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TRANSCRIPTIONAL ACTIVITY OF SEX CHROMOSOMES IN THE OOCYTES OF THE B6.Y^{TIR} SEX-REVERSED FEMALE MOUSE

by

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Abstract

In the B6.Y^{TIR} mouse strain, half of the XY progeny develop bilateral ovaries and the female phenotype. These XY females are infertile mainly due to the death of their embryos. This developmental failure has been attributed to a defect intrinsic to the XY oocyte.

The present study examined the transcriptional activity of the X and Y chromosomes in these oocytes. RT-PCR results show that the *Ube1y* gene is transcribed in the XY ovary at all stages examined and also in growing XY oocytes. The *Sry* gene was transcribed only at the onset of ovarian differentiation whereas the *Zfy* gene was undetectable at all stages during fetal life. The *Xist* gene, which is involved in X inactivation, was not expressed in XY oocytes. We speculate that expression of Y-encoded genes may have a deleterious effect on the quality of the oocytes and thus renders them incompetent for post-fertilization development.

Résumé

Dans la race des souris B6.Y^{TIR} la moitié des progenus XY développe des ovaires, et sont de phénotype féminin. Ces femelles XY sont infertiles essentiellement à cause de l'arrêt du développement de leurs embryons. Cet échec est attribué à une anomalie intrinsèque des ovocytes XY.

Dans cette étude, on s'est intéressé à examiner la transcription des chromosomes X et Y dans ces ovocytes. La RT-PCR démontre que le gêne *Ubely* est transcrit dans tous les ovaires examinés, et au différent stades de différentiation ovocytaire. Alors que le gêne *Sry* n'est transcrit qu' au début de la phase de différenciation ovocytaire, le gêne *Zfy* n'était pas transcrit durant tous les stades fœtaux examinés. D'autre part, le gêne *Xist*, qui est impliqué dans l'inactivation de chromosome X, n'était pas exprimé dans les ovocytes XY. On suppose alors que l'expression des gênes spécifiques par le chromosome Y altère la qualité des ovocytes, et les rendent incapables de se développer après la fertilisation.

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Acknowledgements

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R :-)

1-Introduction

1.1 Sex Determination and Differentiation

Eutherian mammals follow Jost's principle of sexual differentiation (Jost, 1958; Fig.1). Once the genetic sex (XX or XY) is established at the time of fertilization, it governs the developmental fate of the undifferentiated gonad (i.e. gonadal sex).Typically, in XX individuals, the gonadal primordium differentiates into an ovary and the female phenotype develops whereas in XY individuals it develops into a testis and therefore the development of the male phenotype follows.

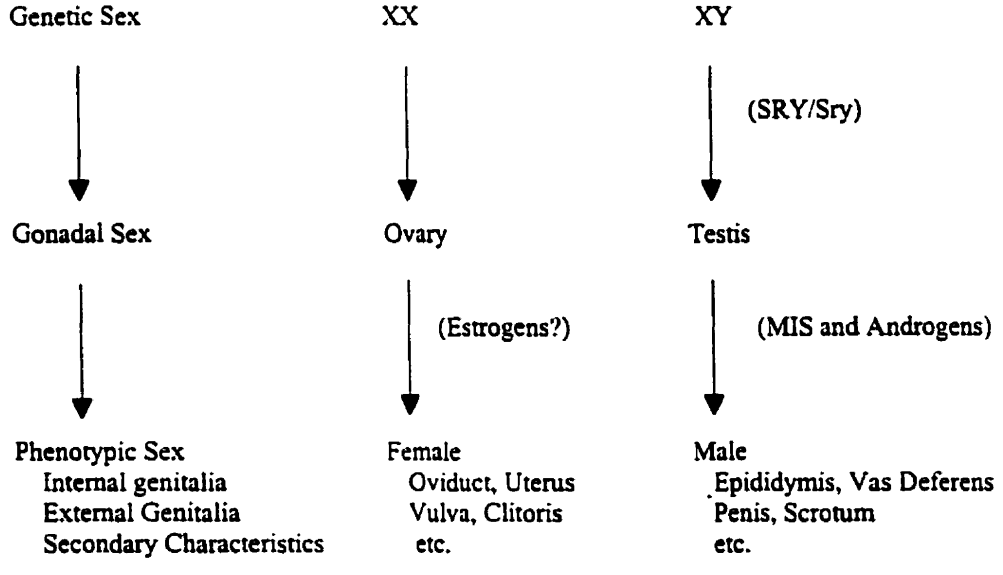

Fig.1 Sexual differentiation in mammals. An elaboration on Jost's paradigm (1958).

Testicular differentiation is the first identifiable step in the male sex determination pathway. At the centre of this event is the expression of the Y- linked gene *SRY/Sry* (humans and mice, respectively) (Sinclair et al., 1990; Gubbay et al., 1990; Bertha et al., 1990; Koopman et al., 1990). The pivotal role of this gene in testis determination was demonstrated in an elegant study where the *Sry* transgene induced testicular differentiation, causing XX fetuses to develop as phenotypic (albeit infertile) males (Koopman et al., 1991).

In addition to the pair of sexually undifferentiated gonads, each embryo also possesses two sets of mesonephric duct systems: the Wolffian and the Müllerian ducts. Once a testis is formed, it produces two hormones that are essential for male phenotypic development. The first is called Müllerian Inhibiting Substance (MIS) or Anti-Müllerian Hormone (AMH). This is a glycoprotein produced by the fetal Sertoli cells and it causes the regression of the Müllerian ducts in the male fetus. The other product is the androgenic steroid testosterone which is secreted by the fetal Leydig cells. Testosterone causes the virilization of the Wolffian ducts into the male internal genitalia, promotes the development of the male external genitalia, and also plays a role in the development of the male secondary sexual characteristics at puberty.

In the absence of a testis and, hence, of its hormones, the Wolffian ducts regress whereas the Müllerian ducts differentiate and give rise to the female reproductive tract. It is not clear whether the fetal ovary produces hormones, and if it does, whether they play a role in the development of the female phenotype in eutherian mammals; it is experimentally not possible to eliminate the exogenous source of female hormones

provided by the mother and the placenta (George and Wilson, 1994). Female hormones do however play a role in the development of the female secondary sexual characteristics at puberty.

1.2 Gametogenesis

The gonad is composed of two cell types: somatic and germ. While the somatic component of the primitive gonad is believed to originate from the coelomic epithelium of the mesonephros, the germ cells arise from an extra-gonadal site.

The primordial germ cells (PGCs) are derived from the primitive ectoderm (also known as the epiblast) (Lawson and Hages, 1994). They are morphologically distinguishable from the somatic cells of the developing embryo because of their large size, large round nuclei, and clear cytoplasm (Politzer, 1928, 1933; Everett, 1943; Witschi, 1948). In addition, they can histochemically be identified by their high alkaline phosphatase and glycogen contents (McKay et al., 1953; Chiquioin, 1954). Using these criteria, PGCs can be recognized in the extraembryonic mesoderm as early as 7- 7.5 days post coitum (dpc) (Ginsburg et al., 1990). From there, they migrate into the embryo, through the hindgut, into the mesoderm of the mesentery, and colonize the genital ridge by 10.5 -11.5 dpc.

While migrating, the PGCs actively proliferate. Their population increases from a mere 10-100 cells to 2500-3000 cells by the time they reach the genital ridge. Once in the gonad, they continue to divide to further increase their population. Following sexual differentiation of the gonad, the fate of PGCs is divided into one of two pathways:

whether to remain mitotic or to enter meiosis. This decision is made by the gonadal environment (i.e. testis or ovary) the PGC inhabits and occurs regardless of the PGC's own chromosomal sex (McLaren, 1983).

Spermatogenesis

If the PGCs are in a testis, they divide mitotically a few more times, differentiate into prespermatogonia[1] and enter mitotic arrest before birth. Soon after birth the prespermatogonia differentiate into spermatogonia and active spermatogonial proliferation begins (Fig. 2). Spermatogonia are stem cells which can therefore both regenerate themselves as well as differentiate into other spermatogenic cell types. Thus, some of the spermatogonia undergo a number of cell divisions and differentiate to become primary spermatocytes.

After the last round of DNA replication, the primary spermatocytes enter the prophase of meiosis I which, for convenience sake, is divided into four progressive stages: leptotene, zygotene, pachytene and diplotene. During this period the homologous chromosomes pair, synapse, and cross over to exchange genetic material. The X and Y chromosomes also pair, albeit their pairing is limited to the pseudoautosomal region (PAR) (Burgoyne, 1982). It has been postulated that X-Y pairing and crossing over (as well as that of the other chromosomes) is essential for the orderly disjunction and segregation of the homologous chromosomes in the subsequent stages of meiosis (Miklos,

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Please note: some authors use different prefixes before the word spermatogonia e.g. pro, M, or T.

