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# Meiotic events at the centromeric heterochromatin: histone H3 phosphorylation, topoisomerase IIα localization and chromosome condensation

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**Abstract.** Mechanisms of chromosome condensation and segregation during the first meiotic division are not well understood. Resolution of recombination events to form chiasmata is important, for it is chiasmata that hold homologous chromosomes together for their oppositional orientation on the meiotic metaphase spindle, thus ensuring their accurate segregation during anaphase I. Events at the centromere are also important in bringing about proper attachment to the spindle apparatus. This study was designed to correlate the presence and activity of two proteins at the centromeric heterochromatin, topoisomerase IIα (TOP2A) and histone H3, with the processes of chromosome condensation and individualization of chiasmate bivalents in murine spermatocytes. We tested the hypothesis that phosphorylation of histone H3 is a key event instigating localization of TOP2A to the centromeric heterochromatin and condensation of chromosomes as spermatocytes exit prophase and progress to metaphase. Activity of topoisomerase II is required for condensation of chromatin at the end of meiotic prophase. Histone H3 becomes phosphorylated at the end of prophase, beginning with its phosphorylation at the centromeric heterochromatin in the diplotene stage. However, it cannot be involved in localization of TOP2A, since TOP2A is localized to the centromeric heterochromatin throughout most of meiotic prophase. This observation suggests a meiotic function for TOP2A in addition to its role in chromatin condensation. The use of kinase inhibitors demonstrates that phosphorylation of histone H3 can be uncoupled from meiotic chromosome condensation; therefore other proteins, such as those constituting metaphase-promoting factor, must be involved. These results define the timing of important meiotic events at the centromeric heterochromatin and provide insight into mechanisms of chromosome condensation for meiotic metaphase.

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**Abbreviations.** *MI* Metaphase I – *OA* Okadaic acid – *MPF* Metaphase-promoting factor – *SC* Synaptonemal complex – *DAPI* 4',6-diamidino-2-phenylindole – *topo II* Topoisomerase II – *TOP2A* Topoisomerase IIα isoform – *TOP2B* Topoisomerase IIβ isoform – *SARs* Scaffold attachment regions – *PROD* Proliferation disrupter – *SMC* Structural maintenance of chromosomes

## Introduction

The completion of meiotic prophase in murine spermatocytes is defined by dramatic transitions in chromatin structure. After homologous chromosomes have been intimately synapsed throughout the pachytene stage, desynapsis begins abruptly during diplonema; chromatin condenses as chiasmate bivalents begin to individualize and the synaptonemal complex (SC) is disassembled. By metaphase I (MI), homologous chromosomes are held together only at their points of recombination, the chiasmata. Accurate segregation of chromosomes at anaphase I requires completion of recombination during the preceding prophase, because it is the chiasmata that hold the homologous chromosomes together for their correct orientation on the MI spindle. Therefore, meiotic recombination and repair events must proceed to a point where they can be successfully resolved as chiasmata before chromosome condensation commences. Events of recombination and cell-cycle progress must be coordinated, perhaps by mechanisms unique to meiosis. The factors and events mediating this meiotic transition are only beginning to be defined, but roles for both topoisomerase II (topo II) enzymes and histone H3 phosphorylation are implicated.

Topo II enzymes remove tangles and catenations in DNA by creating double-strand breaks in one DNA molecule, passing another double helix through the break and then resealing the break. Topo II activity was shown to be required for chromosomes to undergo precocious condensation to an MI configuration after treatment of pachytene spermatocytes with the phosphatase inhibitor

okadaic acid (OA) (Cobb et al. 1997). When topo II activity was inhibited, chromatin condensed in response to OA treatment, with the extent of condensation depending on the particular inhibitor used; however, an MI configuration of individualized bivalents was never observed when topo II was inhibited.

It is not known where the topoisomerase enzymes are localized in spermatocytes or how their localization and action mediate the transition to MI. In rooster spermatocytes, topo II has been reported to be localized in the lateral elements of the SC (Moens and Earnshaw 1989). However, another study on the localization of topo II in spermatogenic cells in the rat utilized an autoimmune serum that uniformly stained the nuclei of spermatogonia and spermatocytes and, to a lesser degree, nuclei of round spermatids (Chen and Longo 1996). This autoimmune serum recognized multiple forms of topo II, but also recognized bands of molecular weights that did not correspond to either of the known isoforms of topo II. The characterized isoforms are topoisomerase (DNA) IIα ($M_r$ 170,000) and topoisomerase (DNA) IIβ ($M_r$ 177,000); also known as topo IIα and topo IIβ, the symbols for which are TOP2A and TOP2B, respectively. In the previous study (Chen and Longo 1996), it was not possible to determine the localization of specific isoforms of topo II. For this reason, the study described here used an antibody specific for the TOP2A isoform of topo II. TOP2A has been shown to associate preferentially with centromeric heterochromatin in mitotic cells in a cell cycle-dependent manner (Rattner et al. 1996). Rattner et al. (1996) showed that TOP2A becomes associated with the centromere during G2, at the time of condensation of centromeric heterochromatin, regions where satellite DNA sequences are present. TOP2A has also been shown to associate with blocks of satellite DNA sequences ectopically present at sites distant from the centromere in the murine L929 cell line (Sumner 1996). From this evidence, it has been hypothesized that TOP2A might be involved in the condensation of regions of satellite DNA sequences (Warburton and Earnshaw 1997). Kallio and Lähdetie (1996) reported that treatment of male mice with the topo II inhibitor etoposide, which prevents religation of topo II-induced double-strand DNA breaks, caused fragmentation of meiotic chromosomes at the centromeric satellite DNA, further suggesting the importance of topo II activity at centromeric regions.

Another chromosomal component potentially important in the condensation of meiotic chromosomes is histone H3 (Hendzel et al. 1997). In mitotic cells, histone H3 is specifically phosphorylated at serine 10 when mitotic chromosomes condense. Reports of histone H3 phosphorylation in meiotic cells are limited to studies on the micronucleus of the ciliated protozoan *Tetrahymena thermophila* (Wei et al. 1998, 1999). The strong correlation of histone H3 phosphorylation with the condensation of chromosomes led to the suggestion that this event drives chromosome condensation. By creating mutants in which serine 10 of histone H3 is replaced by alanine, Wei et al. (1999) showed that phosphorylation of histone H3 is necessary for condensation of both meiotic and mitotic chromosomes in the micronucleus of *Tetrahymena*. During mitosis in mammalian cells, phosphorylation of histone H3 initiates at the centromeric heterochromatin before spreading throughout the chromatin and precedes the accumulation of TOP2A at the centromeric heterochromatin. This temporal sequence gave rise to the hypothesis that phosphorylation of histone H3 might recruit proteins, such as TOP2A, to the heterochromatin and thus catalyze chromosome condensation (Hendzel et al. 1997).

Here, we tested the hypothesis that phosphorylation of histone H3 is a key event instigating both localization of TOP2A to the centromeric heterochromatin and condensation of chromosomes as spermatocytes exit prophase and progress to metaphase. The localization of TOP2A during spermatogenesis and its relationship to H3 phosphorylation were determined. Indirect immunofluorescence was used to stain meiotic cells isolated from mouse testes; this was combined with staining of sectioned tissue to determine stage specificity of TOP2A localization and histone H3 phosphorylation as cells progress through the meiotic cell cycle. These approaches allowed a test of the hypothesis that histone H3 phosphorylation recruits TOP2A to the chromatin. Additionally, the function of histone H3 phosphorylation was investigated by determining whether histone H3 phosphorylation is sufficient to condense MI chromosomes in the absence of metaphase-promoting factor (MPF) activity. For this purpose, we used an experimental system for premature induction of MI by treating pachytene spermatocytes with OA (Wiltshire et al. 1995). The results establish the meiotic timing of histone H3 phosphorylation, define the sequence of TOP2A localization and histone H3 phosphorylation, and demonstrate that histone H3 phosphorylation cannot be solely responsible for condensation of meiotic metaphase chromosomes.

## Materials and methods

### *Animals, cell preparations and culture*

ICR mice (Harlan, Indianapolis, Ind.) were maintained under standard conditions. Mixed germ cells from adult mice were prepared by enzymatic digestion of detunicated testes. The testes were first digested in 0.5 mg/ml collagenase for 20 min at 32°C followed by washing of the seminiferous tubules in Krebs-Ringer bicarbonate solution, and a subsequent digestion in 0.5 mg/ml trypsin for 13 min. The cells were filtered through 80 µm mesh and washed three times. The cells were then fixed for immunofluorescence as described below.

Enriched fractions of particular germ cell types were prepared as previously described (Bellvé 1993). Round spermatids and pachytene spermatocytes were prepared from adult mice. A mixed pool of leptotene and zygotene spermatocytes was prepared from 17 day old mice, and spermatogonia were isolated from 8 day old mice.

