Localisation of histone macroH2A1.2 to the XY-body is not a response to the presence of asynapsed chromosome axes

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Summary

Histone macroH2A1.2 and the murine heterochromatin protein 1, HP1β, have both been implicated in meiotic sex chromosome inactivation (MSCI) and the formation of the XY-body in male meiosis. In order to get a closer insight into the function of histone macroH2A1.2 we have investigated the localisation of macroH2A1.2 in surface spread spermatocytes from normal male mice and in oocytes of XX and XY^{Tdym1} mice. Oocytes of XY^{Tdym1} mice have no XY-body or MSCI despite having an XY chromosome constitution, so the presence or absence of 'XY-body' proteins in association with the X and/or Y chromosome of these oocytes enables some discrimination between potential functions of XY-body located proteins. We demonstrate here that macroH2A1.2 localises to the X and Y chromatin of spermatocytes as they condense to form the XY-body but is not associated with the X and Y chromatin of XY^{Tdym1} early pachytene oocytes. MacroH2A1.2 and HP1β co-localise to autosomal pericentromeric heterochromatin in spermatocytes. However, the two proteins show temporally and spatially distinct patterns of association to X and Y chromatin.

Key words: Histone macroH2A1.2, HP1β, XY-body, Asynapsed chromosome axes

Introduction

Meiosis is a highly specialised cell division during which the diploid chromosome state is reduced to the haploid state. Progression through meiotic prophase is associated with chromosome condensation and synapsis of homologous chromosomes together with reciprocal recombination. The paired homologous chromosomes segregate from each other in the first reduction division. In contrast to the autosomes, which synapse over their entire length, synapsis of the heteromorphic sex chromosomes during male meiosis is restricted to a segment that includes the pseudoautosomal region (PAR). A cardinal feature that distinguishes male from female meiosis in mammals is the progressive condensation of the X and Y chromosomes during meiotic prophase to form the XY-, or sex body (Solari, 1974). The formation of the XY-body has been considered as a morphological manifestation of meiotic sex chromosome inactivation (MSCI) (Monesi, 1965; Kofman-Alfaro and Chandley, 1970; Odartchenko and Pavillard, 1970; Kierszenbaum and Tres, 1974; Latos-Bielenska and Vogel, 1992; McCarrey and Dilworth, 1992). An increasing number of proteins have been identified that locate to the XY-body (Smith and Benavente, 1992; Smith and Benavente, 1995; Calenda et al., 1994; Kralewski et al., 1997; Bauer et al., 1998; Motzkus et al., 1999; Hoyer-Fender et al., 2000; O'Carroll et al., 2000; Parraga and del Mazo, 2000; Richler et al., 2000; Turner et al., 2000) but their significance if any for MSCI or heterochromatinisation are largely unknown. Turner et al. (Turner et al., 2000) have begun to discriminate between potential functions by assaying male XY-body located proteins during XY^{Tdym1} female meiosis. Despite having an XY chromosome constitution, XY^{Tdym1} oocytes have no XY-body or MSCI and their X and Y chromosomes rarely synapse (Turner et al., 2000).

Two proteins that have been implicated in effecting XY-body formation and MSCI are the mammalian HP1-like protein HP1β (M31 or MOD1) (Motzkus et al., 1999) and the unusual core histone macroH2A1.2 (Hoyer-Fender et al., 2000; Richler et al., 2000). HP1β is a member of the highly conserved HP1 class of chromobox genes, whose probable role is to assemble a variety of macromolecular complexes in chromatin (Singh and Huskisson, 1998; Jones et al., 2000). HP1β has therefore been implicated in a wide range of possible functions including transcriptional repression (Ryan et al., 1999), transgene silencing (Festenstein et al., 1999), chromosome segregation (Wang et al., 2000) and nuclear envelope reassembly (Kourmouli et al., 2000). HP1β is predominantly found in pericentromeric constitutive heterochromatin in mitotic (Wregett et al., 1994; Furuta et al., 1997) and meiotic cells (Motzkus et al., 1999) and it is concentrated in the XY-body of late-pachytene and diplotene spermatocytes (Motzkus et al., 1999; Turner et al., 2001). HP1β is a dose-dependent modifier of a variegating position effect (Festenstein et al., 1999). Silencing of gene activity may involve the recruitment of HP1 by transcriptional repressors as described for the KRAB family (Nielsen et al., 1999; Ryan et al., 1999;

Lechner et al., 2000). The mechanism by which HP1 protein represses gene activity may also involve histone modifications. HP1 was described as a methyl-lysine binding protein, which interacts with histone H3, methylated at K9 by the histone methylase SUV39H1, to repress transcription at heterochromatic sites (Rea et al., 2000; Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001; Peters et al., 2002). A model for the temporal events in heterochromatin silencing implies that after deacetylation of histone H3, SUV39H1 methylates H3 at lysine 9 (K9), which then serves as a binding site for the recruitment of HP1 (Strahl and Allis, 2000). HP1 through its shadow chromodomain located at the C terminus can oligomerize (Wang et al., 2000; Zhang and Reinberg, 2001) and it is as oligomers that HP1 proteins are incorporated into heterochromatin-like complexes. In addition alternative pathways for HP1 recruitment may exist as inferred from the recent demonstration of HP1 interaction with nucleosome core particles (Zhao et al., 2000) and histones H4 and H3 (Nielsen et al., 2001; Polioudaki et al., 2001), and by the observation that methylation at K9 of histone H3 is not necessary for HP1 binding to chromosomal DNA (Cowell et al., 2002).

