INTRODUCTION

The relaxed chromatin of interphase is condensed during cell division to allow its segregation on the spindle apparatus. Cytologically, condensation initiates after S-phase and reaches its maximum during early mitosis (Adlakha and Rao, 1986). It is generally thought that the condensation of chromatin occurs through its folding into loops, compaction of the loops, and coiling of the axis upon which the loops are anchored (reviewed by Manuelidis and Chen, 1990; Koshland and Strunnikov, 1996). Additionally, tangles are resolved that arise during DNA replication and by simple diffusion (Sundin and Varshavsky, 1981; Weaver et al., 1985).

Several histones are phosphorylated with chromosome condensation, but a causal role for these modifications in chromatin modeling has not been clearly established (reviewed by Bradbury, 1992). Histone phosphorylations have been implicated in both the initiation and maintenance of mitotic chromosome condensation (Bradbury et al., 1973; Marks et al., 1973; Gurley et al., 1975, 1978), as well as in the silencing of transcription during mitosis (Paulson and Taylor, 1982). More recently it has been proposed that the phosphorylation of histone amino-terminal tails might reduce their affinity for DNA and facilitate the movement of nucleosomes and access of chromatin to condensation factors, transcription factors, and proteases activated during apoptotic cell death (Roth and Allis, 1992; Hendzel et al., 1997; Waring et al., 1997). Additionally, the amino-termini of core histones might be involved in protein-protein interactions and the recruitment of trans-acting factors to specific regions of chromatin (Hendzel et al., 1997).

Site-specific phosphorylation of histone H3 at serine 10 (Ser10) initiates during G2, becomes maximal during metaphase, and diminishes during late anaphase and early telophase (Gurley et al., 1978; Paulson and Taylor, 1982; Hendzel et al., 1997). In Tetrahymena, H3 phosphorylation occurs only in the mitotic micronucleus; H3 is not phosphorylated in the macronucleus, which divides amitotically without obvious chromosome condensation (Allis and Gorovsky, 1981). When mammalian interphase cells are fused with mitotic cells, premature chromosome condensation is induced. The prematurely condensed state induced by okadaic acid treatment during S-phase culminates with H3 phosphorylation throughout the chromatin, but in an absence of mitotic chromosome morphology, indicating that the phosphorylation of H3 is not sufficient for complete condensation. Mild hypotonic treatment of cells arrested in mitosis results in the dephosphorylation of H3 without a cytological loss of chromosome compaction. Hypotonic-treated cells, however, complete mitosis only when H3 is phosphorylated. These observations suggest that H3 phosphorylation is required for cell cycle progression and specifically for the changes in chromatin structure incurred during chromosome condensation.

Key words: Centromere, Heterochromatin, Histone, Mitosis, Okadaic acid, Phosphorylation

SUMMARY

Histone H3 phosphorylation is required for the initiation, but not maintenance, of mammalian chromosome condensation

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specifically in late-replicating/early-condensing chromatin during normal division cycles and when premature condensation is induced. Activity of the kinase that phosphorylates H3 is required for entry into mitosis, but is not required for maintaining the condensation of chromosomes in hypotonically-swollen cells. This modification, then, is associated specifically with cell cycle progression and the dynamics of chromatin modeling in mammalian cells, but may not be required for maintaining the condensed state.

MATERIALS AND METHODS

Cell culture and synchronization
CHO-K1 (Chinese hamster ovary), HeLa, and Indian muntjac (Muntiacus muntjac vaginalis) (IM) cells were cultured in Opti-MEM I (Gibco Laboratories, Grand Island, NY), supplemented with 4% FBS and 1% penicillin/streptomycin. Cultures were maintained in a humidified 37°C incubator with a 5% CO₂ atmosphere. Cell cultures were synchronized in G0 by growing them to confluency in serum-depleted medium, replating to a density of 1×10⁶ cells/28 mm², and incubating in serum-free medium for 66 hours at 37°C, at which time they lacked rounded mitotic cells when examined under the phase contrast microscope (Rao and Engleberg, 1966; Ashihara and Baserga, 1979). The cells were then incubated in serum-containing medium for 4 hours to allow their entry into G1 before adding 2 mM hydroxyurea (HU; Sigma Chemical Co., St. Louis, MO) to inhibit DNA synthesis and arrest cells at the G1/S-phase boundary. Synchronized cultures were released after 16 to 20 hours of block by washing twice with PBS (0.14 M NaCl, 2.5 mM KCl, 8 mM Na₂HPO₄, 1.6 mM KH₂PO₄, pH 7.4) at 37°C and adding fresh medium with serum. Replicating DNA was labeled with the thymidine analog 5-bromo-2¢-deoxyuridine (BrdU; Sigma Chemical Co.), added to the medium of cell cultures to a final concentration of 20 µM. Incorporation was detected using mouse monoclonal anti-BrdU antibody with nuclease (Amersham Life Science, Inc., Piscataway, NJ) and the immunofluorescence procedure given below. PCC was induced in CHO, HeLa, and IM cells with 0.25 to 0.5 µM OA (Gibco BRL, Gaithersburg, MD) for 1.25 to 5 hours. Cells that became detached from the substratum with prolonged treatment were cytospun onto coverslips prior to immunostaining, as described below. Mitotic cell preparations were obtained by exposing asynchronous cultures to the microtubule-depolymerizing drug colcemid (Gibco Laboratories) at 0.1 µg/ml for 4 (HeLa) to 16 (IM) hours. Hypotonic treatment consisted of a 10 minute incubation of cells at 37°C in 75 mM KCl containing multiple protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 µg/ml each of aprotinin, antipain, and pepstatin).

