

Mitotic phosphorylation of histone H3 at threonine 3

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Abstract Nuclear envelope-peripheral heterochromatin fractions contain multiple histone kinase activities. *In vitro* assays and amino-terminal sequencing show that one of these activities co-isolates with heterochromatin protein 1 (HP1) and phosphorylates histone H3 at threonine 3. Antibodies recognizing this post-translational modification reveal that *in vivo* phosphorylation at threonine 3 commences at early prophase in the vicinity of the nuclear envelope, spreads to pericentromeric chromatin during prometaphase and is fully reversed by late anaphase. This spatio-temporal pattern is distinct from H3 phosphorylation at serine 10, which also occurs during cell division, suggesting segregation of differentially phosphorylated chromatin to different regions of mitotic chromosomes.

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1. Introduction

A significant proportion of heterochromatin is located at the periphery of the cell nucleus, in close proximity to the nuclear envelope (NE) [1]. This spatial association, which reflects a multiplicity of interactions between integral proteins of the inner nuclear membrane and components of chromatin (e.g. heterochromatin protein 1 (HP1), barrier to autointegration factor, etc.; see [2,3]), could be interpreted in two different ways. First, peripheral heterochromatin may possess specific epigenetic modifications that dictate NE association (reviewed in [4,5]). Alternatively, domains of non-condensed chromatin could be subjected to 'heterochromatinization' when brought to the vicinity of the NE [6].

NE–chromatin interactions are also relevant to mitotic events. At prophase, condensing chromosomes come into intimate contact with the nuclear surface. Interestingly, the condensation process is not completed until the NE is ruptured and the chromosomes are 'stripped' of membrane remnants by mitotically activated microtubule-based motors [7,8].

Both heterochromatin formation and chromosome condensation involve histone modifications and recruitment of specific regulatory factors [9–12]. One of the most thoroughly analyzed modifications is the phosphorylation of histone H3. The H3 molecule is phosphorylated at four different sites, serines 10/28 and threonines 3/11, all located at its N-terminal tail. Serine 10 phosphorylation during interphase is catalyzed by the RSK-2 and MSK-1 kinases that are induced by mitogenic and stress signals, respectively [13–16]. Mitotic phosphorylation at serines 10 and 28 is mediated by Aurora B kinase ([17–20]; reviewed in [16]), which has close homologues in yeast (Ipl1/AIR-2) and *Aspergillus nidulans* (NIMA). Threonine 11 phosphorylation also occurs during mitosis and involves the Dlk/ZIP kinase, a member of the DAP family [21]. In addition to H3, this enzyme modifies histones H4, H2A and the myosin light chain *in vitro*. Finally, H3 phosphorylation at threonine 3 is mediated by a non-characterized kinase [22,23].

Using NE-associated, peripheral heterochromatin fractions as a source, we have found that the threonine 3-specific kinase activity co-isolates with HP1 and mediates mitotic modification of histone H3 in a spatio-temporal pattern that is distinct from that of serine 10 phosphorylation.

2. Materials and methods

2.1. Antibodies

The anti-M31 rat monoclonal antibody MAC 353 has been described in [24]. Anti-phospho-histone H3 (Ser10) rabbit polyclonal and mouse monoclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA).

2.2. Cell fractionation

NE vesicles were prepared from turkey erythrocyte nuclei, essentially as specified in [25,26] with slight modifications and further purified in flotation gradients (70–50–30–20% sucrose cushions; 100 000×g, 18 h, 4°C), collecting the 50–30% interface. Extracts were prepared by thorough resuspension of NE vesicles or sonicated 'nuclear ghosts' (NGs) in 300–600 mM NaCl, 20 mM Tris–HCl pH 7.5, 260 mM sucrose, 2 mM MgCl₂, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitors, plus or minus 1% Triton X-100 (buffers S and ST, respectively). After ultracentrifugation (200–350 000×g, 30 min, 4°C), the soluble extracts (SE and STE, respectively) were collected and either used immediately, or further fractionated in 5–20% sucrose density gradients (100 000×g, 18 h, 4°C).