Histone macroH2A1.2 is a nucleosomal histone with a size nearly three times that of conventional histone H2A (Pehrson and Fried, 1992). In addition to the N-terminal histone H2A-like region the C-terminal two-thirds of the molecule constitutes the macro domain. In vitro the macro domain acts as a transcriptional repressor (Perche et al., 2000) but its function in vivo in the context of nucleosomes is unknown. MacroH2A1.2 is preferentially concentrated in the inactive X chromosome (Xi) of female mammals (Costanzi and Pehrson, 1998; Mermoud et al., 1999; Costanzi et al., 2000) and requires *Xist* RNA for its localisation to the inactive X (Csankovszki et al., 1999; Beletskii et al., 2001). In male meiosis, macroH2A1.2 localises to centromeric heterochromatin and is concentrated in the developing XY-body in early pachytene spermatocytes (Hoyer-Fender et al., 2000; Richler et al., 2000). Recently, co-localisation of HP1β and histone macroH2A1.2 at the pseudoautosomal region (PAR) has suggested an involvement in reinforcing the attachment between the X and the Y chromosome prior to their disjunction (Turner et al., 2001).

In order to get a closer insight into the function of histone macroH2A1.2 during spermatogenesis we investigated its localisation in surface-spread meiocytes from male and female mice including XY^{Tdym1} females.

Materials and Methods

Mice

Spermatocytes were prepared from adult C57BL/6 XY males using stocks maintained at the University of Göttingen and the National Institute for Medical Research (NIMR). Oocytes were processed at 17.5 and 18.5 days post coitum (dpc) from XX females and XY^{Tdym1} littermates (Lovell-Badge and Robertson, 1990) bred at NIMR on a random-bred MF1 background. The latter mice are female owing to an 11 kb deletion that has removed the testis-determining gene *Sry* (Gubbay et al., 1990; Gubbay et al., 1992). XY^{Tdym1} females were distinguished from XX littermates by cytological examination.

Germ cell preparations

Mouse spermatogenic and oogenic cells were prepared as surface spreads (Peters et al., 1997) or as squashes (Page et al., 1998). Oocytes were fixed in 2% formaldehyde in PBS containing 0.05% Triton X-100 (Page et al., 1998) for 10 minutes at room temperature. Spermatocytes were fixed in 1% paraformaldehyde in 10 mM sodium borate pH 9.2 containing 0.15% Triton X-100 (Peters et al., 1997) for at least 30 minutes at room temperature.

Meiotic staging

Spermatocytes were substaged based on the changing morphology of the autosomes and the XY bivalent (Plug et al., 1998) as revealed by SCP3 staining, and by the changing morphology of the XY-body as revealed by DAPI staining (Turner et al., 2000). Oocytes were substaged as described by Mahadevaiah et al. (Mahadevaiah et al., 1993).

Fluorescence immunostaining

Surface spreads were incubated in PBT (0.15% BSA, 0.1% Tween 20 in PBS) for 60 minutes prior to incubation with primary antibodies diluted in PBT overnight. The polyclonal rabbit anti-rat macroH2A1.2 (Costanzi and Pehrson, 1998), the guinea pig anti-rat SCP3 (Alsheimer and Benavente, 1996), the rat anti-HP1β (Wreggett et al., 1994), the human CREST auto-immunserum (Earnshaw and Rothfield, 1985) and the anti-H4 antibody (sc-10810; Santa Cruz Biotechnology, Santa Cruz, CA) were all used at 1:100. Monoclonal mouse anti-XLR (Calenda et al., 1994) was used at 1:500 and mouse monoclonal anti-γ-H2AX (Upstate Biotechnology, Charlottesville, VA) at 1:12500. We detected the primary antibodies for macroH2A1.2, histone H4, XLR and γ-H2AX using goat anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR), SCP3 using goat anti-guinea pig Cy3 (Jackson ImmunoResearch, West Grove, PA), HP1β using anti-rat Cy3 (Sigma, St Louis, MO), and CREST using goat anti-human Cy5 (Amersham Pharmacia Biotech, Freiburg, Germany). All secondary antibodies were diluted in PBT at 1:100, except goat anti-human Cy5 which was diluted 1:500. DNA was counterstained with 4′,6-diamidine-2′-phenylindoledihydrochloride (DAPI). Slides were air-dried and mounted using Vectashield (Vector, Burlingame, CA). Immunostained cells were examined either on an Olympus IX70 inverted microscope or by confocal microscopy (Zeiss LSM 510). Captured images (spermatocytes n=172, Sertoli cells n=25, XY oocytes n=328, XX oocytes n=67) were processed using Adobe Photoshop 5.0.

Results

MacroH2A1.2 localisation in surface-spread spermatocytes

MacroH2A1.2 was first detected in surface-spread spermatocytes. DAPI staining was used to visualise heterochromatin. For substaging cells an antibody against the synaptonemal complex protein SCP3 was used (Alsheimer and Benavente, 1996). At leptotene, axial elements are first detected as short stretches that extend in length and synapse with homologues during zygotene. Complete synapsis marks the transition to pachytene. In early pachytene X-Y synapsis is more extensive than at later stages and there is no distinct sex body visible by DAPI staining (Turner et al., 2000). By mid pachytene, the extent of X-Y synapsis has reduced and there is a distinct peripheral sex-body visible by DAPI staining. By late pachytene, the SCs have thickened ends and the X and Y are only minimally associated at the PAR telomeric ends. During diplotene, axial elements repel each other, starting at the centromere, which is therefore split in early diplotene. During diakinesis, the axial elements are disassembled except

