Int. J. Dev. Biol. 41: 49-55 (1997)

Original Article

X-chromosome inactivation during the development of the male urogenital ridge of the mouse

ROBYN V. JAMIESON[1], SHEILA X. ZHOU[1], SEONG-SENG TAN[2] and PATRICK P.L. TAM[1]*

[1]*Embryology Unit, Children's Medical Research Institute, Wentworthville and*
[2]*Department of Anatomy and Cell Biology, University of Melbourne, Parkville, Australia*

ABSTRACT In the mouse, the activity of *Sry* (sex-determining gene on the Y chromosome) initiates the transformation of the indifferent gonad into a testis. In humans, a partial Xp21 duplication leads to the development of ovaries instead of testes in XY individuals. This observation indicates that sex determination might also be influenced by a gene-dosage compensation mechanism, in addition to a dominant action of the *Sry* gene. In female mammals, the regulation of X-linked gene dosage at early embryogenesis is achieved through the inactivation of one of the two X chromosomes. Here we have investigated the possibility that inactivation of the X chromosome may play a role in male sex determination. We have shown, using an X-linked *lacZ* transgenic mouse line, that loss of ß-galactosidase activity occurs in certain somatic cells of the developing male urogenital ridge. When changes associated with apoptosis of mesonephric tubules in the developing urogenital ridges are taken into account, expression of the *Xist* (X inactive specific transcript) gene correlates with X inactivation revealed by loss of ß-galactosidase activity in very early mesonephric tubule epithelial cells, gonadal interstitial mesenchymal cells and coelomic epithelial cells.

KEY WORDS: *X-chromosome, inactivation, sex determination, Xist, Sry*

Introduction

In mammals, while the *SRY/Sry* gene is critically important in the sex determination pathway (Koopman *et al.*, 1990, 1991; Sinclair *et al.*, 1990), it is increasingly recognized that other genes are involved in this cascade (Ramkissoon and Goodfellow, 1996). Expression of *Xist*, a gene normally expressed from the inactive X chromosome of female somatic cells (Lyon, 1961; Brockdorff *et al.*, 1992; Brown *et al.*, 1992; Kay *et al.*, 1993), has been reported in developing male urogenital ridges (Dolci *et al.*, 1994). Sex reversal in XY individuals with duplication of Xp21 has led to the postulate that a dosage sensitive sex reversal *(DSS)* locus (Bardoni *et al.*, 1994) must be repressed for *SRY* to exert its full effect on testis differentiation (Ogata and Matsuo, 1994; King *et al.*, 1995). It may be possible that transient X-chromosome inactivation at a critical time point in the developing gonad may play a role in the repression of this X-linked locus in male development (Dolci *et al.*, 1994). X-chromosome inactivation can occur in some male cells, specifically the germ cells during meiosis, as revealed by *XIST/Xist* expression (McCarrey and Dilworth, 1992; Richler *et al.*, 1992; Salido *et al.*, 1992).

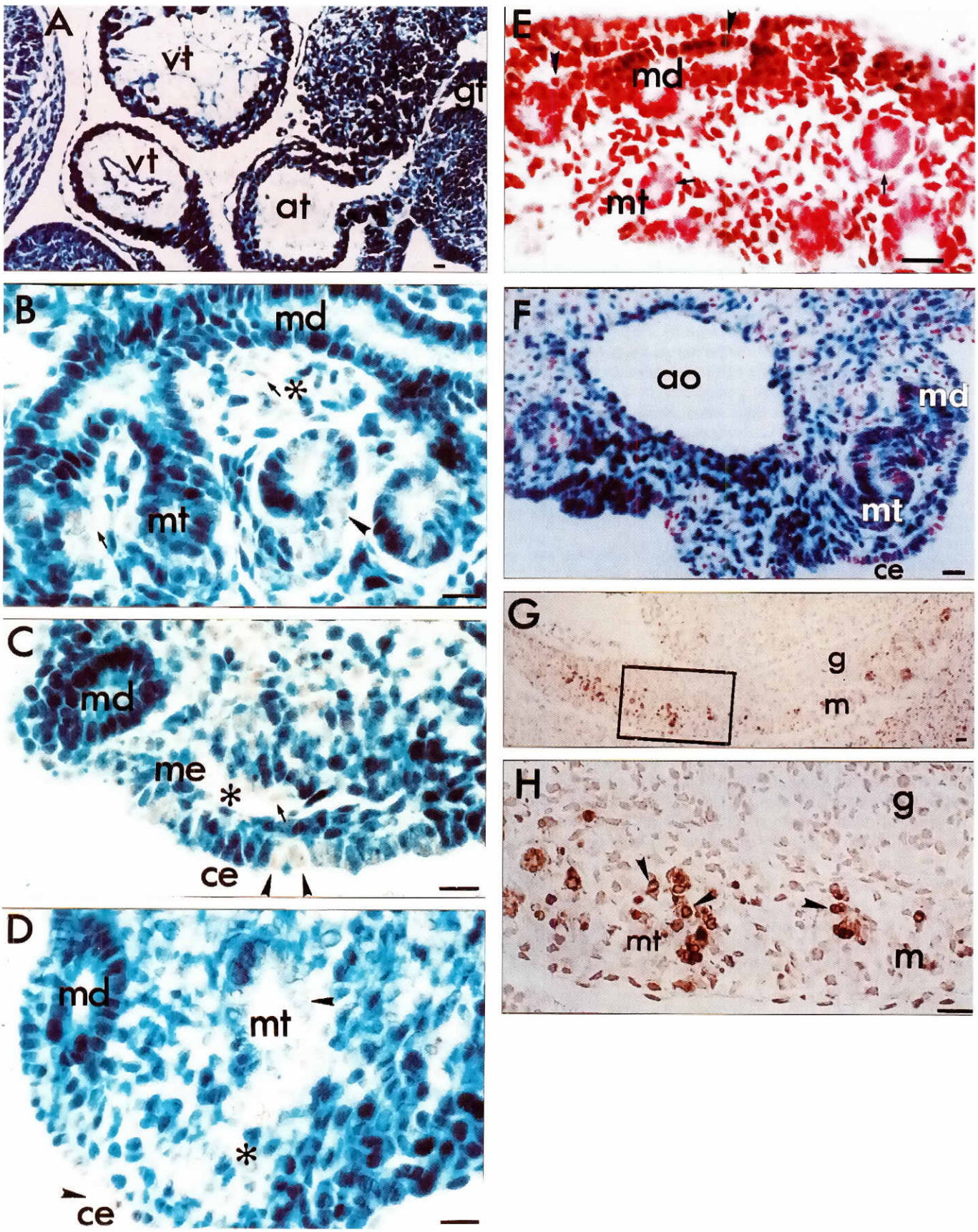
In this study we use a mouse line with an X-linked *lacZ* transgene that is subject to inactivation, so that the loss of β-galactosidase activity provides a direct visualization of X inactivation in individual cells (Tan *et al.*, 1993; Tam *et al.*, 1994a,b). We have taken this approach to pinpoint the cell types that may display X inactivation in the developing male gonad, specifically in the somatic cells which are reputed to be important in sex determination, rather than the germ cells (Merchant, 1975; McCoshen, 1982). In a previous study, the expression of the *Xist* gene was analyzed in tissues isolated from the 12.5 dpc male urogenital ridge. *Xist* transcripts were detected in the somatic tissues of the genital ridge and the adjacent mesonephros, rather than the primordial germ cells (Dolci *et al.*, 1994). In this study, we have further examined the possibility that the inactivation of the chromosome may be restricted to tissues of the genital ridge. We have compared the expression of the *Xist* gene in the mesonephros and the gonadal component of the urogenital ridge at several stages of male development. By detecting the activity of an X-linked *lacZ* transgene, we have been able to pinpoint more precisely the cell populations