Enriched fractions of pachytene spermatocytes were cultured at $2.5\times10^6$ cells/ml at 32°C, 5% $CO_2$ as previously described (Handel et al. 1995). After overnight culture, cells were treated with kinase or topo II inhibitors and/or OA. Kinase and topo II inhibitors were added 30 min before OA. Control cultures were given equivalent volumes of solvents alone. OA (ICN Biomedicals, Costa Mesa, Calif.) was dissolved at 244 µM in ethanol and added

at 5 µM as previously described (Wiltshire et al. 1995). ICRF-193 (a gift of Zenyaku Kogyo Co., Tokyo) was dissolved at 5 mg/ml in dimethyl sulfoxide (DMSO), and treatments and results were as previously reported (Cobb et al. 1997). Butyrolactone I (Biomol, Plymouth Meeting, Pa.) was dissolved at 25 mM in DMSO and added to cultures at 100 µM; staurosporine (Calbiochem, La Jolla, Calif.) was dissolved at 1 mg/ml in DMSO and added at 3.2 µM. Cells were harvested 6 h after OA treatment, and processed for immunoblot analysis, H1 kinase assay, and cytological and immunofluorescent analysis as described below. Unless noted otherwise, all chemicals and enzymes were from Sigma (St. Louis, Mo.).

*Preparation and characterization of monoclonal antibodies*

The cDNA of human TOP2A (kindly provided by Dr. James Wang, Harvard University) was expressed in *Escherichia coli* under the control of a T7 promoter. The protein produced as an inclusion body was mixed with Freund's adjuvant and injected four times every 2 weeks into BALB/c mice. The splenocytes were fused with the myeloma cell line P3-X63Ag8.653 and culture media were screened by the enzyme-linked immunosorbent assay method, using a partially purified TOP2A, TOP2B mixture as antigen. Clone 4E12 was of the IgG1 subtype and recognized TOP2A at an epitope in the first 110 N-terminal amino acids of TOP2A. Clone 4E10 was of the IgG2a subtype and recognized both TOP2A and TOP2B. The 4E10 epitope was localized between amino acids 111 and 175 of TOP2A.

The epitopes recognized by the antibodies were determined by expressing several fragments of TOP2A and TOP2B cDNA (the latter kindly provided by Dr. Ian Hickson, Oxford University) cloned into a T7 expression vector. Each *E. coli* crude extract containing a portion of human TOP2A or TOP2B was analyzed by immunoblotting. The extracts were separated on 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and blocked with 10% nonfat dry milk. Each monoclonal antibody was incubated with the blots at 1:100 dilution of hybridoma culture medium at 4°C overnight. Blots were incubated with peroxidase-conjugated secondary antibody and developed using a 4-chloro-1-naphthol reagent.

*Immunoblot analysis and H1 kinase assay of spermatocyte lysates*

Cells were lysed directly in SDS-PAGE sample buffer to facilitate solubilization of TOP2A and histones. Protein loads were equalized by comparison on Coomassie-stained gels. Lysates were separated on 7% SDS-PAGE for TOP2A and 15% SDS-PAGE for phospho-histone H3 and transferred to nitrocellulose using a Biorad semi-dry apparatus. The 7% gels were transferred with a standard TRIS-glycine buffer, pH 8.3, but 15% gels were transferred with a basic carbonate buffer (Dunn 1986) to facilitate transfer of the basic histones. After transfer, gels were blocked in PBS, 0.1% Tween-20, 3% BSA for 1 h to overnight. Blots were probed with 1:500 anti-TOP2A (4E12 monoclonal antibody or a polyclonal antibody from TopoGen, Columbus, Ohio), or 1:1000 polyclonal anti-phosphorylated serine 10 of histone H3 (phospho-H3) (Upstate Biotechnology, Lake Placid, N.Y.). After washing with PBS, 0.1% Tween-20, blots were incubated with secondary antibody, peroxidase conjugated (Pierce, Rockford, Ill.), at 1:1000 for TOP2A and 1:20,000 for phospho-H3 in blocking solution. Blots were developed by a chemiluminescent technique according to the manufacturer's instructions (Amersham, Arlington, Ill.).

The histone H1 kinase assay for measuring MPF activity in lysates was as previously described (Cobb et al. 1997). After culture, cells were washed twice in PBS before freezing at –80°C. Assays were performed with lysates made from frozen cells using exogenously added histone H1 as a substrate. The assay products were separated on 12.5% SDS-PAGE. Gels were exposed to film to visualize phosphorylation of histone H1.

*Cytological methods*

Chromatin configuration in cultured cells was assessed with preparations made by a modification of the Evans procedure (Evans et al. 1964), as previously described (Wiltshire et al. 1995). Giemsa-stained nuclei were observed and photographed under brightfield illumination with a 100× objective on an Olympus microscope.

Isolated cells from mixed germ cell preparations or enriched germ cell pools were fixed for indirect immunofluorescence in 2% paraformaldehyde, 0.03% SDS as previously described (Cobb et al. 1997). Antibodies and dilutions used were: 1:500–1:1000 4E12 (specific for TOP2A), 1:1000 anti-phospho-H3, 1:500 CREST autoimmune sera, 1:500 Rabbit D anti-SC polyclonal, and 1:500 polyclonal COR1 (which recognizes mouse SYCP3). The latter three antisera were kindly provided by Dr. Peter Moens, York University, Ontario, Canada. Normal mouse and rabbit sera were used as negative controls. Secondary antibodies, used at 1:500 dilution, were conjugated with rhodamine, fluorescein (Pierce, Rockford, Ill.) or 7-amino-4-methylcoumarin-3-acetic acid (Vector Laboratories, Burlingame, Calif.). Immunofluorescence was observed and photographed with a 100× objective. Images were captured to Adobe Photoshop with a Hamamatsu C5810 color-chilled camera.

Testes from adult ICR mice were excised and fixed by overnight immersion in 4% paraformaldehyde in 0.1 M phosphate, pH 7.4. After fixation testes were dehydrated with an ethanol/toluene series and embedded in paraffin according to standard procedures. Sections (3 µm) were dried on albuminized slides, deparaffinized, rehydrated and microwave-treated in 10 mM sodium citrate, pH 6.0 as described (Mizoguchi and Kim 1997). Slides were then processed exactly as those used for indirect immunofluorescent labeling of isolated cells, except antibodies were generally used at higher concentrations (4E12 at 1:100, phospho-H3 at 1:1000, and 1:100 for the secondary antibodies). Normal mouse and rabbit sera were used as negative controls. 4',6-Diamidino-2-phenylindole (DAPI) images were used to identify cell types by appearance and cytological context as previously described (Russell et al. 1990).

## Results

### *Characterization of antibodies and analysis of TOP2A expression*

The TOP2A epitope recognized by monoclonal antibody 4E12 was determined by immunoblotting using N-terminal fragments of human TOP2A and TOP2B expressed in *E. coli* as antigens (Fig. 1A). Human TOP2A is 89% identical to mouse TOP2A and over 90% identical in the N-terminal domain (Adachi et al. 1992). Antibody 4E10 was used as a positive control because it was known to detect the N-terminal domains of both TOP2A and TOP2B. Both antibodies recognized fragments NH and NA, representing the first 494 and 175 amino acids of TOP2A, respectively. However, only 4E12 detected fragment N22, which contains the most N-terminal 110 amino acids of TOP2A. 4E12 did not recognize the peptide N56S containing the corresponding region, the first 187 amino acids, of TOP2B. Therefore 4E12 is specific for the N-terminal domain of TOP2A. This specificity likely arises from the divergence in the sequences of TOP2A and TOP2B within their first 100 amino acids (Jenkins et al. 1992), even though the core 75% of the isozymes are highly homologous, and the most divergent portion is at the C-terminus. The commercial antibody (TopoGen, Columbus, Ohio) used in other studies and in