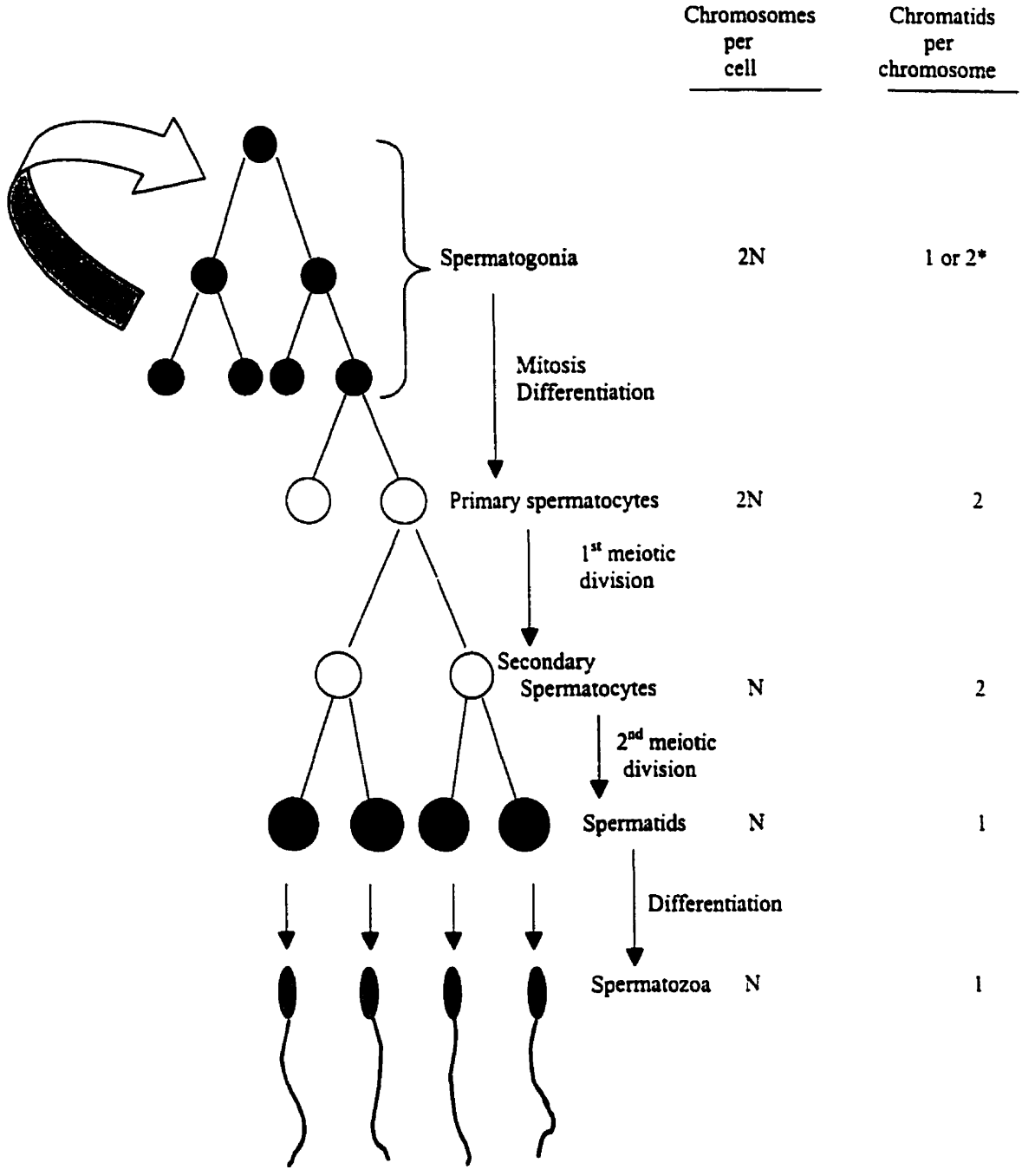

Fig. 2. Spermatogenesis (Adapted from Vander et al., 1994)
* 1 chromatid per chromosome at G_1 and 2 chromatids per chromosome at G_2

1974; Burgoyne , 1982; Evans et al., 1982). A failure to pair and synapse is deleterious to the formation of viable sperm (Burgoyne, 1982, 1986). Interestingly, between the zygotene and mid-pachytene stages of meiotic prophase I, the X-Y pair can be identified as a large condensed body associating with the nuclear envelope. This heterochromatic mass is referred to as the XY body, sex body or sex vesicle.

Upon completion of the first meiotic division, the primary spermatocyte produces two secondary spermatocytes. Each of the latter will in turn complete the second meiotic division to produce two haploid cells called spermatids. The spermatids then undergo a long process of differentiation called spermiogenesis to become functional sperm.

Oogenesis

PGCs in the mouse ovary, referred to as oogonia, also continue to divide mitotically to further increase their population. However, mitotic proliferation of oogonia is limited to the prenatal period (Fig. 3).

Between 13.5 and 15.5 dpc, all oogonia differentiate into primary oocytes and enter meiosis. This is in contrast to the primary spermatocyte, where meiosis is initiated after birth. In addition, upon entry of the primary oocytes into meiosis, no new germ cells are generated whereas only a pool of mitotically-dividing spermatogonia differentiate into primary spermatocytes which enter meiosis.

The primary oocyte progresses through the leptotene, zygotene, and pachytene stages of the first meiotic prophase. Although the distribution of primary oocytes at the first meiotic prophase varies according to different investigators, most agree that by

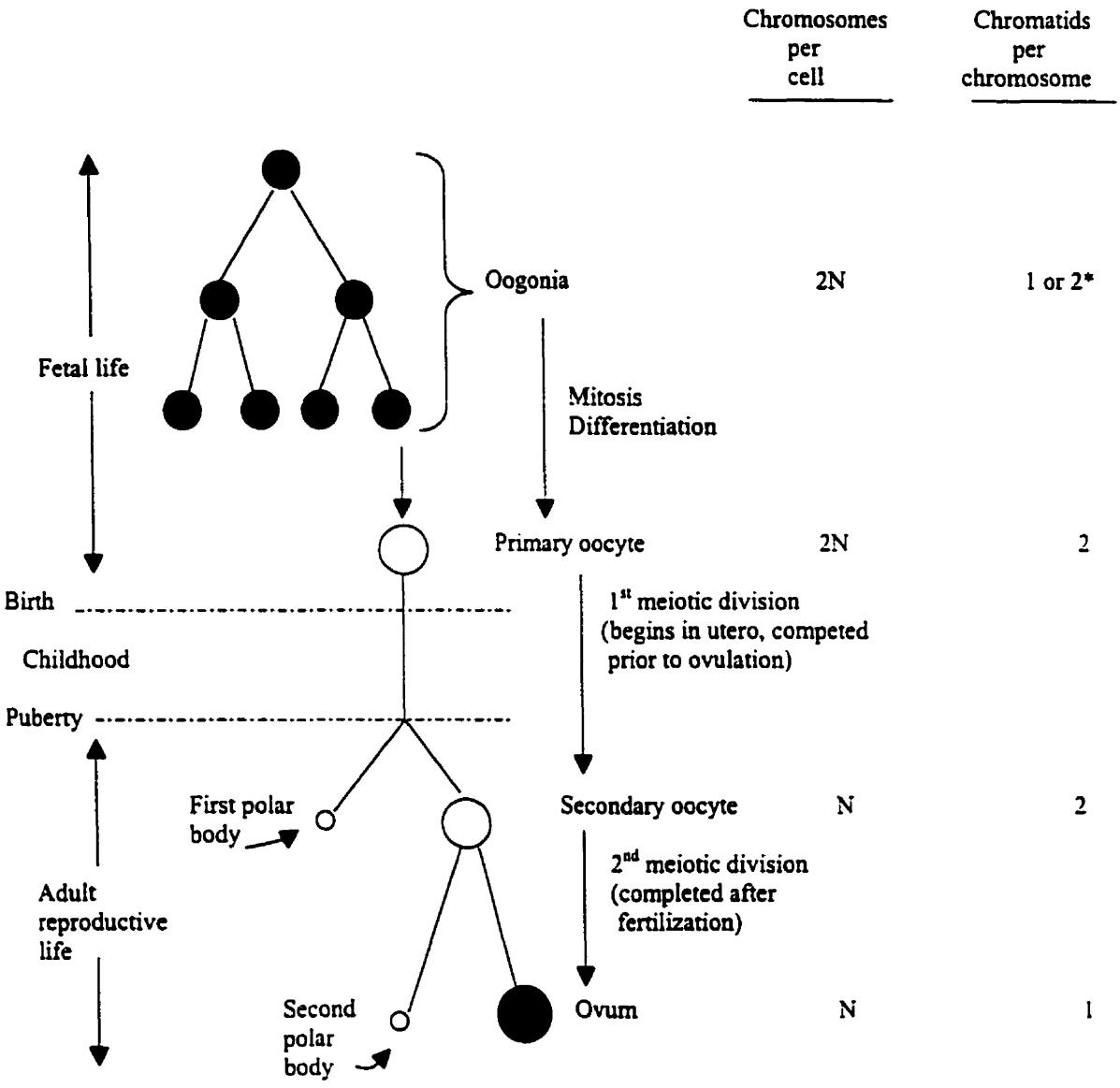

Fig. 3. Oogenesis (Adapted from Vander et al., 1994)
* 1 chromatid per chromosome at G_1 and 2 chromatids per chromosome at G_2

Table I.
Distribution of primary oocytes in the first meiotic prophase (Adapted from Speed, 1982)

Meiotic Stage	Day of gestation							
	13	14	15	16	17	18	19	20
Pre-leptotene	*****	****	**	*	*			
Leptotene		*	**	•	•	•		
Zygotene				•	•	*		
Late Zyg-early pach			*	***	**	***	*	
Pachytene					*	*	**	*
Diplotene-Dictyate							**	****

* Denotes relative abundance at each stage; it does not have any numeric or fractional value such that "*****" means "a large portion of oocytes" and "•" means "a negligible portion".

Table II.
Distribution of primary oocytes in the first meiotic prophase (Based on Borum, 1961, and the review by Wassarman and Kinloch, 1992)

Meiotic Stage	Day of gestation							
	13	14	15	16	17	18	19	20
Pre-leptotene	*****	**						
Leptotene		**						
Zygotene			***					
Late Zyg-early pach			**	•				
Pachytene				****	*****	***	**	
Diplotene-Dictyate						**	****	*****

See Table I for description of symbols.

embryonic day 19 the majority of the oocytes have reached the diplotene stage[2] (Table I and II) (Borum, 1961; Speed, 1982; Wassarman and Kinloch, 1992). The primary oocytes however, do not immediately complete the first meiotic division. Instead they become arrested prior to or soon after birth. At this stage, the diplotene oocyte becomes surrounded by a layer of flattened epithelial cells enclosed by an intact basal lamina. This structure, called a follicle, serves to provide a unique environment for the growth and maturation of the oocyte.

Approximately two weeks after birth, under the influence of the follicle stimulating hormone (FSH) secreted by the pituitary gland, several primordial follicles are recruited for growth. During follicular growth, the primary oocyte also "grows", increasing its size nearly 300-fold, transcribing and accumulating large amounts of mRNA. This RNA is quite stable and will be used to support fertilization and preimplantation development (reviewed by Wassarman and Kinloch, 1992).

Ovulation occurs when the wall of the follicle ruptures and the oocyte is released. This process is under the influence of another pituitary hormone called luteinizing hormone (LH).

It is only immediately before ovulation that the primary oocyte exits the meiotic arrest. Resumption of meiosis is demarcated by germinal vesicle breakdown (GVB) which is the disintegration of the nuclear envelope in the oocytes. Shortly after GVB, the

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Please note that reports on the stages of oocytes vary from one investigator to another. This discrepancy is most likely due to the differences in the mouse strains or the techniques they used to visualise the chromosomes.

primary oocyte completes the first meiotic division by extruding a polar body. The egg is now referred to as a secondary oocyte.

It is only upon fertilization that the secondary oocyte completes the second meiotic division by extruding yet another polar body.

Thus, unlike spermatogenesis, where each primary spermatocyte results in four functional spermatozoa, a primary oocyte produces only a single ovum along with two small, non-functional polar bodies (Fig. 2 and 3).

Role of Sex Chromosomes in Gametogenesis

The early stages of gametogenesis do not appear to be affected by the sex chromosomal composition. As such, XY germ cells can enter meiosis in the fetal ovary and XX germ cells can undergo mitotic arrest in testis cords. Later stages of germ cell development however, do seem to need the presence of the "correct" sex chromosome composition.

