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# EXPRESSION OF THE *Xist* GENE IN UROGENITAL RIDGES OF MIDGESTATION MALE EMBRYOS

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**SUMMARY:** We show by RT-PCR analysis that transcripts from the *Xist* gene, which is normally expressed from the inactive X chromosome of female somatic cells and postnatal male germ cells, are transiently expressed also in male embryos around the time of testis differentiation, mainly in somatic cells of urogenital ridges. In the postnatal testis, we find that *Xist* transcripts are mainly localized within the nucleus of haploid spermatids. These findings suggest that inactivation of the X-chromosome might occur not only in adult male germ cells, but also, transiently, in somatic cells of the male urogenital ridge. Both in the embryonal gonad and in differentiating germ cells *Xist* expression in males overlaps the pattern of expression of the testis determining gene *Sry*. 

Inactivation of all X-chromosomes in excess of one, occurring in early development of female mammals, is a dosage compensation mechanism insuring the same level of expression of X-linked genes in males and females (1). *Xist,* a X-linked gene, is actively transcribed exclusively from the inactive X-chromosome in females (2), and both imprinting and X-chromosome counting mechanisms can regulate its expression (3). Expression of the *Xist* gene has been reported to occur also in germ cells of the postnatal testis (4), in agreement with the theory of X-chromosome inactivation in the male germ cell line, which would be essential for spermatogenesis after birth.

In species where sex-determination is triggered by the X:autosome ratio, X-linked sex determining genes are also involved both in dosage compensation and in germ cell differentiation (5). In mammals the master regulatory gene for sex-

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determination *Sry* (6) is located on the Y-chromosome, and there is no information whether sex-determining genes are involved to any extent with X-chromosome inactivation, which would represent a linkage between mechanisms controlling dosage compensation in females, and both sex-determination and germ cell development in males. *Sry* expression, which occurs with unknown function in spermatogenesis (7), is accompanied by expression of the *Xist* gene (4). Therefore, we wondered whether the expression of *Sry* mRNA that transiently occurs, with obvious function, in somatic cells of the male urogenital ridges at the time of testis determination (8), is also accompanied by a transient *Xist* expression.

## MATERIALS AND METHODS

Mouse germ cell suspensions enriched in primordial germ cells (PGCs), in spermatogonia, in middle-late pachytene spermatocytes, in round spermatids, and primary Sertoli cell enriched cultures, were obtained as previously described (7, 9). Undifferentiated urogenital ridges or the upper part of the body of 10.5-11.5 day post-coitum (dpc) embryos were mechanically collected, and their sex was established through PCR amplification of genomic DNA using primers for the *Zfy* gene. Genital ridges and mesonephroi from mouse embryos between 12.5 and 14.5 dpc, where sexed according to gonadal morphology, which becomes clearly distinguishable starting from 12.5 dpc.

Synthetic oligonucleotides for PCR and RT-PCR analysis were produced with a 391 DNA synthesizer (PCR-MATE EP: Applied Biosystems). Primers used for PCR analysis were: for *Xist* GAGAAATGATACTATTTTTCTGAGG and GCTTTTTGTTCA-GAGTAGCGAGGAC (expected cDNA amplification product, spanning the first two introns of the mouse *Xist* gene: 309 bp); for *Hprt* GAAAGACTTGCT-CGAGATGTCATG and TGGCAACATCAAC-AGGACTCCTCG (expected cDNA amplification product: 570 bp); for *Sry* TGTCTAGAGAG-CATGGAGGGCCAT and AAAATGCCACTCCTCTGTGACACT (expected cDNA amplification product, encompassing the region encoding a DNA binding domain: 278 bp); for *c-Kit* CTGGTGGTTCAGAGTTCCATAGAC and TCAACGACCTTCCCGAAGGCACCA (expected cDNA amplification product: 385 bp); for *Zfy* GACTAGACATG-TCTTAACATCTGTCC and CCTATTGCATGGACAGCAGCTTATG (expected DNA amplification product: 183 bp).

