M31 and macroH2A1.2 colocalise at the pseudoautosomal region during mouse meiosis

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SUMMARY

Progression through meiotic prophase is associated with dramatic changes in chromosome condensation. Two proteins that have been implicated in effecting these changes are the mammalian HP1-like protein M31 (HP1β or MOD1) and the unusual core histone macroH2A1.2. Previous analyses of M31 and macroH2A1.2 localisation in mouse testis sections have indicated that both proteins are components of meiotic centromeric heterochromatin and of the sex body, the transcriptionally inactive domain of the X and Y chromosomes. This second observation has raised the possibility that these proteins co-operate in meiotic sex chromosome inactivation. In order to investigate the roles of M31 and macroH2A1.2 in meiosis in greater detail, we have examined their localisation patterns in surface-spread meiocytes from male and female mice. Using this approach, we report that, in addition to their previous described staining patterns, both proteins localise to a focus within the portion of the pseudoautosomal region (PAR) that contains the *steroid sulphatase* (*Sts*) gene. In light of the timing of its appearance and of its behaviour in sex-chromosomally variant mice, we suggest a role for this heterochromatin focus in preventing complete desynapsis of the terminally associated X and Y chromosomes prior to anaphase I.

Key words: M31, MacroH2A1.2, Heterochromatin proteins, Centromeric heterochromatin, Sex body, Meiotic sex chromosome inactivation, X-Y synapsis, PAR, Non-disjunction

INTRODUCTION

Meiosis is a highly specialised cell division that serves to reduce the diploid to the haploid state. In order to achieve this, homologous chromosomes must first locate and pair with one another, and then engage in reciprocal recombination. Acting as a single unit, homologous chromosomes then migrate to the spindle equator, form attachments to opposite spindle poles and segregate from one another. These unique events are thought to be facilitated both by meiosis-specific structures, notably the synaptonemal complex (Moses, 1956; Fawcett, 1956) and recombination nodules (reviewed by Carpenter, 1994), and by a complex cycle of chromatin expansion and contraction (Hunter et al., 2001).

Events at the centromere might be important at a number of steps in meiosis. For instance, migration of centromeres to the nuclear envelope is the earliest detectable event in meiotic pairing in the mouse (Scherthan et al., 1996). Second, in mouse and *Drosophila*, the centromere harbours a number of proteins with vital roles in chromosome condensation (Cobb et al., 1999 and references therein). Finally, sequences in the centromere might be required for assembly of the functional kinetochore (Wiens and Sorger, 1998). Accordingly, increasing attention has been paid to characterising those proteins that associate with the centromere during meiosis.

M31 is a mammalian member of the highly conserved HP1 protein family, whose probable role is to assemble a variety of macromolecular complexes in chromatin (Jones et al., 2000). This variety has suggested 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 at the end of mitosis (Kormouli et al., 2000). Immunostaining analysis has revealed that M31 is a component of constitutive heterochromatin in both mitotic (Wreggett et al., 1994) and meiotic (Motzkus et al., 1999) cells. In the latter study, M31 was also shown to associate with the sex body, the transcriptionally inactive domain of the X and Y chromosomes, during male meiotic prophase (Motzkus et al., 1999). Specifically, M31 was first seen to associate with the X centromere in early pachytene and then with the Y chromosome in late pachytene. This led to the suggestion that M31 functions in meiotic sex chromosome inactivation (MSCI) and that M31-mediated MSCI initiated on the X chromosome in early pachytene and later spread to the Y chromosome via the synapsed pseudoautosomal region (PAR).

A role in MSCI has also been suggested for the unusual core histone macroH2A1.2 (Hoyer-Fender et al., 2000; Richler et al., 2000). MacroH2A1.2 contains an N terminus that is similar to a conventional histone 2A and a large non-histone domain that acts as a transcriptional repressor in vitro (Perche et al., 2000). Interest in macroH2A1.2 first arose following the demonstration that it localised to the inactive X chromosome in female somatic nuclei (Costanzi and Pehrson, 1998) and that this localisation was *Xist* dependent (Csankovski et al., 1999). Since then, macroH2A1.2 has been shown to localise to the sex body during early pachytene (Hoyer-Fender et al., 2000;

Richler et al., 2000). MacroH2A1.2 also localises to centromeric heterochromatin in meiosis (Hoyer-Fender et al., 2000) but not during mitosis (Costanzi and Pehrson, 1998).

In the present study, we have analysed the role of M31 and macroH2A1.2 in meiosis in greater detail using surface spread preparations from wild-type and sex-chromosomally mutant male and female mice. We have discovered that, in addition to their association with centromeric heterochromatin and with the sex body, both proteins localise to the pseudoautosomal region (PAR), the site of XY pairing during male meiosis. Based on our observations, we hypothesise a role for these proteins in ensuring normal disjunction of the sex chromosomes during metaphase I.

MATERIALS AND METHODS

Mice

All mice were generated on a randomly bred MF1 background using stocks maintained at the National Institute for Medical Research. Adult XY (*n*=5), X^{Y}*O (*n*=2; Odorisio et al., 1998) XY* (*n*=2; Eicher et al., 1991) and X*Sxr*^{a}O (*n*=2; Kot and Handel, 1990; Sutcliffe et al., 1991) males were processed at the age of 2-3 months. XX females (*n*=4) and XY^{Tdym1} littermates (*n*=3; Lovell-Badge and Robertson, 1990), which are female owing to an 11 kb deletion that has removed the testis-determining gene *Sry* (Gubbay et al., 1990; Gubbay et al., 1992), were processed at 18.5 days post coitum (dpc).

