Polycomb group gene silencing proteins are concentrated in the perichromatin compartment of the mammalian nucleus

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Summary

Human Polycomb group (PcG) proteins are involved in cell-type-dependent epigenetic gene silencing in an evolutionarily conserved manner. We have analysed the subnuclear localisation of these regulatory proteins in two different human cell lines and in rat liver tissue by means of light and electron immunomicroscopy using specific antibodies. We find that the PcG proteins HPC2, HPH1, BMI1 and RING1 are highly concentrated in the perichromatin compartment, situated at the surface of condensed chromatin domains. This compartment was demonstrated earlier to be the nuclear site where most pre-mRNA synthesis takes place. Interestingly, these PcG proteins are virtually absent from the interior of condensed chromatin areas. The present observations therefore show that transcriptionally active and PcG-silenced loci occur within the same spatially limited nuclear domain. Our novel high-resolution data strongly support the idea that epigenetic PcG-mediated gene silencing is a local event, rather than affecting large chromatin domains. In addition to being associated with the perichromatin region, PcG proteins also occur in the interchromatin space. Implications of these observations for higher order chromatin structure and for the mechanisms of PcG-mediated gene silencing are discussed.

Key words: PcG proteins, Gene silencing, Chromatin, Immunoelectron microscopy, Transcription

Introduction

In differentiated eukaryotic cells the transcriptional activity of most genes is permanently suppressed. This silenced state is faithfully transmitted to the daughter cells during cell division and constitutes the basis for stable cell differentiation. Polycomb group (PcG) proteins are a heterogeneous class of polypeptides involved in such epigenetic silencing in higher eukaryotes (Jacobs and van Lohuizen, 1999; Lyko and Paro, 1999; Pirrotta, 1998; Satijn and Otte, 1999a). Although identified originally in *Drosophila*, recent work shows that PcG proteins are evolutionarily conserved in mammals (van Lohuizen, 1999; Satijn and Otte, 1999a; Simon and Tamkun, 2002) and probably also in plants (Ma, 1997; Preuss, 1999).

PcG proteins form multimeric complexes (Franke et al., 1992; Satijn and Otte, 1999a; Sewalt et al., 1998; Francis et al., 2001; Simon and Tamkun, 2002) that bind to specific genomic sites, named Polycomb responsive elements (PREs) (Chan et al., 1994; Mihaly et al., 1998; Tillib et al., 1999), resulting in transcriptional silencing of nearby loci in a clonally stable manner. Also, if PcG proteins are artificially targeted to a locus, it becomes transcriptionally inactive (Alkema et al., 1997; Bunker and Kingston, 1994; van der Vlag and Otte, 1999). It is thought that PcG-mediated silencing involves changes in chromatin structure (Paro et al., 1998; Singh, 1994; Singh and Huskisson, 1998; Simon and Tamkun, 2002), putatively inducing a heterochromatin-like structure (Eissenberg and Elgin, 2000).

The fine structural architecture of the cell nucleus has been extensively studied for many years. These studies have allowed one to define several structural compartments or domains in the nucleus and to approach the roles of these domains in nuclear functions. It is now well established that the perichromatin region is a site of DNA replication as well as of transcription (for reviews, see Fakan, 1994; Spector, 1996; Jaunin and Fakan, 2002) and that the perichromatin fibrils are in situ forms of nascent transcripts (Fakan, 1994; Cmarko et al., 1999). Structural components occurring in the interchromatin space such as the interchromatin granules or coiled (Cajal) bodies have been shown to be involved in assembly and storage of pre-mRNA transcription factors (Fakan, 1994; Spector, 1996; Puvion and Puvion-Dutilleul, 1996; Misteli et al., 1997).

There is growing evidence that a tight relationship exists between large-scale chromatin folding and gene activity (Belmont et al., 1999; Tumbar et al., 1999; Francastel et al., 1999; Lundgren et al., 2000). Transcriptionally active loci are located near the surface of compact chromatin domains, possibly looping out towards the interchromatin compartment (Fakan, 1994; Cmarko et al., 1999; Verschure et al., 1999). Buchenau et al. have presented evidence that PcG proteins in *Drosophila* cells do not occur in compact heterochromatin

(Buchenau et al., 1998). Similarly, it has been shown that *Drosophila* PcG proteins collectively bind to numerous sites on polytene chromosomes. These sites are distinct from constitutive heterochromatin marked by heterochromatin protein 1 (HP1), suggesting that PcG proteins are not associated with heterochromatin (Platero et al., 1995; DeCamillis et al., 1992; Rastelli et al., 1993; Sinclair et al., 1998). In contrast, Saurin et al. have shown that some cell types contain nuclear-body-like domains that are enriched in PcG proteins and are associated with pericentromeric heterochromatin (Saurin et al., 1998). Most probably, PcG proteins do not silence loci by incorporating them into constitutive heterochromatin, despite the idea that PcG-protein-mediated silencing induces a heterochromatin-like structure (Eissenberg and Elgin, 2000).

Here we have investigated the subnuclear distribution of four human PcG proteins in two different human cell lines and in rat liver tissue using indirect immunolabelling in combination with light and electron microscopy. We find that each of the PcG proteins is concentrated in the perichromatin compartment, the same nuclear domain where the transcriptionally active genes are situated. This indicates that active and silenced genes are closely associated in space, suggesting that PcG-silencing is a local event, rather than affecting large chromatin domains. In addition to being associated with chromatin, PcG proteins also occur in the interchromatin region. It is not known whether this fraction is associated with looped-out chromatin or whether it is freely diffusing in the interchromatin space.

