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# X-chromosome silencing in the germline of *C. elegans*

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## SUMMARY

**Germline maintenance in the nematode *C. elegans* requires global repressive mechanisms that involve chromatin organization. During meiosis, the X chromosome in both sexes exhibits a striking reduction of histone modifications that correlate with transcriptional activation when compared with the genome as a whole. The histone modification spectrum on the X chromosome corresponds with a lack of transcriptional competence, as measured by reporter transgene arrays. The X chromosome in XO males is structurally analogous to the sex body in mammals, contains a histone modification associated with heterochromatin in other species and is inactivated throughout meiosis. The synapsed X chromosomes in hermaphrodites also appear to be silenced in early meiosis, but genes on the X chromosome are detectably expressed at later stages of oocyte meiosis. Silencing of the sex chromosome during early meiosis is a conserved feature throughout the nematode phylum, and is not limited to hermaphroditic species.**

Key words: *C. elegans*, Germline, Silencing, X-inactivation, Histone modifications, Gametogenesis

## INTRODUCTION

In both *Drosophila* and *C. elegans*, global silencing mechanisms appear to play a crucial role in the specification and maintenance of germ line tissue (Wylie, 1999). In *C. elegans*, these silencing mechanisms can be studied using transgene arrays containing a GFP reporter gene under the control of a promoter normally expressed in all cells (Kelly et al., 1997). The transgene arrays are strongly silenced in germ cells but are reactivated in the soma of each generation. Silencing in the germline can be partially prevented by increasing the 'complexity' of the transgene arrays formed in vivo through the co-injection of excess amounts of random, linear genomic fragments (Kelly et al., 1997).

Germline silencing of transgene arrays requires the action of the MES proteins (maternal effect sterility), MES-2, -3, -4, and -6 (Kelly and Fire, 1998). Two of the mes genes, *mes-2* and *mes-6*, encode worm homologs of the *Drosophila* Polycomb Group proteins, Enhancer of Zeste and Extra Sex Combs, respectively (Holdeman et al., 1998; Korf et al., 1998). The Polycomb Group proteins maintain transcriptional repression of developmentally regulated genes through their ability to modulate chromatin conformation (Kennison, 1995). In addition, MES-2 and MES-4 each contain a SET domain, a conserved feature of many chromatin-interacting proteins. Defects in the *mes* factors cause sterility, owing to germ cell degeneration, and alleviate silencing of transgene arrays in the germline. Similar phenotypes can also result from depletion of a histone H1 isoform, H1.1 (Jedrusik and Schulze, 2001). Gene silencing through the regulation of chromatin conformation is therefore likely to be an essential component of germline maintenance, but the endogenous targets of this regulation are poorly understood. Severity of the germ cell degeneration in *mes* mutant animals increases with X-chromosome dose (Garvin et al., 1998), suggesting that some of the gene targets of MES-induced silencing reside on the X chromosome.

Global gene expression analysis has also suggested that the X chromosome is a possible target of silencing in the *C. elegans* germline. Microarray analyses have identified 1416 germline-enriched genes in *C. elegans*, which were classified into three distinct groups: sperm-enriched, oocyte-enriched and germline-intrinsic genes (defined as genes expressed similarly in the germline regardless of the gamete being made) (Reinke et al., 2000). Strikingly, sperm-enriched and germline-intrinsic genes are almost completely absent from the X chromosome. By contrast, oocyte-enriched genes are present on the X chromosome at a similar frequency to those found on autosomes. *C. elegans* XO males make only sperm and thus would not require expression of oocyte-enriched genes, whereas XX hermaphrodites first produce sperm as L4 larvae and then become strictly oogenic as adults (Schedl, 1997; Hubbard and Greenstein, 2000). The X chromosome in male

germlines may therefore be regulated differently from autosomes in a manner that prohibits the presence of the sperm-enriched and germline-intrinsic genes. Another possibility is that these classes of genes are absent from the X chromosome for some unknown reason, but that the X chromosome is otherwise competent for gene expression.

In support of the first possibility, the distinction of the male X chromosome from the autosomes in the germline of *C. elegans* is reminiscent of sex chromatin formation in other species that bear non-equivalent sex chromosomes (heterogametic: XO or XY). During the pachytene stage of *C. elegans* meiosis in males, the single (and thus unpaired) X chromosome adopts a highly compact morphology analogous to that seen in mammalian spermatocytes (Goldstein, 1982). In the heterogametic sex of diverse species, the male X chromosome in the pachytene stage of meiotic prophase is found in a visually distinct structure called the XY- or sex-body that is transcriptionally inactive (Handel and Hunt, 1992). McKee and Handel (McKee and Handel, 1993) proposed that the condensation of sex chromatin in XY and XO male germlines, and by consequence the transcriptional inactivation of these chromosomes, prevents harmful recombination events between non-equivalent X and Y chromosomes, and prevents loss of a single chromosome lacking a pairing partner in XO animals. In *C. elegans*, both the exclusion of sperm-enriched and germline-intrinsic genes from the X chromosome, and the condensed structure of the X chromosome in the XO male germline suggest that the X chromosome in the male germ line may be targeted for silencing.

If the male X chromosome is silenced in the germline, then one expectation is that it should have a chromatin conformation consistent with decreased transcriptional activity. Chromatin structure can be regulated via differential modification of nucleosomal histone N termini 'tails', which includes acetylation, methylation, phosphorylation and ubiquitination (Strahl and Allis, 2000; Turner, 2000). Combinations of these modifications are proposed to comprise a 'histone code' that determines regional structural properties of chromatin (Strahl and Allis, 2000). The acetylation of lysine residues in the N termini of histones H3 and H4, as well as methylation of lysine 4 in histone H3, generally correlate with a transcriptionally active state. By contrast, methylation of lysine 9 in histone H3 correlates with transcriptional silencing and constitutive heterochromatin formation, and is required for binding of the heterochromatin protein HP1 (Strahl and Allis, 2000; Jenuwein, 2001). Some proteins containing a SET domain are histone methyltransferases that can methylate lysine 9 in histone H3 (Jenuwein, 2001; Jenuwein and Allis, 2001). The general scheme of a 'histone code' has probably undergone specific adaptations in different organisms, but overall remains strongly conserved.

We have used probes specific for histone modifications to study the chromatin organization of the X chromosome in germ cells of *C. elegans* males and hermaphrodites. Both germline-silenced and germline-expressing transgene arrays were used to monitor how the histone modification patterns on these large, extrachromosomal arrays correlate with expression competence. The spectrum of histone modifications on transgene arrays illustrate a consistent correlation with the expression competence of the array in early meiotic germ cells. Moreover, we present evidence that, relative to autosomes, the histones on the X chromosome in male germ cells show a marked reduction in modifications that correlate with transcriptional activation and are enriched in a modification that is associated with heterochromatin.

Strikingly, the X chromosomes in oogenic hermaphrodite germ cells also appear silenced in early meiotic prophase as assessed by their histone modification pattern. Oocyte-enriched genes on the X chromosomes are, on average, expressed at levels significantly lower than oocyte-enriched genes on autosomes. Transcription of several X-linked oocyte genes was only detected in very late meiotic prophase I in the female germline of hermaphrodites.

We also demonstrate that three types of unpaired autosomal sequences are competent to express genes and display activating chromatin modifications: extrachromosomal transgene arrays containing interspersed genomic DNA, unpaired autosomal duplications and the autosomal portions of X:autosome translocations. Each has histone modification patterns more similar to autosomes than to the X chromosome throughout meiosis. These results show that pairing is probably not required for gene expression during meiosis, and suggest that chromatin on autosomes may be refractory to germline silencing. We also show that silencing of the X chromosome is a conserved feature in nematode species with divergent modes of reproduction, and thus does not appear to be a consequence of hermaphroditism.

## MATERIALS AND METHODS

### General methods and strains

The techniques used for animal maintenance and handling were the same as those described by Brenner (Brenner, 1974). Animals were grown at 16°C or 20°C, unless otherwise indicated. The transgenic strain PD7271 (ccEx7271) contains the *pha-1(e2123ts)* mutation and is carrying a highly repetitive array with >100 copies of the plasmid pBK48.1, and ~ 50 copies of a *pha-1* rescuing construct, pC1 (W. K., unpublished). The pBK48.1 plasmid carries a GFP-tagged version of the *let-858* gene, which is normally expressed in all tissues (Kelly et al., 1997). The transgenic strain KW1336 is *unc-4(e120)let-858(cc534)* Ex:pBK48.1cpx, which carries the pBK48.1 plasmid in an array generated by co-injection of excess genomic DNA fragments from *C. elegans* (Kelly et al., 1997). The strain used as wild type in this study is the *C. elegans*, variety Bristol, strain N2 (Brenner 1974). Other *C. elegans* strains used in this study are *mnT10* (X:V), *unc-30(e191) dpy-4(e1166)*(IV);sDp1(IV;f) and *dpy-17(e164) let-809(s2844) ncl-1(e1865) unc-32(e189)*(III); sDp3(III;f). The hermaphrodite (male/hermaphrodite) species used were: *Caenorhabditis briggsae* (AF16), *Oschieus sp*. (CEW1), *Pristionchus pacificus* (PS1843), and *Oscieus myriophila* (EM435). The gonochoristic (male/female) species used were: *Caenorhabditis remanei* (EM464), *Mesorhabditis longespicula* (DF5017) and *Caenorhabditis sp*. (CB516). All of the nematode species other than *C. elegans* and some of the *C. elegans* strains used in this study were provided by the *Caenorhabditis* Genetics Center.

