Identification of a Novel Phosphorylation Site on Histone H3 Coupled with Mitotic Chromosome Condensation*

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Histone H3 (H3) phosphorylation at Ser10 occurs during mitosis in eukaryotes and was recently shown to play an important role in chromosome condensation in Tetrahymena. When producing monoclonal antibodies that recognize glial fibrillary acidic protein phosphorylation at Thr7, we obtained some monoclonal antibodies that cross-reacted with early mitotic chromosomes. They reacted with 15-kDa phosphoprotein specifically in mitotic cell lysate. With microsequencing, this phosphoprotein was proved to be H3. Mutational analysis revealed that they recognized H3 Ser28 phosphorylation. Then we produced a monoclonal antibody, HTA28, using a phosphopeptide corresponding to phosphorylated H3 Ser28. This antibody specifically recognized the phosphorylation of H3 Ser28 but not that of glial fibrillary acidic protein Thr7. Immunocytochemical studies with HTA28 revealed that Ser28 phosphorylation occurred in chromosomes predominantly during early mitosis and coincided with the initiation of mitotic chromosome condensation. Biochemical analyses using 32P-labeled mitotic cells also confirmed that H3 is phosphorylated at Ser28 during early mitosis. In addition, we found that H3 is phosphorylated at Ser28 as well as Ser10 when premature chromosome condensation was induced in tsBN2 cells. These observations suggest that H3 phosphorylation at Ser28, together with Ser10, is a conserved event and is likely to be involved in mitotic chromosome condensation.

During G2 interphase to M phase, the relaxed interphase chromatin is converted into mitotic condensed chromosomes, a process considered to be essential for the following nuclear division to correctly separate parental genetic information into two daughter cells. However, little is known of mechanisms regulating the packing of DNA into mitotic condensed chromosomes (for a review, see Ref. 1).

Histones are major protein constituents of chromatin in eu-
chromosome condensation. Thus, they proposed that other factor(s) or other H3 posttranslational modification(s), including phosphorylation at other site(s), may also play important roles in chromosome condensation (30).

In the present study, we have identified Ser28 as the mitotic H3 phosphorylation site, using immunological and biochemical approaches. H3 Ser28 phosphorylation coincided with mitotic chromosome condensation in several types of cultured cells. In addition, this phosphorylation was also observed when PCC was induced in early S phase-synchronized tsBN2 baby hamster kidney cells.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Production of Antibodies—**GFAP peptides (PG7 (CERRRVpT7SAARR), G7 (CERRRVTPS16)S5AARRS), PG8 (CRRRVT5pS6AARRS), PG13 (CSAARR3pS27YVSSLS), and PG34 (CPGGLRLPS19LARMp)) and histone H3.1 peptides (PH28 (CKAARKSKAPATGGV), H28 (CKAARKSKAPATGGV), PH10 (CQTRARKSGTTGGKAPR), and H10 (CQTRARKSGTTGGKAPR)) (where pT and pS represent phosphothreonine and phosphoserine, respectively) were chemically synthesized by Peptide Institute Inc. (Osaka, Japan). Each rat monoclonal antibody TM-G7 or TA22, which reacted with H3.1, was produced with H28 but not with H28 and PH10, was produced as described (31). Rabbit polyclonal antibody aPH10, which reacted with PH10 but not with H10 and PH28, was produced as described (32) but with slight modifications. aPH10 was purified from immunized rabbit sera by three-step chromatography: affinity chromatography on PH10-coupled Cellulofine (Seikagaku Corp.) and then absorption both in H10-coupled Cellulofine and in PH28-coupled Cellulofine. Immunoblotting was performed using horseradish peroxidase-conjugated secondary antibodies and the ECL Western blotting detection system (Amersham Pharmacia Biotech).

**Cell Culture—**U251 human glioma cells, HeLa human cervical carcinoma cells, NIH 3T3 mouse fibroblastic cells, Madin-Darby bovine kidney cells, baboon hamster kidney (BHK) cells, and tsBN2 cells (a temperature-sensitive mutant of BHK cells; Ref. 33) were grown in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum (Gibco) and then absorption both in H10-coupled Cellulofine and in PH28-coupled Cellulofine. Immunoblotting was performed using horseradish peroxidase-conjugated secondary antibodies and the ECL Western blotting detection system (Amersham Pharmacia Biotech).