Meiotic preparations and fluorescence immunostaining

Mouse spermatogenic and oogenic cells were prepared as surface spreads (Peters et al., 1997), with 2% formaldehyde containing 0.05% Triton X-100 and 0.02% SDS being the fixative of choice. Spermatocytes and oocytes were fixed for 120 minutes and 10 minutes, respectively. Following fixation, slides were incubated in PBT (0.15% BSA, 0.1% Tween-20 in PBS) for 60 minutes prior to incubation overnight at 4°C with primary antibodies diluted in PBT. Rat anti-M31 antibody was used at 1:500. Mouse anti-COR1 antibody was used at 1:250. Rabbit anti-SYCP3 antibody was used at 1:1000. Rabbit anti-macroH2A1.2 antibody was used at 1:100. Human anti-centromere antibody (CREST) was used at 1:5000. Slides were washed three times for 5 minutes in PBS, followed by the application of secondary antibodies. Secondary antibodies used were goat anti-rat Alexa 488 (Molecular Probes), goat anti-rabbit Cy3 (Amersham Pharmacia Biotech), goat anti-rabbit Alexa 488 (Molecular Probes), goat anti-mouse Cy3 (Amersham Pharmacia Biotech), goat anti-mouse Alexa 488 (Molecular Probes), goat anti-rabbit Cy5 (Amersham Pharmacia Biotech) and goat anti-human Cy5 (Amersham Pharmacia Biotech). All secondaries were used at 1:500 in PBS. Following secondary incubations, slides were washed in PBS as described above and placed in a dark chamber to dry for 30 minutes. Slides were subsequently mounted in Vectashield with DAPI (Vector). Where required, controls consisted of omission of primary antibodies and replacement of primary antibodies with pre-immune serum, which resulted in no staining.

Fluorescence in situ hybridisation

Fluorescence in-situ hybridisation (FISH) was carried out using FITC-labelled X chromosome and Cy3-labelled Y chromosome paints, according to manufacturer's instructions (Cambio, Cambridge, UK). Immunostaining was carried out prior to the FISH procedure, because this procedure removes the axial element and M31 immunofluorescent signals. The CREST labelling, by contrast, was not affected by FISH and was therefore used to align the immunostaining and FISH signals for corresponding cells (images having been captured before and after the FISH procedure).

Meiotic substaging

Spermatocytes were substaged based on the changing morphology of the autosomes and of the XY bivalent (Plug et al., 1998). Oocytes were substaged as described by Mahadevaiah et al. (Mahadevaiah et al., 1993).

Image capture

Immunostained cells were examined and digitally imaged on an Olympus IX70 inverted microscope with a 100 W mercury arc lamp, using a 100× 1.35 U-PLAN-APO oil-immersion objective. Each fluorochrome image was captured separately as a 12-bit source image using a computer-assisted (Deltavision) liquid-cooled CCD (Photometrics CH350L; Sensor: Kodak KAF1400, 1317×1035 pixels). A single multiband dichroic mirror was used to eliminate shifts between different filters. Captured images were processed using Adobe Photoshop 5.0.2.

RESULTS

M31 localisation in normal male meiosis

First, we investigated the expression of M31 during XY male meiosis in detail by immunofluorescent labelling of surface-spread spermatocytes (*n*=407). To substage meiosis, we used an antiserum to the axial element protein SYCP3 (Lammers et al., 1994). Axial elements are first detected as short stretches at leptotene. During zygotene, each axial element extends in length and synapses with that of its homologous partner. Completion of synapsis marks the transition to pachytene, the stage during which chiasmata form. During diplotene, the axial elements repel one another and can be seen to associate only at sites of chiasmata. During diakinesis, the axial elements are disassembled and have disappeared completely by metaphase I, except at the centromeres, where axial element remnants remain until anaphase II.

During leptotene, low levels of M31 were localised to the centromeric heterochromatin (Fig. 1A-D). This was also the case during zygotene, at which point M31 could also be seen at a low level throughout the euchromatin (Fig. 1E-H). During early and mid-pachytene, the whole nuclear staining was lost and the centromeric staining was more obvious (Fig. 1I-L). Consistent with the section data of Motzkus et al. (Motzkus et al., 1999), the centromeric heterochromatin of the X chromosome was particularly compact at this stage, and M31 staining of the X centromere was correspondingly bright (Fig. 1I-L). At this stage, about half of the cells analysed also possessed a diffuse focus of M31 in the region of X-Y synapsis (Fig. 1J,K).

At late pachytene and early diplotene, a number of changes in M31 expression were seen. Of particular note was that the autosomal centromeric heterochromatin had become more intensely M31-stained, whereas that of the X chromosome was now only faintly stained or not stained at all (Fig. 1M-P). At this stage, there was the characteristic reduction in the extent of X-Y synapsis, and this was accompanied by an increase in intensity and compaction of M31 within the region of synapsis (Fig. 1N,O). M31 did not associate with the centromeric heterochromatin of the Y chromosome at this or any other stage of pachytene, possibly reflecting a requirement for major satellite DNA, which is absent at the Y centromere (Pardue and Gall, 1970) in binding of M31 to centromeric heterochromatin.