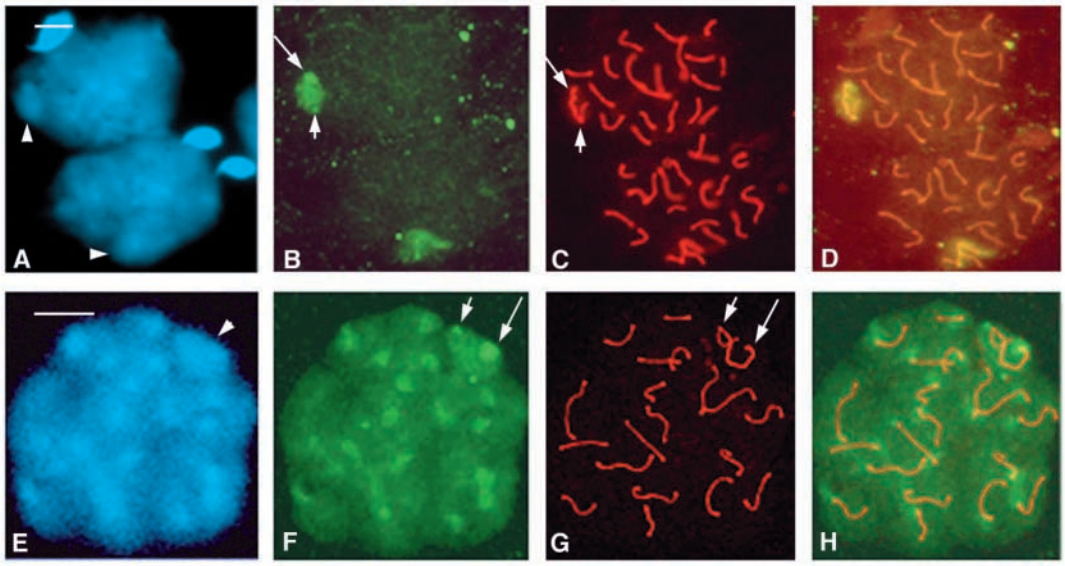

Fig. 1. Localisation of histone macroH2A1.2 in surface spread spermatocytes (blue, DAPI staining; green, anti-macroH2A1.2 antibody; red, anti-SCP3 antibody; yellow/orange, co-localisation of anti-macroH2A1.2 and anti-SCP3 antibodies). (A-D) Histone macroH2A1.2 is preferentially concentrated over the forming XY-body of spermatocytes at early pachytene (lower cell) and at mid-pachytene (upper cell with DAPI dense XY-body – arrowhead in A) with a higher concentration at the X centromere (long arrow) and the synapsed PARs (short arrow) evident in the latter. (E-H) In late pachytene spermatocytes histone macroH2A1.2 is no longer concentrated throughout the XY-body, but remains concentrated on the X centromere and the PARs, and now also localises to the centromeric heterochromatin of autosomes. (Labels as for A-D.) Scale bars: 10 μm.

for SCP3-positive remnants at the centromeres that remain through metaphase I to anaphase II.

MacroH2A1.2 could be seen concentrated in a peripheral nuclear region in early/mid pachytene spermatocytes (Fig. 1A-D) which is in agreement with earlier observations on testis sections (Hoyer-Fender et al., 2000). Detection of synaptonemal complexes by anti-SCP3 antibodies revealed that this concentration of macroH2A1.2 was associated with the X and Y chromatin of the forming XY-body with the brightest staining at the centromere of the X chromosome and within the region of PAR synapsis (Fig. 1B,C). By late pachytene macroH2A1.2 also localised to the heterochromatic pericentromeric regions of autosomes as well as to the centromere of the X chromosome and to the PAR (Fig. 1E-H). However, the rest of the XY-body now showed a staining intensity comparable to the autosomes. Meiotic metaphase chromosomes showed complete decoration with macroH2A1.2 with a higher concentration at the centromeres especially at the centromeres of X and Y chromosomes (not shown). MacroH2A1.2 in spermatocytes therefore seems to be predominantly concentrated in heterochromatic regions.

Co-localisation of macroH2A1.2 and HP1β in surface-spread spermatocytes

Staging of all co-localisation experiments is based on the known distribution of macroH2A1.2 and HP1β during spermatogenic progression (Motzkus et al., 1999; Hoyer-Fender et al., 2000; Turner et al., 2001) as well as on the DAPI staining pattern, which reveals the developing XY-body (Turner et al., 2000).

In early pachytene spermatocytes (Fig. 2A-D) the majority of the chromatin is densely stained with DAPI, so no separate DAPI-dense XY-body domain is apparent. Nevertheless, macroH2A1.2 is preferentially associated with the whole XY-chromatin (Fig. 2A,B). In contrast, HP1β is concentrated in two foci within this domain (Fig. 2C), which Turner et al. (Turner et al., 2001) have shown are located at the X centromere and synapsed PARs. This result is consistent with earlier observations on tissue sections (Motzkus et al., 1999). Remarkably, the antibody against macroH2A1.2 also detects very fine filamentous structures reminiscent of the synaptonemal complex although no antibodies against synaptonemal complex proteins were used (Fig. 2B arrowheads). It seems therefore that macroH2A1.2 may also be present in the condensing euchromatic regions of chromosomes.

In mid/late-pachytene spermatocytes (Fig. 2E-H) the XY-body, together with the pericentromeric autosomal heterochromatin, now appear as distinct DAPI-dense domains. MacroH2A1.2 is localised in the pericentromeric heterochromatin of autosomes as well as in the XY-body where two brightly staining spots were detected (Fig. 2F), the brightest of which most probably represents the centromeric region of the X chromosome. HP1β is similarly localised in the pericentromeric heterochromatin of autosomes as well as in the XY-body where it is also particularly concentrated in one brightly staining spot that co-localises with macroH2A1.2 (Fig. 2G,H). Since the brightly staining spot in the XY-body represents the centromeric region of the X chromosome (Fig. 1F,G), macroH2A1.2 and HP1β co-localise to the X centromere. The second focus of macroH2A1.2 staining is clearly negative for HP1β, and is thus likely to be the Y centromere (Turner et al., 2001).