RNA from adult testicular cells was extracted with GTC and purified on CsCl gradients, whereas RNA from embryonic sources was prepared with a GTC-acid phenol protocol. RNAs were reverse transcribed using random hexamers as primers, using a Perkin Elmer RNA PCR kit. Amplifications of cDNAs were performed with Taq polymerase in a final volume of 100 µl , with a Perkin Elmer-Cetus DNA thermal cycler with 38 step cycles, unless otherwise stated (95°C for 45 sec, 56°-60°C for 45 sec, 72°C for 15 sec), using synthetic oligonucleotides at a final concentration of 0.15 µM.

Genomic DNA was prepared by extractions with neutral phenol and chloroform of samples which had been dissolved in GTC solution. For embryo sexing, 100 ng of genomic DNA was amplified using primers to detect *Zfy* genomic sequences (5' initial denaturation at 95°C followed by 35 step cycles: 1 min at 95°C, 1 min at 58°C, 2 min at 72°C, and 7 min final extension at 72°C) with a final primer concentration of 1 µM in a reaction volume of 50µl.

## RESULTS AND DISCUSSION

Since no *Xist* expression is detectable in male embryos, at least up to 6.5 dpc, even after two rounds of RT-PCR amplification (10), we tested individual embryos at 8.5 dpc.: after a single round of RT-PCR amplification, no *Xist* expression was detectable (not shown). At 10.5 dpc, after a single round of RT-PCR amplification, the *Xist* signal was constantly absent in the upper part of the body in all the males that we analyzed (fig. 1A, top panel). However, a weak *Xist* signal was present in the urogenital ridges in most of the *Zfy*-positive embryos (fig. 1A, bottom panel).

A clear *Xist* signal (comparable to the control *Hprt* signal) was reproducibly observed in urogenital ridges of most of the *Zfy*-positive 11.5 dpc embryos (fig. 1B, middle panel). Most of the male embryos also clearly expressed *Sry* mRNA (fig. 1B, bottom panel), as expected at this developmental stage (8), in which testis determination occurs.

Fig. 1C shows that *Xist* mRNA (confirmed through direct dideoxysequencing of the 309 bp cDNA amplification product) was present also in fetal gonads from 12.5 dpc male embryos. The signal was still present in the differentiated testis at 13.5 dpc, eventhough it appeared somewath reduced if compared to the *Hprt* control, but it was undetectable at 14.5 dpc. These data indicate that the molecular marker of X-chromosome inactivation is transiently expressed in male embryonal gonad from one day before to one-two days after testis determination, with a peak at 11.5 dpc.

To establish whether *Xist* expression in male fetal gonads was due to the somatic or to the PGC component, we compared *Xist* expression in total genital ridges and in enriched population of PGCs from 12.5 dpc embryos. The PGC enrichment was confirmed by the selective expression in this cell preparation of *c-kit*, a specific marker of the germ cell line (fig. 2A, left panel). The *Xist* signal in total male gonads was stronger than that detectable in male PGCs (fig 2A, right panel), eventhough the amount of RNA used for RT-PCR was lower in total gonad samples with respect to PGCs, as evaluated through the intensity of the control *Hprt* band (fig. 2A, left panel). The *Xist* signal was also clearly detectable in the mesonephric component of male urogenital ridges at 12.5 dpc (fig. 2A, right panel), which is devoid of germ cells, and from which Sertoli cells are thought to be derived (11); we have evidence that also the *Sry* gene is expressed in the mesonephric component of urogenital ridges at this developmental stage (not shown). These data suggest that somatic cells, rather than germ cells, of the male fetal gonad transiently express the *Xist* gene around the time of testicular determination. This conclusion is in agreement with previous evidence that no *Xist* expression occurs in germ cells from male fetal testis, at least at 14.5 dpc (4); however, we cannot exclude that also PGCs contribute to *Xist* expression in 12.5 dpc male urogenital ridges. Thus, RT-PCR analysis indicates that *Xist* expression in