Abbreviations used in this paper: AHC, adrenal hypoplasia congenita; ß-gal, ß-galactosidase; *DAX1/Dax1, DSS-AHC critical region on the X*; dpc, days *post coitum*; *DSS*, dosage sensitive sex reversal; *Hprt*, hypoxanthine phosphoribosyl transferase; PCR, polymerase chain reaction; RT, reverse transcription; *SRY/Sry*, sex determining region of the Y; TUNEL, terminal deoxynucleotidyl transferase-mediated 2'-deoxy-uridine-5'-triphosphate-biotin nick end labeling; *XIST/Xist*, X inactive specific transcript.

***Address for reprints:** Embryology Unit, Children's Medical Research Institute, Locked Bag 23, Wentworthville, NSW 2145, Australia. FAX: 61.2.9687 2120. e-mail: patrict@mail.usyd.edu.au

0214-6282/97/$05.00

Printed in Spain


A
vt
gt
vt
at
B
md
mt
C
md
me
ce
D
md
mt
ce
E
md
mt
F
ao
md
mt
ce
G
g
m
H
g
mt
m

that may display X-chromosome inactivation.

Experiments on XX-XY chimeras suggest that of the somatic cells, the Sertoli cell lineage plays a critical role in male sex determination (Burgoyne *et al.*, 1988; Palmer and Burgoyne, 1991). The origin of the Sertoli lineage has not been definitively established with the mesonephric region, the coelomic epithelium and the pre-existing mesenchyme all as possibilities (McLaren, 1991). The mesonephric tubules first develop at about 10 dpc in the mouse and appear to distribute cells from their ventral border to the mesenchyme of the genital ridge at about 11 and 12.5 dpc (Smith and Mackay, 1991). The most caudal tubules begin to show signs of apoptosis (Wyllie *et al.*, 1980) beginning at about 11 dpc (Smith and Mackay, 1991). In the light of the occurrence of cell death, we therefore also examined the influence of cell death in the mesonephros on the β-galactosidase activity in the urogenital tissues during testis formation.

Results

Homozygous female transgenic mice were mated with wild type F1(C57BL/6 x DBA/2) male mice to produce transgenic male and female fetuses that are both hemizygous for the transgene, at 10.5 to 13.5 dpc. Sex was determined by PCR detection of *Sry* in genomic DNA in fetuses aged 10.5 to 12.5 dpc and by morphological characteristics of the fetal gonads at 13.5 dpc. The expression of the transgene was assayed by the activity of *lacZ* encoded β-galactosidase or by the immunocytological detection of the enzyme. As expected from the absence of X inactivation in XY cells, somatic tissues outside the urogenital ridges of the male embryo ubiquitously expressed the *lacZ* transgene (Fig. 1A). Patchy expression of the transgene was noticed however, in the urogenital ridges. In particular, groups of cells in the epithelium of mesonephric tubules, the mesonephric and gonadal mesenchyme and the coelomic epithelium displayed a significantly diminished enzyme activity or lack of immunostaining reaction. The non-expressing cells (*lacZ*-ve) were localized as discrete groups in the mesonephric tubule epithelium amongst the expressing population (Fig. 1B,D,E). The *lacZ*-ve cells were found at every cranio-caudal level of the urogenital ridge. Non-expressing cells were interspersed with *lacZ*-expressing cells in the mesenchyme surrounding the tubules (Fig. 1B) and in the mesenchyme of the developing gonad component (Fig. 1C,D). Non-expressing cells were also seen in the coelomic epithelium of the gonad component of the urogenital ridges (Fig. 1C,D). Tissues in the urogenital ridges of the female embryos showed a mosaic expression pattern which is consistent with X-chromosome inactivation (Fig. 1F). To estimate the preponderance of the non-expressing cells, a cell count was performed for the mesonephric tubules, interstitial mesenchyme and coelomic epithelium in 10.5 and 11.5-day embryos. The non-expressing cells always constituted a minor population in the mesonephros but they were most numerous by proportion at 11.5 days (Table 1). Although the population of non-expressing cells was low in some samples, they were always present in the male urogenital ridges at every stage we examined. In two 10.5-day fetuses, non-expressing cells were absent in the mesonephros, but were found in the mesenchyme and coelomic epithelium.