Immunofluorescence microscopy
Cells were grown on poly-amino acid-coated glass coverslips, or, alternatively, mitotic cells were selectively detached by mitotic shake-off, pelleted at 1,000 g for 5 minutes, and cytocentrifuged (1,000 rpm, 2 minutes) onto coverslips using a Cytospin 3 (Shandon Instruments, Inc., Pittsburgh, PA). Cells were extracted with 0.5% Triton X-100 in PEM (80 mM K-Pipes, pH 6.8, 5 mM EGTA, pH 7.0, 2 mM MgCl₂) for 2 minutes at 4°C. Samples were fixed in 4% formaldehyde (Polysciences, Inc., Warrington, PA) in PEM for 20 minutes at 4°C and permeabilized with 0.5% Triton X-100 in PEM for 30 minutes at RT. Non-specific binding of antibodies was blocked overnight at 4°C with 5% milk in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20). Auto-antisera from an individual with CREST (calcinosis, Raynaud’s phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) variant scleroderma (designated SH) was obtained from the Comprehensive Arthritis Center at the University of Alabama at Birmingham and has been characterized previously (Moroi et al., 1980; Valdivia and Brinkley, 1985). CREST, anti-phosphorylated H3 (Upstate Biotechnology, Lake Placid, NY), and anti-acetylated H3 primary antisera were diluted 1:1,000 in TBS-T and incubated on preparations for 1 hour at 37°C. The samples were then rinsed in TBS-T and blocked again in 5% milk. Fluorophore-labeled goat anti-mouse, anti-rabbit, and anti-human IgG (H+L) secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) were diluted in TBS-T and incubated with samples for 45 minutes at 37°C. The preparations were then rinsed in TBS-T, and DNA was counterstained with 0.1 µg/ml 4’,6-diamidino-2-phenylindole (DAPI) in TBS-T. Coverslips were mounted onto glass slides using Vectashield anti-fade medium (Vector Laboratories, Inc., Burlingame, CA). Fits IIJ and J are composite images obtained with a Deltavision, deconvolution-based optical workstation (Applied Precision, Issaquah, WA). Z-series stacks of multiple focal planes were deconvolved by a constrained iterative algorithm, giving rise to high-resolution optical sections used in rendering 3-D volumes. Fig. 8 was acquired using a Molecular Dynamics (Sunnyvale, CA) Multi Probe 2001 laser scanning confocal microscope with a KrAr dual-line laser and digitally processed using Imagespace software. All other images were collected using a Zeiss Axiophot fluorescence microscope with a Hamamatsu high-resolution/high-sensitivity three chip CCD video camera and digitally processed using Adobe Photoshop.

Microinjections
Unmodified H3 peptide was synthesized corresponding to the histone H3 amino-terminal amino acids 1-20 (A1RTKQTAR-KSTGGKAPRKQL20C). Phosphorylated H3 peptide was synthesized such that it contained a single phosphorylated serine residue at position 10 (A1RTKQTARpSTGGKAPRKQL20C). All peptides contained an artificial cysteine residue at position 21 for coupling purposes. Peptides were diluted in injection buffer (20 mM Tris, 50 mM KCl, pH 7.4) with 1:5 biotinylated anti-goat IgG (H+L) included as a marker, detected by avidin-TXRD (Molecular Probes, Inc., Eugene, OR), and injected into the cytoplasm using a gas-driven microinjector (Eppendorf). Results from repeated measurements were statistically analyzed using Student’s t-test. Statistical significance was determined with a probability of less than 0.05.

RESULTS

Late-replicating/early-condensing chromatin is the first to undergo H3 phosphorylation during G2
Late-replicating chromatin condenses maximally during G2 and early prophase, followed by the condensation of early-replicating chromatin through late metaphase (Kuroiwa, 1971; Francke and Oliver, 1978; Unis, 1980; Drouin et al., 1991). To determine if late-replicating/early-condensing chromatin is the first to undergo histone H3 phosphorylation, cells synchronized in late S-phase of the cell cycle were labeled by BrdU incorporation and in the subsequent G2 phase with an antisera specific for the phosphorylated amino terminus (Ser10) of H3 (Hendzel et al., 1997). CHO cultures become enriched for cells in late S-phase approximately 5 hours after release from HU-block at the G1/S boundary (O’Keefe et al., 1992). At this time, replicating DNA was labeled with BrdU for 6 hours, approximately the time it takes CHO cells to reach late G2 from late S-phase. Immunofluorescence revealed extensive colocalization of anti-BrdU with anti-phosphorylated H3 (anti-PH3) antibodies, indicating that H3 is phosphorylated primarily in late-replicating chromatin during G2 (Fig. 1A-D).
Colocalization was not observed between BrdU incorporated during the first 5 hours of S-phase, immediately following release from HU-block, and anti-PH3 staining 6 hours later in the subsequent G2 (Fig. 1E-H). To additionally determine the specificity of BrdU labeling in the above experiments, we labeled late replicating chromatin and harvested the cells in mitosis, 12 hours after release from HU-block. Sister chromatids were observed to have identical banding patterns, indicative of the semi-conservative incorporation of BrdU during late S-phase (Fig. 1I,J).