At mid-diplotene, M31 appeared throughout the sex body

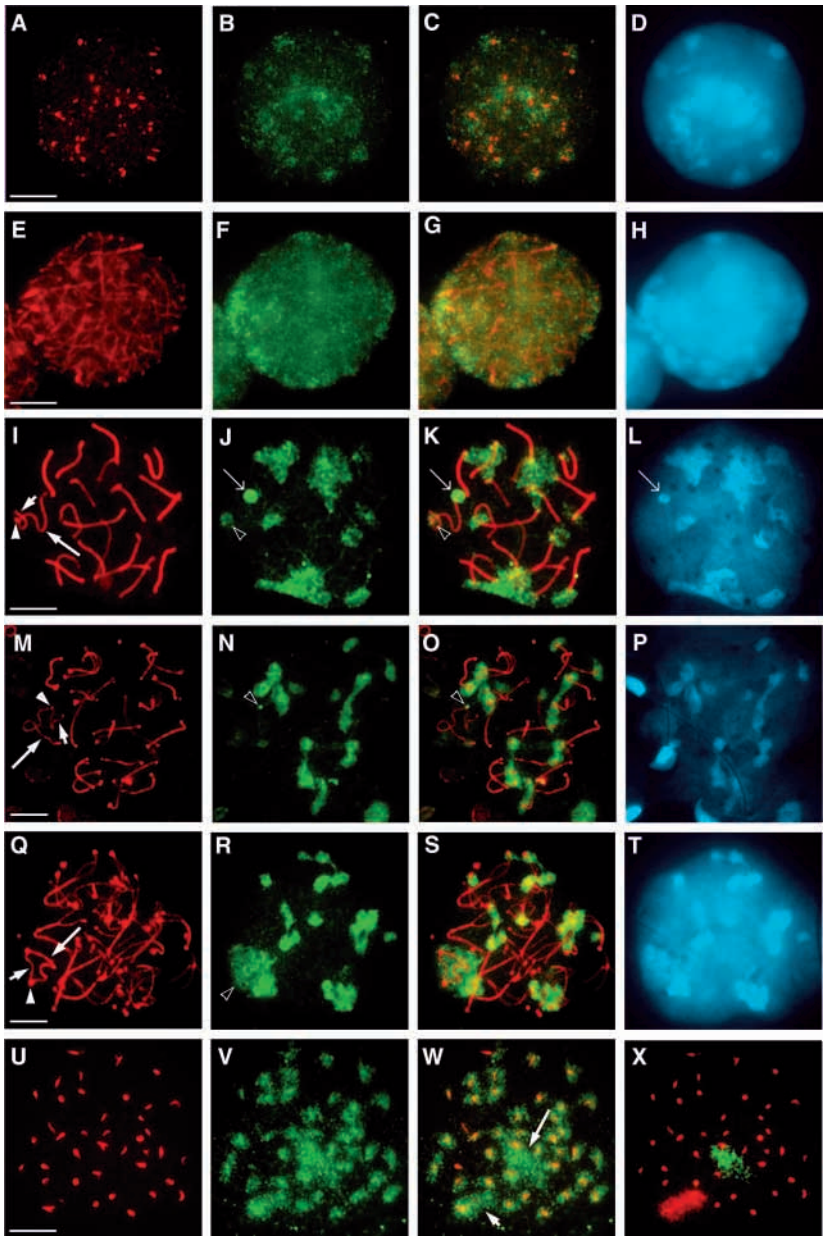

Fig. 1. Expression of M31 during XY male mouse meiosis (M31, green; SYCP3, red; DAPI, blue; long arrow, X chromosome; short arrow, Y chromosome; arrowhead, PAR). (A-D) Leptotene nucleus. (A) Axial element formation has just begun. (B) Same nucleus stained for M31, which is concentrated at the centromeric heterochromatin. (C) Superimposition of (A) and (B). (D) Same nucleus stained with DAPI, which highlights areas of centromeric heterochromatin. (E-H) Zygotene nucleus. (E) Synaptonemal complex (SC) formation has just begun. (F) Same nucleus stained for M31, which is concentrated at the centromeric heterochromatin. (G) Superimposition of (E) and (F). (H) Same nucleus stained with DAPI, which highlights areas of centromeric heterochromatin. (I-L) Mid-pachytene nucleus. (I) In contrast to the autosomes, which synapse over their entire length, the X and Y chromosomes show only limited synapsis that includes the PARs. (J) Same nucleus stained with M31. (K) Superimposition of (I) and (J). At this stage, the centromeric heterochromatin of the X chromosome (thin arrow in J) is very compact in comparison to that of the autosomes, and is correspondingly brightly stained with M31. M31 also assembles in the region of X-Y synapsis (open arrowhead in J,K). (L) Same nucleus stained with DAPI. Notice the compact heterochromatin of the X centromere. (M-P) Early diplotene nucleus; the sex chromosomes have partially desynapsed. (N) Same nucleus stained for M31. (O) Superimposition of (M) and (N). At this stage, autosomal centromeric heterochromatin is more compact and brightly stained with M31, whereas that of the X chromosome is barely stained. The focus in the region of X-Y synapsis (open arrowhead in N,O) decorates the very tip of the SC of the sex chromosomes. (P) Same nucleus stained with DAPI. (Q-T) Mid-diplotene nucleus, showing extensive autosomal desynapsis and marked contraction of the XY bivalent. (R) Same nucleus stained with M31. (S) Superimposition of (Q) and (R). At this stage, M31 associates with the sex body (open arrow in R). (T) Same nucleus stained with DAPI. (U-X) Metaphase I nucleus. At this stage, SYCP3 associates exclusively with the centromeric regions of each bivalent. (V) Same nucleus stained with M31. (W) Superimposition of (U) and (V). As well as centromeric heterochromatin, M31 continues to associate with the X and Y chromatin. (X) Same nucleus with X (green) and Y (red) paints. Bars, 10 μm.

(Fig. 1Q-T). Owing to the high level of M31 throughout the X and Y chromatin at this stage, it was impossible to determine whether the M31 focus was still associated with the region of X-Y synapsis. M31 continued to associate with the sex chromosomes during diakinesis and even metaphase I (Fig. 1U-X).

