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Transcriptional Coactivator PC4, a Chromatin-Associated Protein, Induces Chromatin Condensation▽†

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Human transcriptional coactivator PC4 is a highly abundant multifunctional protein which plays diverse important roles in cellular processes, including transcription, replication, and repair. It is also a unique activator of p53 function. Here we report that PC4 is a bona fide component of chromatin with distinct chromatin organization ability. PC4 is predominantly associated with the chromatin throughout the stages of cell cycle and is broadly distributed on the mitotic chromosome arms in a punctate manner except for the centromere. It selectively interacts with core histones H3 and H2B; this interaction is essential for PC4-mediated chromatin condensation, as demonstrated by micrococcal nuclease (MNase) accessibility assays, circular dichroism spectroscopy, and atomic force microscopy (AFM). The AFM images show that PC4 compacts the 100-kb reconstituted chromatin distinctly compared to the results seen with the linker histone H1. Silencing of PC4 expression in HeLa cells results in chromatin decompaction, as evidenced by the increase in MNase accessibility. Knocking down of PC4 up-regulates several genes, leading to the G_2/M checkpoint arrest of cell cycle, which suggests its physiological role as a chromatin-compacting protein. These results establish PC4 as a new member of chromatin-associated protein family, which plays an important role in chromatin organization.

The eukaryotic genome is organized into a highly complex nucleoprotein structure, the chromatin. This dynamic chromatin structure is regulated by posttranslational modifications of the core histones and histone H1 and also by the direct interaction of nonhistone chromatin-associated proteins (CAPs) with the different components of chromatin, including core octamer and/or linker histones (2, 8, 21, 59, 63). The ATP-dependent chromatin remodeling and histone chaperones (replication dependent and independent) also contribute to the organization of the dynamic chromatin (1, 39). The chromatin fiber-bridging proteins (Sir3p, Tup1, and MENT) (22, 24, 53) and nonhistone CAPs, which include high-mobility-group proteins (HMGs) (2, 8, 11, 45), heterochromatin binding protein 1 (HP1) (37), methyl CpG binding protein 2 (MeCP2) (33) and poly-ADP-ribose polymerase 1 (PARP-1) (29), help in chromatin compaction or decompaction through their direct interaction with core histones and/or DNA. These proteins may also compete or cooperate with histone H1 during this process (11, 56). The interaction of histone H1 with the nucleosomes stabilizes the higher-order compact chromatin structure, restricting the ability of the regulatory factors to access their chromatin binding sites (3, 55, 63). Histone H1 is a key factor to aid in the compaction of the chromatin for mitotic chromosomes.

Transcriptional silencing during mitosis occurs in tandem with numerous structural and biochemical changes, which include chromatin condensation and a massive increase in protein phosphorylation. These changes trigger the dissociation of most of the transcription machinery from the condensed chromatin. Nevertheless, few important transcription regulators, for example, TATA-binding proteins (TBPs) and some TBP-associated factors (TAFs), remain associated with the mitotic chromatin (see references 12 and 50 and references therein). Several TAFs associated with the mitotic chromatin get phosphorylated and consequently cannot modulate activator-dependent transcription, which is restored upon dephosphorylation (50). Apart from transcription factor IID (TFIID), some amount of TFIIB also remains associated with the previously active promoters during mitosis, whereas RNA polymerase II (Pol-II) and NC2 (which can function both as an activator and a repressor) are displaced (12, 13). In general, association of transcription factors with the chromosome and/or chromatin is found to be a highly dynamic process, which depends upon the stages of cell cycle.

The present report focuses on the discovery of a highly abundant, multifunctional transcriptional coactivator, PC4, as a bona fide component of chromatin with distinct functional

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consequences. PC4 plays an important role in transcription, repair, and replication (23, 32, 46, 60). It facilitates activator-dependent transcription by RNA polymerase II to ~85-fold in vitro, through direct interactions with general transcription factors as well as transcriptional activators (23, 26, 32). This 15-kDa protein interacts with free or DNA-bound TFIIA and TBP components of the basal transcription machinery (26) but not with TBP-TFIIB complex or free TFIIB. It cannot interact with highly purified TFIID alone in the absence of TFIIA (23). Apart from its role in transcription, PC4 can interact with TFIIH (20) as well as with the single-stranded DNA (ssDNA), indicating its potential role in the repair pathway. A recent report shows that PC4 directly interacts with XPG, one of the important DNA repair factors specifically required for transcription-coupled repair, and helps in the repair of oxidative DNA damage (60). However, the DNA binding and the interaction with the activators and components of basal transcription machinery are essential for the transcriptional coactivation function of PC4. Interestingly, PC4 inhibits RNA Pol-II phosphorylation and hence Pol-II-mediated transcription (49). Furthermore, PC4 acts as a potent inhibitor of transcription in regions of unpaired double-stranded DNA and ssDNA and on DNA ends (62). PC4-mediated transcription repression can be relieved by ERCC3 helicase activity of TFIIH (19). Its diverse cellular functions also include its ability to interact with TFIIIC, influencing the process of reinitiation and termination in RNA Pol-III-dependent transcription (61). PC4 can interact with CstF64 and thereby has a role in polyadenylation and subsequent transcription termination (10). Recently it has been shown that it also has a role in promoter release and transcription elongation in GAL4- VP16-dependent transcription (20). PC4 can also form complexes with human single-stranded DNA binding protein (HSSB) on ssDNA and markedly affect the replication function of HSSB (46).

PC4 can inhibit self-repression of AP2 in a *ras*-transformed cell line and thus can act as a putative tumor suppressor (27). The tumor suppression activity of PC4 could also be manifested through its ability to enhance the p53 function (4). This functional diversity of PC4 and its similarity to HMGB1 with respect to its DNA-binding properties, involvement in p53 induction, and cellular abundance tempted us to investigate whether PC4 is a CAP. We have found that PC4 is indeed associated with the oligonucleosomes and widely distributed in a punctate manner on the compact metaphase chromosomes. It directly interacts with the core histones H3 and H2B and consequently induces chromatin folding. Significantly, atomic force microscopy (AFM) of PC4 chromatin complexes showed that PC4-mediated chromatin compaction is distinct from the histone H1-induced higher-order fiber formation. Knockdown of PC4 by small interfering RNA (siRNA) in HeLa cells was shown to decondense the chromatin in vivo and facilitate the overexpression of several genes. Furthermore, silencing PC4 gene expression using a vector-based system (30) led to G_2/M checkpoint arrest, suggesting its role in cell cycle progression. These results establish PC4 as a CAP which may play an important role in chromatin compaction and chromatin-mediated transcriptional regulation.

MATERIALS AND METHODS

Expression and purification of recombinant proteins and HeLa core histones. Recombinant N-terminal His_6-tagged PC4 was cloned by PCR-based subcloning using primers complementary to respective 5′ and 3′ ends of the full-length (FL) human PC4. The amplicons were inserted between NdeI and XhoI restriction sites of pET28b vector (Novagen). The His_6-tagged recombinant PC4 was purified using nickel-NTA (Ni-nitrilotriacetic acid) agarose (Novagen) (details available on request). His_6-tagged PC4 deletions 1–62, 1–87, 22–127, 62–127, and 62–87 were cloned in pET28b vector, expressed, and purified to homogeneity (details available on request). The untagged recombinant PC4 was expressed in *Escherichia coli* and purified as described elsewhere (23). The PC4-GST was also expressed in *E. coli*, and PC4-GST Sepharose beads were prepared as described elsewhere (20). His_6-HMGB1 was expressed in *E. coli* and purified as described elsewhere (5). Human core histones were purified from HeLa nuclear pellet as described previously (36). Recombinant core histones (*Xenopus*) H2A, H2B, H3, and H4, which come in inclusion bodies, were purified by denaturation in 8 M urea followed by renaturation as described elsewhere (40). Purification of human linker histone H1 (used in AFM studies) was carried out as described elsewhere (25).

Sucrose gradient fractionation of chromatin fragments. The HeLa cells (~50×10^6) were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum. The nuclei were prepared from packed cells suspended in hypotonic buffer (10 mM Tris-HCl, 10 mM KCl, 15 mM $MgCl_2$) followed by 10 min of incubation at 4°C. The nuclei were digested with micrococcal nuclease (MNase) (0.2 U/μl) for 10 and 15 min at room temperature in nuclei digestion buffer (10% glycerol, 10 mM Tris-HCl [pH 8], 3 mM $CaCl_2$, 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride). MNase digestion was stopped by the addition of 10 mM EDTA, and the digested chromatin was fractionated on a linear sucrose gradient of 15% to 40% in NTE buffer (10 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA) by use of a Beckman ultracentrifuge (SW60Ti rotor) at 28,500 rpm for 14 h. Fractions were analyzed as described in the figure legends.

Immunofluorescence localization of PC4. The HeLa and mouse L cells were cultured as monolayers on poly-L-lysine-coated glass coverslips in Dulbecco's minimal essential medium. Condensed mitotic metaphase chromosomes from mouse L cells were spread using a cytobucket rotor after swelling the cells with 75 mM KCl and probed with purified polyclonal antibody against PC4 followed by secondary antibody conjugated to rhodamine. To stain the chromosomal DNA, Hoechst 33258 (Sigma) was used.

