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Phosphorylation of histone H3 is correlated with changes in the maintenance of sister chromatid cohesion during meiosis in maize, rather than the condensation of the chromatin

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SUMMARY

Meiotic chromosome condensation is a unique process, characterized by dramatic changes in chromosome morphology that are required for the correct progression of pairing, synapsis, recombination and segregation of sister chromatids. We used an antibody that recognizes a ser 10 phosphoepitope on histone H3 to monitor H3 phosphorylation during meiosis in maize meiocytes. H3 phosphorylation has been reported to be an excellent marker for chromosome condensation during mitotic prophase in animal cells. In this study, we find that on maize mitotic chromosomes only pericentromeric regions are stained; there is little staining on the arms. During meiosis, chromosome condensation from leptotene through diplotene occurs in the absence of H3 phosphorylation. Instead, the changes in H3 phosphorylation at different stages of meiosis correlate with the differences in requirements for sister chromatid cohesion at different stages. Just before nuclear envelope breakdown, histone H3 phosphorylation is seen first in the pericentromeric regions and then extends through the arms at metaphase I; at metaphase II only the pericentromeric regions are stained. In *afd1* (absence of first division), a mutant that is defective in many aspects of meiosis including sister chromatid cohesion and has equational separation at metaphase I, staining is restricted to the pericentromeric regions during metaphase I and anaphase I; there is no staining at metaphase II or anaphase II. We conclude that changes in the level of phosphorylation of ser10 in H3 correspond to changes in the cohesion of sister chromatids rather than the extent of chromosome condensation at different stages of meiosis.

Key words: Histone phosphorylation, Meiosis, Sister chromatid cohesion, Chromosome condensation, Maize

INTRODUCTION

During mitosis, chromosome condensation helps to ensure proper segregation of chromosomes by resolving tangles between sister chromatids and nonhomologous chromosomes. Compaction is believed to be achieved by the formation of loops and subsequent higher order folding of these loops (reviewed by Koshland and Strunnikov, 1996). Only a few factors, histones, topoisomerase II, and condensins, have been identified to play a role in condensation.

We know even less about the mechanisms and regulation of meiotic condensation and its function during the successive events of pairing and synapsis. Meiosis differs from mitosis by the requirement for chromosomes to find their homologs and pair prior to disjunction. During the homology search process, there is the formation of a thick tripartite proteinacious structure, the synaptonemal complex (SC), that acts as a glue to hold homologs together (Gillies, 1973; von Wettstein et al., 1984). After recombination, its disassembly is followed by the formation of chiasmata that, because of continuous complete sister cohesion hold the homologs together to insure proper disjunction at anaphase I. In addition, sister chromatid cohesion must remain intact around the kinetochores until anaphase II to insure the formation of balanced gametes (Miyazaki and Orr-Weaver, 1994).

There is a large body of evidence, mostly cytological, suggesting that meiotic chromatin must adopt a specialized morphology to allow the correct progression of meiosis. First, the initiation of pairing just prior to zygotene coincides with a global structural reorganization of the chromatin in maize (Dawe et al., 1994). The first condensation step in leptotene produces a highly condensed fiber, but the homologs stay separate. Then there is a dramatic transformation of the chromosomes at early zygotene: the sister chromatids separate slightly, the surface complexity of the fiber increases, the chromosome volume increases and some heterochromatic elements (the knobs) become long and thin and telomeres cluster on the nuclear envelope. From the time the homologs are fully synapsed at pachytene there is further reduction in length until the SC begins to be taken apart at diplotene.

Finally, after the SC is completely disassembled, the chromosomes align on the metaphase plate. This transition from a diakinesis to a metaphase I chromosome does involve further compaction of the chromatin which would be necessary to keep sister chromatids tightly opposed and to allow accurate segregation at anaphase I. Sister chromatid cohesion should remain intact in the centromeric regions until anaphase II, whereas cohesion along the arms is required until anaphase I.

Very little is known about the factors that are involved in the compaction of chromosomes. A strong correlation exists between the phosphorylation of histone H3 and mitotic chromosome condensation. Histone H3 is not phosphorylated during interphase but becomes phosphorylated at Ser10 just prior to metaphase (Gurley et al., 1978), consistent with a role in a late stage of chromosome condensation (Bradbury, 1992). Site-specific phosphorylation of core histone H3 at serine 10 correlates with mitotic chromosome condensation in mammalian cells and in ciliated protozoa (Ajiro et al., 1996; Hendzel et al., 1997; Wei et al., 1998). In somatic cells, H3 phosphorylation is initiated in pericentromeric heterochromatin in late G_2 and spreads throughout the chromosomes as they condense during mitotic prophase. It then decreases by late anaphase/early telophase as chromosomes begin to decondense. Further evidence for an involvement of H3 phosphorylation was recently shown in strains of *Tetrahymena* in which a mutant H3 gene that fails to phosphorylate contains defects in condensation and chromosome segregation (Wei et al., 1999).

Histone H3 phosphorylation takes place at meiosis in the protozoan *Tetrahymena* (Wei et al., 1998). Phosphorylation is initiated early in prophase I and hyperphosphorylation is triggered at metaphase I. It is not clear whether this represents a general phenomenon especially since meiotic stages are not easy to score in this protozoan. Meiosis in this organism is unique: the nucleus elongates in a crescent like shape during prophase I prior to extreme compaction of the chromosomes at metaphase I. In particular it is not known whether hyperphosphorylation takes place during the later waves of condensation where the fiber becomes hypercondensed at diakinesis or during the transition between diakinesis and metaphase I. Analysis of histone H3 phosphorylation in mouse spermatocytes suggests that this is a late stage event during meiotic prophase, beginning with the centromeric heterochromatin during diplotene (Cobb et al., 1999).

A thorough understanding of the relationship between the states of condensation and meiotic segregation requires high resolution studies of chromatin structure. The superb cytology of maize makes this organism ideal to conduct studies on chromatin condensation. It is easy to identify meiotic chromosomes and to score the early stages of meiosis. For example, at the pachytene stage, chromosomes are long (from 20 to 80 μm), with heterochromatic elements such as knobs, centromeres and chromomeres that are easy to identify to specific chromosomes. Furthermore, maize biology is facilitated by the availability of a large collection of chromosomal rearrangements (translocations, duplications, terminal deletions and inversions) and a large collection of mutants that affect chromosome behavior at meiosis (Carlson, 1988; Golubovskaya, 1989; Staiger and Cande, 1990).