For example, XO germ cells in fetal testes are blocked at the spermatogonial stage. On the other hand, $X_{sxr}O$ germ cells (which contain a translocated segment of the Y chromosome) survive to the later stages of spermatogenesis (Fig. 4, 6). Therefore, it seems that some Y-encoded gene products are essential for germ cell development in the testis. Nonetheless, $X_{sxr}O$ germ cells also fail to form functional sperm mostly because of a spermatogenic block during the first meiotic metaphase (Kot and Handel, 1991; Sutcliffe et al., 1991; Levy and Burgoyne, 1986). It is conceivable that genes on the Y chromosome, other than the Sxr region, are required for the later stages of

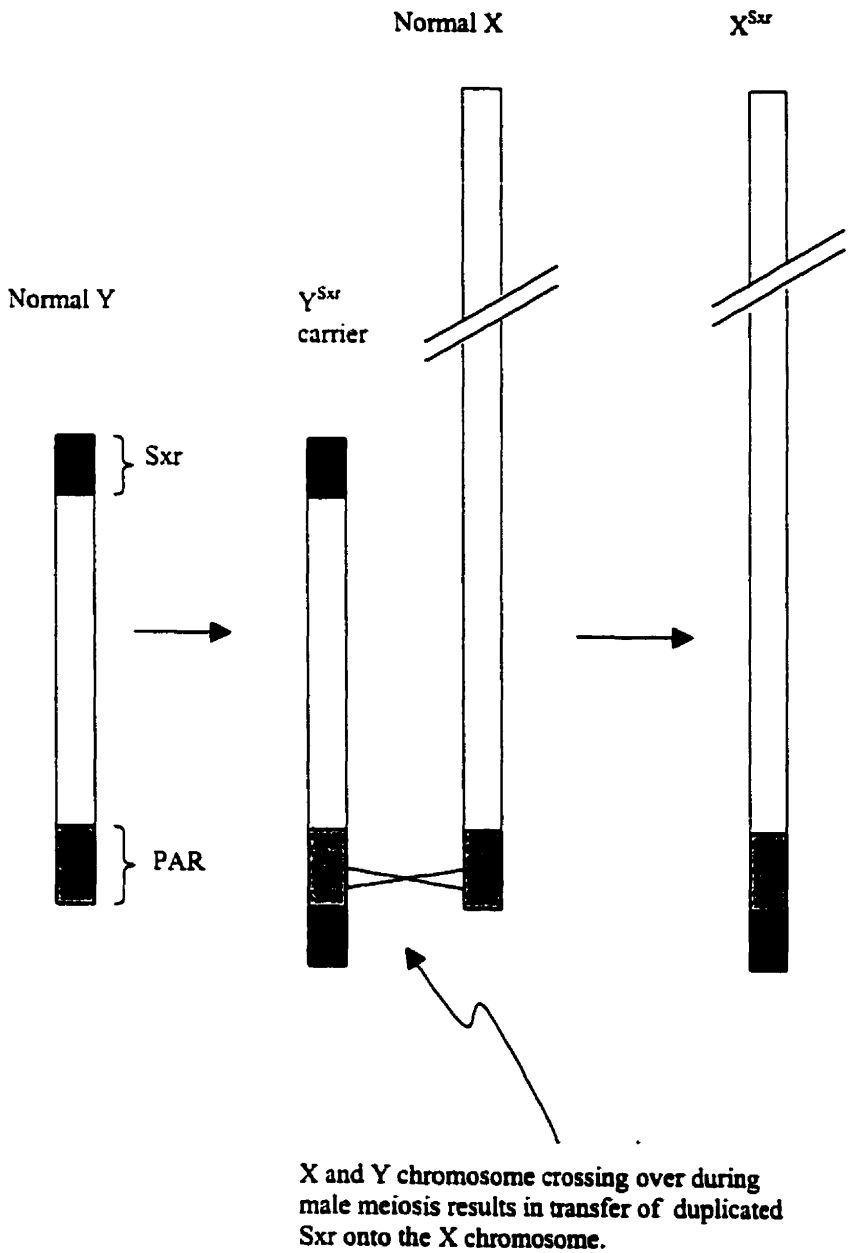

Fig. 4. The Sxr mutation and its mode of inheritance.
PAR = Pseudoautosomal region

spermatogenesis. Furthermore, X-Y pairing also appears to be an important factor for successful spermatogenesis. $X_{sxr}O$ germ cells provided with a Y^{*x}, which has no genes on it and only supplies a pairing partner for the lone X, lifts the meiotic block in these germ cells (Burgoyne et al., 1992). Nonetheless, these mice are still infertile due to abnormal sperm differentiation.

However, the simple presence of a Y chromosome does not guarantee male germ cell development. For instance, individuals possessing an XXY karyotype are infertile (Cattanach, 1961; Russell and Chu, 1961; Jacobs and Strong, 1959). Presumably, double dosage of some X encoded gene products is deleterious to the production of functional sperm (McLaren and Monk; 1981).

Unlike spermatogenesis, oogenesis appears to be more tolerant of sex chromosomal anomalies. For example, XO female mice are fertile (Lyon and Hawker, 1973). They produce smaller litters than XX females presumably because of the loss of their YO embryos and the lower viability of the XO embryos. In addition, the XO female has a shorter reproductive life, presumably because she has a smaller pool of oocytes. This reduction in germ cell number occurs as a result of excessive atresia during the first few days after birth (Burgoyne and Baker 1981, 1985). Furthermore, it has been suggested that this loss is due to the absence of the second X chromosome.

There are also some strains of fertile XY sex-reversed female mice. In one such strain (XY(Sry-)), sex-reversal occurs due the deletion of the *Sry* gene. These mice are fertile, but produce less offspring per litter. Furthermore, the progeny from these females are frequently aneuploid (Lovell-Badge and Robertson, 1990). In another strain (XY^{del}),

the *Sry* locus is intact but its expression is suppressed. These females are fully fertile despite the random segregation of the Y chromosome at meiosis and occasional aneuploidy (Capel et al., 1993; Laval et al., 1995). Therefore, the presence of the Y chromosome is not necessarily deleterious to oogenesis and nor is haplodeficiency of X-encoded gene products incompatible with female fertility. In contrast, some other strains of XY females (B6.Y^{POS} and B6.Y^{TIR}, see below) are infertile. It is expected that their infertility should be attributed to a mechanism other than the mere presence of the Y chromosome or the haplodeficiency of the X dosage.

1.3 Sex-Reversal

The most dramatic consequence of an "error" in the sex differentiation pathway is sex-reversal.

Most cases of XX sex-reversal in humans involve the translocation of the testis determining region (including the *Sry* gene) of the Y chromosome onto the X chromosome or an autosome. A similar situation is seen in the XX_{Sxr} mouse (Cattanach et al., 1971). This sex-reversal mutation (Sxr) arose through a duplication of the region containing the testis determining gene, followed by its translocation on to the PAR of the Y chromosome (Fig. 4). During male meiosis, pairing and crossing over between the sex chromosomes resulted in the transfer of this locus onto the X chromosome (X_{sxr}) (Singh and Jones, 1982; Evans et al., 1982). Due to the presence of the *Sry* gene, the XX_{sxr} progeny develop testis and hence the male phenotype.

XY sex-reversal may involve a defect which affects *Sry* gene expression. For

example, a mutation within its DNA binding domain, or a deletion of its regulatory elements, has been identified in some human XY females as well as in a mutant mouse strain (Lovell-Badge and Robertson, 1990). However, XY sex reversal can also occur in the presence of an intact *Sry* sequence. It is evident that some autosomal gene products also play regulatory roles in gonadal sex differentiation (The reader is referred to the reviews by Wolf (1995) and Werner (1996) for a more comprehensive view of autosomal and X-linked genes involved in sex-determination). One studied example is the *SOX9/Sox9* gene (Foster et al., 1994; Wright et al., 1995; Kwok et al., 1995; Kent et al., 1996; Morais da Silva et al., 1996). Heterozygous mutations of this gene result in a human dwarfism syndrome termed Campomelic dysplasia (CD). In addition to showing general defects in cartilage and bone development, 75% of CD patients also show varying degrees of sex reversal.

Yet another case of XY sex-reversal, this time in the mouse, involves no mutation whatsoever. Here sex-reversal occurs when the X and autosomal chromosomes are from the C57BL/6 (B6) *Mus musculus musculus* strain and the Y chromosome is of *Mus musculus domesticus* origin (Eicher et al. 1982; Nagamine et al., 1987).

1.4 The B6.Y^{TIR} Mouse

In 1982, a case of inherited sex-reversal was reported in the mouse by Eicher and her coworkers. For reasons that have no bearing on the present report, Whitney III and Russell (1978) were trying to transfer a mutation from a certain *Mus musculus domesticus* male onto the C57BL/6 (B6) genetic background. While doing so they noticed that the

sex-ratio of the offspring in generation N3 and onwards was skewed in favour of females. Chromosomal analysis of the progeny revealed that the XX to XY sex ratio followed the 1:1 Mendelian pattern and that some of the females produced were, in fact, of the XY karyotype (Whitney III and Russell, 1978). Most importantly, further analyses revealed that the sex-reversal was due to the origin of the Y chromosome and was not due to the mutation.

Eicher et al. (1982) originally designated the term B6.Y^{DOM} and later B6.Y^{POS} to refer to this consomic strain. Nagamine et al. (1987), who also independently created a similar consomic strain, also referred to theirs as B6.Y^{DOM} but then later changed it to B6.Y^{TIR}. POS stands for Val Poschiavo, Switzerland and TIR stands for Tirano, Italy. These are the geographic regions from which the Y chromosomes of the B6.Y^{POS} and B6.Y^{TIR} , respectively, were obtained. Both regions are fairly close to each other. Furthermore, given the similarity in the behaviour of these Y chromosomes on the B6 background, it is possible that both strains may in fact involve the same mechanism of sex reversal. It is noteworthy that *Mus musculus domesticus* Y chromosomes from several other regions also cause sex-reversal on the B6 background (e.g Y^{POS}, Y^{TIR}, Y^{ORO}, Y^{LIP}, and Y^{APE}) but that not all "*domesticus*" Y chromosomes do (e.g. Y^{SJL}).

Mechanism of Sex-Reversal

The Y^{TIR} or Y^{POS} chromosomes are fully functional in inducing testis formation on other genetic backgrounds than that of the B6 (Eicher and Washburn, 1986; Taketo-Hosotani et al. 1989). On the other hand, the Y chromosome of B6, as well as that from

many other strains, can induce normal testicular development on the B6 background. Therefore, the mechanism of sex-reversal in B6.Y^{POS} and B6.Y^{TIR} is not due to the Y^{TIR}/Y^{POS} chromosomes or the B6 background alone but is due to the combination of both.