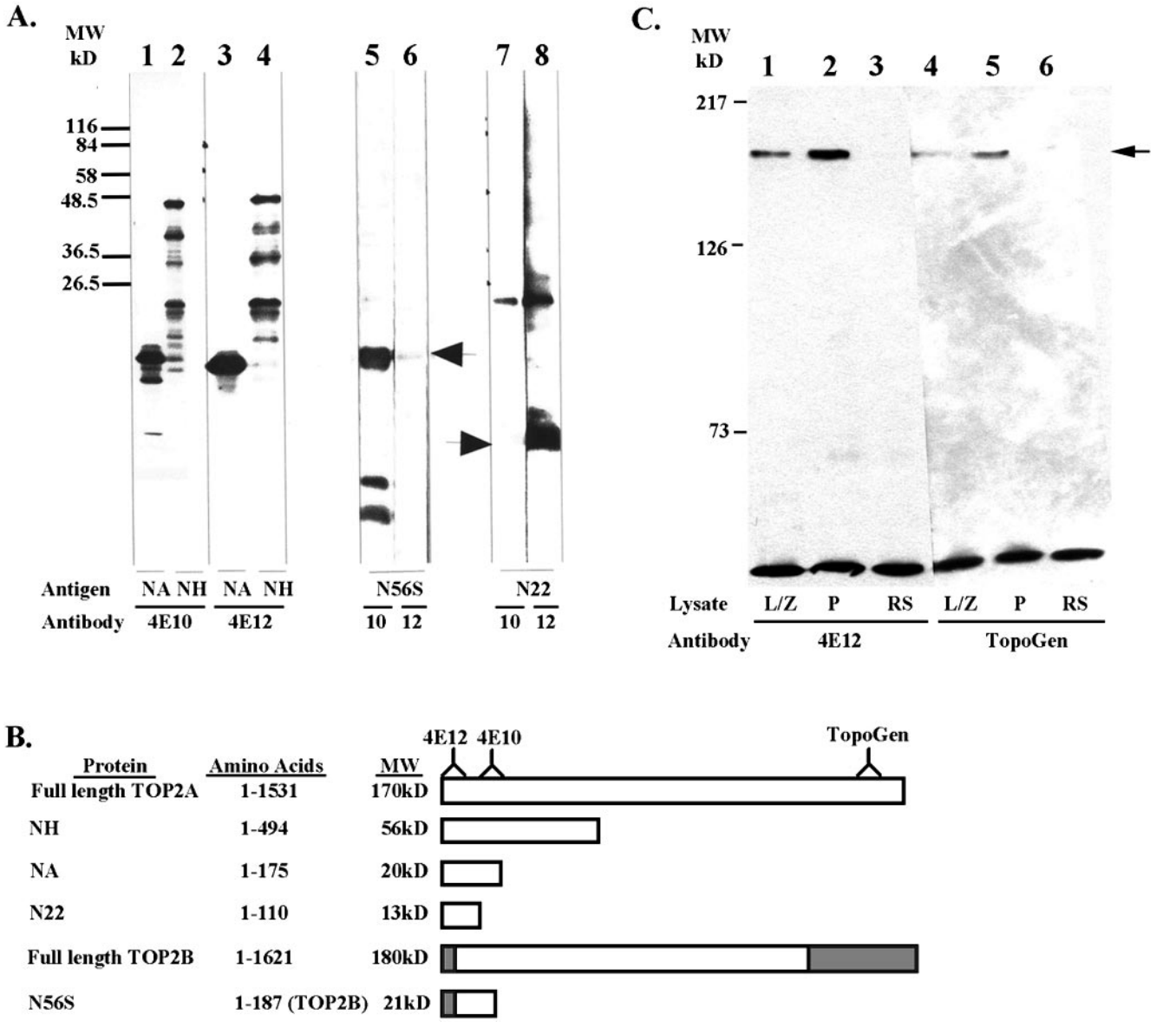


**Fig. 1A–C.** Characterization of TOP2A antibodies and TOP2A expression analysis. **A** Immunoblots used to determine the epitope of TOP2A recognized by antibody 4E12. The antigens are *Escherichia coli* extracts expressing the indicated TOP2A or TOP2B fragments. *Lanes 1, 2, 5* and *7* were probed with antibody 4E10, and *lanes 3, 4, 6* and *8* were probed with antibody 4E12. Antibodies 4E10 and 4E12 recognize the NH peptide (migrating at $M_r$ 48,000) and the NA peptide ($M_r$ 20,000), *lanes 1–4*. Only 4E12 recognizes the N22 peptide ($M_r$ 13,000), which contains the first 110 amino acids of TOP2A (*lane 8*, *arrow*), but not the corresponding N56S peptide from TOP2B (*lane 6*, *arrow*), which is recognized by 4E10 (*lane 5*). Therefore 4E12 is specific for TOP2A. 4E10 was used as a positive control because it recognizes both TOP2A and TOP2B at an epitope that is C-terminal to that of 4E12. **B** Schematic diagram of the protein fragments used in characterizing the TOP2A antibodies. The approximate location of epitopes recognized by the different antibodies is indicated. Those regions of TOP2B that diverge most significantly from TOP2A are indicated in *gray*. For details of the sequences see Jenkins et al. (1992). **C** Immunoblots using the 4E12 antibody (*lanes 1–3*) and the TopoGen antibody (*lanes 4–6*), which both recognize a protein of approximately $M_r$ 170,000 (the molecular weight of TOP2A) in lysates from spermatogenic cells. The band (*arrow*) is present in leptotene/zygotene spermatocytes (*L/Z*) and pachytene spermatocytes (*P*), but much diminished in round spermatids (*RS*). The low molecular weight band at the dye front is recognized by both antibodies and may represent breakdown products of TOP2A

Fig. 1C was prepared against a peptide specific for the C-terminal portion of TOP2A (Rattner et al. 1996).

On immunoblots, both the TopoGen and 4E12 antibodies detected bands of identical molecular weight in lysates from leptotene/zygotene and pachytene spermatocytes (Fig. 1C). This band was at approximately $M_r$ 170,000, the molecular weight of TOP2A. No band was detected at $M_r$ 180,000, the approximate molecular weight of TOP2B. The TOP2A band was greatly diminished in round spermatids, although a light band could be detected with long exposures. These results indicate that TOP2A is expressed in meiotic cells, with the amount of protein decreasing during the post-meiotic spermatid stages. Taken together, the above results indi-

cate that antibody 4E12 is suitable for specifically localizing TOP2A within spermatocytes. Therefore, the 4E12 antibody was used in all indirect immunofluorescence staining described below. The TopoGen antibody produced similar, though less intense, staining patterns, thus verifying the validity of the staining since antibodies directed against epitopes at opposite ends of the TOP2A protein produced similar signals.

The specificity of the phospho-H3 antibody has been described (Hendzel et al. 1997). The antibody does not recognize either acetylated histone H3 or the unphosphorylated protein. Since OA induces phosphorylation, this specificity was supported by the results of Fig. 2A where a single band is seen in immunoblots after, but not before, OA treatment of pachytene spermatocytes.

*Localization of TOP2A during spermatogenesis*

Indirect immunofluorescence staining of spermatogenic cells was used to localize TOP2A during spermatogenesis. Rattner et al. (1996) reported that TOP2A becomes associated with the centromeric region during G2 in a variety of mammalian mitotic cell culture lines. In the present study similar results were obtained for the mitotic cells of the spermatogenic lineage, the spermatogonia. TOP2A is present in many G1 spermatogonia, but it is not closely associated with centromeres (Fig 3A). By G2, TOP2A does colocalize with the centromeres of spermatogonia (Fig. 3B). This confirmed that the 4E12 antibody stained spermatogonia in a pattern similar to that seen in cultured mitotic cell lines.

In the earliest meiotic cells observed, preleptotene or very early leptotene spermatocytes, most TOP2A staining is associated with centromeric heterochromatin, although some staining is dispersed throughout the nucleus (Fig. 3C). Centromeric heterochromatin is coincident with the most intensely DAPI-stained regions. The specificity of this DAPI staining was previously demonstrated by its coincidence with the fluorescence in situ hybridization signal for major satellite DNA probes (Scherthan et al. 1996). Additionally, CREST-staining centromeres are embedded in these heterochromatic patches (see Fig. 5). The association of TOP2A with centromeres is not as specific in leptonema as it is later in meiotic prophase; some centromeres of leptotene spermatocytes, visualized by CREST staining, have no associated TOP2A (data not shown). In contrast, when axial elements of the developing SC first become visible in zygonema, TOP2A staining localizes to a discrete number of patches of heterochromatin (Fig. 3D). At all stages observed there is some diffuse TOP2A staining throughout the chromatin, which is visible in long photographic exposures (arrowhead, Fig. 5A). When homologous chromosomes become fully synapsed at pachynema (Fig. 3E), TOP2A staining remains predominantly associated with the masses of heterochromatin containing the centromeres. The staining in Fig. 5A verifies that CREST-positive centromeres are indeed embedded in these areas of TOP2A staining. The Y chromosome centromere is an exception since it has no associated major satellite DNA or TOP2A. In MI spermatoctyes (Fig. 3F)

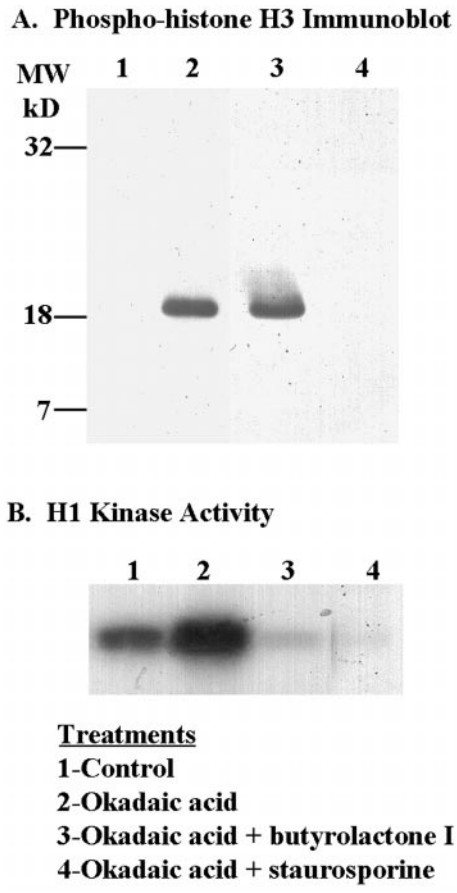


**Fig. 2A, B.** Effects of okadaic acid, butyrolactone I and staurosporine on histone H3 phosphorylation and histone H1 activity in cultured pachytene spermatocytes. **A** Immunoblot using the antibody against phosphorylated histone H3 in lysates of pachytene spermatocytes cultured for 6 h under various conditions. Control cultures (*1*) show only a very weak band after long exposure times. Phosphorylated histone H3 appears in 5 µM okadaic acid (OA) treated cultures (*2*), and the band is not diminished when metaphase-promoting factor (MPF) is inhibited by 100 µM butyrolactone I in the OA-treated cultures (*3*). The phosphorylation induced by OA is inhibited by simultaneous treatment with the kinase inhibitor staurosporine (*4*). **B** H1 kinase activity in the lysates used in **A**. OA stimulates H1 kinase, attributed to MPF, and this activation is blocked by butyrolactone I and staurosporine treatment

both the heterochromatin and TOP2A staining become much more punctate, reflecting individualization of chromosomes and highly condensed heterochromatin at this time. Interestingly, TOP2A staining disappears in the post-meiotic round spermatids where centromeres cluster in a single heterochromatic chromocenter (Fig. 3G). These results show that TOP2A is associated with centromeric heterochromatin in meiotic cells, as in mitotic cells, and that this association is present from early in prophase through division.