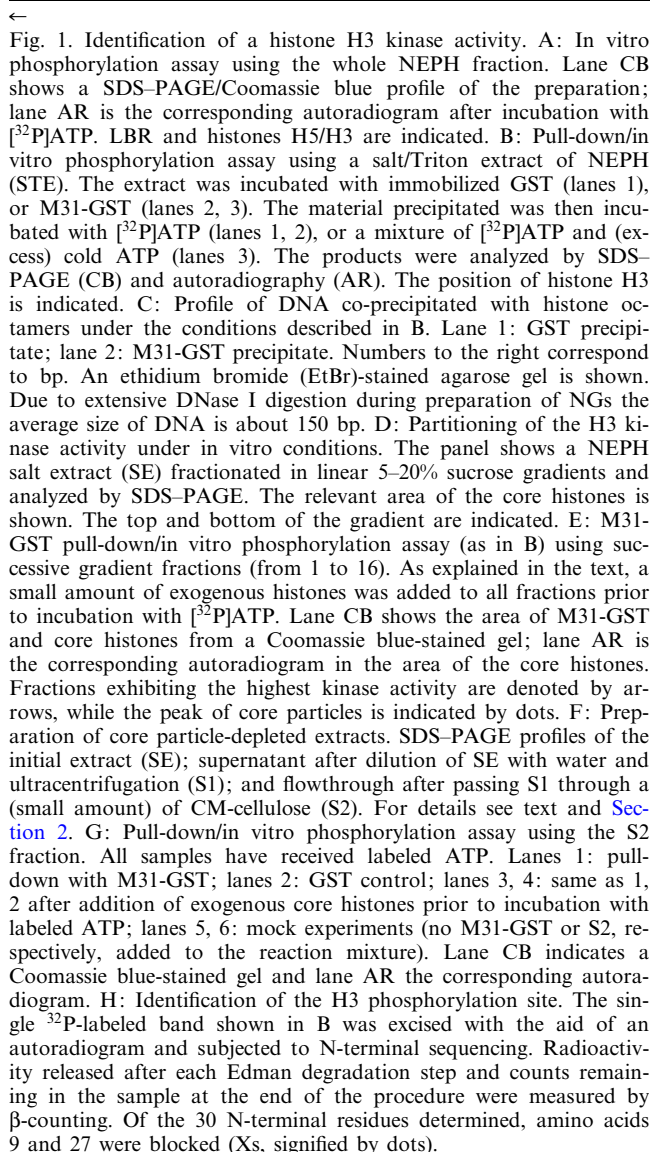
2.3. Microscopy

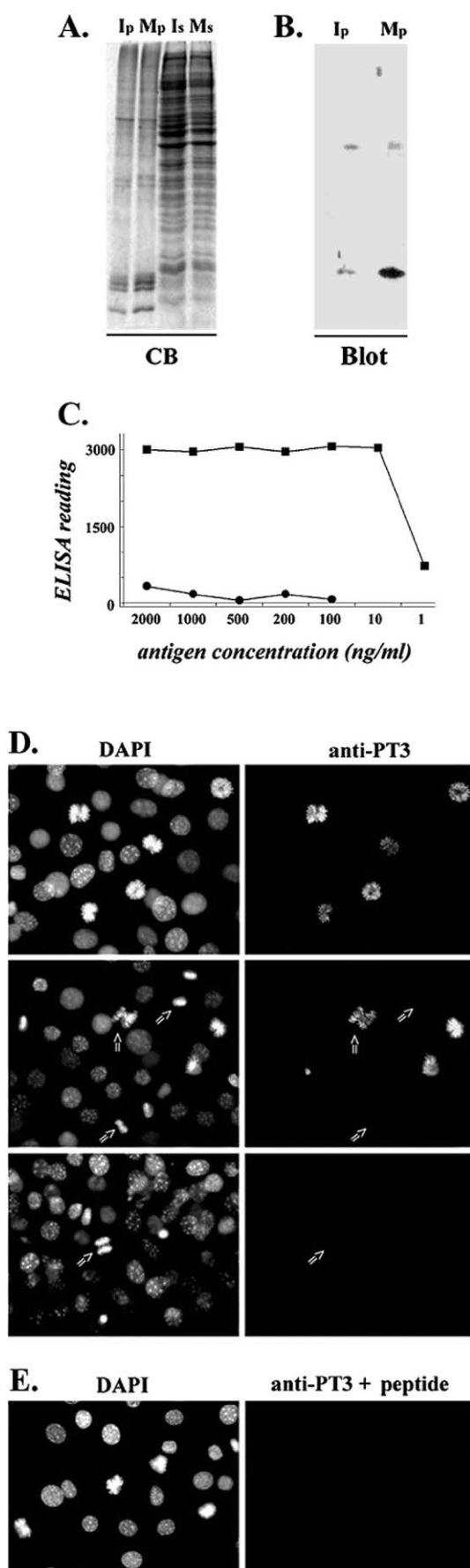
For light microscopy, samples were fixed with 1% formaldehyde in phosphate-buffered saline, permeabilized with 0.2% Triton X-100 and blocked with 1% fish skin gelatin according to [27].