**Immunofluorescence Microscopy—**Cells growing on glass coverslips were fixed with 3.7% formaldehyde in ice-cold PBS for 10 min and then treated with methanol at −20 °C for 10 min. Incubation with primary antibodies diluted in PBS containing 1% sucrose and 1% bovine serum albumin was for 1 h at 37 °C. After washing with PBS, cells were incubated for 1 h with appropriate secondary antibodies diluted 1:100 and subsequently washed with PBS. Then DNA's were stained with 0.5 μg/ml propidium iodide (Sigma) or 0.5 μg/ml 4,6-diamidino-2-phenylindole (Roche Molecular Biochemicals) for 10 min at room temperature. The following antibodies were used for indirect immunofluorescence microscopy: TM-G7 or TA22 (anti-GFAP) rabbit monoclonal antibody diluted 1:20; HTA28 (anti-PG7) rabbit monoclonal antibody diluted 1:20; aPH10 (anti-PH10) rabbit polyclonal antibody diluted 1:100; FITC-conjugated goat anti-rabbit IgG (BIOSOURCE, Camarillo, CA); and Texas Red-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech).

Fluorescently labeled cells were examined, using an Olympus BH2-RFCA microscope or an Olympus LSM-G200 confocal microscope.

**Preparation of Interphase or Early Mitotic Cells—**Just before cells reached confluence, the cells were arrested in early mitosis by adding 50 μM nocodazole for 12 h. Early mitotic cells were collected by mechanical shake off, and the adherent cells served as interphase cells. Cell lysates were prepared as described above. Aliquots of cells were labeled with [32P]orthophosphate at 40 μCi/ml for at least 12 h at 4 °C. Cells were treated with 10% trichloroacetic acid and then collected. Cell pellets were partially lysed with the solution (80 mm NaCl, 20 mm EDTA, and 1% Triton X-100) in the presence or absence of 100 μM okad acid. Total histones were extracted three times with 0.4 N sulfuric acid for 10 min and precipitated with 4 volumes of ethanol as described (24).

**Purification of Histone H3 from Total Histones—**The 3P-labeled total histones described above were fractionated by reverse-phase HPLC (Waters) in a gradient of acetonitrile (40–60%) containing 0.1% trifluoroacetic acid (36) or an RP-8 column (Beckman). H3 fractions were collected and lyophilized.

**Phosphopeptide Mapping—**The 3P-labeled H3 histones was digested with 2% trypsin (Roche Molecular Biochemicals) in 0.1 M ammonium persulfate. The resulting peptides were subjected to two-dimensional peptide mapping, as described (17). Phosphopeptides of H3 histones were identified, using an image analyzer (BAS 2000, Fujix).

**RESULTS**

**One Type of Rat Monoclonal Antibodies Raised against a Phosphopeptide Corresponding to GFAP Phosphorylation at Thr7 Cross-reacts with Early Mitotic Chromosomes—**We earlier reported that GFAP is phosphorylated at Thr7, Ser13, and Ser34 by Rho-associated kinase (Rho-kinase) at cleavage furrow during cytokinesis (37–39). We developed a site- and phosphorylation state-specific monoclonal antibody for Thr7 on GFAP, using synthetic phosphopeptide PG7 (CERRRVTPS16)S5AARRS; also see Fig. 3A) as an antigen for immunization. Then we obtained two types of rat monoclonal antibodies that recognize GFAP phosphorylated at Thr7 but neither unphosphorylated GFAP nor GFAP phosphorylated at other sites including Ser6, Ser13, and Ser14 (Fig. 1, A and B). One type (referred to as TM-G7) specifically reacted with GFAP at the cleavage furrow during cytokinesis (late mitosis) in U251 glioma cells (Fig. 1C). Surprisingly, the immunoreactivity of the other type (referred to as TA22) was observed not only at the cleavage furrow during late mitosis but also at chromosomes during early mitosis in glioma cells (Fig. 1C). Since GFAP is an intermediate filament protein expressed specifically in the cytoplasm of astroglial cells (40–42), TA22 may recognize the antigen(s) other than GFAP during early mitosis.

