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Cell Cycle-dependent Expression and Nucleolar Localization of hCAP-H

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Condensin is a conserved 13S heteropentamer composed of two nonidentical structural maintenance of chromosome (SMC) family proteins, in *Xenopus* XCAP-C and XCAP-E, and three regulatory subunits, XCAP-D2, XCAP-G, and XCAP-H. Both biochemical and genetic analyses have demonstrated an essential role for the 13S condensin complex in mitotic chromosome condensation. Further, a potential requirement for condensin in completion of chromatid arm separation in early anaphase is demonstrated by the mutational phenotypes of the *Drosophila* homologues of *XCAP-H*, *barren* and *XCAP-C*, *DmSMC4*. In this study we have investigated the expression and subcellular distribution of hCAP-H, the human homolog of XCAP-H, in order to better understand its cellular functions. Transcription of *hCAP-H* was restricted to proliferating cells with highest expression during the G_2 phase of the cell cycle. In contrast, cellular hCAP-H protein levels were constant throughout the cell cycle. hCAP-H was found to be associated with mitotic chromosomes exhibiting a nonuniform but symmetric distribution along sister chromatids. The symmetry of hCAP-H association with sister chromatids suggests that there are sequence-dependent domains of condensin aggregation. During interphase hCAP-H, -C, and -E, have distinct punctate nucleolar localization, suggesting that condensin may associate with and modulate the conformation and function of rDNA. hCAP-H association with condensed chromatin was not observed in the early phase of chromosome condensation when histone H3 phosphorylation has already taken place. This finding is consistent with the hypothesis that histone H3 phosphorylation precedes condensin-mediated condensation.

INTRODUCTION

In the process of cell proliferation there is a fundamental requirement for stable distribution of equal complements of the genome to daughter cells. After DNA replication in S phase, DNA from both the template and newly replicated strands must be condensed into compact chromosomes to facilitate their interaction with the mitotic apparatus and to ensure segregation of homologues. In interphase, DNA is ordinarily organized into chromatin through its interaction with various histones and regulatory proteins. The degree of compactness correlates with transcriptional activity and defines active and silent genomic regions. Mitotic condensation requires reorganization of these local chromatin domains into higher order structures and finally into the compact chromosomes. Relatively little is known about the molecular factors controlling high-order chromatin conformation during progression through the cell cycle. However, the condensin complex has been found to play an essential role.

Condensin complexes are structural components of mitotic chromosomes and play a central role in driving chromosome condensation (Hirano *et al.*, 1997; Sutani *et al.*, 1999; Ouspenski *et al.*, 2000). In the *Xenopus* egg extract model the 13S condensin complex is required for ATP-dependent chromatin condensation (Hirano *et al.*, 1997). *Xenopus* 13S condensin is composed of five subunits termed XCAPs (*Xenopus* chromosome–associated proteins). The composition of the condensin complex is conserved in all organisms studied to date. Very recently, Kimura *et al.* (2001) demonstrated that the homologues of *Xenopus* condensin subunits, barren-1/hCAP-H (Cabello *et al.*, 1997), hCAP-C, hCAP-E, CNAP1 (Schmiesing *et al.*, 2000), and hCAP-G form a heteropentamer capable of inducing chromosome condensation in the *Xenopus* egg extract model. In *Saccharomyces cerevisiae* the condensin complex also consists of five subunits: Smc4p (XCAP-C), Smc2p (XCAP-E), Brn1p (XCAP-H), Ycs4p (XCAP-D2), and Ycs5p (XCAP-G). *S. cerevisiae* condensin is required for chromosome condensation, and possibly as a

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direct consequence of this function, it is also necessary for proper sister chromatid separation at anaphase (Freeman *et al.*, 2000).

The *Xenopus* and human condensin complexes consist of two subcomplexes (Hirano *et al.*, 1997, Kimura and Hirano, 2000; Kimura *et al.*, 2001). A stable 8S complex is formed by subunits CAP-C and CAP-E. Sequence analysis indicates that both CAP-C and CAP-E belong to the SMC family of ATPases (Hirano and Mitchison, 1994; Strunnikov *et al.*, 1995; Freeman *et al.*, 2000). Mutants in *Smc2* and *Smc4* in *S. cerevisiae*, and their homologues *cut3* and *cut14* in *Schizosaccaromyces pombe* are defective in proper condensation and segregation of mitotic chromosomes (Guacci *et al.*, 1993; Saka *et al.*, 1994; Strunnikov *et al.*, 1995). Sutani and Yanagida (1997) established that *cut3* and *cut 14* form a stable complex that efficiently renatures DNA and contributes to chromosome condensation in vivo. However, this activity does not require ATP, suggesting that the ATP-dependent increase in condensation activity observed in *Xenopus* and human might be attributed to the other members of the 13S condensin complex. Mitosis-specific phosphorylation appears to play a key role in the chromosomal targeting of the condensin complexes (Hirano *et al.*, 1997; Kimura and Hirano, 1997; Kimura *et al.*, 1999, 2001).

The other three non-SMC subunits, XCAP-D2, XCAP-G, and XCAP-H, form an 11S regulatory complex necessary for activation of the 8S SMC ATPases and promote the association of the 13S holocomplex with mitotic chromatin (Kimura and Hirano, 2000). Although all three 11S subunits are required for proper chromosome condensation and chromatid segregation (Sutani *et al.*, 1999; Freeman *et al.*, 2000; Lavoie *et al.*, 2000; Ouspenski *et al.*, 2000), the specific roles of each of the non-SMC subunits remain to be identified (Kimura and Hirano, 2000). Recently, Kimura and Hirano (2000) have proposed that XCAP-D2 and XCAP-G may participate in the activation of the XCAP-E and XCAP-C ATPases by a process directly involving their HEAT domains or by modifying the conformation of the SMC heterodimer. In *Drosophila* the condensin subunit barren, the homologue of XCAP-H, is required for chromatid arm resolution at anaphase (Bhat *et al.*, 1996). This effect was attributed to barren protein association with topoisomerase II (topoII) and its modulation of topoII activity in vitro. However, in *S. cerevisiae* the barren homologue Brn1p is required for chromatid condensation (Lavoie *et al.*, 2000; Ouspenski *et al.*, 2000) but does not appear to influence topo II activity (Ouspenski, unpublished results; Lavoie *et al.*, 2000).

Surprisingly, in *Drosophila* SMC4 mutants, chromosome condensation does not appear to be affected as indicated by normal compaction of the longitudinal axis during mitosis. However, all *DmSMC4* mutant alleles exhibit a dramatic failure to resolve sister chromatids before anaphase as is manifested by extensive chromatid bridges resulting in chromosome breakage and apoptosis (Steffensen *et al.*, 2001). This phenotype is remarkably similar to the *barren* mutant and *S. pombe cut3* and *cut14.* Taken together, these observations indicate that the condensin complex participates in chromatin remodeling and in anaphase chromatid resolution. The apparently contradictory results regarding the interaction between members of the condensin complex and topoII in different species raises the question of whether the function and regulatory mechanisms of these two pathways are conserved.