In vivo and in vitro histone interaction assays. The in vivo PC4-histone interactions were investigated by performing M2-agarose pulldown assays using FLAG-PC4-transfected HeLa whole-cell extracts followed by immunoblotting by antihistone polyclonal antibodies. The histone interaction ability of PC4 was further characterized by incubating 5 μl of Ni-NTA beads with 1 μg of His_6-PC4 and 200 ng of recombinant (*Xenopus*) individual histones H2A, H2B, H3, and H4 in a final volume of 200 μl in biochemical buffer containing 150 mM KCl supplemented with 30 mM imidazole at 4°C for 2.0 h. The beads were washed five times (1 ml each) with the incubation buffers. The Ni-NTA agarose pulldown complexes were analyzed by Western blotting using anti-H2A, -H2B, -H3, and -H4 polyclonal antibodies. Control experiments were performed with 5 μl of Ni-NTA beads incubated with 200 ng of individual recombinant histones H2A, H2B, H3, and H4 in the same buffer. In order to map the domain of histone H3 or H2B involved in the interactions with PC4, glutathione *S*-transferase (GST) pulldown assays were performed as described elsewhere (35). GST-tagged deletions of each of histone H3 and H2B-NG (N-terminal plus globular), -GC (globular plus C-terminal), and -G (globular) domains were cloned, expressed, and purified (details available on request), and interaction studies were done with native PC4 in the presence of 150 mM NaCl. For scoring the interaction, GST pulldown assays were done followed by probing with anti-PC4 antibodies. The probability of PC4 interaction with the centromeric histone H3 variant CENP-A was verified by immunopulldown assays using antihemagglutinin (anti-HA) antibody and the whole-cell extract prepared from the HeLa cells transfected with HA-centromere protein A (HA–CENP-A) mammalian expression construct.

CD spectroscopy. The circular dichroism (CD) spectra of H1-stripped chromatin (0.6 mg/ml) and complexes with individual different proteins (histone H1, PC4, and HMGB1) were recorded after incubation at 25°C for 90 min or as indicated in the figures in 10 mM Tris-HCl–25 mM NaCl (pH 7.4). The spectra were recorded at room temperature in a JASCO model J715 spectropolarimeter at settings from 250 to 300 nm.

Reconstitution of chromatin template. The 100-kb chromatin was reconstituted using plasmid DNA and highly purified HeLa core histones as described

earlier (25). In brief, equal amounts (0.5 μg) of the purified DNA and the histone octamer were mixed in Hi buffer (10 mM Tris-Cl [pH 7.5], 2 M NaCl, 1 mM EDTA, 0.05% NP-40, 5 mM 2-mercaptoethanol) and placed in a dialysis tube (total volume, 50 μl). The dialysis was started with 150 ml of Hi buffer with stirring at 4°C. Lo buffer (10 mM Tris-Cl [pH 7.5], 1 mM EDTA, 0.05% NP-40, 5 mM 2-mercaptoethanol) was added to the dialysis buffer at a rate of 0.46 ml/min, and the dialysis buffer was simultaneously pumped out at the same speed with a peristaltic pump so that the dialysis buffer contained 50 mM NaCl after 20 h. The sample was collected from the dialysis tube and stored at 4°C. The chromatin template with the tailless core histones was also reconstituted as described above except that the ratio of DNA (0.5 μg) to tailless histones (0.365 μg) was altered to 1.37:1.

AFM. The histone H1 or PC4 was mixed with the reconstituted chromatin and incubated on ice for 5 to approximately 60 min. The samples were diluted 10-fold using fixation buffer containing 0.3% glutaraldehyde, 50 mM NaCl, and 5 mM HEPES-K^+ (pH 7.5). After fixation with glutaraldehyde for 30 min at room temperature, the samples were dropped onto a freshly cleaved mica substrate, which was pretreated with 10 mM spermidine. After 15 min at room temperature, the mica was washed with water and dried under nitrogen. AFM observation was performed using Nanoscope IIIa or IV (Digital Instruments) and a cantilever (OMCL-AC160TS-W2; Olympus) 129 μm in length with a spring constant of 33 to 62 N/m in air under the tapping mode. The scanning frequency was 2 to 3 Hz, and images were captured with the height mode in a 512-by-512 pixel format. The obtained images were processed (plane fitted and flattened) by the program accompanying the imaging module. For the imaging of the DNA with or without PC4, the sample was diluted by the buffer containing 0.3% glutaraldehyde, 5 mM HEPES-K^+ (pH 7.5), and 5 mM $MgCl_2$ and then put on a freshly cleaved mica substrate immediately. After 15 min at room temperature, the mica was washed with water and dried under nitrogen gas. Images of each protein (H1 and PC4) were recorded upon incubation of the proteins (0.2 μg/μl) in the fixation buffer for 30 min as described above (data available on request).

RNA interference. The siRNA sequence targeting the PC4 gene corresponded to the nucleotides 157 of 177 of the coding region relative to the first nucleotide of the start codon (sense, 5′-r[ACAGAGCAGCAGCAGCAGA]dTT-3′; antisense, 5′-r[UCUGCUGCUGCUGCUCUGU]dTT-3′) were synthesized. As a control we used the scrambled RNA with the sequences 5′-r(GAAAGGCAAC GACGGACAC)dTT-3′ (sense) and 5′-r(GCGAACACUAACGUACCUCAU) dTT-3′ (antisense). HeLa cells were transfected using siRNA and scrambled RNA with Lipofectamine 2000 Plus (Invitrogen) according to the manufacturer's protocol. For reverse transcription-PCR (RT-PCR), total mRNA was isolated using TRIzol reagent (Invitrogen). The mRNA was subjected to RT-PCR using the enzyme Superscript II to generate the cDNA library. Subsequently the PCR was performed using gene-specific primers for PC4 and β-actin (loading control). The silencing of PC4 expression was also confirmed by performing Western blotting analysis and immunofluorescence using purified polyclonal antibodies against PC4. Silencing was also done using a vector-based system where PC4 siRNA (sense, 5′-GATCCCCACAGAGCAGCAGCAGCAGATTCAAGAGA TCTGCTGCTGCTGCTCTGTTTTTT-3′; antisense, 5′-AGCTAAAAAACAG AGCAGCAGCAGCAGATCTCTTGAATCTGCTGCTGCTCTGTGGG-3′) was cloned in tandem with a green fluorescent protein (GFP) expression cassette into pGShin2 plasmid (35), a kind gift from Shin-ichiro Kojima. GFP-positive cells were sorted for fluorescence-activated cell sorting (FACS) analysis.

Microarray analysis. Total RNA was isolated using untransfected HeLa cells (control) and PC4 knocked-down HeLa cells (siRNA transfected) and an RNeasy kit (catalog no. 74104; QIAGEN). The RNA samples were quantified by nanodrop (ND1000 spectrophotometer) and analyzed on a formaldehyde-agarose gel. A Micomax TSA indirect labeling kit (Perkin Elmer Life Sciences) was used to synthesize the labeled cDNA from 5 μg of total RNA that was further hybridized on the array by the tyramide signal amplification method. All steps were carried out according to the manufacturer's recommendations.

The microarrays used in this study (human 19kv7) were procured from the Microarray Center, University Health Network, Toronto, Ontario, Canada. Each array carries 19,200 spots from the human genome, arranged in 48 individual arrays of 400 spots each. Measurement of the fluorescence corresponding to hybridization intensities was performed with a ScanArray Express microarray acquisition system (Perkin Elmer). Data were acquired and analyzed by using QUANTARRAY software (version III; Packard Biosciences). Genorm.pl software (Genotypic Technology, Bangalore, India) was used for normalization of the array. Six arrays that included four biological repeats were performed. Each array was done with a control versus PC4 knockdown, including a reverse dye hybridization to control for potential dye bias. After various statistical analyses and rankings were performed, the four best-quality arrays, corresponding to three forward reactions and one dye swap, were selected to calculate the mean severalfold change. Clustering of gene expression data was carried out using CLUSTER (tree and cluster; Eisensoftware). One pair (forward and dye swap) of control arrays was used with RNAs from untransfected HeLa cells versus scrambled RNA from transfected HeLa cells to test whether the global gene expression change was the result of the transfection or not.

Cell cycle analysis. HeLa cells were transfected with pGShin2 (vector) or PG7 (PC4 siRNA cloned into pGShin2 plasmid). Propidium iodide (PI) staining was done as described elsewhere (15). Double-positive cells (for GFP and PI) were sorted and analyzed by flow cytometry for the cell cycle distribution. A three-way statistical analysis of variance was performed using Statistica 5.2B (STATSOFT INC.) software.

The details of the materials and methods regarding synchronization and differential permeabilization of cells, preparation of histone H1 stripped chromatin, and immunofluorescence protocol will be available on request.

RESULTS

PC4 is associated with all the chromatin fractions. The human transcriptional coactivator PC4 is a highly conserved nuclear protein which plays diverse roles in cellular function. On the basis of its ability to (i) bind DNA (62), (ii) undergo posttranslational modifications (acetylation and phosphorylation) (34), and (iii) act as a transcriptional coactivator (23, 32) and also on the basis of its cellular abundance, we speculate that PC4 may perform its nuclear functions by tethering to the chromatin. To examine the association of PC4 to the chromatin, we used sucrose density gradient-fractionated nucleosomal fragments obtained from HeLa nuclei partially digested by MNase. The fractionated nucleosomal fragments were analyzed on a 1% agarose gel (Fig. 1A) to detect the presence of nucleosomal DNA in a particular fraction. The same fractions were also subjected to immunoblotting (Fig. 1B) to confirm PC4 association with the nucleosomes. The results show that PC4 is indeed only present in the fractions where nucleosomes are detected (Fig. 1B, panel I), as validated by the presence of histone H3 (Fig. 1B, panel III). To confirm the proper fractioning of the nucleosomal fragments and associated proteins, we also subjected the fractions to immunoblotting analysis using HMGB1 monoclonal antibody (Fig. 1B, panel II). As reported previously (16), HMGB1 was distributed over all the fractions, unlike PC4. Significantly, the general transcription factor TFIIA was present in the nonchromatin fraction (Fig. 1B, panel IV, lane 16) but not in the chromatin fractions (Fig. 1B, panel IV, lanes 2 to 15), indicating that association of PC4 with the chromatin is not nonspecific. Taken together, these results suggest that PC4 is predominantly associated with the chromatin.