### Antibodies

The following antibodies were used in this work at the indicated dilutions, and were obtained from the indicated sources: rabbit anti-acetylated histone H3 (acetyl-K9, -K14; 1:500), (Upstate Biotechnology); rabbit anti-histone H4 acetyl-K5 (1:400); rabbit anti-histone H4 acetyl-K8 (1:1000) (Serotec); rabbit anti-histone H4 acetyl-K12 (1:400) (Serotec); and rabbit anti-histone H4 acetyl-K16 (1:3000) (Serotec). The following antibodies were a kind gift of Dr

David Allis, University of Virginia, and were used at the indicated dilutions: rabbit anti-histone H3 phospho-S10 (1:2000), rabbit anti-histone H3 dimethyl-K4 (1:1000); rabbit anti-histone H3 dimethyl-K9 (1:500). A manuscript detailing the properties of the α-H3 dimethyl-K4 antibody has been submitted (Briggs et al., 2001). The rabbit anti-histone H1 antibody (1:100 column purified H1.4) was a generous gift from Dr Ekkehard Schulze (Jedrusik and Schulze, 2001), University of Gottigen, Germany; and the sheep anti-phosphoacetylated histone H3 (1:250) was a generous gift from Dr Louis Mahadevan, University of Oxford, UK (Clayton et al., 2000). The monoclonal H5 and H14 antibodies were obtained from Research Diagnostics. Secondary antibodies purchased from Molecular Probes were used at the indicated dilutions: fluorescein isothiocyante (FITC) donkey anti-sheep IgG (1:500), Alexafluor™; 594 goat anti-rabbit IgG (1:500), Alexafluor™; 488 goat anti-mouse IgG (1:500) and FITC goat anti-mouse IgM (1:500).

### Immunocytochemistry

Whole-mount fixation and antibody staining of worms was accomplished by either a paraformaldehyde fixation procedure (Howe et al., 2001) or a methanol/acetone fixation procedure previously described (Strome and Wood, 1983).

Transgene structures were identified in pachytene nuclei by either manual focusing through nuclei or by automated acquisition of z-series images (0.3 μm optical sections) through individual nuclei (Volume Scan (Vaytek) and Image-Pro Plus (Media Cybernetics)). The transgene arrays were distinguished from chromosomal structures by their ball-shaped appearance: focusing through the specimen was required to distinguish the transgene from chromosome ends in each focal plane.

Specimens were observed and images were recorded using either a DeltaVision® system, or a Leica DMRA microscope outfitted with a Cooke Sensicam®. Post-acquisition processing of the images collected using the Leica microscope was accomplished using VayTek.'s MicroTome deconvolution software.

### Microarray analysis

The raw expression values for the 258 oocyte-enriched genes (Reinke et al., 2000) were selected from four replicate microarray hybridization of staged wild-type adult mRNA. For each replicate, the gene expression of all 258 genes was averaged, and then the expression level for each individual gene was normalized to that average (expression of specific gene/average expression). This normalized value is referred to as 'average gene expression' (age) units. The age value for each gene was averaged across all four replicates, and then the genes were separated into groups by chromosome. The mean age value and standard deviation of all oocyte-enriched genes on each chromosome was then calculated. A similar analysis was performed on the 480 somatic genes chosen from the same data set. To select a group of somatically expressed genes of similar expression values and of a similar size as the 258 oocyte-enriched genes, we chose those genes expressed significantly above background whose expression changed less than 1.5-fold between wild type/*glp-4* and *fem-1(lf)*/*fem-3(gf)* microarray hybridization, with $P>0.05$.

### Fluorescence in situ hybridization

For double-label experiments requiring both immunofluorescence and in situ hybridization, antibody staining was carried out before fluorescence in situ hybridization (FISH). Gonads were dissected from adult worms and fixed onto microscope slides in 1% paraformaldehyde in 1× egg buffer (27.5 mM Hepes pH 7.4, 130 mM NaCl, 53 mM KCl, 2.2 mM each $MgCl_2$ and $CaCl_2$) containing 0.05% Tween-20 for 5 minutes. The samples were frozen in liquid nitrogen, the coverslips were removed and slides were immediately transferred to methanol at –20°C. Samples were rehydrated and washed three times in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST). They were blocked in PBST with 0.5 mg/ml bovine serum albumin (BSA) and incubated in a 1:200 dilution of H4Ac12 antibody (Serotec; Raleigh, NC) in block overnight at 4°C. After three washes in PBST, the samples were incubated in a 1:200 dilution of FITC-labeled anti-rabbit IgG (Jackson ImmunoResearch) overnight at 4°C. For in situ hybridization, the samples were post-fixed in 5% paraformaldehyde in PBST, then washed in 2×SSCT (0.3 M NaCl, 0.03 M sodium citrate, 0.1% Tween-20). Probe labeling and FISH were performed as described previously (Dernburg and Sedat, 1998). An X chromosome probe was generated by DOP-PCR amplification and 3′-end-labeling of two YAC clones originating from each end of the X chromosome, kindly provided by the Sanger Center.

### mRNA in situ analyses

cDNAs for four X-linked oocyte enriched genes were amplified by nested RT-PCR. Total RNA (1 μg) from wild-type adult hermaphrodites was converted into first strand cDNA using the 3′-RACE primer (GCGGGATCCTCGAGAAGCTTTTTTTTTTTT) and Superscript II (Lifetech), extracted with phenol/chloroform, precipitated with ethanol and resuspended in 200 μl TE (10 mM Tris, pH 8.0, 1 mM EDTA). 2 μl of the first strand cDNA was used as a template for PCR with outer gene-specific primers (P1) and the 3′-anchor primer (GCGGGATCCTCGAGAAGCTT). The first PCR products were diluted 1:100 with TE (pH 8.0), and re-amplified with inner P2 and 3′-anchor primers. PCR products with the expected sizes were gel-purified and sequenced to confirm identity. Sequences of gene specific primers are listed below. Probes were synthesized using the gel-purified RT-PCR products as described (Seydoux and Fire, 1994). Gonad dissection from 1-day post-L4 adult hermaphrodites, fixation and hybridization were performed as described elsewhere (Kuwabara et al., 2000). Images were captured using a Zeiss Axioskop equipped with a SPOT (Diagnostic Instruments, Inc.) digital CCD and processed with Adobe Photoshop 5.5.

Primers used were as follows: K08A8.1_P1, GGCTCGGAGGACTTGGTGGTG; K08A8.1_P2, GGAGAACTCCGGATATCTCAC; F35C8.7_P1, GTAGTGGCTATTGCAACGTCG; F35C8.7_P2, GCGCTGATTTCCGAATCGAGC; F52D2.2_P1, GTCTATGGCCACCGTTGATCC; F52D2.2_P2, CCATTCCTCGGGAATCGAATG; R09F10.8_P1, GTCTTTATAGTCCCACTGGCG; R09F10.8_P2, GATCTCCGGTCAATTGCCAGC.

The 20 autosomal oocyte-enriched genes examined in the survey of the in situ hybridization screen were T22A3.5, T01G9.5, T01G9.4, B0511.7, T05F1.2 (Chr I), C27A2.6, C27D9.1, C09H10.6 (Chr II), C14B1.9, C16C10.3, F02A9.6, R10E4.4, C38D4.4 (Chr III), C46A5.9, F22B3.4 (Chr IV), C25D7.6, F09G2.8, F38E1.7, T06E6.2 and C29A12.3 (Chr V).

## RESULTS

### Silenced transgenes in germ cells lack histone modifications that correlate with transcriptional activation

*C. elegans* nuclei normally carry transgenic DNA in the form of large, multi-copy arrays that are unlinked to chromosomes (Stinchcomb et al., 1985) (Fig. 1F). Ubiquitously expressed reporters in these repetitive transgenic arrays are strongly silenced in germ cells. This silencing is alleviated by interspersing the reporter transgene with random genomic fragments ('complex' arrays) (Kelly et al., 1997). These repetitive and complex transgene arrays therefore represent large, visually distinctive DNA species that are transcriptionally active or inactive, respectively. Strain PD7271 carries a multi-copy extrachromosomal transgene array of a GFP-tagged ubiquitously expressed gene, *let-858* (Kelly et al.,