Some mesonephric tubule epithelial cells at 11.5 dpc showed blebs (Fig. 1D) characteristic of apoptosis (Wyllie *et al.*, 1980), whereas those at 10.5 dpc did not (Fig. 1B). To examine this further we used the terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxy-uridine-5'-triphosphate (dUTP)-biotin nick end labeling (TUNEL) procedure to visualize areas of increased DNA fragmentation *in situ* (Gavrieli *et al.*, 1992). TUNEL labeling was found in about 41.5% of cells in the mesonephric tubules of the 11.5 dpc male urogenital ridges but not in the gonadal tissue (Fig. 1G,H, and Table 2).

Since Sertoli cells appear to be critical for testis morphogenesis (Palmer and Burgoyne, 1991) we also examined the pre-Sertoli cells in the newly formed seminiferous cords in the 12.5-13.5 day urogenital ridges (Table 1). Only a small number of non-expressing *lacZ* cells were found in this population, which may indicate the ebbing of the non-expressing cell population due to resumption of the X-linked gene activity.

In order to test if the developmental profile of transgene expression is related to X inactivation temporally and spatially, we assayed the expression of the *Xist* gene in urogenital ridges of 10.5 day fetuses and separately in the mesonephric and gonadal components of 11.5 to 13.5 day fetuses. *Xist* mRNAs were detected in the 10.5-day male urogenital ridges (Fig. 2). They were also detected in the gonadal component of the male urogenital ridges at 11.5, 12.5 and 13.5 days but not in the mesonephric component at 11.5 and 12.5 days. Diminished β-gal activity in the mesonephros, interstitial mesenchyme and coelomic epithelium of the 10.5 day male urogenital ridge, along with *Xist* expression in this tissue strongly indicate that X inactivation may occur in the XY somatic cells that contribute to the supporting cells of the seminif-

Fig. 1. X-chromosome activity in the urogenital ridges of H253 embryos visualized by expression of an X-linked *lacZ* transgene in the nucleus of the cells. *In* **(A,B,C,D and F)** *X-gal histochemistry shows cells expressing the X-linked lacZ transgene as blue while non-expressing cells are pink due to a nuclear fast red counterstain. In* **(E)** *immunohistochemistry shows cells positive for β-galactosidase antibody (brown). Negative cells are stained pink by the nuclear fast red counterstain. In* **(A)** *heart and gut tissues are fully expressing the transgene. Some examples of cells showing loss or diminished expression of the transgene are found in* **(B)** *the mesonephric tubule epithelial cells (arrowhead) of the 10.5-day male urogenital ridge and in* **(C)** *discrete patches of cells in the coelomic epithelium of the genital ridge (arrowheads) of the 10.5-day male. About 6% of the cells (arrows) in the mesenchymal compartment (asterisks) investing the mesonephros in* **(B)** *and subjacent to the coelomic epithelium in* **(C)** *also show diminished expression of the lacZ transgene. In the urogenital ridge of the 11.5-day male embryo in* **(D)** *non-expressing cells are encountered in the mesonephric tubule (arrowhead), mesenchyme (asterisk) and the coelomic epithelium of the gonadal part (arrowhead). Cells lining the mesonephric duct always maintain a strong expression of the transgene. In* **(E)** *the absence of the β-galactosidase protein from the mesonephric tubule epithelial cells (arrows) is demonstrated by the lack of diaminobenzidine (DAB) staining of a 11.5-day male embryo after immunostaining with a rabbit anti-E. coli β-galactosidase antibody. Examples of DAB stained cells are indicated by arrowheads. In the urogenital ridge of* **(F)** *a 10.5-day female embryo, a mosaic pattern of expressing and non-expressing cells is found in all tissue types of the urogenital ridge. In situ cell death detection in* **(G)** *shows the presence of apoptotic cells in the mesonephric but not the gonadal part of the urogenital ridge of the 11.5-day male embryo. In* **(H)** *enlargement of the boxed region in* **(G)** *the presence of apoptotic cells (arrowheads) in the mesonephric tubules is shown. Abbreviations: ao, aorta; at, atrium of the heart; ce, coelomic epithelium; g, gonadal part of urogenital ridge; gt, gut; m, mesonephric part of urogenital ridge; md, mesonephric duct; me, urogenital mesenchyme; mt, mesonephric tubule; vt, ventricle of the heart. Bar, 20 μm.*

erous tubules. There is no *Xist* expression in the mesonephric component of the 11.5 and 12.5 day urogenital ridges, whereas *Xist* expression is seen in the gonadal component of these tissues where diminished β-gal activity is also seen in a proportion of interstitial mesenchymal and pre-Sertoli cells.