H3 phosphorylation initiates in pericentric heterochromatin when premature chromosome condensation is induced

The centromere is a principal site for the initiation of chromosome condensation in mammalian cells (He and Brinkley, 1996). H3 phosphorylation concurrently increases in pericentric heterochromatin during G2 and advances outward into the chromosome arms (Hendzel et al., 1997). As shown in Fig. 2A, chromatic regions surrounding prekinetochores are the exclusive sites of H3 phosphorylation during G2 in IM cells, since the number of initiation sites exactly matches the diploid chromosome number (2N=7δ). The centromeres-prekinetochores appear as doublets, stained with CREST auto-antiserum, indicating that the cell has reached G2 (Brenner et al., 1981). To determine if the initiation of H3 phosphorylation follows the same pattern when PCC is induced, asynchronous cell cultures were treated with OA and immunostained with CREST and anti-PH3 antisera. Treatment of mammalian cells with OA, which is a specific inhibitor of the serine/threonine protein phosphatases 1 and 2A, results in PCC and a rapid and stable increase in cellular phosphorylation levels, including that of histone H3 Ser10 (Bialojan and Takai, 1988; Haystead et al., 1989; Cohen et al., 1990; Mahadevan et al., 1991; Ajiro et al., 1996). When PCC is induced in IM cells with 0.25 μM OA, the G2 pattern of pericentric H3 phosphorylation is observed in most cells within 90 minutes (Fig. 2B). The prekinetochores, however, appear as open arrays of CREST-stained subunits, indicating that they have not reached late S-phase/early G2 and are not fully replicated (Brenner et al., 1981; He and Brinkley, 1996). H3 is similarly phosphorylated in pericentric heterochromatin when PCC is induced in CHO and HeLa cells with OA (data not shown). We also examined H3 phosphorylation in cells that were induced to enter mitosis prematurely from the G1/S boundary by caffeine treatment (Schlegel and Pardee, 1986; Brinkley et al., 1988). Prior to entering mitosis, these cells pass through a G2-like state, in which H3 phosphorylation accumulates primarily in pericentric heterochromatin (data not shown).
H3 phosphorylation is not sufficient for chromosome condensation

The effects of OA treatment vary among cell type and cell cycle stage. Exposure of most mammalian interphase cells to the drug induces a mitosis-like state, characterized by PCC, increased cdc2/H1 kinase activity, dispersion of nuclear lamins, and the appearance of mitotic asters (Haystead et al., 1989; Picard et al., 1989; Rime and Ozen, 1990; Yamashita et al., 1990; Zheng et al., 1991). Morphologically normal mitotic chromosome condensation with OA treatment, however, is only observed in a small fraction of unsynchronized cells and has been most clearly demonstrated using mouse cells arrested at G2 (Guo et al., 1995; Ajiro et al., 1996). Similarly, OA-treatment of mitotic cells results in the overcondensation of chromosomes, as well as disruption of the mitotic spindle and metaphase plate formation (Ghosh et al., 1992; Gliksman et al., 1992; Vandré and Wills, 1992). To correlate the extent of H3 phosphorylation with OA-induced PCC, we exposed asynchronous cell populations to 0.5 μM OA for 2.5 hours. Under these conditions, over 70% of HeLa and IM cells appeared rounded up with compact nuclei characteristic of S-phase PCC. The nuclei of these cells displayed high levels of H3 phosphorylation throughout the chromatin (Fig. 3A,B), particularly at the nuclear periphery, resembling prophase cells in untreated cultures (Hendzel et al., 1997). Rudimentary chromosome organization developed in less than 10% of the unsynchronized cells treated under these conditions, indicating that H3 phosphorylation throughout interphase chromatin is not sufficient for mitotic chromosome condensation. The number of cells demonstrating mitotic chromosome organization was reduced to less than 1% when cultures were arrested in early S-phase prior to treatment with OA for 2.5 to 5 hours (Fig. 3C-F). CHO cells synchronized in early S-phase demonstrated PCC and high levels of H3 phosphorylation only after 5 hours of treatment with 0.5 μM OA (data not shown). Similarly, mitotic chromosome morphology was observed in less than 1% of the cells.