Materials and Methods

Cell and tissue processing

Human cell lines SW480 (colorectal adenocarcinoma cells) and T24 (bladder carcinoma cells) were cultured at 37°C under a 10% CO_2 atmosphere in DMEM medium (Gibco, Breda, the Netherlands), supplemented with 10% foetal calf serum (Boehringer, Mannheim, Germany), 2 mM L-Glutamine (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). Liver tissue was obtained from young female Wistar rats.

Antibodies

The cells were immunolabelled with the following monoclonal (mouse) and monospecific polyclonal (rabbit) primary antibodies against human PcG proteins: mouse and rabbit anti-HPC2 (homologue of *Drosophila* Polycomb) (Satijn et al., 1997), rabbit anti-HPH1 (homologue of *Drosophila* Polyhomeotic) (Gunster et al., 1997), rabbit anti-BMI1 (homologue of *Drosophila* Posterior sex combs (Satijn et al., 1997; Satijn and Otte, 1999b), rabbit anti-RING1 (a PcG protein associated polypeptide) (Satijn et al., 1997). For double-labelling experiments, mouse anti-BrdU monoclonal antibody (Partec, Münster, Germany) recognising also BrU in RNA was used. To visualise the primary antibodies, goat anti-mouse and goat anti-rabbit secondary antibodies, conjugated to fluorochromes (Jackson ImmunoResearch Laboratories, West Grove, PA) or with different sizes of colloidal gold (Aurion, Wageningen, The Netherlands, or Jackson ImmunoResearch Laboratories), were used.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes on glass coverslips. The fixed cells were permeabilised with 0.5% Triton-X 100 (Sigma, St. Louis, MO/USA) in PBS and incubated with PBS containing 100 mM glycine (Sigma) for 10 minutes. Subsequently, cells were incubated overnight at 4°C with the primary antibodies. After washing in PBS, cells were incubated with secondary antibodies for 1 hour at room temperature and then washed with PBS. Cells were counterstained with DAPI at room temperature. Slides were mounted in Vectashield (Vector, Burlingame, CA) and were kept at 4°C until evaluation within 24 hours.

Images were recorded with a Zeiss LM510 confocal laser scanning microscope equipped with a 100×/1.23 NA oil immersion lens. A dual-wavelength argon ion laser was used to excite the two fluorochromes simultaneously. Pairs of images, showing the distribution of the antigen and of DAPI, were collected simultaneously. Optical sections through the middle of the nucleus were obtained at a scanning resolution of 512×512 pixels. Each image is the result of the averaging of 16 scans of the same optical section.

Immunoelectron microscopy

Chemical fixation or cryofixation protocols were used. Cells grown on glass coverslips at 50-70% confluency were fixed with 4% paraformaldehyde in 0.1 M Sörensen phosphate buffer, for 1 hour, at 4°C. T24 cells were microinjected with BrUTP and cultured for 10 minutes at 37°C as described previously (Cmarko et al., 1999; Wansink et al., 1993), before fixation and further processing.

Pieces of rat liver were fixed in 4% paraformaldehyde immediately after dissection, for 2 hours on ice. After washing with buffer, free aldehyde groups were blocked by 0.5 M NH_4Cl in PBS for 15 minutes on ice. Cells and tissue were then dehydrated in ethanol, embedded in LRWhite resin, which was allowed to polymerise for 24 hours at 60°C. To overcome some of the potentially deleterious effects of the aldehydic fixation, some specimens were treated using cryopreparative techniques. In short, cells grown on 14 mm Cellagen™ discs (ICN, Aurora, Ohio) were cryofixed by slam freezing using a KF80 apparatus (Reichert, Vienna, Austria). The samples of rat liver were fixed by high pressure freezing (Balzers Union, Balzers, Liechtenstein). The frozen material was cryosubstituted in acetone using a Reichert CS Auto cryosubstitution device and embedded into LRWhite resin (von Schack and Fakan, 1993).

Ultrathin sections were mounted on Formvar/carbon-coated nickel grids and processed for postembedding immunogold labelling, as described previously (Cmarko et al., 1999). Briefly, normal goat-serum-pretreated sections were incubated for 17 hours at 4°C, with the primary antibody or, in the case of double labelling, with a mixture of primary antibodies diluted in PBS with 0.05% Tween 20 (Sigma) and 0.1% BSA (Fluka, Buchs, Switzerland). Colloidal gold-conjugated secondary antibodies were diluted in PBS and reacted with sections for 30 minutes at room temperature. The grids containing sections of chemically fixed cells were submitted to regressive EDTA staining, which is preferential for nuclear ribonucleoprotein constituents (Bernhard, 1969), giving rise to a lower contrast of chromatin. Alternatively, some grids were treated with a 0.2% osmium ammine solution for 1 hour at 20°C to specifically visualise DNA by a Feulgen-type reaction (Cogliati and Gautier, 1973). In cryofixed and cryosubstituted material embedded in LRWhite resin and conventionally contrasted with uranyl-lead staining, the chromatin tends to have a bleached aspect and RNP constituents are well distinguished without the EDTA treatment (von Schack and Fakan, 1993).

In control specimens, sections were treated with the reaction mixture where the primary antibodies were omitted.

The grids were examined with a Philips CM 10 electron microscope at 80 kV using a 30-40 μm objective aperture.

