Chromosoma (2002) 111:22–36
DOI 10.1007/s00412-002-0182-8

ORIGINAL ARTICLE

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Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals

Received: 16 October 2001 / Revised: 3 December 2001 / Accepted: 19 December 2001 / Published online: 31 January 2002

Abstract We show that methylated lysine 9 of histone H3 (Me9H3) is a marker of heterochromatin in divergent animal species. It localises to both constitutive and facultative heterochromatin and replicates late in S-phase of the cell cycle. Significantly, Me9H3 is enriched in the inactive mammalian X chromosome (X_i) in female cells, as well as in the XY body during meiosis in the male, and forms a G-band pattern along the arms of the autosomes. Me9H3 is a constituent of imprinted chromosomes that are repressed. The paternal and maternal pronuclei in one-cell mouse embryos show a striking non-equivalence in Me9H3: the paternal pronucleus contains no immunocytologically detectable Me9H3. The levels of Me9H3 on the parental chromosomes only become equivalent after the two-cell stage. Finally, we provide evidence that Me9H3 is neither necessary nor sufficient for localisation of heterochromatin protein 1 (HP1) to chromosomal DNA.

Edited by: W. Earnshaw

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Introduction

Heterochromatin represents the largest differentiated chromatin compartment within eukaryotic nuclei (reviewed by John 1988). Its study, over many decades, in a variety of organisms, has been crucial for our understanding of how chromatin structure regulates gene expression (Brown 1966). There are two types of heterochromatin, constitutive and facultative. Constitutive heterochromatin is found at distinct chromosome territories, such as the pericentric, telomeric and nucleolar organizer regions (Heitz 1928; McClintock 1934). It also replicates late in S-phase (Lima-de-Faria and Jaworska 1968), is enriched in repetitive sequences (John and Miklos 1979), is depleted of acetylated H4 histone (Jeppesen et al. 1992) and is relatively poor in the number of genes it contains (Pimpinelli et al. 1986). Unlike constitutive heterochromatin, facultative heterochromatin is developmentally regulated and was defined by Brown (1966) on the basis of early work in *Sciara* (reviewed by Metz 1938) and *Coccids*, or mealy bugs (for reviews see Hughes-Schrader 1948; Brown and Nur 1964). In the mealy bug system an entire haploid set of chromosomes becomes facultatively heterochromatinized during the development of males (Chandra and Brown 1975). Be-

cause this set is exclusively paternal in origin this observation was recognized as one of the first examples of parent-of-origin (parental imprinting) effects, which are now extensively studied in mammals (reviewed in Solter 1998).

Recent attention has focused on facultative heterochromatinization of the inactive X (X_i) chromosome in female mammals (Lyon 1961, 1999). X-inactivation in females is thought to be important for maintaining the dosage of X-linked gene products between the sexes. Initiation of X-inactivation requires the expression of a non-coding RNA called *Xist* (Willard and Carrel 2001). Apart from this difference, the X_i has certain similarities to constitutive heterochromatin. X_i is replicated late in S-phase (Morishima et al. 1962), retains a condensed heterochromatic conformation during interphase, called the Barr body (Barr and Bertram 1949), and is hypoacetylated for histone H4 (Jeppesen and Turner 1993). Another parallel between the highly methylated DNA of constitutive heterochromatin (Miller et al. 1974) and the methylation status of CpG islands on the X_i is that cytosines in the CpG islands associated with many X-linked genes are methylated on the X_i but not on the X_a (Grant and Chapman 1988). Hypoacetylation of histone H4 and methylation of the CpG islands are thought to act together to maintain the inactivation status of X_i (Csankovszki et al. 2001). X_i is also enriched for the variant histone macroH2A1.2 (Costanzi and Pehrson 1998).

X-inactivation also occurs in males during spermatogenesis (Solari 1974). During meiosis in males the sex chromosomes become heterochromatic and transcriptionally inactive (McKee and Handel 1993). The condensation occurs in a specific area of the nucleus that is located at the nuclear periphery in close apposition to the nuclear envelope. It is easily discerned morphologically in pachytene/diplotene spermatocytes of male mammals and termed the XY-body (Solari 1974). Suggested functions for the transcriptional inactivation and heterochromatinization include: (1) preventing illegitimate recombination between the sex chromosomes; (2) restricting the presynaptic homology search to the pseudoautosomal regions (PARs), thus facilitating X-Y PAR synapsis; and (3), protecting the asynapsed X and Y non-PAR axes against checkpoint recognition (Turner et al. 2000). The molecular basis of XY-body formation is unknown. The XY-body is, however, enriched for macroH2A1.2 (Hoyer-Fender et al. 2000b; Turner et al. 2001) like the X_i in female cells, although it is not depleted in acetylated H4 histone (Armstrong et al. 1997). Spermatogenesis, and presumably XY-body formation, does not require the activity of the *Xist* gene (Marahrens et al. 1997).

Along with the detailed cytological and molecular characterisation of heterochromatin, many decades of work have shown that heterochromatin (be it constitutive or facultative) is a repressive chromosomal environment (for reviews see Baker 1968; Cattanach 1974; Spofford 1976). The evidence comes from the study of chromosomal rearrangements where a euchromatic segment is brought close to heterochromatin. Such rearrangements often result in variable repression of genes within the segment, which in turn leads to phenotypic variegation in tissues where the rearranged genes are normally expressed, a phenomenon known as position-effect variegation (PEV). In *Drosophila*, PEV has been a very useful tool in the search for second-site mutations that can modify variegation (Grigliatti 1991). These modifiers encode either structural components of heterochromatin or enzymes that modify the components (Reuter and Spierer 1992). The first structural component to be identified was heterochromatin protein 1 (HP1; James and Elgin 1986). HP1 is a dosage-dependent modifier of PEV and allelic to the suppressor mutation *Suvar(2)5* (Eissenberg et al. 1990). The sensitivity of variegating phenotypes to the dosage of modifiers, like HP1, has led to a model where heterochromatin is envisaged as a macromolecular complex that represses gene activity by sequestering regulatory sequences away from the transcriptional machinery (Tartof et al. 1989). This model was extended to the homeotic gene clusters after it was shown that HP1 shared a highly conserved motif, called the chromodomain (CD), with *Drosophila* Polycomb (Pc), which is a repressor of the homeotic genes (Gaunt and Singh 1990; Paro 1990; Paro and Hogness 1991; Singh et al. 1991). Several lines of evidence, including co-immunofluorescence, co-immunoprecipitation and compound mutations in *Polycomb*-Group (*Pc*-G) genes, suggest that *Pc*-G gene products act synergistically and repress the homeotic genes through the assembly of a macromolecular chromatin complex (Bienz and Muller 1995; Pirrotta 1995; Orlando and Paro 1995; Jacobs and van Lohuizen 1999).

HP1 proteins are highly conserved (Singh et al. 1991; Saunders et al. 1993). In the fission yeast an HP1-like protein, swi6p, is involved in silencing of the donor mating type loci (Lorentz et al. 1994). In mammals, three HP1 proteins have been identified, designated HP1α, M31 (HP1β) and M32 (HP1γ) in mouse and HP1α, HP1β and HP1γ in man (Jones et al. 2000). M31 is a component of constitutive heterochromatin and a dosage-dependent modifier of a variegating position effect, like *Drosophila* HP1 (Wreggett et al. 1994; Festenstein et al. 1999). Mammalian transcriptional repressors have utilised this ability to silence gene activity (Jones et al. 2000): several transcriptional repressors, including the KRAB-ZFPs, which are the largest family of repressors in mammals, recruit mammalian HP1 proteins and thereby silence genes (Nielsen et al. 1999; Ryan et al. 1999; Lechner et al. 2000). The mechanism by which HP1 protein represses gene activity has become clearer from a known interaction with the histone methyl-transferase Suv(3)9h1 (Aagaard et al. 1999; Rea et al. 2000). It is thought that methylation of H3 histone at lysine 9 by Suv(3)9h1 creates a binding site for the HP1 CD, resulting in the formation of a repressive protein complex (Bannister et al. 2001; Jacobs et al. 2001; Lachner et al. 2001). This role for HP1 proteins in silencing gene activity is thought to be widespread in the genome and conserved from yeast to man (Wang et al. 2000; Nakayama et al. 2001; Nielsen et al. 2001b).

Acetylation and methylation are just two types of post-translational modification that have been identified in histones (Wu et al. 1986). The "histone code" hypothesis states that such covalent modifications may generate a "code" which can be "deciphered" by chromosomal proteins and thereby regulate tissue-specific patterns of gene expression (Strahl and Allis 2000; Turner 2000). Methylation at lysine 9 in histone H3 (Me9H3) represents one of the most robust histone modifications – the biochemistry suggesting that it is almost permanent in nature (Byvoet et al. 1972; Jenuwein and Allis 2001). In this study we addressed three areas concerning the relationship between heterochromatin, HP1 and Me9H3. First, we explored the relationship of Me9H3 to classical systems of epigenetic silencing, such as X-inactivation in mammals and parental imprinting. Second, we searched for evidence that the mammalian oocyte cytoplasm contains activities that can reprogramme the histone code. This is significant because recent work has shown that differentiated cells types can be "reprogrammed" to an embryonic form after nuclear transfer into enucleated oocytes (Wilmut et al. 1997; Wakayama et al. 1998; Eggan et al. 2000). An important question is whether activities exist within the ooplasm that can remove stable Me9H3 and hence, by extension, reprogramme the histone code. Finally, we investigated the importance of Me9H3 for binding of HP1 to chromosomal DNA.