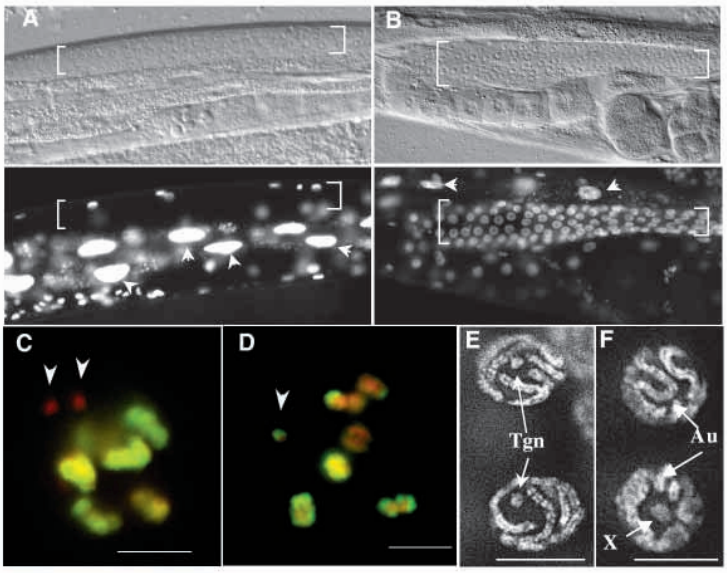


**Fig. 1.** Transgene expression, histone modification and morphology in germ cells. (A,B) Differential interference contrast (top panels) and GFP fluorescence (bottom panels) microscopy of hermaphrodite ovaries from transgenic line PD7271, which carries an extrachromosomal, multi-copy array of a *let-858:gfp* reporter transgene, pBK48.1 (A), and line KW1336, which carries an extrachromosomal 'complex' array with pBK48.1 (B). Brackets indicate germ cell nuclei in one hermaphrodite ovary arm. The arrowheads in the lower panels illustrate GFP fluorescence in intestinal cell nuclei. The lower panel in A represents a longer exposure than that shown in B to demonstrate a complete lack of detectable nuclear GFP expression in germ cells in this transgenic line (brackets; the few fluorescent nuclei within the brackets are from somatic components of the gonad). (C,D) Paraformaldehyde-fixed oocytes from PD7271 (C) and KW1336 (D) hermaphrodites were stained with α-H3 dimethyl-K4 antibody (green) and counterstained with DAPI (red), arrowheads indicate transgene arrays. (E,F) Paraformaldehyde-fixed pachytene stage nuclei from transgenic PD7271 hermaphrodites (E) and N2 males (F) were stained with DAPI and examined by deconvolution fluorescence microscopy. Au, autosomes; X, X chromosome; Tgn, transgene, Scale bars: 5 μm in C-F.

1997). Strong expression of this array is observed in most somatic lineages, but no expression is detected in germ cells at any stage (Fig. 1A). Strain KW1336 contains an extrachromosomal transgene array composed of the same *let-858:gfp* reporter interspersed with a large number of random *C. elegans* genomic fragments (Kelly et al., 1997). The degree of somatic expression of GFP in both strains is equivalent, but germ cell expression is observed only in KW1336 (Fig. 1B). The PD7271 transgene array thus represents chromatin that is silenced in meiotic germ cells, whereas the KW1336 transgene array contains chromatin that is competent for gene expression.

Nucleosomal histone modifications are known to correlate with transcriptional regulation. We used antibodies that recognize specific histone modifications as probes to assess whether global differences in chromatin structure could be identified in 'germ cell-active' (KW1336) and 'germ cell inactive' (PD7271) transgenes. Methylation of histone H3 on lysine 4 (H3 methyl-K4) is a conserved histone modification that correlates with transcriptional activity (Strahl et al., 1999). An antibody that recognizes H3 methyl-K4 was used to label oocyte nuclei, where the diakinetic chromosomes are spatially separate and the extrachromosomal transgene is easily observed. α-H3 dimethyl-K4 stained all six chromosome pairs in both strains, but little or no staining of the germ cell-inactive PD7271 transgene was observed (Fig. 1C). By contrast, α-H3 dimethyl-K4 labeling was readily detectable on the germ cell-active KW1336 transgene (Fig. 1D, arrowhead). In general, histone modifications that have been reported to correlate with transcriptional activation (hereafter referred to as 'activating modifications') were absent from the PD7271 transgene array but detected on the KW1336 transgene array (Table 1). This pattern of transgene histone modification was also observed in earlier stages of meiosis (below and data not shown). Antibodies detecting specific histone modifications are therefore useful probes for identifying chromatin regions that are transcriptionally competent in *C. elegans* germ cells, and

**Table 1. Summary of histone modifications in germ cells of *C. elegans***

| Histone modification | Autosomes (oocytes and sperm) | Male X | Hermaphrodite X | Autosomal duplications | Inactive transgene array | Active transgene array |
|---|---|---|---|---|---|---|
| **Histone H3** | | | | | | |
| Diacetyl (K9,K14) | M, TZ, P, Dp, Di | None | P+/–(?),Dp,Di | M,P, | None | N.D. |
| Phospho-Ser10 | M, TZ, P, Dp, Di | None | Di | N.D. | Di | N.D. |
| Phosphodiacetyl | M, TZ, P, Dp, Di | None | Di* | N.D. | Di | N.D. |
| Methyl K4 | M, TZ, P, Dp, Di | None | Dp, Di | M, TZ, P, Dp, Di | None | M, TZ, P, Dp, Di |
| Methyl K9 | Late P, early Dp | P | P, early Dp | N.D. | P | N.D. |
| **Histone H4** | | | | | | |
| Acetyl-K8 | M, TZ, P, Di, Di | None | Dp, Di | N.D. | None | N.D. |
| Acetyl-K12† | M, TZ, P, Di, Di | N.D. | Di | N.D. | N.D. | N.D. |
| Acetyl-K16 | M, TZ, P, Di, Di | None | Dp, Di | M, TZ, P, Dp, Di | None | M, TZ, P, Dp, Di |

Column 1 indicates the antibody used in each row. Autosomal duplications studied in Column 4 are sDp1 (IV;f) and sDp3 (III;f). Columns 6 and 7 indicate results from strains PD7271 and KW1336, respectively. Histone modifications that correlate with transcriptional activation are in blue, inactivating modifications are in red; the role of acetylation of H4 K12 is currently unclear (Strahl and Allis, 2000). 'None' indicates no significant staining was observed in any region of the gonad (a single unstained chromosome in pre-pachytene cells was presumed to be the unpaired X in male germ cells). M, mitotic region; TZ, transition zone; P, pachytene region; Dp, diplotene region; Di, diakinetic oocytes; N.D., not determined. Little consistent staining was observed with the α-H4 acetyl-K5 antibody which is not included in this table.

*The signal in diakinesis from these antibodies was enriched at or limited to the joined ends of homolog pairs.

†The H4 acetyl-K12 signal was detectable, but consistently lower than what was observed for other modifications.

can be used to characterize such regions in the endogenous chromosomes.

### The male X chromosome lacks histone modifications that correlate with transcriptional activity

Because the condensed nature of the X chromosome in males had been originally noted using electron microscopy (Goldstein, 1982), we wished to determine whether it was also distinguishable from autosomes by light microscopy. The synapsed autosomes exist as ribbons or cords that lie along the nuclear periphery, with little or no spatial overlap (Fig. 1E,F). We found that the male X chromosome is distinguishable from autosomes in DAPI-stained pachytene cells viewed by light microscopy, where it exists as a condensed structure localized to the nuclear periphery (Fig. 1F). This is similar to mammalian meiosis, in which the male X chromosome exists along with the Y chromosome in a condensed structure termed the XY- or sex-body that is thought to be transcriptionally silent (Handel and Hunt, 1992). To determine whether the condensed male X chromosome in *C. elegans* was transcriptionally silent, we used the antibodies described above to test whether the X chromosome in male pachytene cells contained activating histone modifications. Antibody staining of male germline nuclei revealed little or no detectable H3 dimethyl-K4 on the X chromosome in pachytene nuclei, or at any later stage of meiosis (Fig. 2; Table 1). Acetylated forms of histones H3 and H4 were also reduced or absent on the X chromosome in these cells (Table 1). The condensed nuclear structure in cells in the distal (proliferative) zone made it difficult to view individual chromosomes, but a single structure (presumably X) also appeared to be unstained in these earlier stages (not shown). A lack of activating modifications on X chromosome histones was thus observed in all regions of the male gonad (Fig. 2). By contrast, the autosomes exhibited extensive H3 methyl-K4 and other activating modifications distributed along their lengths (Fig. 2; Table 1). The lack of labeling was not due to a general inaccessibility of antibodies to histone epitopes on the X chromosome, as an antibody that recognizes histone H1 variants labeled all chromosomes, including the X chromosome and the transgene arrays (α-H1.4; data not shown). No histone modification assayed was detected in mature spermatids (Fig. 2F). Whether this absence represents a loss of histone modification during sperm chromatin condensation, or a replacement with sperm-specific chromatin proteins is not known.

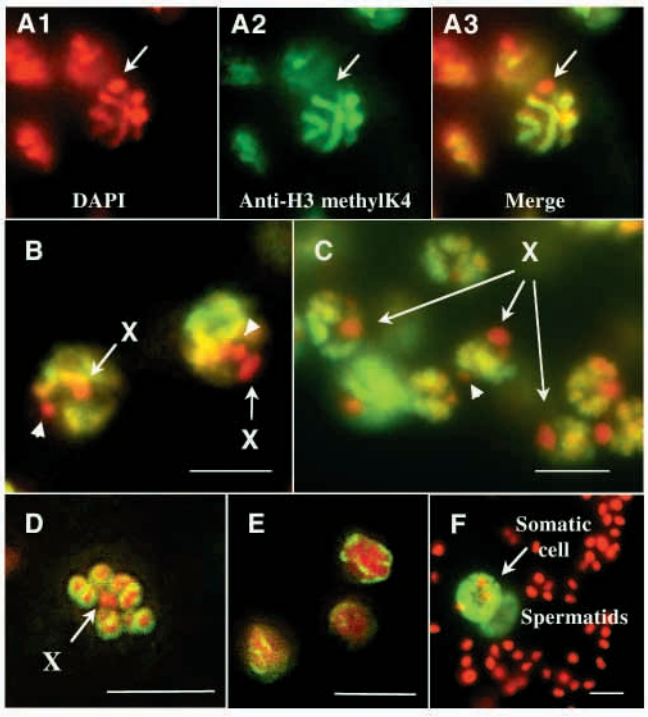


**Fig. 2.** Histone H3 lysine 4 methylation in male germ cells. Gonads from transgenic PD7271 males were fixed in paraformaldehyde and stained with the α-H3 dimethyl K4 antibody (green) and counterstained with DAPI (red). A1-A3 illustrate each signal separately and after merging; (B-F) the merged signals only. Arrows in all panels point to the X chromosome in each nucleus. (A) Pachytene stage; (B-D) progressive stages of primary spermatocytes; (E) condensing secondary spermatocytes; (F) spermatids. Arrowheads in B,C indicate transgenes. Transgenes were identified in all experiments by *z*-axis optical scanning through nuclei to rule out confusion with chromosome ends. Scale bars: 5 μm.