PC4 is broadly distributed on metaphase chromosomes. The direct association of PC4 with the mitotic chromatin was further confirmed by analyzing the PC4 distribution in mitotic chromatin and cytosolic fractions of nocodazole-treated HeLa cells by immunoblotting. Histone H3 and HSC70 antibodies were used as nuclear and cytosolic markers, respectively. As expected, histone H3 was detected only in the mitotic chromatin fraction and interphase nuclear fraction, whereas the cytosolic protein HSC70 was found in cytosolic fractions of mitotic and interphase cells. Interestingly, PC4 was detected only in the nuclear fraction of interphase and mitotic cells and not in the cytosolic fraction. The presence of PC4 in the nuclear fractions prompted us to investigate the strength of the association of PC4 to the chromatin. We have addressed the affinity of PC4 to the chromatin by treating HeLa cells with two dif-

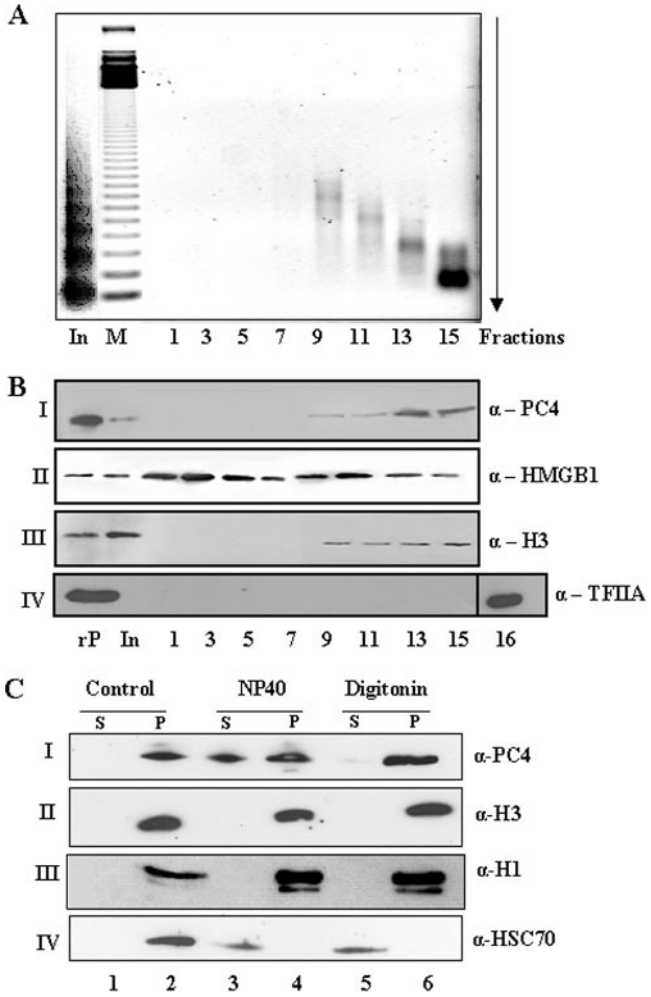

FIG. 1. PC4 cofractionates with HeLa nucleosomes in sucrose gradient. (A) HeLa nuclei were partially digested with MNase and fractionated on a 15% to 40% sucrose gradient. Individual fractions were deproteinized, and the alternative fractions were resolved on a 1% agarose gel and visualized by ethidium bromide staining. (B) Corresponding fractions were analyzed by Western blotting for the presence of PC4 (panel I), HMGB1 (panel II), histone H3 (panel III), and TFIIA (panel IV) by use of the respective antibodies. In panel IV, lane 16 corresponds to the nonchromatin fraction. The lane labeled rP shows the results for the recombinant proteins (PC4/HMGB1/H3/TFIIA). (C) Subcellular localization of PC4 and its relative affinity to chromatin. The cells were incubated in a buffer with 0.1% NP-40 or 40 μg/ml digitonin. After the incubation the supernatants (S) and the remnants of permeabilized cell pellets (P) were analyzed by Western blotting using antibodies against PC4 (panel I), histone H3 (panel II), histone H1 (panel III), and HSC70 (panel IV).

ferent types of detergents with diminishing strengths, NP-40 (Fig. 1C, lanes 3 and 4) and digitonin (Fig. 1C, lanes 5 and 6). NP-40 is a stronger detergent than digitonin (14). Treatment of the cells with buffer alone was used as the control (Fig. 1C, lanes 1 and 2). Though the digitonin treatment could not dissociate PC4 from the chromatin (Fig. 1C, panel I, lanes 5 and 6), the stronger detergent NP-40 could release some amount of PC4 in the supernatant (Fig. 1C, panel I, lanes 3 and 4). This indicates that the binding of PC4 to chromatin is not as strong as core histones or the linker histone H1, which remained associated with the chromatin upon NP-40 treatment (as shown by the Western blotting using histone H3 and H1 antibodies) (Fig. 1C, panel II and III, lanes 3 and 4). The presence of HSC70 (a cytoplasmic marker) in the supernatant fraction alone irrespective of the types of detergent treatment confirms the experimental integrity of the system (Fig. 1C, panel IV, lanes 3 and 5). These data suggest that PC4 is tightly bound to the chromatin, although the binding affinity is not as strong as core histones or linker histone H1.

In order to visualize the chromatin association of PC4, immunofluorescence localization of PC4 was performed using HeLa and mouse L cells with affinity-purified highly specific polyclonal PC4 antibody. The results show a predominant localization of PC4 in the nuclei of both the cell lines, as expected (data not shown). The nuclear association of PC4 was further investigated during the mitotic division of HeLa cells. As depicted in Fig. 2A, PC4 was found to be associated with the chromosomes throughout the different stages of mitosis, indicating its association with individual metaphase chromosomes. To find out the chromosomal distribution of PC4, chromosome spreads were made from metaphase-arrested mouse L cells and HeLa cells and probed with the PC4 antibody. Significantly, it was found that PC4 is distributed throughout the entire chromosome arms in both mouse L cells (Fig. 2B) and HeLa cells (data not shown) in a punctate manner without any apparent chromosome specificity. Interestingly, PC4 is not associated with the chromatin in the centromeric region (Fig. 2B, merged images).

The relative amounts of PC4 in the different stages of cell cycle were also assessed biochemically (48). HeLa cells were arrested in the G_0 and G_1 stages of cell cycle by serum starvation for a period of 3 days followed by serum replenishment for 3 h, and a consistent increase was observed in the amount of PC4 upon serum stimulation (Fig. 2C; compare lanes 3 and 4). Furthermore, the amount of PC4 was also substantially higher when the cells were arrested in the G_1/S phase of cell cycle by a double thymidine and hydroxyurea block (Fig. 2C, lane 5). On the other hand, nocodazole treatment leading to premetaphase arrest showed a large amount of PC4 present in the mitotic stage compared to the results seen for the interphase (Fig. 2C; compare lane 1 to lane 2). These results therefore suggest that although PC4 is present throughout all the stages of cell cycle, as shown by immunofluorescence studies, there are substantial differences in the amounts of the protein in the different stages of cell cycle. The higher amount of PC4 present in the mitotic stage compared to the interphase led us to investigate the strength of interaction of PC4 with the chromatin in these stages of cell cycle. The treatment of chromatin with 0.2% NP-40 also did not lead to a complete removal of PC4 from the chromatin fraction (Fig. 2D, panel III). In fact, it was observed that the amount of PC4 released in the supernatant fraction in the mitotic stage was less than that seen in the interphase stage, indicating a tighter association of PC4 to the mitotic chromatin (Fig. 2D, panel III; compare lanes 1 and 3). In contrast, both histone H3 and histone H1 were found to be tightly bound to the chromatin fraction in the mitotic and interphase stages of the cell cycle (Fig. 2D, panels I and II), as treatment with 0.2% NP-40 could not mobilize these proteins.

Taken together, these data suggest that PC4 is a bona fide nonhistone CAP.