In the absence of OA treatment, H3 phosphorylation was undetectable during early interphase in HeLa cells (Fig. 3G,H). Untreated CHO and IM cells, however, display nuclear foci of anti-PH3 immunoreactivity during early interphase (Fig. 4). The small size and number of these nuclear foci make them distinct from the pericentric pattern of phosphorylation observed during G2. To determine the temporal and spatial occurrences of these foci in interphase nuclei, discrete phases of DNA replication were visualized by pulse-labeling with BrdU at progressive time points through S-phase (O’Keefe et al., 1992). Thirty-two per cent of CHO cells blocked at the G1/S boundary had anti-PH3 foci. This number increased upon release and reached a maximum in late S-phase, when the number of cells with foci was near 70%. The foci vary in number from 1 to 5 at each stage and therefore do not appear to be sequentially replicated. They do not correspond exclusively to early-replicating (Fig. 4A-C) or late-replicating regions of chromatin (Fig. 4D-F). All anti-PH3 immunoreactivity is abolished when the antisem is incubated with peptides corresponding to the phosphorylated form of the H3 amino-terminal tail (Hendzel et al., 1997, and data not shown).

Activity of the kinase that phosphorylates H3 is required for entry into mitosis

The kinase responsible for histone H3 phosphorylation in vivo has not been conclusively identified. It has been shown in a cell-free system that cyclic AMP-dependent protein kinase can phosphorylate H3 on Ser10 (Taylor, 1982; Shibata et al., 1990). Similarly, H3 phosphorylation is linked with elevated cAMP levels during gliotoxin-induced apoptosis (Waring et al., 1997). When H3 phosphorylation is inhibited by the protein kinase inhibitor staurosporine, mammalian cells arrest in late G2 (Crisman et al., 1991; Abe et al., 1991; Th'ng et al., 1994). However, staurosporine is known to inhibit a wide range of kinases, including p34cdc2, which controls mitotic entry (Langan et al., 1989; Herbert et al., 1990; Gadbois et al., 1992). To more directly define the role of the kinase that phosphorylates H3 in cell cycle progression, we microinjected

![Fig. 3. H3 phosphorylation is not sufficient for chromosome condensation.](image)
proliferating cells with peptide corresponding to the unmodified amino-terminal 20 amino acids of H3 to compete for the kinase activity. CHO cells were injected at mid-S-phase, 4 to 5 hours after release from HU-block at the G1/S boundary. Control cells were injected with the phosphorylated (Ser10) form of H3 peptide or with marker only (biotinylated anti-goat IgG). Injected cultures were harvested at a time-point when uninjected cultures became enriched for cells passing or just having passed through mitosis, 12 to 13 hours after release from HU-block. All preparations were masked, and the number of mitotic cells, identified by DAPI staining, was scored at least three times. Increasing concentrations of the unmodified H3 peptide resulted in proportional decreases in mitotic indexes (Fig. 5A). The number of mitotic cells was nearly abolished with the highest concentration of unmodified H3 peptide used. Injection of phosphorylated H3 peptide or marker alone did not significantly reduce the number of cells passing through mitosis when compared to uninjected cells within the same population (Fig. 5A).

To characterize the phenotype of cells inhibited with the H3 competitor peptide, as well as those progressing through mitosis despite injection, we repeated the injections with an intermediate concentration (0.42 μg/μl) of unmodified H3 peptide, empirically determined in the previous experiment. Use of an intermediate dose also reduced the possibility that we were observing effects of peptide toxicity. The number of mitotic, daughter, and binucleate cells was scored morphologically (Fig. 5B). Control preparations were injected with approximately five-times the concentration (2.26 μg/μl) of H3 peptide in the phosphorylated form or with marker only. A statistically significant reduction in the number of mitotic and daughter cells was observed in preparations injected with an intermediate dose of the unmodified H3 peptide when compared to populations injected with the phosphorylated form of H3 peptide or with marker alone (Fig. 5C). When cell populations injected with H3 peptide are normalized against populations injected with marker only, we observed a 38% reduction in the number of mitotic cells and a 36% reduction in the number of daughter cells with an intermediate dose of competitor peptide (Fig. 5D). The number of cells with the focal anti-PH3 staining pattern characteristic of late G2 was not significantly reduced, indicating with the above data that arrest occurred just prior to mitosis. A unique pattern of anti-PH3 staining was observed in 5.5% of the interphase cells injected with unmodified H3 peptide, characterized by high levels of diffuse, predominately nuclear anti-PH3 antibody reactivity in the absence of chromosome condensation (Fig. 5E-G). The percentage of cells with this anti-PH3 staining pattern (5.5%) nearly equals the sum of differences between unmodified H3 and marker only populations in percentages of G2 (1.7-1.3%=0.4%), mitotic (3.9-2.4%=1.5%), and daughter cells (9.0-5.7%=3.3%). Anti-PH3 staining was diffuse throughout the cytoplasm and nucleus of cells injected with the phosphorylated form of H3 peptide, with remarkably high nuclear staining in 1.3% of the cells (data not shown). Significantly, all mitotic cells identified by DAPI staining had H3 highly phosphorylated despite competitive inhibition (Fig. 5E-G), further suggesting that the phosphorylation of H3 is essential for entry into mitosis.

