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

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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, 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 colocalize 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, hp2c. We showed that the hp2c 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 hp2c, we designed an hp2c mutant protein that lacks the C-terminal domain, which is crucial for the ability of hp2c to repress gene activity. Expression of this hp2c mutant in mammalian cell lines results in (i) cellular transformation, (ii) enhanced expression of the c-myc protooncogene, and (iii) apoptosis upon serum deprivation. Our data suggest that hp2c is a repressor of proto-oncogene activity and that interference with hp2c function can lead to derepression of proto-oncogene transcription and subsequently to cellular transformation.

MATERIALS AND METHODS

Isolation of the hp2c gene. For screening a Agt10 human fetal brain cDNA library (Clontech, Palo Alto, Calif.), a probe encompassing the entire coding region of the 3' untranslated region of the longest cDNA from Drosophila melanogaster was used to generate 5' and 3' probes, from which a 32P-labeled 1.6-kb fragment was excised by digestion and used as a probe. The filters were hybridized overnight at 50°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-10× Denhardt's solution-10% dextran sulfate-0.1% sodium dodecyl sulfate (SDS)-100 µg of denatured yeast chromosomal DNA per ml was isolated and blotted, and the expression pattern of hp2c was analyzed. To allow a comparison with the commercial Northern blot, we blotted poly(A)+ RNA of SW480 cells, which are represented on the commercial blot and in which all three genes are strongly expressed.

Analysis of hp2c transcripts. Tissue-specific Northern blots contained approximately 2 µg of poly(A)+ RNA per lane from different human tissues or human cell lines 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 pattern of hp2c was analyzed. To allow a comparison with the commercial Northern blot, we blotted poly(A)+ RNA of SW480 cells, which are represented on the commercial blot and in which all three genes are strongly expressed. The blots were hybridized with [α-32P]dATP-labelled DNA probes and autoradiographed with X-ray films and intensifying screens at −70°C. Northern blot analyses were performed with an hp2c probe that excludes the conserved chromodomains and C-terminal domain (coding for aa 60 to 530).

Production of the M33 and hp2c polyclonal antibodies. A cDNA fragment encoding aa 157 to 432 of M33 was cloned into the expression vector pET-23b (Novagen, Madison, Wis.). This fragment does not encompass the conserved chromodomains or the conserved C-terminal domain. A cDNA fragment encoding aa 60 to 558 of hp2c was cloned in pET-23b (Novagen). This fragment does not encompass the conserved chromodomains and not the conserved C-terminal domain. A cDNA fragment encoding aa 60 to 558 of hp2c was cloned in pET-23b (Novagen). This fragment does not encompass the conserved chromodomains and not the conserved C-terminal domain.

Fusion proteins or extracts of U-2 OS cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. The blots were probed with a 1:10,000 dilution of anti-hPc antibody (Novagen) or a 1:1,000 to 1:5,000 dilution of affinity-purified anti-hPc or anti-M33 antibodies. The secondary alkaline phosphatase-goat anti-rabbit or anti-chicken IgG (H+L) antibodies (Jackson Immunoresearch Laboratories) were diluted 1:10,000, and nitroblue tetrazolium-5-bromo-4-chloro-3-indoly-phosphate toluidinium (NBT/BCIP) (Boehringer) was used as the substrate for detection.

Immunofluorescence staining of tissue culture cells. U-2 OS cells were cultured and labeled as described recently (16). Labeling has been analyzed by confocal laser scanning microscopy, and single optical sections are shown (see Fig. 5). The first two pictures represent the two different detection channels of the dual image, and the third picture represents the false-color overlay. For labeling, donkey anti-rabbit immunoglobulin G coupled to Cy3 (Jackson Immunoresearch Laboratories) and donkey anti-chicken IgG coupled to fluorescein isothiocyanate (FITC) (Jackson) were used.

LexA fusion reporter 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 re- located by the luciferase gene (LUC). U-2 OS cells were cultured in a 25-cm2 flask and cotransfected with the heat shock factor (HSF)-inducible LUC reporter plasmid, 4 µg of the LexA fusion constructs, and 2 µg of the pSVβ-Gal construct (Promega) by the calcium phosphate precipitation method. The HSF-inducible LUC reporter plasmid was activated by exposing the cells at 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 vary between independent experiments. LUC activity in cells transfected with the LUC reporter plasmid only was therefore 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) as specified by the manufacturer, and stably transfected lines were selected by culturing the cells for 2 weeks in medium containing 800 µg of G418 (Gibco) per ml. The surviving cells were cloned by dilution for 2 to 4 weeks in medium containing 250 µg of G418 per ml. Individual cell clones were selected and cultured in individual dishes. After five passages, the cell lines were characterized for hPc/hsf1 and hPc/luciferase activity and gene expression. hPc/hsf1 and hPc/luciferase activity was measured as described previously (26). The numbers were normalized to the pSVβ-Gal activity. 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. Northern blot and RNA Northern analyses were performed by standard procedures. The blots were hybridized with [α-32P]dATP-labelled DNA probes, and the blots were autoradiographed with intensifying screens at −70°C with preflashed X-ray films.