Quantitative evaluation

For quantitative evaluation of the HPC2, BMI1 and RING1 ultrastructural distribution in the nucleus of chemically fixed SW480 cells, 12 micrographs of each group were analysed. Three nuclear regions were considered for quantification: (i) condensed chromatin domains, (ii) the periphery of condensed chromatin, that is, the perichromatin region representing the interface between the condensed chromatin and the interchromatin region, and (iii) the nucleoplasmic area free of condensed chromatin. The width of the perichromatin region is not known and we set it at 40 nm (20 nm on each side of the condensed chromatin edge) taking into account the resolution limits of the indirect immunocytochemical labelling. The micrographs printed at a final magnification of ×52,500 were scanned and the surface area of each of the above-mentioned nuclear regions was morphometrically determined, using the Openlab measurement module (Improvision, Coventry, UK). The number of individual gold particles (12 nm diameter) was counted by hand for each area and the labelling density was expressed as the number of gold particles per μm^2.

Statistical analysis of the labelling density differences between the three above-mentioned compartments in the same experimental group was performed using the Wilcoxon signed ranks test (Siegel and Castellan, 1988). Statistical significance was set at $P<0.01$.

Results

Immunofluorescence microscopic localisation of PcG proteins

Cultured human SW480 and T24 cells were fluorescently labeled using antibodies against the four human Polycomb group (PcG) proteins: HPH1 (homologue of *Drosophila* Ph), BMI1 (homologue of *Drosophila* Psc), HPC2 (homologue of *Drosophila* Pc) and RING1 (a PcG protein associated polypeptide) (Satijn and Otte, 1999a). DNA was counterstained with DAPI. Fig. 1 shows optical sections through the central part of nuclei. Results show that two different PcG proteins (BMI1 and HPC2 in Fig. 1A,B, respectively) do colocalise in PcG-enriched domains previously named PcG bodies (Saurin et al., 1998). Beyond those structures, all four PcG proteins show a diffuse or micropunctuated distribution throughout the nucleoplasm in both SW480 and T24 cells (Fig. 1A,B,F,H,K). Nucleoli exclude PcG proteins (e.g. Fig. 1A,B). No major differences are observed between SW480 and T24 cells with respect to PcG protein localisation, except that the former may more often contain PcG-rich domains. Similarly, double labelling assays with the mouse anti-HPC2 antibody and the two polyclonal probes (anti-RING1 or anti-HPH1) gave results comparable with the above-mentioned anti-HPC2 and anti-BMI1 double labelling experiments (data not shown).

Visual inspection of fluorescence images further shows that, as expected at this spatial resolution, the nucleus seems to be completely filled with DAPI-stained material, that is, DNA. Only the nucleoli contain less DNA. Like DNA, the PcG proteins seem to be present throughout the nucleoplasm with the exception of the nucleoli. However, as the line scans in Fig. 1 show (panels E, J and M), there is no relationship between the detailed spatial distribution of DNA/chromatin (DAPI stained in Fig. 1C,G,I,L) and that of PcG proteins (Fig. 1A,B,F,H,K). Evidently, these four PcG proteins do not simply colocalize with chromatin domains. The resolution limit of light microscopy precludes a more detailed analysis of the localisation of PcG proteins with regards to the fine structure of chromatin areas and particularly of the perichromatin compartment. In order to investigate in more detail the subnuclear distribution of PcG proteins, we have carried out an ultrastructural study using immunoelectron microscopy.

Immunoelectron microscopic localisation of PcG proteins

Sections of cultured cells and rat liver tissue were prepared for electron microscopy either after aldehyde fixation or following cryofixation and cryosubstitution in the absence of chemical fixatives. Particularly the latter method, applied to rat liver tissue, exhibited very good preservation of nuclear fine structure. Immunolabelling of nuclear constituents on ultrathin sections of intact cells is the method of choice for high-resolution in situ localisation of cellular components. In contrast to methods that involve treatment of the specimen with detergents, this procedure avoids protein extraction and possible displacement of antigens in the cell. Moreover, immunolabelling on sections of embedded material circumvents the problem of limited accessibility of antibodies into compact cellular compartments, such as condensed chromatin, because the immunoreaction takes place on the surface of physically sectioned material.

Cell and tissue sections were labelled with antibodies against four human PcG proteins: HPC2, HPH1, BMI1 and RING1. Each of five anti-PcG protein antibodies that were used for immunoelectron microscopy gave a significant signal in the cell nucleus. After EDTA regressive staining of sections, condensed chromatin is relatively electron translucent compared with the RNP-containing perichromatin fibrils and interchromatin compartment (Fig. 2). By contrast, DNA-specific osmium ammine staining resulted in well-contrasted electron dense chromatin areas, leaving the interchromatin domain unstained (Fig. 3A, Fig. 4A).

The immunogold labelling of PcG proteins in paraformaldehyde-fixed material showed intense labelling of the border of condensed chromatin areas, that is, the perichromatin region. The interchromatin compartment was also significantly labelled. Strikingly, almost no label was observed inside condensed chromatin domains (Table 1; Fig. 2, Fig. 3A). Double labelling experiments with the monoclonal anti-HPC2 antibody and rabbit antibodies against the other PcG proteins showed similar distributions for the different PcG proteins, although we did not observe frequent close colocalisation of the two markers (Fig. 4A). This can be explained by steric hindrance occurring within relatively small closely packed molecular assemblies.