Several mutations that affect chromosome condensation at various stages of meiosis have been identified in maize (reviewed by Golubovskaya, 1989) and their effect on histone H3 phosphorylation should clarify its role. One of these mutants, *afd1* (absence of first division) is defective in many aspects of prophase behavior including chromosome condensation and SC formation (Golubovskaya, 1989). Although *afd1* was first described as a mutant which substitutes the second meiotic division for the first (Golubovskaya, 1989), it is characterized by the premature condensation of the chromatin from an interphase fiber directly to a highly compacted diakinesis chromatin; two otherwise normal divisions follow. Sister chromatid cohesion is altered at both metaphase I and metaphase II. Analysis of H3 phosphorylation pattern in this mutant has allowed us to examine the relative roles of H3 phosphorylation in the maintenance of sister chromatid cohesion during metaphase I and II.

Using three-dimensional fluorescence microscopy and an antibody that recognizes a phosphorylated form of histone H3 (Hendzel et al., 1997; Wei et al., 1998), we have monitored changes in chromosome morphology during meiosis in maize. We found that most meiotic chromosome condensation occurs in the absence of ser10 H3 phosphorylation. Only the last wave of condensation is mitotic-like: when chromosomes are fully condensed at metaphase I, they are almost uniformly stained. Chromosomes are completely dephosphorylated by telophase I and phosphorylated at metaphase II. The signals then disappear again by telophase II. The timing of phosphorylation coincides with nuclear envelope breakdown at prometaphase I. The analysis of the meiotic condensation mutation *afd1* assigns a possible role for phosphorylation in the maintenance of sister chromatid cohesion, as opposed to a role in the regulation of chromosome condensation.

MATERIALS AND METHODS

Plant material

The three inbred lines of maize, A344, W23, and W22 were grown for about six weeks under greenhouse conditions and harvested throughout the year. Since the meiotic mutant *afd1* is recessive sterile, it was kept as a heterozygous stock; seeds from those stocks were grown and both the mutants and normal siblings (kept as controls) were studied. *Afd1* (W23) was graciously provided by Dr Inna Golubovskaya (Vavilov Research Institute for Plant Industry, St Petersburg, Russia). Seeds from the maize genotypes A344, B73, W22, and W23, as well as rye (variety Blanco) and the field bean were allowed to germinate and the roots (1 cm from the tip) were harvested. The male meiocytes develop within anthers which are borne in the tassel. The developmental gradient along the tassel branches allows us to score the meiotic stage based on the length of the anther. In addition each anther contains hundreds of meiocytes that undergo the early prophase stages in synchrony (Staiger and Cande, 1990). Tassels were collected at about 6 weeks post germination, and anthers between 1.5 and 4 mm were dissected from their flowers.

Fixation and preparation of somatic cells

Root tip cells were fixed for 1 hour at room temperature in 1 ml of 4% formaldehyde in meiocyte-Buffer-A (15 mM PIPES-NaOH, pH 6.8, 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 2 mM EDTA, 0.15 mM spermine tetra HCl, 0.05 mM spermidine, 1 mM dithiothreitol, 0.32 M sorbitol) for 60 minutes in a gently rotating 1.5 ml microfuge tube. They were then washed 3 times, 10 minutes each, with Buffer-A and stored at 4°C in fresh buffer-A. One or two roots were then cut in several pieces using a razor blade and transferred onto a glass slide

coated with 3-aminopropyltriethoxysilane (Sigma). A coverslip was then put over the roots and pressure was applied on the glass slide with the rubber eraser of a pencil to separate the cells. The coverslip was removed with a razor blade and the slide was immediately spun at 50 ***g*** for 30 seconds in a clinical centrifuge (Sorvall). Although some liquid was lost during the spin, the cells remain intact. About 100 to 300 μl of 1× PBS was added to the slide prior to FISH or immunostaining.

Fixation and preparation of meiocytes

Anthers from pre-emerged tassels were fixed and stored as described for somatic cells. Fixed anthers were then cut open at their tips to release the meiocytes into 100-200 μl of Buffer-A. 10 μl of meiocytes suspended in meiocyte-Buffer-A were then transferred by micropipetting onto a glass slide followed by the immediate addition of 5 μl of activated acrylamide stock. The activated acrylamide stock was made by the addition of 25 μl of 20% ammonium persulfate and 25 μl of 20% sodium sulfite to 500 μl of gel stock (15 mM PIPES-NaOH, pH 6.8, 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 2 mM EDTA, 0.15 mM spermine tetra HCl, 0.05 mM spermidine, 1 mM dithiothreitol, 0.3 M sorbitol, 15% polyacrylamide (from a 30% 29:1 acrylamide:bis acrylamide stock). The slides were rocked and rotated for 15 seconds until the drops mixed, and a coverslip (22×22×1.5 mm) was placed on top for 30 minutes, and then removed with a razor blade, leaving a thin pad of acrylamide with embedded meiocytes attached to the slide.

In situ hybridization and immunolocalization

Newly polymerized acrylamide pads were washed with 1× PBS to remove unpolymerized acrylamide, followed by four equilibration washes with prehybridization buffer (50% deionized formamide, 2× SSC). Hybridization buffer (50% deionized formamide, 2× SSC, 1-3 mg oligonucleotide) was added, removed, and added a second time. The centromeric oligonucleotide was designed from 27 bp 5′-CCTAAAGTAGTGGATTGGGCATGTTCG-3′ of a conserved region of a maize repeated sequence cent-C (Ananiev et al., 1998). The oligonucleotide was labelled by end labelling using the modified base 'Texasred-dUTP' (Genset, France). About 30 μl of probe in hybridization buffer was added, then sealed under a coverslip using rubber cement, and placed in a 40°C warming plate for 30 minutes. The chromosomes were denatured on a PCR block at 90°C for 5 minutes followed by overnight incubation at 37°C. The samples were then washed sequentially with 2× PBS containing 0.1% Tween-20 (4 times), 1× PBS (3 times). Immunofluorescence with the phosphorylated H3 antibody was done by permeabilizing meiocyte-containing pads or root cells for 1 hour in 1× PBS, 1% Triton X-100, 1 mM EDTA, then washing several times in 1× PBS. About 50 μl of diluted (1/500) phosphorylated H3 antibody (gift from C. D. Allis, University of Virginia) was added to the pad. The incubation was conducted for 15 hours in a humid chamber; a cover glass was added to reduce dessication. Washes were conducted in the washing solution 1× PBS, 0.1% Tween-20, 1 mM EDTA several times, for several hours. A secondary antibody was added (FITC-labeled Donkey anti-rabbit; FAB fragment at 1 μg/ml) and allowed to diffuse overnight. Samples were then washed several times overnight with washing solution. A monoclonal antibody that recognizes a component of the nuclear pore complex from rat liver nuclei was used at the 1/200 dilution (Laura and Blobel, 1986). Then a secondary goat anti-mouse antibody labelled with Texas red was added to the slide. Incubation and washes are as indicated above.