Materials and methods

Antibody production

We synthesized a peptide, ARTKQTARKSTGGKAPRKQL of H3 histone, where the underlined K was trimethylated (Bachem). The peptide was rendered immunogenic by coupling to the carrier protein tuberculin PPD (De Silva et al. 1999) using sulfo-SMCC (Pierce Chemical Company). A cysteine residue was added to the carboxyl end of the peptide during synthesis to facilitate conjugation. Two BCG-vaccinated rabbits were injected with the conjugate every 2 weeks and bleeds taken for testing by enzyme-linked immunosorbent assay. Screening the sera on the methyl-K9 H3 peptide and the unmodified peptide revealed that rabbit 2 had generated a specific, high-titre response to the methyl-K9 H3 peptide (data not shown). Sera from this animal were used for all experiments. Before use, the sera were passed over an agarose (Sevenlink Iodoacetyl Agarose) column to which the unmodified peptide was coupled in order to remove any residual activity.

For immunoblotting, various amounts of histones and crude cell extracts were run on SDS-polyacrylamide gels before blotting by standard procedures (Hoyer-Fender et al. 2000a). The amounts used were: 1 µg of recombinant H3 (Upstate); 1 µg of commercial H3 from thymus (Roche); 4 µg of all four histones together (Roche); 20 µg of whole cell extract. The anti-Me9H3 antibody was used at a dilution of 1:500–1:1000 and was detected by sheep anti-rabbit horseradish peroxide-conjugated second stage antibody (Sigma) at a dilution of 1:200. For the competition experiments the anti-Me9H3 antibody was pre-incubated with 100× molar excess of either the methyl-K9 H3 peptide or methyl-K4 H3 peptides.

Immunolocalisation of Me9H3 and acetylated histone H4 (AcH4) on murine metaphase chromosomes

Unfixed metaphase preparations of C127 mouse mammary tumour cells (ATCC CRL 1616) were labelled by indirect immunofluorescence essentially as described previously (Jeppesen and Turner 1993) with some minor modifications. Cell culture, induction of metaphase arrest, and collection of cells by trypsinization were all as described, after which the cells were suspended in 75 mM KCl for hypotonic swelling (15 min at 37°C). The cell density was adjusted to 0.5×10^4 cells/ml with 75 mM KCl, Tween 20 was added to a final concentration of 0.1% (v/v), and 0.5 ml portions were centrifuged onto glass microscope slides at 2000 rpm for 10 min using an Ames Cytotek cytocentrifuge. Cell membranes were solubilized by immersion in KCM [120 mM KCl, 20 mM NaCl, 10 mM TRIS-HCl, pH 7.5, 0.5 mM EDTA, 0.1% (v/v) Triton X-100] for 10 min at room temperature. The experimental techniques used in handling slides and performing antibody reactions have been described in detail elsewhere (Jeppesen et al. 1992; Jeppesen and Turner 1993). Briefly, slides were incubated for 2 h at room temperature simultaneously with rabbit 2 anti-Me9H3 antiserum (see above) diluted 1:100, and sheep antiserum S613, raised against a polypeptide comprising the N-terminal 18 amino acids of histone H4 acetylated at positions serine 1 and lysines 5, 8, 12 and 16 (AcH4; Morrison and Jeppesen, unpublished results) diluted 1:800, in KCM containing 10% normal horse serum (NHS) to reduce non-specific antibody binding. After slides had been washed three times in KCM, primary antibodies were detected by a secondary incubation for 30 min at room temperature with biotin-conjugated donkey anti-sheep IgG (Sigma product B7390) diluted 1:100, and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Scottish Antibody Production Unit) diluted 1:50, in KCM, 10% NHS. Finally, following three more KCM washes, slides were incubated for 30 min at room temperature with tetramethylrhodamine isothiocyanate-conjugated Extravidin (Sigma product E3011) diluted 1:100 in KCM, 10% NHS for visualization of biotin. Post-fixation with formaldehyde and Hoechst 33258 counterstaining for DNA have been described (Jeppesen et al. 1992). Coverslips were mounted in Vectashield anti-fade (Vector) diluted 1:1 with PBS, and images were obtained with a Zeiss Axioskop fluorescence microscope fitted with a Chroma 83000 triple-band-pass filter set and a Photometrics CH250 CCD camera, using IPLab Spectrum v3.1 software for capture and analysis.

Immunofluorescence detection of Me9H3 and sites of DNA synthesis

C127 cells were cultured on coverslips in DMEM supplemented with 10% fetal calf serum and antibiotics. Cells were synchronized at the G1/S-phase border by addition of aphidicolin (Calbiochem) to the culture medium at a final concentration of 5 µg/ml for 12 h. Cells were then released into S-phase by replacing with warm medium as previously described (Wu et al. 1997). At various times after release into S-phase, C127 cells were pulsed labelled with 30 µg/ml 5′-bromo-2′-deoxyuridine (BrdU) for 10 min and then fixed in cold 70% ethanol for 10 min at 4°C. Fixed cells were incubated in blocking buffer (3% BSA, 0.5% Tween 20 in PBS) for 30 min and then incubated with primary anti-Me9H3 antibody diluted 1:1000 in blocking buffer for 1 h at room temperature. Coverslips were washed with 0.5% Tween 20 in PBS and then incubated with secondary antibody [Texas Red-conjugated goat anti-rabbit IgG (Jackson) diluted 1:200 with 10% goat serum in blocking buffer] for 1 h at room temperature. Coverslips were then washed with 0.5% Tween 20 in PBS and bound antibodies were fixed with 4% formaldehyde in PBS for 10 min to preserve them during denaturation. Coverslips were then washed in 0.5% NP40 in PBS for 15 min and detection of BrdU was performed as described (Wu et al. 1997). DNA was stained with 50 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) and coverslips were mounted in Vectashield (Vector). Image acquisition was performed with a Nikon Labophot-2 microscope, a 100× 1.4 NA oil immersion Nikon PlanApo objective and a CCD camera (SPOT RT Slider, Diagnostic Instruments).

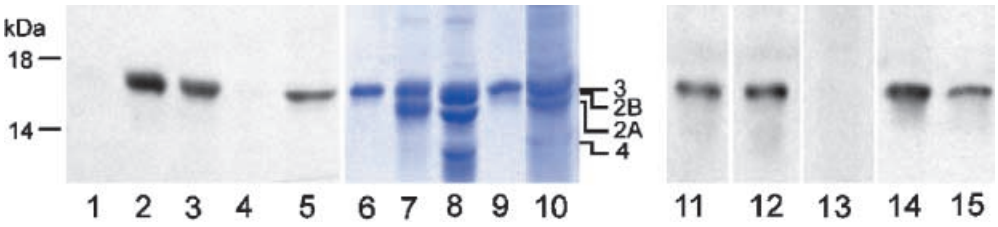


Fig. 1 Specificity of antibody for methylated lysine 9 of histone H3 (Me9H3). The anti-Me9H3 antibody does not recognize recombinant histone H3 (*lanes 1, 4*). Me9H3 is detected in commercial preparations of H3 histone (*lane 2*) and of all four core histones mixed together (*lanes 3, 11, 14*). Me9H3 is detected in extracts of tissue culture cells (*lane 5*) and mature sperm (*lane 15*). Antibody activity is completely ablated by prior incubation with the methyl-K9 H3 peptide (*lane 13*) but not with a methyl-K4 peptide (*lane 12*). *Lanes 6–10* show Commassie blue staining of an identical gel to that blotted and given in *lanes 1–5*

Immunolocalisation of M31 and Me9H3 in surface-spread spermatocytes

Mouse spermatogenic cells were prepared as surface spreads, using the method of Peters et al. (1997) with some modifications. Spermatogenic cells were incubated with hypotonic buffer (pH 8.2) (30 mM TRIS-HCl, 50 mM sucrose, 17 mM trisodium citrate dihydrate and 5 mM EDTA) for 7 min and fixed with 2% formaldehyde containing 0.05% Triton X-100 and 0.02% SDS for 90 to 120 min. Following fixation, slides were incubated in PBT (0.15% BSA, 0.1% Tween-20 in PBS) for 60 min prior to incubation overnight at 37°C with primary antibodies diluted in PBT. Rat anti-M31 was used at 1:500. Rabbit anti-Me9H3 was used at 1:1000. Human anti-centromere antibody (ACA) was used at 1:5000. Slides were washed three times for 5 min in PBS, followed by 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) and goat anti-human Cy5 (Amersham Pharmacia Biotech). All secondary antibodies were used at 1:500 in PBS. Following secondary antibody incubations, slides were washed in PBS as described above and were placed in a dark chamber to dry for 30 min. 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.