Discussion

The somatic tissues of the urogenital ridges, particularly the Sertoli cell lineage, have been shown to be critical in sex determination (Palmer and Burgoyne, 1991). Light and electron-microscopic studies indicate that these cells may be derived from cells of the mesonephric region, the coelomic epithelium and/or the mesenchyme already incorporated into the genital ridge (Upadhyay *et al.*, 1979; McLaren, 1991; Merchant-Larios and Taketo, 1991; Smith and Mackay, 1991; Wartenberg *et al.*, 1991). Our results demonstrate that X-chromosome inactivation may occur in the somatic tissues in the male urogenital ridge at 10.5 dpc. This is supported by the finding of diminished β-galactosidase activity in a proportion of the mesonephric tubule cells, the mesenchymal cells of the urogenital ridge and the coelomic epithelial cells together with *Xist* expression from the 10.5 dpc male urogenital ridges. At 11.5 dpc, decreased β-galactosidase activity is again seen in a proportion of these possible sources of Sertoli cells, but at this stage apoptosis is also demonstrated in the mesonephric tubules. However, apoptosis is not encountered in the gonadal component of the developing urogenital ridge where the somatic components are the gonadal mesenchymal and coelomic epithelial cells. Loss of β-galactosidase activity in the mesonephric tubule epithelial cells at 11.5 dpc could then be attributable to death of these cells, but this cannot account for the decreased transgene activity in the non-apoptotic mesenchymal tissues in the urogenital ridge at this age. Loss of β-galactosidase activity is also seen in a small proportion of Sertoli cells in the 12.5 dpc gonads. That the decreased β-galactosidase activity in these somatic cells of the gonadal portion of the 11.5 dpc and 12.5 dpc urogenital ridges is related to the inactivation of the chromosome is reinforced by our finding of *Xist* expression in the gonadal part but not the mesonephric part of the urogenital ridges. At 13.5 dpc we found loss of β-galactosidase activity again in a small proportion of Sertoli cells. *Xist* expression was present primarily in the gonadal part of the urogenital ridge. To a lesser extent, the mesonephric component also expressed *Xist* but this is possibly related to contamination by gonadal tissues.

Sry is expressed in the male urogenital ridge at 10.5 to 12.5 dpc (Koopman *et al.*, 1990) and interestingly, more recent analysis of its spatial expression indicates that at 12 to 13 dpc it is only expressed in the gonadal portion and not the mesonephric component of the urogenital ridge (Jeske *et al.*, 1995). The temporal and spatial coincidence of decreased β-galactosidase activity, *Xist* expression and *Sry* expression raises the intriguing possibility that these events are linked. It has been postulated that the dosage sensitive sex reversal *(DSS)* locus on the short arm of the human X chromosome (Bardoni *et al.*, 1994) adjacent to the adrenal hypoplasia congenital locus *(AHC)*, must be repressed for *SRY* to exert its full effect on testis differentiation (Ogata and Matsuo, 1994). *DAX1 (DSS-AHC critical region on the X)* is a candidate gene isolated from this region and encodes an orphan nuclear hormone receptor (Zanaria *et al.*, 1994). Mouse *Dax1* is expressed in somatic cells of the genital ridge in males and females at 11.5 dpc but is downregulated in males at 12.5 dpc (Swain *et al.*, 1996). Our results and results from an earlier study of *Xist* expression in urogenital ridges of developing male embryos (Dolci *et al.*, 1994), suggest that the downregulation of this locus may be accomplished by the overall inactivation of the X chromosome in the male at a critical time of sex determination. Whether the *DSS* locus is indeed inactivated during male gonadal development in the human and if *XIST* is involved are not known. If they were, sex reversal could arise if the duplicated Xp interfered with the normal X inactivation process, resulting in an excess of *DSS* gene product which counteracts the activity of the *SRY* gene, and consequently female development.

TABLE 1

THE POPULATION OF β-GAL NON-EXPRESSING CELLS IN 10.5- TO 13.5-DAY MALE AND FEMALE UROGENITAL RIDGES

Age (days p.c.)	% cells with inactive *HMG-lacZ* transgene					
	XY			XX		
	Median	Range	N	Median	Range	N
Mesonephric tubule epithelial cells						
10.5	5.8	0-10	10	59	56-62	6
11.5	12.5	9.5-28	9	57	43-60	7
Interstitial mesenchymal cells						
10.5	3.2	0-5.6	10	50	48-53	5
11.5	3.2	1.6-3.7	9	50	50-54	7
Coelomic epithelium						
10.5	2.0	0-5	10	49	43-54	6
11.5	1.8	0-4	8	50	50-51	7
Supporting cells (Sertoli cells in XY, presumptive follicular cells in XX)						
12.5	0.8	0-3	12	50.5	48-53	6
13.5	3.9	0-10	10	51	47-54	7

N, number of samples. Due to the sample size and the departure of the data from a normal distribution, only the median and the range of the percentages are presented. Results of statistical analyses by the non-parametric Mann-Whitney test show that the cell counts for the mesonephric tubule epithelial cells of the 11.5-day male urogenital ridges are significantly different from those at 10.5 days and the supporting cells at 12.5 and 13.5 days ($p<0.05$, 2-tailed). Cell counts for the mesonephric tubule epithelial cells of the 10.5-day female urogenital ridges are significantly different from the supporting cells at 12.5 and 13.5-days ($p<0.05$, 2-tailed). Cell counts of the interstitial mesenchymal cells and the coelomic epithelial cells do not differ significantly from the supporting cells in the male or female embryos.