The M31 X-Y focus is independent of synapsis and locates to the distal PAR

The association of M31 with the region of X-Y synapsis (Fig. 2B) raised the question of whether it was dependent on X-Y synapsis. To address this, we looked for this M31 focus in two male sex chromosome mutants, X*Sxr*^{a}O (Kot and Handel, 1990; Sutcliffe et al., 1991) and X^{Y}*O (Odorisio et al., 1998), which each have a single sex chromosome, thus precluding X-Y synapsis (Fig. 2A). The X*Sxr*^{a} chromosome comprises an X chromosome to which has been added, distal to the PAR, most of the short arm of the Y chromosome (Evans et al., 1982; Singh and Jones, 1982; McLaren et al., 1988; Roberts et al., 1988). The X^{Y}* chromosome comprises an X chromosome fused to a Y chromosome via a shared PAR (Eicher et al., 1991; Hale et al., 1991). This PAR consists of a region of proximal X-derived PAR and a region of proximal Y-derived PAR in opposite orientations, and lacks distal PAR sequences, including the *steroid sulfatase* (*Sts*) locus (Burgoyne et al., 1998).

We detected a focus of M31 at the distal end of the X*Sxr*^{a} chromosome in all of the late pachytene X*Sxr*^{a}O spermatocytes analysed (n=27). However, this M31 signal was clearly less distal than that of XY males, suggesting that it had retained its

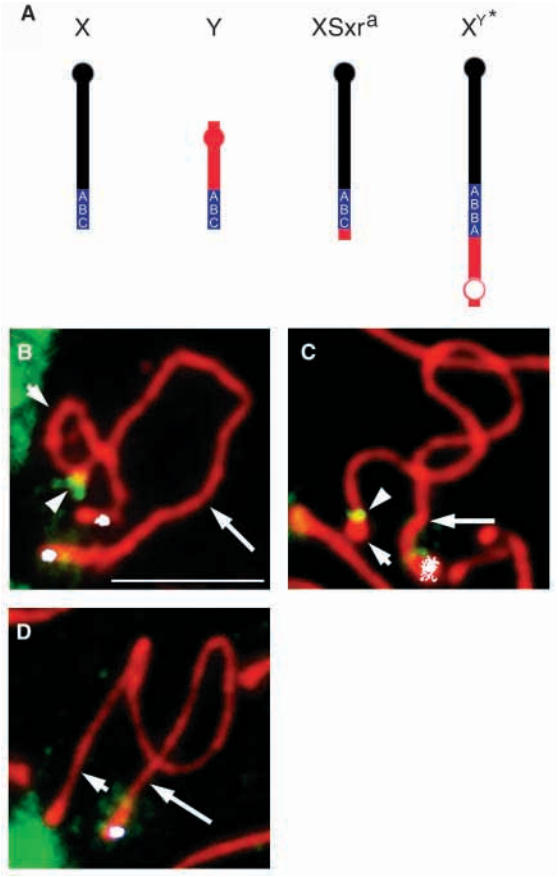

Fig. 2. Association of M31 with the PAR during male meiosis. (M31, green; SYCP3, red; CREST, which labels the functional centromeres, pseudocoloured white; long arrow, X chromosome; short arrow, Y chromosome; arrowhead, PAR). (A) Schematic representation of the X, Y, X*Sxr*^{a} and X^{Y}*O chromosomes; circles represent centromeres. Sequences in the PAR are represented by the letters A-C. The X*Sxr*^{a} chromosome possesses a complete PAR with some additional Y-chromosome short-arm-derived material attached distally. The X^{Y}* chromosome comprises a complete X and Y chromosome, with a compound PAR that is deleted for distal PAR sequence (in this case, 'C'). The open circle represents the original Y centromere, which, in this context, is functionally inactive (for details, see Burgoyne et al., 1998). (B) High power late pachytene XY bivalent. M31 associates with the synapsed sex chromosome PARs; remnants are also present at the centromeric end. (C) Late pachytene X*Sxr*^{a} chromosome, with the X centromeric end marked with CREST. M31 associates with the axial element at a less distal position than in the XY bivalent owing to the presence of most of the Y short arm distal to the PAR (compare with Y short arm in B). (D) Late pachytene X^{Y}* chromosome, with the X centromeric end again marked with CREST (the inactive Y centromere is negative for CREST). An M31 focus is never seen in the vicinity of the PAR on the X^{Y}* chromosome although remnants of M31 remain at the X centromere. Bars, 5 μm.

original position with respect to the PAR (Fig. 2C). This result shows that the M31 focus is not dependent on synapsis and consequently that the location of the M31 focus to this region must be dependent on some other region-specific feature. By contrast, an M31 signal was never detected on the axial element of the X^{Y}* chromosome (*n*=58; Fig. 2D). This surprising finding suggests that the feature leading to the targeting of an M31 focus in this region is located within the distal PAR, because this region is missing from the compound X^{Y}* PAR. The M31 focus was also absent from the Y* PAR (not shown), which has the same structure as the X^{Y}* PAR (Burgoyne et al., 1998).

M31 localisation in XY^{Tdym1} and XX female meiosis

Recently, we (Turner et al., 2000) described the use of XY^{Tdym1} females (which have a Y chromosome deleted for the testis determinant *Sry*; Gubbay et al., 1990) to check for the MSCI- and sex-body-specificity of proteins present in the sex body during male meiosis. These females, despite having an XY chromosome constitution, have no sex body or MSCI and their X and Y chromosomes rarely synapse (Turner et al., 2000). We investigated the sex specificity of the association of M31 with the sex chromosomes by examining its expression in XX oocytes (*n*=75) and XY^{Tdym1} oocytes (*n*=68).