Whole mount immunofluorescent staining of mouse embryos

One-cell embryos were collected from superovulated ($B6C3F_1$× C57Bl/6) female mice, as previously described (Pratt 2001). Embryo culture was performed in M16 medium (Sigma) at 37°C in a humidified atmosphere containing 5% CO_2. Two- to four-cell embryos were flushed out of the oviducts 48 h and 53 h post-HCG, respectively and washed in M2 medium (Sigma).

After 30 min fixation in 2% (v/v) formaldehyde in PBS, all the embryos were permeabilised by incubation in KCM (see above). Then the embryos were placed in blocking solution (3% BSA, 0.1% Tween-20, PBS) for 30 min. Following the blocking step, the embryos were incubated in 1:500 dilution of anti-Me9H3 at 37°C for 1 h and washed extensively in PBS, 1% Triton X100 (v/v). Bound antibody was detected with 1:20 anti-rabbit immunoglobulins-FITC (DAKO F0205) at 37°C for 1 h respectively and washed in at least three drops of PBS, 1% Triton X100. All the above antibodies were diluted in DAKO antibody diluent (DAKO S3022). Then the embryos, with a small volume of wash solution, were placed on the clean slides (2–3 embryos per slide) and mounted very carefully in a drop of mounting medium containing 1.5 µg/ml DAPI (Vector H-1200) just before they dried. Finally a coverslip was placed gently on top and fixed by using Fixogum rubber cement and dried at 4°C. Fluorescence was detected with a Zeiss fluorescent microscope, using filter combinations suitable for the different fluorochromes.

Staining of polytene chromosomes

The simultaneous localisation of MeH3 and *Drosophila* HP1 was obtained by immunostaining of polytene chromosomes of the wild-type Oregon R stock with the anti-HP1 mouse monoclonal C1A9 antibody (1:50 dilution) [The anti-HP-1 monoclonal antibody C1A9 was isolated by James and Elgin (1986) and provided by B. Wakimoto] and the anti-MeH3 rabbit R2B3 antibody (1:50 dilution). The immunostaining was performed according to James et al. (1989). Anti-rabbit Cy3-linked (Jackson) and anti-mouse fluorescein-linked (Amersham) secondary antibodies were used. The slides were incubated in 0.05 µg/ml of DAPI dissolved in 2×SSC for 4 min and mounted in antifading solution. (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate.) Chromosome preparations were analysed using a computer-controlled Nikon E 1000 epifluorescence microscope equipped with a cooled CCD camera (Coolsnap). The fluorescent signals, recorded separately as grey-scale digital images, were pseudocoloured and merged using the Adobe Photoshop program.

Immunolocalisation of Me9H3 and HP1 on mealy bug chromosomes

Chromosome spreads on slides were prepared as previously described (Bongiorni et al. 1999, 2001). Immediately after preparation, slides were washed three times in 1×PBS, 10 min each, treated in 1×PBS, 1% Triton X-100, 10 min at room temperature, and washed again three times in 1×PBS. Blocking was performed with 20% normal goat serum in 1×PBS for 30 min. Slides were simultaneously incubated overnight, at 4°C, with mouse anti-HP1 antibody, diluted 1:10 and rabbit anti-Me9H3 diluted 1:300 in 1×PBS, 0.1% BSA. The anti-HP-1 monoclonal antibody C1A9 was isolated by James and Elgin (1986) and provided by B. Wakimoto. After they had been washed three times in 1× PBS, slides were simultaneously incubated with the secondary antibodies diluted in 1×PBS, 0.1% BSA for 1 h at room temperature. The secondary antibodies (Molecular Probes, Eugene, Ore.) were Alexa 488 goat anti-mouse IgG (H+L) conjugated diluted 1:100, and Alexa 594 goat anti-rabbit IgG (H+L) conjugated diluted 1:600. After incubation the slides were washed three times in 1×PBS and counterstained with 0.2 µg/ml DAPI (Boehringer, Mannheim) in 2×SSC for 5 min. Finally, the slides were mounted in antifade medium (DABCO; Sigma). Immunofluorescent preparations were observed and documented as previously described (Bongiorni et al. 1999, 2001), using filter combinations suitable for the different fluorochromes.

Results

The Me9H3 epitope is enriched in constitutive heterochromatin and the X_i and decorates metaphase chromosomes in a banding pattern

In order to detect Me9H3 in chromosomal DNA we raised an antibody that specifically recognizes methylated K9 of H3 histone (see Materials and methods). Immunoblotting (Fig. 1) showed that the antibody did not rec-

