. Unfortunately, the fact that Pc proteins binds to chromatin and not to naked DNA excludes the use of standard methods, such as DNA footprinting, to assess whether the hPc2 protein is physically associated with the c-myc locus. Association of *Dro-

**TABLE 1.** Colony formation by ΔhPc2-transfected Rat 1A cells in soft agarose

<table>
<thead>
<tr>
<th>Construct</th>
<th>No. of colonies/5 × 10⁶ transduced cells</th>
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<tbody>
<tr>
<td>pRcCMV-c-myc</td>
<td>451 ± 52</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA3-hPc2WT</td>
<td>337 ± 27</td>
</tr>
<tr>
<td>pcDNA3-ΔhPc2</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA3-ΔhPc2-Δchromo</td>
<td>0</td>
</tr>
</tbody>
</table>

* A total of 5 × 10⁶ of each pool of transfected and genetin-selected cells were seeded into 0.4% top agarose, and colonies with diameters of >0.1 mm were counted 14 to 21 days after seeding (34, 40, 44). The entire procedure, including the transfection of the cDNAs, was performed in triplicate, and the mean ± SEM is shown.
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sophila Pc with chromatin of target genes has so far only been proven for chromatin of the homeotic bithorax (Ubx) gene locus (27). In future studies, we will address the question of a potential, direct association of hPc2 with the c-myc locus by employing the in vivo cross-linking method (27). This current lack of knowledge does not, however, detract from our finding that reveals a novel, hitherto unexpected level of regulation of c-myc. This involves regulation of gene activity at the level of changes in chromatin structure, in this case involving one of the PcG proteins.

We observed extensive cell death, with the hallmarks of apoptosis, specifically of C57MG/ΔhPc2 transformed cells after serum deprivation. Apoptosis has been shown to occur upon overexpression of c-myc, in combination with serum deprivation (11–13). Apoptosis therefore appears to be a direct consequence of the deregulated, enhanced c-myc expression in C57MG/ΔhPc2 cells. However, it is noteworthy that mutations in another PcG gene, polyhomeotic (Ph), lead to extensive cell death between 9.5 and 12 h after egg laying, specifically in the ventral epidermis (42, 43). This precisely defined time window and the cell type specificity of cell death point to apoptosis as the underlying cause. It would be of interest to examine this previously described phenomenon in the light of our present results.

Involvement of hPc2 in cellular transformation and apoptosis. We show that interference with hPc2 function by ectopic expression of the hPc2 deletion mutant that lacks the conserved C-terminal domain results in cellular transformation of mammalian cell lines. Concomitantly, the expression of the c-myc proto-oncogene is enhanced in these transformed cells. It is therefore tempting to speculate that one function of the mammalian Pc proteins is to repress the transcription of certain proto-oncogenes. Interference with hPc2 function will then result in derepression of transcriptionally repressed proto-oncogenes and subsequently in cellular transformation. Importantly, our results do not constitute the only link between PcG proteins and cellular transformation. Two other mammalian PcG proteins, bm1 and mel-18, have been shown to be involved in tumorigenesis (5, 18, 46). However, molecular mechanisms that underlie oncogenesis due to changes in chromatin structure have hardly been explored so far. It will be challenging to search for tumor cell lines in which the function of hPc2 is disturbed, either by mutations or by changed expression levels.

ACKNOWLEDGMENTS

D.P.E.S. and D.J.O. contributed equally to this work.

We thank René Bernard for suggesting the apoptosis experiments and for providing probes and the Rat1a cells, Robert Kingston for plasmids, Andy McMahon for the wnt4 probe, Jan Kooter for critically reading the manuscript, and Thijs Hendrix for raising the antibodies. A.P.O. is sponsored by a grant from the Royal Netherlands Academy of Sciences (KNAW).

REFERENCES