H3 phosphorylation is not required for the structural maintenance of isolated chromosomes

Individual metaphase chromosomes can be microscopically identified and examined in much greater detail by incubating cells in water or mild hypotonic solution (75 mM KCl) for 10 minutes (Hsu, 1952). We have found that, though most chromosomes are swollen in hypotonic solution without a detectable alteration in their pattern of anti-PH3 staining, a significant number of cells (up to 40% in IM preparations and 20% in HeLa) have chromosomes in which H3 appears completely unphosphorylated (Fig. 6A,B). No difference in the level of condensation between phosphorylated and unphosphorylated chromosomes within the same preparation is observable by DAPI staining, indicating that H3 phosphorylation is not required for maintaining high levels of chromosome condensation. To determine if the lack of anti-PH3 antibody reactivity with hypotonic treatment was due to alterations in nucleosome structure, we immunostained the preparations with an antibody that reacts with the diacetylated (9, 14) H3 tail throughout the mammalian cell cycle (Boggs et al., 1996). The pattern of anti-diacetylated H3 immunostaining was unaltered in all hypotonic-treated cells, indicating that the Ser10 epitope was likely still accessible and the distribution of H3 unchanged (Fig. 6C,D). All cells arrested in mitosis for prolonged periods (overnight) with colcemid had chromosomes in which H3 was highly phosphorylated, indicating that H3 dephosphorylation did not occur with prolonged mitotic arrest or in the absence of hypotonic treatment (data not shown).

Pericentric heterochromatin is resistant to hypotonic-induced H3 dephosphorylation

Intermediate levels of H3 dephosphorylation were observed in a number (less than 10%) of hypotonically treated IM and HeLa mitotic cells. In each case, pericentric regions of chromatin remained highly phosphorylated, whereas the chromosomal arms were completely dephosphorylated. This retention of H3 phosphorylation is easily observed in the large compound centromeres-kinetochores of IM chromosomes (Fig. 7) that have been stretched by cytocentrifugation following brief hypotonic treatment (Zinkowski et al., 1991). The region of phosphorylated chromatin spans the pairing domain of the centromere-kinetochore complex, where sister chromatids are joined at metaphase, and extends into the chromosomal arms, corresponding to the span of heterochromatin in these chromosomes (Brinkley et al., 1992). Both phosphorylated and dephosphorylated centromeres remain constricted and can be highly elongated, suggesting that H3 phosphorylation is not required for maintaining high levels of chromosome condensation in pericentric heterochromatin and that stretching occurred by a mechanism other than H3 dephosphorylation. Interestingly, the region of chromatin immediately subjacent to the CREST-stained centromere-kinetochore lacks anti-PH3 staining (Fig. 7), similar to results obtained by immunogold EM (Hendzel et al., 1997).

H3 phosphorylation correlates with cell cycle progression after hypotonic treatment

Hypotonic swelling of mammalian cells leads to the loss of kinetochore plate morphology, as well as the disruption of
microtubules, centrioles, and centrosomes (Brinkley et al., 1980; Ris and Witt, 1981). However, if the period of hypotonic swelling is brief (under 15 minutes), the damage can be reversed by returning the cells to an isotonic tissue culture environment (Brinkley et al., 1980). Under these conditions, cell viability is not adversely affected in most cell lines. To determine if H3 phosphorylation is required for progression through mitosis, we exposed mitotic cells to hypotonic conditions, resulting in the dephosphorylation of H3, then released them into tissue culture medium and followed their progress by immunostaining. Congression of IM and HeLa chromosomes to the metaphase plate and entry into anaphase began within 60 minutes of their return to tissue culture medium from hypotonic. Every cell passing through early anaphase had H3 highly phosphorylated, though the chromosomes retained a swollen appearance (Fig. 8). No intermediate stages of H3 phosphorylation were observed after 30 minutes of release. If hypotonic-treated cells were returned to tissue culture medium containing colcemid, every mitotic cell was phosphorylated within 30 minutes. H3 phosphorylation, then, is correlated with cell viability and progression through mitosis following hypotonic treatment.

DISCUSSION

The nucleosomal histones are wrapped by DNA as octamers, consisting of two H2A-H2B dimers and a tetramer of 2 H3 and 2 H4 histones (D’Anna and Isenberg, 1974; Moss et al., 1976; Kornberg, 1977). H3 and H4 amino-terminal tails are exposed from the compact core of the nucleosome and may be involved in regulatory interactions with DNA, histone H1, and other proteins (Glotov et al., 1978; Hardison et al., 1977; Mazen et al., 1987; Roth and Allis, 1992). Interactions of the H3 tail may be modulated by post-translational modifications, including the acetylation of lysine residues 9, 14, 23, and 27, methylation of lysines 9 and 27, and serines 10 and 28 may be phosphorylated (Dixon et al., 1975; Taylor, 1982; Shibata et al., 1990; Bradbury, 1992). The residues involved in the above interactions are largely invariable (Wells and McBride, 1989) and reflect a very high universality in the mechanisms by which chromatin is modeled. The anti-PH3 antiserum used in these studies reacts