In early diplotene spermatocytes (Fig. 2I-L) the XY-body remains DAPI dense. MacroH2A1.2 localises to the autosomal

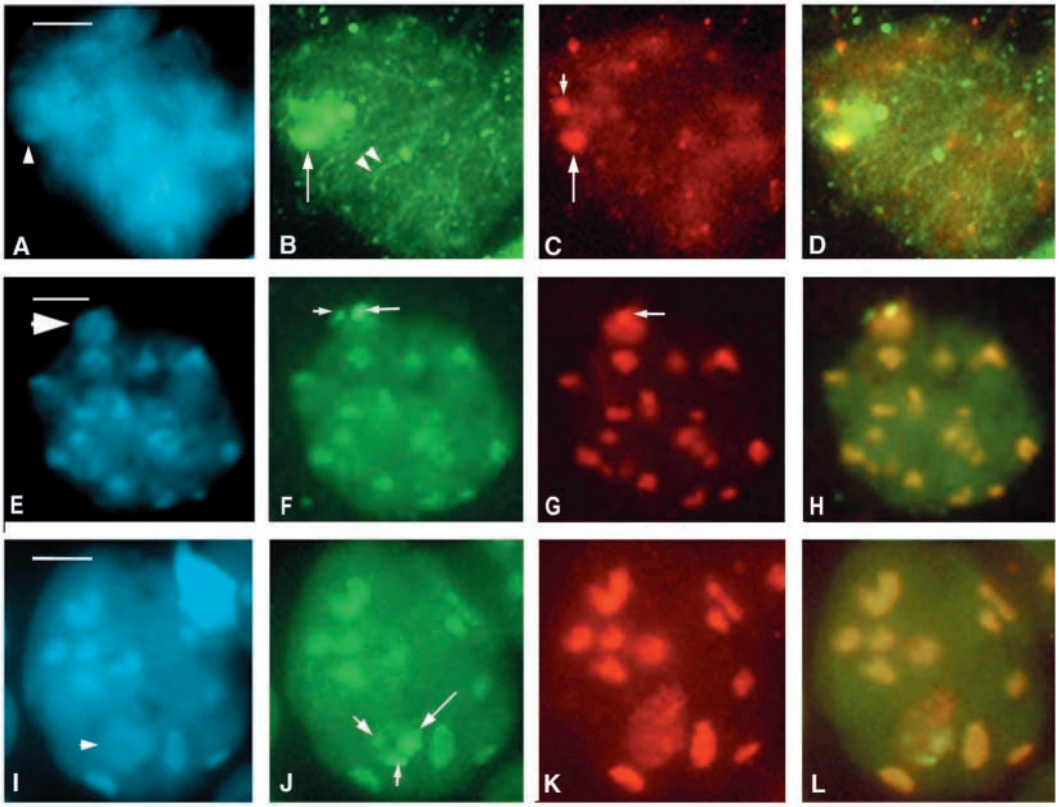

Fig. 2. Co-localisation of macroH2A1.2 and HP1β in spermatocytes. (blue, DAPI staining; green, anti-macroH2A1.2 antibody; red, anti-HP1β antibody; yellow/orange, co-localisation of anti-macroH2A1.2 and anti-HP1β antibodies). (A-D) Histone macroH2A1.2 begins to concentrate over the XY-chromatin in early pachytene spermatocytes (B, arrow) before a DAPI-dense XY-body is detectable (A). HP1β is concentrated in two foci within the XY-body (C, long and short arrows); these foci are presumably associated with the X centromere and synapsed PARs (Turner et al., 2001). The arrowheads in B mark the filamentous structures detected by macroH2A1.2 antibodies. (E-H) In mid/late pachytene spermatocytes (with DAPI dense XY bodies, arrowhead in E) histone macroH2A1.2 and HP1β are now both concentrated throughout the XY-body, with one particularly dense focus, presumably associated with the X centromere (long arrow). Both proteins are also concentrated at the centromeric heterochromatin of autosomes. The XY-body-associated macroH2A1.2-positive/HP1β-negative focus (short arrow) is undoubtedly the Y centromere (Turner et al., 2001). (I-L) In diplotene spermatocytes (with an internalising XY-body, arrowhead in I), macroH2A1.2 and HP1β remain concentrated at autosomal centromeres. However, the XY-body-located staining pattern is now quite distinct, with the concentration of macroH2A1.2 restricted to the X and Y centromeres and the PAR focus (arrows in J), whereas HP1β is uniformly concentrated throughout the XY-body. (Note: a DAPI dense sperm head is apparent in I.) Scale bars: 10 μm.

pericentromeric heterochromatin, and in the XY-body to the X and Y centromeres and the PARs (Fig. 2J). HP1β localises to the autosomal pericentromeric heterochromatin and to the whole of the XY-body with no preferential staining of the X centromere or PARs (Fig. 2K).

In summary, the patterns of staining for the two proteins are similar in the autosomal domain, but are distinct in the gonosomal domain. In the gonosomal domain, macroH2A1.2 begins by staining all of the X and Y chromatin in early pachytene and then becomes progressively concentrated at the X and Y centromeres and the synapsed PARs; staining of the rest of the XY-body reduces to the level of general nuclear staining. HP1β on the other hand begins by being concentrated at the X centromere and PAR, but by diplotene it has become concentrated throughout the whole XY-body domain, X centromeric staining has reduced to background levels and a specific PAR focus is no longer visible.

During the analysis of the spread spermatocytes it was noted that macroH2A1.2 and HP1β also co-localise to the heterochromatin of Sertoli cells, which are the somatic 'supporting' cells within the testis tubules. In mice the nucleus of Sertoli cells characteristically has a nucleolus flanked by two heterochromatic regions making up the clustered centromeres. These concentrations of heterochromatin are clearly visible by DAPI staining and have a high concentration of macroH2A1.2 as well as of HP1β (not shown).