The above-described distribution of PcG proteins observed in chemically fixed cells was confirmed on cryofixed samples. In cultured cells and in rat hepatocytes the proteins were present predominantly at the periphery of condensed chromatin or throughout the interchromatin space, frequently associated with RNP fibrils (Fig. 3B). The perichromatin area, previously shown to be the major site of nucleoplasmic RNA synthesis (Cmarko et al., 1999; Fakan et al., 1976), contains a large number of perichromatin fibrils, which are the in situ forms of nascent heterogeneous RNA transcripts (Fakan, 1994). Each of the four PcG proteins is occasionally observed in close contact with a perichromatin fibril (arrowheads in Fig. 2). This is

confirmed by dual labelling of PcG proteins and of nascent RNA, which was tagged by a brief in vivo incorporation of microinjected BrUTP. Nascent RNA and PcG proteins both occurred in the perichromatin regions and occasional close association of the two signals within this nuclear domain was observed (Fig. 4B). The intranuclear distribution of PcG proteins in the human cell lines and in rat liver cells was similar. Each of the four PcG proteins was also abundantly

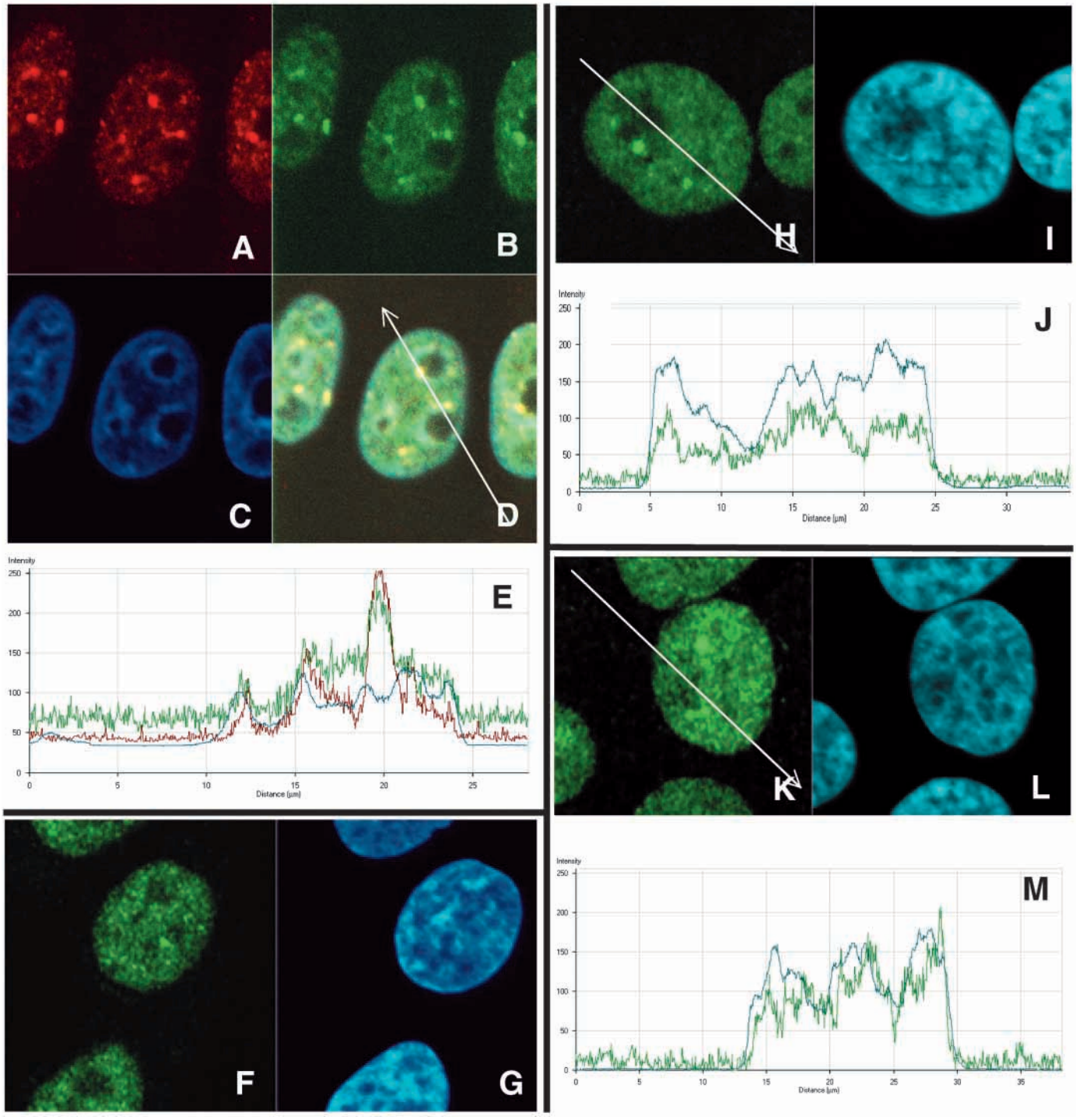

Fig. 1. Immunofluorescent labelling of the PcG proteins in cultured cells. Confocal optical section through the central part of the nucleus. (A-E) SW480 cells labelled with anti-BMI1 (A), anti-HPC2 (B) and DAPI (C); (D) shows the merged image and (E) a line scan along the white arrow in panel D, showing the distribution of BMI1 (red line), HPC2 (green line) and DAPI (blue line). (F-G) SW 480 cells labelled with anti-RING1 (F) and DAPI (G). (H-J) T24 cells labelled with anti-BMI1 (H) and DAPI (I); J shows a line scan along the white arrow in H, showing the distribution of BMI1 (green line) and DAPI (blue line). (K-M) T24 cells labelled with anti-HPH1 (K) and DAPI (L); (M) shows a line scan along the white arrow in K, showing the distribution of HPH1 (green line) and DAPI (blue line).

present in the interchromatin compartment, which is virtually devoid of chromatin. There, PcG proteins were often localised on the periphery of clusters of interchromatin granules whereas the internal area of these clusters remained devoid of label (Fig. 4B). Most probably, the PcG proteins in the perichromatin region are part of multi-protein silencing complexes, whereas those in the interchromatin space may represent a freely diffusing pool of protein molecules.