*Phosphorylation of histone H3 begins at the centromeric heterochromatin during diplonema*

The phospho-H3 antibody was used in indirect immunofluorescence labeling of isolated mixed germ cells from

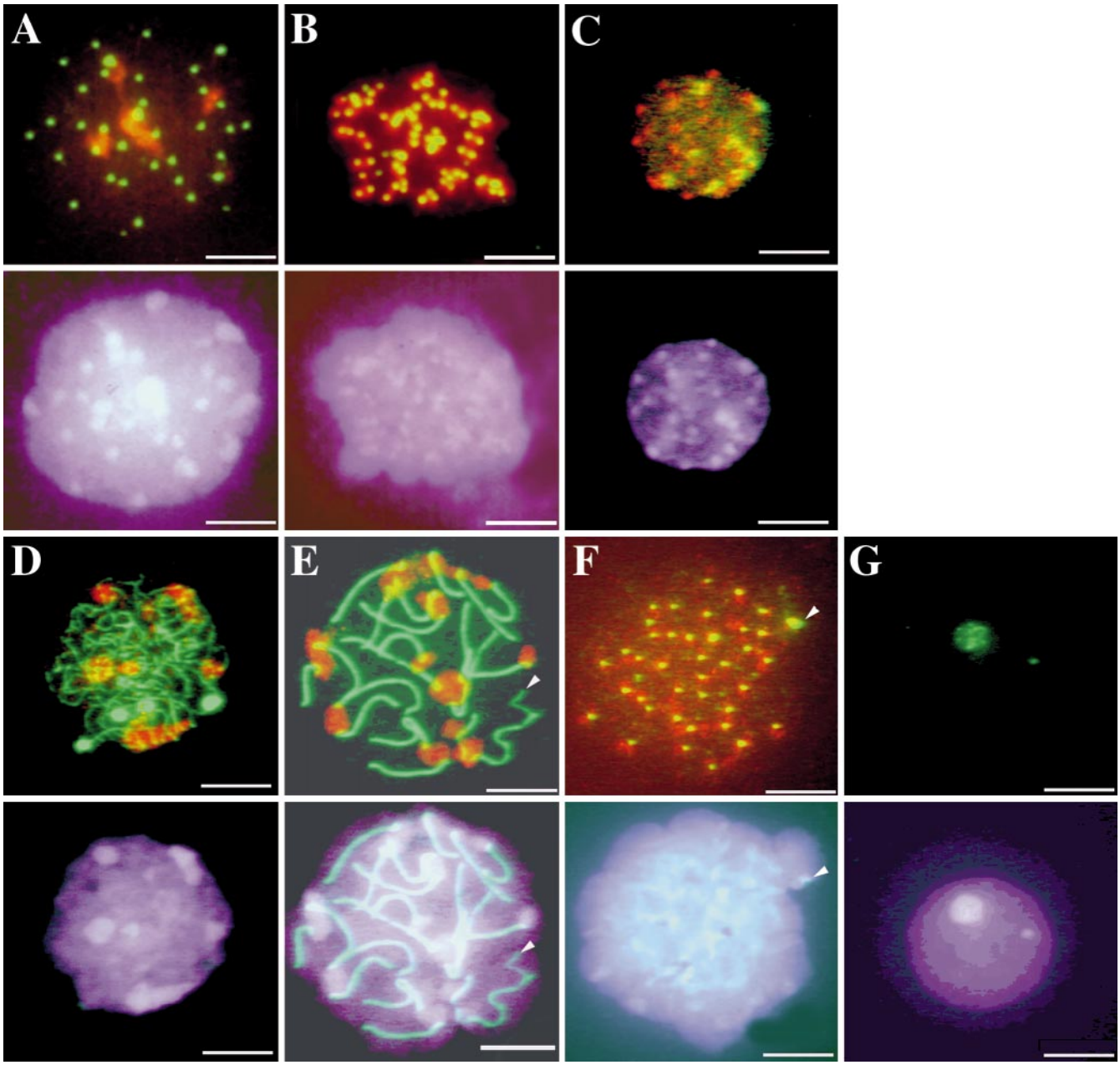


**Fig. 3A–G.** The localization of TOP2A as germ cells progress through stages of spermatogenesis around meiosis. **A–G** represent the different stages, described below. They are arranged according to their temporal order of appearance. In each panel the *red* signal denotes TOP2A. The *green* signal in **A**, **B**, and **G** is CREST antiserum staining of the centromeres. The *green* signal in **C–F** is Rabbit D antibody directed against the synaptonemal complex (SC) (Moens et al. 1992). *Yellow* signals result when the red and green overlap. Each *blue* image is the corresponding 4',6-diamidino-2-phenylindole (DAPI) staining of the nucleus above it, and in **E** the SC image is superimposed on the DAPI image. The intense staining in the DAPI images localizes the centromeric heterochromatin (Scherthan et al. 1996). **A** Type A spermatogonia at G1 (centromeres not duplicated). TOP2A is not localized to centromeres. **B** Type A spermatogonia at G2/M (centromeres are duplicated). Here TOP2A is closely associated with centromeric heterochromatin. **C** Preleptotene or early leptotene spermatocyte. SC proteins are present in aggregates as previously observed (Scherthan et al. 1996), and TOP2A is present in punctate spots associated primarily with the heterochromatin. **D** Zygotene spermatocyte. As chromosomes synapse, the centromeric heterochromatin coalesces into a few patches; the TOP2A staining pattern coincides with the heterochromatin. **E** Pachytene spermatocyte. The SC is completely formed and the TOP2A staining remains associated in clumps, which match aggregates of heterochromatin within which the centromeres are found. *Arrowhead* Y chromosome that lacks centromeric heterochromatin and TOP2A. **F** Metaphase I spermatocyte. The SC has disassembled except for punctate spots of protein at the centromere; TOP2A and heterochromatin are also much more punctate with number of spots equivalent to number of individualized chromosomes. *Arrowhead* X chromosome heterochromatin, centromere. **G** Round spermatid. TOP2A staining has disappeared, and centromeres are clustered in a chromocenter. *Bars* represent 10 μm

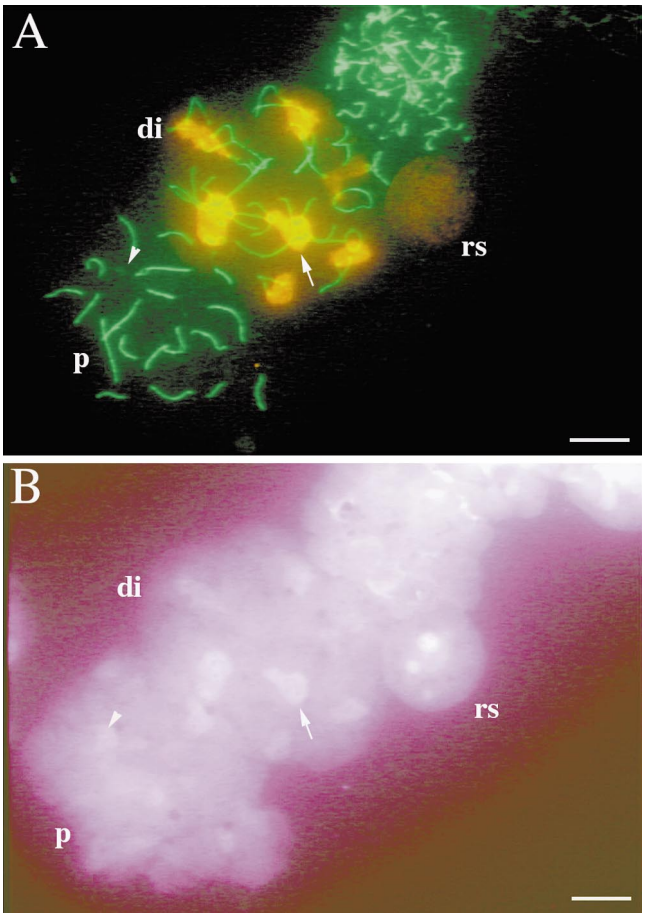


**Fig. 4A, B.** Phosphorylation of histone H3 during meiosis is stage specific. **A** Cluster of adult germ cell nuclei stained with phosphorylated histone H3 antibody (*red*) and COR1 (*green*); **B** is the corresponding DAPI image. When chromosomes are fully synapsed in pachytene spermatocytes (*p*) histone H3 is not phosphorylated. But after chromosomes begin to desynapse in diplotene spermatocytes (*di*), histone H3 is phosphorylated in patches that correspond closely to the brightly staining heterochromatin in the DAPI image (*arrow*). The corresponding heterochromatin patches in the pachytene spermatocyte (*arrowhead*) and in the round spermatid (*rs*) are not phosphorylated. *Bars* represent 10 µm