**TA22 Recognizes the Phosphorylation of Histone H3 in the Early Mitotic Cell Lysate but Not in the Interphase Cell Lysate—**To identify the TA22-reacted protein in the chromosome during early mitotic phase, Western blot analysis of U251 cell lysates was carried out. As shown in Fig. 2A, TA22 immunoreacted with about 15-kDa protein in the early mitotic cell lysate but not in the interphase cell lysate. This TA22 immunoreactivity for the mitotic 15-kDa protein disappeared after treatment of the transferred membrane with the λ protein phospha-
tase (λ-PPase; a dual specificity phosphatase), suggesting that TA22 recognized the phosphoepitope in the 15kDa protein (Fig. 2B). To clarify the molecular identity, we determined three peptide sequences derived from this immunoreacted protein: STELLIR, RVIT, and DIQLARRIRGER (Fig. 2C). They were found within the sequence of the human histone H3.1 or H3.3 (for a review, see Ref. 43). Since histone H3 is one of core histones wrapped by DNA as octamers in eukaryotic nuclei (2–4), TA22 may recognize H3 phosphorylation in the chromatin assembly process. Histones H3.1 and H3.3 represent the sequence of the human histone H3.1 and H3.3, respectively. D and E, recombinant His6-tagged histone H3.3 (wild type; WT) was phosphorylated by A kinase or Cdc2 kinase with or without [γ-32P]ATP in vitro as described under “Experimental Procedures.” Each mutant histone H3.3 in which Ser10, Ser28, or Thr118 was changed to Ala (S10A, S28A, or T118A) was phosphorylated by A kinase as described above. Radiolabeled bands were visualized using autoradiography (32P). After SDS-PAGE, nonradioactive samples were stained with Coomassie Brilliant Blue or transferred onto a PVDF membrane. The membrane was immunoblotted with the antibody TA22 (dilution 1:200). B, after early mitotic U251 cell lysates were resolved by SDS-PAGE, the gel was transferred onto a PVDF membrane as described above. The membrane was preincubated with 1× λ protein phosphatase buffer (50 mM Tris-Cl (pH 7.5), 0.1 mM Na2EDTA, 5 mM dithiothreitol, 0.01% Brij 35, 2 mM MnCl2) in the absence (+ buffer) or the presence (+ λ-PPase) of 100 μg/ml A protein phosphatase (New England Biolabs Inc.) for 1 h at 30 °C. Each treated membrane was immunoblotted with the antibody TA22 (dilution 1:200) and then stained with Coomassie Brilliant Blue. The arrowheads indicate the position of the TA22-immunoreactive band. C, confocal micrographs of U251 glioma cells stained with TM-G7 or TA22 (green). DNAs were stained with propidium iodide (red). Images represent projections of Z-series scans. Bar, 10 μm.

FIG. 1. Immunoreactivities of rat monoclonal anti-PG7 antibodies. A, reactivity of TM-G7 or TA22 to unphosphorylated GFAP (Control) and GFAP phosphorylated by Rho-associated kinase (Rho-kinase). Recombinant GFAP and GFAP phosphorylated by Rho-kinase was prepared as described (38). After SDS-PAGE (100 ng in each lane), samples were transferred onto a polyvinylidene difluoride filter (PVDF) membrane. The membrane was immunoblotted with the antibody TM-G7 or TA22 (dilution 1:200) and then stained with Coomassie Brilliant Blue (CBB). B, specificity of TM-G7 or TA22, determined by a competition assay. GFAP phosphorylated by Rho-kinase was immunoblotted with TM-G7 or TA22 (dilution 1:200) after preincubation with TBS-T. The antibody control, the phosphorylated GFAP was immunoblotted with TM-G7 or TA22 (green). CBB gel was stained with Coomassie Brilliant Blue or transferred onto a PVDF membrane. The membrane was immunoblotted with TA22 (dilution 1:200) after preincubation with TBS-T. The arrowheads indicate the position of GFAP phosphorylated by Rho-kinase. C, confocal micrographs of U251 glioma cells stained with TM-G7 or TA22 (green). DNAs were stained with propidium iodide (red). Images represent projections of Z-series scans. Bar, 10 μm.