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Abbreviations: NEPH, nuclear envelope-peripheral heterochromatin fractions; HP1, heterochromatin protein 1; NE, nuclear envelopes; NG, nuclear ghosts; SE, salt extract; STE, salt/Triton extract





When NEPHs were incubated with [32 P]ATP, three polypeptides with the electrophoretic mobility of histone H5, histone H3 and the lamin B receptor (LBR) were reproducibly phosphorylated (Fig. 1A). Phosphorylation of LBR is known to be catalyzed by a serine-arginine (SRPK1) kinase [30,31]; however, the phosphorylation of histones H5 and H3 could be attributed to a variety of cAMP-dependent and cAMP-independent protein kinases present in nucleated erythrocytes [32].

To enrich for histone H3-specific kinases, we took advantage of the fact that histone H3 binds to HP1 and associated enzymes (e.g. Suvar3, 9) under stringent *in vitro* conditions [33–35]. To this end, NEPHs were extracted with detergent/high salt and the soluble extracts (STE; Fig. 1B) used for pull-down experiments (immunoprecipitation with anti-HP1 antibodies was not considered because avian erythrocytes possess relatively small amounts of endogenous HP1) [36,37]. Data depicted in Fig. 1B,C show that recombinant mouse HP1 β (M31-GST) co-precipitated core particles and a kinase activity (T3K) that apparently phosphorylated histone H3.

To distinguish whether T3K was associated with M31 or nucleosomes, we fractionated salt extracts of NEPHs (SEs; no Triton X-100 included in the buffer to avoid co-extraction of integral membrane proteins such as LBR and associated kinases) in sucrose gradients (Fig. 1D). The gradient fractions were combined with immobilized M31-GST and the complexes formed incubated with [32 P]ATP. To compensate for the fact that the top fractions of the gradient did not contain visible quantities of core histones, a small amount of exogenous octamers was added to all samples at the beginning of the incubation. Results presented in Fig. 1E show clearly that fractions containing nucleosomes did not possess T3K activity.

To examine the alternative possibility, i.e. that T3K associates with M31, we performed pull-down experiments using nucleosome-depleted extracts. To remove chromatin particles, the original SE extract was diluted with water (to lower the NaCl concentration) and ultracentrifuged. In pilot experiments we have found that this 'salt shift' renders the bulk of the core particles pelletable, without affecting other constituents of the preparation. This partially depleted extract (S1 fraction shown in Fig. 1F) was passed through a CM-cellulose column to remove traces of histones (both core and linker; for details see Section 2). Finally, the CM-cellulose flowthrough (S2), adjusted again at high salt, was incubated with M31-GST and used for *in vitro* phosphorylation assays. As shown in Fig. 1G, no labeling of histone H3 was detected when the

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Fig. 2. Characterization of the anti-phosphothreonine 3 antibodies. A,B: Western blot analysis. Ip, Mp: insoluble pellets after homogenization of interphase and nocodazole-arrested C127 cells, respectively; Is, Ms: the corresponding supernatants. CB and Blot show a Coomassie blue-stained gel and the corresponding Western blot. C: ELISA. Different amounts of the antigenic peptide (closed squares) or recombinant H3 (closed circles) were probed by rabbit anti-phosphothreonine 3 antibodies. The OD₄₉₅ values as a function of the antigen concentration are shown in the graph. D,E: Indirect immunofluorescence assay using the anti-phosphothreonine 3 antibodies (anti-PT3), or a mixture of the antibodies and the antigenic peptide, as indicated. The specimens were counterstained with DAPI and photographed at low power in a conventional fluorescence microscope. Arrows point to anaphase or telophase cells. Mitotic figures from prophase to early anaphase are positive, while late anaphase, telophase and interphase cells are negative.

material precipitated from depleted extracts was incubated with [32 P]ATP, or when the experiment was performed in a mock fashion (omitting S2 or M31-GST). However, robust H3 phosphorylation was observed if exogenous core histones were combined with the M31-GST precipitate before addition of [32 P]ATP. Taken together with the previous results, these data demonstrate that T3K associates specifically with M31 under *in vitro* conditions.