The samples were then stained with 10 μg/ml DAPI (4′,6-diamidino-2-phenylindole, dihydrochoride) in 1× PBS for 30 minutes at room temperature. Excess DAPI was removed by washing with 1× PBS (3 times) for a total of 30 minutes. Slides were equilibrated in a fluorochrome stabilizing medium (*p*-phenylene diamine). A coverslip (22×22×1.5 mm) was placed on top of the pad and sealed with transparent nail polish.

Phosphatase assay

Following fixation in 4% formaldehyde, meiocytes were squeezed out of anthers in 1× PP1 buffer (50 mM Tris-HCl, 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% Brij 35). About 0.1 units of protein phosphatase 1 (PP1; New England Biolabs) was added to 15 μl of meiocyte in PP1 buffer (or 0.1 units of PP1 with 10 μM microcystin-LR) in a 500 μl microfuge tube, and incubated for 30 minutes at 37°C. Following the digest about 10 μl of meiocytes were used for immunofluorescence as indicated in the immunofluorescence section.

3-Dimensional microscopy and image processing

All images were recorded on a Delta Vision deconvolution light microscope work station (Applied Precision, Issaquah, WA.) using a Olympus wide-field inverted microscope, equipped with the oil immersion lens 100× Plan-Neofluar or 60× Plan-Apochromat. Data were collected in the X, Y and Z dimensions as described elsewhere (Chen et al., 1995). Following 3-dimensional iterative deconvolution of the original full-sized data sets, individual nuclei were computationally cropped in all dimensions. The images were then adjusted only for brightness and contrast using linear scaling of the minimum and maximum intensities.

RESULTS

Plant mitotic chromosomes are phosphorylated primarily in the pericentromeric heterochromatin during late prophase

We used a polyclonal antibody raised against a synthetic peptide from the amino-terminus of H3 from amino acids 7-22 (ARKSTGGKAPRKLPLC) with a single phosphorylated serine at position 10 (Hendzel et al., 1997). This antibody cross-reacts with maize proteins of the appropriate molecular mass on western blots (J. Thai and W. Z. Cande, unpublished data). In addition the antibody is likely to recognize the same peptide in maize since the maize histone 3 amino acid sequence is identical to the synthetic peptide sequence used for immunization (Chaubet et al., 1987).

We first determined the distribution of phosphorylated H3 antibody in maize mitotic cells. Mitotic cells were obtained from root tips of germinating seeds. During interphase, punctuated staining is observed in the nucleolus organizing region (NOR), but not along the rest of the chromatin (Fig. 1A). It does not appear to stain DNA since there is low or no DAPI staining within the NOR. During prophase as chromosomes condense, phosphorylation takes place in discrete regions of some of the chromosomes (Fig. 1B) and by late prophase, all chromosomes are stained (Fig. 1C). As chromosomes align to the metaphase plate (Fig. 1D) the staining is found on all chromosomes and in many instances it stains regions that coincide to or are very near to the primary constriction. In many cells we find that the number of initiation sites matches the diploid chromosome number ($2n$=20). The distribution of phosphorylated H3 antibody does not change at anaphase (Fig. 1E). Pericentromeric phosphorylation then disappears by telophase (Fig. 1F). These results are in contrast to studies in mammalian cells where the entire lengths of chromosomes are stained by the end of prophase (Hendzel et al., 1997; van Hooser et al., 1998). Different fixation conditions, the use of phosphatase inhibitors during fixation, or very short fixation times using a microwave did not alter the distribution of the phosphorylated H3 antibody. We also obtained similar results

with various maize inbred lines, and other plant species (rye, field bean) (not shown). This suggests that the differences in phosphorylation patterns observed between maize and mammalian mitotic chromosomes is not specific to maize, but is representative of many plant species.

Meiotic chromosome condensation: phosphorylation of histone 3 takes place at the diakinesis-metaphase I transition

Dramatic changes in chromosome compaction take place at the onset of meiotic prophase. We expected that phosphorylation would be initiated during the early stages of compaction. Instead we detect only two signals which localize to the NOR during the leptotene and diakinesis stages of prophase I (Fig. 2A,C), and a single signal at the pachytene stage when the homologs are fully paired (Fig. 2B). Very long exposures did not reveal any phosphorylated H3 signal colocalizing with the chromatin. Different fixation conditions using methanol or acetic acid did not modify the distribution of the antibody (data not shown). The integrity of the nuclear envelope was monitored with an antibody that recognizes a component of the nuclear pore complex (Laura and Blobel, 1986). As expected the nuclear pore staining is seen during prophase I (leptotene to diakinesis) and during telophase I and II (Fig. 2, blue staining). Just prior to metaphase I (Fig. 2D) there is an abrupt transition: the entire lengths of all chromosomes are stained, and the signals persist into anaphase I (Fig. 2E). These signals disappear by telophase I; only one cluster of small spots remains that coincides with the nucleolus (Fig. 2F). Phosphorylation is visible again at metaphase II, but it is found mostly in the pericentromeric regions of all chromosomes (Fig. 2G). Low levels of phosphorylation are detected along chromosome arms. The signal persists during anaphase II (Fig. 2 H). It decreases in intensity by late anaphase II and disappears as the cell enters telophase II (Fig. 2I).