Eicher and Washburn (1986) offered two hypotheses to explain the sex-reversal. In the first they proposed that the POS (or TIR) *Sry* gene cannot interact properly with a B6 autosomal gene(s) which is essential for the testis differentiation pathway. They called this hypothetical autosomal gene *tda-1* (testicular determination autosomal). Furthermore, it was postulated that the B6 *tda-1* gene is recessive to the POS or TIR *Tda-1* gene, since it is only after the second backcross generation that testis differentiation becomes impaired (Nagamine et al., 1987). Their second hypothesis, which was also suggested by Palmer and Burgoyne (1991), stated that "the '*domesticus*' [Y^{POS} and Y^{TIR}] carries a *Tdy* [*Sry*] allele which is later-acting than that on the '*musculus*'-derived Y chromosome of the [B6] inbred strain and that this delay sometimes enables the process of ovary determination to pre-empt Y action." (Palmer and Burgoyne, 1991).

Taketo et al. (1991) confirmed that indeed testicular differentiation is delayed in the B6.Y^{TIR} fetal gonad. However, when the Y^{TIR} is placed onto the SJL inbred background, all the XY progeny developed normal testes without any delay. This finding suggested that the delay in testicular differentiation seen in the B6.Y^{TIR} gonad was not due to the Y^{TIR} itself but that it probably resulted from ineffective interactions between the Y^{TIR} *Sry* and the B6 *Tda-1* allele.

This theory was further confirmed when Lee and Taketo (1994) showed that the

Y^{TIR} *Sry* gene is transcribed in the B6.Y^{TIR} gonad at the proper onset time during fetal development. However, they also noted that the transcription of this gene was prolonged if compared to that of normal male gonads. Furthermore, expression of other genes in the testicular differentiation pathway was delayed in the B6.Y^{TIR} ovotestis and was completely absent in the XY ovary. Therefore, these results seem to suggest that the testis determination pathway in the B6.Y^{TIR} gonad is impaired downstream of *Sry* transcription. As such, given its failure to interact properly with its target, *Sry* transcripts persist and the other genes in the testis determination pathway are not "turned on" at the right time .

Fertility of the B6.Y^{TIR} Mouse

When a B6.Y^{TIR} male mouse is crossed with a B6 female, half of the XY fetuses develop bilateral ovaries along with female internal and external genitalia. The other half develop as hermaphrodites: possessing either an ovary and an ovotestis (a gonad containing both ovarian and testicular tissue) or two ovotestes (Fig. 5) (Eicher et al., 1982; Eicher and Washburn 1983; Taketo-Hosotani et al., 1989).

By puberty, the majority of the ovotestes develop into functional testes. Consequently most B6.Y^{TIR} males can mate with B6 females to produce the next generation of B6.Y^{TIR} progeny.

Their B6.Y^{TIR} female littermates, on the other hand, are infertile. They fail to establish regular estrous cyclicity and hence their chance of reproduction is reduced (Taketo-Hosotani et al., 1989). The defect appears to be restricted to the ovary and does not involve the pituitary gland given that ovariectomized B6.Y^{TIR} females grafted with

XX ovaries initiate regular estrous cyclicity. In contrast, ovariectomized XX females grafted with B6.Y^{TIR} ovaries do not resume their cyclicity. Despite the lack of estrous cyclicity, mature B6.Y^{TIR} females display normal mating behaviour. None, however, have ever become pregnant except for one case reported by Eicher et al. (1982).

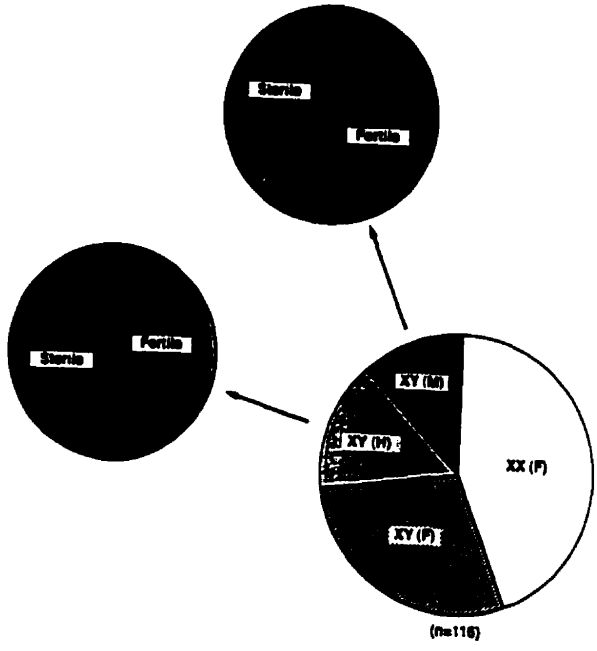

Fig. 5 Fertility of B6XB6.Y^{TIR} progeny

Since the above findings seemed to implicate the ovary in the defect, Taketo-Hosotani et al. (1989) performed a detailed examination of this organ. Their findings showed that, morphologically, the B6.Y^{TIR} ovary is indistinguishable from the XX ovary

until 16.5 dpc. However, for unknown reasons, the XY fetal ovary suffers the loss of all its oocytes in the medullary region between 17.5 and 19.5 dpc. The oocytes in the cortical region on the other hand, survive and continue with their progression through the last stages of meiotic prophase I.

Postnatally, the XY ovary continues to grow and initiates folliculogenesis in the cortical area while the medullary region is occupied with remnants of sterile sex cords surrounded by stromal tissues. Around 2 months after birth, only a few follicles remain in the XY ovary whereas many follicles at various stages of development are present in the control XX ovary.

The B6.Y^{TIR} ovary also develops a number of endocrine problems. In general, the XY ovary produces lower levels of steroids than the XX ovary. Furthermore, administration of PMSG (pregnant mare's serum gonadotropin), which has both FSH and LH activities, fails to increase testosterone production and only slightly increases progesterone levels in the B6.Y^{TIR} ovary. On the other hand, the same treatment increases the synthesis of estradiol from testosterone to a similar extent in both XX and XY ovaries (Villalpando et al., 1993).

Testosterone synthesis is mainly regulated by LH, while follicular growth and aromatase activity is by FSH. Amleh et al. (1996) showed that the XY ovary had a normal distribution for LH receptors. Hence, the lack of response to LH in the XY ovary appears to be at the level of signal transduction or further downstream.

Merchant-Larios et al. (1994) examined the fertility and development of the oocytes derived from B6.Y^{TIR} females. The oocytes ovulated from XY ovaries underwent

fertilization efficiently. However, the majority of the embryos could not proceed beyond the 2-cell stage.

Since it was possible that ovulation failed to yield "healthy" eggs due to endocrine problems, Amleh et al. (1996) collected oocytes directly from younger XY ovaries and examined their maturation, fertilization, and embryonic development in vitro. Their study showed that eggs collected from the juvenile XY ovary are higher in number and have a better developmental potential than if compared to those obtained through ovulation from their adult counterparts. For instance, more zygotes reached the 4- to 8-cell stage and in one experiment two even reached the blastocyst stage. Nonetheless, the majority of the fertilized eggs from the XY ovary still became arrested at the 1- or 2-cell stage.

Thus, the question still remained as to whether the failure in post-fertilization development was due to a defect intrinsic to the oocyte or whether the somatic environment had imposed the defect during oocyte differentiation and growth. By examining XY-XX chimeras composed of B6.Y^{TIR} and XX BALB/c cells, Amleh and Taketo (1998) showed that the presence of XY somatic cells in the ovary is compatible with oogenesis and embryonic development given that live-borns were produce from XX oocytes. However, XY oocytes from the same chimeric females did not result in live-borns. Based on these results, it was concluded that the developmental failure of the XY oocyte is intrinsic to the oocyte itself and not due to its surrounding XY cells.

In addition, nuclear transplantation experiments performed by Amleh et al. (in preparation) have indicated that both the cytoplasmic and nuclear components are defective in the fertilized eggs from B6.Y^{TIR} females. Recent studies in our laboratory

have been focused on identifying the defect(s) present in these oocytes.

1.5 Objective and Rationale

Since, the only genetic difference between the XY oocytes and "normal" XX oocytes is their sex chromosomal compositions, it is likely that one of the possible causes of infertility is the expression of Y-encoded genes in the oocyte.

The objective of the present study is to determine whether the sex chromosomes are transcriptionally active or inactive in XY oocytes.

Most information related to sex chromosomal activity during meiosis and embryonic development has been derived from the analysis of isozymes encoded by the sex chromosomes. Now, with new advances in molecular biology, it has been possible to directly examine the transcriptional activity of the sex chromosomes.

Female eutherian mammals possess two X chromosomes, whereas their male counterparts have only one. In female somatic cells, one of the two X chromosomes is inactivated to compensate for the dosage difference between the two sexes (Lyon, 1961). On the other hand, both sex chromosomes are active in the XX oocyte because the second X chromosome is reactivated when the oocyte enters meiosis (Gartler et al., 1975; Kratzer and Chapman, 1981; Monk and McLaren, 1981). In contrast, in XY germ cells, the X and Y chromosomes are sequestered in the sex body during spermatogenesis (in primary spermatocytes) and are transcriptionally suppressed.[3] This striking sex difference in the

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Note: Both XX and XY germ cells show reduced transcriptional activity at the pachytene stage of meiotic prophase I when maximal chromatin condensation has occurred in order to facilitate chiasmata formation

behaviour of sex chromosomes can be attributed to either the chromosomal composition or the germ cell type or both.

Given all this, we decided to examine transcriptional activity of a few representative genes on the X and Y chromosomes in the XY^{TIR} oocytes. We first examined fetal and adult ovaries at different stages of development. If we detected transcripts for any of the genes, we then collected oocytes and examined them for transcriptional activity

We selected three genes located on the Y chromosome (*Sry*, *Zfy*, *Ube1y*) that have been characterised the best. Most other Y-linked genes are hypothetical or not as much information is known about them. As for the X chromosome, we chose to concentrate our efforts on the *Xist* gene since its transcripts are directly involved in the process of X-inactivation. In addition, *Hprt* was selected as a representative gene on the X chromosome. A brief description of each gene examined is given below.

Sry

Sry, the testis determining gene, is a small intronless gene which contains a DNA binding High Mobility Group (HMG) box domain (Sinclair et al., 1990). Based on its DNA binding properties, it has been suggested that *Sry* may encode a putative transcription factor, and as such, functions in regulating the other genes in the sex-determination pathway.

In mice, *Sry* transcripts can be detected in the male genital ridge between 10.5 and 11.5 dpc. At 12.5 dpc, transcripts are less abundant and from this day onwards they are no

longer detected in fetal testes (Koopman et al., 1990). In adult testes, however, *Sry* transcripts once again become apparent. These, however, are in a circular form and do not seem to be translated into proteins (Capel et al., 1993).