By sequence analysis, we have previously identified the human homologue of XCAP-H/barren and named it human barren-1. We mapped this locus to 2q11.2 (Cabello *et al.*, 1997). For the sake of clarity, we now designate this gene hCAP-H (human chromosome–associated protein H), after the nomenclature forwarded by Hirano *et al.* (1997) for *Xenopus* condensin and Kimura and Hirano (2000) for the human homologues. The composition of the condensin complex is conserved in human cells. Homologues of all five condensin subunits have been identified in the human genome and cDNAs corresponding hCAP-H, hCAP-C, hCAP-E, and the homologue of XCAP-D2, CNAP1, have been cloned (Cabello *et al.*, 1997; Schmiesing *et al.*, 1998; Schmiesing *et al.*, 2000). Schmiesing *et al.* (2000) reported that immunoprecipitation of HeLa cell extracts with CNAP1-specific antibody reveals the association with hCAP-C, hCAP-E, and two unidentified proteins corresponding to the predicted molecular weights of hCAP-H and hCAP-G. Kimura and Hirano (2001) have established the identity of these two proteins by coprecipitation of all five subunits of the 13S holocomplex from HeLa cell extracts

The sequence of events leading to the association of the 13S condensin complex with replicated chromosomes and the pathways that determine the activation of the complex have not been defined. Because the phosphorylation of histone H3 has been associated with the onset of chromosome condensation, Hirano *et al.* (1999) proposed that the association and activation of condensin with mitotic chromosomes may be secondary to the phosphorylation of histone H3. In *Drosophila,* the phosphorylation of histone H3 by Aurora B kinase is required for recruitment of condensin to chromosomes (Giet and Glover, 2001), and Schmiesing *et al.* (2000) have reported that in human cells, a fraction of the SMC heterodimer is associated with discrete chromosomal foci in interphase and that these foci colocalize with the sites of early histone H3 phosphorylation. However, Kimura and Hirano (2001) have demonstrated that the phosphorylation of H3 at Ser-10 is not sufficient to target the 13S condensin complex to chromosomes in vitro, independently of the phosphorylation status of the non-SMC subunits.

In this report, we characterize the cell cycle–dependent expression and localization of hCAP-H. The results are consistent with a role of hCAP-H in mitosis and provide evidence that the association of the condensin complex with human mitotic chromosomes is nonuniform, regulated, and preceded by phosphorylation of histone H3 at Ser-10. Moreover, the results support the hypothesis that hCAP-H, and possibly the condensin complex, play a novel role in interphase.

MATERIALS AND METHODS

Cell Lines

HeLa, REH, Daudi, Raji, HS Sultan, CEM, Jurkat, K562, HL-60, KG1, KG1a, HEL92.1.7, and MEG cell lines were purchased from ATCC and cultured according to ATCC recommendations. Primary skin fibroblasts (LE3352) and EBV-transformed B-lymphoblasts were derived from healthy anonymous subjects and cultured in DMEM and RPMI, respectively, supplemented with 10% bovine calf serum.

Cell Cycle Synchronization

Cells were separated with the use of centrifugal elutriation as previously described (Meistrich, 1983). Size separation was confirmed by counting cells from each fraction with the use of a Coulter Analyzer. Protein and RNA extracts from individual fractions were analyzed by Western and Northern hybridization. Cell cycle distribution of elutriated cells was confirmed by FACS analysis of PI-stained cells.

Northern Blots

Total RNA was isolated from cultured cells with the use of RNAzol B. Ten-microgram RNA samples were electrophoresed on 1% agarose gels containing 8% formaldehyde, blot-transferred onto Hybond membrane, and hybridized to a ^{32}P-labeled *hCAP-H* probe in ExpressHyb (CLONTECH, Palo Alto, CA) after the manufacturer's protocols. After high-stringency wash, membranes were exposed to film or PhosphorImager screen.

Antibody Production

Anti–hCAP-H polyclonal antibodies Ab2573 and Ab2575 were produced in New Zealand White rabbits injected with hCAP-H peptides in the presence of MPL+TDM+CWS adjuvant emulsion (RIBI ImmunoChem Research, Inc. Hamilton, MT). Antigen 2573 was a synthetic peptide identical to the 17-amino acid C terminus of hCAP-H. Antigen 2575 was a recombinant peptide produced in BL-21 bacteria transformed with a plasmid construct consisting of cDNA encoding HCAP-H amino acids 1–453 cloned in vector pET-28c (Novagen, Madison, WI). Recombinant protein was purified after the vector manufacturer's recommended protocol. Both antibodies were antigen-affinity purified. Both purified antibodies recognized a unique band on Western blots corresponding to ~90 kDa. Occasionally, a band of ~45 kDa was detected and was presumed to be proteolysis product of HCAP-H because both bands were undetectable after preincubation with corresponding specific antigens.

Western Blots

Unless noted otherwise, protein extracts were prepared by resuspending known numbers of cells in Laemmli sample buffer. Proteins were separated by electrophoresis on 10% polyacrylamide gels, transferred onto PVDF membrane, and probed with 2575 or 2573 antibodies at a dilution of 1:1000. Bound antibody was detected with ECL (CLONTECH) or SuperSignal (Amersham, Arlington Heights, IL) Western blot kits, following manufacturer's recommended protocols.

Immunofluorescence Microscopy

Normal human skin fibroblasts or HeLa cells were plated and grown on acid-etched, poly-L-lysine–coated glass coverslips for 24 h. Alternatively, metaphase spreads were prepared by treatment of an exponentially growing culture with 100 μg/ml colcemid for 4 h and shake-off recovery of the mitotic fraction. Recovered cells were washed twice in PBS and centrifuged onto glass coverslips. Cells were permeabilized by incubation on the coverslips for 2 min at 4°C in PEM buffer (80 mM K-PIPES, pH 6.8, 5 mM EGTA, pH 7.0, 2 mM $MgCl_2$) containing 0.5% Triton X-100. Subsequently, cells were fixed for 20 min in PEM plus 4% paraformaldehyde at 4°C. A second permeabilization in PEM/0.5% Triton X-100 was conducted for 30 min at room temperature, and coverslips were stored in TBS-T (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) plus 5% milk overnight at 4°C. Cell were immunostained with 1:500 dilutions of primary polyclonal antibodies 2575, 2573, CREST human antisera (Moroi *et al.*, 1980), antinucleolin mAb (Research Diagnostics, Flanders, NJ), or mAb recognizing histone H3 phosphorylated on serine 10 (kindly provided by Dr. W.M. James, Intergen, Gaithersburg, MD) for 60 min at 37°C. After a thorough rinse, coverslips were incubated with Texas Red– (TXRD) or FITC-conjugated goat anti-human or anti-rabbit secondary antibodies, counterstained with DAPI for 30 s, and subsequently mounted onto glass slides, with the use of Vectashield antifade medium (Vector Laboratories Inc., Burlingame, CA). Immunofluorescence analysis was conducted on a DeltaVision deconvolution microscope.