In control assays, where the primary antibody was omitted, the level of labelling over cell sections was negligible.

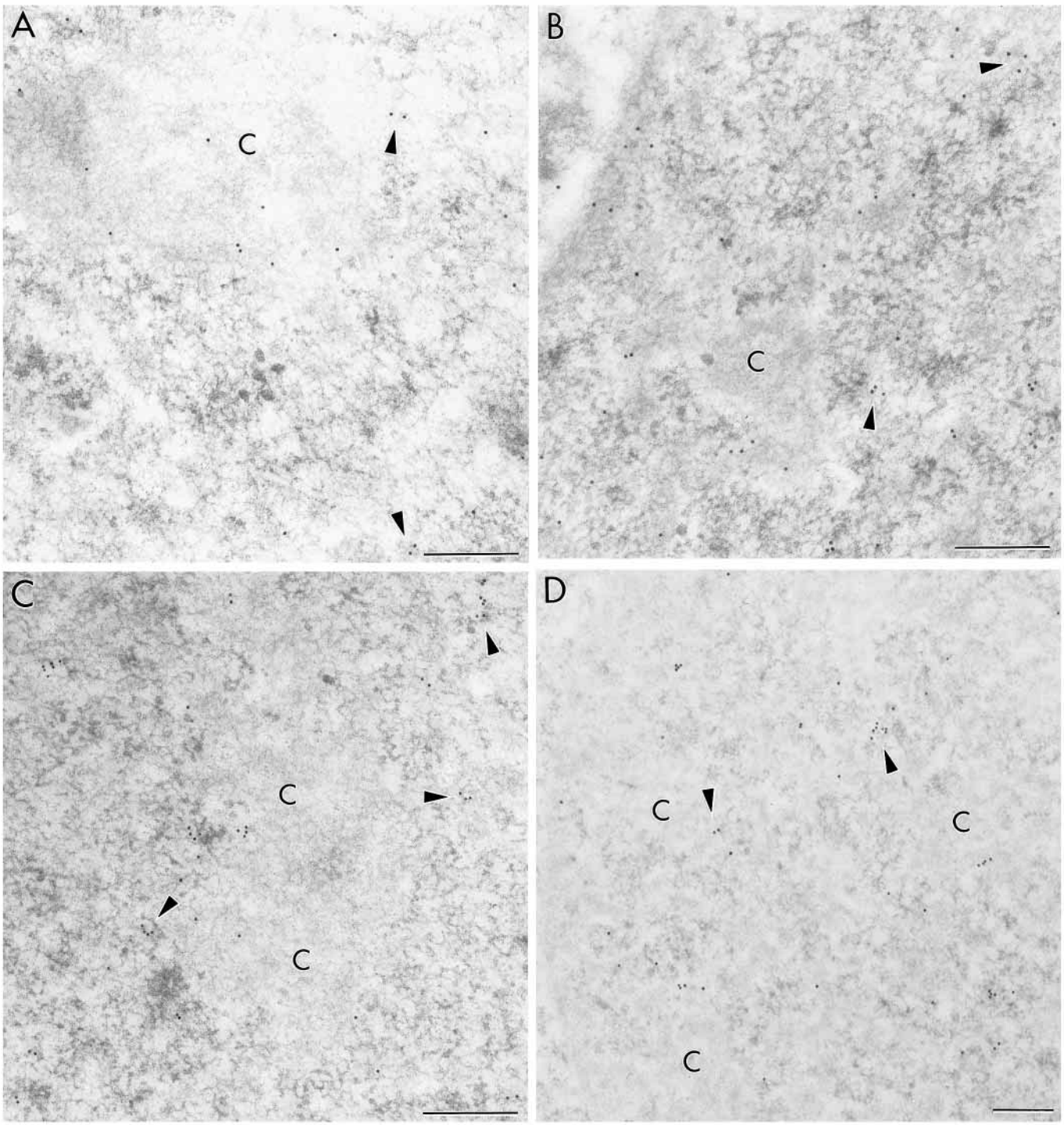

Fig. 2. Immunoelectron microscopic visualisation of PcG protein distribution in sections of paraformaldehyde-fixed SW480 cells, following the EDTA staining procedure. (A) Rabbit anti-HPH1 antibody revealed by goat anti-rabbit secondary antibody conjugated with 12 nm colloidal gold particles; (B) rabbit anti-HPC2 antibody and 12 nm particles; (C) rabbit anti-RING1 and 12 nm particles and (D) rabbit anti-BMI1 and 15 nm particles. Nuclear distribution of PcG proteins frequently occurs on electron-dense RNP-containing fibrils, which are distributed in the nucleoplasm (some fibrils indicated by arrowheads). Condensed chromatin (c) is virtually devoid of signal. Bars, 0.3 μm.