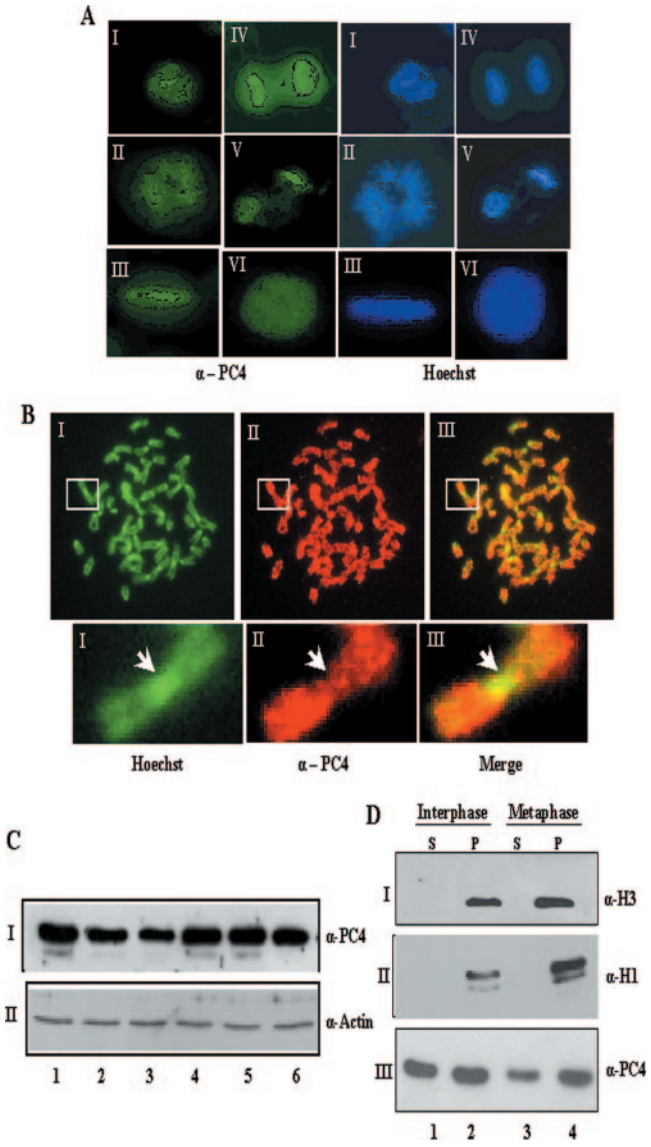

FIG. 2. Association of PC4 to the chromosome in different stages of mitosis. (A) HeLa cells were fixed and stained for PC4 with the purified polyclonal antibody against PC4 followed by fluorescein isothiocyanate-conjugated secondary antibody and for DNA with Hoechst. Representative cells at different stages during mitosis, including prophase (panel I), prometaphase (panel II), metaphase (panel III), anaphase (panel IV), telophase (panel V), and interphase (panel VI), are shown. Green, chromosome stained with PC4 antibodies; blue, DNA stained with Hoechst. (B) Distribution of PC4 on mitotic chromosomes. The condensed mitotic metaphase chromosomes from mouse L cells were spread on a slide and stained with Hoechst for DNA (panel I). The chromosomes were probed with purified polyclonal antibody against PC4 followed by secondary antibody conjugated to rhodamine (panel II). Panel III shows a merge of the antibody and DNA stained images. One of the individual chromosomes has been highlighted to indicate the centromere (arrows) in the bottom panels. (C) Distribution of PC4 throughout the different stages of cell cycle. Relative amounts of PC4 present during mitosis (lane 1), interphase (lane 2), G_0/G_1 arrest (by serum starvation) (lane 3), release of G_0/G_1 arrest (upon serum stimulation) (lane 4), and G_1/S arrest (lane 5) in comparison to an asynchronous cell population (lane 6) were assessed by probing with anti-PC4 antibodies in Western blotting analysis. As a loading control, Western blotting analysis was done with antiactin antibodies (panel II). (D) Comparative affinity of PC4 to the chromatin during interphase and mitotic stages of cell cycle. The mitotic and interphase stage cells were incubated in a buffer with 0.2% NP-40. After the incubation the supernatants (S) (lanes 1 and 3) and the remnants of permeabilized cell pellets (P) (lanes 2 and 4) were analyzed by Western blotting using antibodies against PC4 (panel I), histone H1 (panel II), and histone H3 (panel III).

Preferential interactions of PC4 with different core histones. The stable association of PC4 to the chromatin could occur through its nonspecific DNA binding ability (34), interaction with bookmarked general transcription factors (12, 13, 50) or other nonhistone chromatin-associated proteins (33, 37), or direct interactions with the core histones. Direct interactions of several nonhistone CAPs with the core histones have been shown to contribute to their association with the chromatin (43, 54). In order to examine the histone-interacting ability of PC4 in vivo the FLAG-tagged PC4 mammalian expression vector was transfected into the HeLa cells. The expression of this construct was confirmed by Western blotting analysis using both anti-FLAG and anti-PC4 antibodies (data not shown). FLAG-tagged PC4 was pulled down by M2-agarose beads from whole-cell lysates prepared from transfected cells, and the pulldown complex was analyzed by immunoblotting with highly specific antihistone antibodies. It was found that PC4 could efficiently pull down all the core histones (Fig. 3A, lane 2). Furthermore, the PC4-GST construct could also pull down the core histones (Fig. 3A, lane 4) from the whole-cell extract but not the GST alone (Fig. 3A, lane 3). In order to find out the specific site of interaction(s) of PC4 on the nucleosome, histone interaction experiments were carried out using recombinant individual core histones and His_6-PC4. The results show that PC4 bound to the Ni-NTA beads could predominantly pull down histone H3 and H2B (Fig. 3B, panels II and III, lane 3). The amounts of histones H2A and H4 pulled down by PC4 were found to be almost negligible compared to the amounts seen with H3 and H2B (Fig. 3B; compare lane 3 of panels I and IV to lane 3 of panels II and III). These data argue that PC4 directly interacts with histones, with a distinct preference for histone H3 and H2B. Interestingly, PC4 did not show any interaction with histone H1 (Fig. 3B, panel V, lane 3). We further analyzed the relative strength of PC4 interaction with the core histones. For this purpose the PC4-core histone complex was washed with increasing concentrations of salt in the washing buffer. The PC4-histone interaction was found to be quite stable up to a 200 mM salt concentration, beyond which the complex could barely be detected (Fig. 3C; compare lane 4 to lane 5).

The site of interaction on the core histone occasionally determines the structural and functional role of chromatin-interacting nonhistone proteins. Therefore, we investigated the domains of histone H3 and H2B involved in the PC4 and histone contact. Three GST-fused deletion mutants, consisting of NG, GC, and G domains of each of histone H3 and H2B, were constructed. The Western blotting analysis shows that PC4 interacts quite efficiently with the GC and G domains of both histone H3 and histone H2B compared to the results seen with the FL domain (Fig. 3D, panel I and II; compare lane 2 to lanes 4 and 5). This indicates that the globular domain of histone H3 and H2B is the preferred interaction site for PC4.

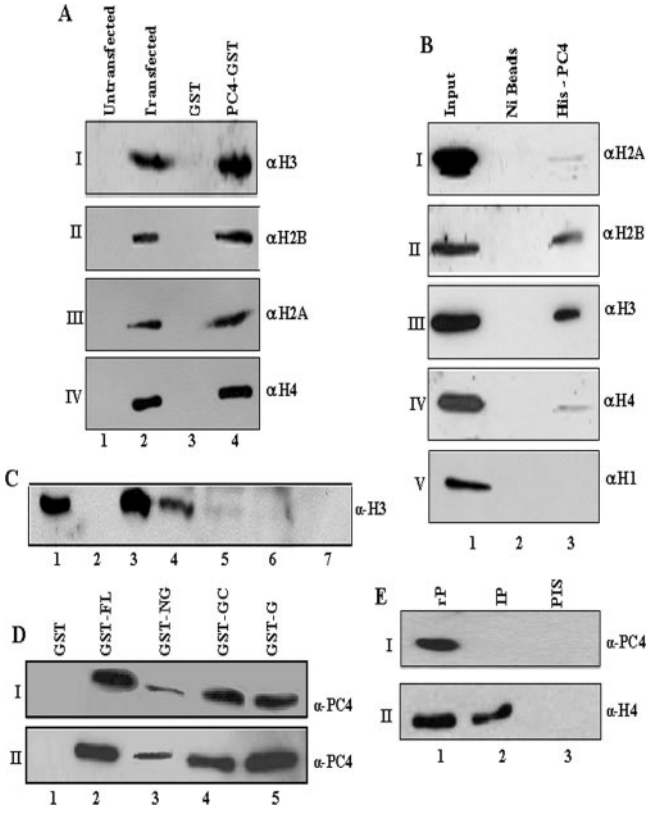

FIG. 3. PC4 interacts with histones. (A) To find out the histone interaction ability of PC4 in vivo, HeLa cells were transfected with a FLAG-PC4 (F-PC4) mammalian expression construct. The expressed F-PC4 was pulled down by M2-agarose beads, and the complex was subjected to Western blotting analysis using different antibodies as indicated (lane 2). Lane 1, untransfected control, lanes 3 and 4, pull-down complexes obtained from HeLa whole-cell extract incubated with GST and PC4-GST. (B) The in vitro interactions were assessed by incubating 1 μg of His_6-PC4 bound to Ni-NTA beads with 200 ng of individual recombinant core histones (H2A, H2B, H3, and H4) and the linker histone H1. The complexes were pulled down and analyzed by Western blotting. Lane 1, individual histones (input); lane 2, the histones incubated with Ni-NTA agarose alone; lane 3, individual histone incubated with Ni-NTA agarose bound to His_6-PC4. (C) The strength of interaction of PC4 with the histones was checked by stringency washes with buffer containing increasing salt concentrations at 100 (lane 3), 200 (lane 4), 300 (lane 5), 400 (lane 6), and 500 (lane 7) mM. (D) Mapping the domain(s) of core histones H3 (panel I) and H2B (panel II) involved in the interaction with PC4. Different deletion mutants were subjected to GST pulldown followed by Western blotting analysis using anti-PC4 polyclonal antibodies. The results for PC4 incubated with GST (lane 1) and the FL (lane 2), NG (lane 3), GC (lane 4), and G (lane 5) domains of the deletion mutants of H3 (panel I) and H2B (panel II) are shown. (E) CENP-A does not interact with PC4. An HA-tagged CENP-A construct was transfected into HeLa cells, the expressed protein was pulled down by anti-HA antibody, and the presence of interacting proteins, for example, PC4 (panel I, lane 2) and histone H4 (panel II, lane 2), was analyzed by Western blotting. rP, IP, and PIS indicate recombinant protein, immunopulldown, and preimmune serum control, respectively. All the interactions were done in the presence of 150 mM NaCl.

Interestingly, the presence of an N-terminal tail along with the globular domain (i.e., NG) significantly inhibits the interaction of PC4 with the core histones alone (Fig. 3D; compare lane 2 to lane 4), indicating that the N-terminal tail plays a negative role in this phenomenon.

