Histone H3 Tails Containing Dimethylated Lysine and Adjacent Phosphorylated Serine Modifications Adopt a Specific Conformation during Mitosis and Meiosis^{∇†}

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Condensation of chromatin, mediated in part by posttranslational modifications of histones, is essential for cell division during mitosis. Histone H3 tails are dimethylated on lysine (Kme2) and become phosphorylated on serine (Sp) residues during mitosis. We have explored the possibility that these double modifications are involved in the establishment of H3 tail conformations during the cell cycle. Here we describe a specific chromatin conformation occurring at Kme2 and adjacently phosphorylated S of H3 tails upon formation of a hydrogen bond. This conformation appears exclusively between early prophase and early anaphase of the mitosis, when chromatin condensation is highest. Moreover, we observed that the conformed H3Kme2Sp tail is present at the diplotene and metaphase stages in spermatocytes and oocytes. Our data together with results obtained by cryoelectron microscopy suggest that the conformation of Kme2Sp-modified H3 tails changes during mitosis and meiosis. This is supported by biostructural modeling of a modified histone H3 tail bound by an antibody, indicating that Kme2Sp-modified H3 tails can adopt at least two different conformations. Thus, the H3K9me2S10p and the H3K27me2S28p sites are involved in the acquisition of specific chromatin conformations during chromatin condensation for cell division.

Chromatin is the physiologically relevant substrate for all genetic processes inside the nuclei of eukaryotic cells. Dynamic changes in the local and global organization of chromatin are regulators of genomic function. To fit in the nucleus, the genomic DNA has to be compacted to form the chromatin. The basic unit of the chromatin is the nucleosome, which is composed of two copies of H2A, H2B, H3, and H4 core histone proteins, which arrange in octamers and around which wraps about 146 bp of DNA (2, 31). Due to the nucleosomes, the DNA reaches a sixfold compaction and forms the 30-nm chromatin fiber. At this step, the chromatin fiber accounts only for 40-fold compaction. The remaining about 500-fold compaction is achieved through mechanisms which are still not well understood (5, 37, 55). The chromatin undergoes various levels of condensation-decondensation during the life of the cell:

chromatin condensation is essential during mitosis and meiosis, whereas chromatin decondensation is necessary for replication, repair, recombination, and transcription.

Histones are important actors in regulating chromatin processes. In fact, their N-terminal tails are the targets of numerous posttranslational modifications. These modifications include acetylation or methylation of lysines (K), methylation of arginines (R), phosphorylation of serines (S) and threonines (T), and ubiquitination of lysines (24, 50, 51). The modifications, depending on their nature, the moment when they appear, and the modified amino acid, could play a role in the condensation or decondensation of the chromatin.

Additionally, posttranslational modifications of the histone tails can influence other modifications (11, 14, 38). Many of the histone modifier enzymes display a high degree of specificity not only toward a particular site but also toward the preexisting modification state of their substrate. So far the amino-terminal tail of H3 has the highest density of posttranslational modifications mapped among histones and thus gives rise to a complex pattern of coexisting or mutually exclusive combinations of marks. Contrary to what was believed before (42), it has been recently shown that the methylation on K9 of histone H3 and the phosphorylation on the neighboring S10 can occur on the same histone tails. Indeed, according to the methyl/phospho binary-switch hypothesis (15), even though trimethylation

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of H3K9 persists during mitosis, the additional transient modification of histone H3 by phosphorylation of S10 is sufficient to eject HP1 (heterochromatin protein 1) proteins from their binding sites (14, 23, 30).

The S10 of histone H3 is a residue conserved across eukaryotes, and it becomes highly phosphorylated in mitotic and meiotic cells (8, 19, 27, 39, 53). Apart from S10, S28 and T11 of histone H3 are phosphorylated during mitosis (18, 40). However, the precise role of these phosphorylation events is still unclear. During G₂ interphase to M phase the chromatin condenses gradually and finally reaches its most compacted state at metaphase to allow the formation of mitotic chromosomes. This step is essential for good sharing of the genetic information during cell division (22). It has been shown that mitotic H3 phosphorylation at S10 plays an important role in chromosome condensation and segregation in *Tetrahymena* (54) and *Xenopus* eggs (12). Moreover, S10 phosphorylation appears to be involved in the initiation of mammalian chromosome condensation (52). During mitosis, S10 phosphorylation seems to be associated with inactive chromatin. However, some degree of S10 phosphorylation has also been found in interphasic cells, where it is implicated in gene activation (4, 10, 13, 25).

The modification of histone tails by themselves may not be sufficient for the complex and fine regulation of chromatin state and the consequent gene expression changes. Although histone tails were often considered to be in a permanently "unstructured" state, a structure of histone tails could also participate in this regulation. This hypothesis would predict that, at least in the case of chromatin condensation, H3S10p ("p" indicates phosphorylation) could act in concert with another modification(s) and alter the structure of the histone tails.

Here we describe a specific chromatin conformation that occurs at dimethylated lysine (Kme2) and adjacently phosphorylated serine (Sp) residues of histone H3 tails. This chromatin conformation occurs exclusively between the early prophase and the early anaphase of the mitosis. Our data suggest that this conformation is different from the primary doubly modified sequence of the histone H3 tail and involves a specific charged structure of the H3K9me2S10p and/or of the H3K27me2S28p doubly modified H3 histone tails. These data together with results obtained by cryoelectron microscopy suggest that the conformation of Kme2Sp-modified histone H3 tails changes during mitosis. Molecular dynamics computer simulations indicate that the K9me2S10p histone H3 tail can adopt a favorable "turn" conformation containing an H bond that would be recognized by the hypervariable region of the monoclonal antibody (MAb) that we developed. Together, our observations suggest that the H3K9me2S10p and H3K27me2S28p sites are involved in the acquisition of a specific chromatin conformation during mitosis.

MATERIALS AND METHODS

Antibody production. To generate the anti-MAb, a peptide corresponding to amino acids 180 to 195 of human TAF10 dimethylated at lysine 189 was synthesized, coupled to ovalbumin as a carrier protein, and used for immunization. Immunization and MAb production were essentially as described previously (7).

Whole-cell extract and histone preparations. Whole-cell extracts were prepared by resuspending cells in 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol,

20% glycerol, 0.4 M or 1 M KCl, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (2.5 µg/ml of leupeptin, pepstatin, chymostatin, aprotinin) and by lysing them by three cycles of freeze-thaw. Cell debris was eliminated by centrifugation.

HeLa cells synchronized or not in G₂/M or treated with phosphatase inhibitors (10 mM β-glycerophosphate, 2 mM orthovanadate, 10 mM sodium fluoride) were used for histone preparations using a hydroxyapatite column as previously described (9).

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blot analysis were as described by Frontini et al. (17). For Western blotting, anti-dimeK9-pS10 polyclonal antibody (33) was used at a dilution of 1:10,000 and anti-H3K9ac, anti-H3K4me3, anti-H3K9me2, and anti-H3K27me3 polyclonal antibodies (PABs; Abcam) and 51TA2H12 MAb were used at a dilution of 1:1,000.