Subcellular Localization of GFP-tagged HCAP-H

A GFP fusion to the NH_2-terminus of hCAP-H was created by ligation of the KIAA0074 cDNA (Nomura *et al.*, 1994) between the *Hin*dIII and *Sac*II sites of pEGFP-C2 (CLONTECH) in frame with the initiation codon of hCAP-H. HeLa or LE3352 cells were transfected with this construct with the use of Superfect (Qiagen, Santa Clarita, CA). Cultured cells were protected from light for the remainder of the procedures. After 20-h recovery, cells were resuspended by trypsin treatment and plated onto glass coverslips. Some coverslips were processed for analysis 4 h after plating (24 h posttransfection), and others were analyzed at 48 h posttransfection. Coverslips were directly inverted onto glass slides for immediate visualization of live cells or processed as described for immunofluorescence. Coverslips were mounted on Vectashield and sealed. Stable transfectants were generated by cotransfection of EGFP-hCAP-H with pGK/Puro that confers resistance to puromycin. After 48 h posttransfection, cells are exposed to 5 mg/ml puromycin. One week after initial exposure to puromycin, 17 individual colonies showing various levels of fluorescence intensity were selected and maintained subsequently in medium containing puromycin.

Subcellular Colocalization of DsRed1-tagged HCAP-C and EGFP-tagged hCAP-H

An hCAP-C cDNA was kindly provided by Drs. T. Nishiwaki and Y. Nakamura (Human Genome Center, Institute of Medical Science, The University of Tokyo). A DsRed1 in-frame fusion to the C terminus of hCAP-C was created by ligation in pDsRed1-N1 (CLONTECH). HeLa cells were cotransfected with this construct and pEGFP-HCAP-H with the use of Superfect (Qiagen). Cultured cells were protected from light for the remainder of the procedures. After 20 h recovery, cells were resuspended by trypsin treatment and plated onto glass coverslips. Coverslips were processed for analysis 48 h posttransfection as described for immunofluorescence. Coverslips were mounted on Vectashield and sealed. Simultaneous expression of the two fusion proteins was monitored and documented with the DeltaVision deconvolution microscope.

RESULTS

hCAP-H Is Conserved through Evolution

CAP-H genes and proteins have been found in simple and complex eukaryotes (Cabello *et al.*, 1997; Hirano *et al.*, 1997; Lavoie *et al.*, 2000; Ouspenski *et al.*, 2000). Database analysis demonstrated numerous candidate homologues with obvious primary amino acid sequence conservation. Interestingly, each organism appeared to have only a single homologous gene, and there was no evidence of functional duplication and divergence in the genomes of *S. cerevisiae*, *C. elegans*, *D. melanogaster*, or humans in which near complete sequence information was available. Amino acid sequence alignments of predicted proteins demonstrated four highly conserved domains suggesting that these segments may serve specific conserved functions (Figure 1A). The individual motifs are unique to the CAP-H family and do not suggest known functions.

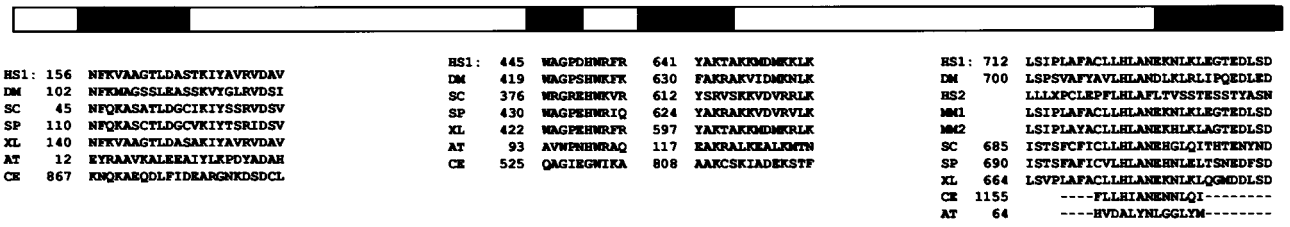

Figure 1. Conservation of hCAP-H. Sequence alignment and schematic position of the four conserved domains in the barren/CAP-H family of proteins. HS, human CAP-H GenBank BAA07556; MM, mouse GenBank AC074224; DM, *D. melanogaster* GenBank AAB40125; SC, *S. cerevisiae*; SP, *S. pombe* GenBank BAA82625; CE, *C. elegans* GenBank CAB03149; XL, *Xenopus* GenBank AAC60203; AT, Arabidopsis GenBank AAC25941. Multiple sequence alignment was produced by global progressive alignment in ClustalW.

Cell Cycle-dependent Expression of hCAP-H

hCAP-H cDNA was initially identified in the myeloid precursor cell line KG1 (Nomura *et al.*, 1994). An *hCAP-H* transcript of ~4 kb was detected in a variety of tissue culture cell lines (Figure 2A), consistent with a predicted requirement during cell proliferation. To establish whether *hCAP-H* is expressed in adult tissues, we performed Northern blot analysis. Although low-level expression was detected in lung and placenta, *hCAP-H* transcript was not detectable in heart, skeletal muscle, pancreas, kidney, and liver. These results are consistent with the concept that *hCAP-H* is not transcribed or is transcribed at very low levels in quiescent cells.