PC4 is broadly distributed over all the chromosome arms except the centromeric region, as evidenced by chromosomal localization of PC4 (by immunofluorescence). If the chromosomal localization of PC4 were a result of its interaction with histone H3, the absence of PC4 over the centromere could be attributed to its inability to interact with the centromeric variant of histone H3, CENP-A. Therefore, we were interested to investigate whether PC4 interacts with CENP-A. The mammalian expression construct of an HA-tagged CENP-A clone was transfected into the HeLa cells, and the expressed protein was pulled down by anti-HA-Sepharose beads. Immunoblotting of the pulled-down complex with PC4 and histone H4 antibodies revealed that CENP-A could efficiently interact with histone H4 (Fig. 3E, panel II, lane 2) (65), while PC4 did not show any detectable interaction with CENP-A (Fig. 3E, panel I, lane 2). Taken together, these results suggest that PC4 binds to the chromatin through direct interaction with the globular domain of core histones H2B and H3 but not with the centromeric variant of histone H3, CENP-A.

PC4 induces chromatin condensation. The stable chromatin association, direct interaction with the core histones, and uniform (punctate) distribution over the metaphase chromosome arms suggest that PC4 may have a specific role to play in chromatin organization. The effect of PC4 in the chromatin organization was addressed by employing CD spectroscopy using H1-stripped chromatin fiber. Incubation of PC4 with H1-stripped chromatin decreased the molar ellipticity (peak) value of the chromatin spectra, indicating that PC4 was inducing condensation of the chromatin (Fig. 4A). This observation was further confirmed by the addition of equimolar amounts of histone H1 in a separate reaction using an equivalent amount of H1-stripped chromatin. The results show that histone H1 decreases the ellipticity value to the same extent as PC4. Addition of HMGB1, which dynamically interacts with chromatin, could not alter the chromatin spectra as expected (11) (Fig. 4A). Interestingly, an equimolar amount of PC4 could not alter the ellipticity peak value of total DNA isolated from the HeLa cells, indicating the general necessity of a chromatin template (specifically histones) for PC4 to induce the chromatin compaction (Fig. 4B). In order to visualize the PC4-mediated chromatin condensation, we subjected the 100-kb reconstituted chromatin (Fig. 4C) with either PC4 or H1 complexes to AFM. Significantly, though histone H1 induced the formation of the expected higher-ordered fiber structure (Fig. 4D), incubation of the reconstituted chromatin with PC4 led to the formation of distinct compact globular structures (Fig. 4E). In agreement with the CD spectroscopic data, addition of recombinant PC4 to the purified DNA (Fig. 4G) had no visual effect on the folding of the DNA molecules (Fig. 4G versus F).

The distinct differences in the AFM images of histone H1-mediated chromatin folding and PC4-induced compaction tempted us to investigate further the mechanistic details of the chromatin organization by these two proteins. In order to quantitate the chromatin condensation, dose-dependent condensation of the histone H1-stripped chromatin from HeLa cells was compared between H1 and PC4 by CD spectroscopy (Fig. 5A and B). Though PC4 seems to be less efficient than histone H1, gradual increases in the protein concentration decreased the ellipticity value in a regular fashion (Fig. 5A versus 5B). The AFM images obtained with similar experi-

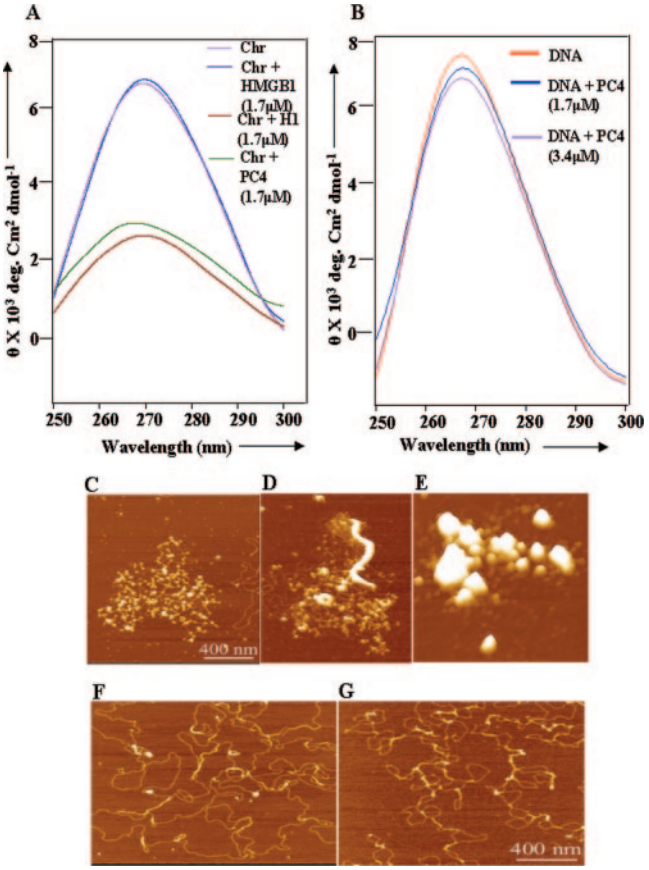

FIG. 4. PC4 induces chromatin (Chr) condensation. (A and B) CD spectra of histone H1-stripped chromatin incubated with PC4, H1, and HMGB1 (A) and CD spectra of DNA incubated with increasing concentrations of PC4 (B) are shown. (C to E) PC4 condenses the chromatin fiber into a distinct globular structure. AFM images of the 106-kbp reconstituted chromatin fibers (C) incubated with histone H1 (D) and PC4 (E) are shown. The molar ratio of histone H1 (or PC4) to the histone octamer was 1:1. Upon 60 min of incubation on ice the complexes were fixed by 0.3% glutaraldehyde, mounted on mica, and observed using AFM. The 106-kb plasmid DNA was similarly incubated with (F) or without (G) PC4 at the same ratio and processed for AFM imaging (see Materials and Methods).

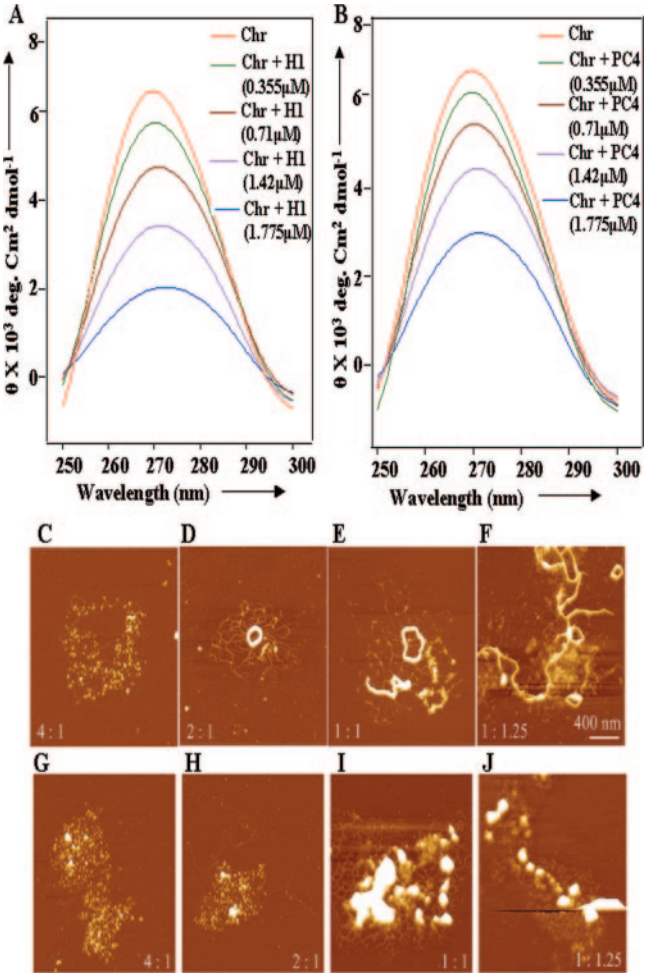

FIG. 5. Comparative dose-dependent condensation of chromatin (Chr) fibers by histone H1 and PC4. (A and B) Effects of increasing concentrations of histone H1 (A) and PC4 (B) on the CD spectra of histone H1-stripped HeLa chromatin. (C to J) AFM images of the chromatin incubated with various amounts of histone H1 (C to F) or PC4 (G to J). PC4 or H1 was mixed with the reconstituted chromatin in 50 mM NaCl at molar ratios of the histone octamer to PC4 or H1 of 4:1, 2:1, 1:1, and 1:1.25, respectively.

ments using the 100-kb reconstituted chromatin and various concentrations of histone H1 and PC4 (expressed in the ratios of core histone, H1, or PC4) (Fig. 5C to F or 5G to J) showed that PC4-mediated chromatin globule formation is achieved optimally at the equimolar ratio of core histone and PC4 (Fig. 5I). A further increase in the concentration of PC4 did not increase the size of the globule (Fig. 5I versus 5J). However, when the core histone/histone H1 molar ratio was increased to 1:1.25, a highly folded fiber structure could be observed (Fig. 5E versus 5F).

Furthermore, we have also analyzed the time-dependent chromatin organization induced by the presence of PC4 and the linker histone H1. Interestingly, histone H1 could fold the chromatin very rapidly (within 5 min), as revealed by both the CD spectroscopic analysis (Fig. 6A) and the AFM images (Fig. 6C to F). On the other hand, chromatin compaction (formation of globular structure) by PC4 was found to be a gradual process; at least 15 min was required to initiate the compaction process (Fig. 6B and 6G to J). These results suggest that though both histones H1 and PC4 induce chromatin condensation, the types and modes of action are distinctly different.

