Meiotic sex chromosome inactivation in male mice with targeted disruptions of *Xist*

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Summary

X chromosome inactivation occurs twice during the life cycle of placental mammals. In normal females, one X chromosome in each cell is inactivated early in embryogenesis, while in the male, the X chromosome is inactivated together with the Y chromosome in spermatogenic cells shortly before or during early meiotic prophase. Inactivation of one X chromosome in somatic cells of females serves to equalise X-linked gene dosage between males and females, but the role of male meiotic sex chromosome inactivation (MSCI) is unknown. The inactive X-chromosome of somatic cells and male meiotic cells share similar properties such as late replication and enrichment for histone macroH2A1.2, suggesting a common mechanism of inactivation. This possibility is supported by the fact that *Xist* RNA that mediates somatic X-inactivation is expressed in the testis of male mice and humans. In the present study we show that both *Xist* RNA and *Tsix* RNA, an antisense RNA that controls *Xist* function in the soma, are expressed in the testis in a germ-cell-dependent manner. However, our finding that MSCI and sex-body formation are unaltered in mice with targeted mutations of *Xist* that prevent somatic X inactivation suggests that somatic X-inactivation and MSCI occur by fundamentally different mechanisms.

Key words: *Xist/Tsix*, Germline expression, *Xist* mutants, MacroH2A1.2, Meiotic sex chromosome inactivation, Sex body, Spermatogenesis

Introduction

Meiotic sex chromosome inactivation (MSCI) is the process by which the X and Y chromosomes of primary spermatocytes are transcriptionally inactivated; the associated formation of a heteropyknotic XY- or sex body has been viewed as the morphological manifestation of this inactivation process (Solari, 1974; McKee and Handel, 1993). Little is known of the molecular basis or biological role of MSCI. Lifschytz and Lindsley suggested that certain sex-linked genes may be inhibitory to spermatogenesis and that MSCI might serve to silence such genes (Lifschytz and Lindsley, 1972). Others have viewed MSCI as an unfortunate consequence of a need to mask the non-synapsed regions of the sex chromosomes, either in order to avoid the checkpoint that causes germ cell death in response to defective chromosome synapsis (Jablonka and Lamb, 1988; Odorisio et al., 1998), to prevent the initiation of recombination events in the non-synapsed regions of the sex chromosomes (McKee and Handel, 1993), or to ensure efficient sex chromosome synapsis (Turner et al., 2000).

It has been suggested that MSCI may have the same molecular basis as the somatic X-inactivation process that ensures X-linked dosage compensation between male (XY) and female (XX) mammals. Somatic X-inactivation requires the cis-acting *Xist* RNA, that coats one of the two X-chromosomes in each female cell (Penny et al., 1996; Marahrens et al., 1997; Brockdorff, 1998; Jaenisch et al., 1998; Lyon, 1999). The choice of which X chromosome to silence is mediated in-cis by the antisense *Tsix* RNA (Lee et al., 1999; Lee and Lu, 1999; Luikenhuis et al., 2001). Recent mutation analysis has demonstrated that the domain responsible for X-silencing resides at the 5′-end of the *Xist* RNA, while localisation of *Xist* RNA to the X chromosome is mediated by functionally redundant sequences dispersed throughout the rest of the gene (Wutz et al., 2002). In the male, *Xist* expression is restricted to the testis, the site at which MSCI takes place (Richler et al., 1992; Salido et al., 1992; McCarrey and Dilworth, 1992). Furthermore, it has been reported that *Xist* transcripts coat the X and Y chromosomal axes in the sex body of pachytene spermatocytes (Ayoub et al., 1997). The inactive somatic X chromosome, and the inactive X and Y chromosomes of spermatocytes, also share other features such as late replication (Priest et al., 1967; Kofman-Alfaro and Chandley, 1970; Odartchenko and Pavillard, 1970) and enrichment for the H2A variant macroH2A1.2 (Costanzi and Pehrson, 1998; Hoyer-Fender et al., 2000; Richler et al., 2000). Ayoub et al. have proposed that MSCI is brought about by *Xist* RNA-mediated spreading of inactivation from the X chromosome to the Y chromosome, via the region of X-Y pairing (Ayoub et al., 1997).

Despite the circumstantial evidence in favour of a common

mechanism, a striking argument against a requirement for *Xist* function in spermatogenesis comes from the demonstration that fertility is not impaired in males with a targeted *Xist* mutation, although this mutation disrupts somatic X-inactivation (Marahrens et al., 1997). This suggests either that MSCI is *Xist*-independent, or that MSCI is *Xist*-dependent but is not required for efficient spermatogenesis. Here, we differentiate between these two alternatives by examining sex body formation and MSCI in male mice with two different *Xist* disruptions (Marahrens et al., 1997; Csankovszki et al., 1999). We also examine the expression profile of *Xist* and *Tsix* during normal spermatogenesis.

Materials and Methods

Mice

The targeted *Xist* mutations, here termed $Xist^{trun}$ and $Xist^{1lox}$ (Fig. 1) were those described by Marahrens et al. (Marahrens et al., 1997) and Csankovszki et al. (Csankovszki et al., 1999), respectively. Both mutations abolish somatic X-inactivation. $Xist^{trun}$ has a 15 kb deletion encompassing most of exon 1 and all of exons 2-5, while leaving both exon 6 and the somatic P_1 promoter intact and gives rise to a truncated transcript (Marahrens et al., 1997). $Xist^{1lox}$ has a deletion that spans 5 kb upstream of P_1 to intron 3 (Csankovszki et al., 1999). This deletion includes the domain shown by Wutz et al. (Wutz et al., 2002) to be essential for X chromosome inactivation. Due to loss of P_1, *Xist* transcription is abolished in embryonic fibroblasts harbouring the $Xist^{1lox}$ deletion (Csankovszki et al., 1999). However, we found by strand-specific RTPCR that *Xist* transcription in testes of normal males utilises (at least in part) a start site upstream of P_1; a truncated transcript is therefore also generated in $Xist^{1lox}$ testis (see supplementary figure, http://jcs.biologists.org/supplemental). Five $Xist^{trun}$ and 3 $Xist^{1lox}$ males were used for analysis of MSCI and sex body formation. All males were fertile with testis weights in the normal range (93-164 mg). A testis from one male of each genotype was processed for histology and found to be normal (data not shown).