Determination of the histone H3 phosphorylation site was accomplished by N-terminal sequencing. The bulk (ca. 90%) of radioactivity was released in the first 10 Edman degradation steps and 32 P peaked at amino acid 3 (a threonine; Fig. 1H). No other peaks in the N-terminal sequence, or the C-terminal segment of histone H3 were detected, but we did notice ‘trailing’ of 32 P in residues 4 and 5 (a lysine and a glutamine, respectively) in both microsequencing attempts we made. This seems to be a common shortcoming of the technique (see also [38–40]) and can be explained by non-specific sticking of cleaved material to adjacent residues. Interestingly, the N-terminal sequence of histone H3 was ‘blocked’ in two positions (Fig. 1G, residues denoted by Xs). This is in line with mass spectrometry and immunohistochemical data which indicate that NEPH-associated chromatin, being largely heterochromatic, is extensively methylated at lysines 9 and 27 (our unpublished observations; see also [37]).

To examine the *in vivo* significance of H3 phosphorylation, we raised polyclonal antibodies using as an antigen a synthetic histone H3 peptide (15-mer) phosphorylated at threonine 3. In Western blots the antibodies reacted strongly with mitotically modified histone H3, but gave only a background signal with interphase H3 (Fig. 2A,B). ELISAs confirmed that the antibodies were of high affinity and did not react with recombinant (i.e. unmodified) histone H3 (Fig. 2C).

Indirect immunofluorescence showed that threonine 3-specific phosphorylation occurs only during the early phases of mitosis (Fig. 2D,E). The distribution of threonine 3-phosphorylated histone H3 was very similar to that reported for threonine 11-phosphorylated H3 [21]. However, examination of mitotic cells by confocal microscopy revealed a significant difference between serine 10- and threonine 3-specific H3 phosphorylation. During late anaphase and telophase no staining with anti-phosphothreonine 3 antibodies could be discerned, while phosphoserine 10-histone H3 was clearly detectable at the periphery of chromosome packages and reforming nuclei (Fig. 3A,B, anaphase and telophase). Furthermore, although both modifications appeared to commence at areas of prophase chromosomes neighboring with the NE (Fig. 3A,B, prophase), upon progression of mitosis the two signals became distinct: threonine 3-phosphorylated H3 was more concentrated in the central region of the metaphase plate and extended to chromosome arms, while serine 10-phosphorylated H3 was more prominent in the periphery of the metaphase plate (Fig. 3A,B, prometaphase and metaphase).

Double labeling with anti-phosphoserine 10 and phosphothreonine 3 antibodies was not feasible for technical reasons. Nonetheless, double immunofluorescence with anti-phosphoH3 and CREST antibodies showed that threonine 3- and serine 10-specific phosphorylation did not involve centromeric foci (Fig. 4B, panels prophase). The rim-fluorescence pattern of serine 10 phosphorylation in prophase cells was much more uniform than the pattern of threonine 3 phosphorylation (Fig.

4A,B, prophase; see also Fig. 3). Upon progression of mitosis, threonine 3 phosphorylation spread to the chromosome arms and pericentromeric chromatin, whereas serine 10 phosphorylation was focused at the distal segments of the chromosomes and was much less in other points (Fig. 4B, metaphase).