Interaction with core histones is essential to induce chromatin compaction by PC4. PC4 interacts with core histones H3 and H2B in vitro and induces chromatin condensation. However, the functional requirement of histone interaction in this phenomenon needs to be established. In order to address the connection between histone interaction and chromatin condensation by PC4, we made different deletion constructs of PC4 (deletions 1–62, 1–87, 22–127, and 62–127). It was found that except for PC4 (1–62), all the PC4 deletion mutants could interact with both the core histones H3 and H2B. Based on these results, an internal deletion construct of PC4, PC4 Δ62–87, was made (Fig. 7A). As expected, PC4 Δ62–87 could not interact with core histones H3 and H2B (Fig. 7B, panels I and II, lane 3). These deletion mutants of PC4 were then used in

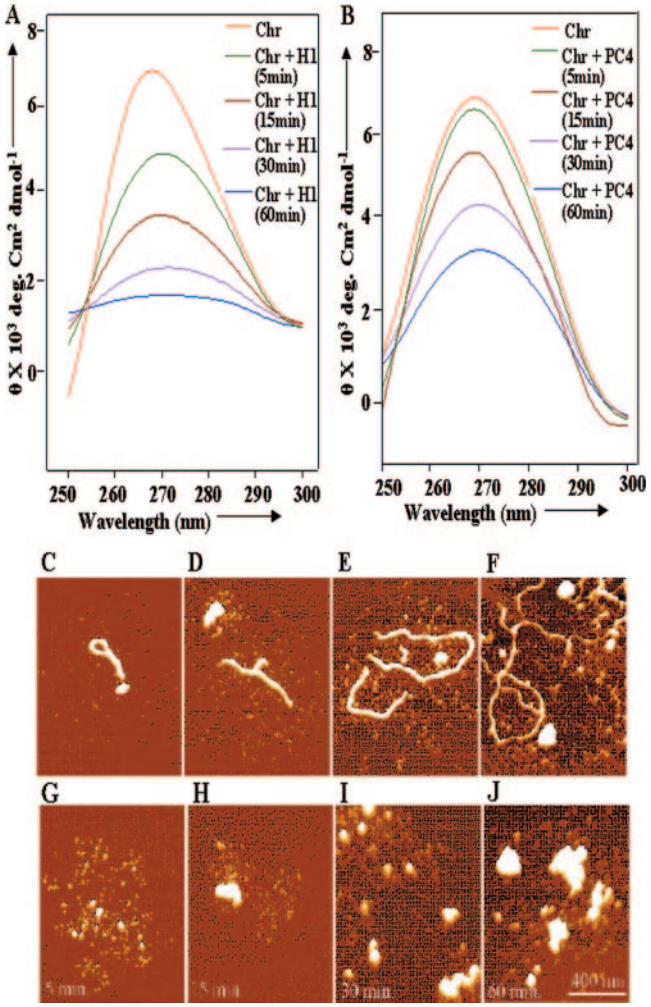

FIG. 6. PC4-induced chromatin (Chr) condensation is a slow process. (A and B) Histone H1 (A) and PC4 (B) were incubated with H1-stripped HeLa chromatin at different time points (5, 15, 30, and 60 min) and subjected to CD spectroscopy. (C to J) In order to visualize the time-dependent condensation brought about by PC4, salt-dialyzed reconstituted chromatin and H1 (C to F) or PC4 (G to J) were mixed at a 1:1 molar ratio of the histone octamer to PC4 or H1. After being kept on ice for 5 min, 15 min, 30 min, and 60 min, they were fixed by 0.3% glutaraldehyde and observed using AFM.

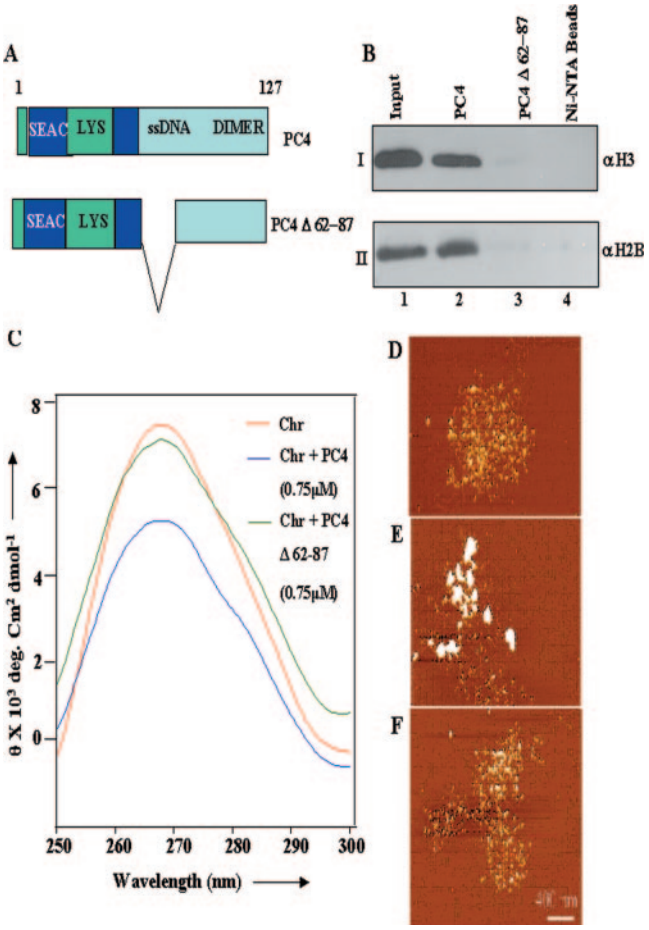

FIG. 7. Histone interaction ability is essential for chromatin condensation by PC4. (A and B) Full-length and mutant PC4 (A) was incubated with core histones and analyzed by Western blotting with antibodies against histone H3 (B, panel I) and H2B (B, panel II). (C) Comparative analysis of chromatin (Chr)-condensing ability of PC4 and PC4 Δ62–87 visualized through CD spectroscopy. (D to F) AFM images of the reconstituted chromatin (D) with PC4 (E) and histone interaction-deficient PC4 mutant (F). PC4 or PC4 mutant were incubated for 90 min with the reconstituted chromatin at a molar ratio of the histone octamer to PC4 of 1:1, and the samples were processed for AFM as described above.

the CD spectroscopic analysis. Interestingly, it was observed that except for PC4 (1–62) (data not shown) and PC4 Δ62–87 (Fig. 7C), all the mutants could induce chromatin condensation with various abilities to condense chromatin compared to the results seen with the full-length protein. The AFM images obtained using reconstituted chromatin and PC4 Δ62–87 further confirm these results. Though the equimolar amount of PC4 could efficiently induce chromatin globule formation (Fig. 7E), the addition of PC4 Δ62–87 showed a negligible effect on the reconstituted chromatin images (Fig. 7F), suggesting that PC4 induces chromatin compaction through direct interactions with the core histones.

PC4 interacts with the tailless globular domains of histones (H3 and H2B) quite efficiently (Fig. 3D), and the role of the N-terminal tail is rather negative. To investigate the functional validity of these interactions, 100 kb chromatin template was reconstituted using tailless octameric histones. As reported previously, tailless histone could be organized into a chromatin template similar to that seen with the wild-type histones (18) (compare Fig. 8A to 8B). In agreement with the histone interaction data, we observed that PC4 could efficiently condense the chromatin reconstituted with the tailless histones (Fig. 8C). Thus, the flexible N-terminal tails of histones may not be essential for PC4-mediated chromatin compaction.

Knocking down of PC4 expression alters chromatin organization (in vivo), gene expression, and cell cycle progression. In order to validate the chromatin condensation by PC4 in vivo, PC4 expression was knocked down by RNA interference, using a double-stranded (21-bp) RNA duplex homologous to PC4 mRNA. A scrambled RNA of same base composition and similar length was used as a control for these experiments. The knockdown of PC4 was confirmed by immunoblotting (Fig. 9A), immunofluorescence, and RT-PCR (data not shown). We

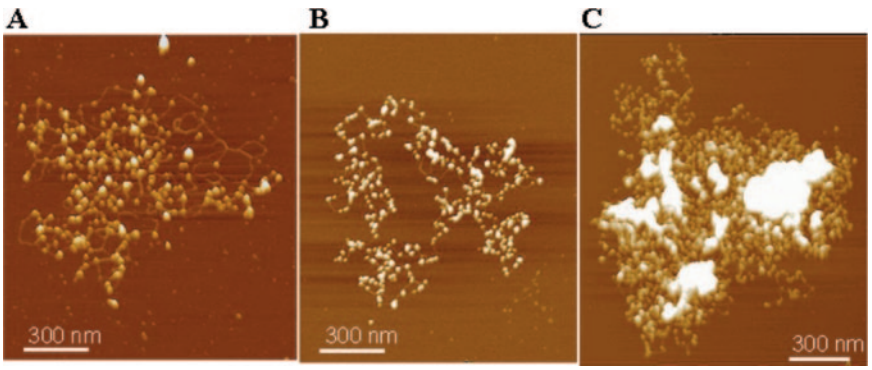

FIG. 8. Histone tails are not essential for PC4-mediated chromatin compaction. (A to C) AFM images of the reconstituted chromatin with wild-type histones (A) and tailless histones (B) and the effect of adding PC4 to the chromatin reconstituted with tailless histones (C). The molar ratio of the histone octamer to PC4 was 4:1.