As in the male, M31 was present throughout the whole nucleus during early stages of meiosis in XX and XY^{Tdym1} oocytes (not shown), and then in association with the centromeric heterochromatin during pachytene (Fig. 3A,B,D,E). In contrast to the male, in XY^{Tdym1} oocytes, M31 was present over the whole of the chromatin of the Y^{Tdym1} chromosome (but not the X chromosome) at pachytene (Fig. 3B,G-L) and diplotene; furthermore, there was no preferential association of M31 with the centromeric heterochromatin of the X chromosome at early pachytene, or subsequent loss at late pachytene (not shown). However, as in the male, M31 did associate with the axial elements of the X and Y^{Tdym1} chromosome PARs, even though they were usually asynapsed, thus confirming that the M31 PAR focus is not dependent on synapsis (Fig. 3A-C,G-L). A distally located M31 signal was also seen on one bivalent in each XX oocyte. This was shown to be the XX bivalent by X chromosome painting (Fig. 3D-F). These observations demonstrate that association of M31 with X chromatin is male specific but that its association with Y chromatin and the PAR is not.

MacroH2A1.2 colocalises with M31 at the PAR during late prophase

Next, we analysed the localisation of a second heterochromatin-associating protein, macroH2A1.2, during male meiosis by immunostaining with antisera to macroH2A1.2 and the axial element component COR1 (which is raised against guinea-pig COR1 protein but recognises the homologous mouse SYCP3 protein; Dobson et al., 1994). MacroH2A1.2 was present in the sex body at early pachytene and appeared in the euchromatin and at higher levels in the centromeric heterochromatin at late pachytene/diplotene (not shown). Like M31, macroH2A1.2 also localised to the PAR from mid pachytene onwards (*n*=85; Fig. 4A-F), suggesting that the PAR is a site that recruits a heterochromatin focus during meiosis which includes both M31 and macroH2A1.2. The macroH2A1.2 PAR focus was maintained throughout diplotene and diakinesis (Fig. 4D-F), but had become indistinguishable above the general chromatin staining by metaphase I (Fig. 4G-I), the stage that immediately precedes X-Y disjunction. That M31 and macroH2A1.2 colocalised at the PAR was verified by triple labelling of late pachytene spermatocytes with antisera to the axial element protein COR1, M31 and macroH2A1.2 (*n*=25, Fig. 5A-C). We also established from an analysis of X^{Y}*O males (Fig. 2A), and of XX and XY

females, that there was the same regional location of macroH2A1.2 within the PAR and lack of sex specificity as seen with M31 (not shown).

Unlike M31, macroH2A1.2 was highly concentrated at the centromeric heterochromatin of both the X and the Y chromosome from late pachytene and continued to associate preferentially with the X and Y centromeres during the first meiotic division (Fig. 4A-I). Intriguingly, we noted a single, punctate focus of macroH2A1.2 that colocalised with a centromere signal (as stained with CREST) in approximately half of round spermatids analysed (Fig. 4J,L). In order to determine whether this might represent continued association of macroH2A1.2 with the X or the Y centromere, we stained these round spermatids with X- and Y-specific paints, and found that the signal was retained on the Y centromere (Fig. 4K). Thus, while preferential binding of macroH2A1.2 to the X centromere is lost between metaphase I and completion of meiosis, binding to the Y centromere is maintained. This allows discrimination of X- and Y-bearing spermatids: of 80 round spermatids analysed by macroH2A1.2 staining and sex chromosome painting, 98% of them could be correctly identified as X or Y bearing based on macroH2A1.2 immunostaining alone.