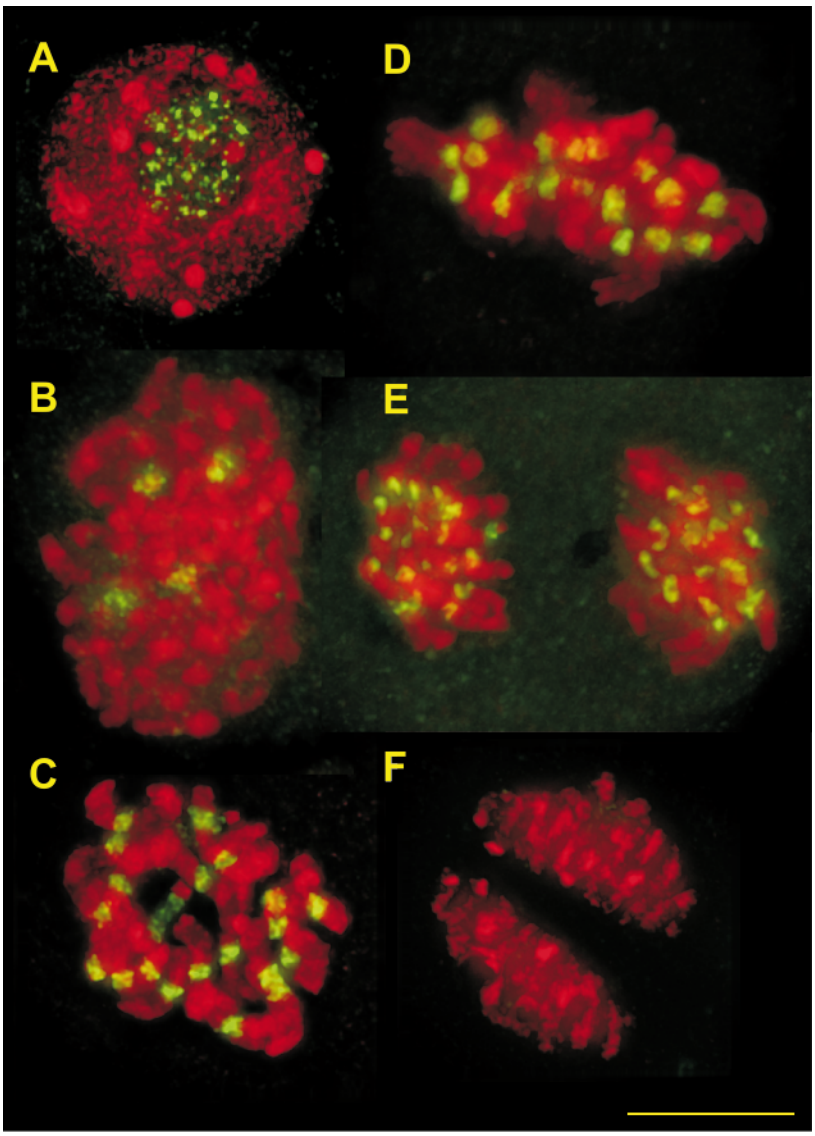

Fig. 1. Distribution of phosphorylated histone H3 antibody in maize somatic nuclei. Projections of several optical sections of wild-type maize mitotic root cell nuclei. DAPI-stained chromosomes are red, and the polyclonal antibody raised against phosphorylated ser-10 histone H3 is green and yellow where it overlaps the red chromatin. (A) A344 interphase nucleus. The staining is restricted to the NOR. (B) Early prophase stage: phosphorylation is found at discrete locations on several chromosomes. (C) Late prophase stage: phosphorylation of H3 is found at the pericentromeric regions of most chromosomes. (D and E) Metaphase and anaphase, respectively: phosphorylation is found mostly in the pericentromeric regions, very little is detected along chromosome arms. (F) Telophase: phosphorylation is no longer detected on the chromosomes, but only on the reforming nucleoli. Bar, 10 μm, for all figures.

Protein phosphatase 1 treatment of meiocytes leads to the disappearance of H3 phosphorylation on condensed chromosomes

To further demonstrate that the phosphorylated H3 antibody recognizes the phosphate moiety on serine 10 residue, protein phosphatase 1 (PP1) was applied to fixed meiocytes. PP1 preferentially releases phosphate groups from phosphoserine/threonine residues in proteins. Following incubation of PP1 with meiocytes extruded from anthers, meiocytes were stained with the phosphorylated H3 antibody. No signal is detected on segregating chromosomes during metaphase I, anaphase I, metaphase II or anaphase II (Fig. 3A, metaphase I). Only signals that colocalize with the NOR remain at pachytene and diakinesis, as well as in adjacent tapetal cells (not shown). This could indicate that either the phosphorylated H3 antibody recognizes other epitopes, or that phosphate residues in the nucleolus are not accessible to PP1 in some manner.

As a control for specific activity of PP1 on phosphate groups, we used PP1 in combination with the specific inhibitor microcystin-LR. In meiocytes treated with PP1 with Microcystin-LR and stained with phosphorylated H3 antibody, chromosomes are stained as in normal meiocytes, at all stages of meiosis I and II (Fig. 3B, metaphase I). These observations strongly