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Material immunoprecipitated using antibody 51TA2H12 was separated on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Protein bands were visualized by Coomassie R250 (Bio-Rad) staining, excised, and in-gel digested with trypsin. Preparation of gel-eluted peptides, mass measurements, and analysis of the data were as described by Robert et al. (43).

ELISA, dot blot analysis, and peptide competition experiments. Standard enzyme-linked immunosorbent assay (ELISA) was performed using 2 µg/ml peptides. Bound antibody was detected with a secondary antibody conjugated to alkaline phosphatase, and the color reaction was developed with *p*-nitrophenylphosphate.

For dot blot analysis, 5 µl of peptides (10 mg/ml) was directly loaded onto a nitrocellulose membrane and dried for 15 min at room temperature (RT). The membrane was then processed for Western blot analysis as described above. Anti-dimeK9-pS10 PAB (33) was used at a dilution of 1:10,000, and anti-S10p and anti-S28p PABs (Upstate), as well as 51TA2H12 MAb, were used at a dilution of 1:1,000.

Competition experiments were performed by incubating antibodies at a final concentration of 20 µg/ml with the different peptides at a final concentration of 5 µg/ml in 20 µl for 45 min at RT before using them for Western blot analysis (data not shown) or confocal imaging.

Immunofluorescence of testis sections and oocytes. Testes were dissected from 8-week-old CD1 mice housed under a 12-h light/dark period. Testes were immediately fixed in 4% paraformaldehyde, processed for embedding in paraffin, and sectioned using standard histological protocols. Immunofluorescence was performed on 5-µm-thick sections. Briefly, tissues were deparaffinized with Citrisolve, rehydrated through three changes of alcohol, and washed in PBS-T (phosphate-buffered saline with 0.05% Triton X). Antigen retrieval was performed by incubation in sodium citrate buffer and heating in the microwave until boiling, followed by cooling for 30 min at RT. Slides were then rinsed in PBS-T, blocked in 3% bovine serum albumin and 10% normal goat serum in PBS-T for 1 hour, and incubated overnight at 4°C with either polyclonal rabbit anti-H3-S10p (1/250), mouse monoclonal 51TA2H12 (1/50), control rabbit immunoglobulin G (IgG), or mouse IgG (Jackson ImmunoResearch Laboratories Inc) with rocking. After a washing, sections were incubated with secondary Alexa 488-coupled anti-mouse or Alexa 594-coupled anti-rabbit antibodies (Invitrogen) for 1 h at RT. Tissues were counterstained with 4',6'-diamidino-2-phenylindole (DAPI). Spermatogenic stages were determined as described by Russell et al. (44).

Oocytes at the germinal vesicle (GV) stage were obtained from 6-week-old CD1 females. To obtain metaphase I oocytes, GV oocytes were allowed to undergo spontaneous maturation by culturing in M16 medium supplemented with 5% fetal calf serum at 37°C under 5% CO₂. Metaphase II oocytes were collected from ampullae of mice superovulated with intraperitoneal injection of pregnant mare serum gonadotropin and human chorionic gonadotropin. After removal of the zona pellucida with acid Tyrode's solution (Sigma), oocytes were washed and fixed as described previously (49). Oocytes were incubated with the 51TA2H12 antibody for ~12 h at 4°C, washed, incubated with Alexa 488-coupled anti-mouse antibody, and mounted in Vectashield (Vector Laboratories). Confocal microscopy was performed using a 63× oil objective in an inverted Leica SP2 confocal laser microscope. At least 10 oocytes/stage were analyzed.

Immunofluorescence and confocal imaging. NIH 3T3 cells were grown on coverslips. Cells were fixed in 2% paraformaldehyde for 5 min, permeabilized in 0.5% Triton X-100 twice for 5 min each, and incubated with primary antibody followed by Cy3-labeled anti-mouse or anti-rabbit secondary antibody (1:500; Jackson Laboratories) or Alexa488-labeled anti-rat secondary antibody (1:500; Invitrogen). When applicable, nuclei were stained with Hoechst 33258 at 5 µg/ml. Images were analyzed by using either a wide-field fluorescence Leica

(DMIRBE) microscope with a Cool Snap Ropers camera or a Leica-based confocal microscope.

Confocal imaging, was performed using a Leica TCS SP2 confocal microscope operating with a 40-mW argon laser tuned to line 488 when used with Alexa 488 fluorochrome or with a helium-neon laser tuned to line 543 when used with Cy3 fluorochrome. All imaging was done with a 100×/1.4-numerical-aperture oil immersion objective, 5× zoom, and a pinhole of 1 Airy unit. Images were processed in parallel with Photoshop (Adobe) software. To compare the intensities of labeling between antibodies, the power of the laser was set to the most intense labeling for each antibody (on metaphasic cells [see Fig. 2 and 3] or cells labeled with antibodies without peptides [see Fig. 5]) and the same conditions were used for all images for a given antibody.

Correlative light microscopy and EM. Semithin (for light microscopy and confocal microscopy) and ultrathin (for electron microscopy [EM]) cryosections of NIH 3T3 cells synchronized in the G₂/M phase with nocodazole were prepared and immunolabeled according to previously described methods (48). Briefly, cells were fixed with 2.5% paraformaldehyde and 0.1% glutaraldehyde for 1 hour. The free aldehydes were blocked with 0.2% glycine in phosphate buffer. The cells were then embedded in 10% gelatin and immediately pelleted. Gelatin-embedded cell pellets were sectioned into cubes deposited in 2.3 M ice-cold sucrose and agitated in a cold room for at least 2 h. Each cube was mounted on a pin and rapidly frozen in liquid nitrogen. One-hundred-nanometer ultrathin sections were cut with a cryoimmuno diamond knife (Diatome) using a cryoultramicrotome (UM6-Leica). Sections were deposited on nickel Formvar-carbon-coated 50-hexagonal-mesh grids and incubated over droplets of either anti-dimeK9-pS10 antibody (diluted at 1:10,000) or 51TA2H12 antibody (diluted at 1:1,000) using an automatic labeling apparatus (IGL; Leica). Primary antibodies were detected using protein A coupled to 10-nm colloidal gold (after bridging with a goat anti-rabbit or a rabbit anti-mouse antibody, respectively, at a dilution of 1:500). Sections were embedded and stained with methylcellulose-uranyl acetate and observed using a Philips CM 120 transmission electron microscope with a current voltage of 80 kV.

Cloning of 51TA2H12 heavy and light chain variable-region genes. cDNAs encoding variable heavy and light domains of the 51TA2H12 antibody were cloned from hybridoma cells. Total RNA was prepared using the TRI Reagent RNA isolation reagent (Sigma-Aldrich), and cDNA was prepared using a SMART rapid amplification of cDNA ends (RACE) kit (BD Biosciences Clontech). Variable heavy and light domains were PCR amplified from the cDNA using 5' RACE primer (NUP; BD Biosciences Clontech) and the following 3' primers complementary to murine immunoglobulin k and g1 constant domains: k, 5'-GAGCACCGCAACAGTGGTAGGTCG-3'; g1, 5'-TATTGCAGGGTCC CAAGGCAGTG-3'. The PCR products were ligated into the TA cloning vector pGEM-T (Promega). The resulting plasmids were then subjected to DNA sequencing to determine the variable heavy and light sequences for 51TA2H12. The cDNA sequences were translated and the predicted amino acid sequence determined (see Fig. S4 in the supplemental material).