RNA extraction and RTPCR

Total RNA was extracted using Trizol® (Gibco BRL) exactly according to manufacturer's instructions. Where DNAse treatment was required, 1μg total RNA was incubated with 1.5 U RQ DNAse 1 (Promega) buffered with 5× First Strand cDNA Synthesis buffer (Gibco BRL) at 37°C for 60 minutes, with subsequent heat inactivation at 95°C for 5 minutes. For *Xist* and *Tsix*, strand-specific reverse transcription was carried out according to Lee et al. (Lee et al., 1999). Briefly, 3 pmol *Xist* primer (MIX20 – Kay et al., 1993) or *Tsix* primer (JT4 – 5′ CGA CCT ATT CCC TTT GAC GA 3′) was added to 0.2-2μg RNA. The resulting mixture was heated to 70°C for 5 minutes, and equilibrated to 50°C. First strand cDNA was synthesised with Superscript II reverse transcriptase (200 U, Gibco BRL) for 1 hour at 50°C. The enzyme was then heat inactivated at 90°C for 5 minutes. *Xist*/*Tsix* RTPCR conditions were 1 cycle of 95°C 2 minutes, 35 cycles of 96°C 0.5 minutes, 55°C 0.5 minutes, 72°C 1 minute and 1 cycle 72°C 10 minutes. For *Hprt* and *Dazla*, reverse transcription was primed with oligo-dT primers and RTPCR conditions were as described by Mahadevaiah et al. (Mahadevaiah et al., 1998). *Hprt* and *Dazla* primers were those described by Mahadevaiah et al. (Mahadevaiah et al., 1998) and Cooke et al. (Cooke et al., 1996), respectively. In all cases an 'H_2O + master mix' control was included in which the cDNA was omitted from the reaction. All these RTPCRs utilise primers from two exons so any amplification from contaminating DNA would give rise to a distinctly larger product.

Testis preparations and fluorescence immunostaining

Mouse spermatogenic cells were prepared as squash (Page et al., 1998) or surface spread preparations (Peters et al., 1997). Following fixation, slides were incubated in PBT (0.15% BSA, 0.1% Tween-20 in PBS) for 60 minutes prior to incubation overnight at 37°C with primary antibodies diluted in PBT. Mouse anti-COR1 (that recognises mouse SYCP3, Dobson et al., 1994) was used at 1:10. Rabbit anti-SYCP3 (Lammers et al., 1994) was used at 1:1000. Rabbit anti-macroH2A1.2 (Costanzi and Pehrson, 1998) was used at 1:100. Mouse anti-XY77 (Kralewski et al., 1997) was used undiluted. Mouse anti-XLR (Calenda et al., 1994) was used at 1:500. Rabbit anti-POLII [C-21, Santa Cruz (Turner et al., 2000)] was used at 1:100. Rat anti-M31 (Turner et al., 2001) was used at 1:500. The rabbit anti-RBMY was a polyclonal antibody raised as follows: amino acids 113-232 of mouse RBMY fused with GST or thioredoxin were expressed and purified from E. coli BL21 cells as described previously (Elliott et al., 2000). A rabbit was immunised with the GST fusion protein, and the resulting antiserum affinity purified using the thioredoxin fusion protein immobilised on a Sulfolink affinity column (Pierce). The antibody recognised a single testis-specific band of the appropriate molecular mass that was preabsorbable with the immunising protein (data not shown). Furthermore, as with a previously reported RBMY antibody (Mahadevaiah et al., 1998), staining of spermatogonia was seen in normal males but was abolished in XY^{d1}*Sry* mice in which most copies of *Rbmy* are deleted (J. M. A. Turner, An investigation into the role of sex chromosome synapsis in meiotic sex chromosome inactivation and fertility, PhD thesis, University College London, 2000). This RBMY antibody was used at 1:100. Slides were washed three times for 5 minutes in PBS, followed by application of secondary antibodies. Secondary antibodies used were 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) and goat anti-rat Alexa 488 (Molecular Probes). All secondaries were used at 1:500 in PBS for 60 minutes at room temperature. Slides were then washed in PBS as described above and were placed in a dark chamber for 10 minutes. Slides were mounted with Vectashield with DAPI (Vector). Controls consisted of omission of primary antibodies and replacement of primary antibodies with preimmune sera. Immunostained cells were examined and digitally imaged on an Olympus IX70 inverted microscope with a 100 W mercury arc lamp,

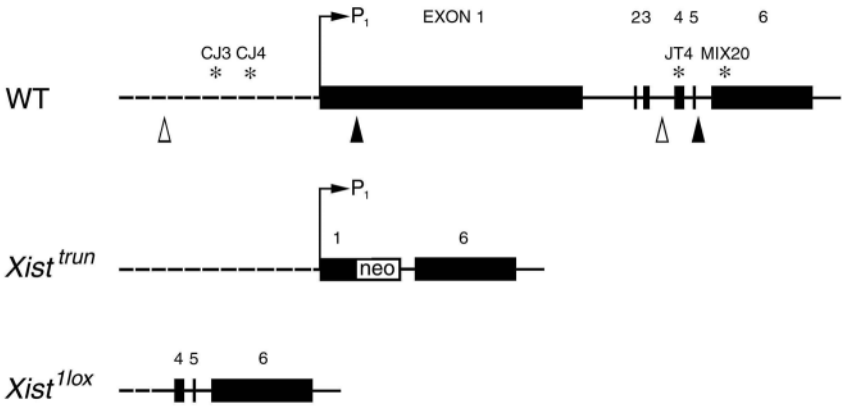

Fig. 1. Schematic of the *Xist* disruptions and primer pairs used in the present study. Closed arrowheads indicate the interval deleted in $Xist^{trun}$ males and open arrowheads indicate that deleted in $Xist^{1lox}$ males. Asterisks denote primer positions (see Materials and Methods).