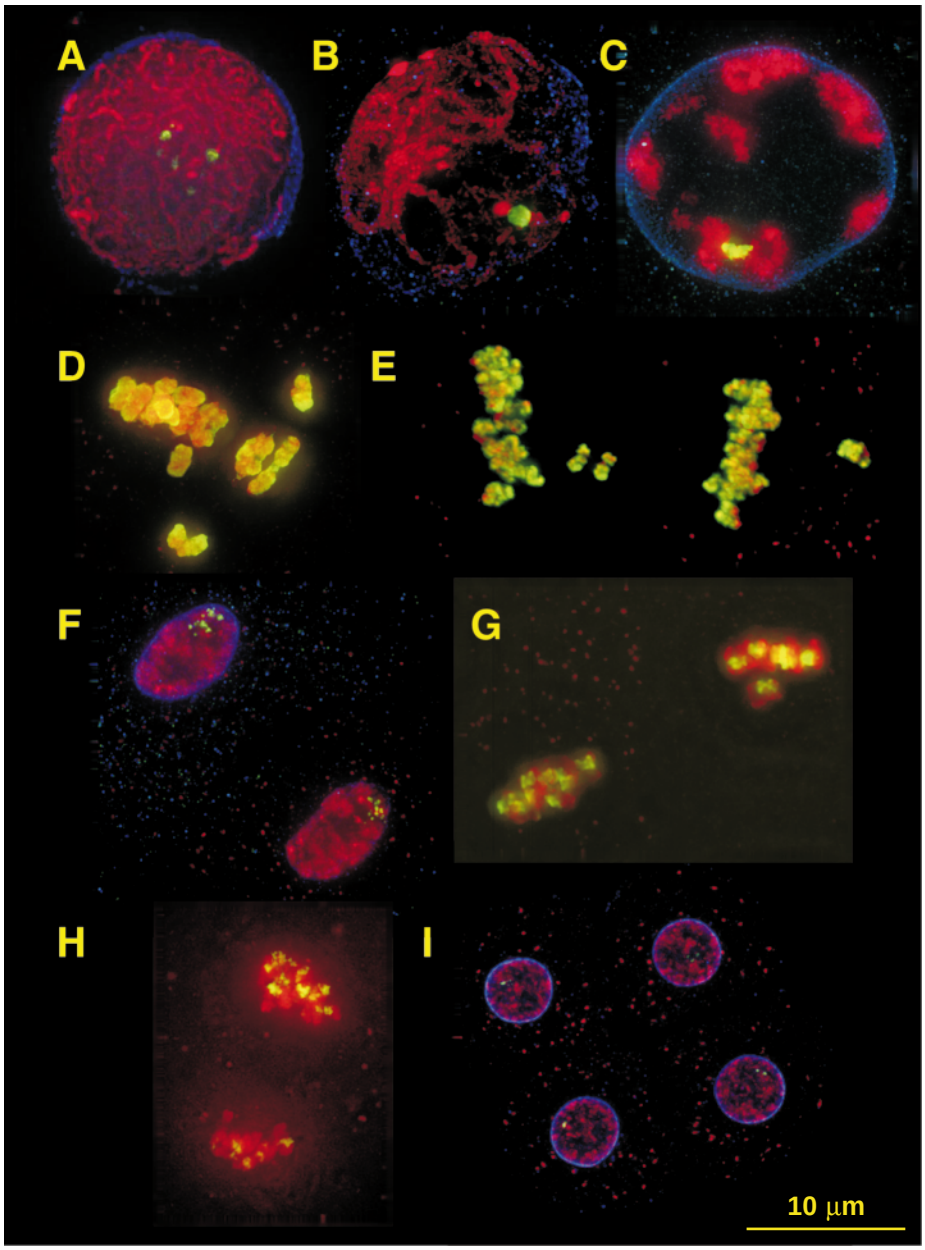

Fig. 2. Distribution of phosphorylated histone H3 antibody correlates with late meiotic condensation in maize normal meiocytes. Projections of several optical sections of wild-type maize meiocytes. The DAPI-stained chromosomes are red, the polyclonal antibody raised against phosphorylated ser-10 histone H3 is green or yellow, the monoclonal antibody raised against a component of the nuclear pore complex from rat liver nuclei is shown in blue. Substages of meiosis are shown in successive order. (A) Leptotene. (B) Pachytene. (C) Diakinesis. (D) Metaphase I. (E) Anaphase I. (F) Telophase I. (G) Metaphase II. (H) Anaphase II. (I) Telophase II.

suggest that PP1 activity is responsible for the removal of phosphate groups, leading to decreased staining of chromosomes.

Phosphorylation of histone 3 coincides with the breakdown of the nuclear envelope

To precisely time the initiation of phosphorylation in relation to nuclear envelope breakdown, we used an antibody that recognizes a component of the nuclear pore complex from rat liver nuclei (Laura and Blobel, 1986); it cross-reacts with maize proteins (J. Thai and Z. Cande, unpublished data). This antibody is a good marker for the integrity of the nuclear envelope since nuclear pores are structural components of the nuclear envelope and that are dispersed during nuclear envelope breakdown. Nuclear envelope breakdown takes place at the transition to prometaphase and it coincides with a redistribution of microtubules to form the bipolar spindle (reviewed by Gant and Wilson, 1997; Chan and Cande, 1998). We used indirect immunofluorescence to analyze the distribution of nuclear pores and its relation to the extent of H3 phosphorylation from leptotene until telophase II.

At leptotene the nuclear pore antibody recognizes the entire contour of the nuclear envelope (Fig. 2A). The staining is punctuated revealing that nuclear pores are present throughout the nuclear envelope. At diakinesis the chromosomes are arranged along the nuclear envelope and they are frequently in contact with the envelope (Fig. 2C). From leptotene to diakinesis H3 phosphorylation is detected only in the nucleolus (Fig. 2A-2C). We found in a few diakinesis cells phosphorylated H3 staining in the pericentromeric regions of all chromosomes (Fig. 4, top). Furthermore nuclear pore staining is closely associated with the chromatin. These cells very likely represent a stage late in diakinesis. It is a transient stage since only 8 such cells were found out 101 meiocytes that were dissected from a single anther (Table 1). Within one anther, all meiocytes are in a similar stage of development. Prometaphase I is also a transient stage (7 out of 101 meiocytes; Fig. 4 bottom). Chromosomes appear more compacted but they are not completely aligned onto the metaphase plate, and

Table 1. Phosphorylation of histone H3 initiates rapidly between the diakinesis and prometaphase I stages

	Antibody staining		
Stage	Phosphorylated H3	Nuclear pore	Sample (*n*)
Diakinesis	No	Yes	44
Late diakinesis	Yes	Yes	8
Prometaphase I	Yes	No	7
Metaphase I	Yes	No	32

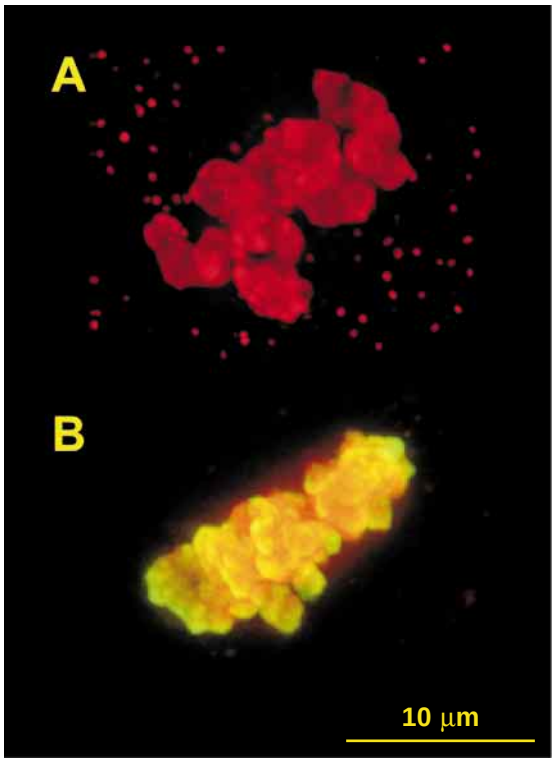

Fig. 3. Protein phosphatase 1 treatment of meiocytes leads to the disappearance of the phosphorylated H3 signal. Projections of several optical sections of wild-type maize meiocytes. The DAPI-stained chromosomes are red, and the polyclonal antibody raised against phosphorylated ser-10 histone H3 is green or yellow. (A) Metaphase I chromosomes treated with 0.1 unit of PP1. No phosphorylated H3 signal is detected. (B) Metaphase I chromosomes treated with 0.1 unit of PP1 in the presence of 10 uM microcystin-LR, a serine-threonine phosphatase inhibitor. The phosphorylated H3 signal is restored.

they are stained along their entire length with the phosphorylated H3 antibody. Maximum phosphorylation coincides with the pericentromeric regions. The nuclear pore signal has now disappeared, indicating that the nuclear membrane has disassembled. Nuclear pore staining is observed again at telophase I (Fig. 2F), coincident with decondensed chromatin. As chromosomes recondense at metaphase II, the nuclear pore signal disappears again, and it reappears at telophase II (Fig. 2I). These results suggest that H3 phosphorylation and dephosphorylation are coincident to the events of nuclear envelope disassembly and reformation respectively.