### The male X chromosome and a silenced transgene contain a histone H3 modification that correlates with heterochromatin

Methylation of histone H3 on lysine 9 (H3 methyl-K9) correlates with transcriptional silencing and the association of heterochromatin proteins with silenced DNA (Rea et al., 2000; Nakayama et al., 2001; Bannister et al., 2001; Lachner et al., 2001; Jenuwein, 2001). We used an antibody that specifically recognizes H3 methyl-K9 to test for this modification on the X chromosome and transgene array in males. In contrast to the activating modifications, H3 methyl-K9 was restricted to a single chromosome in male germ cells (Table 1; Fig. 3). This epitope became most pronounced in germ cells during pachytene, with little or no detectable staining before or after this stage (Fig. 3A).

In order to ascertain that the chromosome staining with the H3 methyl-K9 antibody was indeed the X chromosome, we co-stained with a second antibody, α-phosphoacetyl-H3, that recognizes an activating modification and does not detectably stain the male X chromosome in fixation conditions that preserve the male X chromosome structure (not shown). This antibody was raised in sheep, whereas the α-H3 dimethyl-K9 is from rabbit sera, allowing simultaneous staining with both reagents. The single chromosome that contained widespread H3 methyl-K9 also exhibited reduced phosphoacetyl-H3 modification, indicating that it is indeed the X chromosome (Fig. 3C,D).

Pachytene nuclei in male animals carrying the silenced PD7271 transgene array exhibited a second, smaller region containing H3 methyl-K9 (Fig. 3D, arrowheads). This second region was not observed in non-transgenic control animals (Fig. 3A-C), identifying this staining body as the transgene array. These results demonstrate that histone H3 on the male X chromosome and silenced transgene arrays has an elevated level of methylation on lysine 9 that is widely distributed in the DNA of both.

### Oocyte-enriched genes on the X chromosome have reduced expression relative to those on autosomes

Microarray analysis has previously shown that sperm-enriched

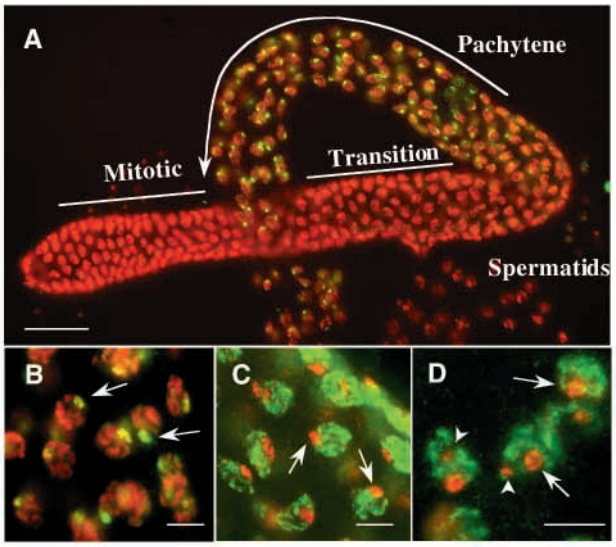


**Fig. 3.** Histone H3 lysine 9 methylation in male germ cells. Methanol/acetone fixed gonads from N2 (A-C) and PD7271 transgenic males (D) were stained with α-H3 methyl-K9 alone (green) and counter stained with DAPI (red; A,B), or stained with both α-H3 methyl-K9 (red) and α-phosphoacetyl H3 (green; C,D). The curved arrow in A shows the direction of meiotic progression. Arrows in B-D indicate a chromosome enriched in H3 modified by methyl-K9 (green in B, red in C), and under-modified by the phosphoacetyl epitope (green in C). Arrowheads in D indicate inactive transgenes. Scale bars: 5 μm.

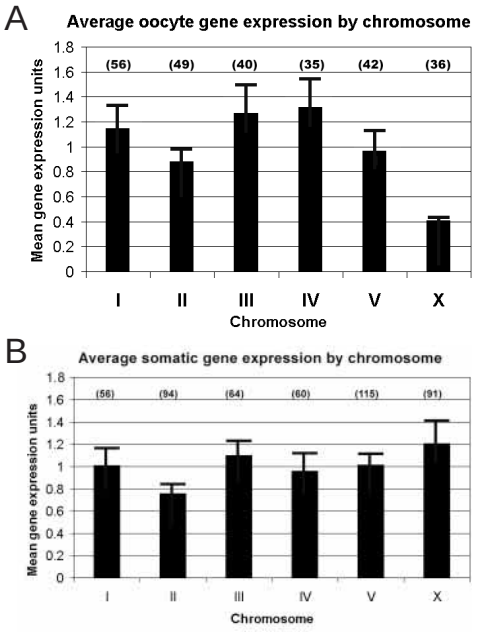


**Fig. 4.** Reduced expression of X-linked oocyte-specific genes. Relative abundance of autosomal- and X-linked oocyte-enriched mRNAs (A) and autosomal- and X-linked somatic mRNAs (B) were determined for each chromosome as described in Materials and Methods, and plotted with standard errors. The number of genes used in the calculation for each chromosome is listed in parentheses.

and germ cell-intrinsic genes are under-represented on the X chromosome (Reinke et al., 2000). Oocyte-enriched genes are found on the X chromosome at a frequency comparable with autosomes, suggesting that the X chromosomes in the oogenic hermaphrodite germline can support gene expression. We analyzed these microarray data further to determine whether the X-linked oocyte-enriched genes were expressed at levels comparable with oocyte-enriched genes on autosomes. We used the raw expression values from four microarray experiments that corresponded to staged wild-type young adult hermaphrodites (Reinke et al., 2000). After normalization of the raw values to allow averaging of experiments, we compared the average expression level of the oocyte-enriched genes from each chromosome with each other. Surprisingly, mean expression of oocyte-enriched genes on the X chromosome is significantly lower than the mean for oocyte-enriched genes on any autosome (Fig. 4A). To determine if this decreased expression from the X chromosome relative to autosomes is restricted to germline-enriched genes, we performed the same analysis on a set of genes that show no germline enrichment in the microarray results, and as such are likely to be expressed in somatic tissues (Materials and Methods). In contrast to the germline-enriched genes, these 'somatic' genes were expressed at similar levels from the X chromosome and all autosomes (Fig. 4B), demonstrating that the reduced X-linked expression relative to autosomes is specific to germ cell-expressed genes.

## Histones on the hermaphrodite X chromosome also lack activating modifications

The microarray data indicated that X-linked oocyte-enriched gene expression is reduced relative to autosomes. We therefore examined pachytene stage germ cells from adult hermaphrodites with α-H3 dimethyl-K4 to compare the patterns observed with those seen in males (Fig. 5A). Each hermaphrodite germ cell nucleus exhibited a single, exceptional chromosome pair that was strongly under-stained along its length, relative to the other chromosomes in the same nucleus (Fig. 5A3, arrows). Other activating modifications were also undetectable or greatly decreased on this chromosome pair: acetylated H3, phosphorylated H3 and multiple acetylated forms of histone H4 (Table 1; Fig. 5B-E). This chromosome pair was also under-stained by mAb H5 (Fig. 5F,G), which recognizes a phospho-epitope that is enriched on actively transcribing RNA polymerase II (POL II) (Dahmus, 1996; Kim et al., 1997; Seydoux and Dunn, 1997). A similar lack of staining was observed with mAb H14 (not shown), which recognizes a phospho-epitope on POL II that is thought to define an earlier step in transcription initiation (Dahmus, 1996).