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

Xist/Tsix expression in normal spermatogenesis

We began our study by analysing the expression profile of *Xist* and *Tsix* during normal mouse spermatogenesis using a primer pair that spans exons 4 to 6 of the *Xist* locus (JT4/MIX20). *Xist*^{trun} testes that are deleted for these exons, and wild type male undifferentiated ES cells that express both *Xist* and *Tsix* (Lee et al., 1999), were used as negative and positive controls, respectively. We detected both *Xist* and *Tsix* transcripts in testis but not in male somatic tissues such as liver (Fig. 2a). Consistent with a previous report (Richler et al., 1992), we repeatedly found a low level of *Xist* expression in the male heart (Fig. 2a).

Having verified that *Xist* was expressed in the testis, we then wished to establish the time course of its expression and whether expression was germ cell-dependent, as would be expected if it were involved in MSCI. *Xist* and *Tsix* transcripts were detected at all ages from 11.5 days post coitum (dpc) up to and including adult (Fig. 2b). Notably, *Tsix* transcript levels were always considerably lower than those of *Xist*, although we cannot discount the possibility that this reflects differences in the efficiency of JT4 and MIX20 as primers for strand-specific reverse transcription.

To assess whether transcription of *Xist* and *Tsix* was germ cell-dependent, we analysed *Xist* and *Tsix* expression in embryonic *W*^{e}*/W*^{e} testis and testis from adult XO males transgenic for the testis determinant *Sry*, both of which are deficient in germ cells (Mahadevaiah et al., 1998; Mazeyrat et al., 2001). The degree of germ cell deficiency was monitored by carrying out RTPCR for *Dazla*, an autosomally encoded germ cell-specific gene (Cooke et al., 1996), and *Dazla*-positive samples were excluded. Neither *Xist* nor *Tsix* transcripts were detectable in *Dazla*-negative *W*^{e}*/W*^{e} and XO*Sry* testes (Fig. 2c). Collectively, these results indicate that both *Xist* and *Tsix* are expressed in the testis, that expression of both genes begins from a very early stage of testis development and that expression of both genes is germ cell-dependent.

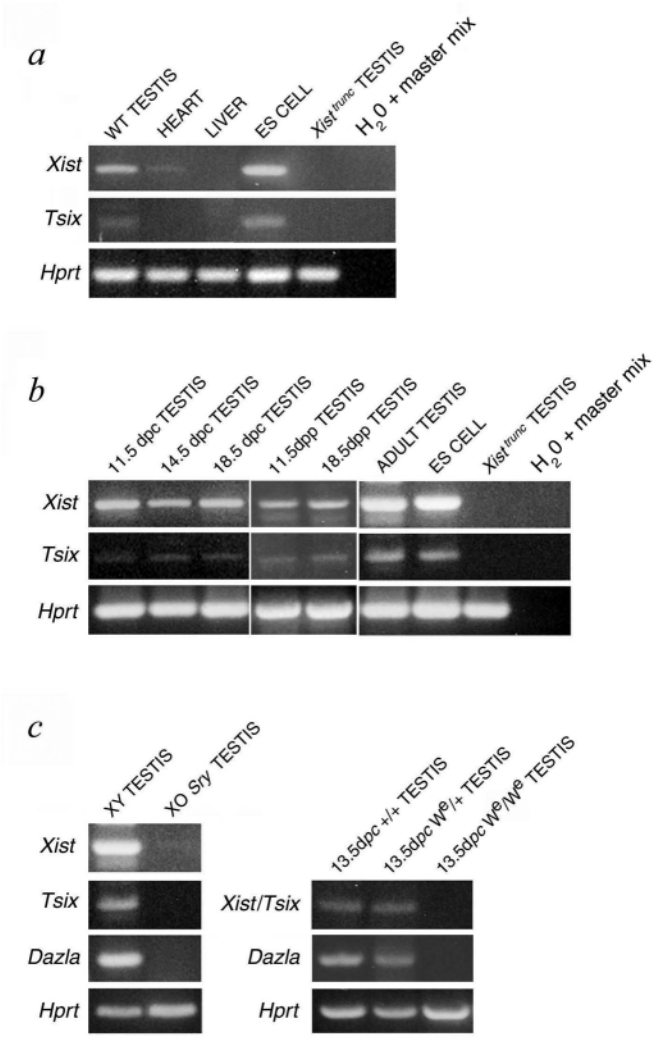

Fig. 2. *Xist* and *Tsix* expression during spermatogenesis analysed by strand-specific RTPCR using primer pair JT4/MIX20 (product 230 bp). ES cells serve as *Xist* and *Tsix* positive controls, and testes from *Xist*^{trun} males serve as negative controls. *Hprt* serves as RTPCR control. (a) In the male mouse, *Xist* is expressed in the testis and at very low levels in the heart, while *Tsix* is detected only in the testis. (b) Expression of both *Xist* and *Tsix* is detectable from 11.5 dpc. (c) Germ cell-dependency of *Xist* and *Tsix*, with germ cells detected by *Dazla* RTPCR. Wild-type adult testis, 13.5 dpc wild type and *W*^{e}/+ heterozygous testis all express *Xist* and *Tsix*, while neither are detectable in adult XO*Sry* and embryonic *W*^{e}*/ W*^{e} testes that lack germ cells as evidenced by the absence of *Dazla* transcripts.