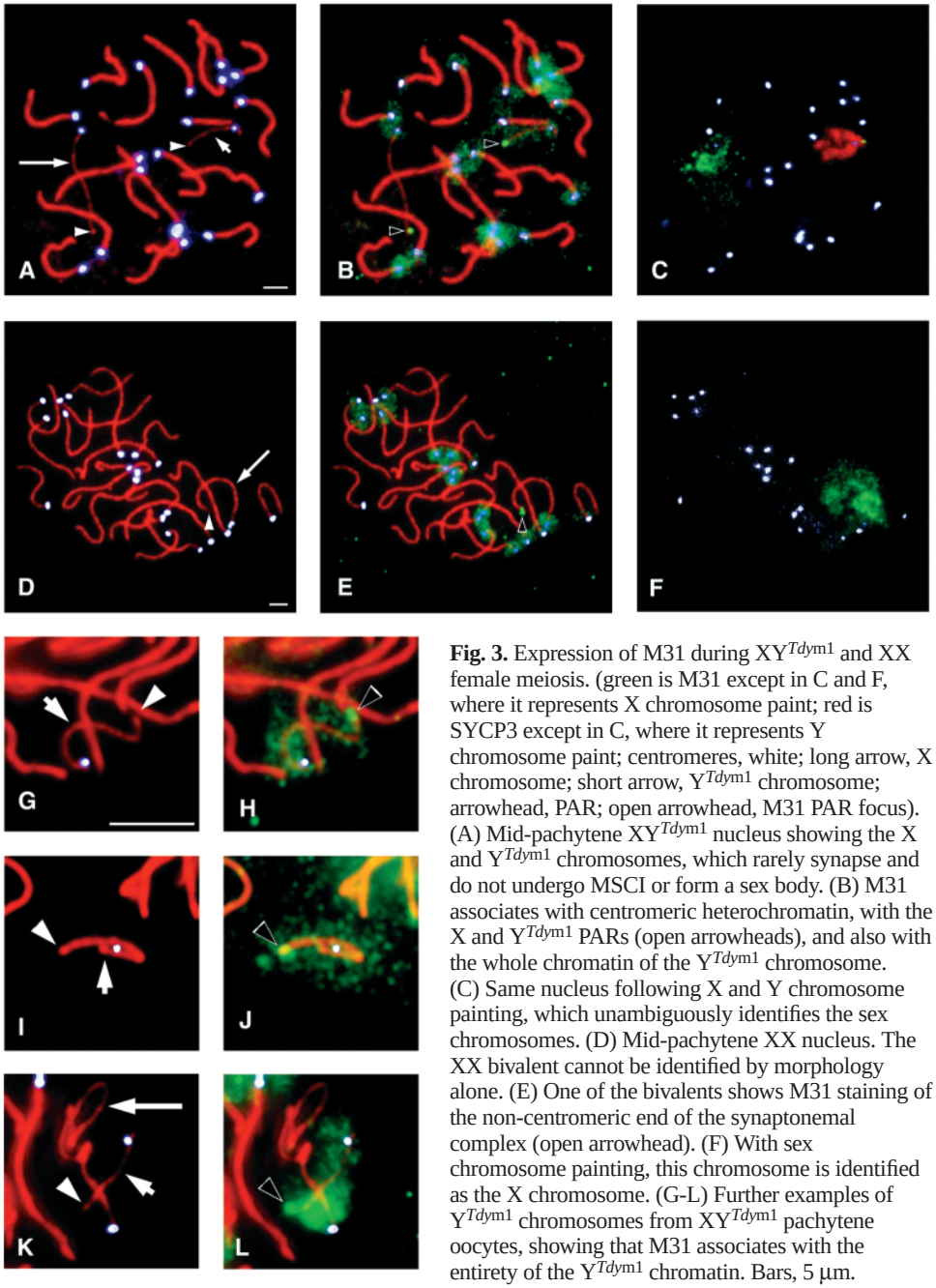

Fig. 3. Expression of M31 during XY^{Tdym1} and XX female meiosis. (green is M31 except in C and F, where it represents X chromosome paint; red is SYCP3 except in C, where it represents Y chromosome paint; centromeres, white; long arrow, X chromosome; short arrow, Y^{Tdym1} chromosome; arrowhead, PAR; open arrowhead, M31 PAR focus). (A) Mid-pachytene XY^{Tdym1} nucleus showing the X and Y^{Tdym1} chromosomes, which rarely synapse and do not undergo MSCI or form a sex body. (B) M31 associates with centromeric heterochromatin, with the X and Y^{Tdym1} PARs (open arrowheads), and also with the whole chromatin of the Y^{Tdym1} chromosome. (C) Same nucleus following X and Y chromosome painting, which unambiguously identifies the sex chromosomes. (D) Mid-pachytene XX nucleus. The XX bivalent cannot be identified by morphology alone. (E) One of the bivalents shows M31 staining of the non-centromeric end of the synaptonemal complex (open arrowhead). (F) With sex chromosome painting, this chromosome is identified as the X chromosome. (G-L) Further examples of Y^{Tdym1} chromosomes from XY^{Tdym1} pachytene oocytes, showing that M31 associates with the entirety of the Y^{Tdym1} chromatin. Bars, 5 μm.

DISCUSSION

In the present study, we have analysed the localisation of M31 and macroH2A1.2, both of which are components of heterochromatin, during male and female meiosis. Our results suggest that both proteins localise to four distinct domains during male meiosis, namely the centromeric heterochromatin, the euchromatin, the sex body and the PAR.

In our experiments, M31 localised to centromeric heterochromatin and euchromatin from leptotene/zygotene, whereas macroH2A1.2 localised to these sites from late pachytene/diplotene. Current ideas on meiosis suggest that chromosome contraction and relaxation might be important in controlling interactions between homologous chromosomes (Hunter et al., 2001). Thus, chromosome condensation at zygotene facilitates synapsis and recombination, whereas condensation at diplotene allows resolution of chiasmata and individualisation of chromosomes prior to disjunction (Hunter et al., 2001; Cobb et al., 1999). Viewed in this way, M31 might partly control the early condensation step, whereas macroH2A1.2 and M31 might together be involved in controlling the later step. Interestingly, these expression patterns are highly reminiscent of those of topoisomerase IIα (similar to M31) and phosphorylated histone H3 (similar to macroH2A1.2), both of which have been implicated in chromosome condensation (Cobb et al., 1999). M31 and macroH2A1.2 show complementary centromeric localisation patterns at metaphase I, M31 associates with the autosomal centromeres, whereas macroH2A1.2 associates with those of the sex chromosomes. This centromeric localisation might indicate a role for these proteins in kinetochore assembly at this stage.