The α-H3 phospho-S10 antibody consistently labeled one end of the chromosome pair in hermaphrodites (arrow in Fig. 5C). This chromosome pair also reliably showed a slight increase in labeling with the α-acetyl-H3 (acetyl-K9, -K14) antibody relative to the other probes tested (Fig. 5B). The patterns described above were also seen in the pachytene germ cells of L4 stage hermaphrodite larvae, in which the germ cells are spermatogenic (not shown). We wished to verify that the histone modifications we saw on this chromosome pair in hermaphrodite germ cells correlate with transcriptional competence, so we examined staining of the transcriptionally silent and active transgene arrays. Similar to the staining pattern observed in males, the silenced transgene array was

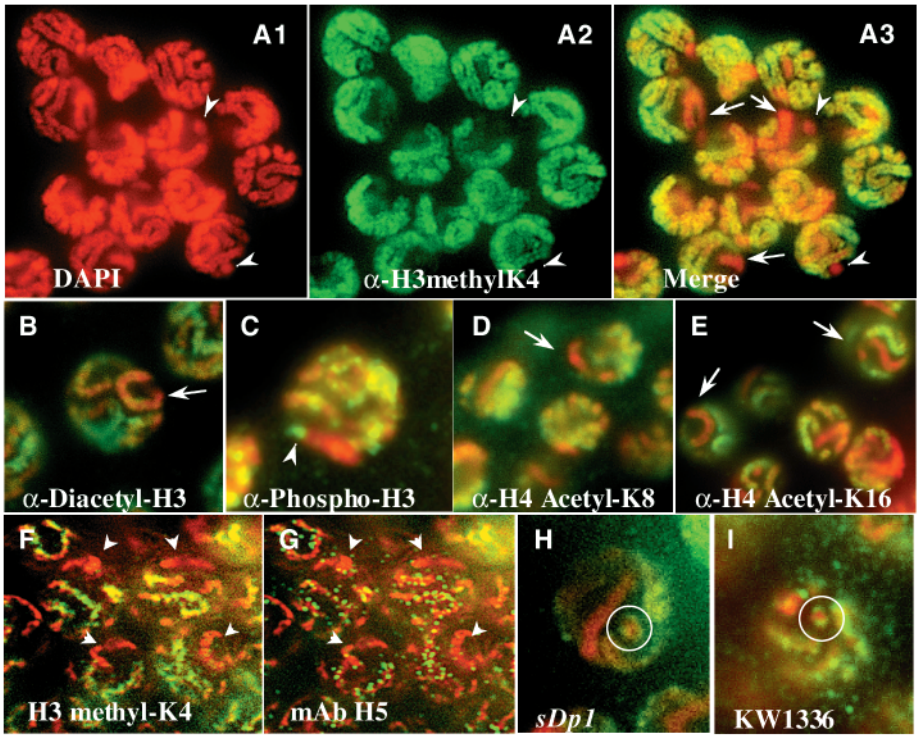


**Fig. 5.** Decreased activating histone modifications on one hermaphrodite chromosome pair. (A-E) PD7271 (A1-A3) or N2 (B-G) hermaphrodite ovaries were fixed with paraformaldehyde and stained with antibodies recognizing histone H3 methyl-K4 (A), H3 acetyl-K9, –14 (B), H3 phospho-S10 (C), histone H4 acetyl-K8 (D) and H4 acetyl-K16 (E). In A-E, antibody staining is false-colored green and DAPI counterstain is false-colored red. Arrowheads in A1-A3 point to transgenes identified as in Fig. 2. Arrows in A3-E point to the under-labeled chromosome; arrowhead in C points out a concentration of the H3 phospho-S10 modification that is otherwise absent from this chromosome. (F,G) N2 hermaphrodite germ cells were simultaneously stained with α-H3 dimethyl-K4 (F; green) and mAb H5 (G; green), and counterstained with DAPI (F,G; red). Arrowheads indicate chromosomes under-stained by both antibodies. (H,I) α-H3 dimethyl-K4 staining of pachytene nuclei in strain carrying autosomal duplication sDp1 (H) and the activated transgene (KW1336; I). The free duplication and the transgene are circled in H,I, respectively.

also strongly under-stained in hermaphrodite germ cells (Fig. 5A, arrowheads). None of the activating modifications were detected on the inactive transgene array, but all were consistently observed on free autosomal duplications (e.g. sDp1 (IV;f; Fig. 5H) and sDp3 (III;f, not shown)) and the activated transgene array (Fig. 5I).

We next tested whether this chromosome pair was the X chromosome by examining animals carrying an X:autosome fusion. Hermaphrodites that are homozygous for the reciprocal translocation mnT10 carry two such fusion chromosomes, each comprising a large portion of the X chromosome fused with part of chromosome V (Herman et al., 1982). Two chromosome pairs in pachytene nuclei from these animals each displayed partial modification by H3 dimethyl-K4 (Fig. 6A). The parts containing the modification appeared to have a discrete border in the hybrid chromosomes. Fluorescent in situ hybridization (FISH) analysis was also performed (in conjunction with antibody labeling) on wild-type (N2 Bristol) animals using probes specific for each end of the X chromosome (Fig. 6B). The under-stained chromosome was labeled by the X-specific FISH probes, which demonstrates unambiguously that the hermaphrodite chromosome lacking the activating histone modifications is the X chromosome. In addition, triplo-X hermaphrodite offspring from *him-5* animals exhibited an additional chromosome that lacked activating histone modifications (not shown).

### H3 lysine 9 methylation of the X chromosome in hermaphrodite germ cells differs from male germ cells

We also stained hermaphrodite germ cells with α-H3 dimethyl-K9, which correlates with transcriptional silencing (Table 1). The H3 methyl-K9 modification observed in hermaphrodites was highest in pachytene nuclei (Fig. 7A). Early in pachytene, each nucleus exhibited a single major focus of antibody binding that was observed to localize to an apparent terminus of one chromosome pair (Fig. 7B, arrowheads). The nature of the major signal is unknown, although it localized to one end of the same chromosome that was under-stained by the α-phosphoacetyl H3 antibody, indicating that it is the X chromosome (data not shown). As the cells progressed through pachytene into diplotene, we noticed a transient accumulation of the H3 methyl-K9 modification in numerous chromosomal regions, which rapidly disappeared in later stages (Fig. 7A, curved arrow). This dynamic pattern was not observed in male germ cells (Fig. 3A). As the nuclei progressed through

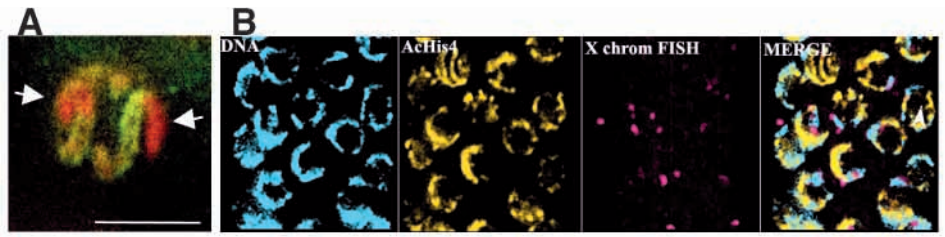


**Fig. 6.** The under-modified chromosome is the X chromosome. (A) Hermaphrodite animals homozygous for an X:autosome reciprocal translocation [mnT10(X:V)] were stained with α-H3 dimethyl-K4 (green) and counterstained with DAPI (red). Arrows point to chromosomes in a nucleus that are each half-labeled by the antibody. Scale bar: 5 μm.
(B) Hermaphrodite germ cells were probed simultaneously by antibody α-H4 acetyl-K12; ('AcHis4') and by fluorescent in situ hybridization (FISH) using probes to both ends of the X chromosome. Scale bar: 5 μm in A.

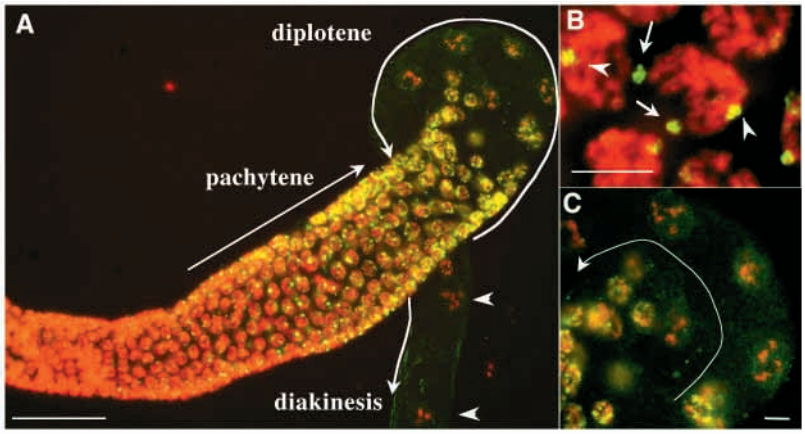


**Fig. 7.** Histone H3 lysine 9 methylation in hermaphrodites. N2 (Bristol) (A,C) and transgenic strain PD7271 (B) hermaphrodite ovaries were fixed in methanol/acetone and stained with anti-H3 dimethyl-K9 antibody (green) and counter-stained with DAPI (red). The progression of the nuclei through meiotic prophase I is indicated by labels and arrows in A. Arrowheads in B indicate the major staining foci seen in early pachytene nuclei in all strains; the arrows show additional nuclear foci unique to the PD7271 strain, which correspond to the transgene arrays. (C) An enlargement of the pachytene-to-diplotene transition region from A. Scale bars: 30 μm in A; 5 μm in B,C.

diplotene into diakinesis, the H3 methyl-K9 modification was lost from all chromosomes (Fig. 7B, arrowheads, and 7C). We also noted staining of the inactive transgene array with α-H3 dimethyl K9, although the intensity of the staining was variable. The arrays shown in Fig. 7B illustrate the highest level of staining observed (arrows).