Sex body formation in *Xist*^{trun} and *Xist*^{1lox} spermatocytes

According to Solari (Solari, 1974), sex body formation in the mouse begins during zygotene; at the light microscope level a morphologically distinct sex body is apparent by mid-pachytene (Turner et al., 2000). During diplotene, the sex chromosomes migrate towards the centre of the nucleus and, by metaphase I, they exhibit a similar condensation state to that of the autosomes. We wished to ascertain if sex bodies were formed in *Xist*^{trun} and *Xist*^{1lox} spermatocytes and, if they were, whether the timing of their formation and disassembly resembled that of controls. To do this, we performed marker analysis using immunostaining for proteins known to associate preferentially or specifically with sex chromosomes during different stages of meiosis. In each case, spermatocytes were substaged by immunostaining for the axial element protein SYCP3 (Lammers et al., 1994).

In order to assess the full time course of sex body formation and loss we first used immunostaining for the phosphorylated form of histone H2AX, termed γ-H2AX (Rogakou et al., 1999). γ-H2AX marks the chromatin of the X and Y-chromosomes as they first condense in late zygotene/early pachytene and continues to do so until the sex body is lost during diakinesis/metaphase I (Mahadevaiah et al., 2001). In *Xist*^{trun} (*n*=142) and *Xist*^{1lox} (*n*=167) spermatocytes just as in normal spermatocytes (*n*=107), γ-H2AX staining appeared on the sex

chromosomes in late zygotene and persisted throughout pachytene, before being lost at metaphase I (Fig. 3).

As a more specific sex body marker, we used immunostaining for the XY77 protein, which associates with the chromatin of the sex chromosomes of late pachytene spermatocytes (Kralewski et al., 1997). This protein does not localise to meiotic sex chromosomes in circumstances when they are not inactivated, such as in XY female oocytes (Turner et al., 2000). This immunostaining requires squash preparations in which the sex body is less clearly demarcated, the sex body can nonetheless be identified by DAPI staining. Sex bodies identified by this criterion in *Xist*^{trun} (*n*=75) and *Xist*^{1lox} (*n*=80) spermatocytes stained positively for XY77, just as in normal males (*n*=81, Fig. 4).

Next we analysed the sex body status of diplotene *Xist*^{trun} and *Xist*^{1lox} spermatocytes using immunostaining for M31. M31 is a mammalian HP1-like protein that has been implicated in transcriptional repression, transgene silencing and MSCI (Jones et al., 2000; Motzkus et al., 1999). In testis sections, M31 has been shown to localise to the sex body during late prophase (Motzkus et al., 1999), specifically during diplotene (Turner et al., 2001). Once again, we detected sex bodies by DAPI staining in wild type (*n*=42), *Xist*^{trun} (*n*=45) and *Xist*^{1lox} (*n*=40) spermatocytes and in each case these stained positively for M31 (Fig. 4).

Finally, we analysed the sex bodies of *Xist* mutant males by immunostaining for the core histone macroH2A1.2. MacroH2A1.2 is concentrated on the somatic inactive X chromosome (Costanzi and Pehrson, 1998; Mermoud et al., 1999; Rasmussen et al., 2000) in an *Xist*-dependent manner (Csankovszki et al., 1999) and is also enriched on the chromatin of the sex chromosomes of early pachytene spermatocytes (Hoyer-Fender et al., 2000; Richler et al., 2000). Consistent with the section data of Hoyer-Fender et al. (Hoyer-Fender et al., 2000), we detected macroH2A1.2 in the sex body of early pachytene spermatocytes from normal males (*n*=56) and this localisation was maintained in *Xist*^{trun} (*n*=62) and *Xist*^{1lox} spermatocytes (*n*=82, Fig. 5).

Together, these experiments show that sex body formation proceeds normally in spermatocytes harbouring *Xist* disruptions.

MSCI in *Xist*^{trun} and *Xist*^{1lox} spermatocytes

Transcriptional inactivation of the sex chromosomes during meiosis can be readily demonstrated by visualising the distribution of transcriptional and pre-mRNA splicing factors in the autosomal versus the sex chromosome domains (Richler et al., 1994). For example, RNA polymerase II (RNA POLII) is present at high concentration throughout the autosomal chromatin but is virtually absent in the sex body during pachytene (Richler et al., 1994; Turner et al., 2000). In contrast, RNA POLII uniformly coats all chromosomes in XX and XY female oocytes, in which no sex bodies are formed (Turner et al., 2000). We therefore examined RNA POLII staining of the sex chromosomes in *Xist*^{trun} and *Xist*^{1lox} spermatocytes (Fig. 6). During wild type spermatogenesis, RNA POLII levels were found to be highest during late pachytene/early diplotene and it was at this stage that exclusion of RNA POLII from the sex body could be most easily visualised (data not shown). Analysis of *Xist*^{trun} (*n*=45) and *Xist*^{1lox} (*n*=49) late pachytene/early diplotene surface spread spermatocytes (identified by anti-SYCP3 staining) revealed marked exclusion or absence of RNA POLII from the sex chromosome domain in all cells analysed (Fig. 6). We conclude that global transcriptional inactivation of the sex chromosomes is achieved in *Xist*^{trun} and *Xist*^{1lox} spermatocytes.