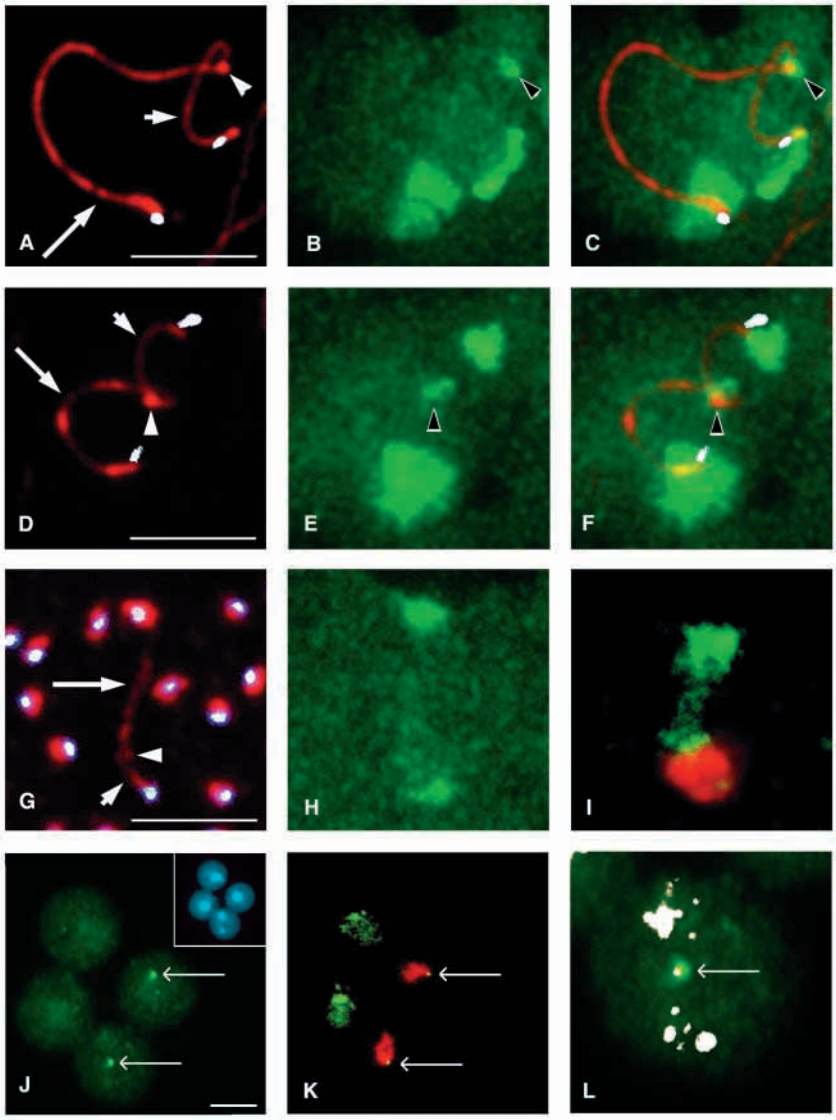

Fig. 4. Association of macroH2A1.2 with the XY bivalent during male meiosis (green is macroH2A1.2 except in I and K, where it represents X chromosome paint; red is COR1 except in I and K, where it represents Y chromosome paint; centromeres, white; DAPI, blue; long arrow, X chromosome; short arrow, Y chromosome; arrowhead, PAR; open arrowhead, macroH2A1.2 PAR focus). (A) Early diplotene XY bivalent. (B) Same nucleus stained for macroH2A1.2. (C) Superimposition of (A) and (B). MacroH2A1.2 forms a sharp focus at the PARs, and, in contrast to M31 at this stage, is abundant at the centromeric heterochromatin of the X and Y chromosomes. (D) Diakinesis XY bivalent. (E) Same nucleus stained for macroH2A1.2. (F) Superimposition of (D) and (E). MacroH2A1.2 continues to associate with the PAR and the heterochromatin of the X and Y chromosome. (G) Metaphase I XY bivalent. (H) Same nucleus stained for macroH2A1.2, which no longer preferentially associates the PAR, but continues to associate with the X and Y centromeric heterochromatin. (I) Same nucleus stained with X and Y chromosome paints, which discriminates these chromosomes from one another. (J) Round spermatids stained for macroH2A1.2, which forms a sharp focus (open arrows) in half of the cells analysed (inset DAPI stained image). (K) Same spermatids stained with X and Y chromosome paints, which demonstrate that the sharp focus is present in Y-bearing, but not X-bearing, spermatids (notice that this focus is preserved after the FISH procedure and appears yellow in this image). (L) The region of heterochromatin from the lowest spermatid stained with CREST and macroH2A1.2. The heterochromatin is due to the clustering of centromeres, and the macroH2A1.2 is closely associated with a single (Y) centromere. Bars, 5 μm.

MacroH2A1.2 and M31 were also found to localise to the sex body from early pachytene and diplotene, respectively. In a previous study, it was shown that M31 exhibited dynamic relocalisation from the X centromere to the entire sex body during meiotic prophase (Motzkus et al., 1999). These observations led Motzkus et al. (1999) to suggest that MSCI-associated heterochromatinisation spreads from the X chromosome to the Y chromosome via the region of X-Y synapsis and that M31 is involved in this process. From our present results, it could be argued that the PAR M31 focus that we observed represents the point at which inactivation spreads from the X chromosome to the Y chromosome, thereby satisfying a prediction of the trans-heterochromatinisation model (Motzkus et al., 1999). However, there are important caveats. (1) If inactivation were to spread from the X centromere to the PAR between early and late pachytene, cells

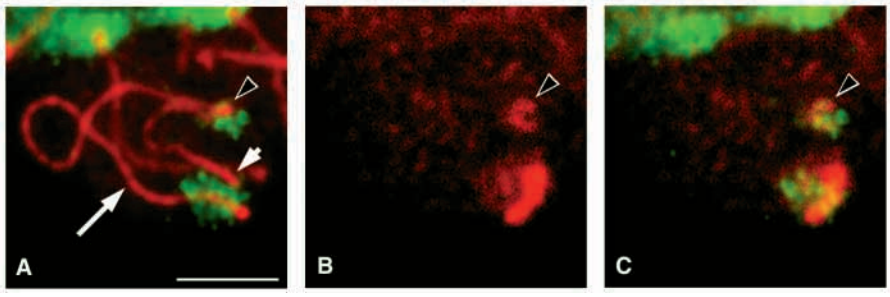

Fig. 5. M31 and macroH2A1.2 colocalise at the PAR (green is M31, red is COR1 in A and macroH2A1.2 in B and C; long arrow, X chromosome; short arrow, Y chromosome; open arrowhead, M31/macroH2A1.2 PAR focus). (A) Late pachytene XY bivalent showing M31 at the PAR and at the X-centromeric heterochromatin. (B) Same nucleus stained for macroH2A1.2, which localises to the PAR and to the X and Y centromeric heterochromatin. (C) Same nucleus stained for M31 and macroH2A1.2, which shows colocalisation at the PAR and at the centromeric heterochromatin of the X but not the Y chromosome. Bars, 5 μm.

at mid-pachytene would be expected to display M31 staining throughout the chromatin of the X chromosome. This is not the case. In surface-spread preparations, association of M31 with the X chromatin was not apparent until mid-diplotene (Fig. 1Q-S). (2) Because the PAR is thought to escape MSCI (McKee and Handel, 1993), a locus that transmits inactivation from the X chromosome to the Y chromosome might be expected to lie proximal to but not within the PAR. The M31 focus that we observed did reside within the PAR. (3) Association of M31 with the X chromosome, the Y chromosome and the PAR should be male specific. Although association of M31 with the X chromosome in the XY body was male specific (Fig. 1Q-S; Fig. 3A-C), its association with the Y chromosome and with the PAR was not (Fig. 3A-C,G-L). Indeed, the PAR focus can be observed on the XX bivalent in normal female oocytes (Fig. 3D-F).