MacroH2A1.2 is not concentrated on the X and Y chromatin of XY oocytes

In order to get a closer insight into the possible function of macroH2A1.2 in XY-body formation we investigated the localisation of macroH2A1.2 in surface-spread oocytes from XX and XY^{Tdym1} females in which there is no XY-body formation or MSCI. The centromeres were detected with CREST. In XX early pachytene oocytes macroH2A1.2 localised to the clustered pericentromeric heterochromatin of autosomes as well as gonosomes (Fig. 3A,B). The XX bivalent could be identified by the brightly staining PAR focus at the non-centromeric end of the SC (Turner et al., 2001) (Fig. 3B, arrow). In XY^{Tdym1} early pachytene oocytes macroH2A1.2 also localises to the pericentromeric heterochromatin (Fig. 3C,D). The asynapsed gonosomal axes could be identified by their fainter SCP3 staining (Fig. 3C) and by the presence of the brightly staining PAR focus at their non-centromeric ends. Aside from the centromeric ends, the X as well as the Y axis appeared to show no concentration of macroH2A1.2 (Fig. 3C,D). In order to confirm this we prepared squash preparations of XX and

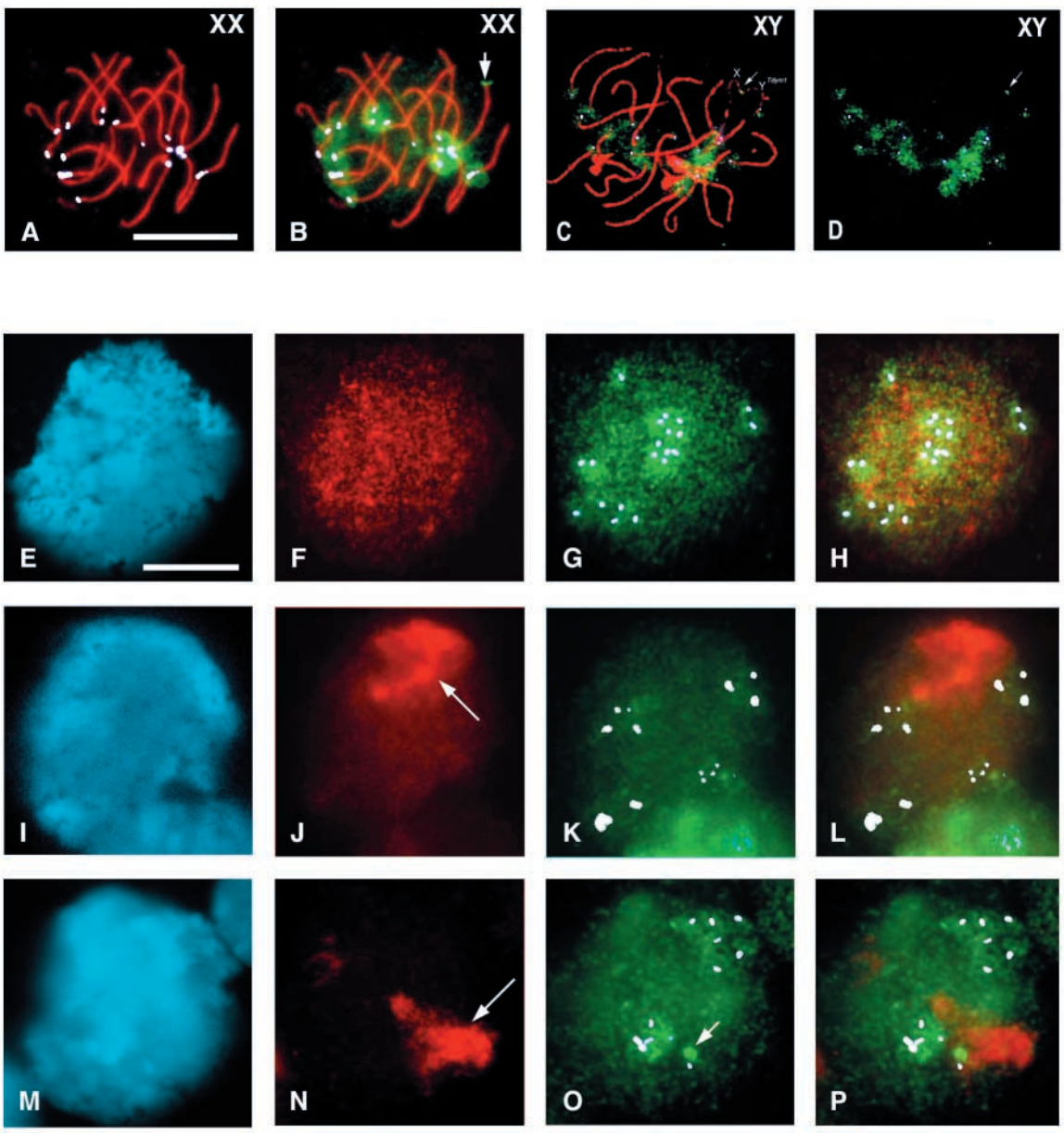

Fig. 3. Histone macroH2A1.2 is not concentrated in X and Y chromatin of early pachytene XY oocytes. (Blue, DAPI; red, anti-SCP3 in A-D and anti-XLR in E-P; green, anti-macroH2A1.2 antibody; yellow/orange, co-localisation of anti-macroH2A1.2 with anti-SCP3 or anti-XLR; white, CREST antibody, which marks the centromeres). (A,B) In spread oocytes from XX mice, macroH2A1.2 is concentrated at the centromeric heterochromatin of all bivalents as well as at the non-centromeric end of one bivalent (arrow); this bivalent must be the X which has a PAR focus (Turner et al., 2001). (C,D) In this spread oocyte from an XY^{Tdym1} mouse in which the X and Y are synapsed, the PAR focus of macroH2A1.2 is clearly visible (arrow). The thin asynapsed non-PAR axes of the X and Y are also distinguishable, but only the centromeric region of the X has a high concentration of histone macroH2A1.2. (E-H) In XX early pachytene oocyte squashes, anti-XLR antibody produces a granular but fairly uniform staining thoughout the nucleus. MacroH2A1.2, however, is concentrated in the regions where the centromeres are clustered. (I-L) In early pachytene XY oocytes, anti-XLR antibody now concentrates on the chromatin of the asynapsed X (arrow). (M-P) In late pachytene XY oocytes, macroH2A1.2 preferentially stains the X centromere (arrow in O) and the PAR focus. Arrow in N indicates the chromatin of asynapsed X. Scale bar: (A-D) 21 μm; (E-P) 7 μm.