Homology model building. The overall fold of the 51TA2H12 MAb has been modeled according to the crystal complex of fluorescein bound to the high-affinity anti-fluorescein 4-4-20 Fab fragment determined at 1.85 Å (1FLR). The loops of the heavy chain (1FLR-H) that differ significantly between 51TA2H12 and 1FLR-H have been generated according to other structures. The loops H1 (10 amino acids; over 80% sequence identity) and H2 (20 amino acids; over 80% sequence identity) have been generated according to 12E8 (a monoclonal 2E8 Fab antibody fragment specific for the low-density lipoprotein), and the loop H3 (23 amino acids; 50% sequence identity) has been generated according to 1AFV (antibody Fab25.3 fragment from the crystal structure of dimeric human immunodeficiency virus type 1 capsid protein). Ten conformations have been generated with the Modeler package (version 8).

Computational studies. (i) Peptide-antibody complexes. Using a homology model of the 51TA2H12 antibody as the target structure, the multiple-copy simultaneous search (MCSS) method (35) combined with a postprocessing analysis (46) was used to exhaustively map the N-terminal part of the 51TA2H12 antibody surface for possible binding sites for small functional groups. The functional groups used in this study were chemical fragments corresponding to side chains of amino acids found at the N-terminal end of histone H3. New functional groups representing trimethyl-ammonium (TRIM) and methylphosphate (PHO2) were developed to represent the side chain extremities of dimethylated lysine and phosphorylated serine, respectively. The details concerning development of these groups will be described elsewhere (C. Grauffel et al., unpublished data). Details of the homology modeling and of the MCSS procedure are given in the supplementary material.

The docking procedure identified possible binding sites for the side chains of Kme2 (TRIM) and Sp (PHO2) that were used as anchor points to build dipep-

TABLE 1. List of molecular dynamics run for the unbound peptides^a

Sequence	Length (ns)	%8/10	%9/10
A7-R8-K9me2-S10p-T11-G12	10	33.7	3.1
A7-R8-K9me2-S10p*-T11-G12	10	3.7	1.3
A7-R8-K9me2-D10-T11-G12	10	6.5	1.5
A7-R8-K9me3-S10p-T11-G12	4	23.4	0.0
A25-R26-K27me2-S28p-A29-P30-A31	10	28.5	3.0

^a Sequences and durations of the simulations are indicated. For each sequence, the temperature was 1,200 K. %8/10, percentage of conformations from the simulation that showed internal hydrogen bonds between the side chain of amino acid at position 8 (R8 in wild-type [WT] H3) and the side chain at position 10 (S10 in WT H3); %9/10, percentage of conformations from the simulation that showed internal hydrogen bonds between the side chain of amino acid at position 9 (K9 in WT H3) and the side chain at position 10 (S10 in WT H3). S10p* indicates a monoanionic phosphorylated serine. The starting structure was always an extended conformation.

tides (with neutral acetyl N-terminal and C-terminal ends) in complex with the 51TA2H12 antibody. Two different complexes were constructed and then subjected to molecular dynamics simulations to further assess their stability; details of the simulations are given in the supplementary material. Only one complex for which the peptide stayed bound at the surface of the antibody for the duration (1.5 ns) of the simulation was retained. A tetrapeptide having the sequence R-Kme2-Sp-T (with neutral N terminus/C terminus) was constructed at the surface of the antibody using the stable dipeptide-antibody complex identified by this simulation procedure. The R side chain in the tetrapeptide was positioned using an MCSS docking of R side chains as a guide. The stability of the antibody-tetrapeptide complex was then further assessed in a 2.1-ns molecular dynamics simulation. To further characterize the specificity of the binding pocket identified above, we performed simulations of the tetrapeptides R-Kme2-D-T, R-Kme2-Sp(-1)-T, and R-Kme3-Sp-T bound to the antibody. These peptides were built using the initial stable position of the R-Kme2-Sp(-2)-T peptide by replacing the corresponding atoms.

(ii) Molecular dynamics simulations of the isolated peptides. To explore the conformational space of the native and modified histone tail peptides, molecular dynamics simulations of several peptides in solution were performed. The simulations were done using explicit water (TIP3P) with periodic boundary conditions in a cubic box of 36 Å. Counterions (Na⁺ and Cl⁻) were added to give an ionic strength of approximately 0.2 M. Further details of the simulation procedure are provided in the supplementary material. A list of the simulations performed is given in Table 1, together with the temperature and initial configuration used for the simulations. For all simulations, atomic coordinates were stored each 1,000 steps (1 ps) for subsequent analysis. The H bond selection was made using a distance criterion of less than 1.8 Å between the hydrogen atoms (of R, Kme2) and the oxygens (of Sp, D).

RESULTS

The 51TA2H12 antibody recognizes doubly modified histone H3 in mitotic cells. In our attempt to raise antibodies against posttranslationally modified TATA binding protein-associated factors, we raised an antibody against the ASGSSRSKSKme2DR KYTLC dimethylated peptide (see Materials and Methods). The obtained 51TA2H12 mouse MAb strongly recognized an epitope in mitotic cells (Fig. 1A). Since only mitotic cells were labeled by immunofluorescence experiments (Fig. 1A) and since the staining resembled a staining obtained by using an antibody raised against phosphorylated S10 of histone H3 (data not shown), next we tested whether the antibody would recognize purified histones from HeLa cells. We first performed Western blot analysis using whole-cell extracts as well as purified histone preparations (Fig. 1B and C). This showed that this new antibody recognizes specifically histone H3 (Fig. 1C). Moreover, histones purified from G₂/M-synchronized cells were more efficiently recognized (Fig. 1B, lanes 1 and 3, and C, top) and immunopurified by the

FIG. 1. The 51TA2H12 antibody recognizes a doubly modified form of histone H3. (A) F9 cells stained with the 51TA2H12 antibody (left). A merge image with Hoechst DNA staining is shown on the right. Note that only mitotic cells are labeled. Bar, 150 μ m. (B) Western blot analysis on 1 M KCl whole-cell extract (WCE) with 51TA2H12. WCEs from HeLa G₂/M (lanes 1 and 3)- or G₁/S-synchronized cells (lanes 2 and 4) were

51TA2H12 MAb than histones purified from unsynchronized cells (Fig. 1C, middle; compare lanes 1 and 2). Finally, recombinant *Xenopus* histones produced in bacteria, e.g., unmodified, could not be recognized or immunopurified by the 51TA2H12 antibody (Fig. 1C, lane 3). Thus, our results indicate that the 51TA2H12 MAb specifically recognizes mitotically modified histone H3.