Fig. 4. Foci of phosphorylated H3 form in CHO nuclei during early interphase. G1(A) and G2 (not shown) phases of the cell cycle were distinguished by a 5-hour BrdU-labeling of DNA replication. The cells were then immunostained with anti-PH3 (red) and anti-BrdU (green) antibodies. DNA was counterstained with DAPI (blue). To visualize discrete phases of DNA replication, cells were arrested at the G1/S boundary with HU, released for 0.5 (B), 2 (C), 5 (D), 7 (E), or 9 hours (F) and pulse-labeled for 20 minutes with BrdU prior to immunostaining. Bars, 5 μm.

Fig. 5. Delayed entry into mitosis with the competitive inhibition of the kinase that phosphorylates H3. (A) Synchronized CHO cells were injected at late S-phase with increasing concentrations of peptide corresponding to the unmodified (•) or phosphorylated (○) H3 amino terminus. The mitotic index of each injected population (~125 cells each) was divided by the mitotic index of uninjected cells on the same coverslip to derive normalized values on the y-axis. (B) Injected populations were stained for coinjected marker antibody, and the number of mitotic (filled arrow), recently divided daughter (arrowhead), and binucleate cells (open arrow) was scored. G2 cells were scored by their characteristic, focal pattern of anti-PH3 immunostaining (see Figs 1, 2). Bar, 50 μm. (C) Cells were injected with marker only (n=4), with 0.42 μg/μl unmodified H3 competitor peptide (n=6), or with 2.26 μg/μl of the phosphorylated form of the H3 peptide (n=2), where n is the number of separate experiments consisting of ~200 injected cells each. (D) Values from C were normalized by dividing the number of cells injected with unmodified (black bars) or phosphorylated (hatched bars) H3 peptide by the number of cells injected with marker only (white bars) at the same stage within the same preparation. Injected cells were immunostained for coinjected marker antibody (E), anti-PH3 antibody (F), and DAPI (G). All mitotic chromosomes had H3 phosphorylated (arrow). A number of cells injected with unmodified H3 peptide had high levels of diffuse, predominately nuclear anti-PH3 staining in the absence of chromosome condensation (arrowhead). Bar, 25 μm.
with the N-terminal phosphorylated form of Ser/Thr 10 in all eukaryotes examined, staining with the highest intensity during mitotic and, in *Tetrahymena*, meiotic chromosome condensation (Hendzel et al., 1997; Wei et al., 1998). Unphosphorylated chromosomes appeared condensed to the same degree as phosphorylated chromosomes within the same preparation, as resolved by DAPI staining (asterisks). Bar, 50 μm. (C) Anti-diacylated H3 immunoreactivity (red) was observed in all hypotonically swollen IM chromosomes (m). Interphase cells (i) stain more intensely with the antibody, and low levels of cytoplasmic staining are observed regardless of hypotonic treatment. Bar, 25 μm. (D) A higher magnification view of hypotonically-swollen IM chromosomes immunostained with anti-diacylated H3 antibody (red). Bar, 10 μm.

Fig. 6. H3 phosphorylation is not required for the maintenance of condensed chromosomes. (A,B) IM cells were arrested in mitosis and incubated in hypotonic solution. Preparations were immunostained with anti-PH3 antibody (red), and DNA was counterstained with DAPI (blue). Unphosphorylated chromosomes appeared condensed to the same degree as phosphorylated chromosomes within the same preparation, as resolved by DAPI staining (asterisks). Bar, 50 μm. (C) Anti-diacylated H3 immunoreactivity (red) was observed in all hypotonically swollen IM chromosomes (m). Interphase cells (i) stain more intensely with the antibody, and low levels of cytoplasmic staining are observed regardless of hypotonic treatment. Bar, 25 μm. (D) A higher magnification view of hypotonically-swollen IM chromosomes immunostained with anti-diacylated H3 antibody (red). Bar, 10 μm.

Heterochromatic regions of chromosomes, including centromeres and telomeres, remain highly condensed throughout the cell cycle and likely contain elements that direct chromosome condensation (Koshland and Strunnikov, 1996). The centromere is a principal site for the initiation of chromosome condensation at the onset of mitosis in mammalian cells (He and Brinkley, 1996). Consistent with a role in the initiation of mammalian cell chromosome condensation, H3 phosphorylation dramatically increases during G2 exclusively in the late-replicating/early-condensing heterochromatin surrounding centromeres. The same pattern of H3 phosphorylation is observed when PCC is induced by the phosphatase inhibitor OA and in cells induced to enter mitosis prematurely. These results provide additional evidence of the tight coupling between H3 phosphorylation and the initiation of chromosome condensation near centromeres-prekinetochores. H3 appears highly phosphorylated throughout the chromatid of OA-treated cells, but mitotic chromosome morphology does not develop. This result indicates that H3 phosphorylation is not sufficient for chromosome condensation.