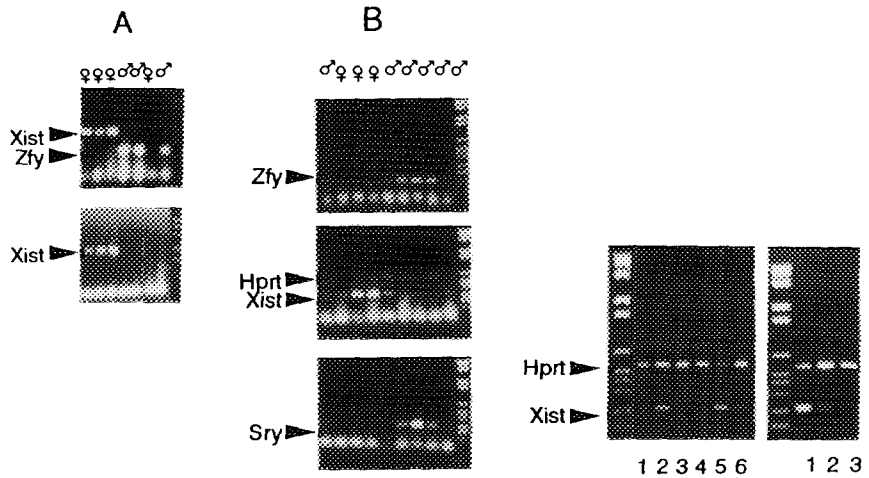


Figure 1. **A**: *Weak Xist expression is detected in urogenital ridges, but not in the cranial region of the body of individually sexed male mouse embryos just before the time of sex determination. Top panel.* RT-PCR amplification, using *Xist* primers of total RNA from the upper part of the body of 6 individual 10.5 dpc embryos. The RT-PCR reactions were mixed with the product of *Zfy* PCR reactions performed on genomic DNA prepared from the same samples for embryo sexing. *Bottom panel.* RT-PCR amplification, using *Xist* primers of total RNA from urogenital ridges of the same embryos. Size of DNA MW markers in decreasing order: 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, and 154 bp. **B**: *Clear Xist signals are detected in urogenital ridges of individually sexed male mouse embryos at the time of testicular determination. Top panel.* Sexing of 9 individual 11.5 dpc mouse embryos using primers for PCR detection of *Zfy* genomic sequences. *Middle panel.* RT-PCR amplification, using contemporaneously *Hprt* and *Xist* primers of total RNA from urogenital ridges of the same embryos. *Bottom panel.* RT-PCR amplification using *Sry* primers of total RNA from urogenital ridges of the same embryos. No signal was obtained omitting reverse transcriptase from the reaction (not shown).DNA MW markers as in A.
**C**: *Expression of the Xist gene in male embryonal gonad declines after testicular determination. Left panel.* RT-PCR amplification, using contemporaneously mouse *Hprt* and *Xist* primers, of total cellular RNA from genital ridges of: 1) 12.5 dpc male embryos; 2) 12.5 dpc female embryos; 3) 13.5 dpc male embryos; 4) 14.5 dpc male embryos; 5) 14.5 dpc female embryos. Lane 6: *Hprt/Xist* RT-PCR of total cellular RNA from spermatogonia of 7 day old mice. *Right panel.* RT-PCR amplification, using contemporaneously *Hprt* and *Xist* primers of total cellular RNA from a different preparation of genital ridges from 12.5 dpc female (lane 1), or male (lane 2) embryos, and from primary cultures of Sertoli cells from prepuberal testis (lane 3). DNA MW markers as in A.

male midgestation embryos is both temporally and spatially defined. *In situ* hybridization studies on sections from individually sexed embryos, using as a probes both sense and antisense RNAs generated from a *Xist* cDNA containing plasmid, showed strong positivity only with the antisense probe in all tissues of female embryos at all analyzed ages, and also in seminiferous tubules from adult testis, as expected (not shown). We could not extend morphologically the results