To obtain more information on the nature of the posttranslational modification of histone H3 that is recognized by the 51TA2H12 MAb, we performed immunoprecipitations with our antibody and subjected the eluted material to mass spectrometric analysis. This analysis first showed that the immunopurified material contained a peptide of 929.5 Da corresponding to histone H3 from amino acid 9 to amino acid 17 and carrying a dimethylation (Fig. 1D). This further confirmed that our antibody is specific for H3. As this endogenous peptide possesses two lysines that could be potentially dimethylated, we compared its fragmentation pattern by MALDI-TOF to those of two synthetic 9-17 H3 peptides carrying a dimethylation on either lysine 9 or lysine 14 (Fig. 1E). The similarity between the peptide recognized by our antibody and the K9me2 H3 synthetic peptide (Fig. 1E) indicated that the dimethyl lysine modification involved in the recognition of the histone tail by the 51TA2H12 MAb occurred on lysine 9 of histone H3. In parallel, another peptide of 1,461.8 Da, corresponding to histone H3 amino acids 27 to 40 carrying a dimethylation, was found in the immunopurified material. Complementary analysis has shown that the dimethylation occurred on lysine 27 of this peptide (data not shown). Since the phosphorylation is a labile modification, difficult to analyze by MALDI-TOF mass spectrometry, by this method we were unable to determine whether the epitopes were also phosphorylated. However, we showed by several other methods that the 51TA2H12 MAb recognizes S10- or S28-phosphorylated histone H3, but only when doubly modified with H3K9me2 or H3K27me2, respectively (see below).

To further investigate the specificity of our antibody, we tested its immunoreactivity using different synthetic H3 peptides. By ELISA and dot blot analyses we demonstrated that the 51TA2H12 MAb specifically recognizes the H3 peptide that is simultaneously dimethylated on K9 and phosphorylated on S10 (Fig. 1F; see Fig. S1, graph A, in the supplemental

material). In contrast, the 51TA2H12 MAb does not recognize the unmodified H3 tail peptide or the S10 phosphorylated peptide alone (Fig. 1F; see Fig. S1, graph B, in the supplemental material) and recognizes only weakly the peptide that is exclusively dimethylated on K9 (see Fig. S1, graph C, in the supplemental material). A similar weak recognition has also been observed for the H3 peptide carrying a dimethylation on K9 and a phosphorylation on T11 (see Fig. S2 in the supplemental material). Moreover, the phosphorylation of T11 on the H3K9me2S10p peptide does not impair the recognition of the peptide by 51TA2H12 antibody (see Fig. S2 in the supplemental material). Importantly, the 51TA2H12 MAb does not recognize histone H3 peptide trimethylated on K9 and phosphorylated on S10 (Fig. 1F, bottom). Since on the histone H3 tail there is a second epitope where lysine dimethylation and serine phosphorylation occur at adjacent sites (K27me2S28p), we tested whether the corresponding synthetic peptide with the K27me2S28p modification would be recognized by the 51TA2H12 MAb. As shown in Fig. 1F, the 51TA2H12 antibody readily recognizes also this double modification. Nevertheless, our antibody does not recognize the same Kme2Sp motif, which can occur on K26 and S27 of histone H1 (11) (see Fig. S3 in the supplemental material). In addition, commercial antibodies characterized and raised either against S10-phosphorylated histone H3 (21) or against S28-phosphorylated histone H3 (18) gave a distinctive recognition pattern compared to the 51TA2H12 MAb in these in vitro tests (Fig. 1E and see below). Interestingly, a polyclonal rabbit antibody raised against a H3K9me2S10p peptide (anti-dimeK9-pS10 pAb) (33) gave comparable, but not identical, results to the one obtained with the 51TA2H12 MAb in ELISA and dot blot tests (Fig. 1E; see Fig. S1 in the supplemental material). These results together indicate that the 51TA2H12 MAb specifically recognizes the doubly modified K9me2S10p and the K27me2S28p forms of the histone H3 tail.

Histone H3 tails dimethylated on lysine and phosphorylated on the adjacent serine exhibit different conformations during mitosis and meiosis. In order to further characterize the 51TA2H12 antibody in vivo, we next compared the previously characterized antibodies (anti-S10p, anti-S28p, and anti-dimeK9pS10 PABs), recognizing specific cell cycle stages (18, 21, 33), with the 51TA2H12 antibody. We examined the rec-

blotted and revealed with the 51TA2H12 antibody (lanes 1 and 2) and, later, with an anti-TAF15 antibody as a positive control (lanes 3 and 4). Note that only H3 was recognized by 51TA2H12 antibody and in a bigger amount in the G₂/M WCE although the same amount of total proteins was loaded. α , anti. (C) Western blot (WB) analysis of purified histones and histones immunopurified with the 51TA2H12 MAb from HeLa cells. Purified histones from cycling cells (lane 1) or G₂/M-synchronized cells (lane 2) were blotted and revealed with the 51TA2H12 antibody (top). The same histone preparations were immunoprecipitated (IP) with the 51TA2H12 and analyzed by Western blotting (middle). The bottom panel shows the Coomassie blue R250 (Coom.) staining of the top panel. In lane 3, recombinant histone H3 overexpressed in *Escherichia coli* (e.g., unmodified) was used as negative control. (D) Spectra of histone H3 obtained from mass spectrometry analysis from different samples as indicated at the right of each spectrum. Three of the samples (SN, IP 51TA2H12, and input) derive from the 51TA2H12 immunoprecipitation of purified HeLa cell histones. SN corresponds to the spectrum obtained for the supernatant after immunoprecipitation, IP 51TA2H12 to the immunoprecipitated material, and input to the input material before immunoprecipitation. The bacterially expressed (exp.) H3 spectrum was obtained using recombinant histone H3. Masses of ions corresponding to unmodified H3 peptides (unmod.) or dimethylated peptides (me2) are indicated. Ions arising from nonhistone peptides or contaminations are indicated by an asterisk. (E) Tandem mass spectrometry spectra for two synthetic dimethylated H3 peptides (top and bottom plots) and for the 929.5-Da endogenous immunopurified H3 peptide including amino acids 9 to 17, carrying two methylations (see me2 peptide on the IP 51TA2H12 spectrum of panel C). Ions allowing the comparison of the different peptides are highlighted in red boxes. (F) Specificity of the 51TA2H12 antibody for the doubly Kme2/Sp-modified H3 peptides. ELISA and dot blot analyses of various H3 unmodified and modified peptides with the 51TA2H12 antibody are shown. Antibodies recognizing S10p, S28p, or dimeK9-pS10 were also included in the analysis.

FIG. 2. 51TA2H12 labeling is specific to well-defined stages of mitosis. (A) NIH 3T3 cells were stained with the indicated antibodies and analyzed under confocal microscopy. Below each image is shown the corresponding Hoechst DNA labeling, and the cell cycle phase is indicated at the top. α , anti. Bar, 10 μ m. (B) Schematic representation of the data shown in panel A. The variation of the intensity of the colored bar corresponds to the variation in the labeling of the different antibodies along the cell cycle. At least 50 cells were analyzed per stage for each antibody.

ognition pattern of these antibodies on NIH 3T3 cells in different stages of the cell cycle by confocal microscopy (Fig. 2A). While the anti-S10p and the anti-dimeK9-pS10 PAbs stained almost all phases of the mitosis (Fig. 2A, bottom four rows), the anti-S28p PAb stained cells only from prophase to anaphase (Fig. 2A, top two rows). Interestingly, the 51TA2H12 MAb staining was different from that of all the tested antibodies, since it labeled cells first in early prophase and the labeling disappeared specifically at the early anaphase (Fig. 2A, third

and fourth rows). Note that we obtained the same results also with HeLa and COS-1 cells (data not shown). Thus, these four different antibodies all recognized distinct epitopes throughout mitosis (Fig. 2B). Further, the 51TA2H12 and the anti-dimeK9-pS10 antibodies also recognized different stages of mitosis, in spite of the fact that they both recognize the synthetic doubly modified H3K9me2S10p and H3K27me2S28p peptides in vitro.