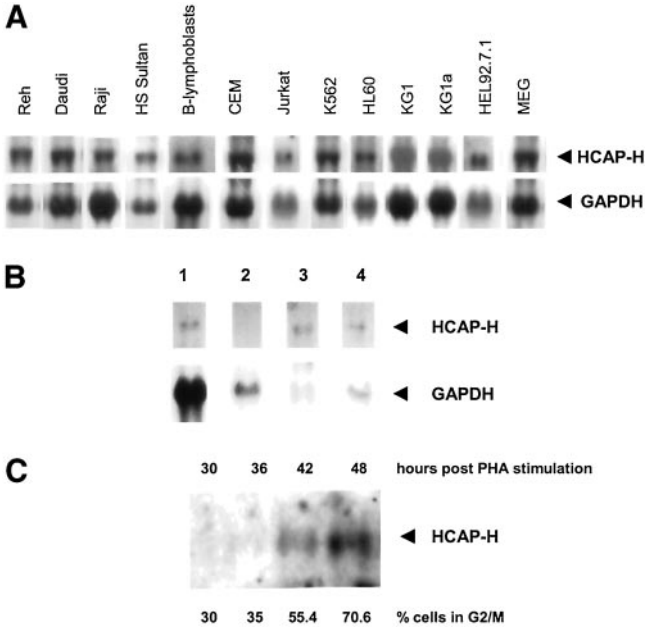

Figure 2. Expression pattern of hCAP-H. (A) Northern hybridization of hCAP-H on total RNA purified from indicated cell lines. (B) Northern hybridization of *hCAP-H* to total RNA extracted from peripheral blood lymphocytes at the time of isolation (quiescent) (2) and 72 h poststimulation with PHA (see MATERIALS AND METHODS) (3). A single transcript of similar size was detected on lanes loaded with total RNA derived from transformed B-lymphoblasts (1) and K562 cells. (C) Time course of *hCAP-H* expression in PBL after PHA stimulation. Total RNA was isolated from PBL before stimulation (control) and every 6 h poststimulation. Top lane labels indicate the number of hours after addition of PHA to cultures. Bottom labels indicates percentage of PI-stained cells in G_2-M as indicated by FACS profile: control, 2.0%: 30 h, 30%; 36 h, 35%; 42 h, 55.4%; 48 h, 70.6%.

Many genes that participate in cell cycle–specific processes exhibit cyclically modulated expression levels. To test whether *hCAP-H* expression is cell cycle regulated, we determined its expression in quiescent tissues and proliferating cells. To establish whether *hCAP-H* transcription is dependent upon proliferative activity, we assessed its expression in primary and phytohemaglutinin (PHA)-stimulated peripheral blood lymphocytes. Although *hCAP-H* mRNA is not detected by Northern hybridization in quiescent peripheral blood lymphocytes (PBLs), expression was induced by mitogenic stimulation with PHA (Figure 2B). These data demonstrate that induction of *hCAP-H* transcription correlates with proliferative activity.

To determine whether the transcription of *hCAP-H* varies through the cell cycle, we analyzed *hCAP-H* mRNA levels in samples taken at fixed intervals after PHA stimulation. Parallel samples were fixed in 100% ethanol and stained with propidium iodide in the presence of RNAse A for subsequent analysis of DNA content by flow cytometry. *hCAP-H* transcription was not detectable by Northern analysis until 36 h poststimulation, coinciding with progress of the cell population toward mitosis (Figure 2C). These data indicate that *hCAP-H* is transcribed after the replication (S) phase and are consistent with its role in mitosis. To determine whether *hCAP-H* transcription is cyclical in continuously growing cells, we determined the mRNA content by Northern blot analyses on Molt-4 cells separated by elutriation (Figure 3A). *hCAP-H* message was detectable only in fractions corresponding to G2 and M phases, indicating that hCAP-H transcription is cell cycle regulated and restricted to G2/M (Figure 3B).

Affinity purified anti-C′ terminal peptide (Ab2573) and anti–recombinant peptide 1–435 (Ab2575) antibodies recognize a single band of ~97 kDa on Western blots of HeLa and Molt-4 cell extracts. Binding specificity was demonstrated on blots preincubated with corresponding antigens on which no bands were detected. To establish whether hCAP-H protein levels are cell cycle-dependent, we probed Western blots of Molt-4 elutriated cells with Ab2575. A single 97-kDa band of invariant intensity was detected in all fractions indicating that, in contrast with mRNA levels, hCAP-H protein levels remain stable throughout the cell cycle in proliferating cells (Figure 3, C and

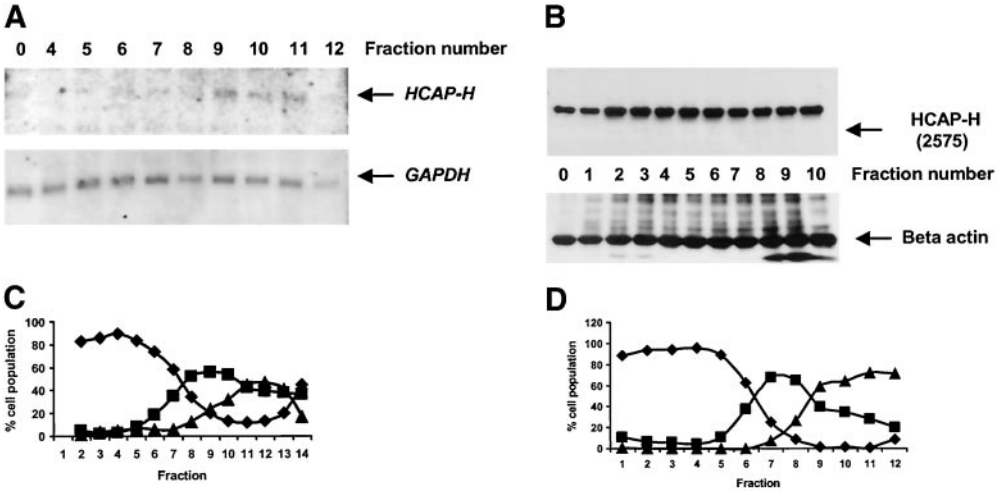

Figure 3. Expression of hCAP-H through the cell cycle. (A) Northern blot of total RNA derived from elutriated Molt-4 cells. Probes were prepared by random priming of full HCAP-H and GAPDH cDNA. hCAP-H is present in fractions 8–12, containing the largest populations of mitotic cells as indicated by elutriation profile (B). (C) Western blot of whole cell protein extracts prepared from elutriated Molt-4 cells. hCAP-H levels are independent of cell cycle stage-specific populations as indicated by elutriation profile. (D) Blots were probed with antibeta actin and Ab2575 antisera as described in MATERIALS AND METHODS. Vertical axis indicates percentage of elutriated cells in G_0/G_1 (♦), S (□), and G_2/M (▲).

D). Similar results were obtained with the use of Hoechst-stained FACS sorted cells.

Localization of hCAP-H in Mitosis

To determine the subcellular localization of hCAP-H, HeLa cells and primary diploid human skin fibroblasts (LE3352) were immunostained with antihCAP-H1–435 (Ab2575) and anti–hCAP-H C′ terminal peptide (Ab2573) antibodies and analyzed by fluorescence microscopy. Staining with Ab2575 demonstrated association of hCAP-H with mitotic chromosomes from early prophase through telophase (Figure 4). However, Ab2573 fails to recognize epitopes that localize to chromatin during mitosis in HeLa cells. These results are consistent with a role of hCAP-H in mitotic chromosome condensation. Deconvolution immunofluorescence microscopy of HeLa cells stained with Ab2575 revealed a discontinuous distribution of hCAP-H along the condensed chromosomes, defining discrete domains along the chromatin (Figure 5, A and B). A similar distribution pattern was obtained by visualization of EGFP-hCAP-H fusion protein transiently expressed in HeLa cells (Figure 5C). Symmetric staining patterns were consistently observed in HeLa and LE3352 metaphase spread preparations. To further analyze the distribution of hCAP-H on condensed chromatin, metaphase spreads of LE3352 cells were stained with Ab2575. The patterns of Immunofluorescence signals did not correspond to the heterochromatin banding pattern.