Consistent with previous observations [17], double labeling

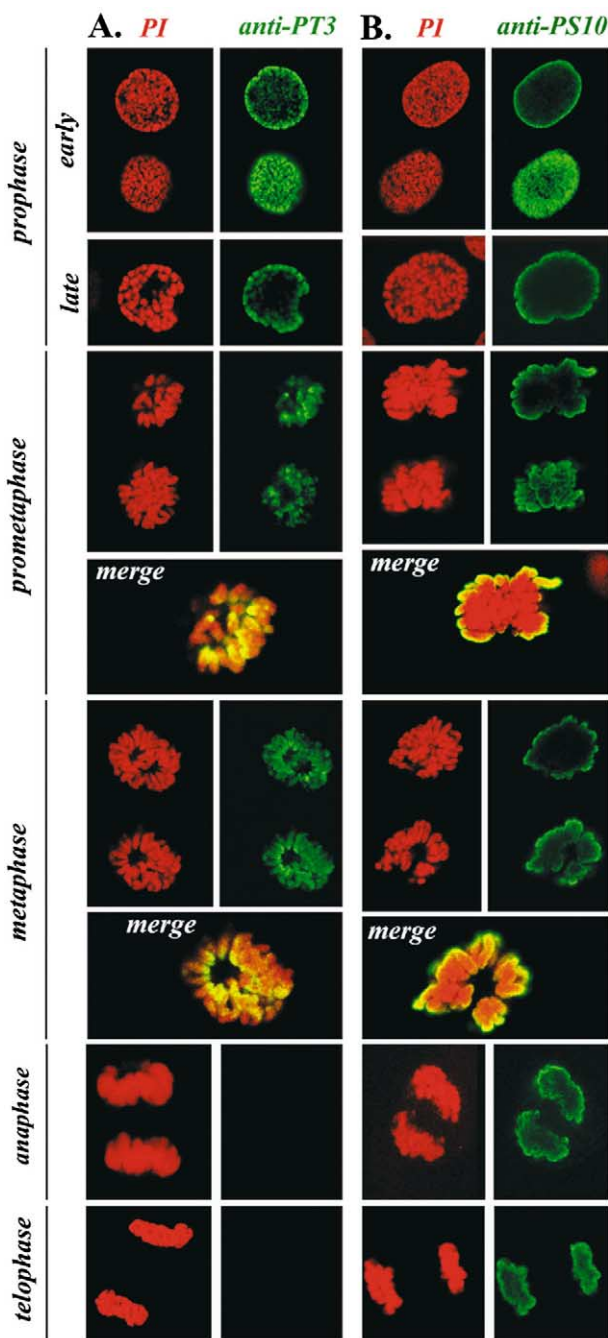


Fig. 3. Comparison of threonine 3- and serine 10-specific phosphorylation during mitosis. A: Immunostaining of C127 cells with anti-phosphothreonine 3-specific antibodies (anti-PT3). B: Immunostaining of C127 cells with anti-phosphoserine 10-specific antibodies (anti-PS10). The phases of mitosis are indicated on the left. For early prophase, prometaphase and metaphase two different confocal sections (one equatorial and one more tangential) are shown. The specimens were counterstained with propidium iodide (PI). For clarity, merged images have been slightly magnified in relation to individual sections.