Because these antibodies do not recognize exactly the same

FIG. 3. The 51TA2H12 antibody displays a pattern distinct from that for phosphorylated S10 and S28 throughout mitosis. Shown is a comparison between 51TA2H12 and either S10p or S28p labeling during mitosis in NIH 3T3 cells. Colabeling of NIH 3T3 cells with 51TA2H12 and S10p (A) or S28p (B) antibodies was analyzed by confocal microscopy. The corresponding merge image and Hoechst DNA labeling are shown at the bottom for each panel. Bar, 10 μ m.

epitope, we next colabeled NIH 3T3 cells in different cell cycle stages with a combination of antibodies in parallel. These experiments confirmed that the epitopes recognized by either the anti-S10p or the anti-S28p antibodies only partially overlap with that recognized by the 51TA2H12 MAb and indicated that the 51TA2H12 MAb labeled mostly peripheral heterochromatin (Fig. 3A and B). Indeed, in prometaphasic and metaphasic cells, although anti-S10p antibody labels also peripheral heterochromatin, some regions are stained only by 51TA2H12 (Fig. 3A, red or merge images). Moreover, we observed that cells in late G₂, anaphase, and telophase are exclusively labeled by anti-S10p PAb (Fig. 3A), consistent with results shown on Fig. 2A. On the other hand, the timings of appearance and disappearance of 51TA2H12 MAb and anti-S28p PAb appeared to be more similar (Fig. 3B). Nevertheless, the localization of the two antibodies was clearly exclusive,

especially in prophasic cells (Fig. 3B, merge). These data further confirm that these antibodies do not recognize the same epitopes on the chromatin throughout mitosis. As both antibodies are specific to mitosis, the phase of the cell cycle where the chromatin is the most condensed, we analyzed their distribution during meiosis to examine whether the labeling of 51TA2H12 is also associated with chromatin compaction during gametogenesis. To characterize the distribution of 51TA2H12 MAb in mammalian spermatogenesis, we compared the staining of the previously characterized antibody recognizing histone H3S10p, shown to recognize specific meiotic cell types (8, 27), to that of the 51TA2H12 antibody. The distribution obtained using the 51TA2H12 MAb exhibited a tightly controlled temporal and spatial appearance during spermatogenesis (Fig. 4A). The specific conformation of histone H3 detected using the 51TA2H12

FIG. 4. Metaphasic meiotic chromosomes are marked by the 51TA2H12 antibody. (A) The 51TA2H12 antibody displays a tightly regulated distribution during murine meiosis that differs from that displayed by phosphorylated S10. Shown are colocalization of MAb 51TA2H12 (green) and anti-S10p (red) in cross sections of seminiferous epithelium (A to G) and colocalization of MAb 51TA2H12 (green) and the synaptonemal complex protein 3 (SCP3; red) in pachytene spermatocytes. Spermatogonia (SG), leptotene spermatocytes (L), zygotene spermatocytes (Z), pachytene spermatocytes (P), diplotene spermatocytes (D), metaphase spermatocytes (M), anaphase spermatocytes (A), secondary spermatocytes (sp^2), and elongating spermatids (eS) are shown. Sections were counterstained with DAPI. Roman numerals indicate the stage of spermatogenesis. (B) Distribution of the 51TA2H12 antibody in metaphase I and metaphase II oocytes. Oocytes were stained with the 51TA2H12 antibody (green) and DAPI and analyzed by confocal microscopy. Shown are single sections of representative metaphasic chromosomes of oocytes analyzed in parallel and under the same confocal parameters. Bar, 30 μ m.

MAb was observed at the onset of spermatogenesis, with moderate-to-strong signals present in spermatogonia (Fig. 4A). Unlike the meiotic localization observed for H3S10p, the H3 conformation detected by 51TA2H12 MAb was first observed at the onset of meiosis in preleptotene spermatocytes, where it was strongly up-regulated and persisted to mid-meiosis (zygotene through pachytene) (Fig. 4A, panels A to C). Similar to H3S10p staining, the 51TA2H12 signal was highest in diplotene spermatocytes and metaphase spermatocytes (Fig. 4A, panels D to F). At anaphase, signals for 51TA2H12 displayed

a punctuate distribution in contrast to the widespread localization of histone H3S10p, which is dispersed on the chromosomes (Fig. 4A, panels G to I). No reactivity to the 51TA2H12 MAb was detected in postmeiotic round and elongating spermatids (Fig. 4A, panels A to C).

We also analyzed the distribution pattern of the 51TA2H12 MAb during oogenesis. Fully grown preovulatory oocytes at the GV stage are arrested at the diplotene phase of the first meiotic division. Upon ovulation meiosis resumes and oocytes proceed until the metaphase of the second meiotic division.

FIG. 5. The charge and side chain of the serine in combination with the neighboring methylated lysine are essential for the recognition of H3 by the 51TA2H12 antibody. Analysis of the recognition of wild-type and mutated H3 peptides by the 51TA2H12 and anti-dimeK9-pS10 antibodies. (A) ELISA and dot blot analyses of the recognition of various H3 peptides by the antibodies (51TA2H12, anti-S10p, anti-S28p, or anti-dimeK9-pS10) as indicated at the bottom are shown. α, anti. (B) Competition tests of either the 51TA2H12 or anti-dimeK9-pS10 antibodies with the indicated H3 peptides by immunofluorescence and subsequent confocal microscopy. Below each image is shown the corresponding Hoechst DNA labeling. Bar, 10 μm. Note that the peptide bearing the serine-to-aspartic acid (D) mutation (which mimics the side chain and the charge of a phosphorylated serine) fully competes the recognition of the 51TA2H12 antibody in vitro and in vivo during mitosis.

The oocyte remains arrested at this stage until fertilization. We detected a bright staining with the 51TA2H12 MAb on metaphase I chromosomes (Fig. 4B, panels A to C). The pattern of staining displayed by the antibody was very similar to that observed in mitotic cells, with stronger intensity in the periphery of the condensed chromosomes. Similarly, in metaphase II-arrested oocytes the 51TA2H12 MAb stained the peripheral regions of the chromosomes, albeit with a lower intensity than for metaphase I chromosomes (Fig. 4B, panels D to F). We did not detect any signal with the 51TA2H12 MAb in oocytes at the GV stage prior to resumption of meiosis (not shown).

Thus, the specific conformation recognized by the 51TA2H12 MAb is also present in meiotic chromosomes, in particular during the phases of meiosis when chromatin condensation is highest.