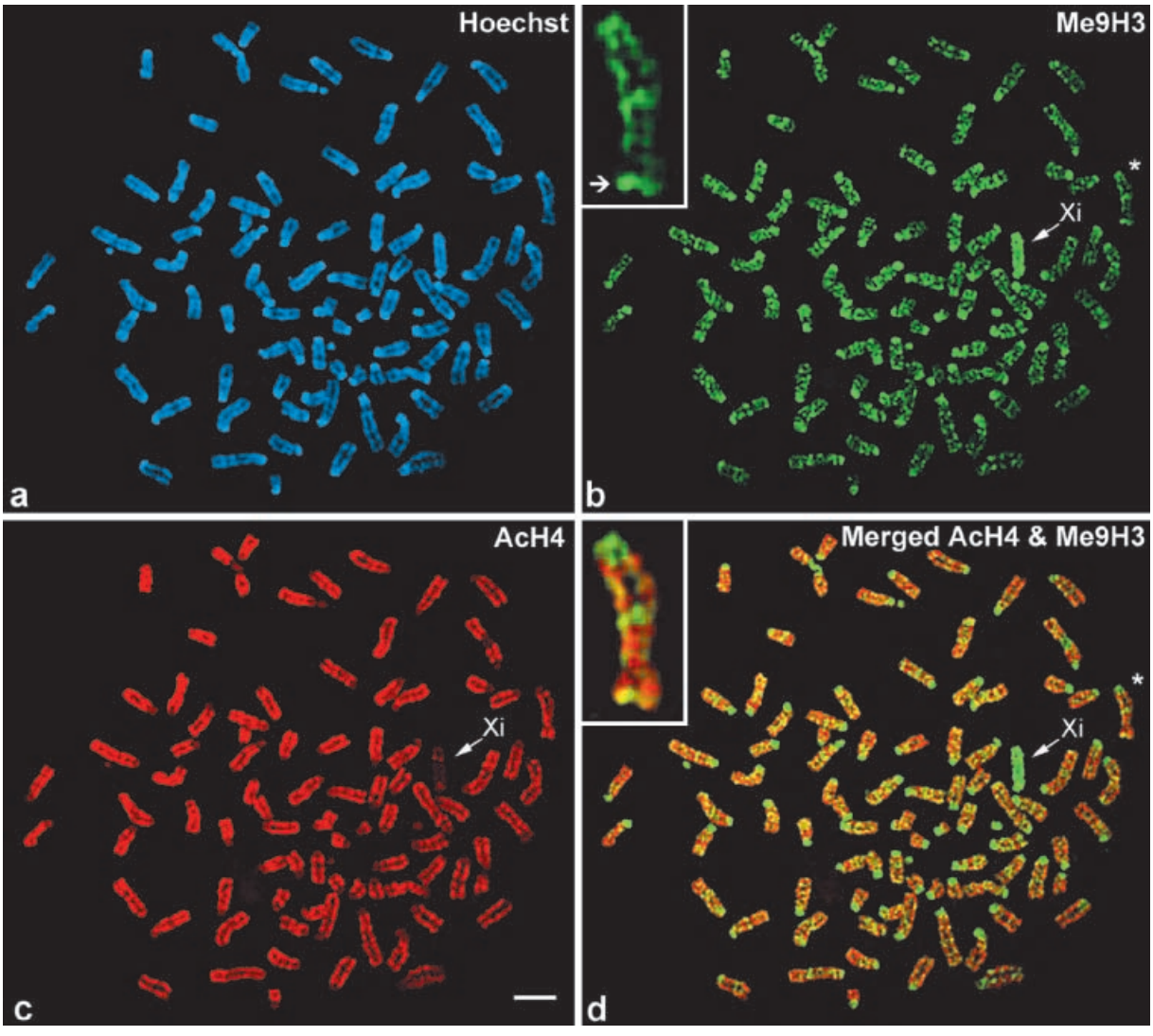


Fig. 2a–d Distribution of acetylated histone H4 (AcH4) and Me9H3 in murine metaphase chromosomes. Unfixed metaphase spread of mouse C127 cell simultaneously labelled with rabbit anti-Me9H3 and sheep S613 anti-AcH4 antisera: **a** Hoechst 33258 fluorescence (*blue*); **b** distribution of Me9H3 by indirect immunofluorescence [fluorescein isothiocyanate (FITC), *green*]; **c** distribution of AcH4 by indirect immunofluorescence (tetramethylrhodamine isothiocyanate, *red*); **d** merged images (**b**+**c**). (Monochrome CCD images have been pseudocoloured for clarity.) Binding of the anti-Me9H3 (**b**) is enriched at the centromeres, at some of the telomeres and is non-uniform along the chromosome arms. The Me9H3 banding pattern is most clearly seen in the chromosome, marked with an *asterisk*, that is enlarged approximately fourfold in the *insert*. The *arrow in the insert* points to the telomeres. One larger chromosome, the putative inactivated X (X_i), is also distinguishable by its intense immunofluorescence. We confirmed that this strongly labelled chromosome was the X_i by comparison with the staining pattern obtained with the anti-AcH4 antibody (acetylated lysines 5, 8, 12 and 16; **c**). The intensely Me9H3-staining chromosome in **b** was virtually depleted in AcH4, which is a diagnostic feature of the X_i (Jeppesen and Turner 1993). Centromeric heterochromatin is also depleted in AcH4. A comparison of the banding patterns obtained with antibodies to AcH4 and Me9H3 is shown in the merged image in **d** and most clearly seen in the *insert*, where the chromosome marked with the *asterisk* is enlarged. The enlarged chromosome shows generally non-overlappingbands of *red* (AcH4) and *green* (Me9H3), which correlate with R- and G-bands, respectively (see text for details). The *bar* in **c** represents 5 μm

ognize recombinant H3 (lanes 1 and 4), but did recognize Me9H3 in commercial preparations of H3 histone (lane 2) and of all four histones (lanes 3, 11 and 14). Me9H3 was also found in crude extracts from tissue culture cells (lane 5) and mature sperm (lane 15). Binding to H3 histone preparation was completely ablated by preincubation with a methyl-K9 H3 peptide (lane 13), but not by a methyl-K4 H3 peptide (lane 12). This antibody was then used in all the experiments described below.

Figure 2 shows a metaphase spread prepared from a mouse mammary gland tumour cell line, C127, stained with Hoechst 33258, which preferentially stains AT-rich DNA (Fig. 2a), and the same spread simultaneously labelled by indirect immunofluorescence with antibodies to acetylated H4 histone (acetylated on lysines 5, 8, 12 and 16; Fig. 2c) and antibodies to histone H3 methylated at lysine 9 (Me9H3; Fig. 2b). As previously reported (Jeppesen and Turner 1993), the X_i is generally under-

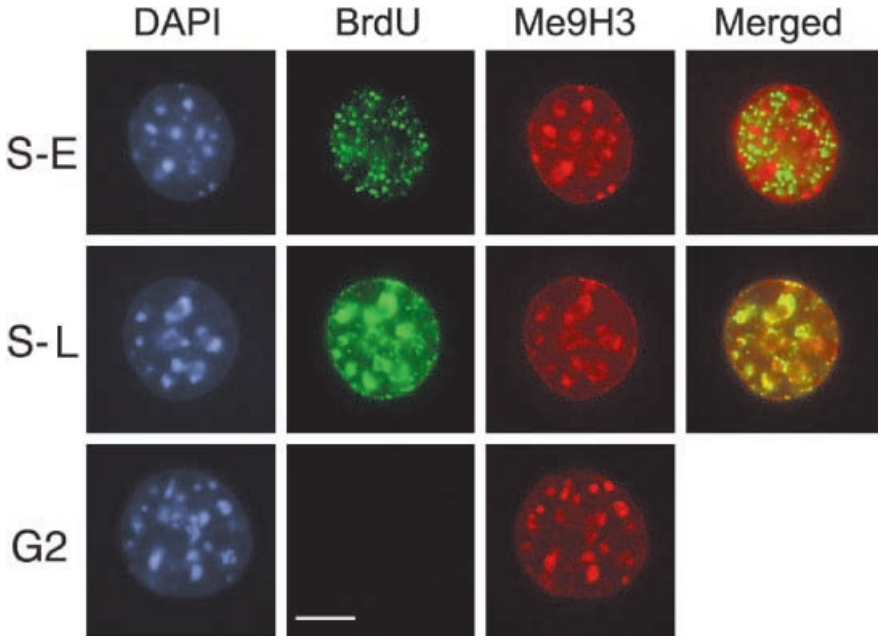


Fig. 3 Me9H3 replicates late in S-phase of the cell cycle. C127 cells were arrested at the G1/S-phase border with aphidicolin and then released into S-phase. Cells were pulse labelled with 5-bromo-2′-deoxyuridine (BrdU) either early (1 h post release, *S-E*) or late (5 h post-release; *S-L*) in S-phase, or as cells were starting to leave S-phase (9 h post-release; *G2*) and were then stained for 4′,6-diamidino-2-phenylindole (DAPI) (*blue*), BrdU (*green*) and Me9H3 (*red*). The BrdU and Me9H3 staining patterns were merged in the far right column (*Merged*). This showed that the BrdU and Me9H3 staining patterns co-localise late in S-phase (*S-L*). The *bar* represents 5 µm

acetylated and can clearly be identified by its characteristically weak fluorescence in Fig. 2c. [The three bands of H4 hyperacetylation evident on the mouse X_i have been discussed by Jeppesen and Turner (1993).] In addition to X_i, centromeric heterochromatin is also underacetylated (Fig. 2c). By contrast, the distribution pattern of Me9H3 is very different (Fig. 2b). Centromeric heterochromatin is enriched in Me9H3 and the banding pattern along the chromosome arms is particularly striking. In some chromosomes the telomeres are also labelled (see insert in Fig. 2b). Finally, inspection of a large number of chromosome spreads showed that the underacetylated X_i was consistently more intensely stained with the anti-Me9H3 antibody than the rest of the chromosomes (see X_i in Fig. 2b, d). These differences between the staining patterns of acetylated H4 (as determined using our antibody to AcH4) and methylated H3 histones are most clearly seen in the merged image in Fig. 2d (magnified for an autosome in the insert), where an alternating red (H4 acetylation) and green (H3 methylation) banding pattern is suggested along the length of the chromosome arms. In contrast, the X_i fluoresces almost homogeneously bright green through an overall enrichment in Me9H3. In short, Me9H3 distribution appears to be the complement of H4 acetylation (as detected with our antibody to AcH4).

The H4 acetylation bands (in red; see insert in Fig. 2d) are known to correlate with R-bands (Jeppesen 1997). The apparent alternation of these bands with those labelled by the anti-Me9H3 antibody (in green, see inserts in Fig. 2b, d) suggests that the Me9H3 bands are coincident with G- (or Giemsa) bands. This G-band-like pattern is also observed in human metaphase chromosomes, as is the Me9H3 enrichment in the X_i (unpublished results).

Me9H3 is replicated with heterochromatin late in S-phase

A diagnostic feature of both constitutive and facultative heterochromatin is that they are replicated late in S-phase of the cell-cycle (Morishima et al. 1962; Lima-de-Faria and Jaworska 1968). Because we had shown that Me9H3 is enriched in constitutive heterochromatin, particularly around the centromeres and at some telomeres (Fig. 2b), and was also enriched in the facultatively heterochromatic X_i (Fig. 2b, d), we investigated whether the presence of Me9H3 is generally coincident with late S-phase replication, the characteristic hallmark of heterochromatin. Accordingly, we determined the time at which chromatin enriched in Me9H3 replicates using dual labelling with BrdU and anti-Me9H3 antibodies. As seen in Fig. 3, cells pulse-labelled with BrdU late in S-phase (S-L; 5 h post-release from the aphidicolin block) showed co-localised staining with anti-BrdU and anti-Me9H3 antibodies, confirming that Me9H3 replicates late in S-phase. No overlap between BrdU and Me9H3 staining was observed at early S-phase (S-E; 1 h post-release), or in G2-phase (G2; 9 h post-release).

Me9H3 staining of the XY-body precedes staining with M31 and Me9H3 is retained in the developing sperm head

The distribution of M31 during meiosis in males has been described (see Hoyer-Fender et al. 2000a; O'Carroll et al. 2000; Turner et al. 2001). Thus at mid-pachytene, autosomal (ACA-positive) centromeric heterochromatin is M31 stained, while the XY-body,

which contains the X and Y chromosomes, is only faintly stained or not stained at all (Fig. 4 c; arrow points to XY-body). When the same nucleus is stained with the anti-Me9H3 antibody, Me9H3 is found to co-localise with M31 at centromeric heterochromatin and, quite strikingly, the entire XY-body is also clearly stained (see arrow in Fig. 4b and the merged image in Fig. 4d). Thus, enrichment of Me9H3 within the XY-body at mid-pachytene is not sufficient for the recruitment of M31, even though, within the same nucleus, Me9H3 and M31 co-localise with the ACA-positive heterochromatic blocks (see merged image in Fig. 4d). M31 eventually co-localises with Me9H3 at the XY-body during late pachytene/diplotene (determined by SCP3 staining; Turner et al. 2001), as seen in the diplotene nucleus shown in Fig. 4e–h (especially the merged image in Fig. 4h; arrow points to XY-body). Me9H3 staining is also detectable post-meiotically throughout all spermiogenic stages except the most mature sperm. The absence of staining in mature sperm may be due to lack of antibody penetration, since Me9H3 is still detectable in mature sperm by immunoblotting (Fig. 1, lane 14). It is known that a small fraction of histones remains associated with the sperm, the amount depending on species (around 15% in man; Gatewood et al. 1987; Hoyer-Fender et al. 2000a). In elongating spermatids (Fig. 4i, j), Me9H3 co-localises with the DAPI- and ACA-positive centromeric DNA.