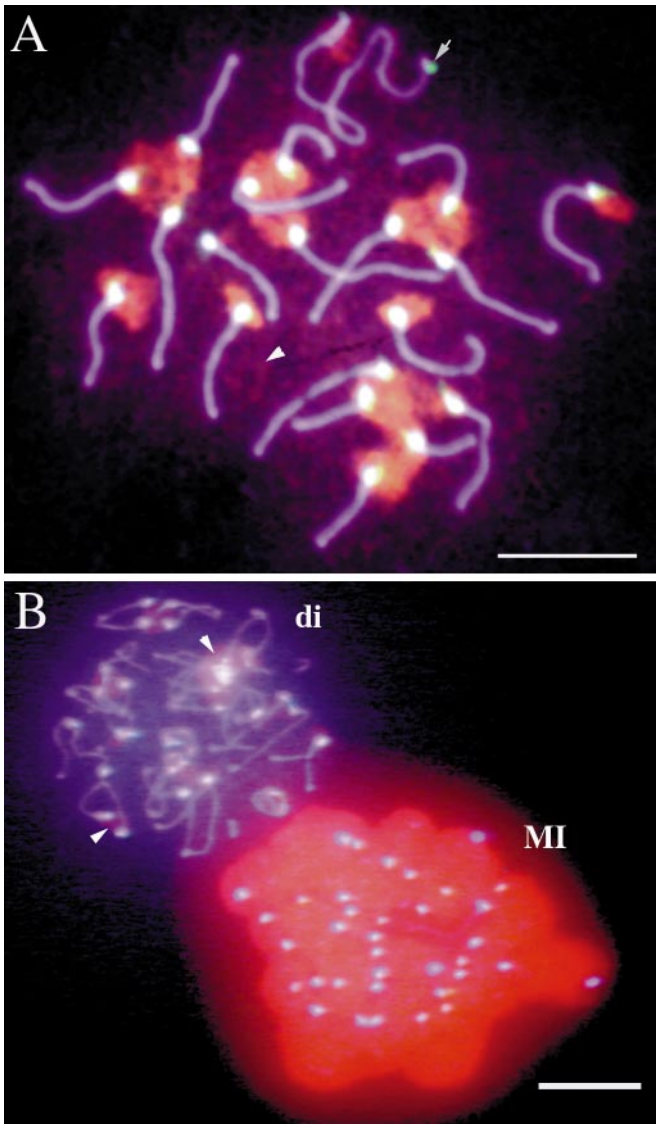


**Fig. 5A, B.** Centromeres are embedded in the heterochromatic regions where TOP2A is localized and histone H3 is first phosphorylated. **A** Triple-labeled pachytene spermatocyte with TOP2A (*red*), SC antibody (*blue*) and CREST antibody staining centromeres (*green*). Centromeres appear *white* where the three signals overlap. All centromeres are associated with TOP2A, except the Y chromosome (*arrow*). Note diffuse light TOP2A staining throughout chromatin (*arrowhead*). **B** Triple labeled diplotene (*di*) and metaphase I (*MI*) spermatocytes with phosphorylated H3 (*red*), SC (*blue*) and CREST (*green*). Centromeres are associated with the areas where histone H3 is first phosphorylated (*arrowheads*) before phosphorylation spreads throughout the chromatin at MI. *Bars* represent 10 µm

adult mice to determine whether histone H3 is phosphorylated during meiotic chromosome condensation as it is in mitotic cells. Most spermatocytes were negative for phosphorylated histone H3 staining. Pachytene spermatocytes showed no evidence of staining (Fig. 4A), in agreement with the immunoblotting results (Fig. 2A). Diplonema, when chromosomes are desynapsing, was the earliest stage where staining was seen (Figs. 4A, 5B). This initial staining exactly corresponds to areas of centromeric heterochromatin. This pattern of phospho-H3 localization is apparently quite transient, as cells with the staining pattern seen in Fig. 4A were relatively rare. Most diplotene spermatocytes showed more diffuse phospho-H3 staining, throughout their chromatin, while the earliest diplotene spermatocytes, in which desynapsis was just beginning, showed no phospho-H3 (not shown). The most intense staining was seen throughout the chromatin in metaphase spermatocytes (Fig. 5B). No MI nuclei were observed without intense phospho-H3 staining throughout their chromatin.

### *Simultaneous localization of TOP2A and phosphorylated histone H3 in sectioned testes*

In the previous experiments using isolated cells, phospho-H3 staining was not seen until diplonema, al-

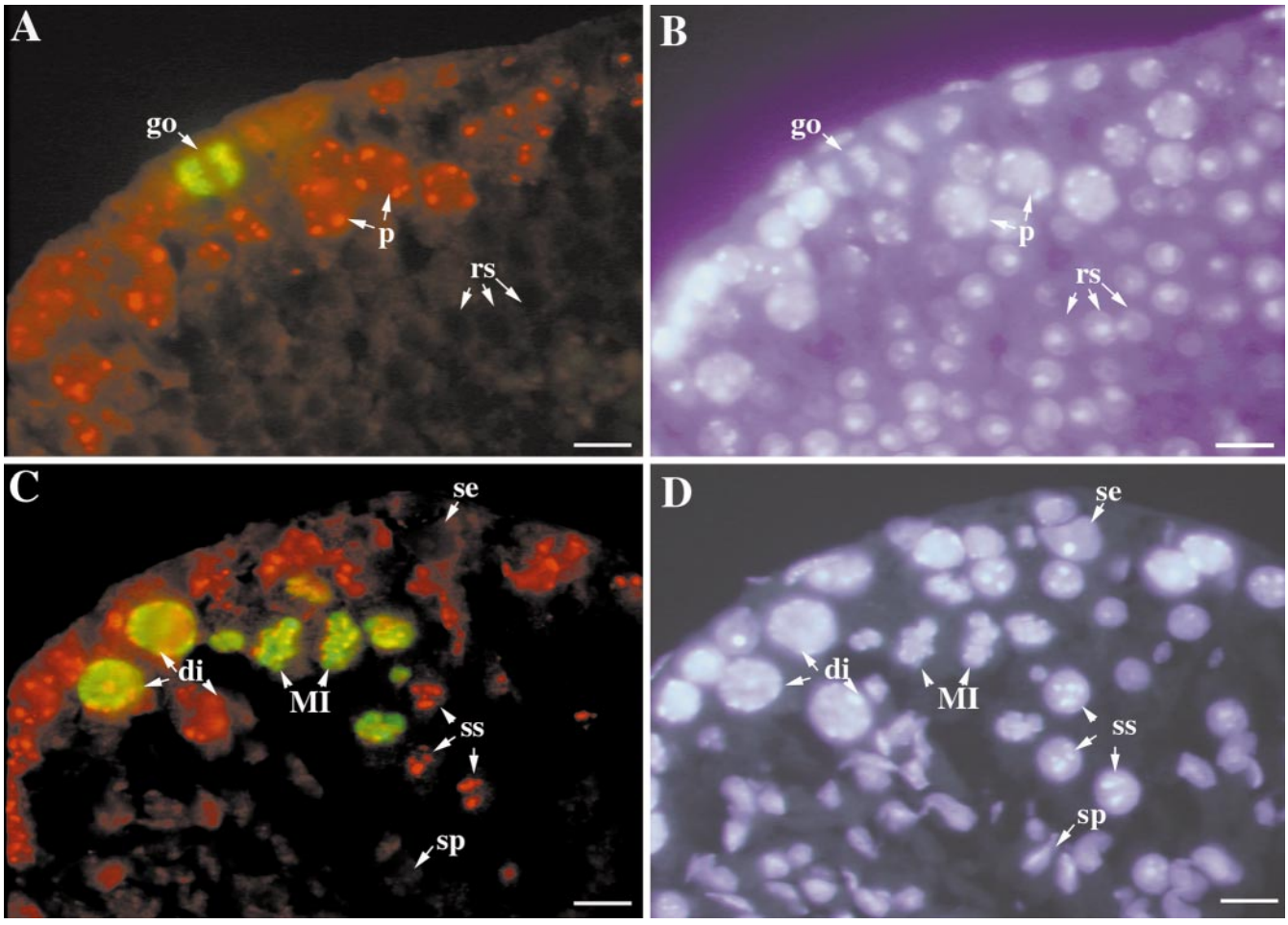


**Fig. 6.** Indirect immunofluorescence staining (**A**, **C**) of phosphorylated histone H3 (*green*) and TOP2A (*red*) in testis sections from adult mice with corresponding DAPI images (**B**, **D**). **A** In this tubule (approximately stage VII–VIII) only one type A spermatogonium (*go*) in anaphase is positive for phosphorylated histone H3. In this cell TOP2A antibody stains only at the leading edge of the chromosomal mass (presumably the centromere/kinetochore), where the overlapping green and red give a *yellow* signal. The pachytene spermatocytes (*p*) in this section have intense TOP2A staining in the heterochromatin, corresponding to the bright DAPI patches in **B**. Round spermatids (*rs*) are negative with both antibodies. **C** In this stage XII tubule there is extensive phosphorylated histone H3 in diplotene (*di*) and MI spermatocytes, but the staining has disappeared in secondary spermatocytes (*ss*). The lower diplotene spermatocyte has not yet acquired phosphorylated histone H3. TOP2A staining, by contrast, is present at the centromeric heterochromatin of all meiotic cells, but not in Sertoli cells (*se*) or spermatids (*sp*). *Bars* represent 10 µm