Similarly, the background labelling observed over the resin outside the cells was very low.

Quantitative evaluation of immunogold labelling distribution

Quantitative evaluation of the labelling within condensed chromatin, the perichromatin region (here defined as a 40 nm rim around condensed chromatin domains) and the interchromatin space demonstrates that PcG proteins are highly concentrated in the perichromatin region (Table 1). Only a few gold particles were found in condensed chromatin domains. The differences in labelling densities between the three nuclear compartments are statistically significant (Wilcoxon signed rank test, $P<0.01$).

Discussion

A remarkably strict relationship exists between higher order chromatin folding and gene activity. In the cell nucleus, chromatin is organised in what seem in optical or physical sections numerous compact chromatin domains, representing cross sections through the interphase chromosomal fibre. These domains should not be confused with classic constitutive heterochromatin, which is mainly pericentromeric chromatin, often located near the nuclear envelope and around nucleoli. Most genes that become associated with that type of heterochromatin are silenced (Brown et al., 1997; Francastel et al., 1999; Lundgren et al., 2000). In contrast to the heterochromatin, transcriptionally active loci predominantly occur at the periphery of compact chromatin domains, that is, in the perichromatin compartment (Fakan, 1994; Puvion and Puvion-Dutilleul, 1996; Spector, 1996; Cmarko et al., 1999).

To further explore the relationship between gene activity and epigenetic gene silencing on the one hand and chromatin organisation on the other hand, we have analysed the localisation of four members of the human PcG protein family in the cell nucleus. PcG proteins were originally identified

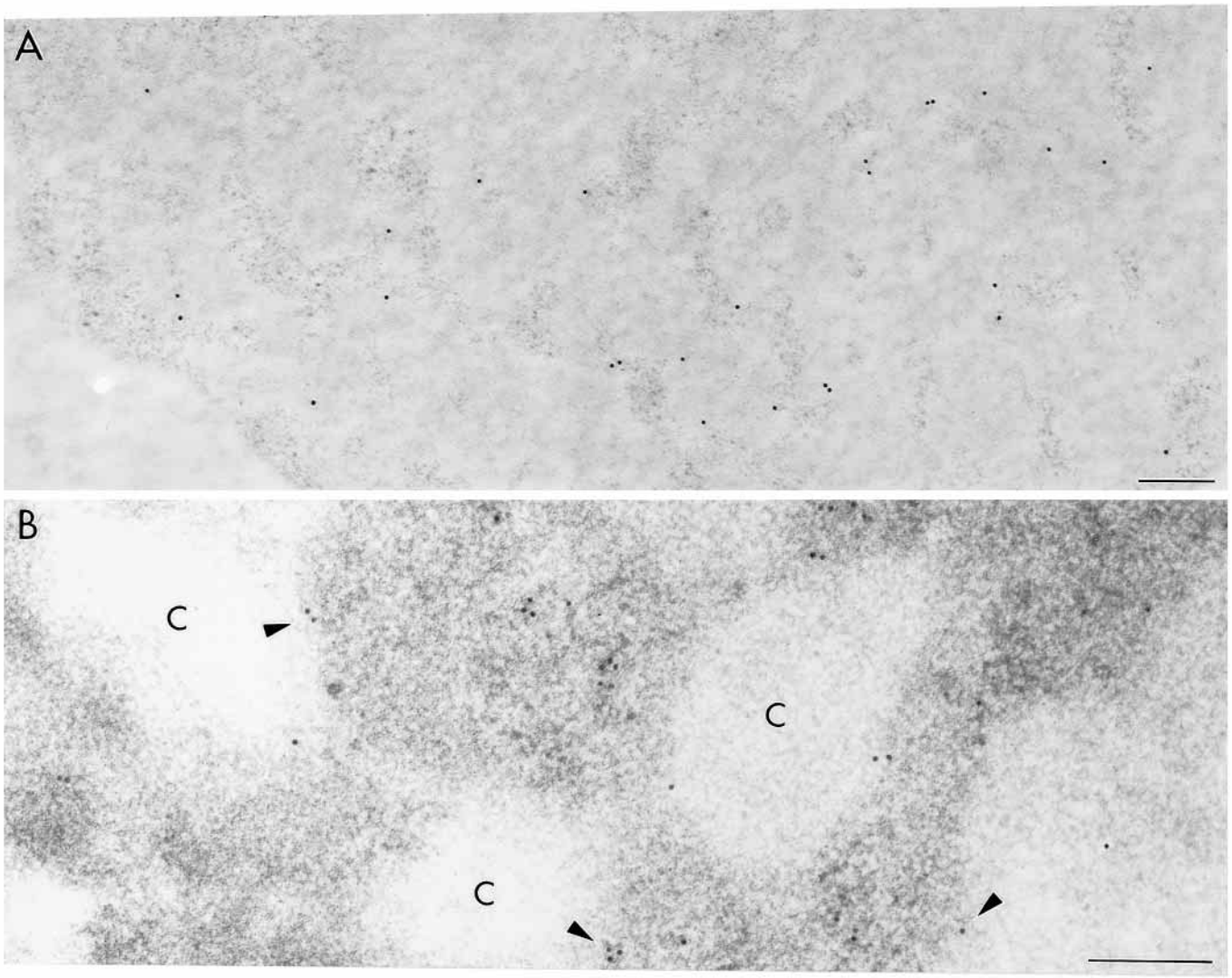

Fig. 3. Immunoelectron microscopic visualisation of PcG proteins in (A) an osmium-ammine-stained section of a paraformaldehyde-fixed SW480 cell (anti-HPC2 labelling) and (B) in high pressure frozen and cryosubstituted rat liver tissue (anti-BMI labelling); uranyl-lead stained. The primary antibodies were revealed by goat anti-rabbit secondary antibody conjugated with 12 nm colloidal gold particles. PcG proteins mainly occur at the periphery of condensed chromatin (c). Some perichromatin fibrils are indicated by arrowheads. Bars, 0.2 μm.