H3 phosphorylation is initiated in the pericentromeric regions of all chromosomes during mitosis and meiosis

Previous studies indicate that histone H3 phosphorylation is initiated in pericentromeric heterochromatin and advances outward into the chromosome arms (Hendzel et al., 1997; van Hooser et al., 1998). We find that in mitotic maize cells, phosphorylation is coincident with the primary constriction of chromosomes (Fig. 1C). To determine whether phosphorylation is also initiated in the centromeric regions of meiotic chromosomes, we combined immunofluorescence and

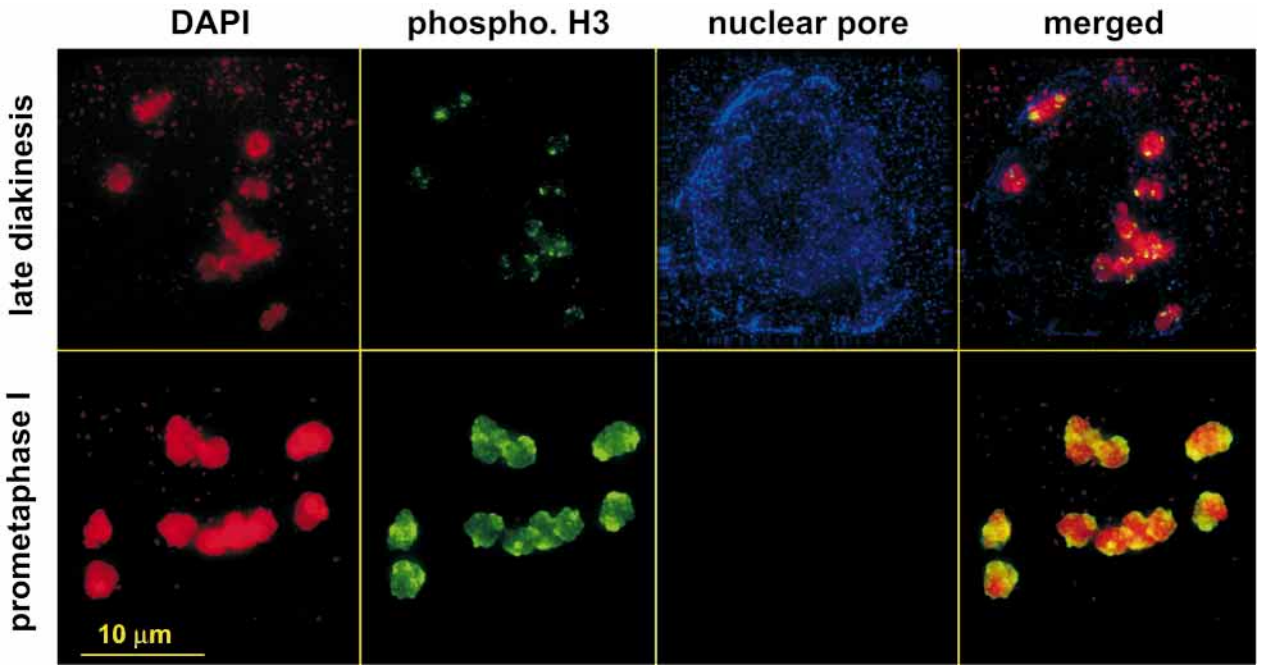

Fig. 4. H3 phosphorylation coincides with the breakdown of the nuclear envelope. Projections of optical sections from wide-field fluorescence microscopy of wild-type maize meiocytes. The DAPI-stained chromosomes are red, the polyclonal antibody raised against phosphorylated ser-10 histone H3 is yellow, the monoclonal antibody raised against a component of the nuclear pore complex from rat liver nuclei is blue. Phosphorylation is initiated in pericentromeric regions of the chromosomes during late diakinesis (top), and the signal spreads along the arms as seen during prometaphase I (bottom).

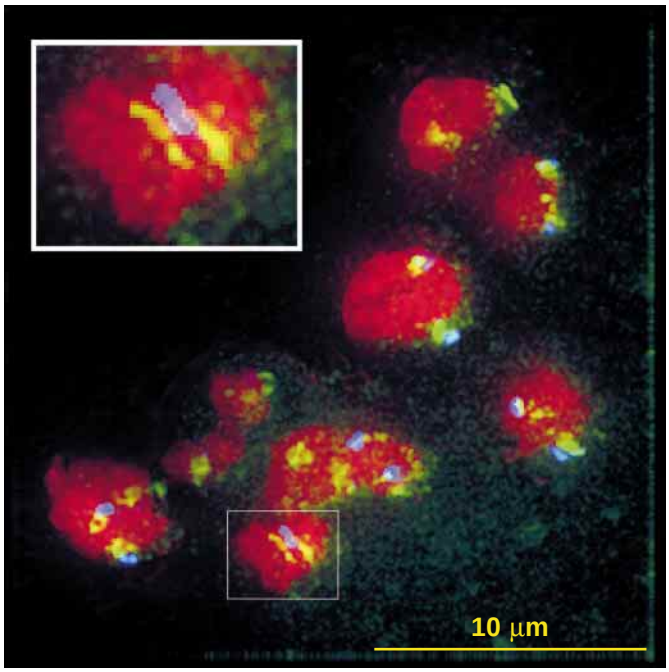

Fig. 5. H3 phosphorylation initiates in the pericentromeric regions of all chromosomes. Projections of several optical sections of wild-type maize meiocytes. The DAPI-stained chromosomes are red, the polyclonal antibody raised against phosphorylated ser-10 histone H3 is green or yellow, the FISH probe corresponding to the cent-C maize centromeric repeat is blue. The image in the corner corresponds to a magnification of one chromosome.

FISH using a probe specific to all maize chromosome centromeres (Ananiev et al., 1998). At prometaphase I chromosomes are phosphorylated at both ends of each pair of homologs where centromeres lie (Fig. 5). This is the exact same stage seen in Fig. 4 (prometaphase I, merged signal). Overlapping FISH and H3 phosphorylated signals indicate that phosphorylation is highest in all centromeric regions. Furthermore phosphorylation is mostly confined to pericentromeric regions because the signals flank the FISH centromeric signals as shown in the magnified insert (the centromeric signal appears blue with its flanking two stained regions of phosphorylation in yellow). We conclude that, as in vertebrates, H3 phosphorylation initiation is pericentromeric in maize.