### Gene expression from the hermaphrodite X chromosome is detected late in meiotic prophase

A lack of activating histone modifications on the X chromosome was observed from the mitotic region through mid-pachytene, in both oogenic (adult hermaphrodite) and spermatogenic (adult male and L4 hermaphrodite) germ cells. This absence of activating modifications persisted in later stages of meiosis in males and L4 hermaphrodites (Fig. 2 and data not shown). In oogenic hermaphrodites, however, the X chromosome became increasingly decorated with activating histone modifications as germ cells progressed into the diplotene stage of oogenesis (Fig. 8A). By diakinesis, all of the oocyte chromosomes exhibited detectable staining (Fig. 8B). Histones on the inactive transgene array completely lacked detectable activating modifications throughout meiosis, with the exception of the H3 phospho-S10 epitope, which is present on the inactivated transgene array in diakinesis (Table 1). Histones on the activated transgene (Fig. 1D) and the autosomal duplications continued to be modified in a manner similar to the autosomes in these stages (Fig. 8C). Histones on the third X chromosome in triplo-X hermaphrodites also acquired activating modifications at diplotene (not shown).

The accumulation of activating modifications at diplotene suggested that the X chromosome becomes activated at this stage in oogenesis. Therefore, the apparent lower relative expression levels of X-linked oocyte genes (Fig. 4) could be due to a transient burst of activation in the subset of oogonial cells passing through diplotene, rather than a sustained lower expression throughout meiosis. We used in situ hybridization analysis to determine when mRNA synthesis from a set of X-linked oocyte-enriched genes could be detected. The three genes tested were chosen at random from a set of genes showing strong enrichment in oogenic germ lines and relatively high levels of expression (Reinke et al., 2000). We could detect transcripts for these genes beginning with the last few nuclei showing pachytene DNA morphology (Fig. 9). The late-pachytene expression pattern strongly contrasts with in situ results of many different autosomal-linked germ cell-intrinsic and oocyte-specific genes previously examined, which show extensive RNA accumulation throughout pachytene and earlier stages (Jones et al., 1996; Lee and Schedl, 2001) (M.-H. L. and T. Schedl, unpublished). To determine the strength of this correlation, we selected a priori 30 autosomal oocyte-enriched genes under the same criteria used for the three X-linked oocyte-enriched genes analyzed by in situ hybridization. We surveyed the expression pattern of these 30 in an unpublished large-scale in situ hybridization screen (Y. Kohara, National Institute of Genetics, Japan; http://nematode.lab.nig.ac.jp/). Of the 20 that were detectably expressed in the germline, all 20 demonstrated considerable or preferential staining in the distal portion of the germline, in contrast to the pattern seen for the three X-linked oocyte genes shown here. These results support a conclusion that oocyte-enriched genes on the X chromosome become transcriptionally competent at a late stage in meiotic prophase.

Previous experiments have demonstrated that the bulk of RNA synthesis occurs in the pachytene region of the hermaphrodite gonad, with progressively less synthesis as the nuclei progress through diplotene into diakinesis (Starck, 1977; Schisa et al., 2001). By diakinesis, homologs have desynapsed but remain attached to their partners through chiasmata, and

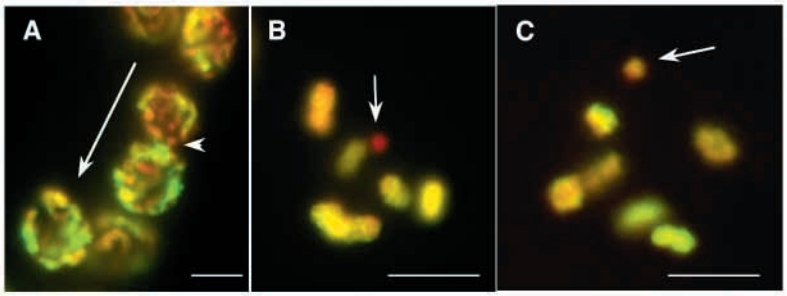


**Fig. 8.** Post-pachytene histone modification in hermaphrodites. Hermaphrodite ovaries were fixed in paraformaldehyde, stained with α-H3 dimethyl-K4 (green) and counter-stained with DAPI (red). (A,B) Transgenic strain PD7271 with inactive transgene. (A) Nuclei progressing into diplotene, with the direction of progression indicated by the long arrow; arrowhead points to a transgene. (B) An oocyte in diakinesis with six attached homolog pairs and transgene indicated (arrow). (C) Oocyte in diakinesis from sDp1 strain, with duplication indicated (arrow). Scale bars: 5 μm.

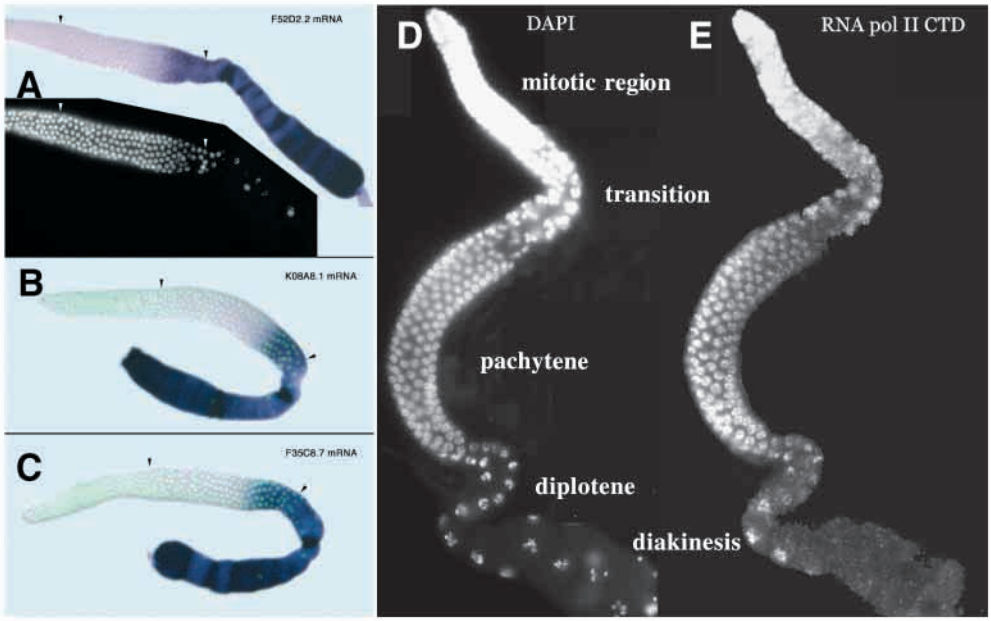


**Fig. 9.** In situ analysis of X-linked oocyte gene expression. (A-C) Antisense probes were generated for several X-linked genes that exhibited an oocyte-enriched profile by microarray analysis (Reinke et al., 2000). The probes were then hybridized to wild-type ovaries to determine where the corresponding mRNAs begin to accumulate. In each panel, the mitotic (distal) region is towards the left and maturing oocytes in the most proximal region of each ovary are towards the right. The arrowheads indicate the boundaries of the pachytene region of each ovary. DAPI and *lacZ* are shown separately in A; these signals have been merged in B,C. (D,E) Distribution of transcription-competent RNA POL II. A methanol/acetone fixed hermaphrodite gonad was stained with DAPI (D) and also with monoclonal antibody H14 (E).

the chromosomes are highly condensed (Albertson et al., 1997). We obtained additional evidence supporting the notion that germ nuclei in diakinesis stage are not actively transcribing genes, by labeling hermaphrodite germ cells with the monoclonal antibody H14, which recognizes a phospho-epitope on the large subunit of RNA polymerase II that correlates with transcriptional competence (Dahmus, 1996; Kim et al., 1997; Seydoux and Dunn, 1997). We found that the H14 antibody labeled nuclei up to and including pachytene stage, but the signal became undetectable as the cells progress through diplotene into diakinesis (Fig. 9D,E). Our result agrees with the previous 3H-uridine incorporation experiments, demonstrating that transcription from any chromosome is unlikely to be occurring in late meiosis I in oocytes. The X-linked oocyte genes tested therefore appear to have a narrow window of time during which they can be expressed.

## Conservation of germline X-chromosome silencing in hermaphroditic and gonochoristic nematodes

The hermaphrodite germline in *C. elegans* first undergoes spermatogenesis in L4 larvae, but switches entirely to egg production after the last larval molt and remains oogenic (female) for the reproductive lifespan of the adult (Schedl, 1997; Hubbard and Greenstein, 2000). The silenced X chromosome in the adult hermaphrodite oogenic germ line could be the result of briefly adopting a male mode of reproduction in the L4 stage, as L4 hermaphrodite germ cells exhibit a similar histone antibody staining pattern as that seen in males (data not shown). The decreased amount of histone modification on the hermaphrodite X chromosome in the adult might thus be specific to the hermaphrodite mode of reproduction. We therefore studied whether the germline X chromosome silencing seen in *C. elegans* was restricted to hermaphroditic nematode species. Germ cells from a variety of divergent nematode genera and species, representing both gonochoristic (male/female) and hermaphroditic species, were analyzed for the presence of H3 methyl-K4 (Fig. 10), as well as H4 acetyl-K8 and -K16 (not shown). Remarkably, all of the species examined exhibited a staining pattern similar to that seen in *C. elegans*; i.e. one meiotic chromosome (or one chromosome pair) in all sexes examined appeared under-