Western blot analysis of hp2c and c-myc proteins. The expression levels of the hp2c and c-myc proteins were analyzed by using cell lysates of the stably transfected C57MG and U-2 OS clones (35). For hp2c detection, the blots were incubated with a 1:5,000 dilution of affinity-purified rabbit anti-hPc2 antibodies. The monoclonal 9E10 antibody was used for the detection of c-myc (8). Equal amounts of proteins were loaded, as measured by the bichinonic 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 hp2c/WT, the C-terminal deletion mutant (hp2cΔA), and a mutant lacking the chromodomains (Δchromo), which were all cloned in the pcDNA3 vector. As a positive control, the c-myc/pcDNA3, which was cloned in the pRc-CMV vector was transfected. The cells were subjected to selection with 500 µg of G418 per ml. The cells were then cultured for 14 days, at which time the G418-resistant clones were counted. The clones were trypsinized, and cells were counted. A total of 5 × 105 cells in 5 ml of 10% Dulbecco’s modified Eagle’s medium containing 0.4% (wt/vol) agarose was seeded in 5-cm petri dishes containing 1% (wt/vol) 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 pcDNAs, 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 (Nexins 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 still viable, since they excluded propidium iodine (36). The assay was repeated five times with similar results. For annexin V staining, C57MG-representant clones were counted. The clones were trypsinized, and cells were counted. A total of 5 × 105 cells in 5 ml of 10% Dulbecco’s modified Eagle’s medium containing 0.4% (wt/vol) agarose was seeded in 5-cm petri dishes containing 1% (wt/vol) 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 PCDNAs, was performed in triplicate.

RESULTS

Isolation and characterization of a novel human Pc homolog, hp2c. 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 chromodomains (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 chromodomains, which is 96% identical to the homologous region of the mouse Pc homolog, M33, and 55% identical to the Drosophila Pc chromodomains (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 homolog. 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. 2). 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 only in the conserved C-terminal domain and presumably also in the chromodomain. The hPc 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.

FIG. 1. Nucleotide sequence of hPc2 and its predicted amino acid sequence. The chromodomain 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.
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). 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 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 nucleus 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 HPH1 (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

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**FIG. 2.** Comparison of the hPc2 protein with other *Pc* homologs. (A) The sequence is aligned to *Xenopus Pc, Pc, M33,* and the CBX2 (hPc1) protein sequences. Identical amino acids are shown, and nonidentical amino acids are indicated by dashes. The conserved chromodomain 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). We tested the ability of an hPc2 deletion mutant protein, ΔhPc2, that lacks the last 30 aa of the C terminus (Fig. 2) to repress gene activity. U-2 OS cells were transfected with a construct containing a tandem of four LexA operators, binding sites for the HSF transcriptional activator, and the hsp70 TATA promoter immediately upstream of the LUC reporter gene. The endogenous HSF was used as a transcriptional activator. In the absence of HSF, no LUC activity was observed (36). Maximum LUC activity in the presence of HSF was set at 100% (Fig. 6, Control). Cotransfection of LexA alone had no significant influence on HSF-induced (Fig. 6, 97% ± 6% [mean ± SEM], n = 4) LUC activity. We found that LexA-hPc2 repressed LUC activity to approximately 20% (14% ± 4% [mean ± SEM], n = 4). When LexA-ΔhPc2 is targeted to the reporter gene, LUC activity is not significantly repressed (90% ± 9% [mean ± SEM], n = 4). The results show that the C-terminal deletion mutant, unlike hPc2, is not able to repress HSF-induced LUC activity. This is in agreement with earlier data which also showed that a *Drosophila* Pc protein which lacks the C-terminal domain is unable to repress gene activity (4). This indicates that the conserved C-terminal region of hPc2 is involved in mediating gene repression.

**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 [3H]thy-midine 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.
cells with cDNA fragments of several oncogenes. We found that in C57MG/ΔhPc2 cells the expression of the c-myc protooncogene 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...
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 oncoprotein, 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 (Rat 1a-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 phosphatidylserine 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 phosphatidylserine (20, 22). After 4 to 6 h of serum deprivation, we observed a substantial increase in the 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. 9. Changes in gene expression and growth characteristics in C57MG/ΔhPc2 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/ΔhPc2 (lane 5) was Northern blotted and probed with a fragment of the wnt4 gene. To verify equal RNA loading, the filter was hybridized with a GAPDH probe. (B) Untransfected C57MG cells and C57MG/NEO, C57MG/wnt4, C57MG/hPc2WT, and C57MG/ΔhPc2 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.

FIG. 10. c-myc expression is enhanced in C57MG/ΔhPc2 cells. Poly(A)⁺ mRNA of C57MG control cells (lane 1) and C57MG/wnt1 (lane 2), C57MG/hPc2WT (lane 3), and C57MG/ΔhPc2 cells (lane 4) was Northern blotted and probed with fragments of the c-myc and wnt4 genes. To verify equal RNA loading, the filter was hybridized with a GAPDH probe.
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, Pc. It is important, however, to note that when Drosophila PcG protein Polyhomeotic-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 Drosophila PcG proteins with c-myc suggests that hPc2 may function as an auto- or trans-repressor for the c-myc locus in the mammalian cell lines.

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 Bml1 (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 BMI1 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 two 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.

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

<table>
<thead>
<tr>
<th>Construct</th>
<th>No. of colonies/5 × 10⁴ transfected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRCMV c-myc</td>
<td>451 ± 52</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA3-hPc2WT</td>
<td>0</td>
</tr>
<tr>
<td>pcDNA3-ΔhPc2</td>
<td>337 ± 27</td>
</tr>
<tr>
<td>pcDNA3-Δchromo</td>
<td>0</td>
</tr>
</tbody>
</table>

* A total of 5 × 10⁴ 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.
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/hPc2 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/hPc2 cells. Apoptosis has not been described for Drosophila Pc mutants. 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 hPc 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, bmi1 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.

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

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