Me9H3 is depleted in the paternal pronucleus and the genomes only become equivalent for Me9H3 after the two-cell stage

Guided by the observation that developing spermatozoa contain Me9H3 (Fig. 4j), and that by immunoblotting Me9H3 is also detectable in mature sperm (Fig. 1, lane 15), we next investigated the distribution of Me9H3 in the unfertilized oocyte. We also studied the distribution of Me9H3 during fertilization, pronuclear formation and the early cleavage divisions. The maternal (ma) chromosomes in a metaphase II-arrested oocyte stain strongly for Me9H3 (Fig. 5a–c). Immediately after fertilization, the spermatozoon (sp) can be observed as a DAPI-stained crescent at the periphery of the oocyte (sp; bottom of Fig. 5d); the maternal chromosomes are still at metaphase II. The sperm appears to contain no immunocytologically detectable Me9H3, while Me9H3 is enriched within the polar body (pb) and the maternal chromosomes (Fig. 5e, f). One possibility was that the absence of Me9H3 in the fertilizing spermatozoon was an artefact due to antibody exclusion or to slightly different fixation conditions, which results in the loss of the

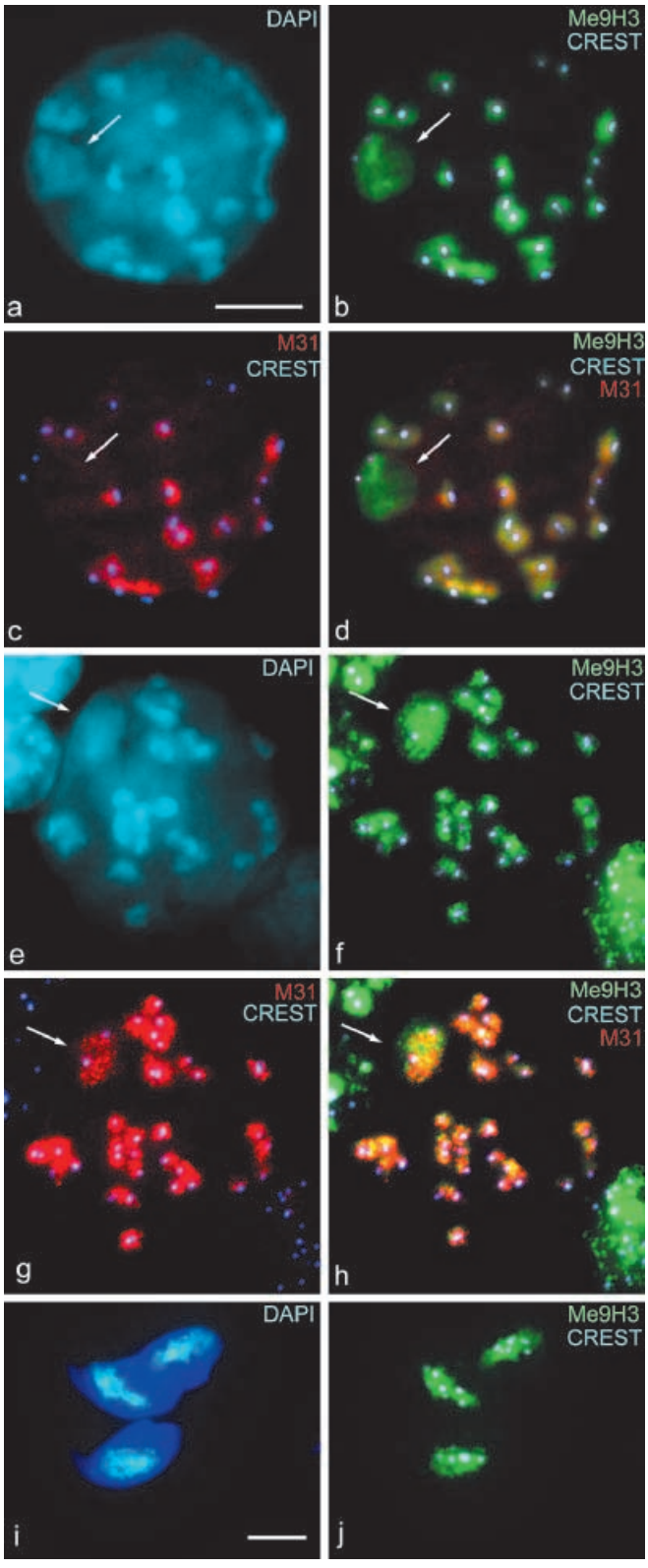


Fig. 4a–j Me9H3 localises to the XY-body and is present in developing sperm. Mid-pachytene (**a**), diplotene (**e**) spermatocytes and hypotonically decondensed developing sperm (**i**) are stained with DAPI. Anti-centromere antibody (ACA)-positive centromeric DNA (*white*) co-localises with M31 (*red*) and Me9H3 (*green*) at most positions within the nuclei. In the mid-pachytene nucleus there is no co-localisation of Me9H3 (**b**) with M31 (**c**) over the XY-body (see merged image in **d**; *arrow* points to XY-body). By contrast, in the diplotene nucleus both Me9H3 (**f**) and M31 (**g**) co-localise over the XY-body (see merged image in **h**; *arrow* points to XY-body). In hypotonically swollen developing sperm the Me9H3 (**j**) co-localises with bright (AT-rich) DAPI staining blocks (**i**) and the ACA-positive signals (merged image in **j**). *Bars* in **a** and **i** represent 5 µm