TABLE 2

THE POPULATION OF APOPTOTIC CELLS IN MESONEPHRIC TUBULE EPITHELIAL CELLS OF 11.5-DAY MALE UROGENITAL RIDGES

% TUNEL positive cells		
Median	Values	N
41.5	35,40,43,45	4

N, number of samples.

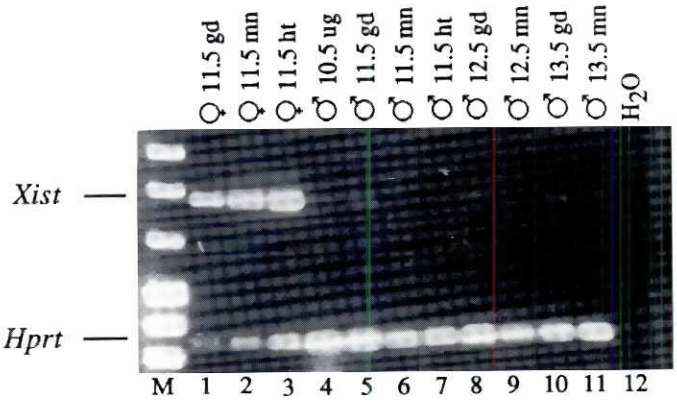


Fig. 2. *Xist* expression, analyzed by reverse transcription and PCR, in urogenital ridges, dissected gonadal and mesonephric components of urogenital ridges and gonads, and hearts of male and female 10.5- to 13.5-day fetuses. *The Xist transcript gives a 864 base pair (bp) RT-PCR product. An Xist signal was detected in male urogenital ridges at 10.5 dpc* **(lane 4)** *and the gonadal components* **(lanes 5 and 8)** *but not the mesonephric components of urogenital ridges at 11.5 dpc and 12.5 dpc* **(lanes 6 and 9)**. *At 13.5 dpc a signal was detected in both components* **(lanes 10 and 11)** *but was weaker in the mesonephric component* **(lane 11)**. *Xist transcript was absent in male heart tissue* **(lane 7)**. *The 864 bp Xist band was always present in female urogenital ridges, both gonadal and mesonephric components* **(lanes 1 and 2)** *and heart tissue* **(lane 3)**. *A Hprt band of 352 bp was present in all samples showing comparable efficiency has been achieved in the isolation of RNA from different tissue samples. In this experiment, RT-PCR was performed using* **(lane 1)** *total RNA from gonadal component of urogenital ridges of one 11.5-day female,* **(lane 2)** *total RNA from mesonephric component of urogenital ridges of one 11.5-day female,* **(lane 3)** *total RNA from heart of one 11.5-day female,* **(lane 4)** *total RNA from urogenital ridges of ten 10.5-day male embryos,* **(lane 5)** *total RNA from gonadal components of urogenital ridges of six 11.5-day male embryos,* **(lane 6)** *total RNA from mesonephric components of urogenital ridges of six 11.5-day male embryos,* **(lane 7)** *total RNA from heart of one 11.5-day male,* **(lane 8)** *total RNA from gonadal components of urogenital ridges of six 12.5-day male embryos,* **(lane 9)** *total RNA from mesonephric components of urogenital ridges of six 12.5-day male embryos,* **(lane 10)** *total RNA from gonadal components of gonads of four 13.5-day male embryos,* **(lane 11)** *total RNA from mesonephric components of gonads of four 13.5-day male embryos,* **(lane 12)** *H_2O. The* **(M lane)** *displays the molecular markers with sizes ranging from 1,114 to 320 base length. gd, gonadal component of urogenital ridges (11.5-12.5-day) or gonads (13.5-day); mn, mesonephric component of urogenital ridges or gonads; ug, urogenital ridges (10.5-day); ht, heart.*

Our results indicate that if X-chromosome inactivation is playing a role in somatic cells in the developing male gonad at a critical time, this may occur only in a relatively small population of the cells. In the adult testes of XX-XY chimeric mice, the XX and XY components contribute randomly to the somatic cell populations except for the Sertoli cells which are predominantly XY (Burgoyne *et al.*, 1988). Interestingly, in chimeric fetuses examined at 13.5 to 16.5 dpc, the Sertoli cell population may contain as much as 21% XX cells (Palmer and Burgoyne, 1991). This indicates that in the early stages of urogenital ridge development the XY supporting cells are able to recruit XX cells into the pre-Sertoli cell population. Perhaps at an earlier gestation the XY pre-Sertoli population may be an even smaller percentage, since the XX cells appear to be selected against with time (Palmer and Burgoyne, 1991). In the male urogenital ridge perhaps only the activity of a small population of XY cells is critical in the sex determination pathway, and these may constitute the cell population that has to inactivate the X chromosome or specific locus. These cells, then fully expressing *Sry* and other sex-determining genes, may lead the morphogenesis of the testis by recruiting other somatic cells to the male differentiation pathway (Capel, 1995).

Materials and Methods

Embryos

Transgenic H253 mice used in this study carry on their X chromosome a *lacZ* transgene under the control of the 5' regulatory elements of the mouse *3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG)* gene (Tan *et al.*, 1993; Tam *et al.*, 1994a,b). Homozygous female transgenic H253 mice, carrying the X-linked *lacZ* transgene on both X chromosomes (Tam and Tan, 1992), were mated with wild type F1(C57BL/6 x DBA/2) males. In this mating all offspring are transgenic and are of two types: transgenic males (X-chromosome carrying the transgene) and hemizygous females (one of the two X chromosomes carrying the transgene). Fetuses were collected from pregnant mice at 10.5 to 13.5 dpc. To distinguish males and females, the cephalic regions of the embryos were isolated and stained with X-gal (Tan *et al.*, 1993). Females can be distinguished from males by their characteristic mosaic staining pattern when examined under the dissection microscope using incident illumination. For 13.5-day specimens, the sex of the fetal specimen was confirmed by the morphology of the fetal gonad. For 10.5-12.5-day specimens, the genotype as revealed by X-gal staining reaction was confirmed by PCR analysis for *Sry* on genomic DNA extracted from somatic tissues outside the urogenital ridge.