The 51TA2H12 and anti-dimeK9-pS10 antibodies do not recognize the same conformation of doubly modified histone H3 tails. To determine the specific differences in the epitopes

of the two antibodies (51TA2H12 MAb and the anti-dimeK9-pS10 PAb), which both recognize the H3K9me2S10p and H3K27me2S28p peptides in vitro (Fig. 1F), and to explore a possible conformational context in their respective recognition of histone H3 tails, we tested these antibodies systematically on several mutated H3 peptides. We also compared them to the anti-S10p and the anti-S28p antibodies. Mutated peptides in which K9 or K27 was dimethylated but either the phosphorylated S10 or S28 was replaced with an aspartic acid (D) to mimic the side chain and the charge of the respective phosphorylated serines were synthesized (Fig. 5A). These mutations did not decrease the affinity of the 51TA2H12 antibody for the corresponding H3 peptides, while in contrast the mutations did abolish the different H3 tail recognition by the anti-S10p, anti-S28p, and the anti-dimeK9-pS10 antibodies (Fig. 5A). These results suggest that the anti-dimeK9-pS10 and the 51TA2H12 antibodies do not recognize the same confor-

mation of the doubly modified histone H3 tails. Moreover, the charge and the side chain of the modified peptides seem to be crucial to allow their recognition by the 51TA2H12 antibody.

We then examined whether these potential conformational differences in the epitopes of the 51TA2H12 antibody and the anti-dimeK9-pS10 antibody occur also *in vivo* during mitosis. To this end we performed immunofluorescence combined with peptide competition tests on mitotic NIH 3T3 cells (Fig. 5B). The staining of the two antibodies on the metaphasic NIH 3T3 cells remained unchanged when challenged with a H3 tail peptide that was phosphorylated only on S10 (Fig. 5B, panels a, b, g, and h). In contrast, both antibodies were competed by the H3K9me2S10p or the H3K27me2S28p peptides and thus show no or only weak labeling on the metaphasic cells (Fig. 5B, panels c, e, i, and k). Importantly, the mutated H3K9me2D10 or H3K27me2D28 peptides, in which S10 or S28 has been replaced by an aspartic acid, fully competed the recognition of the 51TA2H12 MAb (Fig. 5B, panels d and f) but did not affect the staining of the anti-dimeK9-pS10 PAb (Fig. 5B, panels j and l). These results further confirm that the two antibodies react differently with the doubly modified epitopes during mitosis, suggesting that the 51TA2H12 antibody recognizes a specific conformation of the H3K9me2S10p and/or the H3K27me2S28p modified histone tails, while the anti-dimeK9-pS10 antibody seems to be more specific to the primary sequence of the same modified histone H3 tail.

Locating a potential binding site for the H3K9me2S10p epitope on 51TA2H12 MAb: modeling of the structure of the histone H3 tail-antibody complex. To determine the nature of the conformational differences recognized by the 51TA2H12 antibody, we set out to define the structure of its epitope. The cDNA encoding the variable regions of the light (VL) and heavy (VH) chains of 51TA2H12 antibody were cloned from the hybridoma cells by 5' RACE and sequenced. The deduced amino acid sequences of VH and VL chains of the MAb were used to build the homology model of the 51TA2H12 antibody.

Using this structure, a docking procedure was used to identify favorable binding positions for TRIM, a mimic of dimethyl lysine, and PHO2, a mimic of phosphoserine. Two regions where TRIM was preferentially localized were identified, while for PHO2, a larger number of potential binding sites were located. However, only one site for the TRIM/PHO2 pair that allowed the construction of a dipeptide bound at the surface of the antibody was identified. Subsequent molecular dynamics simulations of the antibody/dipeptide complex as well as the analogous antibody/tetrapeptide (R8-K9me2-S10p-T11) complex showed these structures to be stable for the duration of their respective simulations. The characteristics of this binding site are consistent with those observed in crystal structures of other protein domains known to bind methylated lysines (6, 16, 26, 29) in that the methylated lysine is bound in an aromatic cage, which is formed by W41 and Y24 from the antibody heavy chain and is stabilized by cation-aromatic cage interactions (Fig. 6A). The phosphate group of the peptide interacts with heavy chain H95 and light chain K35 and Y37 (Fig. 6B). This interaction with two charged residues is similar to that observed in other complexes involving phosphates (16).

To better correlate the docking results with the affinity tests performed on different peptides *in vitro*, we constructed mutants of the tetrapeptide and used these in molecular dynamics

simulations of the peptide/antibody complex. The mutations affected both the Sp (replaced by a monoanionic phosphate group or by an aspartate) and the Kme2 (mutated into a Kme3). From the simulations, we observed that the peptides R-Kme2-D-T and R-Kme2-Sp(-1)-T stayed bound in a position very similar to that of R-Kme2-Sp(-2)-T for the duration (1.5 ns) of the simulations, with only some changes in the local interaction networks as a result of the substitutions (Table 1). This is in agreement with the experimental observation that the R-Kme2-D-T peptide is strongly recognized by the antibody. It furthermore supports the idea that the protonation state of the phosphate group during binding could be either -1 or -2, which are the two forms present at physiological pH. Note that the stability of the peptide-antibody complex carrying the Kme3 mutation is seriously compromised (Table 1). This is in good agreement with the experimental observation that the R-Kme3-S-T peptide is not recognized by the antibody. Together, these data suggest that the 51TA2H12 antibody recognizes a conformation of the modified histone H3 tail peptide where the side chain of Kme2 and Sp are in close proximity and interact with each other (Fig. 6C).

Isolated histone H3 tail peptides adopt preferential conformations. To further assess the relevance of the proposed structure of the histone-antibody complex, molecular dynamics simulations of the unbound modified and unmodified peptides were performed. High-temperature molecular dynamics simulations, starting from fully extended conformations of the histone peptides, were used to enhance the conformational sampling and to generate a large set of representative conformations of the peptides. During the simulations, internal structure formed due mainly to the formation of hydrogen bonds between the phosphorylated serine (S10p, with a dianionic phosphate) and either the methylated lysine (K9me2) or the arginine (R8) (Table 1). When the S10p was replaced by either D or a monoanionic phosphate, the internal structure remained during the high-temperature simulations, however to a lesser extent. The stability of the internal K9me2-S10p hydrogen bond was further tested by a simulation at 300 K of a peptide where the K9me2-S10p hydrogen bond was initially present. Simulations were performed for both monoanionic and dianionic phosphate. During these simulations, the internal H bond was maintained for several hundred picoseconds with both monoanionic and dianionic phosphate (data not shown).

Thus, the molecular dynamics simulations of the histone peptides indicate that the H3 tail peptide can adopt a conformation in solution that maintains an internal structure between Kme2 and Sp stabilized by an internal H bond (Fig. 6C and D). Importantly, this conformation is similar to that of the above-described doubly modified H3 peptide bound to the model of the 51TA2H12 antibody. Our results together thus suggest that this structure, formed by an internal H bond, is specifically recognized by the 51TA2H12 antibody.