Localization of hCAP-H in Interphase

hCAP-H subcellular localization was found to be cell cycle dependent. Unexpectedly, hCAP-H was clearly detected with Ab2573 and Ab2575 in interphase nucleoli in HeLa and Le3352 cells, with a distinct punctate distribution (Figure 6, A and C). This nucleolar localization was consistently observed in all cells in interphase. The specificity of these immunostaining patterns was demonstrated by epitope competition (Figure 6B,D) and control experiments in which primary antibody was omitted or substituted by nonreactive rabbit IgG. To confirm the identity of the nucleoli in interphase cells, HeLa cells were simultaneously stained with monoclonal antinucleolin antibody and Ab2575 (Figure 6, E and H). The superimposed images indicate that nucleolin delineates the nucleolar perimeter and that hCAP-H is localized within the nucleolus. To determine whether the nucleolar localization of hCAP-H reflects the localization of the condensin holocomplex in interphase, we examined the localization of the 8S condensin subcomplex as indicated by localization of hCAP-C. HeLa cells were transiently cotransfected with pEGFP-hCAP-H, and pDsRed1-hCAP-C. Those cells that expressed both fusion proteins demonstrated colocalization of the 2 proteins in the nucleoli (Figure 6, I and L). Similar results were obtained in HeLa cells transfected with EGFP-hCAP-H, in which the fluorescence signal localized to interphase nucleoli 48 h posttransfection. hCAP-H was also detected in the nucleoli of G_0 quiescent lymphocytes, indicating that it must have persisted after a previous period of transcriptional activity.

Association of hCAP-H with Chromatin Relative to H3 Phosphorylation

The phosphorylation of histone H3 is considered the earliest event defining the onset of mitotic condensation (Sauve *et al.*, 1997; Wei *et al.*, 1999). Schmiesing *et al.* (2000) have proposed that H3 phosphorylation is initiated at chromatin sites on which hCAP-E is associated throughout interphase. To establish the timing of association of hCAP-H with relative to the initiation of chromatin condensation, HeLa cells were stained simultaneously with a mouse mAb specific to histone H3 phos-

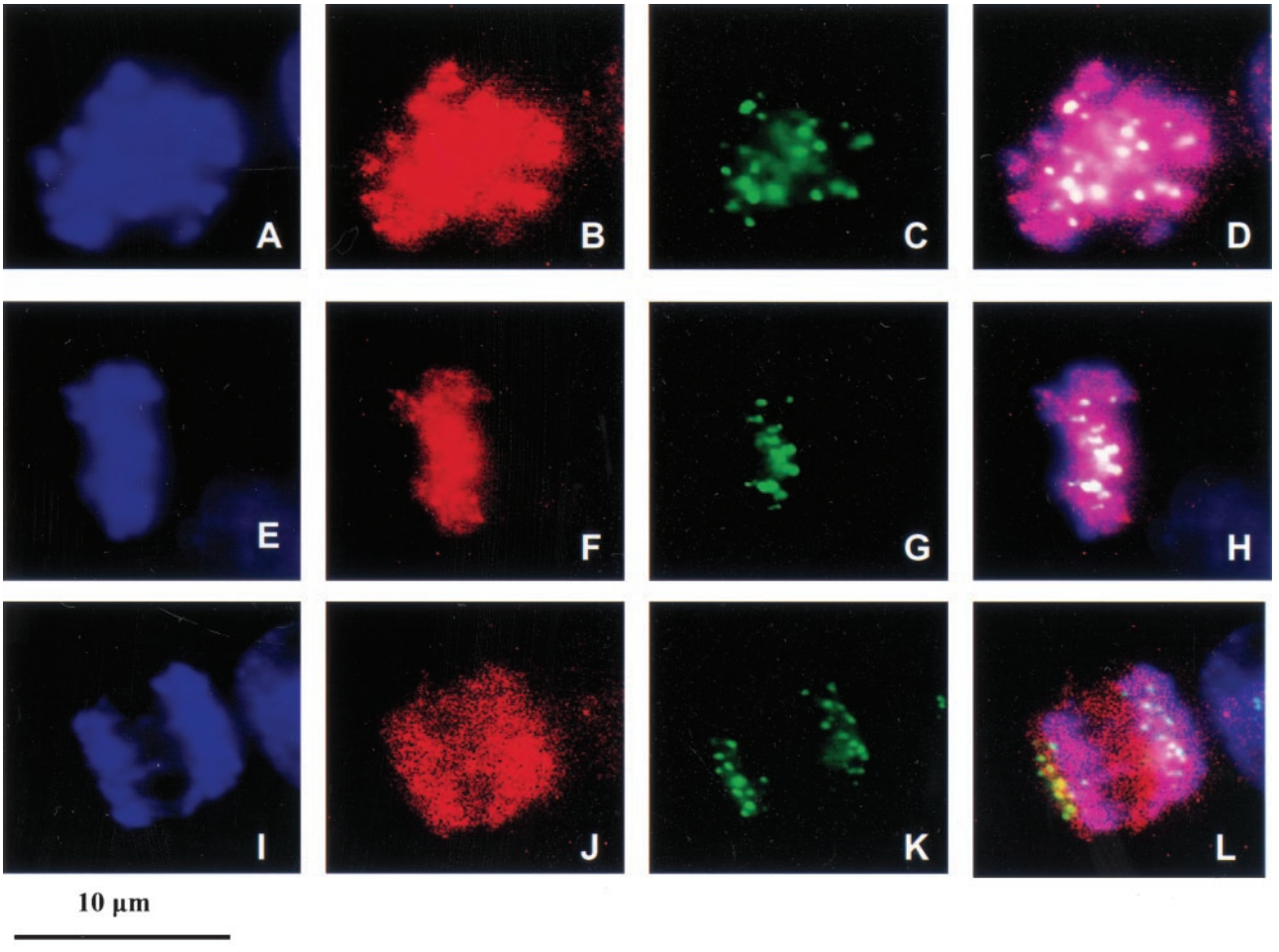

Figure 4. Immunofluorescence localization of HCAP-H in mitosis. hCAP-H is associated with DAPI-stained chromatin (detected at 418 nm; visible blue spectrum; A, E, and I) from prophase (A–D), through metaphase (E–H), until late telophase (I–L). Immunostaining of mitotic HeLa cells with hCAP-H–specific antibody Ab2575, detected as fluorescence at 617 nm (visible red spectrum) with Texas red–conjugated goat anti-rabbit $F(ab')_2$ as described in MATERIALS AND METHODS (B, F, and J). Centromeres are immunostained with CREST antisera and detected at 528 nm (visible green spectrum) with FITC-conjugated goat anti-rabbit $F(ab)_2$ as described in MATERIALS AND METHODS (C, G, and K). Merged images (D, H, and L) indicate colocalization of Ab2575 with mitotic chromatin.

phorylated on serine 10 (Sauve *et al.*, 1997) and with rabbit Ab2575. Interestingly, deconvolved images of cells in prophase indicated that although hCAP-H was clearly detected in the nucleoli, it could not be detected on nascent condensed chromatin marked by H3 phosphorylation (Figure 7).