FIG. 2. Identification of a TA22-cross-reacted protein during early mitosis. A, interphase (I) or early mitotic (M) U251 cell lysates were prepared as described under “Experimental Procedures.” Lysates of 1×105 cells were loaded on lanes and resolved by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (CBB) or transferred onto a PVDF membrane. The membrane was immunoblotted with TA22 (dilution 1:200). B, after early mitotic U251 cell lysates were resolved by SDS-PAGE, the gel was transferred onto a PVDF membrane as described above. The membrane was preincubated with 1× λ protein phosphatase buffer (50 mM Tris-Cl (pH 7.5), 0.1 mM Na2EDTA, 5 mM dithiothreitol, 0.01% Brij 35, 2 mM MnCl2) in the absence (+ buffer) or the presence (+ λ-PPase) of 100 μg/ml A protein phosphatase (New England Biolabs Inc.) for 1 h at 30 °C. Each treated membrane was immunoblotted with the antibody TA22 (dilution 1:200) and then stained with Coomassie Brilliant Blue. The arrowheads indicate the position of the TA22-immunoreactive band. C, the TA22-immunoreactive band at 15 kDa in the mitotic U251 cell lysate was cut from transferred membrane and digested with lysyl-endopeptidase (57). The resulting peptides were fractionated by C18 column chromatography and subjected to amino acid sequence analysis. Three peptide sequences derived from this 15kDa protein were determined (square; H3.1 and H3.3 represent the sequence of the human histone H3.1 and H3.3, respectively. D and E, recombinant His6-tagged histone H3.3 (wild type; WT) was phosphorylated by A kinase or Cdc2 kinase with or without γ-32P[ATP in vitro as described under “Experimental Procedures.” Each mutant histone H3.3 in which Ser10, Ser28, or Thr118 was changed to Ala (S10A, S28A, or T118A) was phosphorylated by A kinase as described above. Radiolabeled bands were visualized using autoradiography (32P). After SDS-PAGE, nonradioactive samples were stained with Coomassie Brilliant Blue or transferred onto a PVDF membrane. The membrane was immunoblotted with the antibody TA22 (dilution 1:200).
mitotic cell lysate (Fig. 4) concerning H3 Ser28 phosphorylation in cells, because there is the possibility that it might recognize several phosphoproteins, including H3 Ser28 (Fig. 1 and 2), much like monoclonal antibody MPM-2, which can recognize phosphorylated forms of several proteins during mitosis (46). There-fore, with this antibody it is difficult to obtain clear data concerning H3 Ser28 phosphorylation in cells, including H3, in mitotic chromosomes.

To overcome this difficulty, we produced a site- and phosphoprotein specific antibody HTA28 for H3 Ser28. The rat monoclonal antibody TA22 recognized the phosphorylation of at least two proteins including H3 Ser28 (Fig. 1 and 2), much like monoclonal antibody MPM-2, which can recognize phosphorylated forms of several proteins during mitosis (46). Therefore, with this antibody it is difficult to obtain clear data concerning H3 Ser28 phosphorylation in cells, because there is the possibility that it might recognize several phosphoproteins, including H3, in mitotic chromosomes.

Next, we examined mitotic H3 phosphorylation sites, using total histones extracted from mitotic HeLa cells. Total histones were extracted with or without okadaic acid (one of the serine/threonine phosphatase inhibitors) from mitotic HeLa cells. As shown in Fig. 6A, aPH10 immunoreacted with the band at 15 kDa corresponding to the position of H3 in mitotic cell lysate (lane d) and in total histones (lanes e and f). In contrast, although HTA28 immunoreacted with the band at 15 kDa in mitotic cell lysate (lane g), no signal or only a faint signal at 15 kDa was detected in total histones extracted without okadaic acid (lane h). However, the HTA28-immunoreactive band at 15 kDa was observed in total histones extracted with okadaic acid (lane i). Fig. 6B shows tryptic phosphopeptide mapping of H3 histones purified from 32P-labeled total histones by HPLC. Ser10-phosphorylated peptides (arrowheads) were detected, regardless of whether total histones were extracted with (b) or without (a) okadaic acid from 32P-labeled mitotic HeLa cells (Fig. 6B). However, Ser28-phosphorylated peptide (arrow) was detected only when the extraction procedure was performed in the presence of okadaic acid (Fig. 6B). These results indicated that H3 may be dephosphorylated at Ser28 (but not excessively at Ser10) on the extraction procedure of total histones, and our immunological observations that H3 phosphorylation occurs not only at Ser10 but also at Ser28 during mitosis were thus confirmed.