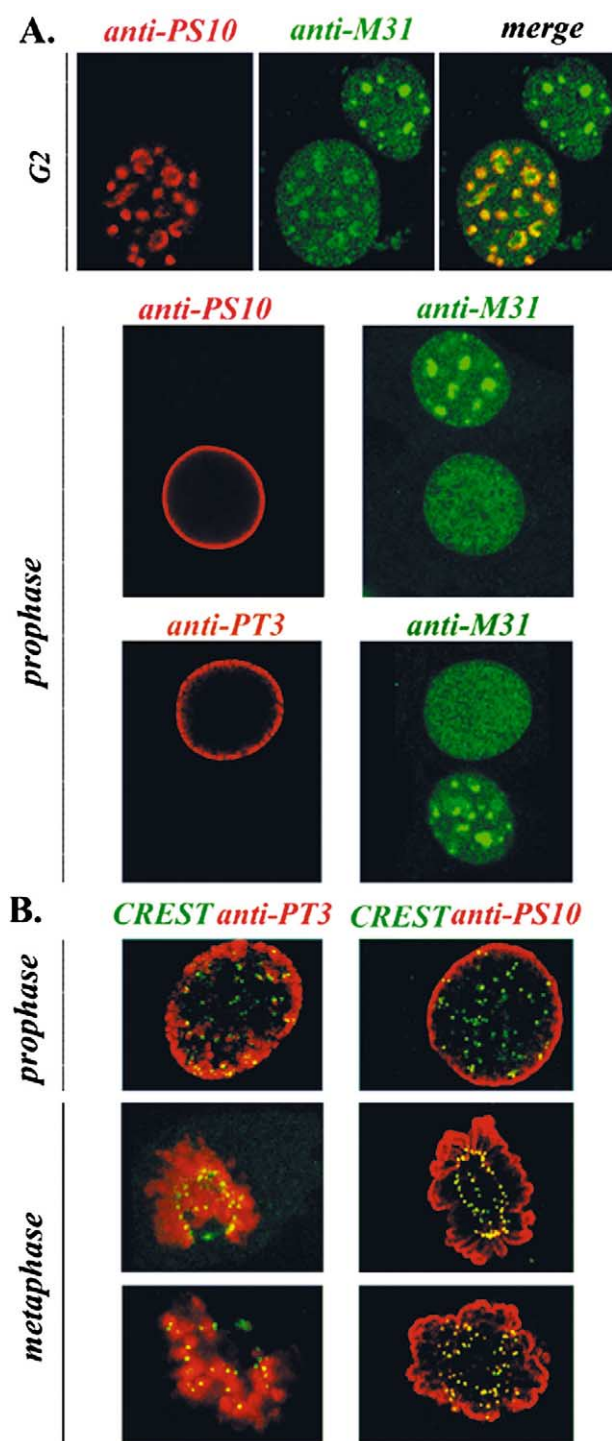


Fig. 4. Double labeling of interphase and mitotic cells with different antibodies. A: Staining of G2 and prophase C127 cells by anti-phosphoserine 10/anti-phosphothreonine 3 and anti-M31 antibodies (anti-PS10, anti-PT3, anti-M31, respectively), as detected by confocal microscopy. Interphase cells (including those in G2 phase) were not decorated by anti-phosphothreonine 3 antibodies and are not shown here (see Fig. 2D). B: Staining of prophase and metaphase cells by anti-phosphoserine 10/anti-phosphothreonine 3 and CREST antibodies, as indicated.

with anti-M31 and anti-phospho H3 antibodies showed that serine 10 phosphorylation begins in the G2 phase around heterochromatic foci containing M31 (Fig. 4A, G2). At this point, a fluorescence signal with anti-phosphothreonine 3 anti-

bodies could not be discerned (see also Fig. 2D). Finally, in prophase cells, threonine 3- and serine 10-phosphorylated H3 were distributed differently from M31, which exhibited a diffuse nucleoplasmic fluorescence (Fig. 4A, prophase). From this it can be concluded that M31 no longer associates with any of the two phosphorylated histone H3 species during mitosis.

A protein kinase that phosphorylates threonine 3 of histone H3 in vitro has been previously identified in bovine thymus nuclei by Shoemaker and Chalkley [22,23]. Using more refined methods and material we have extended these findings, demonstrating that threonine 3 phosphorylation occurs in vivo. Due to the scarcity of material we have not been able to purify T3K and study directly its distribution. However, from the currently available data there are strong indications that this enzyme is tightly associated with peripheral structures of the cell nucleus: (1) threonine 3 phosphorylation during early prophase clearly initiates in the neighborhood of the NE; (2) the H3 kinase activity can be detected in highly purified NEPH preparations; (3) T3K associates with HP1, a heterochromatin-specific protein known to interact with the NE [41–43].

The patterns of threonine 3 and threonine 11 phosphorylation clearly differ from that of serine 10 phosphorylation ([21] and this report). Interestingly, all three modifications commence at chromosomal regions attaching to the NE during prophase, but 'focus' quickly on different territories. This would suggest that 'waves' of phosphorylation and dephosphorylation sweep over the chromosomes at early phases of mitosis and create zones containing different forms of phosphorylated H3. The alternative explanation, i.e. that anti-phosphoserine and anti-threonine antibodies do not have access to the same territories of metaphase chromosomes (because the corresponding epitopes are differentially masked) is not as likely: a variety of experiments that we have performed using different sample preparation and fixation conditions confirm that threonine 3 and serine 10 phosphorylated histone H3 species are always distributed in different areas of mitotic chromosomes (data not shown).

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