In a previous study, Ayoub et al. used an in-situ RTPCR technique to demonstrate that *Xist* was present in the sex body during pachytene where it coated the axes of both the X and Y

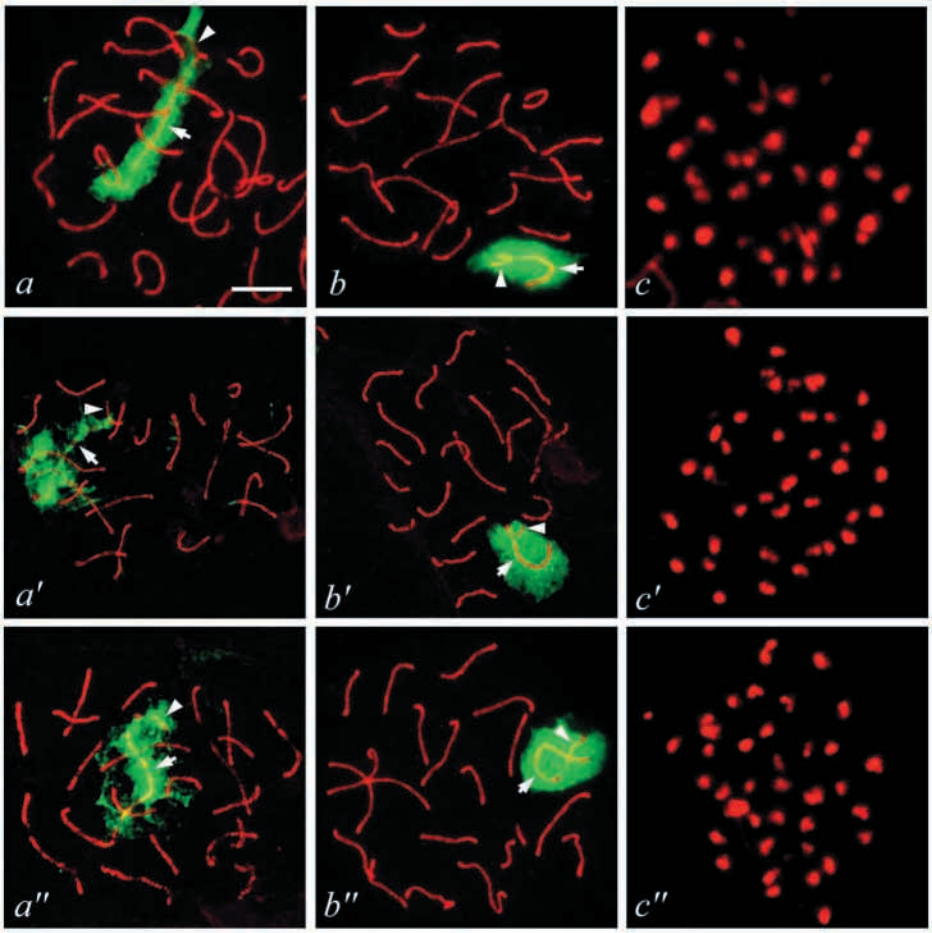

Fig. 3. Analysis of sex body formation and disappearance in wild type (a-c), *Xist*^{trun} (a′-c′) and *Xist*^{1lox} (a″-c″) spermatocytes using the sex body marker γ-H2AX. (γ-H2AX, green; SYCP3, red; X chromosome, short arrow; Y chromosome, arrowhead). γ-H2AX labels the sex chromosomes prior to their incorporation into the sex body (a,a′,a″) and then associates with the sex body throughout pachytene (b,b′,b″). It finally disappears as sex bodies are disassembled, at metaphase I (c,c′,c″). Bar, 10 μm.

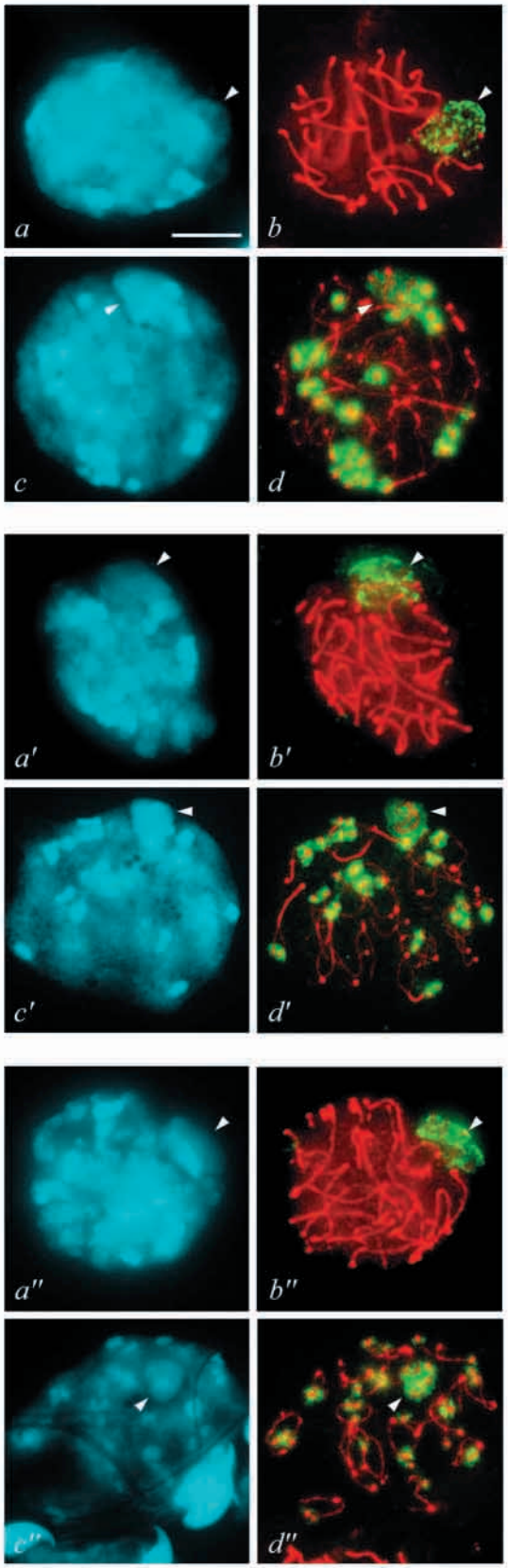

Fig. 4. Analysis of sex bodies in wild type (a-d), *Xist*^{trun} (a′-d′) and *Xist*^{1lox} (a″-d″) late pachytene and diplotene spermatocytes by DAPI staining and immunostaining for XY77 and M31 (DAPI, blue; SYCP3, red; green is XY77 in b,b′,b″ and M31 in d,d′,d″; sex body, arrowhead). In late pachytene wild type, *Xist*^{trun} and *Xist*^{1lox} spermatocytes the sex body protrudes from the edge of the nucleus (a,a′,a″) and stains positively for XY77 (b,b′,b″). Later, during diplotene, in wild type, *Xist*^{trun} and *Xist*^{1lox} spermatocytes the sex body becomes more densely staining for DAPI (c,c′,c″) and in all three genotypes is M31-positive (d,d′,d′). Bar, 5 μm.