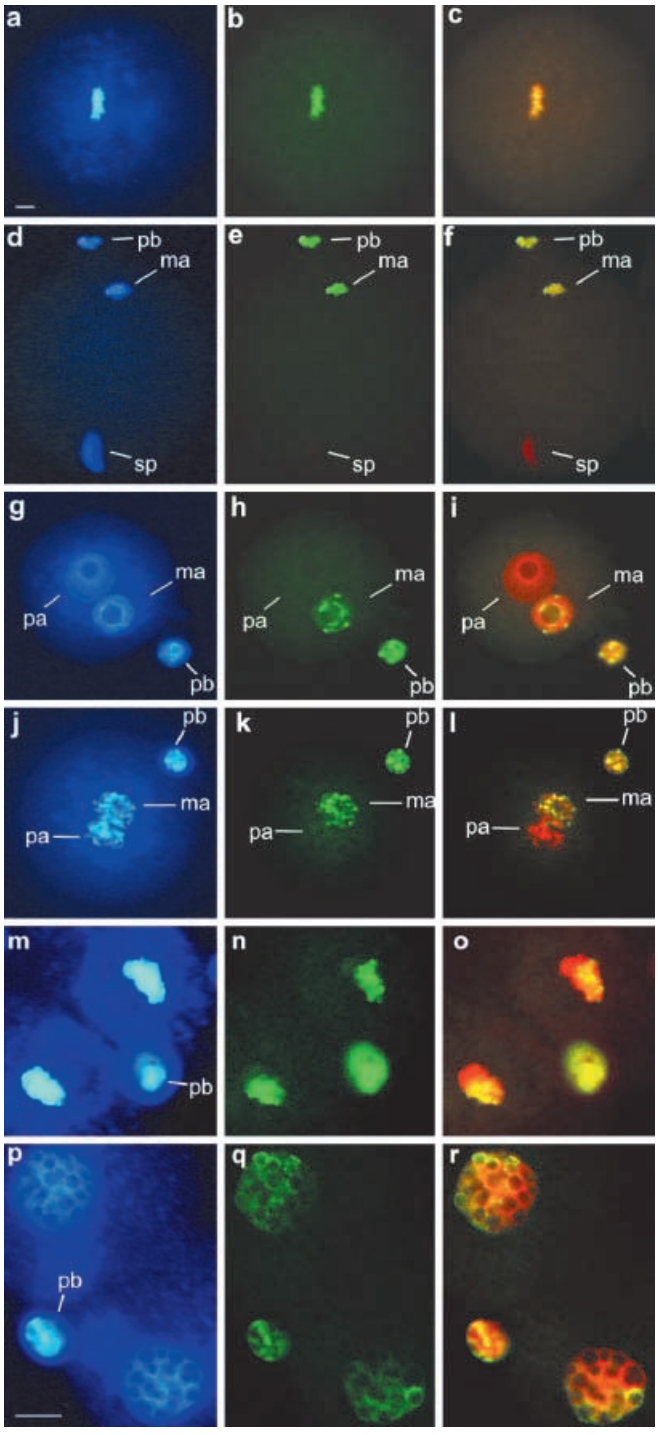


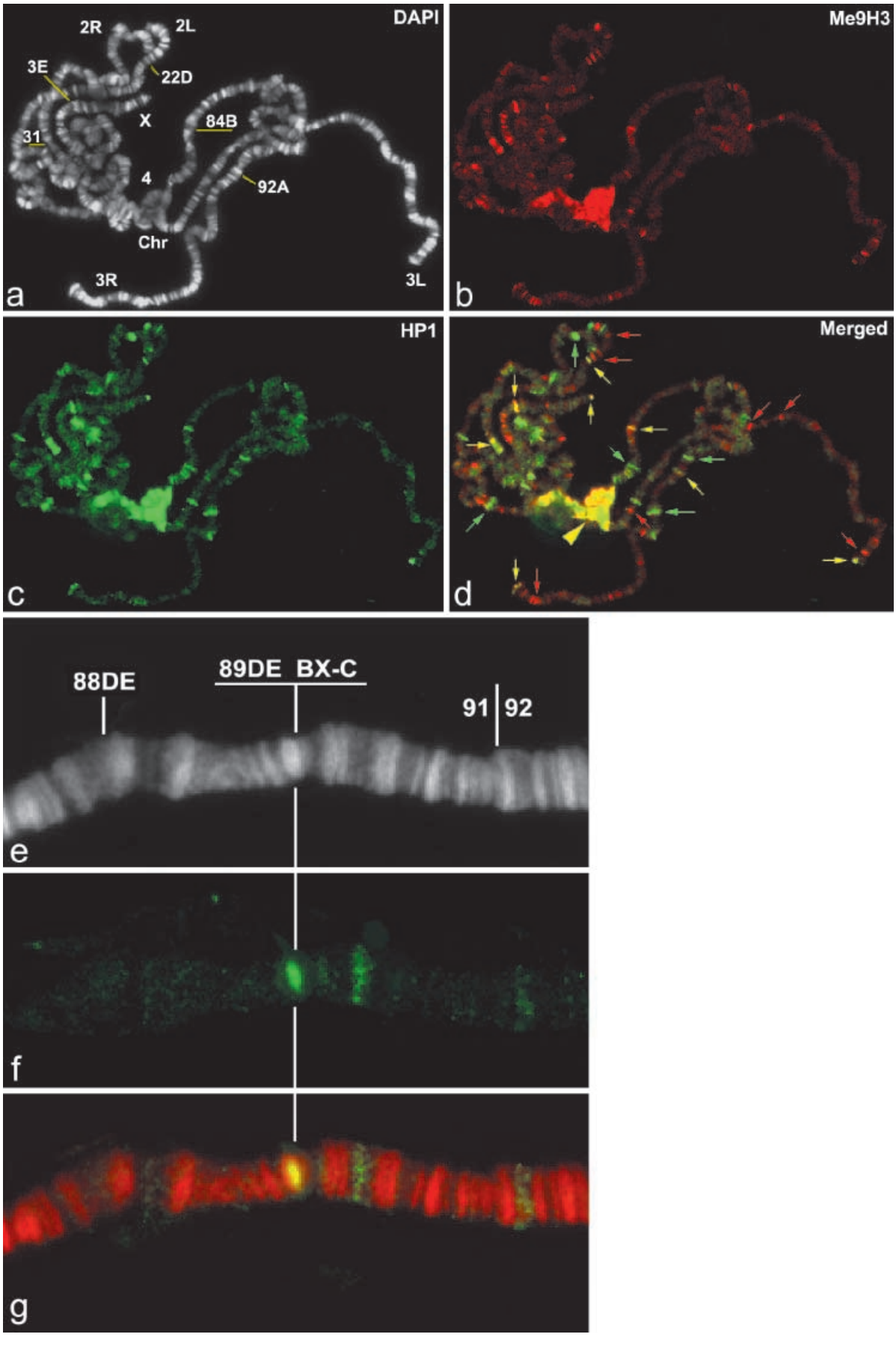
Fig. 5a–r Distribution of Me9H3 in oocytes and one- and two-cell mouse embryos. Oocytes and one- and two-cell embryos stained with DAPI are shown in the left-hand column (**a**, **d**, **g**, **j**, **m** and **p**). The sperm (*sp*), maternal (*ma*), paternal (*pa*) and polar body (*pb*) DNA stains intensely with DAPI, compared with the cytoplasm. The same embryos were stained using the anti-Me9H3 antibody and the immunofluorescence (FITC; *green*) micrographs are given in the middle column (**b**, **e**, **h**, **k**, **n**, **q**). The right-hand column (**c**, **f**, **i**, **l**, **o**, **r**) depicts the merged images of the embryos given in the left and middle columns. DAPI is pseudocoloured in *red*; co-localisation is *yellow*. Me9H3 is enriched in the maternal chromosomes of a metaphase II-arrested oocyte (**a–c**; *n*=15). Approximately 1 h after fertilization (*n*=10) the sperm nucleus is found in the ooplasm and lacks Me9H3, while the polar body and the maternal chromosomes contain Me9H3 (**d–f**). Me9H3 is present in the maternal pronucleus but undetectable in the paternal pronucleus (pronuclear stage; *n*=32) (**g–i**). At syngamy (*n*=33), the difference in Me9H3 between the parental chromosomes is clear (**j–l**). Me9H3 staining is partitioned in the late telophase/early G1-phase two-cell embryo (early two-cell; *n*=15) (**m–o**). Late two-cell embryos (*n*=35) also exhibit partitioning of Me9H3 staining (**p–r**). The magnification of **a–l** is the same and the *bar* in **a** represents 10 µm. The magnification of **m–r** is the same and the *bar* in **p** represents 10 µm

Me9H3 epitope (see Materials and Methods for conditions). However, we were dissuaded from making this interpretation because: (1) the Me9H3 is still detectable in the condensed metaphase II maternal chromosomes; and (2), we examined many stages, from sperm entry through sperm head decondensation to the formation of a well-defined pronucleus, and found that the paternal chromosomes always lacked detectable Me9H3. An example is given in Fig. 5g–i, where the somewhat larger paternal (pa) pronucleus does not contain detectable Me9H3, while Me9H3 can clearly be observed in the smaller maternal (ma) pronucleus as punctate blocks, some of which are in close apposition to the nucleolus. Thus, in the same environment, under the same fixation conditions, the parental pronuclei exhibit a striking non-equivalence in terms of Me9H3.

The difference in parental Me9H3 levels is also observed at subsequent stages of development. It is very clear at syngamy, which is the stage at which the parental chromosomes condense and come together to form a unique metaphase plate, just before the embryo cleaves to form two cells. At syngamy, the paternal chromosome set, which has undergone DNA replication and is now diploid, is devoid of Me9H3, while the diploid maternal set is clearly labelled with the anti-Me9H3 antibody (Fig. 5j–l). After the first mitosis, the difference between the parental chromosomes can also be observed in the two-cell embryo; each cell contains a diploid nucleus with a set of maternal and paternal chromosomes. As seen, Me9H3 localises to a portion of the reforming early G1-phase nuclei (Fig. 5m–o; especially the merged image in 5o). Because it is the maternal chromosomes that are stained in all previous stages we suspect that the Me9H3 is preferentially associated with the maternal chromosome set. This uneven staining of nuclei is also observed in late two-cell stage embryos (Fig. 5p–r). At the four-cell stage no partitioning of Me9H3 can be observed within the nuclei (data not shown).