DISCUSSION

Conservation of the Condensin Complex

The condensin complex has been identified as a conserved heteropentamer required for mitotic chromosome condensation in *S. cerevisiae* (Freeman *et al.*, 2000), *S. pombe* (Sutani *et al.* 1999), and *Xenopus* (Hirano *et al.*, 1997). The five-subunit composition of the condensin complex is conserved in human cells (Kimura *et al.*, 2001). The two SMC-type subunits are capable of binding DNA, and it has been proposed that by an ATP-dependent hinge-like action they promote the high-order compaction of chromatin at mitosis (Kimura *et al.*, 1999). The specific functions in activating or regulating condensin activity of the remaining three non-SMC subunits are not known (Kimura and Hirano, 2001). One of these proteins, barren, is required for sister chromatid separation at anaphase and appeared to modulate the activity of topoisomerase II (Bhat *et al.*, 1996). The characteristic failure to resolve and separate sister chromatids at anaphase of *barren* mutants was mimicked by the phenotype was observed in mutants of *DmSMC4.* Further investigation of functional connections between chromosome condensation and sister chromatid decatenation will be required to understand these linked processes.

Cell Cycle–dependent Expression of hCAP-H

The functions of XCAP-H in *Xenopus* (Hirano *et al.*, 1997) and barren in *Drosophila* (Bhat *et al.*, 1996) appear mitosis

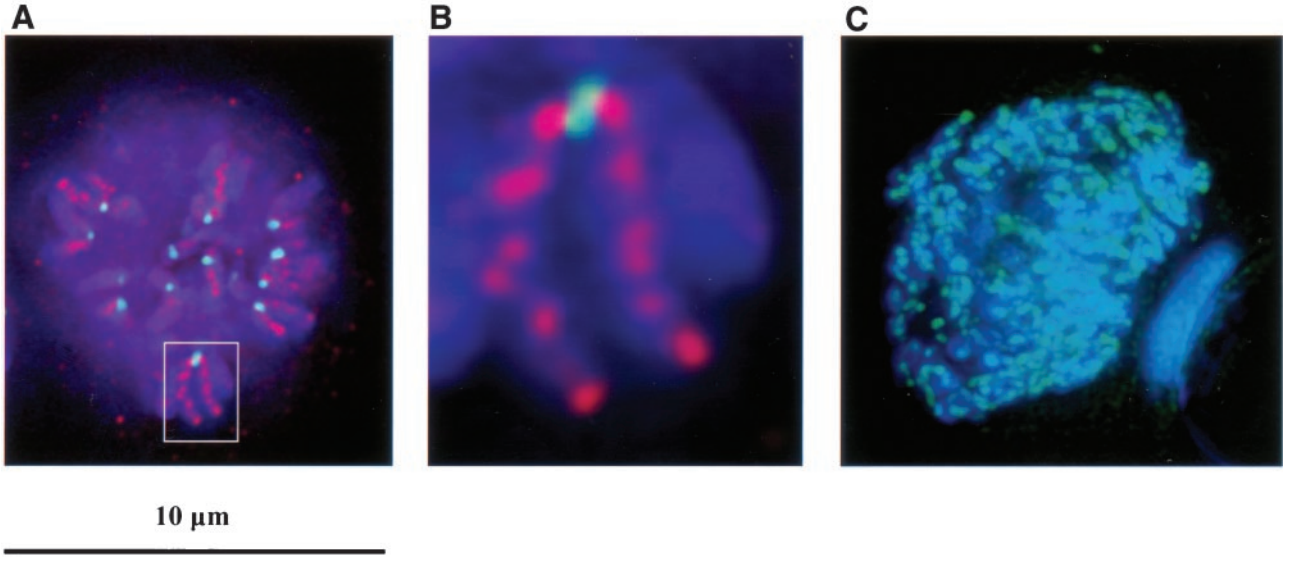

Figure 5. Cluster distribution of HCAP-H on condensed chromosomes. (A) Deconvolved image of mitotic HeLa cells immunostained with hCAP-H–specific antibody Ab2575, detected as fluorescence at 617 nm (visible red spectrum) with Texas red–conjugated goat anti-rabbit $F(ab')_2$ as described in MATERIALS AND METHODS. hCAP-H is associated with DAPI-stained chromatin (detected at 418 nm; visible blue spectrum). Centromeres are immunostained with CREST antisera and detected at 528 nm (visible green spectrum) with FITC-conjugated goat anti-rabbit $F(ab)_2$ as described in MATERIALS AND METHODS. (B) Symmetric distribution of Ab2575 staining on an acrocentric chromosome identified on the inset in A. (C) Deconvolved image of a HeLa cell in anaphase, transiently expressing EGFP-hCAP-H 24 h posttransfection with pEGFP-hCAP-H. EGFP-hCAP-H, detected at 528 nm (visible green spectrum), is associated with DAPI-stained chromatin, detected at 418 nm (visible blue spectrum). Merged image represents 50 superimposed 200-nm-thick optical sections.

specific. The expression analysis in cell lines and adult tissues revealed that hCAP-H transcript is present in proliferating cells but not in quiescent cells. The data derived from synchronized and elutriated cells indicate that *hCAPH* mRNA is transcribed during G_2 and degraded upon completion of mitosis. This is consistent with the cell cycle dependence of *BRN1* mRNA levels, which are maximum at G_2/M, in *S. cerevisiae* (Cho *et al.*, 1998). However, Western blots indicated that unlike most proteins subject to cell cycle–regulated transcription, levels of hCAP-H protein remained constant throughout the cell cycle. These data are consistent with the detection by immunofluorescence of hCAP-H protein and during interphase (see below). The occurrence of cell cycle–regulated expression of *hCAP-H* transcription is interesting given that the protein appears to persist into interphase. This could indicate that interphase hCAP-H is either quantitatively insufficient or not functionally available for the mitotic activity of condensin. We recently reported that in *S. cerevisiae* Brn1p is present throughout the cell cycle, although cyclical fluctuations in the protein level were detected by Western blots (Ouspenski *et al.*, 2000). These results may reflect the fact that in yeast the steady state pool of Brn1p is smaller than the cycling pool, whereas in mammalian cells the cycling pool resulting from the premitotic *hCAPH* transcription is minimal compared with the high levels of the steady state pool.