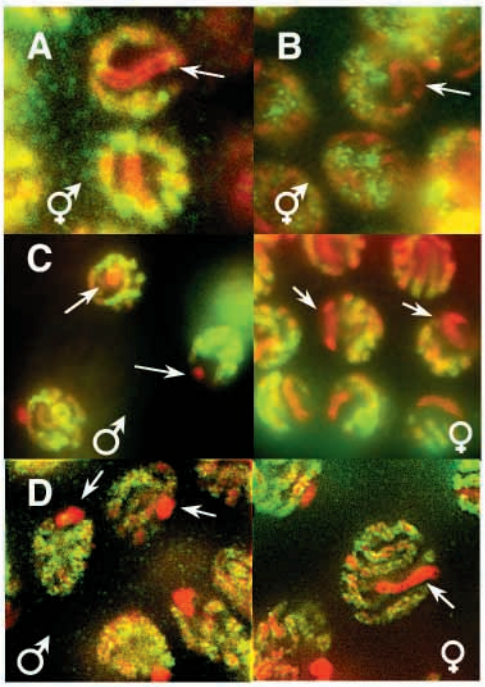


**Fig. 10.** Evolutionary conservation of meiotic silencing. Gonads from divergent species of nematodes were fixed in paraformaldehyde and stained with α-H3 dimethyl-K4. Hermaphrodite species tested include *Oscheius sp.* (A), *Pristionchus pacificus* (B) and *C. briggsae* and *Oschieus myriophila* (not shown). Gonochoristic species include *C. remanei* (C), *Caenorhabditis sp.* (D) and *Mesorhabditis longespicula* (not shown). Arrows in each panel indicate the 'under-modified' chromosome.

stained. These results suggest that X chromosome silencing in the germ cells of both sexes is widely conserved in the nematode phylum. Its presence does not correlate with the hermaphrodite mode of reproduction, and is thus unlikely to be a 'vestige' in oogonia of a spermatogenic process.

## DISCUSSION

### Germ cell repression and X-chromosome silencing

We have demonstrated that the X chromosome in germ cells of both sexes of *C. elegans* has numerous global attributes associated with silent chromatin. The pattern of histone modifications on the X chromosome in both germlines is consistent with a chromosome-wide reduction in transcriptional competence. The correlation of both activating and inactivating histone modifications consistently follows the germline expression competence of reporter transgene arrays used for comparison. The male X chromosome not only lacks activating histone modifications (Fig. 2), it is also enriched for a modification that is associated with heterochromatin in other species, histone H3 lysine 9 methylation (Fig. 3).

Oocyte-enriched genes, whose expression would only be required in the hermaphrodite, are present on the X chromosome with a frequency similar to that found on autosomes (Reinke et al., 2000). One might have therefore predicted that germline genes on the X chromosome would exhibit expression properties similar to those of autosomal germline genes in hermaphrodite germ cells. We were thus surprised to find that the mean expression of X-linked oocyte-enriched genes was significantly decreased compared with the number of autosomal oocyte-enriched genes (Fig. 4). Additionally, the paired X chromosomes in adult hermaphrodites, like the male X chromosome and inactive transgene arrays, shows a marked reduction of activating histone modifications (Fig. 5). The hermaphrodite X chromosome is also under-stained by antibodies that recognize transcriptionally active POLII, further correlating the lack of activating histone modifications with transcriptional inactivity.

These results support a conclusion that the X chromosome is silenced in both sexes up to and including the pachytene stage in meiosis. The identical pattern of histone modifications occurs during spermatogenesis in both XX hermaphrodite larvae and adult XO males through the pachytene stage. In contrast to the male X chromosome, however, the X chromosomes in the female adult germline accumulate activating histone modifications as the nuclei progress through diplotene (Fig. 8), presumably allowing expression of the X-linked oocyte-enriched genes as the cells enter oogenesis. This conclusion is supported by our in situ results, which demonstrate that transcription of several X-linked genes does not begin until the cells progress towards diplotene (Fig. 9).

The X chromosomes in both sexes are under-represented by a wide variety of chromatin modifications. By direct measurement of X-linked oocyte gene expression, in situ hybridization and correlation with transgene expression, we have demonstrated that most expression of X-linked genes is likely to be greatly reduced. It must be emphasized, however, that we are not proposing an inactivation of the X chromosome in toto for either sex. In mammals, one of the X chromosomes is 'inactivated' in somatic cells, yet expression from numerous loci on the 'inactive' X chromosome has been detected in soma (Carrel et al., 1999; Sudbrak et al., 2001). The term 'inactive', when applied to an entire chromosome, thus more reasonably defines a global state of inactivation with certain local domains that are refractory to such silencing. Indeed histone H1.1 has been shown to be expressed in germ cells, is required for germ line silencing of transgenes, and is an X-linked gene (Jedrusik and Schulze, 2001). Histone transcripts are normally transcribed and translated by special mechanisms, yet it is likely there are other X-linked genes that are expressed in germ cells. For example, the expression and abundance of X-linked 'housekeeping' genes in germ cells have not yet been directly analyzed in *C. elegans*. However in a survey of 350 ovary-expressed genes, none of the 81 genes whose depletion by RNAi caused scoreable phenotypes were X-linked (Piano et al., 2000).

### Facultative heterochromatin on the X chromosome

The morphology and the transcriptional silencing of the meiotic male X chromosome we observe in *C. elegans* are reminiscent of the male X chromosome in mammals. In mammals, the X and Y chromosomes form a condensed XY (sex) body that is transcriptionally inactive (Handel and Hunt, 1992). The presence of the H3 methyl-K9 modification in particular is strongly linked to the epigenetic establishment of silenced chromatin (Rea et al., 2000; Nakayama et al., 2001; Jenuwein, 2001). The methylation of histone H3 on lysine 9 by SU(VAR)3-9 creates a binding site for the heterochromatin protein, HP1, which binds to chromatin through its chromodomain, a conserved domain found on numerous chromatin-associated proteins (Bannister et al., 2001; Lachner et al., 2001). Mouse homologs of both HP1 (M31) and SU(VAR)3-9 (Suv39h2) are found in the inactive XY body through the pachytene stage of sperm meiosis (Motzkus et al., 1999; O'Carroll et al., 2000). A recent report demonstrates that disrupting Suv39h histone methyltransferase activity in mice results in poor viability, with survivors exhibiting aberrant sex chromosome segregation during male meiosis (Peters et al., 2001). *Drosophila* SU(VAR)3-9 associates with and regulates heterochromatin formation in flies, and a *Su(var)3-9* homologue in *S. pombe* (*clr-4*) is a key player in maintaining heritably stable heterochromatic regions of yeast genome via H3 lysine-9 methylation (Kennison, 1995; Grewal, 2000). The apparent uniform distribution of H3 methyl-K9 on the worm male X chromosome might initiate a particularly potent form of inactivation that is not reversed until after fertilization. Post-fertilization reactivation of the male X chromosome chromatin is probably made possible by the erasure of this mark during spermatogenesis.

In hermaphrodites, the H3 methyl-K9 epitope is initially concentrated on one end of a single set of paired homologues, which appears to be the paired X chromosome. The focal H3 methyl-K9 modification later appears to transiently increase in many regions of the genome as the cells progress through late pachytene into diplotene; it then rapidly disappears in diakinesis. The reasons for this dynamic regulation are unclear, but the H3 methyl-K9 modification may either prepare the genome for, or be made unnecessary by, other modes of chromatin condensation that occur during diakinesis.

As discussed above, methylation of H3 lysine-9 creates a binding site for the heterochromatin protein HP1. Recent

results have demonstrated that the inactivation of a *C. elegans* HP-1 homolog, *hpl-2*, results in both de-silencing of inactive transgene arrays in germ cells and temperature-sensitive defects in hermaphrodite fertility (F. Couteau, F. Guerry, F. Müller, and F. Palladino, personal communication). In addition, the *C. elegans* genome contains at least 28 genes containing a recognizable SET domain, which is a conserved domain present in Su(var)3-9 and other predicted silencing proteins. The role of these genes in silencing of the X chromosome in the germline is currently being investigated.

### Why are the male and hermaphrodite X chromosomes silenced?

McKee and Handel (McKee and Handel, 1993) have proposed that sex chromosome condensation is a meiotic adaptation that prevents damaging recombination events between non-homologous sex chromosomes (XY) or loss of a partner-less chromosome (XO). Correlative data from mammals (XY), *Drosophila* (XY) and now *C. elegans* (XO) support this model. Meiotic chromosomes in male mammals undergo pairing and recombination, and spermatocytes contain condensed sex heterochromatin that does not incorporate $^{3}$H-uridine during meiosis (Solari 1974; Henderson 1964). Strikingly, a recent report showed a relative enrichment of early spermatogenesis genes on the X and Y chromosomes, suggesting that the sex chromosomes are transcriptionally competent during early stages of spermatogenesis in mammals. In keeping with the model that the X and Y chromosomes are not transcriptionally active during meiosis, however, they did not recover any known genes specific to meiotic germ cells (Wang et al., 2001).

By contrast, meiotic chromosomes in *Drosophila* males do not undergo recombination and the X and Y pair does not form a condensed body (Meyer 1960). Instead, X and Y pair through the association of the tandemly repeated rDNA region in common between the two chromosomes (McKee and Karpen, 1990). Microarray analysis of *Drosophila* development has identified many male germline genes, and found a much less dramatic bias in the distribution of these genes on the sex chromosomes than is seen in *C. elegans* (K. White, personal communication).