Even if the trans-heterochromatinisation model is not tenable, the presence of M31 throughout the sex body at diplotene, together with the fact that this staining is male specific, does suggest an MSCI- or sex-body-related role for this protein. We (Turner et al., 2000) have recently discussed the possible functions of MSCI and sex-body formation and have suggested that they might be functionally and temporally distinct phenomena. Thus, MSCI is almost certainly initiated early, before entry into meiotic prophase, and we have suggested that MSCI might be an unfortunate by-product of a need to focus the 'homology search' on the X and Y PARs. The appearance of a morphologically distinct 'sex body' does not occur until pachytene, and it is in conjunction with or subsequent to the formation of this specialised nuclear compartment that 'sex-body' proteins such as M31 concentrate on the X and Y chromatin. Some of these proteins (such as ASY (Turner et al., 2000)) seem to be targeted to the chromatin of asynapsed chromosome regions and might play a role in a 'pachytene' or 'synapsis' checkpoint (Odorisio et al., 1998). Others, such as XY77 (Kralewski et al., 1997; Turner et al., 2000), might play a role in protecting the X and Y non-PAR axes (which are asynapsed in normal male meiosis) from checkpoint recognition. In spread preparations, M31 does not appear until diplotene, which is too late for either of the proposed checkpoint-related functions. It is possible that M31 is associated with the further condensation of the X and Y that occurs during diplotene, which (as with the autosomes) might be a necessary prelude to their disjunction (Hunter et al., 2001; Cobb et al., 1999). It is intriguing that Y chromatin (but not X chromatin) also accumulates M31 during female meiosis, perhaps reflecting the inherently heterochromatic nature of much of this chromosome (Singh et al., 1994).

Whether the localisation of macroH2A1.2 to the X chromatin is male specific is currently under investigation (S. Hoyer-Fender et al., unpublished). If it is then its localisation to the sex chromosomes at the point at which the sex body is first apparent would support a role for macroH2A1.2 in sex-body formation.

With respect to the M31/macroH2A2.1 focus, it is hard to conceive of an X-chromosome-specific function during normal female meiosis, because the X bivalent is indistinguishable from the autosomal bivalents in its morphology and behaviour. We therefore suspect that the PAR focus in female meiosis is a by-product of specificity for the PAR imposed by some male-limited function. We do not know how M31 and macroH2A1.2 are targeted to the PAR; M31 at least is thought not to bind DNA (Ball et al., 1997). As to the function in male meiosis, we are attracted to the idea that the M31/macroH2A1.2 PAR focus might play a role in ensuring efficient disjunction of the X and Y chromosome at the first meiotic metaphase. Several studies in different organisms have demonstrated an association between distally located chiasmata and disjunctional errors at metaphase I (Koehler et al., 1996; Lamb et al., 1996; Ross et al., 1996; Lamb et al., 1997). These and other analyses have led to the proposal that chiasmata are prevented from migrating off the ends of the chromosomes by sister chromatid cohesion distal to the chiasmata. In this model, distally located chiasmata are more prone to terminal loss because of the limited extent of the region of cohesion (Moore and Orr-Weaver, 1998). In the case of the male XY bivalent, there is a single chiasma that is always in an extreme distal location and thus might be inherently prone to disjunctional errors. The M31/macroH2A1.2 heterochromatin focus might therefore act to strengthen sister chromatid cohesion within each PAR axis distal to the obligatory chiasma, which would require that the focus is always distal to the chiasma. This might in fact be the case because the *Sts* locus, which lies within the PAR region to which the focus locates, shows the 50% recombination frequency predicted for a locus distal to the obligatory chiasma (Keitges et al., 1985). The fact that the focus is closely associated with the X and Y PAR axes, even when they are not synapsed, is also consistent with a role in sister chromatid cohesion. A role for heterochromatin in sister chromatid cohesion has been suggested previously (Allshire, 1997). Alternatively, the PAR chromatin complex could prevent chiasma loss by providing an interhomologue heterochromatin link similar to that postulated for the achiasmate chromosome 4 bivalent in *Drosophila melanogaster* (Dernberg et al., 1996). Some circumstantial evidence in support of a role for the PAR focus in preventing premature X-Y separation comes from data suggesting increased X-Y separation in XY* males (Hale et al., 1991), which we have shown lack a focus on the Y* PAR.

Finally, the time course of appearance and disappearance of the PAR heterochromatin complex is compatible with a role in reinforcing the X-Y association. Thus, it first appears at around the same time as MLH1, a mismatch-repair protein that locates to the sites of chiasmata (Baker et al., 1996), and then disappears at the first meiotic metaphase, just prior to the point at which the sex chromosomes disjoin.

In summary, we have characterised in detail the expression patterns of the heterochromatin proteins M31 and macroH2A1.2 during male and female meiosis. In doing so, we have shown that both localise to the distal PAR. Although the role of this M31/macroH2A1.2 focus remains unknown, various properties of its binding support a role in reinforcing the attachment between the X and the Y chromosome prior to their disjunction. Conditional testis-specific mutagenesis of M31, currently under way, should provide a test of this hypothesis. In the meantime, it would be interesting to know whether other heterochromatin proteins, particularly those that associate with either M31 or macroH2A1.2, also contribute to this PAR focus. For instance, Suv39h interacts with M31 (Aagaard et al., 1999) and a testis-specific isoform, Suv39h2 has been shown to localise to the sex body (O'Carroll et al., 2000).

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