EM reveals a different localization of the epitopes recognized by 51TA2H12 and anti-dimeK9-pS10 antibodies *in vivo*. In order to explore a possible link of the different conformations of the modified H3 tails that we describe and the degree of chromatin compaction, cryosections of metaphasic NIH 3T3 cells were labeled with either the 51TA2H12 or the anti-dimeK9-pS10 antibody and observed by EM with gold-labeled

FIG. 6. Analysis of the conformation of the H3K9me2S10p histone tail recognized by 51TA2H12. (A) Binding pocket for dimethylated K9 on the surface of 51TA2H12 antibody. The hydrophobic amino acids (Y24 and W41 of the heavy chain) involved in K9me2 recognition are shown in pink, while the peptide is in yellow. The antibody-peptide interactions are mediated primarily by the methyl groups of the lysine side chain. (B) Binding pocket of phosphorylated S10 on the surface of the 51TA2H12 antibody. The amino acids involved in electrostatic interaction with S10p are shown in green for the charged residues (K35 of the light chain, H95 of the heavy chain) and in pink for the hydrophobic amino acid (Y37 of the light chain). (C) General view of the tetrapeptide R-Kme2-Sp-T docked at the surface of the 51TA2H12 antibody. For the antibody, the amino acids are shown as in panels A and B. (D) Cross-eyed stereo view of a representative structure of the unbound A7-R8-K9me2-S10p-T11-G12 H3 peptide (with neutral N- and C-terminal ends) as determined by high-temperature molecular dynamics simulations in solution (in green) is superimposed with the peptide structure obtained after docking the same peptide at the epitope of the antibody (in pink; see also panel C). Note that in both histone H3 tail peptide structures the side chains of Kme2 and Sp are in close proximity and interact with each other forming an H bond.

FIG. 7. Localization of the structured doubly modified histone tail on chromatin and analysis of the modifications associated with H3Kme2Sp. (A) The 51TA2H12 and the anti-dimeK9-pS10 antibodies do not recognize the same structures in mitotic cells when observed by EM. Localization of the epitopes recognized by 51TA2H12 (right) and anti-dimeK9-pS10 (left) antibodies was analyzed by cryoelectron microscopy of metaphasic NIH 3T3 cells. Gold particles can be distinguished as small dots. Condensed chromosomes appear as dense, black masses. Arrows on the right image indicate specific fibers recognized by 51TA2H12. α , anti. Bar, 200 nm. Note that the 51TA2H12 antibody recognizes specific stretches of chromatin fibers. (B) Some other histone posttranslational modifications can occur simultaneously with the structured H3Kme2Sp. Western blot analysis of purified histones and histones immunoprecipitated with the 51TA2H12 MAb from HeLa cells treated with phosphatase inhibitors is shown. Histone preparations were immunoprecipitated with the 51TA2H12 MAb and analyzed by Western blotting. Immunoprecipitated material (IP 51TA2H12) was blotted and revealed with the indicated antibodies. Recombinant histone H3 overexpressed in *E. coli* (e.g., unmodified) was used as a negative control (Bact. exp. H3), and a histone preparation used for the immunoprecipitation (input) was used as a positive control. The ratio of the IP signal over that of the input (IP/input) is indicated on the right of each panel. The upper left panel shows the Coomassie blue (Coom.) staining of the material that was tested on all the other panels as loading control. Note that, under the conditions used for our purification and subsequent immunoprecipitation, we recover H3-H4 tetramers, and hence H4 is visible on the gel.

protein A. For the two antibodies, the labeling was specifically restricted to metaphasic chromosomes and was distributed all over the chromosome section (Fig. 7A). However, the distributions of the gold particles on the mitotic chromosome for the two antibodies were significantly different, reinforcing our observations that the two antibodies recognize different epitopes or distinct conformations of the same protein (Fig. 7A). The anti-dimeK9-pS10 labeling appeared to be evenly distributed all over the mitotic chromosome, whereas in cells labeled with 51TA2H12 MAb, the gold particles were densely distributed along 30-nm large fiber-like structures and large areas of the

chromosome were devoid of labeling. Importantly, the size of these fibers is consistent with highly condensed 30-nm chromatin fibers. Interestingly, the cellular sections contained fibers that did not show any labeling, suggesting that the 51TA2H12 antibody recognizes specific stretches of chromatin fibers.

Certain modifications are preferentially associated with the structured H3Kme2Sp histone tail. To investigate the presence of other posttranslational histone modifications associated with H3 bearing the conformation recognized by 51TA2H12, we performed immunoprecipitations on histone

preparations from HeLa cells using the 51TA2H12 MAb. The association of other histone modifications with the structured immunoprecipitated H3Kme2Sp tails was then tested by subsequent Western blot analysis with antibodies directed against several histone modifications (Fig. 7B). Although these experiments would not allow us to distinguish between the presence of other histone modifications on the same tail of H3 versus on the same nucleosome particle, they can reveal preferential association of marks in nucleosomes enriched with the conformation recognized by our antibody. We again observed that the double modification recognized by 51TA2H12 was highly enriched in the immunoprecipitated material (about 12-fold) compared to the input material. In contrast, in the immunoprecipitated doubly modified histone H3 sample anti-dimeK9-pS10 PAb recognition was poorly enriched (less than twofold; Fig. 7B). These results suggest that both modifications occur rarely on the histone H3 tails of the same nucleosome.

In spite of the fact that none of the tested H3 tail modifications were enriched to the same extent as the one recognized by 51TA2H12, other modifications appeared to be enriched significantly in the immunoprecipitated material. Indeed, H3K27me3 appeared often associated with the structured H3Kme2Sp histone tail, as well as H3K9ac and, at a lower level, H4K20me3 (Fig. 7B). All other modifications examined were not significantly enriched in the immunoprecipitated material, suggesting that the structured H3Kme2Sp histone tails are associated with specific, but not all, histone posttranslational modifications.

DISCUSSION

Here we describe a specific chromatin conformation that occurs at dimethylated lysine and adjacently phosphorylated serine residues of the histone H3 tail. This chromatin conformation is present only between the early prophase and the early anaphase of the mitosis. Moreover, this specific histone conformation is also detected in high levels in diplotene and metaphase stages of meiosis. Our data indicate that this conformation is not recognized by another antibody raised against H3K9me2S10p, which appears to be more specific for the primary sequence of the modified histone H3 tail. In contrast, our novel antibody recognizes a specific charged structure of the H3K9me2S10p and/or the H3K27me2S28p doubly modified H3 histone tail stabilized by intramolecular interactions between the Kme2 and Sp. Thus, the chemically modified Kme2Sp histone H3 tail adopts an ensemble of conformations, which are distinct from that of unmodified H3 tails, and one of these conformations is specifically recognized between the early prophase and the early anaphase of the mitosis and meiosis. Our observations suggest that H3K9me2S10p and/or H3K27me2S28p sites could adopt specific chromatin conformations during mitosis and meiosis that may participate in mitotic and meiotic chromatin condensation.

Histone posttranslational modifications are crucial to regulate the global structure of the chromosomes and gene transcription. The unique presence of the posttranslational modifications does not seem to be sufficient to regulate transcription. Their association with other histone modifications and the fact that proteins are proposed to "read" these modifications were, it was suggested, necessary to lead to chro-

matin condensation or decondensation (38, 47). Methylated K9 of histone H3, for example, constitutes the docking site of the protein HP1, necessary for the formation of a proper heterochromatin. Interestingly, the addition of a phosphate group on the adjacent S10 on the same histone tail seems to be important for the chromatin dynamics that is necessary for the cells to enter into mitosis (14, 23; this study).