Zfy

The human *ZFY* (zinc-finger gene on the Y chromosome) was one of the first genes to be mapped in the testis determination region and was thus a candidate for testis determining gene (Page et al., 1987). However, the subsequent discovery of *SRY* as the testis determining gene eliminated *ZFY*'s candidacy.

In the mouse, four homologues of *ZFY* exist: *Zfy-1* and *Zfy-2* on the Y chromosome, *Zfx* on the X chromosome, and *Zfa* on chromosome 10. *Zfy-1* and *-2* genes encode similar proteins containing zinc-binding fingers (Ashworth et al., 1989; Mardon and Page, 1989). The function of these genes are at present still unknown.

The pattern of expression for the two murine Y-encoded zinc finger genes has been described by various authors with slight variations. According to Koopman et al. (1989), *Zfy-1* expression is seen in differentiating embryonic male testes from 10.5 to 14.5 dpc, whereas *Zfy-2* transcripts are detected in adult testes. Nagamine et al. (1990), on the other hand, reported that both *Zfy-1* and *-2* are expressed in fetal testes as well as in adult testes but that the levels of *Zfy-1* are higher than *Zfy-2* in fetal testes, whereas the opposite pattern is seen in the adult testes.

Ube1y

Ube1y (also known as *Sby*) encodes a ubiquitin activating enzyme E1 (Ube1) similar to the X-linked human UBE1 (Mitchell et al., 1991; Kay et al., 1991; Handley et al. 1991). UBE1 plays an important role in the activation of ubiquitin, a key protein in the progression of the eukaryotic cell cycle.

Ube1y is present on the Y chromosome of a wide range of mammals, including mice, but excluding old world primates such as humans and chimpanzees. On the other hand, *Ube1y's* homologue on the X-chromosome, called *Ube1x* or *Sbx*, is present in all mammals. *Ube1y* has been suggested as a candidate for the hypothetical spermatogonial proliferation gene (Spy) in the mouse (Sutcliffe and Burgoyne, 1989; Mitchell et al., 1991; Kay et al., 1991). It is speculated that *Ube1y* products are required in addition to *Ube1x*'s to promote the active proliferation of spermatogonia.

Ube1y transcripts have been detected only in the testis at all stages of development (Odorisio et al. , 1996).

Xist

A gene called *XIST* (X-inactive specific transcript) in humans, and *Xist* in mice, is expressed exclusively from the inactive X chromosome while it is silenced from the active X chromosome (Brown et al., 1991; Borsani et al., 1991).

The *XIST/Xist* gene does not contain a conserved open reading frame (ORF), nor does it seem to encode a protein. Given that the majority of its transcripts are located in the nucleus, it has been hypothesised that *XIST/Xist* must encode a functional RNA that physically mediates X-inactivation (Brown et al. 1992, Brockdorff et al. 1992).

Xist expression in the germ cells also correlates with the stages when an inactive X chromosome is present (McCarrey and Dilworth, 1992; Richler et al., 1992; Salido et al., 1992). Briefly, *Xist* transcripts disappear in XX oocytes between 12.5 and 13.5 dpc when the second X chromosome is reactivated. On the other hand, *Xist* transcripts become detectable in spermatogonia, primary spermatocytes, and at low levels in post meiotic spermatids (McCarrey and Dilworth, 1992).

Hprt

The X chromosome is home to many "housekeeping" genes essential for the survival of the cell. The enzyme hypoxanthine phosphoribosyl transferase (HPRT/Hprt) is one of these genes.

HPRT is involved in the salvage pathway of purine synthesis. Mutations resulting in HPRT deficiency cause Lesch-Nyhan Syndrome and gouty arteritis in humans.

Like most genes on the X chromosome, it is subjected to X inactivation in the murine somatic cell. Accordingly, consistent levels of *Hprt* expression are detected in all tissues of both sexes. This is a reason why *Hprt* is often used as a positive control for RT-PCR. On the other hand, in the female germ cells both X chromosomes are active and one would expect a double dosage of transcripts for this gene. In the male germ cells, the single X is suppressed during spermatogenesis and instead a coping mechanism is used whereby the *Hprt* gene products are stabilized.

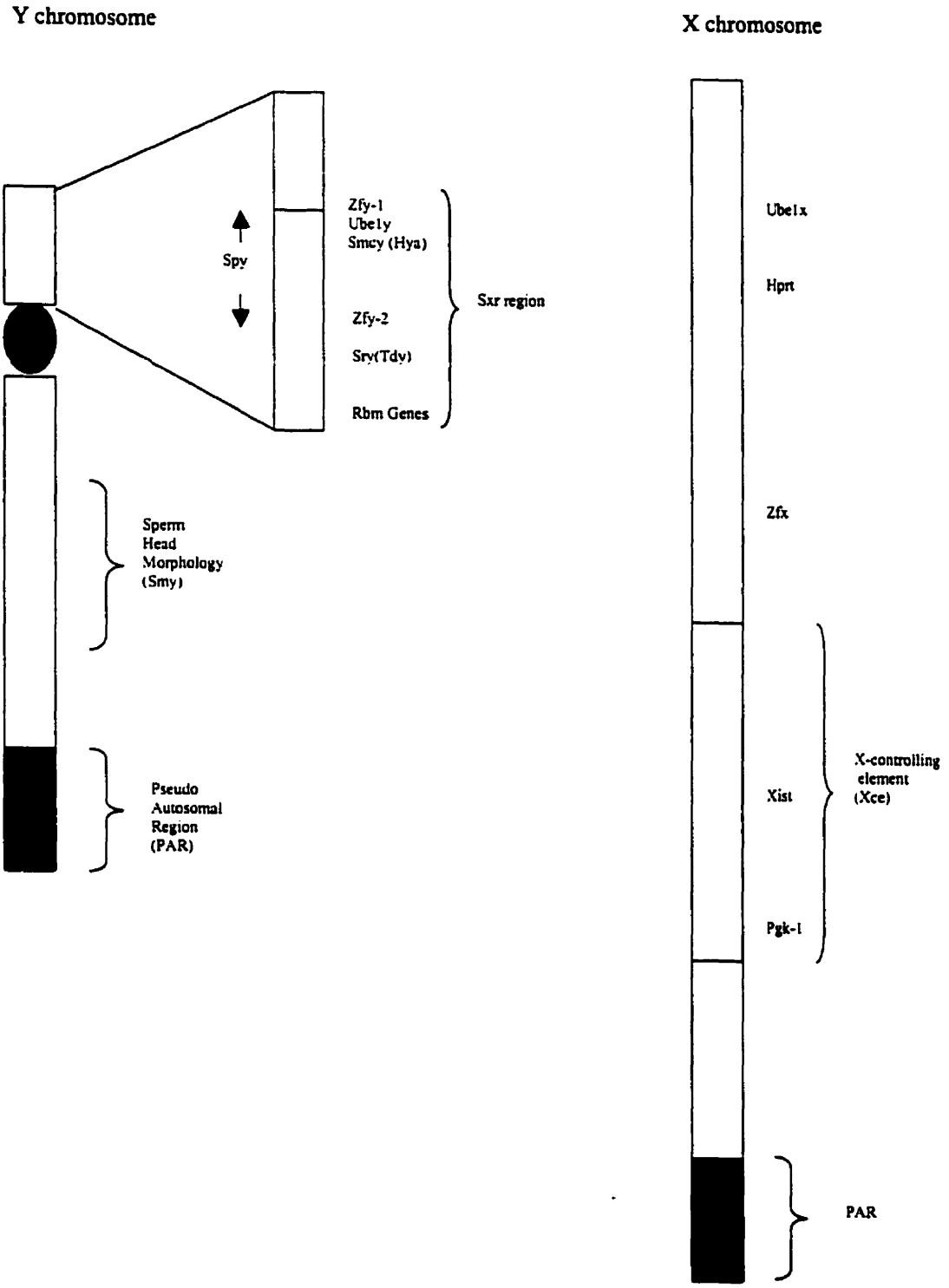


Fig. 6. A summary of genes mapping to the mouse Y and X chromosomes. Adapted from Affara et al. (1996), Disteche (1995), and Knowles and Cooley (1994)

2-Materials and Methods

2.1 Preparation of B6.Y^{TIR} Mice

The consomic B6.Y^{TIR} strain (previously called B6.Y^{DOM}) has been established by Nagamine et al. (1987). A *Mus musculus domesticus* male trapped in Tirano (Italy) was crossed with B6 females (Jackson Laboratories, Bar Harbor, Me) and the resultant XY progeny were backcrossed to B6 females. This backcross has been repeated in our mouse colony and the N28- N32 backcross generations were used in this study.

The B6.Y^{TIR} progeny were produced by caging one to three B6 females with each B6.Y^{TIR} male overnight. The presence or absence of copulation plugs (cp) was examined every morning between 8:00 and 11:00 am for up to 4-5 days. The female mouse that had mated was housed singly and this day was defined as 0.5 days post coitum (dpc). Pregnant females were either sacrificed at 12.5, 14.5 or 17.5 dpc or allowed to deliver and nurse their offspring. The pups were weaned at 25 days post partum (dpp) and sacrificed between 26 and 30 dpp.

2.2 Genotyping

To determine the chromosomal sex of individual fetuses and postnatal mice, tissue from the liver and ear punch, respectively, was digested overnight in a lysis buffer containing 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Tween 20, 0.5% NP-40, and 0.4 mg/ml proteinase K (Boehringer-Mannheim, Germany). The lysate was then subjected to PCR amplification for the Y-encoded *Zfy* gene using the conditions and

primers described by Nagamine et. al (1989; also see below).

2.3 Isolation of Gonads

Pregnant B6 females at 12.5, 14.5, and 17.5 dpc were sacrificed and their fetuses were removed. Fetal gonads were dissected out and isolated from the mesonephroi under the dissecting microscope in Eagle's minimum essential medium (MEM with Hank's salts; Gibco BRL, Life Technologies, Grand Island, N.Y.). Kidneys were similarly isolated from some of the fetuses. Each tissue was then rinsed in 0.9% NaCl, individually flash frozen in liquid nitrogen and stored at -80°C until further use.

XX and XY female offspring between 25 and 30 dpp were injected intraperitoneally with 5 IU of pregnant mare's serum gonadotropin (PMSG; Sigma Chemical, St. Louis, MO) and their ovaries were removed 16-20 hr later. The ovaries were isolated from surrounding tissues in MEM under the dissecting microscope and stored at -80°C as described above. In addition, kidneys from XX and XY females as well as testes and kidneys from B6.Y^{TIR} males (30-70 dpp) were isolated and stored as described above.