though TOP2A was associated with the centromeric heterochromatin early in meiotic prophase. This implies that TOP2A is associated with centromeres long before histone H3 is phosphorylated. These results were verified by staining testis tissue sections simultaneously with phospho-H3 and TOP2A antibodies. This approach is valuable because, in sectioned testes, the stage of meiotic germ cells can be deduced by their location in the tubule, their appearance, and their stage-specific association with easily identified spermiogenic cells (Russell et al. 1990). Staining of sections with the 4E12 antibody showed TOP2A localization in the mitotic and meiotic cells, but staining was not present in round and elongating spermatids (Fig. 6). This staining precisely correlated with that seen in isolated cells (Fig. 3), as careful examination showed that the staining in pachytene spermatocytes closely matches the heterochromatin patches in the DAPI image. However, no phosphorylated H3 was seen, except in mitotic spermatogonia in the basal region of the tubules, in any seminiferous tubule stages before stage XI and XII (Fig. 6A). Phospho-H3 staining abruptly appears as meiotic prophase cells approach the division stage. Initially, light staining appears at the centromeric heterochromatin in diplotene spermatocytes, in a pattern identical to that of the TOP2A staining (Fig. 7). While TOP2A staining stays associated with the heterochromatin, phosphorylation of histone H3 spreads throughout the chromatin and MI chromosomes are heavily phosphorylated (Fig. 6C). The histone becomes dephosphorylated in secondary spermatocytes, while TOP2A remains localized in the heterochromatic regions of these cells. TOP2A staining is present immediately after the second meiotic division, in very early round spermatids (not shown), but the staining disappears quickly from all subsequent round spermatids (Fig. 6A).

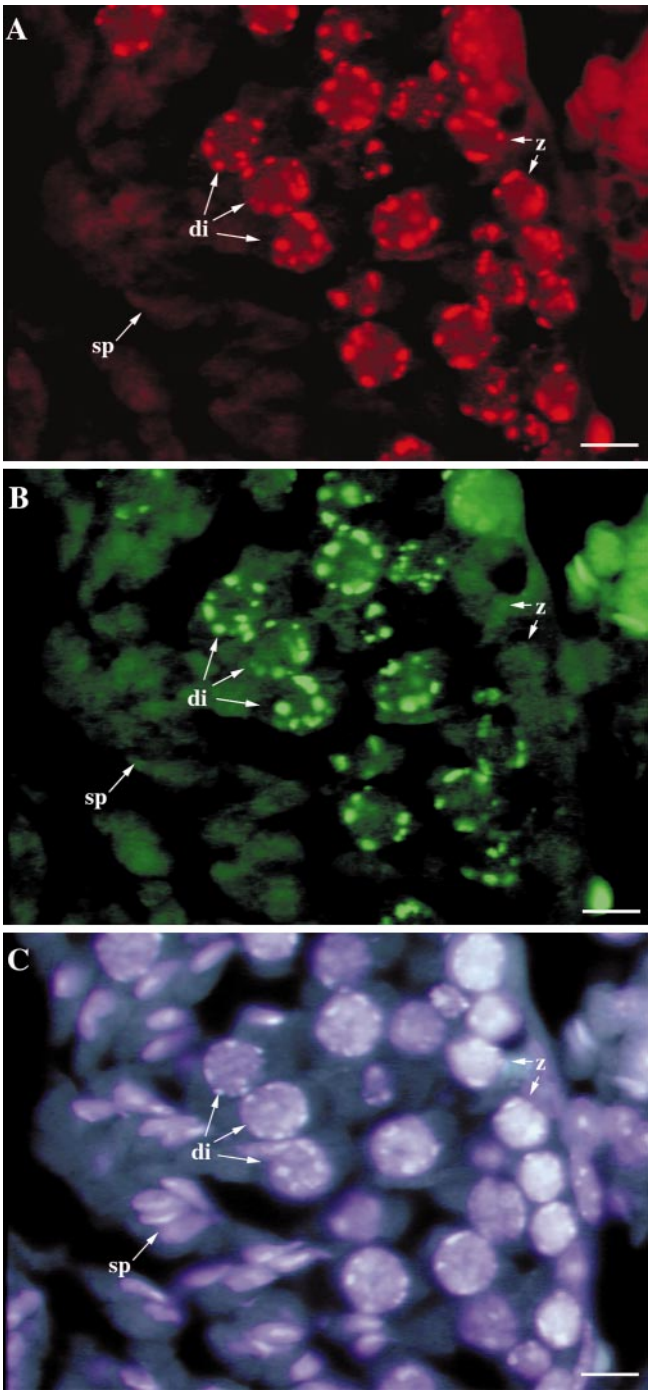


**Fig. 7A–C.** Indirect immunofluorescence staining in a stage XI seminiferous tubule section from an adult mouse. **A** TOP2A antibody (*red*) stains the heterochromatin in zygotene (*z*) and diplotene (*di*) spermatocytes, but it does not stain elongating spermatids (*sp*). **B** Phosphorylated histone H3 (*green*) is present only in the heterochromatin of diplotene spermatocytes, and this staining pattern appears identical to that of the TOP2A staining in these spermatoctyes. **C** The DAPI image confirms that the staining in **A** and **B** corresponds to the brightly staining heterochromatin at the nuclear periphery. *Bars* represent 10 μm

*Topoisomerase II activity is required, but histone H3 phosphorylation without MPF activation is not sufficient to condense MI chromosomes*

Meiosis can be analyzed in vitro by treating pachytene spermatocytes with OA to induce the precocious condensation of MI chromosomes. A previous study reported that topo II activity is required for OA-induced condensation of MI chromosomes (Cobb et al. 1997). When topo II activity is blocked by the inhibitor ICRF-193, OA treatment leads to SC disassembly to a late diplotene-like configuration (Cobb et al. 1997) and chromatin condenses into elongated, paired strands (Fig. 8C), unlike the MI chromosomes seen after OA treatment alone (Fig. 8B). Pachytene spermatocytes cultured with ICRF-193 alone do not lose their centromeric localization of TOP2A. In spermatocytes treated with OA and ICRF-193 histone H3 is phosphorylated throughout the nuclei, as verified by immunofluorescence staining (not shown), and a significant number of the cells show TOP2A staining along the strands of chromatin rather than at the centromeric heterochromatin (not shown).

The role of histone H3 phosphorylation in the condensation of MI chromosomes was investigated by using kinase inhibitors in combination with OA treatment. Butyrolactone I is a cell-permeable inhibitor specific for cyclin-dependent kinases such as the CDC2 component of MPF (Kitagawa et al. 1993). MPF is activated by OA treatment of pachytene spermatocytes (Wiltshire et al. 1995). MPF activation that occurs within spermatocytes treated with OA is stable so that the activation can be assayed in lysates from the treated cells (Fig. 2B). Treatment of spermatocytes with butyrolactone I inhibited the normal activation of MPF by OA (Fig. 2B). Others have shown with a similar assay that the inhibition of MPF within cells is essentially irreversible (Poon et al. 1997). In contrast to the effect on MPF activation, butyrolactone I treatment did not significantly inhibit histone H3 phosphorylation within spermatocytes (Fig. 2A), and this assay was confirmed by indirect immunofluorescence of treated nuclei showing that histone H3 is phosphorylated throughout the chromatin after OA and butyrolactone I treatment (not shown). Although histone H3 phosphorylation was not quantitated precisely, any large changes in phosphorylation would have been detected because the ECL immunoblotting system used in Fig. 2A shows approximately quantitative linearity (verified by comparing signals from different volumes of lysates, not shown). The broad-spectrum protein kinase inhibitor staurosporine (Tamaoki et al. 1986) did block both OA-induced H3 phosphorylation and MPF activity. The staurosporine/OA-treated spermatocytes appeared identical to control pachytene spermatocytes (Fig. 8A). Interestingly, cells treated with both butyrolactone I and OA underwent initial stages of chromatin condensation (Fig. 8D), but as when topo II is inhibited, individualized MI chromosomes did not form in butyrolactone/OA-treated spermatocytes. Thus, treatment of pachytene spermatocytes with butyrolactone I and OA results in phosphorylation of histone H3 but failure in condensation of MI chromosomes. Therefore H3 phos-