through their involvement in the epigenetic silencing of homeotic genes in *Drosophila* (see Pirrotta, 1998). More recently, it has been shown that PcG proteins are evolutionarily conserved and play an important role in the epigenetic repression of many other genes in higher eukaryotes, including plants and animals (Jacobs and van Lohuizen, 1999; Satijn and Otte, 1999a). PcG protein complexes bind to genomic sequence elements called Polycomb response elements (PREs), thus repressing nearby genes in cis (Eissenberg and Elgin, 2000; Lyko and Paro, 1999; Jacobs and van Lohuizen, 1999). Here, we investigated in two different human cell lines and in rat liver tissue the subnuclear distribution of four human PcG proteins, that is, HPC2, HPH1, BMI1 and RING1, with respect to the distribution of condensed chromatin and sites of transcription. We carried out indirect immunofluorescent and immunogold electron microscopic labelling of these proteins.

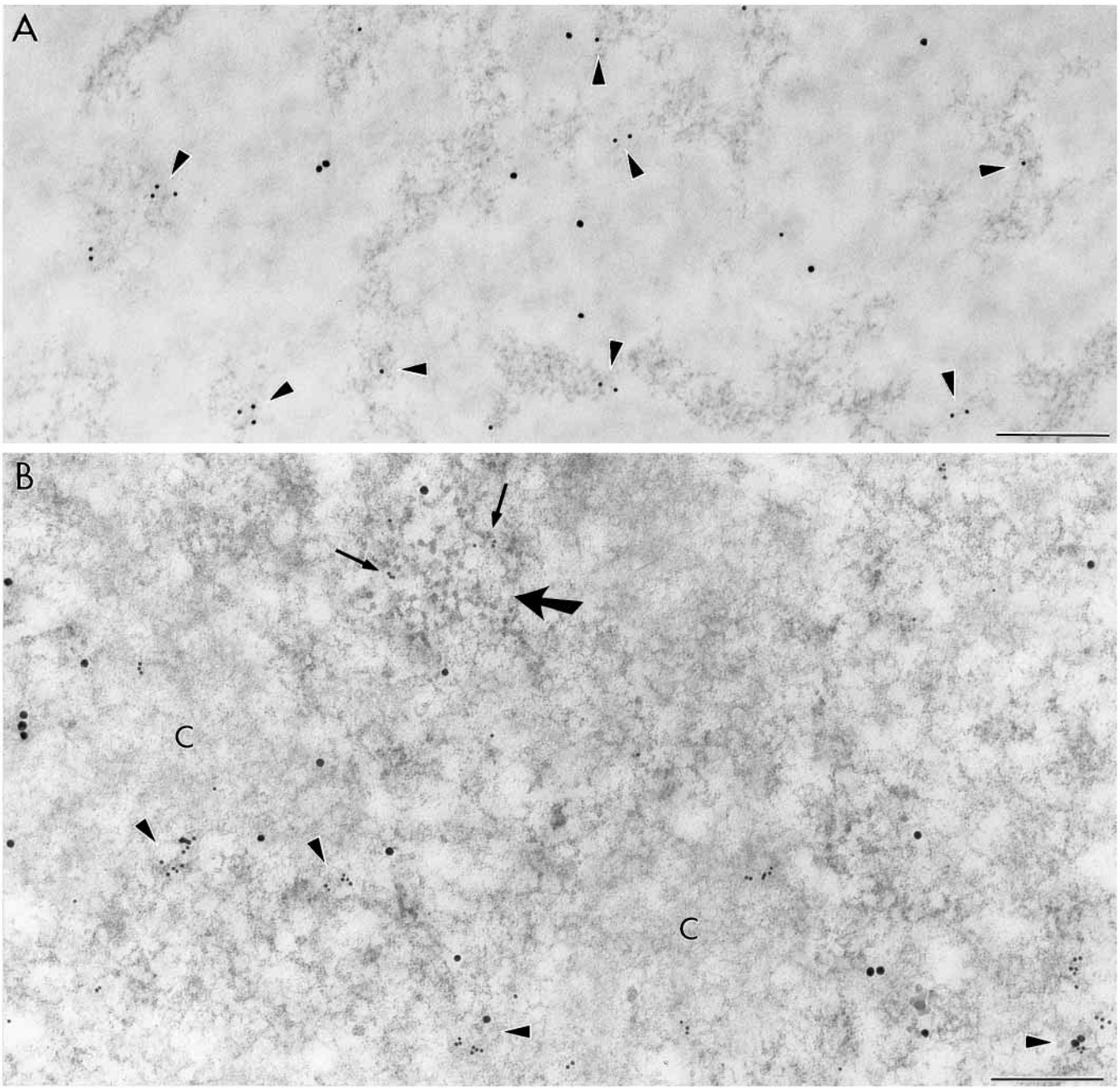

Fig. 4. Double immunolabelling. (A) Double-labelling with mouse monoclonal anti-HPC2 antibody (15 nm gold particles) and rabbit anti-BMI1 antibody (10 nm gold particles, some indicated by arrowheads) in an SW480 cell stained with osmium ammine specific for DNA; most signal is observed on the periphery or outside DNA-rich regions. (B) Double labelling experiment with PcG protein BMI1 (6 nm gold particles) and nascent BrU-containing RNA (15 nm particles) in a T24 cell; EDTA staining. Both labels mostly occur near the border of condensed chromatin regions (c), sometimes in association with perichromatin fibrils (some indicated by arrowheads). A cluster of interchromatin granules (large arrow) exhibits anti-PcG protein label only on its periphery (small arrows). Bars, 0.2 μm.

Table 1. Quantitative evaluation of electron microscopic immunodetection of PcG proteins in SW480 cells

Evaluated area	anti-HPC2	anti-BMI1	anti-RING1
Condensed chromatin	0.79±0.20/81.2	0.39±0.06/110.9	0.26±0.06/101.8
Perichromatin region	27.58±4.35/20.8	35.12±5.35/14.6	40.26±5.76/11.6
Interchromatin region	6.05±0.67/180.2	4.12±0.56/218.8	5.92±1.18/185.5

Twelve images were quantitatively evaluated for each PcG protein. For each of three analysed nuclear compartments the total number of gold grains and the total surface of each nuclear area were determined and the labelling density (number of gold particles per square micrometer) was calculated. The data in the table represent the labelling density±s.e. followed by the total surface (μm^2) considered for each nuclear compartment. The perichromatin region is defined as a 40 nm zone representing the interface between the condensed chromatin domains and the interchromatin region (see Materials and Methods).

Condensed chromatin was visualised by counterstaining with DAPI in confocal light microscopy and using the osmium ammine staining for DNA or the EDTA regressive staining in electron microscopy.

Our results for all four PcG proteins are identical. The four antigens were almost exclusively localized in the nucleus. Unexpectedly, HPC2, HPH1, BMI1, RING1 proteins were found highly concentrated in the perichromatin compartment, representing the border of condensed chromatin domains (Table 1). In addition, the PcG proteins also occur in the interchromatin space although at a five- to ten-fold lower concentration (Table 1), possibly as freely diffusing protein molecules. Strikingly, PcG proteins were almost absent from condensed chromatin (Table 1). This lack of label is not due to inaccessibility of antigens