2.4 Isolation of Oocytes from Prepubertal Ovaries

As described above, ovaries were dissected out from 26 and 30 dpp, PMSG treated, XX and XY females. Oocytes at the growth phase were collected according to the method of Mangia and Epstein (1975) with several modifications. Briefly, each pair of ovaries were digested at 37°C for 15 min in a 300 µl droplet of Ca^{2+}, Mg^{2+}-free

Dulbecco's phosphate buffered saline (DPBS; Gibco BRL, Life Technologies) containing 0.5 mg/ml collagenease (Worthington Diagnostic Systems Inc, Freehold, NJ), 0.5 mg/ml trypsin (Worthington Diagnostic Systems Inc) and 0.5 mg/ml hyaluronidase (Sigma). In earlier studies, lysozyme was used instead of trypsin. After digestion, the follicles were punctured with two 25-gauge needles to release the oocytes. The mixture was diluted by the addition of 3 ml of Ca^{2+}, Mg^{2+}-free DPBS, followed by pipetting up and down to further dissociate the oocytes from the somatic cells. Germinal vesicle-stage oocytes, free of somatic cells, were collected with a mouth-controlled micropipette and transferred to a droplet of Ca^{2+}, Mg^{2+}-free DPBS containing 3% bovine serum albumin (BSA, ICN Biomedicals Inc., Aurora, Ohio). Isolated oocytes were washed at least twice more in fresh droplets of Ca^{2+}, Mg^{2+}-free DPBS containing 3% BSA, flash frozen in liquid N_2 and stored at -80°C until further use.

2.5 RNA Isolation and RT-PCR

Total RNA was isolated using the method described by Chomczynski and Sacchi (1987) and dissolved in the appropriate amount of DEPC-treated water (Table III).

After denaturation at 90°C for 5 min, aliquots of the RNA sample were subjected to cDNA synthesis in a total volume of 10 µl containing 16.2-21.0 units of RNA Guard (Pharmacia, Milwaukee, Wisconsin), 100 units of M-MLV reverse transcriptase (Gibco BRL), 1 mM each dNTPs (Pharmacia), 2.5 µM random hexamers (Pharmacia), 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, and 50 mM KCl. For PCR analysis of *Sry*, *Zfy*, *Xist(b)*, and *Xist(k)*, negative controls were prepared by omitting the reverse transcriptase. The

reaction mixture was first incubated at 25°C for 10 min and then at 42°C for 60 min. Finally, the enzyme was inactivated by incubation at 99°C for 5 min.

Table III.
The concentration of RNA used for RT-PCR reactions.

Tissue	Amount of water used to dissolve RNA isolated from each tissue (µl)	Amount used per reaction		
		Zfy	*β-actin*	Other
Fetal ovary	16	3 µl	1µl	2µl
Fetal testis	16	3 µl	1µl	2µl
Fetal kidney	32	3 µl	1µl	2µl
Adult ovary	32	3 µl	1µl	2µl
Adult testis	*X*	0.5 µg	0.5 µg	0.5 µg
Adult kidney	*X*	0.5 µg	0.5 µg	0.5 µg
Oocytes	*X*	15-20 oocytes	15-20 oocytes	15-20 oocytes

X = Amount of water that will result in the concentration mentioned in the third column.

PCR amplification was performed with a Perkin-Elmer Cetus thermocycler (Model 9600) after adding a set of, 20 pmole each, sense and antisense primers (Table IV), 2.5 units *Taq* DNA polymerase (Boehringer-Mannheim), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$ for *Zfy*, 1.5 mM $MgCl_2$ for *Xist(k)* or 2.0 mM $MgCl_2$ for all other primers, in a total volume of 50 µl. For fetal and adult gonads, as well as kidneys, the PCR was cycled 30 times for *Sry* and 35 times for all other primers used. Oocyte samples were amplified for 45 cycles. For all primers except *Zfy* the amplification cycle consisted of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 30 sec except that the first denaturation was extended to 5-7 min and the last extension was for 7-10 min. For *Zfy* amplification an annealing temperature

(Tm) of 60°C was used.

After PCR amplification, 20 µl of each reaction mixture was applied to a 2% agarose gel (Gibco BRL or Boehringer Mannheim), electrophoresed in 1X TAE buffer (Sambrooke et al., 1989), and visualized with ethidium bromide staining. Gels were photographed with a Filmless Eagle Eye Camera (PDI Bioscience, Aurora, Ontario). Each band was judged in a positive or negative manner. Only those samples that had successfully amplified β-actin were considered for further analyses.

Table IV.
Primers used in the RT-PCR assays

Gene	*Primers used*	*Length of PCR product*	*Primer sequence source (primary accession #)*
Sry	5'-CTGGTGACAATTGTCTAGAG-3' 5'-TGTGGGTTCCTGTCCCACTG-3'	349 bp	Gubbay et al., 1990
Sry'	5'- CTTACTAACAGCTGACATCACTG-3' 5'TGTGGGTTCCTGTCCCACTG-3'	451 bp	Capel et al., 1993
Zfy[&&]	5'-AAGATAAGCTTAGATAATCACATGGA-3' 5'-CCTATGAAATCCTTTCGTGCACATGT-3'	618 pb	Nagamine et. al., 1987
Ubely(a)[*]	5'-GACCCCAAGTTCATGGAG-3' 5'-CCTCCTAGTCCGTATGTCTGA-3'	335 bp	Hendriksen et al., 1995
Ubely(b)[**]	5'-GACCCCAAGTTCATGGAG-3' 5'-TGGCTGTCATCAATGGTG-3'	473 bp	Mitchell et al., 1991 (X62581)
Ubelx[**]	5'-GACCCCAAGTTCATGGAG-3' 5'-GTCTTGGGATCCAGTCAAC-3'	345 bp	Mitchell et al., 1991 (X62580)
Xist(b)	5'-ATCTAAGACAAAATACATCATTCCG-3' 5'-CTTGGACTTAGCTCAGGTTTTGTGTC-3'	251 bp	Borsani et al., 1991
Xist(k)	5'-ACTGCCAGCAGCCTATACAG-3' 5'-GTTGATCCTCGGGTCATTTA-3'	578 bp	Kay et al., 1993
Hprt	5'-CCTGCTGGATTACATTAAAGCACTG -3' 5'-GTCAAGGGCATATCCAACAACAAAC-3'	352 bp	Melton et al., 1984
B-actin[**]	5'-CCTAGGCACCAGGGTGTGAT-3' 5'-TCACGGTTGGCCTTAGGGTT-3'	239 bp	Tokunga et al., 1986 (X03672)

[*] modified by 3 bases from original design
[**] designed from cDNA sequence
[&&] *Zfy* primers do not differentiate between the *Zfy*-1 and -2 homologous sequences.

2.6 DNA Sequencing

RT-PCR products of *Ubely(a)* from fetal XY ovaries and ovotestes were purified using the QIAquick PCR purification kit (QIAGEN, Mississauga, Ontario) and sequenced using [gamma-^{32}P]dATP and the *fmol*® DNA cycle sequencing system (Promega, Germany) according to the manufacturer's protocol. The samples were run on a 6% denaturing PAGE (Sambrooke et al., 1989) and visualised by exposing the gel to a BioMax MS film (Estman Kodak Company Rochester, NY).

RT-PCR products of *Ube1x* from prepubertal ovaries and adult testes were purified using QIAquick gel extraction kit (QIAGEN) and sequenced by the Sheldon Biotechnology Center (McGill University).

2.7 Restriction Endonuclease Digestion Analysis

To verify the *Ube1y(o)* RT-PCR products, the amplified sample was purified using the method mentioned above and digested with *Sca*I (Promega) according to the manufacturer's instructions. The digested products were run on a 12% non-denaturing PAGE (Sambrooke et al., 1989) and visualized by ethidium bromide staining.

2.8 Data Analysis

All primer sequences were verified with the database in GenBank at the National Center for Biotechnology Information (NCBI) homepage (http://www.ncbi.nlm.nih.gov/) using the BLAST Sequence Similarity Searching programme. Sequences for *Ubely* and *Ube1x* were also retrieved from the same source and examined using the alignment

programme ALIGN offered by Pedro's Research Tools

(http://www.public.iastate.edu/~pedro/research_tools.html).

3-Results

The transcription of genes on the X and Y chromosomes was examined in B6.Y^{TIR} gonads and control B6 XX ovaries at various stages of development. The number of gonads in which RT-PCR products were detectable is given in Table V. The total number of gonads analysed are indicated in parenthesis. For simplicity, these results are interpreted and summarised in Table VI by using the following criteria. "+++" indicates that the transcripts were consistently detected in most samples and with a relatively strong intensity (as compared to β-actin). "++" indicates that the transcripts were detected with relatively strong intensity but that the sample size was small. "+" was used for those genes whose RT-PCR results consistently yielded a signal but at a lower intensity. "+/-" indicates that transcripts were rarely detected, and is irrespective of the band intensity. A "-" denotes that either RT-PCR products were absent in all or in the majority of the samples examined and that the sample size was large.

In addition, the results for RT-PCR analysis of growing oocyte samples are presented separately in table VIII.

The results for each gene and considerations given to data analyses are described below.

3.1 Gonads and Growing oocytes

Sry

Low transcript levels for this gene were detected in three out of seven XY gonads examined at 12.5 dpc and in the two XY ovaries examined at 14.5 dpc. We did not detect

any transcripts in the XY ovotestis at 14.5 dpc. As well, no transcripts were detected in either gonadal type at 17.5 dpc. In adult gonads, only one out of three XY ovaries showed a band with a low intensity (data not shown), whereas all XY testes consistently produced relatively bright RT-PCR bands (Fig. 8 lane 3). No *Sry* transcripts were detected in XY oocytes (Table VIII). None of the samples from XX females produced correct size RT-PCR amplification products.

The *Sry'* primers detected the circular form of *Sry* transcripts in the adult XY testis (Fig. 8, lane 5) but not in fetal XY gonads (data not shown).

Zfy

A RT-PCR band for this gene was not detected in the majority of XY fetal gonads. In the rare few cases in which a signal for *Zfy* was detected, a band with a more or less similar intensity was also detected in the RT negative control sample (Fig. 7, lane 1 vs 2). Therefore, these *Zfy* samples were assumed to be negative for *Zfy* transcripts. In two instances, a *Zfy* band was seen only in the positive reaction and not in the negative control reaction or there was a significant difference between the intensities of the bands. These results were considered to be positive (data not shown).

In four reactions with 12.5 dpc XY gonads, where higher amounts of RNA were used in RT-PCR reactions, a positive band was detected (data not shown). In these instances too, a band with similar intensity was sometimes seen in the negative control reaction.

In adult XY testes, a correct size band was consistently detected in all four

samples (Fig. 8, lane 1). The single adult XY ovary examined was negative for *Zfy*. In addition, *Zfy* transcripts were not detected in growing XY oocytes (Fig. 13, lane 10).

As well, examination of control XX gonads and XY kidneys did not detect a signal.