H3 Ser28 Phosphorylation Coincided with Chromosome Condensation—H3 was phosphorylated at Ser28 specifically during early mitosis. This phosphorylation appeared to correlate spatiotemporally with the initiation of the chromosome condensation (Figs. 4 and 5). To further confirm this possibility, we used tsBN2 cells, a temperature-sensitive mutant of BHK cells: these cells exhibit PCC at nonpermissive temperature (33). PCC under this condition mimics normal chromosome condensation, as evidenced by premature activation of Cdc2 kinase and the association of mitosis-specific antigen recognized by a monoclonal antibody MPM-2 with PCC chromosome (48). To determine if H3 phosphorylation occurs at Ser10 and Ser28 accompanied with PCC, tsBN2 and BHK parental cells were synchronized in early S phase by adding aphidicolin at 32.5 °C (permissive temperature). Upon temperature shift up to 41 °C (nonpermissive temperature) for 4 h, PCC was induced in about 10% of tsBN2 cells, but not in any BHK cells (Fig. 7). Upon incubating at 32.5 °C (permissive temperature) for an additional 4 h, no PCC was observed in each cell line (Fig. 7). H3 phosphorylation at Ser10 and Ser28 was observed only in tsBN2 cells where PCC was induced at 41 °C (Fig. 7). Together with data shown in Figs. 4 and 5, these results suggested that H3 phosphorylation at Ser28, as well as Ser10, correlated closely with chromosome condensation.

**DISCUSSION**

A major new finding in this study is that histone H3 is phosphorylated not only at Ser10 but also at Ser28 during mitosis. This H3 phosphorylation at Ser28, together with that at Ser10, correlates with mitotic chromosome condensation in various types of cultured cells and with PCC induced in tsBN2 cells.
H3 was considered to be phosphorylated only at Ser10 during mitosis (9, 25, 28, 29). Why had only Ser10 been identified as the mitosis-specific H3 phosphorylation site? It may be due to the H3 purification procedure from mitotic cells. Most studies determining the *in vivo* H3 phosphorylation site used the total histones extracted from the isolated chromatin in 32P-labeled mitotic cells. By using the method similar to the reported one (9, 25), we could also detect only Ser10 as the mitosis-specific H3 phosphorylation site (Fig. 6). However, both Ser10 and Ser28 were detectable when the extraction of total histones from mitotic cells was done in the presence of phosphatase 1 and 2A inhibitor, okadaic acid (Fig. 6). Thus, H3 may be dephosphorylated at Ser28 (but not excessively at Ser10) upon extracting total histones from mitotic cells. Therefore, Ser28 may be first identified as one of the mitosis-specific phosphorylation sites by using the site- and phosphorylation state-specific antibody.

Identification of a novel H3 phosphorylation site raised the question about the ratio of phosphorylated H3 Ser28 to phosphorylated H3 Ser10 or total H3 histones in early mitotic cells. Biological analyses revealed that the phosphorylation level of H3 Ser28 was lower than that of H3 Ser10 in early mitotic cells (Fig. 6B). However, H3 Ser28 was more sensitive to phosphatase(s) than H3 Ser10 and dephosphorylated to some extent during the purification even in the presence of the phosphatase inhibitor (Fig. 6A, lanes g and i). Thus, we consider that Ser28 phosphorylation also occurs to some extent, although the phosphorylation level of H3 Ser28 may be slightly lower than that of H3 Ser10.

H3 Ser10 phosphorylation correlated closely with chromosome condensation and was required for proper chromosome condensation and segregation (see Introduction). Recently, this phosphorylation was reported to be required for the initiation,
but not maintenance, of mammalian chromosome condensation (47). H3 Ser28 phosphorylation occurred specifically during early mitosis, at least in mammalian cells and correlated closely with mitotic chromosome condensation and PCC induced in tsBN2 cells (Figs. 4–7). While the biological significance of H3 Ser28 phosphorylation remains unknown, these observations raised the possibility that H3 phosphorylation at Ser28, as well as Ser10, may play an important role in chromosome condensation in mammalian cells.

SMC (structural maintenance of chromosomes) proteins are also revealed to play important roles in chromosome condensation; these proteins are probably directly involved in chromosome condensation (for reviews, see Ref. 1 and 49–52). However, the relationship between H3 phosphorylation and SMC proteins remains unknown. Recently, mitotic H3 phosphorylation was reported to promote the disassociation between the H3 amino-terminal tail and DNA (53). Since the H3 amino-terminal tail emerged from the nucleosome at the entry and exist points of DNA (4), the phosphorylation of the H3 amino-terminal tail at Ser10 and Ser28 might reduce their affinity for DNA.

**FIG. 5.** Indirect immunofluorescence of mitotic U251 (A) or HeLa (B) cells stained with HTA28. DNAs were stained with propidium iodide (PI). Bars, 10 μm.