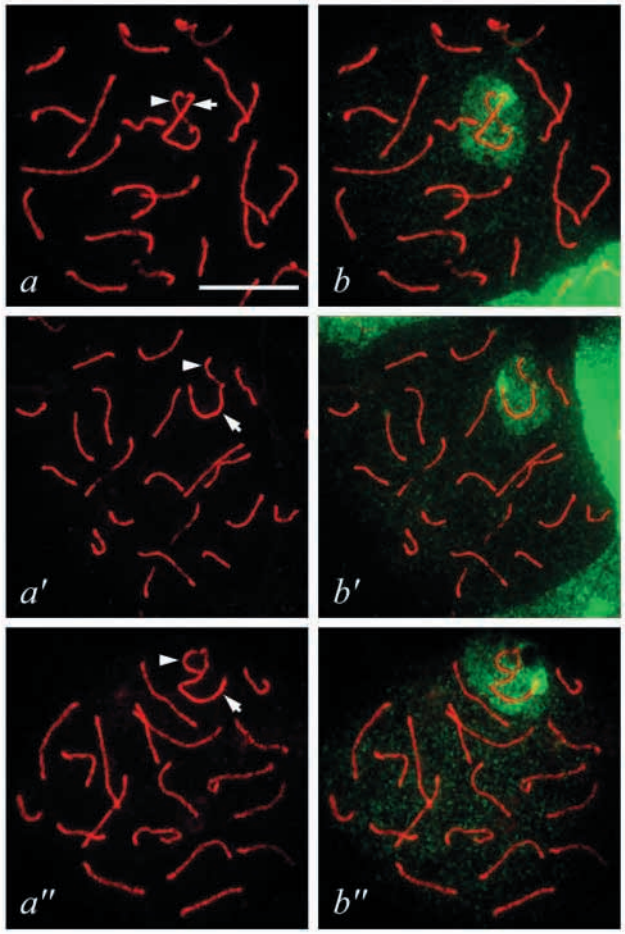

Fig. 5. Analysis of macroH2A1.2 localisation in wild type (a,b), *Xist*^{trun} (a′,b′) and *Xist*^{1lox} (a″,b″) pachytene spermatocytes. (MacroH2A1.2, green; SYCP3, red; X chromosome, short arrow; Y chromosome, arrowhead). MacroH2A1.2 is enriched in the chromatin of the sex chromosomes during early pachytene in all three genotypes. Bar, 10 μm.

chromosome (Ayoub et al., 1997). They hypothesised that inactivation of the Y chromosome was achieved by '*quasi*-cis' spreading of *Xist* transcripts from the X chromosome to the Y chromosome via the X-Y pairing (pseudoautosomal) region. In this model, Y-linked genes would fail to undergo MSCI in *Xist*-disrupted spermatocytes. To test this hypothesis, we examined expression of the multiple-copy Y-linked gene *Rbmy* during *Xist*^{trun} and *Xist*^{1lox} spermatogenesis. In normal males, *Rbmy* is strongly expressed in spermatogonia, is transcriptionally repressed during meiosis and is reactivated post-meiotically, suggesting that it is subject to MSCI (Mahadevaiah et al., 1998). This is supported by our finding that inappropriate

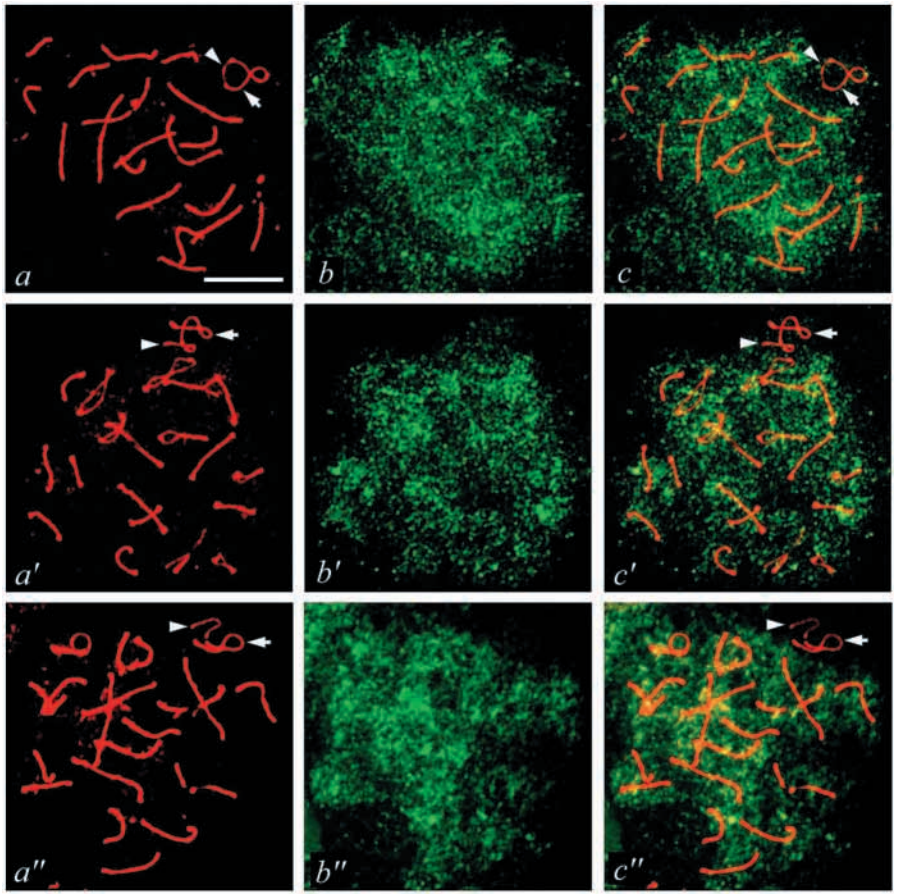

Fig. 6. Analysis of MSCI by exclusion of RNA POLII from the sex body in wild type (a-c), *Xist*^{trun} (a′-c′) and *Xist*^{1lox} (a″-c″) late pachytene/early diplotene spermatocytes (RNA POLII, green; SYCP3, red; X chromosome, short arrow; Y chromosome, arrowhead). RNA POLII is excluded from the sex body in all three genotypes. Bar, 10 μm.