next investigated the effect of PC4 repression on the global chromatin folding in human cells by the use of an MNase accessibility assay. The equal amounts of chromatin used in the experiment were confirmed by Western blotting using antibodies against different core histones and histone H1 (data not shown). The results showed that while the MNase pattern of the chromatin isolated from scrambled RNA (scRNA)-transfected HeLa cells resembled that obtained with the untransfected control, the chromatin of the siRNA-transfected HeLa cells was more susceptible to the MNase digestion (Fig. 9B, lanes 2 and 3 versus lane 4). Taken together, these data suggest that the silencing of PC4 decompacts the higher-ordered chromatin structure in vivo. These results were further confirmed by subjecting the chromatin isolated from siRNA- and scRNA-

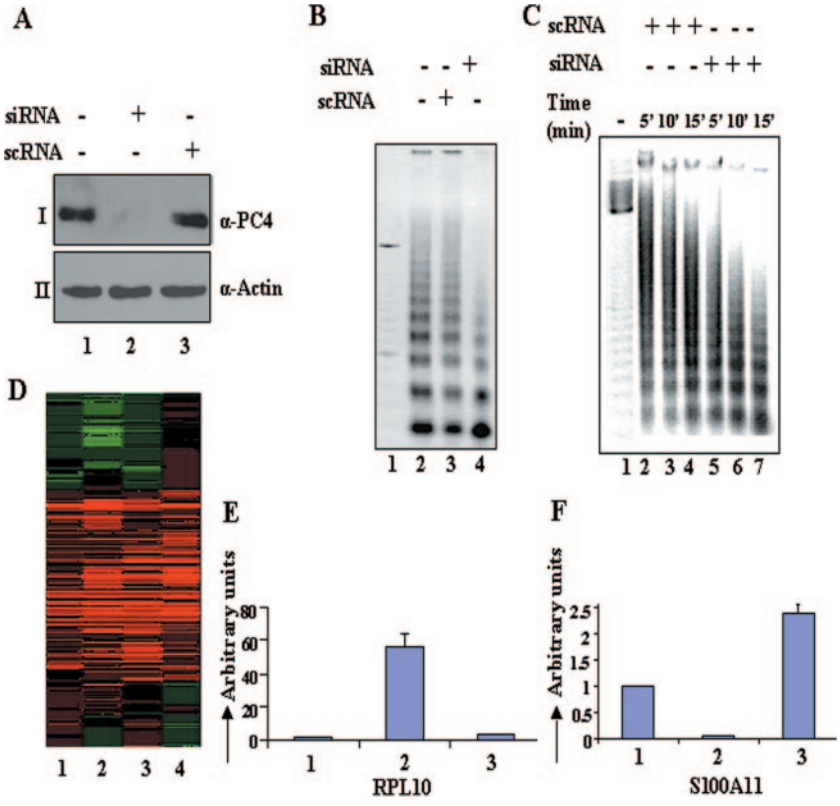

FIG. 9. siRNA-mediated knocking down of PC4 expression induces chromatin decompaction (in vivo) and global gene expression. (A) The expression of PC4 after transfection of siRNA and scRNA was verified by Western blotting analysis. (B) After knocking down of PC4, chromatin was isolated from untransfected (lane 2), scRNA-transfected (lane 3), and siRNA-transfected (lane 4) HeLa cells and were subjected to partial MNase digestion and analyzed on a 1% agarose gel. (C) Similar MNase digestions were also carried out at three different time points with the chromatin isolated from siRNA- and scRNA-transfected HeLa cells. Lane 1, 123 bp ladder; lanes 2 to 4, chromatin isolated from scRNA-transfected HeLa cells subjected to 5, 10, and 15 min of MNase digestion; lanes 5 to 7, same time points of MNase digestions carried out with chromatin isolated from siRNA-transfected HeLa cells. (D) Microarray analysis of gene expression upon PC4 knockdown by siRNA. Hierarchical clustering of the gene expression profiling data obtained by cDNA microarray analysis of siRNA-mediated PC4 knockdown HeLa cells. Lanes 1 to 3, forward reaction; lane 4, dye swap. (E and F) Real-time PCR analysis of the up-regulated and down-regulated candidate genes RPL10 and S100A11 upon knocking down of PC4 expression validates the microarray data.

transfected cells to a multiple-time-point MNase digestion assay. In agreement with the single time point of digestion, the chromatin isolated from siRNA-transfected cells was found to be more accessible to MNase (Fig. 9C, lanes 2 to 4 versus lanes 5 to 7).

Since siRNA knockdown of PC4 opens up the chromatin, as evidenced in the MNase accessibility assays, the absence of PC4 would presumably up-regulate a substantial number of genes in the cells. To investigate the effect of PC4 knockdown on global gene expression, we carried out genome-wide differential expression analysis using siRNA-transfected HeLa cells and a microarray. The expression profile analysis identified 128 up-regulated genes and 49 down-regulated genes in response to PC4 knockdown. In all experiments, a substantial number of the affected genes were of unknown function. We have clustered the genes according to the levels of their expression (Fig. 9D). An extensive table with all of the differentially expressed genes arranged into functional groups is available (see Table S1 in the supplemental material). The control experiment was carried out with the untransfected HeLa cells, and scrambled RNA-transfected HeLa cells did not show any differential regulation (data not shown). In order to validate the microarray data, two candidate genes were chosen, and after knocking down of PC4 expression, their expression levels were compared by real-time PCR analysis. It was found that compared to the results seen with the scRNA-transfected HeLa cells, there was an enhancement in the expression of the RPL10 gene upon PC4 siRNA transfection (Fig. 9E). On the other hand, S100A11 gene expression was reduced upon PC4 siRNA transfection compared to the results seen with the scRNA-transfected cells (Fig. 9F). These results were in agreement with the microarray data. The down-regulation of several genes in the absence of PC4 is not surprising, since it is a positive coactivator. The up-regulation of a large number of genes suggests that, at least partially, knocking down of PC4 results in a global opening of the chromatin.

The altered gene expression pattern seen upon knocking down of PC4 in HeLa cells suggests that it may play a significant role in cell cycle regulation. We designed a vector-based knocking-down system to probe into the role of PC4 in the cell cycle. In agreement with the short hairpin RNA vector-mediated silencing of the PC4 gene (Fig. 10A), Hoechst staining followed by confocal microscopic imaging (Fig. 10B) of the control (vector transfected) and knock down of PC4 (PG7 transfected) showed differential densities of compaction of chromatin DNA (Fig. 10B; compare panel I to panel II). The PC4 knockdown cells lost most of the densely packed chromatin (Fig. 10B, panel II). Furthermore, we also observed a significant reduction in the number of metaphase plates upon silencing PC4 gene expression in comparison to the control results (data not shown). However, after the control and PG7 transfection, GFP-positive cells were sorted and demarcated as subpopulation R1, as represented in the dot plot analysis (Fig. 10C, panels I and II). Cell cycle analysis of the R1 population showed that the percentages of cells in the G_1/S phase of cell cycle were 52.4% for the control and 27.95% upon PC4 knockdown. On the other hand, there was an increase in G_2/M cell populations from 13.73% for the control to 46.12% after PG7 transfection. A drop in the pre-G_1 cell population was also observed—33.87% and 25.94% for the control and after PG7

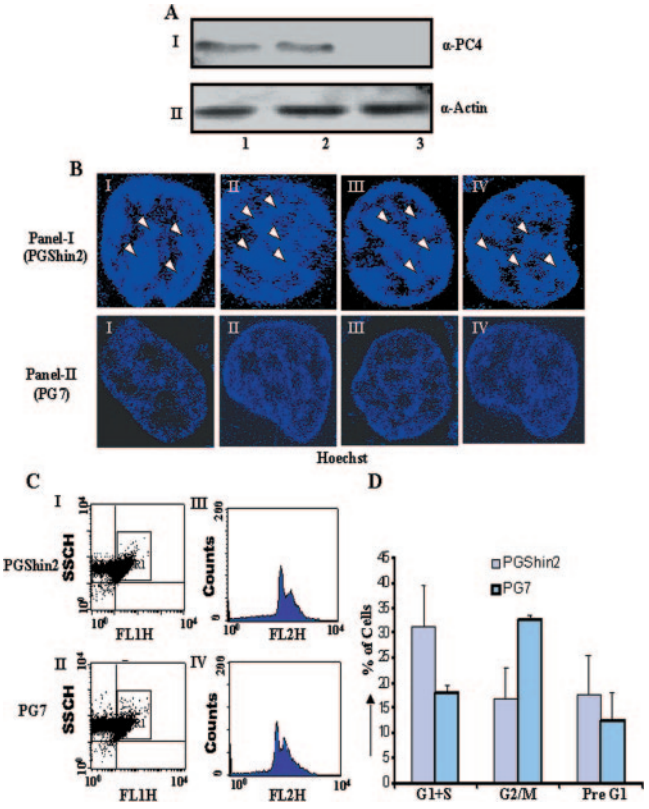

FIG. 10. Effect of knocking down of PC4 on the cell cycle. PC4 expression was silenced by using a vector-based system with GFP in the expression cassette. (A) Silencing of PC4 expression was checked by Western blotting analysis using anti-PC4 antibodies. Lane 1, untransfected; lane 2, vector (pGShin2) transfected; lane 3, PG7 (vector with PC4 siRNA) transfected. (B) Hoechst staining images of pGShin2 (panel I)- and PG7 (panel II)-transfected HeLa cells. (C) FACS analysis of HeLa cells upon knocking down PC4 gene expression. GFP-positive cells were sorted, and then PI-stained cells were analyzed from this subpopulation to look into the effect of silencing PC4 gene expression. Dot plot analysis (panels II and I) and histogram analysis (panels III and IV) of pGShin2- and PG7-transfected cells are presented. (D) The differences in G_1/S, G_2/M, and pre-G_1 cell populations upon transfection of pGShin2 and PG7 are shown in a bar graph.

transfection, respectively. These results are represented in the histogram analysis (Fig. 10C, panels III and IV). Three consecutive repeats of the experiment indicated that upon PC4 knockdown there is an approximately twofold drop in the G_1/S phase and a consecutive (approximately threefold) increase in G_2/M cell population (Fig. 10D), suggesting G_2/M cell cycle arrest. Statistical analysis of the FACS results by analysis of variance indicated that the observation made is significant, as reflected by the standard parameters $F_{2,4} = 11.12$ ($P < 0.02$). These results reflect that the nonhistone chromatin component PC4 is involved in chromatin compaction and has a significant role to play in maintenance of cell cycle.