Phosphorylation of histone H3 in *afd1* is restricted to pericentromeric regions at metaphase I and it and does not occur at metaphase II

Phosphorylation is initiated during a late step of condensation at the breakdown of the nuclear envelope. We next examined the distribution of phosphorylated H3 antibody in the meiotic mutant *afd1* (absence of first division). The early steps of condensation (leptotene to pachytene) are omitted in *afd1*, leading to 20 univalents at metaphase (Golubovskaya 1989; Chan and Cande, 1998). Since there is no formation of a leptotene fiber at early prophase, homologs cannot pair or undergo recombination. A very low level of synapsis is usually observed. In addition there are changes in chromatin structure during prophase I. A low level of compaction is observed before diakinesis where one can follow the path of the chromosomes (Fig. 6A,B). Diakinesis chromosomes are less condensed than in the wild-type (compare Fig. 6B and Fig. 2C), and thin fibers connect different chromosomes. This stage (Fig. 6B) is equivalent to diplotene and early diakinesis and the chromosomes do not display any phosphorylation. Therefore phosphorylation does not occur prematurely in this mutant and it reinforces our observations that phosphorylation is not correlated with the changes in condensation during meiotic prophase. Then there is an abrupt transition that takes place at metaphase I: all univalent chromosomes are phosphorylated in the pericentromeric regions (Fig. 6C). The staining is very similar

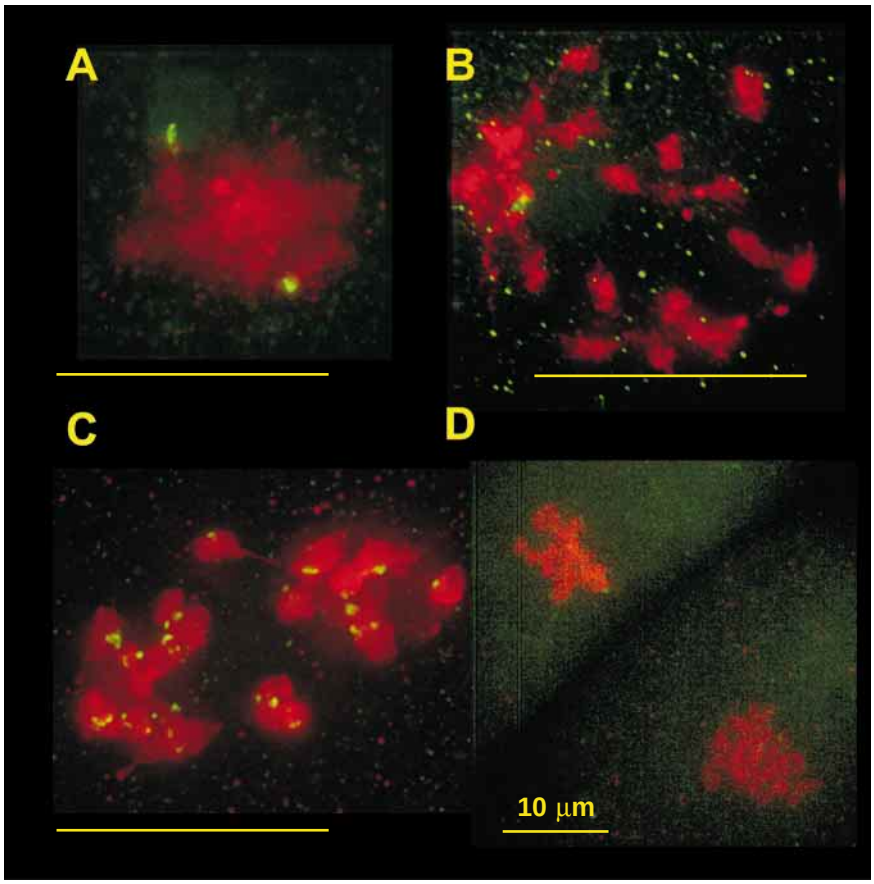

Fig. 6. H3 hyperphosphorylation is restricted to pericentromeric regions in the *afd1* mutation. Projections of several optical sections of *afd1* meiocytes. The DAPI-stained chromosomes stained are red, and the polyclonal antibody raised against phosphorylated ser-10 histone H3 is yellow. No antibody signal is found on the chromosomes at an early stage of prophase (A), or later, i.e. diakinesis (B). Phosphorylation is first detected at metaphase I (C). It is not found on metaphase II chromosomes (D).

to what is observed in univalent chromosomes in normal meiocytes at metaphase II. The twenty univalents in *afd* segregate equationally at anaphase I. The sister chromatids separate as a result of premature division of the centromeres, and the chromatids move to opposite poles. The staining has disappeared at late anaphase I (not shown). Phosphorylation at metaphase II and anaphase II was examined and we found that no phosphorylation was detected on any region of the chromosomes including pericentromeric regions (see Fig. 6D). Positive signals on adjacent metaphase I and anaphase I cells confirmed the lack of phosphorylation at meiosis II in *afd* (data not shown).

DISCUSSION

Meiotic chromosome condensation in maize mitotic and meiotic cells was followed by the use of an antibody that recognizes a phosphorylated form of ser 10 in histone H3. The pattern of H3 phosphorylation was determined in wild-type and in the meiotic condensation mutation *afd1*. The pattern of H3 phosphorylation correlates strongly with the maintenance of sister-chromatid cohesion, but not with a role in meiotic condensation per se. Histone H3 phosphorylation is tightly correlated with the condensation of mitotic chromosomes in animal cells (Hendzel et al., 1997; Wei et al., 1998; van Hooser et al., 1998): phosphorylation initiates late in prophase and is maximal at metaphase. Dephosphorylation takes place as chromosomes decondense in late anaphase. We wanted to know the distribution of a phosphorylated histone H3 antibody vis a vis meiotic chromosome compaction because the compaction of chromosomes during the meiotic cell cycle is unique. Indeed, dramatic changes in condensation take place during prophase I. In particular, the transition from paired chromosomes (pachytene) to the diassembly of the SC (diplotene/diakinesis) results in a very high level of compaction. Maize chromosomes contract more than 5 times along the axial core from early pachytene to late diakinesis (E. Kaszás and Z. Cande, unpublished observations). Metaphase I represents the highest level of compaction with chromosomes that are smaller than their mitotic counterparts. In this study we unexpectedly find that phosphorylation initiates at the diakinesis-prometaphase I transition. Thus, phosphorylation occurs late at a time when chromosomes are already highly compacted and when homologs are ready to align to the metaphase plate. This suggests that the dramatic changes in morphology for the initial steps in condensation from leptotene to early diakinesis do not require H3 phosphorylation. Recently, Cobb et al. (1999), using mice spermatocytes and the same antibody that we used in this study, came to a similar conclusion. They demonstrated that H3 phosphorylation does not occur until late diplotene and that it is initiated at the centromeric heterochromatin and spreads along the chromosome arms. Our results contradict meiotic studies in *Tetrahymena*, where phosphorylation initiates at a stage equivalent to zygotene (initiation of pairing) (Wei et al., 1998). Meiotic prophase in *Tetrahymena* is unusual with an elongation of the cell in a crescent-like shape. Because of the unusual alignment of meiotic chromosomes in this protozoan the chromatin may adopt a different mode of compaction.