expression of RBMY occurs in pachytene cells of XYY males, in which there is evidence of disrupted sex body formation (J. M. A. Turner, PhD thesis, University College London, 2000). RBMY staining was performed on squash preparations in which spermatocytes were substaged on the basis of immunostaining for the spermatocyte-specific XMR protein (Calenda et al., 1994). The pattern of RBMY staining in *Xist*^{trun} and *Xist*^{1lox} spermatocytes was indistinguishable from controls (Fig. 7). Taken together, these results suggest that MSCI proceeds normally in males with targeted *Xist*-disruptions.

Discussion

Almost ten years have elapsed since the first studies indicating a role for *Xist* in MSCI were published (Richler et al., 1992;

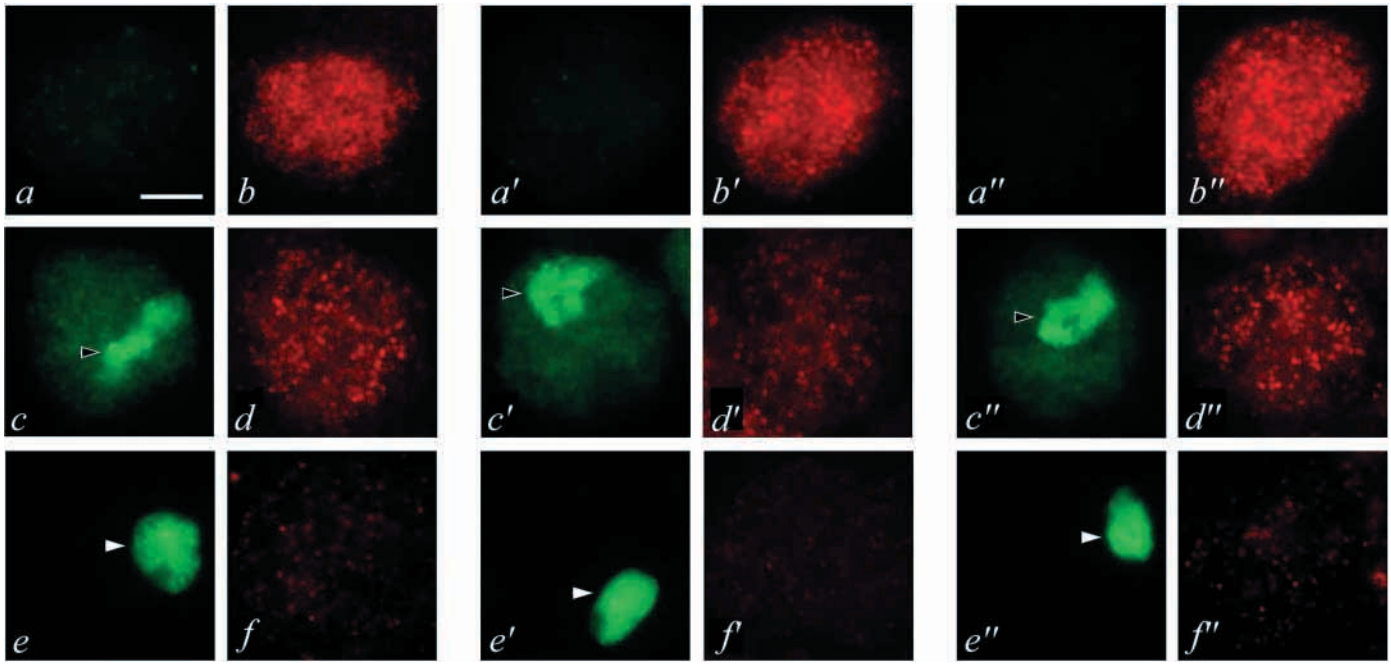

Fig. 7. Analysis of MSCI by *Rbmy*-inactivation in wild type (a-f), *Xist*^{trun} (a′-f′) and *Xist*^{1lox} (a″-f″) spermatogenic cells (RBMY, red; XMR, green; forming sex body, black arrowhead, mature sex body, white arrowhead). Spermatogonia, identified by their lack of staining for XMR (a,a′,a″) abundantly express RBMY (b,b′,b″). Late zygotene spermatocytes, identified by their low level nuclear XMR staining with brighter superimposed sex chromosome XMR staining (c,c′,c″), express RBMY at lower levels (d,d′,d″). Pachytene spermatocytes, identified by their mature XMR-positive sex bodies and absent whole nuclear XMR staining (e,e′,e″) contain undetectable levels of RBMY (f,f′,f″) in all three genotypes. Bar, 5 μm.

Salido et al., 1992; McCarrey and Dilworth, 1992). Since then, a number of reports have appeared that either support or contest this claim (Kay et al., 1993; Armstrong et al., 1994; Norris et al., 1994; Rastan, 1994; Hendriksen et al., 1995; Ayoub et al., 1997; Richler et al., 2000). We have addressed the role of *Xist* in MSCI by recording the expression pattern of *Xist* and *Tsix* in normal spermatogenesis and by examining sex body formation and MSCI in males with two different *Xist* disruptions that abolish somatic X-inactivation (Marahrens et al., 1997; Csankovszki et al., 1999).