Ubely

The *Ubely(a)* primers consistently amplified the product of expected molecular weight in the majority of the XY fetal gonads examined regardless of the gonadal phenotype (Fig. 7, lane 3). In contrast, no transcripts were detected in XX fetal gonads. *Ubely(a)* primers also amplified an intense band with the correct size in both growing XY oocytes samples examined. Partial sequencing of pooled *Ubely* amplicons from XY fetal ovaries at 17.5 dpc confirmed its identity (appendix A).

Ubely(a) primers also detected transcripts in adult XY testes and ovaries as well as XY oocytes. However, the majority of the adult XX ovaries also produced a correct size band (335 bp), albeit the band intensity was generally lower than that obtained from the XY testes and ovaries (Fig. 9, lane 4). In addition, *Ubely(a)* primers also amplified a correct size band in 9 out of 12 XX oocytes samples examined (Fig. 12a, lane 4); here, the intensity of the detected band was quite high, in 5 out of the 9 samples, if compared to bands seen that in adult XX ovaries. Since the *Ubely* gene does not exist in the XX tissue, the term *pseudo-Ubely* will be used to refer to this amplification product from the XX ovary and oocytes.

We had prepared the *Ubely(a)* primers based on Hendriksen et al. (1995), who

had examined the postmeiotic transcriptional activity of this gene during male spermatogenesis. However, we slightly modified the downstream primer sequence by extending it from 18 bases to 21 bases at the 3' end in order to increase the Tm value for the PCR reaction. Verification of the *Ube1y(a)* primer sequences in GenBank (NCBI homepage) had only identified the *Mus musculus musculus* (B6 male) cDNA sequence for the *Ube1y* gene (Mitchell et al. 1991). Thus, we were taken by surprise when *Ube1y(a)* primers amplified correct size bands in XX adult ovaries and oocytes.

Since, the *Ube1y* has an X-linked homologue called *Ube1x*, and that growing oocytes are transcriptionally very active, it was conceivable that the *Ube1y(a)* primers amplified *Ube1x* transcripts in the absence of their own target. Another search on the Internet revealed a cDNA sequence for the *Ube1x* gene from a 129/sv/Pas strain, which is also of *Mus musculus musculus* origin (Mitchell et al., 1991). Using the ALIGN programme we compared the *Ube1y* and *Ube1x* sequences. The alignment results showed a 75% homology between the two sequences (Appendix B). Most importantly, the 3'ends of the *Ube1y(a)* primers bore a perfect match to both the *Ube1y* and *Ube1x* sequences (appendix B).

To increase the specificity of the primers to the *Ube1y* sequence, we redesigned the downstream *Ube1y* primer, targeting an area of the sequence that showed the least amount of homology to the *Ube1x* sequence (Appendix B). This new *Ube1y* primer set, designated as *Ube1y(b)*, was not very efficient in detecting *Ube1y* products in XY samples if compared with the *Ube1y(a)* primers (Fig. 10, lane 6 vs 4; Fig. 13, lane 5 vs 4). In addition, they did not amplify a product from XX adult ovaries and oocytes as

frequently or as intensely as the *Ube1y(a)* primers did (Fig. 9, lane 5 vs 4). Changing the Tm value and $MgCl_2$ concentrations helped improve the efficiency of amplification in XY samples sometimes but not always. We therefore needed a better method to verify the RT-PCR products.

To verify the identity of the *pseudo-Ube1y* as *Ube1x*, we used the *ScaI* restriction enzyme (RE). Based on the available sequences for *Ube1y* and *Ube1x*, from B6 and 129/sv/Pas respectively, the RE *ScaI* would digest the 335 bp *Ube1y* amplicon into fragments of 279 and 56 bps, whereas it would digest the *pseudo-Ube1y* amplicon into 187 bp and 148 bp fragments if it were *Ube1x*. Fig. 11 shows representative results of the RE digestion.

ScaI digestion of the *Ube1y* amplicon from XY samples yielded the two expected fragments (Fig. 11, lanes 1 and 2). The results from the *pseudo-Ube1y* were, however, not very conclusive in that *ScaI* did not seem to cut the amplicon into any fragments (Fig. 11, lane 3). We believe the ambiguity to be due to sequence differences between the two strains. The available sequence for the *Ube1x* gene is for the 129/sv/Pas strain whereas our mice have the B6 *Ube1x* gene. Thus, although both strains are of the *Mus musculus musculus* genus, there may be sufficient amounts of polymorphisims in their genome.

One way to test this hypothesis would be to RT-PCR amplify RNA isolated from adult 129/sv/Pas ovaries using the *Ube1y(a)* primers and to see if digestion with *ScaI* results in the expected 187 and 148 bp fragments.

An alternative approach would be to sequence the B6 *Ube1x* cDNA to see whether

it is missing that particular *ScaI* recognition site. The latter approach is more appealing because *pseudo-Ube1y* transcripts were not always detected in adult XX ovary and oocyte samples. Therefore, examining 129/sv/Pas adult ovaries would not necessarily be fruitful.

Therefore, under the circumstances, the best mode of identification of *Ube1y* transcripts is with *Ube1y(a)* primers followed by RE digestion. Based on this analysis, it appears that XY ovaries and oocytes contain *Ube1y* transcripts, whereas XX ovaries and oocytes do not.

Ube1x

Given that the *Ube1y(a)* primers bore a perfect match to the *Ube1x* sequence in their 3'end, we decided to examine the transcriptional activity of this gene. It is known that oocytes in the growth phase have a high transcriptional activity (Wassarman and Kinloch, 1992). Furthermore, both X chromosomes are transcriptionally active, contributing X-encoded products to the pool of mRNA in the oocyte. Therefore, it is plausible that high levels of *Ube1x* transcripts are present in growing oocytes and given their sequence similarity to *Ube1y*, the *Ube1y(a)* primers anneal to them in the absence of their own target. This leaves open the possibility of the same thing occurring in the XY ovaries and oocytes. Implying that what we observed as *Ubely* could in fact have been a combination of transcripts from both *Ube1y* and *Ube1x* or even simply *Ube1x* by itself. It was for this reason that we tried designing new primers for both *Ube1y* and *Ube1x*, but given the sequence similarities between the two this was not an easy task. The sequences and the regions where we chose the primers from are given in appendix B.

The *Ube1x* primer set was successful in amplifying a band in both XX and XY adult samples examined. However, the major band it consistently amplified appeared to have a molecular weigh of approximately 400 bp. In addition, at least two other fainter bands of lower molecular weight were also detected in most of the samples, one of which appeared to be ~300 bp (Fig. 9b, lane 8). The expected size of amplicon, based on the *Ube1x* sequence reported in 129/sv/Pas mouse, is 345 bp. Therefore, none of the bands matched the expected size. Varying the Tm value and/or $MgCl_2$ concentration of the PCR reaction did not change the results.

As such, the ~400 bp band from XY adult testes and XX adult ovaries was isolated and sequenced (Appendix C). Searching the data base with this sequence showed that it bore great homology to the mouse male-enhanced antigen-2 (*Mea-2*). However, the *Ube1x* primer sequences we had designed did not match the *Mea-2* cDNA sequences flanking the ~400 bp amplified region.

Xist

In the present study we used two different sets of primers to detect *Xist* transcripts. Initially, we used the primers *Xist(b)* designed by Borsani et al (1991) (these primers were also used by McCarrey and Dilworth (1992) when they examined the germ cells). Later, however, we switched to the primer set we called *Xist(k)*, which was designed by Kay et al. (1993). This switch was necessitated because the *Xist(b)* primers were in exon 6 of the gene and did not flank an intron (Kay et al., 1993). Consequently, a negative control RT-PCR reaction had to be performed each time to exclude the possibility of DNA

amplification. The *Xist(k)* primers, on the other hand, were in exons 3 and 6, respectively, spanning 3 introns. Thus, theoretically the presence of DNA contamination could be detected by amplifying for a higher molecular weight band and therefore there would not have been a need to perform a negative control RT reaction. This was particularly useful when we examined the fetal gonads since the amount of RNA was limited. This was the major reason why some of our RT-PCR experiments with the *Xist(b)* primers were not validated with a negative control RT-PCR reaction. Therefore, the new primers would have allowed us to safely judge the authenticity of any band observed in the positive RT-PCR reaction.

RT-PCR using the *Xist(k)* primers detected an ~600 bp (correct size 578 bp) band in XX fetal ovaries and sometimes a very faint one in XY ovaries and ovotestes. Wishing to get an approximate idea of the size of the band resulting from DNA contamination of the RNA sample, we performed a PCR reaction with the primers using genomic DNA as our template. We detected two bands: One higher molecular weight band, which we interpreted as corresponding to both exons and introns combined, and lower molecular weight band, which, to our surprise, corresponded to the size of amplicon we expected for *Xist* in the absence of introns. In addition, RNase digestion of the DNA samples did not change the observed results. Therefore, in order to be safe rather than sorry, we chose to perform RT negative control reactions when testing for *Xist* transcripts even with the *Xist(k)* primers

RT-PCR experiments with the *Xist(b)* primer set detected *Xist* transcripts in both XX and XY fetal gonads, but given that at the time a negative control reaction could not

be performed due to limited amount of available RNA, these results have been compiled into a separate table (Table VII) whereas those experiments where a negative control reaction was also carried out are listed in Table (V). In the former case, a faint band was observed in some of the XY gonads at 14.5 and 17.5 dpc, whereas all XX gonads examined between 12.5 and 17.5 dpc produced a bright band with these primers. In the experiments where a negative control RT-PCR reaction was carried out along side the positive reaction, only XX fetal gonads produced the band.

With the *Xist(k)* primer set on the other hand, faint bands were detected in one each XY fetal ovary and ovotestis at 17.5 dpc. In addition, a correct size band was also amplified from two out of three adult XY ovaries, albeit at much lower intensity if compared to the signal from XX ovaries (data not shown). In contrast, *Xist* transcripts were abundant in XX fetal and adult ovaries.

Xist transcripts were also detected in all adult XY testes examined. The level of transcripts were more abundant in the testis than the kidney of the same genotype (Fig. 16, lanes 1 and 3 vs lanes 2 and 4).

XX growing oocytes were examined for *Xist* transcripts using the *Xist(k)* primers. In the 6 (out of 14) XX samples, where the enzyme lysozyme had been used for the dissociation of the ovarian cells (see materials and methods), an *Xist* band was consistently detected. In the 8 samples where trypsin had been used, various results were obtained. In one experiment, a faint but correct size band was seen, whereas in another, an additional two lower molecular weight bands were also seen. In a third sample, multiple bands were amplified along with a right size band. In yet another two experiments,

multiple lower molecular weight bands (similar to the ones in the other experiments) were obtained but not one with the correct molecular weight. In an additional three experiments, no bands were detected with *Xist(k)* primers.