β-gal histochemistry and immunohistochemistry

β-gal is seen as a blue reaction product in the nucleus following histochemical reaction with the X-gal substrate. For β-gal histochemistry of the urogenital ridges, the trunk of the fetus was dissected to expose the urogenital ridges. In 11.5-13.5-day fetuses, most of the flank and neural and somitic structures dorsal to the urogenital ridges were removed to facilitate penetration of the histochemical reagents. The urogenital ridges were fixed by 4% paraformaldehyde for 2 min at room temperature, washed several times in PBS and stained at 37°C overnight with X-gal staining solution (Tan *et al.*, 1993). The stained specimens were processed for wax histology. Serial 8 μm sections were cut either longitudinally or transversely to the urogenital ridges.

For the immunohistochemical detection of β-galactosidase in the 11.5-day male embryos, the trunks were dissected in 4% paraformaldehyde fixative (in Ca^{++}/Mg^{++}-free phosphate buffer) and fixed for 4 h. Sixty μm thick slices were obtained using a cryostat and incubated overnight with rabbit anti-β galactosidase antibody (Cappel, 1:1000 dilution). Secondary antibodies were biotinylated goat anti-rabbit IgG (Vector, 1:400 dilution) and biotin-avidin complex (Vector Elite ABC kit, 1:100 dilution) in antibody buffer solution containing 0.2% Triton X. The immunohistochemical reaction was revealed with diaminobenzidine (0.5 mg/ml) in the presence of 0.01% H_2O_2. The specimens were processed for wax histology. Sections were cut at 8 μm and counterstained with 0.1% (w/v) nuclear fast red.

In situ cell death detection

A modification of the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) procedure (Gavrieli *et al.*, 1992) was used to detect cells containing fragmented DNA. The trunks of 11.5-day male embryos were dissected, fixed in 4% paraformaldehyde for 2 h and processed for wax histology. Serial sections of 8 μm thickness were cut longitudinally to the urogenital ridges. Sections were dewaxed, rehydrated and endogenous peroxidase blocked with 3% (v/v) hydrogen peroxide. Sections were incubated with Proteinase K (20 μg/ml in Tris/HCl) and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Slides were incubated with TUNEL reaction mixture (Boehringer Mannheim, Cat. No. 1684817) of terminal deoxynucleotidyl transferase (TdT) from calf

thymus and nucleotide mixture with fluorescein for 60 min at 37°C. TdT catalyzes the polymerization of nucleotides to the free 3'-OH DNA ends which are seen with the cleavage of genomic DNA that occurs during apoptosis. Sections were incubated with anti-fluorescein antibody Fab fragments from sheep conjugated with horse-radish peroxidase and then reacted with diaminobenzidine (DAB, 1 mg/ml) and hydrogen peroxide (0.0225%) for 2-7 min for color development. Stained sections were dehydrated and mounted in Entellan (Merck).

RT-PCR

RNA preparations were made from urogenital ridges at 10.5 dpc, the genital and mesonephric components of urogenital ridges at 11.5-13.5 dpc and hearts dissected from individual embryos, using a modification of the method described by Chomczynski and Sacchi (1987). Tissues were lysed in 100 μl of TRIzol reagent (Gibco BRL) containing guanidine isothiocyanate and phenol. Chloroform (20 μl) was added, the aqueous phase collected and the RNA was precipitated with 50 μl of isopropanolol. First strand cDNA was synthesized using a SuperScript II kit (Gibco BRL, Cat. No. 18089-011) with random hexamers to prime the synthesis. The 20 μl reaction mix contained the total RNA sample from the urogenital ridges, the genital or mesonephric components, or from the heart, 50 ng of random hexamers, 10 mM dithiothreitol, 1xSuperScript buffer, 0.5 mM dNTP mix, and 200 U of SuperScript II reverse transcriptase (Gibco BRL). The reaction was carried out at 42°C for 60 min and terminated by heating to 70°C for 15 min. Any RNA/DNA hybrids that might be present were digested with 0.5 U of RNase H at 37°C for 20 min and terminated by incubation at 95°C for 5 min. The entire cDNA product was used for PCR amplification in a 50 μl reaction containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.25 μM primers and 2.5 U of Taq polymerase. Amplifications consisted of an initial 5 min denaturation at 95°C, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by a 15 min extension at 72°C. A 20 μl aliquot was analyzed on gels of 1.5% agarose and ethidium-stained gels were photographed directly. Primers which spanned intron and exon boundaries were (5'-3'): *Xist* (Kay *et al.*, 1993) CATCACAACAGCAGTTCTCC and TTTAAGATGCTGCAGTCAGG and *Hprt* (Koopman *et al.*, 1989) CCTGCTGGATTACATTAAAGCACTG and GTCAAGGGCATATCCAACAACAAAC.