XY^{Tdym1} pachytene oocytes (Fig. 3E-P) and stained them for macroH2A1.2 and with an anti-XLR antibody (Calenda et al., 1994) that stains the whole X chromatin when the X is asynapsed [Turner, J. M. A. (2000). *An Investigation into The Role of Sex Chromosome Synapsis in Meiotic Sex Chromosome Inactivation and Fertility.* PhD Thesis. University of London]. This confirmed that, in contrast to the male, there is no macroH2A1.2 staining of the X chromatin during early pachytene in XY females. In late pachytene XY oocytes, as in the male, macroH2A1.2 preferentially stained the X centromere and the PAR focus. This result establishes that the presence of macroH2A1.2 on gonosomal chromatin in early pachytene

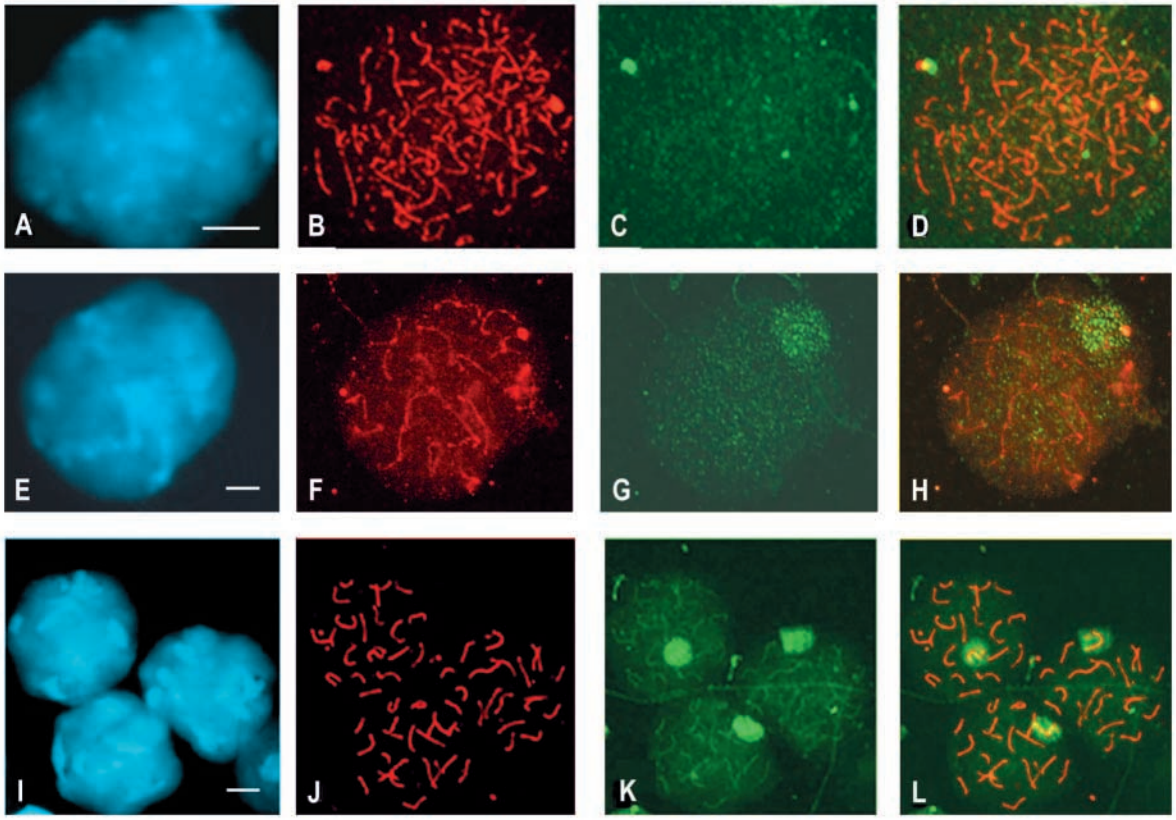

Fig. 4. Condensation of gonosomal chromatin starts at zygotene/pachytene transition in spread spermatocytes (blue, DAPI; red, anti-SCP3; green, anti-histone H4). At leptotene (not shown) and zygotene (A-D) histone H4 showed uniform nuclear distribution. At zygotene/pachytene transition (E-H) histone H4 is slightly more concentrated in the forming XY-body located at the nuclear periphery. At mid-pachytene (I-L) and diplotene (not shown) the XY-bodies have a higher histone H4 concentration than the autosomal chromatin. Scale bars: 10 μm.

spermatocytes is not simply due to the presence of gonosomal chromatin per se and is also not a response to the presence of asynapsed chromosomal axes.

Coincidence of gonosomal chromatin condensation with histone macroH2A1.2 concentration

In early pachytene spermatocytes histone macroH2A1.2 is preferentially associated with the gonosomal chromatin before a separate DAPI-dense XY-body is apparent. To determine whether this uneven distribution inside the nucleus reflects a differential chromatin packaging between gonosomes and autosomes we investigated the distribution of histone H4 in spread spermatocytes. The antibody used for histone H4 detection is directed against recombinant histone H4. In early meiotic prophase stages (leptotene, not shown, and zygotene, Fig. 4A-D) histone H4 is evenly distributed in the whole nucleus without preferential staining of any nuclear compartment. But in the period from the zygotene/pachytene transition (Fig. 4E-H) to mid pachytene (Fig. 4I-L) histone H4 is found to be progressively concentrated in the whole of the XY-body. Since the pattern of H4 staining is comparable to that for macroH2A1.2, this suggests that the progressive localisation of both to the sex body is largely a reflection of the increasing concentration of the chromatin. This view was supported by double labelling spread spermatocytes for macroH2A1.2 and phosphorylated H2AX (γ-H2AX); γ-H2AX localises to gonosomal chromatin in late zygotene before condensation, forming a tadpole-like structure and is then retained through to diplotene (Mahadevaiah et al., 2001; Fernandez-Capetillo et al., 2003). When the uncondensed gonosomal chromatin is first highlighted by γ-H2AX in late zygotene (Fig. 5A-D) macroH2A1.2 showed no preferential association with the gonosomal chromatin; concentration of macroH2A1.2 to gonosomal chromatin starts at early pachytene coincident with the first stages of condensation (Fig. 5E-H).