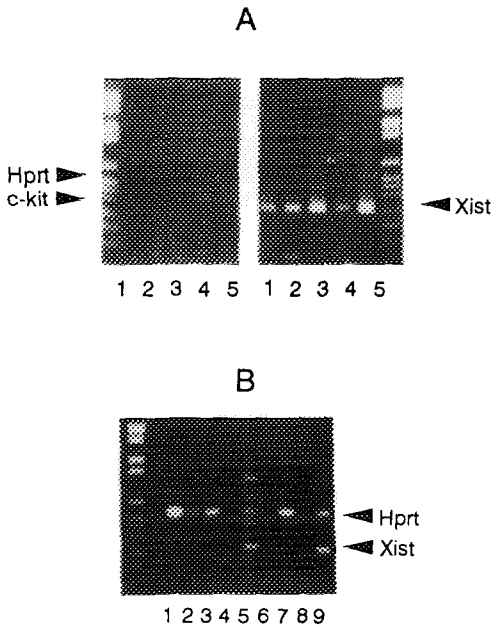


Figure 2. **A**: *Xist transcripts are present in somatic cells, rather than in germ cells, of the male fetal gonads at 12.5 dpc.* RT-PCR amplification, using contemporaneously *Hprt* and mouse *c-Kit* primers (32 step cycles )(*left panel*) or *Xist* primers (*right panel*) of total cellular RNAs of the following tissues from 12.5 dpc mouse urogenital ridges: 1) male mesonephroi; 2) total male gonads; 3) total female gonads; 4) enriched populations of male PGCs; 5) enriched populations of female PGCs. DNA MW markers as in fig. 1A.
**B**: *Xist transcripts in the adult testis are mainly expressed in round spermatids and localized within the nucleus.* RT-PCR amplification (33 step cycles), using contemporaneously mouse *Xist* and mouse *Hprt* primers of: 1) 1 µg total cellular RNA from primary cultures of Sertoli cells from 13 day old mice; 2) 0.3 µg total cellular RNA from spermatogonia from 7 day old mice; 3) 1 µg total cellular RNA from middle-late pachytene spermatocytes (95% homogeneous); 4) same as in lane 3, but omitting reverse transcriptase from the reaction; 5) 1 µg total cellular RNA from round spermatids (98% homogeneous); 6) same as in lane 5, but omitting reverse transcriptase from the reaction; 7) 1 µg cytoplasmic RNA from round spermatids (80% homogeneous); 8) 0.1 µg double selected polyA+ RNA from total cellular RNA preparations from round spermatids (98% homogeneous); 9) 1 µg total cellular RNA from female fetal gonads at 12.5 dpc. DNA MW markers as in fig. 1A.

of RT-PCR analysis in male embryos, possibly due to the low level of *Xist* expression in the male urogenital ridge, or to the fact that it is limited to a subset of somatic cells.

To establish whether *Sry* and *Xist* are expressed in the adult testis with the same spatial and temporal correlation observed in the fetal gonad, we investigated by RT-PCR analysis *Xist* RNA expression in different postnatal testicular cell types. Fig.2B shows the highest intensity of the *Xist* signal in total cellular RNA

preparations from round spermatids, which are also the site of maximal *Sry* expression (7). No *Xist* transcripts are detectable in cytoplasmic RNA and polyA + RNAs from the same source, indicating that, in haploid germ cells, *Xist* mRNA is poorly polyadenylated and is localized within the nucleus, as it has been described for female somatic cells (2). Using a higher number of cycles for RT-PCR amplification, the *Xist* signal was detectable also in spermatogonia (see fig.1C, left panel, lane 6), whereas it was completely absent in highly homogeneous populations of primary Sertoli cell cultures (fig 2B, see also fig. 1C, right panel, lane 3). Our finding that round spermatids, instead than late pachytene spermatocytes, as previously reported (4), are the site of maximal *Xist* transcription, was confirmed through *in situ* hybridization studies with antisense riboprobes, showing that the highest concentration of *Xist* transcripts is present in the proximity of the lumen of seminiferous tubules from adult testis (not shown).

Since a direct involvment of the *Xist* gene product in the initiation of the process of X-inactivation is likely (10), inactivation of the single X-chromosome might occur not only in postnatal male germ cells, but also, transiently, in somatic cells of the developing gonad in male embryos, around the time of testicular differentiation. A possible involvment of X-chromosome inactivation in mechanisms controlling sex-determination in mammals is suggested by the existence of genes on the X-chromosome influencing testis differentiation in humans (12, 13) and rodents (14). Very recently, a dosage sensitive locus on the short arm of the human X chromosome has been found, which is involved in male to female sex reversal (15). We have now shown that the pattern of expression of the molecular marker of X-inactivation in both somatic cells of the male urogenital ridges, and in germ cells from the adult testis, overlaps the developmental and tissue-specific pattern of expression observed for *Sry* mRNA. The focus of future studies should be to investigate whether there is any correlation between the expression of the sex determining Y-linked *Sry* gene and of the *Xist* gene in males, and whether the transient *Xist* expression in somatic male gonadal cells is coupled effectively to transient silencing of some X-linked genes.

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