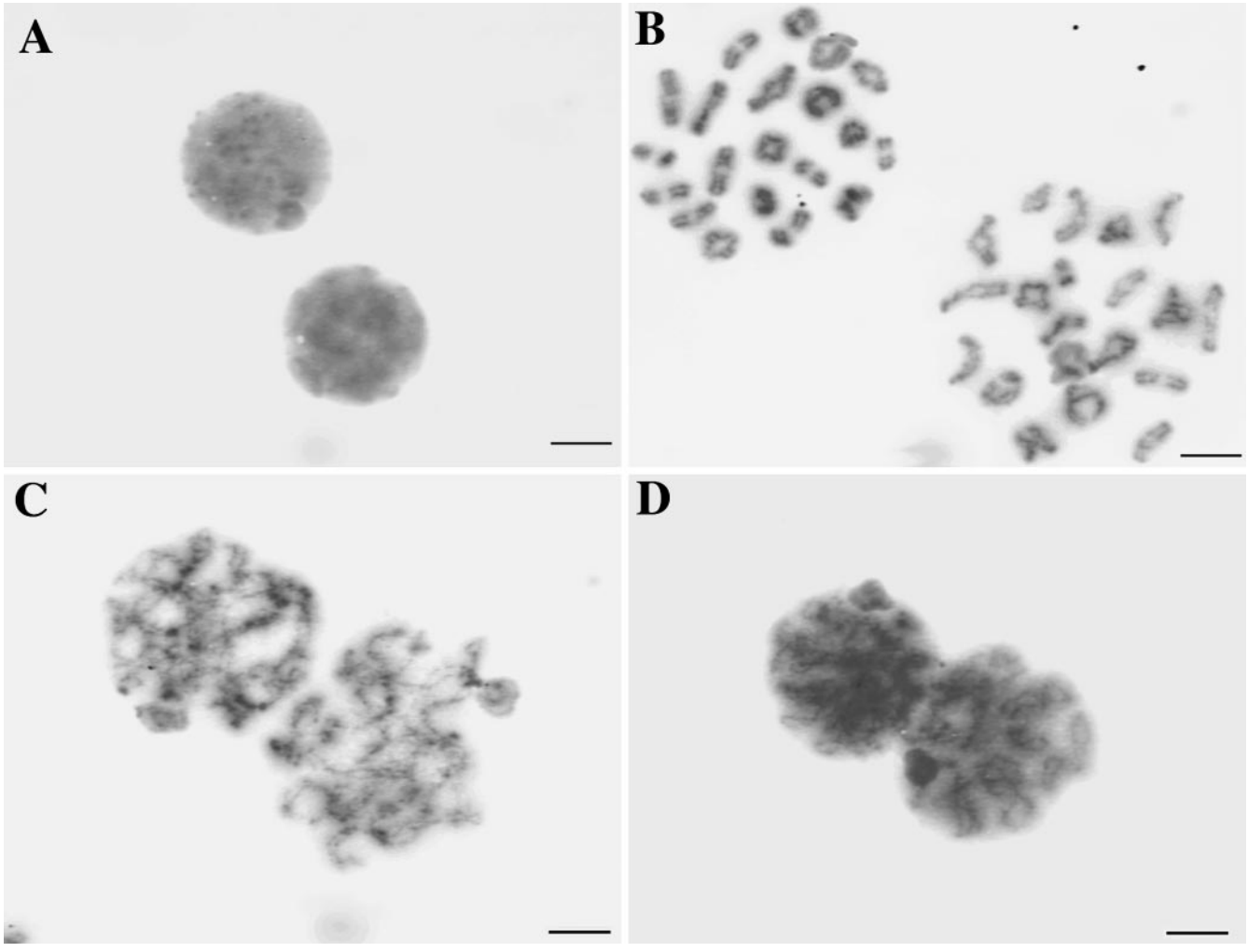


**Fig. 8A–D.** Air-dried nuclear preparations showing chromatin configuration of pachytene spermatocytes after 6 h of culture. **A** Untreated control pachytene spermatocytes with uncondensed chromatin. **B** Pachytene spermatocytes treated with 5 µM OA showing condensed chiasmate bivalent chromosomes in an MI configuration. **C** Pachytene spermatocytes treated with OA and 50 µM ICRF-193 to inhibit topoisomerase II activity; although individualized MI chromosomes are not formed, chromatin condenses into elongated strands. **D** Pachytene spermatocytes simultaneously treated with OA and 100 µM butyrolactone I, which inhibits MPF activation but not histone H3 phosphorylation. Progression to MI is blocked, but some chromatin condensation is visible. *Bars* represent 10 µm

phorylation induced by OA treatment may be necessary, but is not sufficient to condense MI chromosomes in cultured pachytene spermatocytes. These results are consistent with findings reported elsewhere (Cobb et al. 1999) that histone H3 phosphorylation in cultured leptotene/zygotene spermatocytes also does not lead to the condensation of individualized chromosomes.

## Discussion

The proteins mediating the unique structural changes and movements of mammalian meiotic chromosomes are only beginning to be identified, and most emphasis thus far has been on the proteins of the SC (Dobson et al. 1994; Moens and Spyropoulos 1995). The localization and potential meiotic function of two other likely participants, TOP2A and phosphorylated histone H3, are described here. As shown previously, topo II activity is required when pachytene spermatocytes condense MI chromosomes after OA treatment (Cobb et al. 1997). Here it was found that TOP2A is present in the centromeric heterochromatin throughout meiotic prophase, suggesting that it may have other meiotic functions in addition to being required for the condensation of chromosomes at the end of meiotic prophase. In contrast, histone H3 becomes phosphorylated only at the end of meiotic prophase, implying that its meiotic function could be limited to chromosome condensation or other events of the meiotic G2/M transition. Interestingly, in *Tetrahymena* histone H3 is phosphorylated much earlier in meiotic prophase, at a stage corresponding to zygonema (Wei et al. 1998), suggesting a role in chromosome condensation in early meiotic prophase. In murine spermatocytes the initial phosphorylation of histone H3 during diplonema is confined to the centromeric heterochro-

matin. However, the phosphorylation of histone H3 cannot be responsible for recruiting TOP2A to this location because TOP2A is localized to the same site early in meiotic prophase and remains there. Moreover, the use of kinase inhibitors in conjunction with OA treatment of pachytene spermatocytes demonstrated that histone H3 phosphorylation can be uncoupled from complete chromosome condensation; thus although phosphorylation of this protein normally occurs at the transition to meiotic metaphase, it is not sufficient to condense MI chromosomes. These results are relevant to the roles of these proteins in meiotic chromosome condensation and to the function of the centromeric heterochromatin in meiosis. They also suggest the possibility of a role for TOP2A earlier in meiotic prophase.

Mechanisms and key players in many of the events governing condensation and orientation of chromosomes at metaphase are becoming well understood (Nicklas 1997). Metaphase I of meiosis presents some special problems in chromosome mechanics. During the preceding prophase, homologous chromosomes have been intimately synapsed and have undergone recombination events that link the homologs together. The physical "glue" of the chiasma is important for correct orientation and segregation of homologs during the first meiotic division (Koehler et al. 1996). Because of homologous synapsis and recombination, preparation for the meiotic divisions involves untangling chromosome associations far more complex than those of mitosis. Furthermore, in mitosis, the centromeres provide the connection that holds chromosomes together and ensures their orientation on the spindle apparatus. Such is not the case in the first meiotic division, where non-sister centromeres are not linked.

### *Roles of TOP2A and phosphorylated histone H3 in meiotic chromosome condensation*

Three classes of proteins have been implicated in the process of chromosome condensation: the histones, the SMC (structural maintenance of chromosomes) family of ATPases and the topo II enzymes (Koshland and Strunnikov 1996). It has been proposed that mitotic chromosome condensation comprises two processes: resolution and compaction (Hirano 1995). Resolution is necessary to remove tangles between chromatids before they can be compacted to form individualized chromosomes. These processes are hypothesized to be synergistic; resolution of tangles must occur before compaction, and initiation of compaction promotes the resolution of further tangles. In this model, topo II activity is responsible for resolution of chromatin tangles and SMC ATPases are responsible for compaction. Phosphorylation of histone H3 has also been suggested as an agent in compaction because of the timing of its appearance (Hendzel et al. 1997). During meiosis, tangles may exist between synapsed homologs, sister or non-sister chromatids and nonhomologous chromosomes. OA treatment induces full resolution and compaction of pachytene chromosomes to an MI configuration of chiasmate bivalents (Fig. 8B). However, in the absence of topo II activity (as when inhibited by ICRF-193), OA treatment causes condensation of chromatin into paired fibers. These fibers are not fully compacted along their axes (Fig. 8C). This observation is consistent with the model of Hirano; compaction may halt when topo II activity is inhibited because tangles remain in the chromatin fibers.