During meiosis in *C. elegans* XO males, autosomes pair and recombine, while the unpaired X chromosome forms a condensed body, analogous to the mammalian sex body (Goldstein, 1982) (this study). Spermatogenesis genes are largely absent from the X chromosome (Reinke et al., 2000), and we have shown in this work that a major hallmark of heterochromatin formation, the H3 methyl-K9 modification, is specifically located on the X chromosome during male meiosis. Sex chromosome condensation could result in silencing of the X chromosome in males and therefore prohibit the X-linkage of genes whose activity is required in germ cells for proliferation, meiosis or sperm development and function. The correlation between the reliance on recombination for homolog segregation, the condensation of sex chromatin, and gene expression (or lack thereof) in all of these species continues to support the model that sex chromosome inactivation in the heterogametic sex occurs to promote orderly segregation of sex chromosomes (McKee and Handel, 1993). In mammals, it may serve to restrict recombination to a small region of homology between the X and Y chromosomes. In worms, the inactive condensation state of X chromosome may play a role in ensuring that the chromosome will reach one or the other spindle pole despite lacking a partner and a chiasma.

If condensation and decreased gene expression of the male X chromosome in the germline of *C. elegans* is a consequence of lack of a pairing partner as suggested by the above model, then why are the hermaphrodite X chromosome homologs, which do align, synapse and recombine, also silenced in the germline? We considered the possibility that silencing was simply a consequence of hermaphrodites briefly adopting a male mode of gametogenesis. However, our data indicating that a single chromosome pair lacking detectable activating histone modifications is present in the germlines of obligate female nematodes suggests that such is not the case (Fig. 10). Another possibility is that because many germline-enriched genes are largely excluded from the X chromosome, the hermaphrodite X chromosomes exhibit sub-threshold levels of activating chromatin modifications simply as a consequence of the absence of this class of genes. The rapid accumulation of activating modifications during diplotene would thus be due to the expression of oocyte-enriched genes at that time. This hypothesis is formally possible, as we know nothing about the relative abundance of common essential (‘housekeeping’) genes on the X chromosome, or the pattern of their expression during meiosis. However, if the frequency of housekeeping genes on the X chromosome is not different from autosomes, and their expression occurs in meiotic stages earlier than diplotene, the above hypothesis would predict little difference in chromatin modification patterns between the X chromosome and autosomes.

Alternatively, one could propose that the lack of histone modifications and apparent gene expression from the hermaphrodite X chromosome is caused by active processes that keep the X chromosome silent in early meiosis for reasons required for proper germ cell function. Intriguingly, pairing and recombination between the hermaphrodite X chromosome homologs does not appear equivalent to the pairing and recombination that occurs between autosomal homologs. Most exchange events tend to occur in the terminal 30% of autosome arms, while the X chromosome displays a more uniform distribution of crossovers along its length. Additionally, some mutations that cause chromosome nondisjunction, such as those of *him-5* and *him-8*, preferentially affect the X chromosome (Broverman and Meneely, 1994). Thus, one possibility for the reduced X-linked gene expression seen in hermaphrodite germ cells could be that special requirements of a unique meiotic machinery acting specifically on the X chromosome prohibit gene expression in the earlier stages of meiotic prophase, but no longer do so once synapsis and recombination have occurred. The converse is equally plausible: the special recombination attributes of the hermaphrodite X chromosome could result from structural constraints arising from a requirement for silencing the X chromosome.

One such requirement for silencing the X chromosome in hermaphrodites could arise from a need to prevent the activation of dosage compensation in the germline. The absence of sperm-enriched and germ cell-intrinsic genes and the silencing of the X chromosome in both sexes together remove a requirement for dosage compensation in germ cells, because few genes on the X chromosome will be expressed in the germ lines of both sexes. Any genes that escape this

silencing in the germline (e.g. 'housekeeping' genes) may not require equalization in the two gametes, as the oocyte dramatically expands its cytoplasmic components, while mature sperm expel most of theirs. The canonical *C. elegans* dosage compensation complex (DCC), which is restricted to the X chromosome in somatic cells, does not localize specifically to the X chromosome in the hermaphrodite germline (Lieb et al., 1996). Instead, several components of the dosage compensation complex in worms are found on all chromosomes in germ cells, and are required during meiosis for proper segregation of all chromosomes (Meyer, 2000). Coincident activation of meiosis and dosage compensation could conceivably be fatal to both processes through competition for limited shared components. The DCC does not assemble onto the X chromosome until well past fertilization – after the meiotic requirements for the shared factors have passed and zygotic activation of the genome can decrease the competition (Meyer, 2000).

While it is clear that a lack of germline-expressed genes on the X chromosome could obviate the need for dosage compensation in the germ line, it is also possible that a requirement for the absence of the DCC from the germline could have resulted in the exclusion of a subset of these genes from the X chromosome in the first place. Sex in *C. elegans* is determined by a mechanism that 'counts' X chromosomes: a binary switch is achieved through amplification of signals resulting from initial twofold differences in the expression of several X-linked genes that function as counted signal elements (Meyer, 2000). The very early embryo cannot employ mechanisms that equalize the expression of X-linked genes between XX and XO embryos; to do so would equalize expression of signal elements and thus prevent their interpretation by the sex determination pathway. To avoid this equalization, it may have been advantageous to exclude the DCC from the X chromosome in the germline, and consequently any germ cell-specific genes that would require equal expression between the two sexes.

Both X chromosomes in female mammals become active at meiotic prophase, although the extent of the activation is unclear (Handel and Hunt, 1992), suggesting that dosage compensation is not required or engaged during meiosis in mammals. Interestingly, Xist RNA, which is required for dosage compensation in the somatic tissues of female mammals, is also concentrated in the XY body in mammalian testes, leading to the suggestion that Xist-mediated dosage compensation evolved from meiotic inactivation mechanisms (Ayoub, 1997). Additionally, the activity of the sperm Suv39h2 methyltransferase has been proposed to establish an epigenetic imprint through its role in organizing meiotic heterochromatin (O'Carroll et al., 2000). Marking of regions of the X chromosome in germ cells as facultative heterochromatin, perhaps through H3 K9-methylation, could serve as an epigenetic mark that is later targeted by the somatic DCC. The concentration of H3 methyl-K9 on one end of the hermaphrodite X chromosome is reminiscent of asymmetries observed for the human X chromosome in the female soma. The arm of the X chromosome which contains the X-inactivation center, Xq, is enriched for interspersed repeated LINE-1 elements which themselves are enriched in α-heterochromatin (Bailey et al., 2000). Conversely, genes that escape X-inactivation are enriched on the other arm, Xp (Carrel et al., 1999). An asymmetry in epigenetic regulation of the X chromosome may be a conserved feature in sex chromosome evolution.

### Protection of the autosomes from germline silencing

The inactivated transgene array in PD7271 contains no X-linked DNA sequences, yet mimics the modes of X chromosome silencing observed in pachytene germ cells of both sexes. However, in the diplotene stage of meiosis I in oocytes, when the X chromosome becomes transcriptionally active, the inactivated repetitive transgene array fails to accumulate any activating histone modifications or display detectable GFP expression. Experiments with other silenced transgene arrays also show this result, suggesting that this is a general property of repetitive transgene arrays (W. G. K., unpublished). Unpaired autosomal duplications, the (presumably) autosomal region of autosomal:X translocations, and complex transgene arrays composed of random genomic fragments, by contrast, all carry activating histone modifications: furthermore, all three types of sequences support gene expression in germ cells. These results suggest that germline silencing mechanisms are not specifically targeted to X-linked sequences, but may be targeted by default through the absence of sequences present on autosomal DNA. Autosomal chromosomes, and regions of autosomal chromosomes, are somehow refractory to the silencing mechanisms. One could thus hypothesize that the transcriptional activity that is limited to the autosomes in pachytene nuclei is accomplished by specifically preventing silencing in essential regions along these chromosomes. Theoretically, this could be accomplished via boundary-type *cis* elements or 'barriers' (Gerasimova and Corces, 1996), present only on autosomes, which are specifically recognized by anti-silencing factors (e.g. de-repressors). Silenced transgene arrays corresponding to autosomal genes, such as the *let-858* gene used as a reporter in this study, may frequently not include such a barrier element, which may reside some distance away in the chromosomal locus. Another possibility is that repetitive transgene arrays are also targeted by either parallel or overlapping mechanisms that recognize and silence unusual repetitive sequences during normal genome defense. Such mechanisms may preclude the addition of histone modifications during diplotene and diakinesis to repetitive transgene arrays (Fig. 8B), but not to their more complex counterparts (Fig. 8D).

What are the silencing mechanisms that target the X chromosome for silencing in *C. elegans* germ cells? Previous studies have shown that silencing of transgene arrays can be disrupted by mutations in four different mes genes (Kelly and Fire, 1998). Interestingly, the activity of the MES proteins is sensitive to X-chromosome dose, independent of the sex of the animal (Garvin et al., 1998). XX and XXX hermaphrodites with mutations in any of the mes genes show correspondingly increased degeneration of germ cells. This fact has led to the proposal that at least some of the target genes requiring MES repression are found on the X chromosome. Our results may lend support to this proposal, and suggest that silencing of the X chromosome, potentially through the action of the MES protein products, is indeed an important aspect of maintaining germ cell viability. The nature of the mes phenotype, however, has made analysis of the effect of *mes* mutations on chromatin

organization difficult: the generation exhibiting the maternal effect sterility presents few germ cells to examine, all of which are in various stages of degeneration. Analysis of the preceding generation, which exhibits no germ cell defects, predictably shows little or no consistent disruption in chromatin organization (W. G. K. and C. E. S., unpublished). We are actively pursuing other methods for investigating the role of MES proteins and other factors in X chromosome silencing in the germline.

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