Discussion

In surface spread spermatocytes from normal male mice we found co-localisation of histone macroH2A1.2 and of HP1β in the constitutive pericentromeric heterochromatin of autosomes as well as in the heterochromatic regions of the somatic Sertoli cells. In contrast to the constitutive heterochromatin of autosomes, there is a remarkable difference in the temporal and spatial distribution of macroH2A1.2 and of HP1β in the facultative heterochromatin of the XY-body that forms during male meiotic prophase. Whereas macroH2A1.2 is preferentially located to the X and Y chromatin when condensation starts to form the XY-body and then becomes concentrated at the X centromere [and Y centromere (see Turner et al., 2001)] and the PARs at the end of meiotic prophase, HP1β is first restricted to the X centromere and PARs

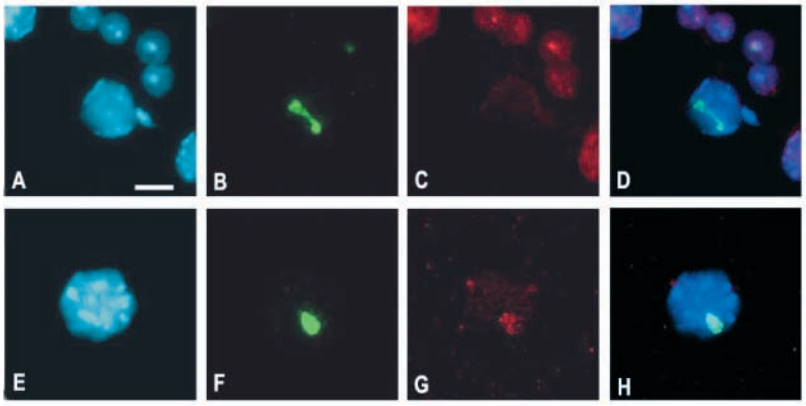

Fig. 5. Histone macroH2A1.2 begins to concentrate in the XY-body at early pachytene of spermatocytes (blue, DAPI; green, anti-gammaH2AX; red, anti-macroH2A1.2). The gammaH2AX antibody stains the X and Y chromatin before it condenses at late zygotene (A-D) where a 'tadpole-shaped' structure is visible. At this stage no detectable concentration of macroH2A1.2 could be found. However, once condensation has started at early pachytene (E-H) histone macroH2A1.2 begins to appear over the XY-body. Scale bars: 25 μm.

and then spreads to include the whole of the XY-body with no preferential concentration at the X centromere or the PARs.

Turner et al. (Turner et al., 2000) (and unpublished data) have found that the location of some proteins to gonosomal chromatin in pachytene spermatocytes is associated with chromosomal asynapsis (of the non-PAR axes), rather than being XY-body specific. We therefore asked whether the location of histone macroH2A1.2 to gonosomal chromatin might also be a response to the asynapsis of the non-PAR axes. To answer this, we examined XY^{Tdym1} oocytes, which have no XY-body or MSCI and their X and Y chromosomes rarely synapse. Our results demonstrated that aside from the X and Y centromeres and the X and Y PARs, macroH2A1.2 is not concentrated in the chromatin of the X and Y of XY^{Tdym1} oocytes. The preferential concentration of macroH2A1.2 in the developing XY-body in early pachytene spermatocytes therefore seems not to be a response to the presence of gonosomal chromatin per se or to the presence of asynapsed chromosome axes.

While it is clear that preferential localisation of macroH2A1.2 to X and Y chromatin in meiotic prophase is a male-limited phenomenon, we considered it possible that this could simply reflect the condensation of the chromatin to form an XY-body in spermatocytes but not in XY oocytes (Turner et al., 2000). The concentration of macroH2A1.2 to the inactive X chromosome of female mammals as a simple measure of the degree of chromatin condensation has been suggested recently (Perche et al., 2000). Several results have since been published disagreeing with this conclusion but instead presenting evidence that macroH2A1.2 density on the Xi is not a simple measure of DNA compaction (Chadwick et al., 2001; Costanzi and Pehrson, 2001; Rasmussen et al., 2001). However, our comparison of the time course for macroH2A1.2 staining of the gonosomal domain in primary spermatocytes, with that for histone H4 and γ-H2AX supported the possibility of a reflection of chromatin density. The γ-H2AX staining of the gonosomal domain covers the period from late zygotene to early pachytene during which the chromatin becomes spatially restricted (condensed) from a former tadpole-like structure in late zygotene to form a nascent vesicle-like XY-body (Mahadevaiah et al., 2001). Since concentration of both histone H4 and macroH2A1.2 is coincident with this spatial restriction, we consider it likely to largely be a reflection of increasing chromatin condensation. However, for the histone H4 antibody staining different accessibilities because of altered chromatin conformation has to be kept in mind. Using DAPI staining as an indicator of chromatin density there is a period in early pachytene when macroH2A1.2 staining is primarily restricted to the gonosomal chromatin, with little staining of other regions of similar or greater chromatin density including the centromeric regions. This result indicates that at least at this stage the concentration of macroH2A1.2 may not be merely a reflection of chromatin density. By the mid-pachytene stage, macroH2A1.2 staining changes to a pattern that is similar to the distribution of DNA as measured by DAPI.