DISCUSSION

Multifunctional, highly abundant, nuclear proteins are often associated with chromatin having distinct functional consequences (9, 31, 41). Though PC4 was originally discovered as a

positive coactivator for RNA Pol-II-driven, activator-dependent transcription from the DNA template, further analysis showed that PC4 is also needed for replication (46), repair (60), and the proper termination of multiple rounds of Pol-III transcription (61). This functional diversity prompted us to investigate PC4 from a broader perspective, and in agreement with our speculation, we have found that PC4 is indeed stably associated with the chromatin through all the stages of the cell cycle. The distinct and punctate appearance of PC4 on the metaphase chromosomes suggests its role in chromatin organization. We have shown that PC4 induces chromatin compaction and the formation of a very distinct type of globular structure, as revealed by CD spectroscopy and AFM analysis. Knocking down of PC4 by siRNA rendered the in vivo chromatin much more accessible to MNase and led to up-regulation of several genes, suggesting the cellular role of PC4 as a nonhistone chromatin-organizing protein. Silencing of PC4 gene expression (by a vector-based system, PG7) also caused G_2/M checkpoint arrest, indicating its function in cell cycle progression.

The stable association of PC4 to the chromatin was confirmed by the fact that on treatment with a weak detergent, digitonin (14), PC4 remained associated with the chromatin (Fig. 1C, panel I, lane 6). It was also observed that upon treatment with NP-40, a substantial amount of PC4 was still bound to the chromatin. In the case of HMGB1, though digitonin treatment could release the protein from chromatin to a lesser extent, exposure to NP-40 led to the dissociation of more than 70% of HMGB1 (16). Taken together, these data suggest that PC4 is more stably associated with chromatin than with HMGB1. However, the affinity of PC4 to the chromatin is not as high as that seen with histone H3 or H1. The treatment with NP-40 could not mobilize core histone H3 and the linker histone H1 from the chromatin (Fig. 1C, panels II and III). The mechanism of high-affinity association of PC4 to the chromatin has yet to be elucidated. We have shown that PC4 preferentially interacts with core histones H3 and H2B (Fig. 3B). Most of the other CAPs also interact with the core histones directly (42, 43, 47, 54, 58), with the exception of HMGN2, which does not interact with the free histones (8). However, it is not known whether interaction with the core histones is important for chromatin association and the consequent function of these proteins. Detailed domain analysis showed that the globular domain of histone H3 or H2B is the preferential docking site of PC4 (Fig. 3D). The N-terminal tails of the histones were found to have an inhibitory effect on the interaction with PC4. In case of the polycomb group of protein PRC1, the bridging of nucleosomes was also found to be independent of the histone N-terminal domain (51). The functional role of the flexible N-terminal tail of histones was further underscored when it was observed that PC4 could condense chromatin reconstituted with the tailless histones, as visualized by AFM (tailless core octamer/PC4 ratio, 4:1) (Fig. 8C). In fact, when the stoichiometry of the tailless octamer/PC4 ratio at 1:1 was investigated, individual nucleosomes could not be observed (data not shown); rather, the entire chromatin fiber condensed into a large globule unlike the distinct condensed zones observed with intact core octamer used in the chromatin reconstitution while maintaining the same stoichiometry (Fig. 5I). The presence of PC4 throughout the chromosome arms with the exception of centromeric region (Fig. 2B) strongly argues that PC4 is associated with the metaphase chromatin through its interaction with the histones. The fact that the centromeric region contains an altered form of nucleosomes (with H3 being replaced by its variant CENP-A) (52) and our finding that PC4 predominantly interacts with histone H3 but not with the centromeric variant CENP-A strongly supports this hypothesis. Furthermore, it also suggests that in vivo PC4 prefers to interact with the canonical nucleosomes rather the centromeric nucleosomes (7) containing the histone variants CENP-A.

The data regarding the stable and regular association of PC4 with the chromatin definitely suggest a significant role of PC4 in chromatin organization. By employing CD spectroscopy (which measures the conformational change of the chromatin-DNA) (28, 38), visualization of chromatin compaction (upon ectopic addition of PC4) by AFM, and RNA-interference-mediated knockdown of PC4, we have shown that PC4 indeed stimulates the chromatin condensation both in vitro and in vivo. The CD spectral data showed that PC4 folds the histone H1-stripped chromatin to the same extent as seen with histone H1. Although the role of histone H1 in the chromatin condensation is not clearly understood, per the general consensus the linker histone-induced contraction of the internucleosomal angle (not the bending of the linker DNA) is responsible for the organization of the solenoid structure and its further folding (57). However, PC4 folds the chromatin into a very distinct type of higher-ordered globular structure unlike that of the linker histone H1-induced folded fiber (compare Fig. 4D to 4E). There are few chromatin-interacting proteins that are known to form this kind of structure; these include the polycomb group of proteins (18) and MENT protein (53). Both of these protein types cause chromatin condensation in vivo and in vitro. The functional cooperation of these types of proteins, including PC4 with the linker histone H1, presumably establishes the cell cycle-specific physiological organization of chromatin domains. We have found that the PC4 mutants that are not capable of interacting with the core histones H3 and H2B could not fold the H1-stripped chromatin. These data clearly indicate that interaction with nucleosomal histones is essential to induce the chromatin condensation by PC4. The possible mechanism of PC4-mediated chromatin condensation could be through the linking of different widely separated nucleosomes by PC4 through the direct interaction with the histones, resulting in looping out of chromatin. These loops may be further condensed by PC4 in a similar manner, giving rise to the large globular structures observed in our AFM studies. Further investigation is necessary to elucidate the molecular details of the condensation process.

The expression of PC4 was knocked down efficiently by duplex siRNA or a vector-based system (PG7) (30) in HeLa cells. As expected, knocking down of PC4 significantly increased the accessibility of MNase to the HeLa chromatin, indicating that PC4 is involved in the global compaction of the chromatin. The Hoechst-stained images of the nuclei obtained after knocking down PC4 by PG7 also show chromatin decompaction unlike the distinct condensed regions observed in the control (vector transfected). These data demonstrate that the multifunctional coactivator is indeed involved in the organization of higher-order chromatin structure.

The established functions of PC4 suggested that it could be

an essential gene for the cells. Therefore, knocking down of PC4 was expected to cause the down-regulation of a vast majority of genes. However, the data presented in the Fig. 10 (see also Table S1 in the supplemental material) clearly indicate that the number of genes that are up-regulated by siRNA-mediated knockdown of PC4 is threefold greater than the number of down-regulated genes. To best explain these observations we propose that the absence of PC4 causes at least partial opening of different chromatin territories and facilitates transcription. Though a negative role of PC4 in transcription has previously been scarcely reported (19, 64), the number and the expression (*n*-fold) of upregulated genes prompt us to suggest that PC4 strongly interacts with the core histones and thereby induces chromatin condensation to repress the gene expression. Surprisingly, we noticed that, although PC4 is a multifunctional general transcription coactivator and chromatin-organizing protein, knocking down of it affects relatively fewer numbers of genes. Presumably, the functional redundancy of other transcriptional coactivator and chromatin proteins with PC4 could help to restore the regulation of several genes under this condition. Significantly, knocking down of three H1 genes (H1c, H1d, and H1e) (50% of the total H1) in mouse embryonic stem cells caused a dramatic change in chromatin organization but, in agreement with our present observation, affected fewer genes (29 genes) (17) than PC4 (177 genes). It would be interesting to find out the alteration of global gene expression upon knocking down of both PC4 and these H1 genes.

Detailed analysis of the candidate genes picked up in a microarray upon knocking down PC4 revealed that there are a number of cell cycle-regulatory genes (such as CDC10) and of genes belonging to the signal transduction cascades (such as MAPK4, MAP3K7IP1, and WNT5B) that are differentially expressed. Interestingly, CDC10 is down-regulated, which is an important component of the transcription complex in the S phase of cell cycle (6). Furthermore, there are two candidates belonging to the CAP family, STK4 and SAFB, which are also up-regulated upon PC4 knockdown. SAFB induces chromatin condensation and has an inhibitory role in cell proliferation (44). FACS analysis after PC4 knockdown shows a drop in the G_1/S population and an increase in the G_2/M population of the cell cycle, establishing its role in cell cycle progression.

The present finding that the global transcriptional coactivator PC4 is a CAP inducing chromatin folding in vitro as well as in vivo reveals a new facet of this highly conserved nuclear protein. Its ability to interact with histones suggests that this versatile nuclear factor could play a significant role in chromatin dynamics, regulating replication, repair, and transcription. In order to understand the mechanism of PC4 function in the chromatin context, the functional correlation of PC4 with histone H1 and other nonhistone chromatin proteins (for example, HP1, HMGs, and PARP-1) should be addressed. In this context it could be speculated that the posttranslational modifications of PC4 may regulate its multifunctional activity, ranging from chromatin organization to transcription.

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