Me9H3 and its relation to silenced domains on *Drosophila* polytene chromosomes

Our observation that Me9H3 localises to chromosome regions (centromeres, telomeres and G-bands) and to whole chromosomes (the X_i) that are repressive in nature (Fig. 2) prompted us to investigate whether Me9H3 is a general


DAPI
2R
2L
3E
22D
X
84B
31
4
92A
Chr
3R
3L
a
Me9H3
b
HP1
c
Merged
d
88DE
89DE
BX-C
91
92
e
f
g

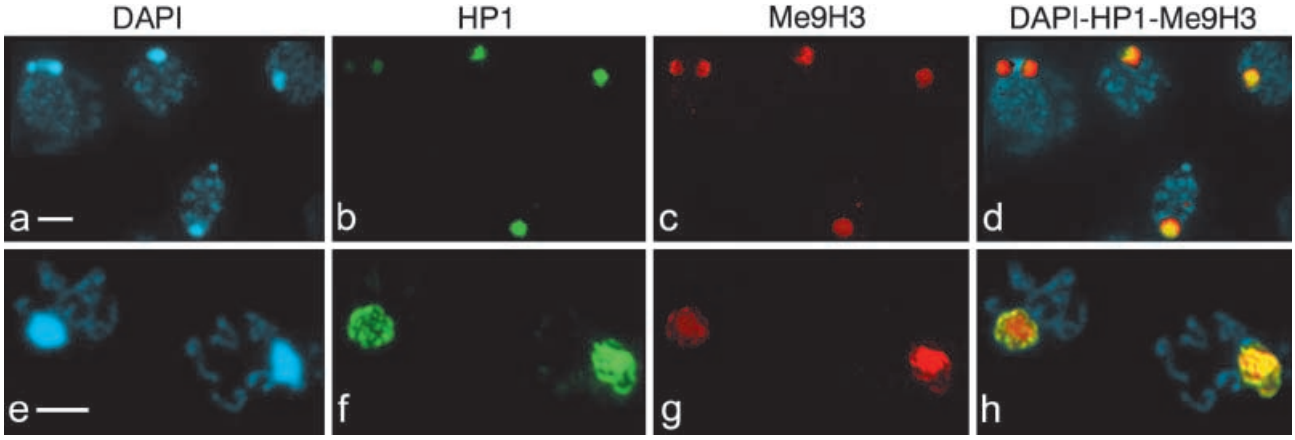


Fig. 7a–h HP1 and Me9H3 distribution in interphase and pro-metaphase cells of male mealy bugs. Interphase and pro-metaphase cells from mealy bugs stained with DAPI are shown in **a** and **e**, respectively, and reveal the strongly stained paternal heterochromatic chromocentre. Immunoflorescence labelling of the interphase nuclei with antibodies to HP1 (*green spots* in **b**) and Me9H3 (*red* in **c**) showed a remarkable coincidence with the strong DAPI staining spots in **a**, **d**. Likewise, staining of the pro-metaphase with antibodies to HP1(*green spots* in **f**) and Me9H3 (*red* in **g**) showed coincident staining with the intense DAPI-stained chromocentre in **e**, **h**. The magnification of **a–d** is the same and the *bar* in **a** represents 5 µm. The magnification of **e–h** is the same and the *bar* in **e** represents 5 µm

component of repressed chromatin. We chose to address this issue by immunolabelling *Drosophila* polytene chromosomes, which allow a much greater cytological resolution than that obtained with mammalian metaphase chromosomes. We also attempted to correlate the Me9H3 staining pattern with HP1 binding sites in order to explore further the relationship between Me9H3 and HP1.

Staining of polytene chromosomes with Me9H3 antibody showed enrichment in the chromocentre (Chr), chromosome *4* and some of the telomeres (Fig. 6a, b). Enrichment at these sites was coincident with intense HP1 staining (cf. Fig. 6b with 6c; yellow arrows 6d). We also found Me9H3 sites in the euchromatic arms, especially at the interval encompassing band 31 on chromosome *2L*, which is a known euchromatic site for HP1 binding (James et al. 1989; cf. Fig. 6a–c; yellow arrows in 6d). Genes that reside at this location are repressed by HP1 (Hwang et al. 2001) and our data indicate that Me9H3 may be part of the mechanism of repression. Similar coincident sites are found at other euchromatic loci, the most prominent being band 22D on *2L*, band 3E on the *X* and bands 84B and 92A on *3R* (Fig. 6a for map positions; Fig. 6d for merged image where yellow arrows denote the overlap). It remains to be demonstrated whether HP1 is involved in repression of genes at these loci.

There are euchromatic Me9H3 sites that do not co-localise with HP1 binding sites. This is most clearly seen in the merged image in Fig. 6d, where the HP1 binding sites are given in green (see green arrows in 6d). The converse is also true; there are HP1 binding sites that do not co-localise with Me9H3 sites (see red arrows in 6d). These results indicate that the mere presence of Me9H3 is neither necessary nor sufficient for HP1 binding to polytene chromosomes.

During the mapping of euchromatic Me9H3 sites we observed strong Me9H3 staining of band 89DE on chromosome *3* (Fig. 6e–g). This is significant because the Bithorax complex (BX-C) is in this band (Spierer et al. 1983). The BX-C contains the homeotic genes, which are crucial determinants of developmental fate and known to be repressed in the anterior regions of the embryo, such as the salivary glands, by the *Pc*-G complex (Zink and Paro 1989). The *Pc*-G complex epigenetically silences the homeotic genes in regions outside their normal realm of activity (for review see Pirrotta 1995; Orlando and Paro 1995). Our data indicate that the mechanism of *Pc*-G silencing is likely to involve a histone methyltransferase.

◀ **Fig. 6a–g** HP1 and Me9H3 localisation in wild-type *Drosophila* polytene chromosomes. A polytene chromosome spread stained with DAPI is shown in **a**. The indirect immunofluorescent labelling (Cy3; *red*) of the same spread with rabbit anti-Me9H3 is given in **b**, and labelling (FITC; *green*) with mouse anti-HP1 monoclonal is given in **c**. The images in **b** and **c** are merged in **d**. The localisation of Me9H3 (**b**) shows strong accumulation in the chromocentre (*Chr*) and at multiple euchromatic sites. All the telomeres are labelled. The localisation of HP1 (**c**) gives a similar picture, where HP1 is enriched in the chromocentre and at several euchromatic sites. Again the telomeres are labelled. The merged image (**d**) confirms that Me9H3 and HP1 co-localise at the chromocentre, at the telomeres and at several of the euchromatic sites (highlighted by the *yellow arrows*). The map positions for the co-localising bands are given in **a**. However, there are some Me9H3 sites that do not localise with HP1 sites (the *red arrows* in **d**) and some HP1 sites that do not localise with Me9H3 sites (the *green arrows* in **d**). An enlarged region encompassing polytene regions 89DE, which contains the bithorax complex (*BX-C*), is stained with DAPI and given in **e**. The same region stained (FITC; *green*) with the rabbit anti-Me9H3 antibody (**f**) shows a strong signal at map position 89DE. The merged image (**g**), where the DNA is pseudocoloured in *red*, shows a strong *yellow* band at 89DE, which confirms co-localisation of Me9H3 with the BX-C

Me9H3 and parental imprinting in mealy bugs

We explored the role of Me9H3 in parental imprinting by examining Me9H3 within the paternal chromosome set in mealy bugs. Cellular suspensions of embryonic tissues

were prepared by dissection from gravid females. Both male and female embryonic cells are present in these preparations, but the male tissues can be clearly distinguished by DAPI staining, because the heterochromatic chromosomes stain intensely as a brightly stained chromocentre in interphase nuclei (Fig. 7a, e; Epstein et al. 1992; Bongiorni et al. 1999, 2001). When male interphase cells were simultaneously labelled with antibodies to *Drosophila* HP1 (Fig. 7b) and Me9H3 (Fig. 7c), we found that there was a striking coincidence (see merged image in Fig. 7d). Only small "flecks" of Me9H3 were sometimes observed in the euchromatin. The co-localisation of HP1 with Me9H3 was observed throughout the cell cycle. For example, at pro-metaphase, the HP1 signal was present only in the paternal heterochromatic set, while the euchromatic chromosomes derived from the mother were unlabelled (Fig. 7e–h).

In female interphase and mitotic cells HP1 and Me9H3 were scattered throughout the chromatin; there were no differences between the homologous chromosomes (data not shown).

Discussion

Immunofluorescence using an antibody specific for Me9H3 has shown that both constitutive (centromeres and telomeres) and facultative (X_i) heterochromatin in mammalian cells is enriched in Me9H3 (Fig. 2). This complements previous work, which showed that another defining feature of mammalian heterochromatin is depletion of AcH4 (Jeppesen et al. 1992; Jeppesen and Turner 1993). The reciprocity of AcH4 and Me9H3 staining patterns can be extended to the banding patterns found in the chromosome arms (Fig. 2b, d, especially the insert in d). Me9H3 decorates the chromosome axes in a G-band-like pattern, which alternates with an AcH4 pattern that duplicates R-bands (Fig. 2c; Jeppesen 1997). This reciprocity is likely to reflect the general properties of G- and R-bands (for reviews see Holmquist 1992; Cross and Bird 1995; Gardiner 1995). G-bands share characteristics with heterochromatin: they are late replicating, contain a low number of tissue-specific genes and are enriched in long interspersed repetitive elements. By contrast, R-bands replicate early, possess high CpG island density, contain a large number of both housekeeping and tissue-specific genes, and are replete with short interspersed repeats. The correlation of AcH4 and Me9H3 with R- and G-bands, respectively, indicates that histone modifications may reflect an organizing principle by which interphase chromatin is assembled into metaphase chromosomes.