**FIG. 6.** Identification of mitotic H3 phosphorylation sites. A, total histones were extracted with (lanes c, f, and i) or without (lanes b, e, and h) okadaic acid from mitotic HeLa cells as described under “Experimental Procedures.” Mitotic HeLa cell lysates (12.5 μg of protein/lane; lanes a, d, and g) and total histones (2 μg of protein/lane) were loaded on lanes and resolved by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (CBB) or transferred onto a PVDF membrane. The membrane was immunoblotted with the antibody αPH10 (dilution 1:10,000) and HTA28 (dilution 1:200). B, total histones were extracted with (b) or without (a) okadaic acid from 32P-labeled mitotic HeLa cells. Then H3 histones were separated from total histones by reverse-phase HPLC and subjected to tryptic phosphopeptide mapping, as described under "Experimental Procedures" (horizontal dimension, electrophoresis with butanol/acetic acid/water/pyridine (50:25:900:25), pH 4.7) at 550 V for 50 min (vertical dimension, ascending chromatography in butanol/acetic acid/water/pyridine (48.8:15.2:60.4:75.6)). The bottom left corner shows the spot origin. The arrowheads and an arrow indicate the 32P spot identified as peptides containing Ser10 and Ser28 residue, respectively, in an earlier study (45).

**FIG. 7.** Indirect double immunofluorescence of S phase-synchronized BHK or tsBN2 baby hamster kidney cells stained with αPH10 and HTA28. BHK or tsBN2 cells (a temperature-sensitive mutant of BHK cells; Ref. 33) were grown at 32.5 °C (permissive temperature). Just before cells reached confluence, BHK cells and tsBN2 cells were arrested in early S-phase by the addition of 15 μM aphidicolin at 32.5 °C (permissive temperature) for 18 h. Then, cells were cultured at 32.5 °C (permissive temperature) or 41 °C (nonpermissive temperature) for an additional 4 h. DNAs were stained with 4',6-diamidino-2-phenylindole (DAPI).
and facilitate the targeting of condensing factors including SMC proteins.

H3 Ser$^{10}$ phosphorylation also occurred in some interphase nuclei where H3 Ser$^{28}$ phosphorylation was not observed (Fig. 4, arrows). Other groups reported similar observations that H3 Ser$^{10}$ phosphorylation initiated during late S phase or G2 phase in mammalian cells (28, 47). In contrast, H3 Ser$^{28}$ phosphorylation appeared to be initiated at the onset of mitosis, prophase (Fig. 4 and 5). These observations raise the question about mechanisms regulating the phosphorylation at these two sites on H3. We consider two possibilities. 1) One possibility is the existence of at least two H3 kinase activities in a cell cycle-dependent manner. Protein kinase(s) activated during S or G2 interphase might phosphorylate H3 at Ser$^{10}$ but not at Ser$^{28}$. Since Ser$^{10}$ and Ser$^{28}$ on H3 were phosphorylated in tsBN2 cells where Cdc2 kinase was prematurely activated (Fig. 7), other H3 kinase(s) might phosphorylate H3 at both Ser$^{10}$ and Ser$^{28}$ after the activation of Cdc2 kinase. This mitotic H3 kinase might not be Cdc2 kinase, because H3 Ser$^{28}$ was not phosphorylated by Cdc2 kinase in vitro (Fig. 2D). 2) The second possibility is a different site specificity of H3 phosphatase(s). Since H3 was dephosphorylated exclusively at Ser$^{28}$ during the extraction procedure of total histones (Fig. 6), Ser$^{28}$ might be more sensitive to phosphatase(s) than Ser$^{10}$ even in cells. Thus, during S or G2 interphase, the Ser$^{28}$ phosphorylation level might be too low to detect, although both Ser$^{10}$ and Ser$^{28}$ could be phosphorylated by H3 kinase activity. Since phosphatase 1 was reported to be phosphorylated and inactivated by Cdc2 kinase during mitosis (54–56), the phosphorylation level at Ser$^{28}$ as well as Ser$^{10}$ might be elevated by inactivation of phosphatase(s) during mitosis.

In conclusion, we first found that H3 Ser$^{28}$ phosphorylation occurred specifically during early mitosis and coincided with chromosome condensation. Further analyses of protein kinase(s) and phosphatase(s) regulating H3 phosphorylation level at Ser$^{10}$ and Ser$^{28}$ will help to elucidate the molecular mechanism of some mitotic events including chromosome condensation.

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