in compact chromatin, because labeling takes place on the surface of physically sectioned specimens. Our results show that PcG proteins are preferentially located in the perichromatin compartment. This compartment also contains transcriptionally active genes (Cmarko et al., 1999; Verschure et al., 1999), as further confirmed by dual labeling of PcG proteins with nascent RNA (Fig. 4B). These results strongly suggest that loci silenced by PcG proteins are spatially interspersed with transcriptionally active genes, both in space, that is, in the perichromatin compartment, and probably also on the linear genome. We conclude that PcG proteins act only locally rather than involving larger chromatin domains.

Our findings argue against a mechanism in which silencing of genes by binding PcG proteins to PREs gives rise to repositioning of silenced loci inside compact chromatin domains, that is, away from the perichromatin compartment. Clearly, the state of chromatin after silencing by PcG proteins is different from that in heterochromatin that seems to be involved in silencing loci by, for instance, Ikaros-related gene product (Brown et al., 1997; Fisher and Merkenschlager, 2002) or by inactivation of certain enhancer functions (Francastel et al., 1999).

The present findings, revealing that PcG proteins are preferentially associated with the periphery of condensed chromatin and therefore probably exert their gene silencing function in the same compartment that contains transcriptionally active chromatin, are in agreement with observations showing that PcG proteins occupy different positions on polytene *Drosophila* chromosomes than HP1, a typical marker for constitutive heterochromatin (Platero et al., 1995; Sinclair et al., 1998).

Our data also agree with the previous suggestion, based on light microscopic observations, that the distribution of PcG proteins does not correlate with local high concentrations of DNA (Buchenau et al., 1998) and that PcG proteins occur in a punctated pattern (Buchenau et al., 1998; Dietzel et al., 1999). However, in contrast to the conclusion of Buchenau et al. (Buchenau et al., 1998), we demonstrate that PcG proteins accumulate in the same compartment where transcription takes place. This discrepancy is probably due to the fact that the above authors used the hnRNP-K protein as a marker for transcription sites rather then nascent RNA.

We observed that each of the four PcG proteins is occasionally associated with RNP fibrils in perichromatin regions and interchromatin space (arrowheads in Fig, 2, Fig. 3B). It suggests that PcG proteins may interact with RNA-containing nuclear components. This is in line with a recent finding suggesting that chromodomain proteins preferentially interact with RNA (Akhtar et al., 2000).

These and earlier observations raise important questions about higher order chromatin folding in relation to gene transcription and gene silencing. The emerging picture is that in interphase nuclei chromatin is organised in such a way that active genes, as well as PcG silenced loci, are localised at the periphery of the compact chromatin domain. This seems to impose severe constraints on the way the chromatin fibre is folded in vivo. It further raises the question as to what sequences are inside compact chromatin domains. Is there only repetitive, non-coding DNA or are there also coding sequences that are silenced by mechanisms that do not involve PcG proteins? Present work is focusing on these questions.

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