Though chromatin-associated H3 provides a significantly better substrate, free H3 monomers are phosphorylated in vitro by cyclic AMP-dependent protein kinase (Belyavsky et al., 1980; Shibata et al., 1990). We therefore expected injected H3 amino-terminal peptides containing the unmodified Ser10 epitope to compete with, but not necessarily abolish chromatin-associated H3 phosphorylation. This competition would likely become less significant with the onset of mitosis and increased kinase activity and/or chromatin-associated substrate accessibility. Injection of unmodified H3 peptide into cells at mid-S-phase resulted in a marked reduction of cells entering mitosis when compared to cells injected with the phosphorylated form of H3 peptide or with marker only. Though reduced in number, all injected cells passing through mitosis had H3 highly phosphorylated, suggesting that the modification is essential for entry into mitosis. Since injection of H3 tails in the phosphorylated form did not result in the same reduction of cells entering mitosis, competition for histone

Fig. 7. Pericentric heterochromatin is resistant to hypotonic-induced H3 dephosphorylation. IM cells were arrested in mitosis and incubated in hypotonic solution prior to immunostaining with anti-PH3 (A) and CREST antisera (B). DNA was counterstained with DAPI (C). (D) Intermediate levels of H3 phosphorylation were observed in some cells in which pericentric heterochromatin remained highly phosphorylated, whereas chromosome arms were dephosphorylated. Both regions remained highly condensed. Bar, 5 μm.

Fig. 8. Cells do not pass from metaphase to anaphase with H3 dephosphorylated. (A) Cells arrested in mitosis were incubated briefly in hypotonic solution to induce the dephosphorylation of H3 and released into tissue culture medium. The cells were collected 60 minutes after release and immunostained with anti-PH3 antibody (red) and CREST auto-antiserum (green). All cells entering anaphase after hypotonic treatment had H3 phosphorylated throughout their chromosomes, though they retained a swollen appearance relative to untreated preparations (B). Bar, 10 μm.
acetylase activity did not result in cell cycle arrest under the conditions of our experiment. It remains possible that the injected N-terminal peptides were not suitable substrates for acetylation; or, interestingly, phosphorylated H3 tails are not substrates for acetylation.

Concomitant with a reduction in the number of cells entering mitosis in populations injected with unmodified H3 peptide, there was an equivalent accumulation of interphase cells with high levels of anti-PH3 reactivity diffuse throughout uncondensed nuclei. We postulate that this fraction of cells represents the cell cycle stage at which H3 kinase activity is elevated, resulting in the phosphorylation of competitor peptides in the nucleus. Together with the fact that the number of cells in late G2 was not significantly reduced in injected populations, this suggests that the competitive inhibition of H3 phosphorylation prevented chromosome condensation and entry into mitosis. Our conclusions support earlier studies, in which the protein kinase inhibitor staurosporine was found to block H3 phosphorylation and arrest mammalian cells in late G2 (Crisman et al., 1991; Abe et al., 1991; Th'ng et al., 1994). However, it remains possible that inhibition of the kinase that phosphorylates H3 prevented the phosphorylation of other targets essential for entry into mitosis.

Collectively, the above evidence implicates a role for H3 phosphorylation in the initiation of chromosome condensation and entry into mitosis. This modification, however, may not have a critical role in the maintenance of chromosome condensation, as chromosomes can be hypotonically swollen with H3 remaining phosphorylated, and the dephosphorylation of H3 does not result in decondensation. It is important to note that hypotonic treatment results in extensive physiological disruption of cells, albeit reversible in most cases. The primary conclusion that can be drawn from the above data, then, is that H3 phosphorylation is not required for maintaining the condensation of isolated chromosomes. However, this result also confirms the in vivo observations of Rao and colleagues, who found that the fusion of mammalian mitotic cells with interphase cells results in a large reduction of H1 and H3 phosphorylations without a loss of chromosome condensation (Johnson and Rao, 1970; Hanks et al., 1983). It also suggests that the decondensation of metaphase chromosomes observed following treatment with the protein kinase inhibitors staurosporine (Th’ng et al., 1994) and N-6 dimethylaminopurine (Ajiji et al., 1996) may not have been produced exclusively by H3 dephosphorylation. This is easily conceived, as staurosporine is a potent inhibitor of all kinases tested in vitro, though its action in vivo may be narrower (Herbert et al., 1990; Th’ng et al., 1994); and N-6 dimethylaminopurine is a non-specific kinase inhibitor in vitro, capable of affecting multiple phases of the cell cycle (Vesely et al., 1994; Simili et al., 1997).

Though hypotonic treatment resulted in a large number of cells with dephosphorylated chromosomal H3, when these cells were returned to an isotonic tissue culture environment, they were not observed to enter anaphase with H3 dephosphorylated. It is not structural inhibition that prevents cells with dephosphorylated chromosomes from progressing through mitosis, as these chromosomes appear as compact as those with H3 phosphorylated that undergo anaphase within the same preparation. It is both possible that hypotonically-treated cells rephosphorylate chromosomal H3 upon their return to an isotonic environment, or simply that cells with dephosphorylated chromosomes are not viable. Suggestive of the latter, IM cells are particularly susceptible to H3 dephosphorylation and recover poorly from hypotonic treatment relative to other mammalian cell lines (B.R.B., unpublished observations).