Me9H3 in relation to X-inactivation and parental imprinting

The enrichment of Me9H3 in the X_i (Fig. 2b, d) is likely to be one of the mechanisms, along with CpG methylation and deacetylation of H3 and H4 histones, that maintain the X_i in a silent state (Jeppesen and Turner 1993; Boggs et al. 1996; Csankovszki et al. 2001). This view stems from evidence that deacetylation of histones on the X_i is a secondary event during X-inactivation, detectable after *Xist* upregulation and the establishment of transcriptional repression and late replication (Keohane et al. 1996). Because deacetylation of histones, especially of lysines 9 and 14 on H3, is required for methylation of lysine 9 on histone H3 (Nakayama et al. 2001), it would place the enrichment of Me9H3 in the X_i as a later event towards the end of the inactivation process.

As well as an association with the X_i, our data indicate that Me9H3 is likely to be important for the cell-to-cell inheritance of silenced states associated with parental imprinting phenomena. In the classical *Coccid* system, we have shown that in males the silenced paternal chromosome set is associated with Me9H3 and HP1 (Fig. 7). This association may extend to mammalian imprinted genes that lie within similar repressed chromosomal domains (Banerjee et al. 2000).

(Re)programming Me9H3 patterns in the mammalian oocyte

Our observation that fertilizing sperm lacks detectable Me9H3 (Fig. 5d–f) may result from additional histone modification(s) that block reactivity of our antibody to Me9H3 or that Me9H3 associated with mature sperm (see lane 15 in Fig. 1) is removed by activities in the oocyte cytoplasm. Such activities, which could involve specific histone demethylases or proteolytic enzymes that degrade Me9H3, would be important for the epigenetic reprogramming of lineage-specific patterns of Me9H3 that must occur during the animal cloning procedure (Wilmut et al. 1997).

We suggest that the patterns observed for Me9H3 in Fig. 5 are likely to be related to three aspects of early mouse development: (1) the staining pattern of Me9H3 may reflect its involvement in keeping the parental genomes separate during the first two cleavage divisions (Mayer et al. 2000a). It has been shown that HP1 associates with the nuclear envelope as part of a ternary complex that includes HP1, both core histones, H3 and H4, and the integral nuclear envelope protein, the Lamin B receptor (LBR) (Kourmouli et al. 2000, 2001; Polioudaki et al. 2001). Given recent work demonstrating that HP1 binds preferentially to Me9H3 (see Introduction), the maternal genome may form a more stable HP1:H3/H4:LBR complex at the nuclear envelope, compared with the paternal genome, which lacks Me9H3. (2) The apparent lack of Me9H3 on paternal chromatin may facilitate active 5-methylcytosine (MeC) demethylation of the paternal genome. During pre-implantation development the MeCs in the paternal chromosomes are actively demethylated and this active demethylation begins at around 6 h after fertilization – by 8 h the paternal chromosomes are much depleted in MeC (Mayer et al.

2000b). Our results indicate that active MeC demethylation, either by specific demethylases or by excision repair mechanisms, may be a consequence of greater accessibility to the paternal chromatin because it lacks Me9H3. Acetylation is unlikely to be involved because by 6 h post-fertilization the acetylation status of histones in the pronuclei is equivalent (Worrad et al. 1995; Adenot et al. 1997). (3) The increase in Me9H3 levels during the two-cell stage may be the reason that genes need to use enhancers for their activity. It is known that the transition from a one-cell embryo to a four-cell embryo, which encompasses the period of zygotic genome activation, is associated with a gradual increase in chromatin-mediated repression of promoters and origins of replication (Martinez-Salas et al. 1988; Majumder and DePamphilis 1995; Wiekowski et al. 1997). As shown (Fig. 5g–r), the levels of Me9H3 are increasing during this period and we suggest that Me9H3 is part of the mechanism responsible for the increase in repression seen during the first two cleavage divisions. The increase in repression seen during pre-implantation development is unlikely to be due to MeC because global levels of MeC are decreasing during this time (Monk et al. 1987; Brandeis et al. 1993; Razin and Shemer 1995).

The relationship of Me9H3 to HP1

Staining of mouse metaphase chromosomes with the anti-Me9H3 antibody revealed more Me9H3 sites (Fig. 2b, d) than known HP1 sites (Nicol and Jeppesen 1994; Wreggett et al. 1994; Minc et al. 1999, 2000). Mammalian HP1 proteins have been found at the centromeres, but they have not been localised to the X_i (P.J. and P.B.S., unpublished data). Although there is a suggestion of telomeric localisation in some chromosomes (Nicol and Jeppesen 1994), there does not appear to be a strong G-band association as shown by Me9H3. These data indicate that the relationship of Me9H3 to HP1 is not an absolute one and is likely to be regulated. Clearly, the mere presence of Me9H3 is not, on its own, sufficient for HP1 binding to chromosomal DNA.

We favour the view that Me9H3 is a robust epigenetic "mark", which interacts with CD proteins, like HP1, and that the affinity of this rather weak association (a dissociation constant of 100 μM; Jacobs et al. 2001) can be modulated by further, more regulatable, modifications of CD proteins and histones. At metaphase, much of the chromatin-bound M31 (HP1β) and M32 (HP1γ) is dispersed into the mitotic cytoplasm (Kourmouli et al. 2000, 2001), and different phosphorylation states of HP1 (Huang et al. 1998; Minc et al. 1999), which are known to be cell cycle regulated (Minc et al. 1999), could determine the interaction of HP1 proteins with metaphase chromatin. Re-binding of the HP1 in the following interphase may utilise Me9H3 as an epigenetic mark that enables the inheritance of silenced states through mitosis. A similar but complementary role for histone acetylation was postulated previously (Jeppesen 1997), where it was suggested that histone hyperacetylation marks transcriptionally active chromatin domains. Thus core histone acetylation and H3 methylation can be envisaged as different facets of the histone code, but with a common aim – to mark active and repressed chromatin domains, respectively, at cell division.

Additional histone modifications might also affect the interaction of HP1 with Me9H3. For example, Me9H3 localises to blocks of constitutive heterochromatin and the XY-body in mid-pachytene spermatocytes (Fig. 4b). Yet, in the same nucleus, M31 only localises to the heterochromatic blocks and is conspicuously absent from the XY-body (Fig. 4c, d). Importantly, the XY-body in pachytene cells differs from the heterochromatic blocks and the X_i in females in one respect – it is enriched in AcH4 (Armstrong et al. 1997). Since HP1 proteins can interact with H4 (Zhao et al. 2000; Polioudaki et al. 2001), acetylation of H4, alone or in conjunction with other modifications, might reduce the affinity of HP1 for chromosomal DNA in the XY-body.

Further support for a general role in epigenetic marking of silenced states comes from the localisation of Me9H3 to the BX-C on polytene chromosomes (Fig. 6e–g). The BX-C contains the homeotic genes, which are crucial determinants of cellular fate that direct the development of structures and appendages at appropriate positions along the anterior-posterior (A-P) axis of the embryo (Akam 1987). They are expressed in precise, spatially restricted patterns that are confined to particular regions along the A-P axis by the activity of regulatory elements called polycomb response elements (PREs; Simon et al. 1993). PREs are DNA sequences to which the *Pc*-G complex binds and represses the activity of homeotic genes in regions of the embryo where they are not normally expressed (Bienz and Muller 1995; Orlando and Paro 1995; Pirrotta 1995). Two lines of evidence, derived from immunolocalisation (Wang et al. 1997; Buchenau et al. 1998) and knockout/supply (Beuchle et al. 2001) experiments, indicate that binding of the *Pc*-G to the PRE during the larval stages involves the recognition of an epigenetic mark at the PRE that is acquired early in development. We suggest that the Me9H3 we have detected at the BX-C might be this mark. A *Pc*-G gene product that could act as the histone methyltransferase is E(z), which contains a SET domain (Jenuwein et al. 1998). However, recent work on its mammalian homologue, EZH2, failed to reveal a histone methylase activity (Rea et al. 2000). The most likely *Pc*-G protein that recognizes the Me9H3 is Pc itself, which contains a classical CD, similar to that found in HP1 (Jones et al. 2000).

We also observed HP1 binding sites that do not localise with cytologically detectable Me9H3 sites (Fig. 6b–d). Bearing in mind the limitations of this type of microscopic analysis, it would seem that Me9H3 is not necessary for HP1 binding to chromosomal DNA. This would be consistent with evidence that HP1 can interact with a number of chromatin-associated proteins, including: CAF1 (Murzina et al. 1999), ORC (Pak et al.

1997), histone H4 (Zhao et al. 2000; Polioudaki et al. 2001) and with the histone fold of H3 (Nielsen et al. 2001a). Of these, mutations in ORC2 lead to a partial dissociation of HP1 from centromeric heterochromatin as well as from some euchromatic sites (Huang et al. 1998). Thus, the localisation of HP1 within the nucleus is probably due to a combination of (low-affinity) protein:protein interactions, of which the most notable, and perhaps the most common, is Me9H3.

Acknowledgements We thank Dr. W.C. Earnshaw for the anti-ACA antibody and members of the Singh laboratory for many helpful discussions. The PBS laboratory is funded by a BBSRC Core Strategic Grant.

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