Phosphorylated H3 does not correlate with condensation of the chromatin

We find that in mitotic plant cells ser 10 H3 phosphorylation initiates at late prophase and it is maximal at metaphase; it then disappears by late anaphase/telophase. The observation that ser10 H3 phosphorylation occurs late during mitotic prophase suggests that it is unlikely that H3 phosphorylation plays an important role in mitotic chromosome condensation in plant cells. In parallel to studies in mammalian cells (Hendzel et al., 1997; Wei et al., 1998), phosphorylation is first visible in pericentromeric regions. However, it occurs during late prophase. Nevertheless there are several significant differences. First, the arms are considerably less phosphorylated relative to pericentromeric regions on mitotic metaphase chromosomes. Secondly, the timing of phosphorylation is shifted: phosphorylation is first visible at metaphase. Histone H3 phosphorylation is not uniform on maize chromosomes during mitosis and meiosis II. There is phosphorylation around the centromeres and very little or none on the rest of the chromosomes. However, during metaphase I of meiosis the staining of the chromosomes is much more uniform along their lengths. This contrasts with studies of mitotic chromosome condensation in other organisms (Hendzel et al., 1997; Wei et al., 1998).

Phosphorylation of histone H3 correlates with the maintenance of sister chromatid cohesion

The distribution and timing of phosphorylation of histone H3 coincides with the sites and the time at which sister chromatid cohesion is maintained. It can not be involved with its establishment since sister chromatid cohesion can be detected during leptotene, although it is transiently altered during zygotene (Dawe et al., 1994). Pericentromeric regions are the major sites of H3 phosphorylation during both mitosis and meiosis II. Indeed sister chromatid cohesion is maintained in the centromeric regions until anaphase in chromosomes during both mitosis and meiosis II (Miyazaki and Orr-Weaver, 1994). During meiosis I cohesion is maintained throughout the entire lengths of the chromosomes. Sister arm cohesion is released at anaphase I but sister centromere cohesion is only released at anaphase II to allow segregation of the single sister chromatids. The distribution of H3 phosphorylation correlates very well with the maintenance and release of sister chromatid cohesion. In normal meiocytes we observe staining along the lengths of all chromosomes at metaphase I. Then the staining is found mostly in pericentromeric regions at metaphase II and anaphase II. Further support for a role of H3 phosphorylation in cohesion is provided by the study of *afd1*. In this mutant the initial steps of meiotic condensation do not take place, leading to a lack of homologous pairing and to the formation of 20 univalents. Unlike desynaptic mutants such as *dsy1*, sister chromatid cohesion is altered in *afd1*. The *afd1* chromosomes undergo sister chromatid separation at anaphase I exactly like mitotic chromosomes (Golubovskaya, 1989; Chan and Cande, 1999). As chromosomes migrate to the metaphase I plate, phosphorylation is found only in the pericentromeric regions. At the second meiotic division, *afd1* chromosomes consist of individual sister chromatids and there is no sister chromatid cohesion. This is correlated with an absence of phosphorylation in the pericentromeric regions. These results

strongly suggest that H3 phosphorylation is involved in regulation or maintenance of cohesion in the centromeric regions. There is no sister chromatid cohesion in *afd1* at metaphase II, and no phosphorylation is observed.

On the basis of these observations we can suggest at least two possible models for the role of serine 10 phosphorylation during meiosis: first, phosphorylation could protect sister chromatid cohesion locally, perhaps by stabilizing cohesin binding to chromatin during metaphase. This regulation would be required since most cohesins dissociate from chromosomes during prophase-prometaphase (Losada et al., 1998). There has be some way to prevent their dissociation from the chromosomes around the centromeres during metaphase I. Alternatively, phosphorylation could prepare the chromosomes for the destruction of cohesion at the metaphase to anaphase transition. The signals remaining at anaphase would imply that endogenous phosphatases have not yet acted on the phosphorylated sites.

Our findings on the timing and distribution of the phosphorylated H3 antibody are in accordance with studies on the *Drosophila* protein MEI-S332. MEI-S332 is required for cohesion between the centromeres of the sister chromatids during meiosis and mitosis (Kerrebrock et al., 1995). MEI-S332 does not localize on the chromosomes in the prophase I karyosome. Instead it assembles at a time when the nuclear envelope breaks down and the spindle begins to form. Furthermore, the MEI-S332 protein localizes to the centromeric regions during mitosis and it dissociates from the chromosomes at the onset of anaphase when the sister chromatids separate from one another (Moore et al., 1998; Tang et al., 1998). Our results show that phosphorylation of H3 is detected late during mitotic and meiotic prophase and not on the chromosomes immediately after DNA replication when cohesion has been established. Consequently, histone H3 phosphorylation can not be involved in the initiation of sister chromatid cohesion. Altogether our results indicate that histone 3 phosphorylation could be involved in the maintenance of sister chromatid cohesion.

Concluding remarks

In this study, the distribution of a phosphorylated histone H3 antibody is used as a marker for the transition from an already highly condensed chromatin (diakinesis) to a maximum level of compaction (metaphase I). Phosphorylation also coincides with the events of condensation during meiosis II.

Studies of mutations that affect the normal condensation of the meiotic chromatin at different substages suggest a new role of phosphorylation in sister chromatid cohesion. Alteration of histone H3 phosphorylation distribution in *afd1* at metaphase I and anaphase I and its absence at metaphase II and anaphase II suggest its participation in the maintenance of sister chromatid cohesion (Fig. 6, *afd1*). The recent discovery of cohesion proteins and their interaction with members of the SMC (condensin) family of proteins (Michaelis et al., 1997; Losada et al., 1998) suggest a link between both processes. A meiosis specific cohesin complex has been identified in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Klein et al., 1999; Watanabe and Nurse, 1999). The Rec8 protein is present as punctuate foci in early prophase I and subsequently localizes to the centromere and adjacent regions. After the metaphase I/anaphase I transition, the amount of Rec8 protein is reduced and that remaining is present solely at the centromere. Rec8 is no longer observed after sister-chromatid separation at anaphase II. The distribution and timing of Rec8 staining strikingly parallels the distribution of phosphorylated H3 antibody in maize meiotic chromosomes.

During meiosis the mechanism underlying cohesion of sister chromatids along their arms is clearly different from that responsible for cohesion in the centromere region. Studies in yeast (reviewed by Orr-Weaver, 1999) and vertebrates (Rieder and Cole, 1999) show that the chromatids on a mitotic chromosome are also tethered along their arms and in the centromere by different mechanisms. In maize the preferred localization of phosphorylated H3 is in the pericentromeric regions. It is initiated at prometaphase and dephosphorylation is first observed when chromosomes have separated: this strongly suggests a primary role of H3 phosphorylation in the maintenance of sister chromatid cohesion at the centromere. High levels on chromosome arms on metaphase I chromosomes suggest that it may also play a role in cohesion of the chromatids along the arms.

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