In early pachytene staining of macroH2A1.2 occurs primarily in the XY body. But from the mid-pachytene stage on histone macroH2A1.2 showed a remarkable background staining throughout the nucleus. This contrasted with the anti-HP1β staining which from mid-pachytene onwards was almost exclusively restricted to pericentromeric heterochromatin and then to the XY-body (see for example Fig. 2). These results support the view that macroH2A1.2 is a general chromosomal protein, with the apparent concentration at heterochromatic regions, whereas HP1β is recruited to specific chromosomal regions in conjunction with heterochromatinisation. This recruitment of HP1β is thought to be a response to the methylation of histone H3 at lysine 9, which has been shown to occur in pericentric regions and in the XY-body (Peters et al., 2001; Cowell et al., 2002). However, differences in the formation as well as in the structural organisation has been demonstrated for the pericentromeric autosomal heterochromatin and the facultative heterochromatin of the Xi in female mammals (Maison et al., 2002). Our results with polyclonal antibodies raised against histone H4 suggest that the structural organisation of XY gonosomal heterochromatin may also differ from pericentromeric heterochromatin. While the XY-body was stained with these anti-H4 antibodies starting in early pachytene, the pericentromeric heterochromatin was not stained by these antibodies at any stage. DAPI staining suggests that the chromatin density of the centromeric regions is at least as high as that of the XY-body and, therefore, the lack of centromeric H4-staining appears to be due to epitope inaccessibility.

It has previously been reported that HP1β localises to the PARs of the X and Y of spermatocytes, and of XX and XY oocytes even when the X and Y fail to synapse (Turner et al., 2001). We show here that macroH2A1.2 is also present at the PAR focus. This focus of heterochromatin may aid sister chromatid cohesion (Allshire, 1997). Improved sister chromatid adhesion distal to the X-Y cross-over may be important to reinforce the X-Y association thus ensuring proper X-Y segregation (Dernburg et al., 1996; Turner et al., 2001).

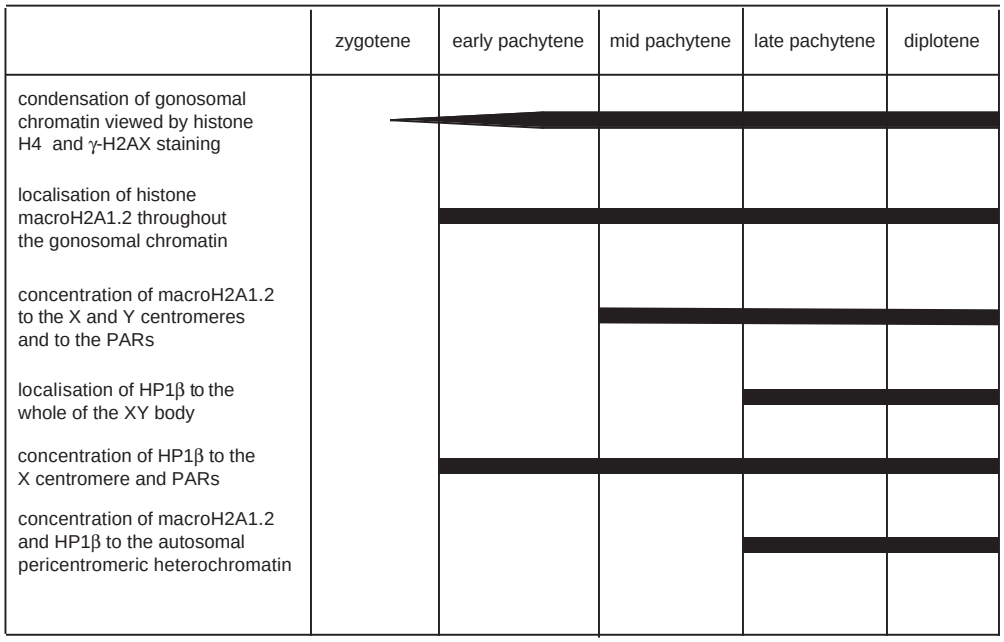

Fig. 6. Spatial and temporal patterns of histone macroH2A1.2 and HP1β localisation during prophase progression in male meiosis. The gonosome condensation describes the events as marked by anti-histone H4 and anti-γH2AX staining but a DAPI dense XY-body first become apparent at mid-pachytene.

MacroH2A1.2 also co-localises with HP1β in pericentromeric heterochromatin of autosomes and of the X chromosome in mid to late pachytene spermatocytes, and to heterochromatic regions of somatic Sertoli cells. This suggests that histone macroH2A1.2, in addition to HP1β, may have a function in heterochromatin formation at these sites. The spatial and temporal patterns of macroH2A1.2 and HP1β staining (Fig. 6) suggest that macroH2A1.2 could have a role in the recruitment of HP1β-containing protein complexes and the regulation of chromatin function. Whereas the formation of the nucleosome is independent of the histone tails, they are required for nucleosome-nucleosome interaction and for establishing transcriptionally repressive chromatin (Luger et al., 1997). The unusual large C-terminal tail of histone macroH2A1.2 may therefore be viewed as a special histone tail modification important for the recruitment of proteins or protein complexes to regulate chromatin functions as the histone code hypothesis predicts (Strahl and Allis, 2000), thus extending the predicted histone code hypothesis for the regulation of chromatin function to histone variants. HP1β, formerly viewed as a stable component of heterochromatin is, on the contrary, a protein with very dynamic binding (Festenstein et al., 2003; Cheutin et al., 2003). Stable repression of heterochromatin therefore might rely on components stably integrated into chromosomes. MacroH2A1.2 is an integral component of nucleosomes (Pehrson and Fried, 1992) and hence could function in the recruitment of protein complexes, including HP1β, which in turn promote heterochromatin formation. However, in the XY-body condensation and transcriptional silencing precede the appearance of HP1β throughout the gonosomal domain, suggesting that interaction between macroH2A1.2 and HP1β is not involved in XY-body formation.

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