Kimura *et al.* (1998) have demonstrated that 13S condensin purified from interphase cells can be activated for condensation activity by Cdc2-catalyzed phosphorylation. It is, therefore, unlikely that phosphorylation state alone could explain a requirement for cell cycle–regulated transcription of hCAP-H. However, access to hCAP-H by the appropriate kinase(s) might play a role. The phosphatase that mediates physiological dephosphorylation of CAP-H at anaphase is unknown.

Cell Cycle–dependent Subcellular Localization of hCAP-H

The association of hCAP-H with mitotic chromosomes is consistent with its role in mitotic condensation. As predicted, hCAP-H, like hCAP-C, and -E (Schmiesing *et al.*, 1998, Figure 6) is bound to metaphase chromosomes. Deconvolution fluorescence microscopy demonstrated that hCAP-H-containing condensin complexes are not uniformly distributed along mitotic chromosomes. The finding of discontinuous binding or aggregation of supramolecular condensin complexes onto mitotic chromatin was unanticipated. The remarkably symmetric patterns of hCAP-H staining on metaphase sister chromatids may reflect a sequence-specific association of 13S condensin with chromatin, as has been reported for the other SMC-containing complex, cohesin (Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Losada *et al.*, 1998; Blat and Kleckner, 1999; Tanaka *et al.*, 1999). In *S cerevisiae* we found that sustained Brn1p protein expression is required for the maintenance of condensation (Ouspenski *et al.*, 2000). However, it would appear from the discrete association pattern of hCAP-H with mitotic chromosomes, that chromatin association with hCAP-H and perhaps the 11S subcomplex is required for mitotic chromosome condensation but not for the maintenance of the condensed state in human cells. The discontinuous pattern of chromosomal

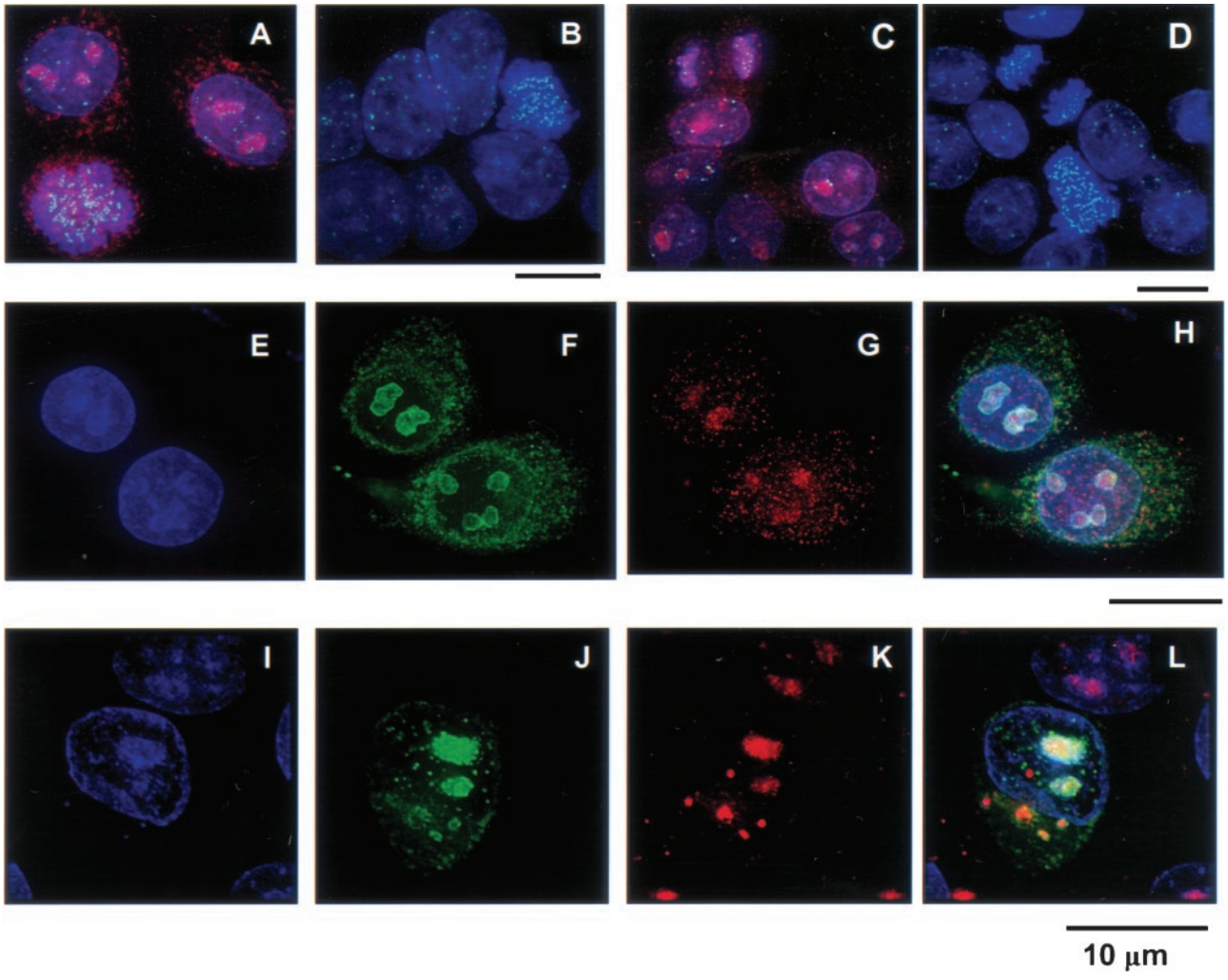

Figure 6. Localization of HCAPH in interphase nucleoli. DAPI counterstaining identifies HeLa cell nuclei (B, D, E, H, I, and L). Cells were stained with Ab2575 (A and B) and Ab2573 (C and D) as described in MATERIALS AND METHODS. Nonreactivity in the presence of corresponding epitopes, truncated recombinant hCAP-H (B), and synthetic hCAP-H C terminus (D) indicates specificity of the nucleolar staining. hCAP-H staining with Ab2575 (G; detected with TXRD-conjugated goat anti-rabbit secondary antibody) colocalizes (H) with antinucleolin mAb (F; detected with anti FITC conjugated goat anti-mouse IgG secondary antibody). EGFP-hCAP-H (J) and DRS1-Red-hCAP-C (K) expressed in HeLa cells colocalize (L) in interphase nucleoli. Scale bars, 10 μm.

staining with anti–hCAP-H antibodies was consistent with immunofluorescence experiments, indicating that hCAP-C exhibits a punctate distribution throughout the entire chromosome during metaphase (Schmiesing *et al.*, 2000). This suggests condensin regulatory mechanisms may have evolved to maintain established condensation of periodic macrodomains in the human genome. Thus, the mitotic activity of condensin could be regulated in at least three levels: mitosis-specific phosphorylation (Kimura *et al.*, 1998, 1999; Kimura and Hirano, 2000) mitosis-specific transcription, and mitosis-specific chromosome localization.