PCR

For *Sry* PCR analysis on the embryos whose tissues were used for X-gal histochemistry, 0.1 μg genomic DNA was added to a 25 μl reaction mix containing 1 mM each dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM primers and 0.5 U Taq polymerase. Amplifications consisted of a 5 min denaturation at 95°C, followed by 40 cycles of 53°C for 40 sec, 72°C for 1 min, and 94°C for 30 sec, followed by a 10 min extension at 72°C. *Sry* primers 5'-3' were: CTGTGTAAGATCTTCAATC and GTGGTGAGAGGCACAAGT (Gubbay *et al.*, 1990). For *Sry* PCR analysis in 10.5-12.5-day embryos used for *Xist* RT-PCR and 11.5-day embryos used for *in situ* cell death detection, 0.1 μg genomic DNA was added to a 50 μl reaction mix containing 1 mM each dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM primers and 0.5 U Taq polymerase. Amplifications consisted of a 5 min denaturation at 95°C, followed by 35 cycles of 53°C for 40 sec, 72°C for 1 min, and 93°C for 30 sec, followed by a 15 min extension at 72°C. *Sry* primers 5'-3' were: TTCAGCCCTACAGCCACATGA and ATGTGGGTTCCTGTCCCACTG (Mulder *et al.*, 1993). For control, PCR of *Hprt* was performed using the following primers (5'-3'): ATACAAAGCCTAAGATGAGC and TATTCCACTGAGCAAAACCT.

Scoring for non-expressing cells

The population of non-expressing *lacZ*-transgenic cells was determined by scoring the number of nuclear-fast red stained cells in randomly selected areas of the epithelium of the mesonephric tubule, interstitial mesenchyme and coelomic epithelium of 10.5-11.5-day male and female urogenital ridges. In 12.5 and 13.5-day fetuses, the Sertoli cells in the newly formed testicular cord (recognizable as seminiferous tubules by 13.5 days) and the cells surrounding the oocytes in the fetal ovary were analyzed. Between 60 to 100 cells were counted in several sections of each specimen and the number of expressing (blue nucleus) and non-expressing cells (red nucleus) was scored. The size of the non-expressing cell population was then computed as the percentage of the total number of cells counted. In the female fetuses, the non-expressing population is expected to be close to 50% if random inactivation of the X chromosome has occurred. The X chromosome of the male is normally expected to remain active. The presence of non-expressing cells in the transgenic male animal therefore indicates that these cells may have switched off the only X chromosome and become nullisomic for X-linked activity.

Scoring for apoptotic cells

The population of apoptotic cells in the mesonephric tubule epithelium of the 11.5-day male urogenital ridges was determined by scoring the number of TUNEL positive and TUNEL negative cells in randomly selected areas of the epithelium. Sixty to 100 cells were counted in each specimen and the size of the TUNEL positive population was computed as a percentage of the total number of cells counted.

Acknowledgments

We thank Peter Rowe, Graham Kay, Peter Koopman and Anne McLaren for their helpful comments on the manuscript, and Bev Faulkner-Jones for performing the immunohistochemistry. RVJ was a Medical Postgraduate Scholar of the National Health and Medical Research Council (NH & MRC) of Australia. Our work was supported by the NH & MRC and Mr James Fairfax.

References

BARDONI, B., ZANARIA, E., GUIOLI, S., FLORIDIA, G., WORLEY, K.C., TONINI, G., FERRANTE, E., CHIUMELLO, G., McCABE, E.R.B., FRACCARO, M., ZUFFARDI, O. and CAMERINO, G. (1994). A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. *Nature Genet. 7:* 497-501.

BROCKDORFF, N., ASHWORTH, A., KAY, G.F., McCABE, V.M., NORRIS, D.P., COOPER, P.J., SWIFT, S. and RASTAN, S. (1992). The product of the mouse *Xist* gene is a 15kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell 71:* 515-526.

BROWN, C.J., HENDRICH, B.D., RUPERT, J.L., LAFRENIERE, R.G., XING, Y., LAWRENCE, J. and WILLARD, H.F. (1992). The human *XIST* gene: analysis of a 17kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell 71:* 527-542.

BURGOYNE, P.S., BUEHR, M., KOOPMAN, P., ROSSANT, J. and McLAREN, A. (1988). Cell-autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XX-XY chimaeric mouse testes. *Development 102:* 443-450.

CAPEL, B. (1995). New bedfellows in the mammalian sex-determination affair. *Trends Genet. 11:* 161-163.

CHOMCZYNSKI, P. and SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem. 162:* 156-159.

DOLCI, S., GEREMIA, R., ALBANESI, C. and ROSSI, P. (1994). Expression of the *Xist* in urogenital ridges of midgestation male embryos. *Biochem. Biophys. Res. Commun. 205:* 334-340.

GAVRIELI, Y., SHERMAN, Y. and BEN-SASSON, S.A. (1992). Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol. 119:* 493-501.

GUBBAY, J., COLLIGNON, J., KOOPMAN, P., CAPEL, B., ECONOMOU, A., MUNSTERBERG, A., VIVIAN, N., GOODFELLOW, P. and LOVELL-BADGE, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature 346:* 245-250.

JESKE, Y.W.A., BOWLES, J., GREENFIELD, A. and KOOPMAN, P. (1995). Expression of a linear *Sry* transcript in the mouse genital ridge. *Nature Genet. 10:* 480-482.

KAY, G.F., PENNY, G.D., PATEL, D., ASHWORTH, A., BROCKDORFF, N. and RASTAN, S. (1993). Expression of *Xist* during mouse development suggests a role in the initiation of X chromosome inactivation. *Cell 72:* 171-182.

KING, V., KORN, R., KWOK, C., RAMKISSOON, Y., WUNDERLE, V. and GOODFELLOW, P. (1995). One for a boy, two for a girl? *Curr. Biol. 5:* 37-39.