Chemical modifications of histone tails by themselves may not be sufficient for the regulation of chromatin structure and the consequent changes in gene expression. The structure of the histone tails could also participate in this regulation, but the mechanism of such a regulation is less well understood and thus remains more controversial. Indeed, histone tails have been often considered to be in a permanently unstructured state. However, at present increasing experimental evidence favors the possibility that histone tails could be structured. Supporting this idea, it has been proposed, using circular dichroism or molecular dynamics computer simulations, that histone H3 N-terminal tails can adopt an α -helical conformation when bound to DNA (3, 28). This binding to DNA occurs principally in interphase but is reduced in mitosis (45). Moreover, recent data suggest that this binding between the histone H3 tail and DNA would depend on the charge and the dimension of the protein (1). Indeed, our results show that the recognition of Kme2Sp-modified histone H3 tails by the 51TA2H12 antibody requires not only the biochemical properties carried by the dimethylation and adjacent phosphorylation of the epitope but also the formation of a hydrogen bond between the K9me2 and S10p or K27me2 and S28p of the histone H3 tail. The formation of this internal hydrogen bond in the Kme2Sp-modified histone H3 tail and its importance in the 51TA2H12 antibody recognition of the epitope were confirmed by using the Kme2D-modified peptides, in which this internal hydrogen bond can also form. Importantly, the Kme2D-modified H3 tail peptides are not recognized by the anti-dimeK9-pS10 antibody. In contrast, the Kme3Sp-modified peptide (which cannot form the internal H bond) is recognized only by the anti-dimeK9-pS10 antibody, not by 51TA2H12 MAb, further indicating that the recognition of specific chromatin conformations is a specific feature of the monoclonal 51TA2H12 antibody. This suggests that intratail interactions resulting from concurrent dimethylation and phosphorylation at adjacent K-S sites could confer specific conformations to the H3 tail during mitosis.

We found by using molecular dynamics simulations that the unbound histone tail peptide can adopt a stable conformation that maintains an internal structure between Kme2 and Sp. Interestingly, the formation of an internal structure in modified histone H3 tail peptides has already been observed in a complex with the protein 14-3-3 (32). In this complex, an internal hydrogen bond between the arginine R8 and a phosphorylated pS10 in an R8-K9ac-S10p octapeptide was observed. In contrast, the anti-dimeK9-pS10 antibody seems to recognize a conformation of the Kme2Sp-modified histone H3 in which the internal hydrogen bond does not seem to be present.

We observed a very specific labeling of the mitotic cells by the 51TA2H12 MAb that was different from that by the anti-dimeK9-pS10 antibody. The appearance of anti-dimeK9-pS10 signal in late G₂ stage reveals the presence of simultaneously

FIG. 8. Model for the function of H3K9me2S10p structured histone tail in the condensation of the chromatin during mitosis. Only the H3 tail is represented on each nucleosome. Red dots correspond to dimethylation of K9 or K27, green dots to phosphorylation of S10 or S28, and gray dots to acetylation of K or R. The hydrogen bond between adjacent Kme2 and Sp is represented by a black link. HDAC, histone deacetylase; HMT, histone methyltransferase; CF, condensation factor. See Discussion for details.

labeled H3Kme2Sp tails. However, these tails are not recognized by the 51TA2H12 antibody until the early prophase (Fig. 2B). These results suggest that the H3Kme2Sp tails present in late G_2 cells are not in the appropriate conformation to allow their recognition by 51TA2H12 antibody. Thus, *in vivo* the conformation containing an internal hydrogen bond formed between the K9me2 and S10p or K27me2 and S28p occurs exclusively during certain phases of mitosis, as this conformation is the only one that is recognized by the 51TA2H12 MAb. Importantly, this specific Kme2Sp conformation seems to occur specifically in the H3 tail context, as histone H1, which can have a similarly modified sequence (K26me2S27p), is not recognized by our antibody (see Fig. S3 in the supplemental material). Moreover, these structured double modifications on H3 tails seem to be specific for a subset of 30-nm mitotic chromatin fibers because in the high-magnification EM pictures of mitotic cells only some fibers were labeled by the 51TA2H12 MAb.

What is the function of such structured doubly modified Kme2Sp H3 tails? We demonstrate that the labeling of the mitotic and the meiotic cells by the 51TA2H12 MAb is the strongest in the cell cycle phases where the chromatin is the most condensed. Cells treated by Hesperadin (Aurora kinase B inhibitor) and observed by confocal microscopy have difficulty in passing the metaphasic stage of the mitosis (our unpublished results; 20). Moreover, Aurora kinase B has been shown to be required for maximal chromosome compaction in anaphase in mammalian cells (36). These data are in agreement with the fact that phosphorylation has been involved in the chromosome condensation process (12, 52, 53) and are consistent with

a potential role of the structured doubly modified histone tail in the compaction of the chromatin in mitosis. However, if the biochemical properties of phosphorylation participate in the opening of the chromatin in interphasic cells, due to the repulsion of the negative charges carried by both the modification of the tail and by the DNA, how could the condensation of the chromatin be explained by the same modification? Several models have been proposed to answer this question (41). We hypothesize that, in addition to the charge, the conformation change of the histone H3 tail is the way by which phosphorylation can promote condensation of the chromatin (Fig. 8). According to our model the specific stable conformation of the H3 tail containing the internal hydrogen bond resulting from the presence of the Kme2 and the adjacent phosphorylation of the serine may favor the proximity of residues either on the same histone tail or between several histone tails. This assembly of histone tails could in turn constitute specific docking sites for proteins able to condense chromatin (Fig. 8, right), different from topoisomerase 2 or condensin complexes. This would be in good agreement with previous data suggesting that redundant pathways for mitotic chromosome condensation exist and that the function of condensins may primarily be to stabilize and/or properly shape the compacted metaphase chromosomes (references 5 and 12 and references therein). Furthermore, the assembly of histone tails could improve the efficiency of the quick action of different histone-modifying enzymes during mitosis and meiosis (Fig. 8, left). This hypothesis would suggest an increase of global histone methylation or deacetylation in mitosis to favor chromatin condensation. Consistent with this, our data revealed an association of the struc-

tured H3Kme2Sp modification with the trimethylation of H3K27 and H4K20. The presence of the H3K9ac that we also observed could help in the dissociation of the histone tail from the DNA by some electrostatic mechanism. Moreover, trimethylation of H3K9 has been shown to be more important during mitosis (34). Alternatively, but not exclusively, the closeness of the modified residues in the histone H3 tails brought about by the acquisition of a particular conformation may, on its own, promote condensation in a chain reaction fashion extending toward the neighboring tails.

Specific recognition of doubly modified histone H3 tails phosphorylated on S10 and acetylated on K9 has been documented (32). Our data suggest the existence of a yet-unidentified protein or complex that could affect chromatin condensation by specifically binding to the doubly modified and structured H3Kme2Sp tails that we describe here. In the future, the identification of such an activity would be extremely important to understand mitotic chromatin condensation.

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