Were *Xist* to mediate MSCI, then we would expect *Xist* expression to begin in the germ cells around the time that MSCI commences. Our data, which show that *Xist* transcription is germ cell-dependent, are consistent with those of Salido et al. (Salido et al., 1992) but not with those of Kay et al. (Kay et al., 1993), who detected *Xist* transcripts in germ cell deficient W^v/W^v testes (Lyon and Searle, 1989). However, W^vW^v testes invariably have some germ cells remaining, and even with the more penetrant W^e allele we have encountered some *Dazla*-positive (germ cells still present), *Xist*-positive testes. There also appears to be a discrepancy between different reports regarding the timing of *Xist* expression. Both the present study and that of Jamieson et al. (Jamieson et al., 1997) detected *Xist* transcripts from as early as 11.5 dpc. This clearly does not fit well with the timing of MSCI, which has been estimated by quantitative RTPCR for selected X-linked genes to commence no earlier than the spermatogonial A and B stages, i.e. around 6-8 dpp (Singer-Sam et al., 1990; McCarrey et al., 1992). In contrast to our results, McCarrey and Dilworth (McCarrey and Dilworth, 1992) did not detect *Xist* transcripts until after birth, around the time that primitive type A spermatogonia appear, which more closely corresponds to the timing of onset of MSCI. However, their RNA samples came from purified germ cell populations, and it is possible that the low levels of *Xist* transcripts are depleted during the purification procedure.

A second prediction of the hypothesis that MSCI is *Xist*-dependent is that disruption of *Xist* function should abolish MSCI and sex body formation. We have not found this to be the case. We encountered no difference in the timing of sex body formation, in the protein content of sex bodies as judged by marker analysis, or in MSCI between wild type, $Xist^{trun}$ and $Xist^{1lox}$ spermatocytes. Interestingly, the $Xist^{1lox}$ mutation disrupts localisation of macroH2A1.2 to the somatic inactive X-chromosome (Csankovszki et al., 1999) but not to the sex body. A possible explanation for this is that the transcript produced in male spermatogenic cells (see supplementary figure) but not female somatic cells (Csankovszki et al., 1999) carrying this mutation, retains the 3′ region of the transcript necessary for macroH2A1.2 recruitment (Wutz et al., 2002). The role of macroH2A1.2 in somatic X-inactivation remains undetermined, but the timing of its appearance suggests that macroH2A1.2 functions downstream of the initiation of X-inactivation (Mermoud et al., 1999; Rasmussen et al., 2000). In the male macroH2A1.2 similarly appears after the initiation of MSCI. Perhaps in both cases, macroH2A1.2 plays a role in initiating heterochromatinisation. However, Perche et al. (Perche et al., 2000) have suggested that macroH2A1.2 localisation to the Barr body is merely a reflection of increased nucleosomal density resulting from heterochromatinisation (see also Rasmussen et al., 2001; Constanzi and Pehrson, 2001). During spermatogenesis it is our impression that macroH2A1.2 localisation to X and Y chromatin immediately precedes condensation and heterochromatinisation but this needs to be confirmed with a careful time course for these processes.

Since the males with both *Xist* disruptions studied here produce truncated *Xist* transcripts, it could be that the truncated transcripts are functioning to bring about MSCI. However, this seems unlikely in the light of the recent mapping of functional domains within the *Xist* transcript by Wutz et al. (Wutz et al., 2002). The 5′ repeat domain shown to be required for transcriptional silencing is deleted in the $Xist^{1lox}$ males and in both mutations the sequences required for localisation of the transcripts to the X chromosome in somatic cells, are sufficiently depleted to prevent *Xist* function in females. If these truncated transcripts are functioning in MSCI, it must be by a very different mechanism than that involved in somatic X-inactivation.

If MSCI is indeed completely *Xist*-independent, the question of why *Xist* and *Tsix* are expressed in the testis deserves consideration. One explanation is that *Xist* and *Tsix* may be transcribed illegitimately as a result of the programmed demethylation of the *Xist* locus that occurs in the male germline (Norris et al., 1994). This demethylation event has been proposed to underlie the imprinted paternal *Xist* expression that occurs in the trophectoderm and primitive endoderm during early female embryogenesis (Kay et al., 1993; Norris et al., 1994; Rastan, 1994). Both Kay et al. (Kay et al., 1993) and McCarrey and Dilworth (McCarrey and Dilworth, 1992) have highlighted the fact that the expression level of *Xist* in the testis is considerably lower than that of female somatic cells. These observations, together with the inability to detect *Xist* transcripts by conventional in-situ analysis (Salido et al., 1992; Ayoub et al., 1997) throw doubt on the possible functional significance of such low-level *Xist* expression, and we have found the *Tsix* transcript levels to be even lower than those of *Xist.*

Our study highlights an important mechanistic difference between female somatic and male germline X-inactivation and poses the question of how MSCI is controlled. Progress in this direction is slow in coming and has to date relied on identification of proteins that preferentially associate with the sex body (Smith and Benavente, 1992; Calenda et al., 1994; Smith and Benavente, 1995; Kralewski et al., 1997; Bauer et al., 1998; Motzkus et al., 1999; Parraga and del Mazo, 2000; Hoyer-Fender et al., 2000; Richler et al., 2000; O'Carroll et al., 2000; Turner et al., 2000). Perhaps the most promising candidates are those with proven roles in transcriptional repression and/or heterochromatinisation, such as M31 (Motzkus et al., 1999), macroH2A1.2 (Hoyer-Fender et al., 2000; Richler et al., 2000) and Suv39h2 (O'Carroll et al., 2000). Conditional disruption within the male germ line of the genes encoding these and other sex body-associating proteins may provide insights into the mechanism, and even more importantly, the role of MSCI.

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