Others have shown that OA treatment leads to phosphorylation of histone H3 in mitotic cells (Guo et al. 1995; Ajiro et al. 1996). Guo et al. (1995) showed that even when MPF activity was ablated by a temperature-sensitive mutation, histone H3 is phosphorylated and mitotic chromosomes condense after treatment with the phosphatase inhibitors OA or fostriecin. In the present study MPF activity was eliminated with a chemical inhibitor, butyrolactone I. Because of previous results (Guo et al. 1995), it was anticipated that histone H3 phosphorylation in the absence of MPF activity might be sufficient to condense meiotic chromosomes as well. Surprisingly, even though histone H3 is phosphorylated, butyrolactone/OA-treated spermatocytes do not condense MI chromosomes (Fig. 8D). Instead chromatin forms thin, incompletely condensed fibers, even less distinct than those in the ICRF-193/OA-treated cells. Therefore, while histone H3 phosphorylation may be necessary, it is not sufficient for MI chromosome condensation. This result indicates a potential difference between the condensation of meiotic and mitotic chromosomes, but, ideally, should be confirmed without the use of chemical inhibitors. These results from analysis of meiotic spermatocytes are similar to those reported elsewhere (Van Hooser et al. 1998) in which treatment of S-phase cells with OA caused H3 phosphorylation throughout the chromatin, but mitotic chromosomes did not condense. However, the inhibition of chromosome condensation here cannot be due to incomplete DNA replication since pachytene spermatocytes completed S-phase before entering meiotic prophase.

### *The centromeric heterochromatin during meiosis*

Centromeric heterochromatin appears to be the center for both TOP2A localization and the initial phosphorylation of histone H3 in meiosis, but it is not clear what function this implies. The major satellite DNA sequences are known to be at the centromeric ends of all the murine chromosomes except the Y (Pardue and Gall 1970), but the function of satellite DNA is unknown. Although it has been suggested that these sequences may be important for localization of the kinetochore (Csink and Henikoff 1998; Wiens and Sorger 1998), the murine Y chromosome maintains a functional kinetochore without either satellite DNA or detectable amounts of TOP2A (arrows in Figs. 3E and 5A). This implies that TOP2A could function in the formation and/or maintenance of heterochromatin, but is not necessarily required for organization of all kinetochores. Nonetheless, a role for TOP2A in the correct formation of kinetochore structure in mitotic cells has been suggested. Rattner et al. (1996)

showed that, when the activity of topo II is inhibited by ICRF-193 in Indian muntjac cells, the structure of the kinetochore is abnormal; TOP2A does not accumulate in the centromeric region, and the centromeric heterochromatin is less compact. The mechanism by which TOP2A facilitates kinetochore formation is not known. It could be an indirect role; for example, its activity might lead to condensation of chromatin in this region.

Several *Drosophila* proteins have been described that bind to the repeated satellite sequences of centromeric heterochromatin (Csink and Henikoff 1998). Several of these proteins bind to dispersed euchromatic sites during interphase, but bind to specific sequences of the centromeric heterochromatin during mitosis. The GAGA factor, for example, acts in transcriptional regulation at multiple euchromatic sites during interphase, but at mitosis it changes localization to the centromeric heterochromatin and is required for chromosome condensation (Bhat et al. 1996). The PROD (proliferation disrupter) protein similarly changes localization to the centromeric heterochromatin at mitosis and is required for chromosome condensation, especially in the centromeric regions (Torok et al. 1997). These examples provide precedents for centromeric heterochromatin localization of proteins required for chromosome condensation.

*Drosophila* topo II binds to a 359 bp repeat of the centromeric heterochromatin of the *X* chromosome as well as other sites with similar sequences in the euchromatin (Kas and Laemmli 1992). Topo II preferentially binds and cleaves DNA at sites called scaffold attachment regions (SARs) where chromatin loops are anchored to the nuclear scaffold in both *Drosophila* and vertebrates (Poljak and Kas 1995). In *Drosophila*, SAR sites have a sequence similar to the satellite heterochromatin sites where topo II binds (Kas and Laemmli 1992). Specific binding sites for TOP2A have not yet been identified in vertebrate satellite sequences, but TOP2A could function in both euchromatic sites along the chromosome arms as well as heterochromatic sites near the centromeres. In many studies TOP2A staining has been observed along chromatid arms (Sumner 1996), and, in the study reported here, diffuse staining throughout the nucleus was consistently seen. The intense staining at the centromeric heterochromatin may reflect a much higher concentration of the enzyme at this site, but euchromatic sites may require lower concentrations.

In contrast to results reported in mitotic cells (Hendzel et al. 1997), this report clearly shows that TOP2A is localized to the centromeric heterochromatin many days before histone H3 is phosphorylated at this site. This was verified in both isolated germ cells and in sectioned tissue. Therefore, phosphorylation of histone H3 is unlikely to recruit TOP2A to this region. Nonetheless, the precise colocalization of the initial histone H3 phosphorylation events and TOP2A at diplonema is intriguing. Could decatenation and phosphorylation events at the centromeric heterochromatin trigger chromosome condensation? At this point any proposed functions are speculative, but recent evidence indicates that phosphorylation may release the N-terminal tail of histone H3 from DNA (Sauvé et al. 1999). Such a change in the chromatin could presumably alter the accessibility of substrates of TOP2A at the centromeric heterochromatin. Likewise, changes in chromatin configuration could release TOP2A to act at other sites.

### *Other meiotic functions for TOP2A*

If TOP2A is present only to aid in chromosome condensation and segregation why is it present so early in meiotic prophase? The enzyme may also be important for other chromosomal functions. Meiotic chromosome pairing during spermatogenesis is correlated with large movements of chromosome centromeres and telomeres (Scherthan et al. 1996). Pairing apparently initiates at the bouquet stage in early zygonema when all telomeres are clustered at one end of the nuclear envelope. Decatenation events could be important in these chromosomal movements. Critical testing of this idea would require the ability to manipulate spermatocytes experimentally during the pairing process in early meiosis. The discovery that achiasmate pairing sites are in heterochromatic regions of repetitive DNA in *Drosophila* demonstrates that these regions can be important in homolog recognition (Renauld and Gasser 1997; McKee 1998). However, a similar role for heterochromatin in vertebrates has not been identified. Normal meiotic pairing and synapsis, followed by pachytene arrest was observed in the absence of topo II activity in *Saccharomyces cerevisiae* (Rose and Holm 1993). This could suggest that topo II activity might not be needed for early meiotic events in this species; however, the arrest in pachynema could also be the manifestation of failed events earlier in prophase.

Moens and Earnshaw (1989) reported that antibodies directed against topo II (not specific for either isoform) labeled the lateral elements of the SCs in rooster spermatocytes, and a similar localization in *Saccharomyces* meiotic chromosomes has been found (Klein et al. 1992). However, in the present study no specific TOP2A labeling was observed along the axes of the SCs of murine spermatocytes. The differences in these results could be due to species-specific differences in either TOP2A localization or the relative abundance of TOP2A in different nuclear locations. Although TOP2A can be localized in the axes of metaphase chromosomes in mitotic cells (Rattner et al. 1996), this too may be species specific, since another report (Sumner 1996) found that human mitotic metaphase chromosomes usually have TOP2A axial staining, but mouse chromosomes do not. In the present study, there was no specific localization of TOP2A to axes of meiotic metaphase chromosomes, although chromatin of the chromosomal arms was diffusely stained. Thus, TOP2A may be less abundant or accessible in mouse chromosomes, but it cannot be concluded that the protein is completely absent from the axes of metaphase chromosomes.

Although TOP2A and phosphorylated histone H3 can be specifically localized in meiosis, neither is present when chromatin is drastically remodeled and compacted during post-meiotic spermiogenic differentiation. The

abrupt disappearance of TOP2A as cells exit meiosis is interesting since topo II activity has been detected in round spermatids (Cobb et al. 1997). However, the decatenation activity detected could be due to TOP2B. Recent studies on TOP2B are reviewed elsewhere (Austin and Marsh 1998), and future studies should determine the role and localization of TOP2B during spermiogenic chromatin remodeling.

### *Conclusion*

When induced by OA to enter meiotic metaphase I prematurely, pachytene spermatocytes do not completely condense chromosomes when MPF activity is inhibited, even though histone H3 is phosphorylated in these conditions. This suggests that although phosphorylation of histone H3, which normally occurs during the transition to metaphase, may be necessary for condensation of meiotic chromosomes, it is not sufficient. This result also indicates that MPF is not the kinase that phosphorylates histone H3 at the end of meiotic prophase. This study shows that TOP2A and phosphorylated histone H3 are co-localized in the centromeric heterochromatin as spermatocytes exit meiotic prophase and enter the division phase. However, phosphorylation of histone H3 cannot be responsible for the localization of TOP2A since TOP2A associates with centromeric heterochromatin early in meiotic prophase and remains there through the division phase. Thus the role of histone H3 phosphorylation in MI chromosome condensation is not yet clear. It is likely that identification of the signal that leads to temporally and spatially specific phosphorylation of histone H3 will be important in elucidating the interplay among MPF activation, histone H3 phosphorylation, topo IIα activity and condensation of meiotic chromosomes.

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