Intermediate levels of H3 phosphorylation were observed in hypotonic-treated chromosomes of IM and HeLa cells, in which pericentric heterochromatin remained phosphorylated and chromosome arms were dephosphorylated. Since pericentric heterochromatin is more resistant to hypotonic swelling than other chromosomal regions (Brinkley et al., 1980), H3 phosphorylation may have a role in maintaining the highest levels of chromosome condensation. Similarly, we found that the chromosomes of cells arrested in mitosis with colcemid were more resistant to hypotonic-induced dephosphorylation of H3 than those of unarrested mitotic cells, perhaps related to the varying degrees of hypercondensation observed in colcemid treated preparations (see Rieder and Palazzo, 1992). H3 phosphorylation, then, correlates with the most condensed regions of chromatin during hypotonic treatment, though its removal does not result in decondensation.

The periodicity of interaction between DNA and nuclear matrix proteins appears to hold interphase chromatin in loops of approximately 60 kb, which may be folded to form the ~200 nm wide chromatin fiber observed by electron microscopy (Marsden and Laemmli, 1979; Rattner and Lin, 1985; Fey et al., 1986; Nelson et al., 1986). Euchromatin is compacted from a ~200 nm diameter interphase fiber to a 700 nm metaphase structure. H3 phosphorylation correlates best with the initial events of condensation: specifically, compaction of the ~200 nm chromatid fiber (Hendzel et al., 1997). However, heterochromatin remains at an approximately equivalent level of compaction (700 nm) throughout the cell cycle (reviewed by Manuelidis and Chen, 1990). The fact that metaphase chromosomes can have H3 hypotonically dephosphorylated without a cytological loss of chromosome condensation is consistent with the fact that heterochromatin is dephosphorylated at the end of mitosis, yet maintains a high level of compaction. Why then is heterochromatin phosphorylated? One possibility is that H3 phosphorylation in pericentric heterochromatin may be required for the release of sister chromatid cohesion at the metaphase to anaphase transition, a process interrelated with chromosome condensation (Guacci et al., 1997). Additionally H3 phosphorylation may be required throughout early mitosis for the initiation of chromosome condensation, which continues until late metaphase (Adlakha and Rao, 1986; Drouin et al., 1991).

Correlating morphological chromatin data with the quantification of histone phosphorylations led to the suggestion that H3 phosphorylation occurs during the final organization and maintenance of chromosomes (Gurley et al., 1978). More recently, however, the phosphorylation of H3 has been observed to precede detectable chromosome condensation and its dephosphorylation to precede detectable chromosome decondensation (Hendzel et al., 1997). Additionally, H3 phosphorylation may be associated with the relatively relaxed state of transcriptionally active chromatin coincident with the mitogenic induction of early-response genes, as well as make chromatin accessible to nucleases involved in apoptotic pathways (Halegoua and Patrick, 1980; Mahadevan et al., 1988, 1991; Barratt et al., 1994; Waring et al., 1997). An attractive hypothesis that reconciles these apparently divergent properties is that the phosphorylation of histone N-terminal tails reduces their affinity...
for DNA and facilitates the movement of nucleosomes and targeting of factors involved in condensation, transcription, and apoptosis (Roth and Allis, 1992; Hendzel et al., 1997; Waring et al., 1997). In this way, H3 phosphorylation would work in conjunction with other mechanisms involved in the modeling of active chromatin, including the phosphorylation and depletion of histone H1 and the enrichment of hyperacetylated histones H3 and H4 (Allegra et al., 1987; Kamakaka and Thomas, 1990; Roth and Allis, 1992; Mizzen and Allis, 1998). Our data supports this role for histone phosphorylation in chromatin modeling, in that the phosphorylation of H3 appears to be required for dynamic changes in chromatin structure, but not for their maintenance.

The molecular mechanisms by which heterochromatin is kept refractory from the dynamic changes of other chromosomal regions remain largely unknown, as do the mechanisms by which centromeres-prekinetochores direct the initiation of chromosome condensation in mammalian cells during G2, and why. Seemingly disparate events coalesce at the nuclear envelope. H3 phosphorylation initiates with chromosome condensation in pericentric heterochromatin, often observed to be at the nuclear periphery (Comings and Okada, 1970; Robbins et al., 1970). Similarly, chromosome condensation initiates in foci on the nuclear envelope during the early embryonic divisions of Drosophila nuclei, though these regions are distinct from centromeres and telomeres (Hiraoka et al., 1989). The highest levels of H3 phosphorylation in mammalian prophase nuclei are consistently observed at the periphery of the nucleus. Just as the nuclear lamina is phosphorylated and disassembles, kinetochores plates first appear. In this way, nuclear structures are central components of the signaling cascades leading to mitosis.

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