KOOPMAN, P., GUBBAY, J., COLLIGNON, J. and LOVELL-BADGE, R. (1989). *Zfy* gene expression patterns are not compatible with a primary role in mouse sex determination. *Nature 342:* 940-942.

KOOPMAN, P., GUBBAY, J., VIVIAN, N., GOODFELLOW, P. and LOVELL-BADGE, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. *Nature 351:* 117-121.

KOOPMAN, P., MUNSTERBERG, A., CAPEL, B., VIVIAN, N. and LOVELL-BADGE, R. (1990) Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature 348:* 450-452.

LYON, M. F. (1961). Gene action in the X chromosome of the mouse *(Mus musculus L.). Nature 190:* 372-373.

McCARREY, J.R. and DILWORTH, D.D. (1992). Expression of *Xist* in mouse germ cells correlates with X-chromosome inactivation. *Nature Genet. 2:* 200-203.

McCOSHEN, J.A. (1982). *In vivo* sex differentiation of congenic germinal cell aplastic gonads. *Am. J. Obstet. Gynecol. 142:* 83-88.

McLAREN, A. (1991). Development of the mammalian gonad: the fate of the supporting cell lineage. *BioEssays 13:* 151-156.

MERCHANT, H. (1975). Rat gonadal and ovarian organogenesis with and without germ cells. An ultrastructural study. *Dev. Biol. 44:* 1-21.

MERCHANT-LARIOS, H. and TAKETO, T. (1991). Testicular differentiation in mammals under normal and experimental conditions. *J. Electron. Microsc. Tech. 19:* 158-171.

MULDER, L.C., SACCO, M.G., MANGIARINI, L., BROWN, J., COLLOTTA, A., VILLA, A., DE GIOVANNI, A.M., VEZZONI, P. and CLERICI, L. (1993). Preimplantation embryo sexing by polymerase chain reaction amplification of the *sry* gene on single mouse blastomeres. *Genet. Analysis Tech. Appl. 10:* 147-149.

OGATA, T. and MATSUO, N. (1994). Testis determining gene(s) on the X chromosome short arm: chromosomal localisation and possible role in testis determination. *J. Med. Genet. 31:* 349-350.

PALMER, S.J. and BURGOYNE, P.S. (1991). *In situ* analysis of fetal, prepuberal and adult XX-XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. *Development 112:* 265-268.

RAMKISSOON, Y. and GOODFELLOW, P. (1996). Early steps in mammalian sex determination. *Curr. Opin. Genet. Dev. 6:* 316-321.

RICHLER, C., SOREQ, H. and WAHRMAN, J. (1992). X inactivation in mammalian testis is correlated with inactive X-specific transcription. *Nature Genet. 2:* 192-195.

SALIDO, E.C., YEN, P.H., MOHANDAS, T.K. and SHAPIRO, L.J. (1992). Expression of the X-inactivation-associated gene *XIST* during spermatogenesis. *Nature Genet. 2:* 196-199.

SINCLAIR, A.H., BERTA, P., PALMER, M.S., HAWKINS, J.R., GRIFFITHS, B.L., SMITH, M.J., FOSTER, J.W., FRISCHAUF, A., LOVELL-BADGE, R. and GOODFELLOW, P.N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature 346:* 240-244.

SMITH, C. and MACKAY, S. (1991). Morphological development and fate of the mouse mesonephros. *J. Anat. 174:* 171-184.

SWAIN, A., ZANARIA, E., HACKER, A., LOVELL-BADGE, R. and CAMERINO, G. (1996). Mouse *Dax1* expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. *Nature Genet. 12:* 404-409.

TAM, P.P.L. and TAN, S-S. (1992). The somitogenetic potential of cells in the primitive streak and the tail bud of the organogenesis-stage mouse embryo. *Development 115:* 703-715.

TAM, P.P.L., WILLIAMS, E.A. and TAN, S-S. (1994a). Expression of an X-linked *HMG-lacZ* transgene in mouse embryos: implication of chromosomal imprinting and lineage-specific X-chromosome activity. *Dev. Genet. 15:* 491-503.

TAM, P.P.L., ZHOU, S.X. and TAN, S-S. (1994b). X-chromosome activity of the mouse primordial germ cells revealed by the expression of an X-linked *lacZ* transgene. *Development 120:* 2925-2932.

TAN, S-S., WILLIAMS, E.A. and TAM, P.P.L. (1993). X-chromosome inactivation occurs at different times in different tissues of the post-implantation mouse embryo. *Nature Genet. 3:* 170-174.

UPADHYAY, S., LUCIANI, J.M. and ZAMBONI, L. (1979). The role of the mesonephros in the development of indifferent gonads and ovaries of the mouse. *Ann. Biol. Anim. Biochim. Biophys. 19(4B):* 1179-1196.

WARTENBERG, H., KINSKY, I., VIEBAHN, C. and SCHMOLKE, C. (1991). Fine structural characteristics of testicular cord formation in the developing rabbit gonad. *J. Electron. Microsc. Tech. 19:* 133-157.

WYLLIE, A.H., KERR, J.F.R. and CURRIE, A.R. (1980). Cell death: the significance of apoptosis. *Int. Rev. Cytol. 68:* 251-306.

ZANARIA, E., MUSCATELLI, F., BARDONI, B., STROM, T.M., GUIOLI, S., GUO, W., LALLI, E., MOSER, C., WALKER, A.P., McCABE, E.R.B., MEITINGER, T., MONACO, A.P., SASSONE-CORSI, P. and CAMERINO, G. (1994). An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. *Nature 372:* 635-641.

Received: October 1996
Accepted for publication: December 1996