Detection of the hCAP-H C′ terminal epitope recognized by Ab2573 within nucleoli but not on mitotic chromatin suggests that the C terminus of hCAP-H may be unavailable for binding during chromosomal condensation. This observation is consistent with a recent report by Kimura and Hirano (2000) indicating that an antibody increased against the C-terminal tail of XCAP-H was unable to precipitate the 13S condensin complex, whereas an antibody increased against a recombinant XCAP-H fragment (Hirano *et al.*, 1997) precipitated all five subunits. These authors concluded that the C-terminal tail of XCAP-H is buried inside the 13S condensin complex. The organization of condensin components in the nucleolus is of interest and it is possible that hCAP-H or the 11S complex are involved in alternative molecular interactions.

Accumulation of hCAP-H, in interphase nucleoli of HeLa and LE-3352 cells is consistent with the data, indicating that hCAP-H protein levels remain elevated through the cell cycle. The nucleolar localization of hCAP-H is not unique and is consistent with the nucleolar localization of HCAP-C and anti–hCAP-E (Cabello and

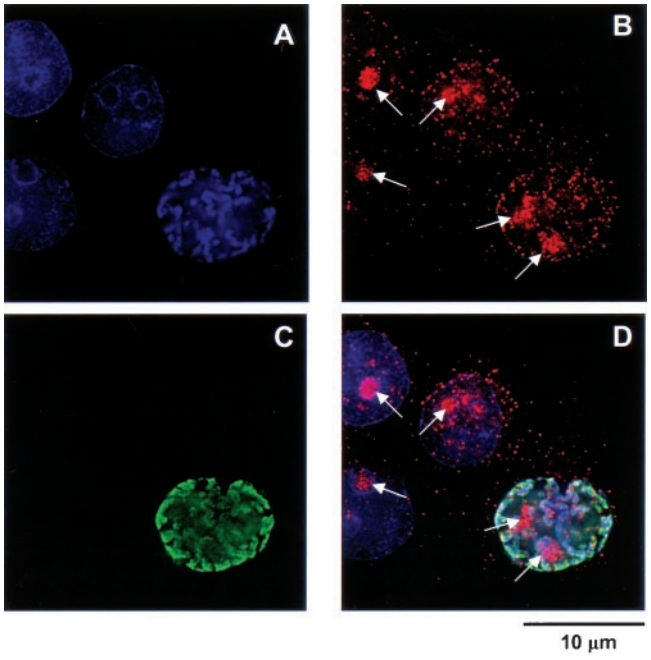

Figure 7. Histone H3 phosphorylation and HCAP-H association with mitotic chromatin. (A) DAPI stained detected at 418 nm; visible blue spectrum. The cell in the lower right corner presents partially condensed chromosomes and nuclear clefting. (B) Immunostaining with Ab2575 detected as fluorescence at 617 nm (visible red spectrum) with Texas red–conjugated goat anti-rabbit F(ab')$_2$; nucleoli are indicated by arrows. (C) Immunostaining with mAb against histone H3 phosphorylated on S10, detected as fluorescence at 528 nm (visible green spectrum) with FITC goat anti-mouse IgG. (D) Merged image represents 50 superimposed 200-nm-thick optical sections.

Belmont, unpublished results). These data suggest that the condensin complex may remain associated with nucleolar chromatin domains, perhaps participating in chromatin remodeling and condensation-dependent silencing of rDNA loci. Alternatively, it is possible that active sequestration of condensin subunits into the nucleoli serves to remove them from condensed chromatin, facilitating the decondensation of chromatin during telophase (Bachant and Elledge, 1999). The persistence of hCAP-H in the nucleoli of quiescent cells tends to favor an active role of condensin rather than passive sequestration, which might be expected to be evanescent. The punctate nucleolar localization of hCAP-H, -C, and -E suggests the novel possibility that condensin may associate with and modulate rDNA conformation. These observations are consistent with the chromatin immunoprecipitation analysis reported by Freeman *et al.* (2000), indicating the physical association of condensin with rDNA in *S. cerevisiae*. The difference between the size of the steady state pool in human and yeast cells may reflect the marked difference between the number and complexity of rDNA repeats found in the genomes of the two species. The condensin complex represents the first example of a DNA conformation modulator that localizes to interphase nucleoli and specifically binds rDNA.

Sequence of Initiation of Chromatin Condensation

It is generally accepted that the committing event in mitotic condensation is the phosphorylation of histone H3 (Hendzel *et al.*, 1997; Wei *et al.*, 1999). This has led to the suggestion that the phosphorylation of histone H3 recruits condensin to mitotic chromatin (Hirano, 1999). However, Schmiesing *et al.* (2000) have proposed that association of hCAP-E with interphase chromatin defines the initiation sites of H3 phosphorylation. We tested the timing of association of hCAP-H with mitotic chromatin with respect to the phosphorylation of H3 by simultaneous immunolocalization of H3 phosphorylated on Ser10 and hCAP-H in HeLa cells. The results indicate that Ser10-H3 phosphorylation (Figure 7C) precedes the disintegration of the nucleoli that can be identified by the localization of hCAP-H (Figure 7B,D) and, importantly, also precedes the association of detectable amounts of hCAP-H with chromatin. Although hCAP-E appears to be associated with chromatin early in prophase (Schmiesing *et al.*, 2000), we could detect hCAP-H on mitotic chromatin only relatively late in prophase (Figure 4). This could simply be explained by the sensitivity of the detection methods and the density of the hCAP-E complexes on the chromatin. On the other hand, assuming that hCAP-H reflects the presence of the 11S condensin complex, these results could also suggest the hypothesis that delayed association of the 11S subcomplex confers another activation step in the condensation process. Giet and Glover (2001) have demonstrated that histone H3 phosphorylation at serine 10 by Aurora B kinase is necessary for the recruitment of barren to mitotic chromosomes and that the association of barren with chromosomes follows the same dynamics as H3 phosphorylation. However, the simultaneous immunolocalization of phosphorylated histone H3 and chromatin-associated hCAP-H indicate a temporal dissociation of the two phenomena and are consistent with the observation that in vitro phosphorylation of histone H3 at serine 10 is not sufficient to account for mitosis-specific targeting of 13S condensin to chromatin (Kimura and Hirano, 2000).

In summary, the hCAP-H localization indicates that condensin associates with discrete domains onto mitotic chromatin in human cells and that it is found associated with nucleoli during interphase. These results suggest that hCAP-H and perhaps the condensin complex may participate in rDNA remodeling. The results are consistent with the data indicating that the mitotic function of condensin is dependent on the phosphorylation of histone H3 on serine 10 and suggest that this dependence is mediated by a yet unidentified event. Further investigation of the pathways that target and activate the condensin complex will be required to understand integration of the process of chromosome condensation with the regulation of cell cycle progression at the onset of mitosis.

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