As for XY oocytes, a total of 8 samples were examined, where 4 were negative for *Xist(k)* RT-PCR products and the other 4 amplified multiple lower molecular weight bands.

Hprt

This product was examined only in adult gonads and isolated oocytes. RT-PCR products were consistently detected in all tissues examined. In one half of the samples (both XX and XY), the levels of this gene in oocytes, appeared to be lower than *β-actin* levels, whereas in the other half, it appeared to be much higher than *β-actin*.

3.2 Quantitation

Our attempts at quantitation of the RT-PCR products were not successful. Competitive RT-PCR between *Ube1y* with *β-actin* was not effective given that the latter out-competed the former. In addition, comparison of the band intensity of a given RT-PCR product with *β-actin* from the same RNA source did not yield a consistent ratio from one experiment to another when the same gonadal type and stage of development were used.

Table V. Total number of gonads examined at various stages of development.

Gene	Gonad	Age			
		12.5 dpc**	14.5 dpc	17.5 dpc	25-70 dpp
		No. of samples that amplified a band (No. gonads examined)			
Sry	XX ovary	0(3)	0(2)	0(4)	0(6)
	XY ovary	3(7)	2(2)	0(3)	1(3)
	XY testis*		0(3)	0(4)	4(4)
Zfy	XX ovary	0(3)	0(3)	0(3)	0(4)
	XY ovary	0(3)	1(8)	1(9)	0(1)
	XY testis*		1(5)	1(6)	4(4)
Ube1y(a)	XX ovary	0(3)	0(3)	0(3)faint	6(10)faint
	XY ovary	5(7)	8(8)	8(10)	3(3)
	XY testis*		3(4)	5(7)	4(4)
Ube1y(b)	XX ovary	(0)	(0)	0(2)	3(5)faint
	XY ovary	(0)	(0)	1(1)	1(1)
	XY testis*		(0)	1(1)	4(4)
Xist(b)[&&]	XX ovary	(0)	2(2)	1(1)	(0)
	XY ovary	(0)	0(4)	0(6)	(0)
	XY testis*		0(2)	0(4)	(0)
Xist(k)	XX ovary	(0)	(0)	2(2)	10(10)
	XY ovary	(0)	(0)	1(1)faint	2(3)faint
	XY testis*		(0)	1(1)faint	4(4)
ß-actin	XX ovary	3(3)	4(4)	6(6)	10(10)
	XY ovary	7(7)	8(8)	10(10)	3(3)
	XY testis*		5(5)	7(7)	4(4)

*Ovotestis in fetal life.

**At 12.5 dpc B6.Y^{TIR} gonads are sexually undifferentiated.

[&&] More gonads were examined with this set of primers than indicated in the table. However, given that a negative control reaction was not done for them they are represented in a separate table (Table VII).

Table VI. Summary of gene expression in gonads at various stages of development.

Gene	Gonad	Age			
		12.5 dpc**	14.5 dpc	17.5 dpc	25-70 dpp
Sry	XX ovary	-	-	-	-
	XY ovary	+/-	+/-	-	-
	XY testis*		-	-	+++
Zfy	XX ovary	-	-	-	-
	XY ovary	-	-	-	-
	XY testis*		-	-	+++
Ube1y [&]	XX ovary	-	-	-	+
	XY ovary	+++	+++	+++	+++
	XY testis*		+++	+++	+++
Xist	XX ovary	+	+	++	+++
	XY ovary	-	-	+/-	+/-
	XY testis*		-	+/-	++
ß-actin	XX ovary	+++	+++	+++	+++
	XY ovary	+++	+++	+++	+++
	XY testis*		+++	+++	+++

*Ovotestis in fetal life
**At 12.5 dpc B6.Y^{TIR} gonads are sexually undifferentiated.
[&] The *speudo-Ube1y* amplification products detected in XX adult ovarian samples were later confirmed as not being *Ube1y* transcripts.

Legend:

+++	Detected in most samples, strong band.
++	Detected in most samples, strong band, small sample size.
+	Detected in most samples, faint band.
+/-	Detected in a few samples, faint or strong band.
-	Not detected in most samples, faint or strong band, large sample size.

Table VII. Total number of gonads examined with *Xist(b)* primers without a negative control RT-PCR reaction.

Gene	Gonad	Age		
		12.5 dpc**	14.5 dpc	17.5 dpc
		(No.gonads that showed a band (No. gonads examined)		
Xist(b)	XX ovary	3(3)	2(2)	3(3)
	XY ovary	0(5)	2(4)faint	1(3)faint
	XY testis*		2(3)	1(1)

*Ovotestis in fetal life.

**At 12.5 dpc B6.Y^{TIR} gonads are sexually undifferentiated.

Table VIII. Gene expression in oocytes isolated from prepubertal ovaries.

Gene	Genotype of oocytes	No. of samples with detectable RT-PCR bands (No. of samples examined)
Sry	XX	0(1)
	XY	0(3)
Zfy	XX	(0)
	XY	0(1)
Ubely(a)	XX	9(12)
	XY	2(2)
Ubely(b)	XX	2(8)
	XY	5(6)
Xist (k)	XX	8(14)
	XY	0(8)
Hprt	XX	14(15)
	XY	6(6)
ß-actin	XX	15(15)
	XY	8(8)

Fig 7. **RT-PCR results for a fetal XY ovary at 14.5 dpc.**
The primers used are indicated above each lane. Briefly, the expected fragment sizes are: *Zfy* (618 pb), *Ube1y(a)* (335 bp), *Xist(b)* (251 bp) and *ß-actin* (239 bp). The "+" and "-" signs indicate whether reverse transcriptase was used or not, respectively, in the cDNA synthesis step. Note the similar intensities observed between lanes 1 and 2 for *Zfy*. This ovary is considered "negative" for *Zfy* transcripts.

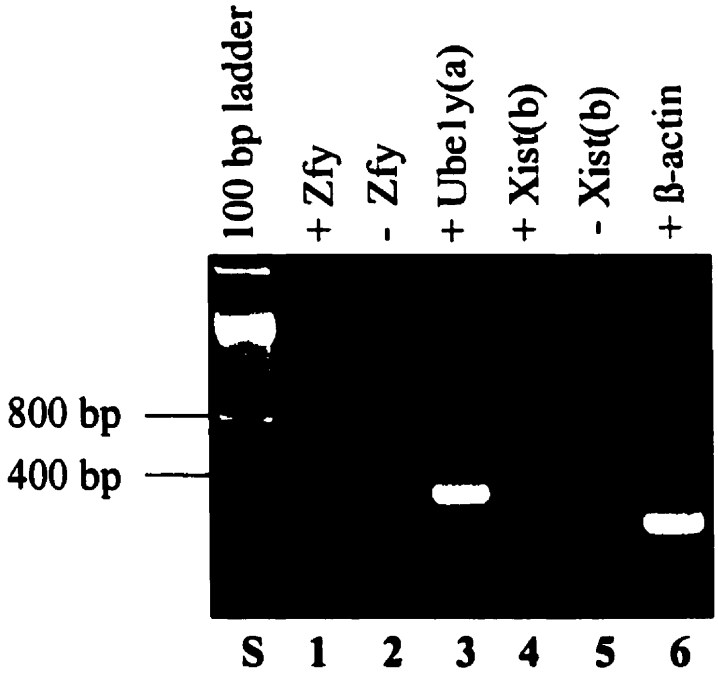

100 bp ladder
+ Zfy
- Zfy
+ Ube1y(a)
+ Xist(b)
- Xist(b)
+ ß-actin
800 bp
400 bp
S
1
2
3
4
5
6

Fig. 8. **Representative RT-PCR results for adult B6.Y^{TIR} testes.** The primers used are indicated above each lane. Briefly, the expected fragment sizes are: *Zfy* (618 pb), *Sry* (linear; 349 bp), *Sry'* (circular; 451 bp), *Ube1y(a)* (335 bp), *Ube1y(b)* (473 bp), *Ube1x* (345 bp), *Xist(k)* (578 bp), *Hprt* (352 bp) and *ß-actin* (239 bp). The "+" and "-" signs indicate whether reverse transcriptase was used or not, respectively, in the cDNA synthesis step. Note that the *Ube1x* amplicon is ~400 bp.

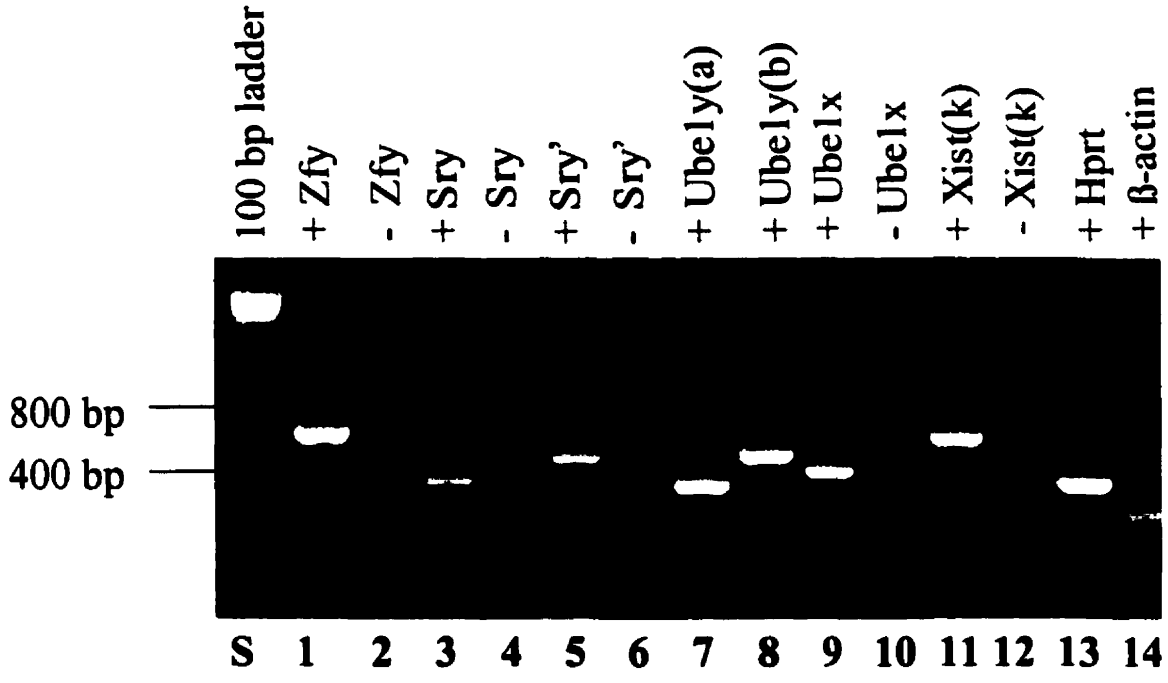
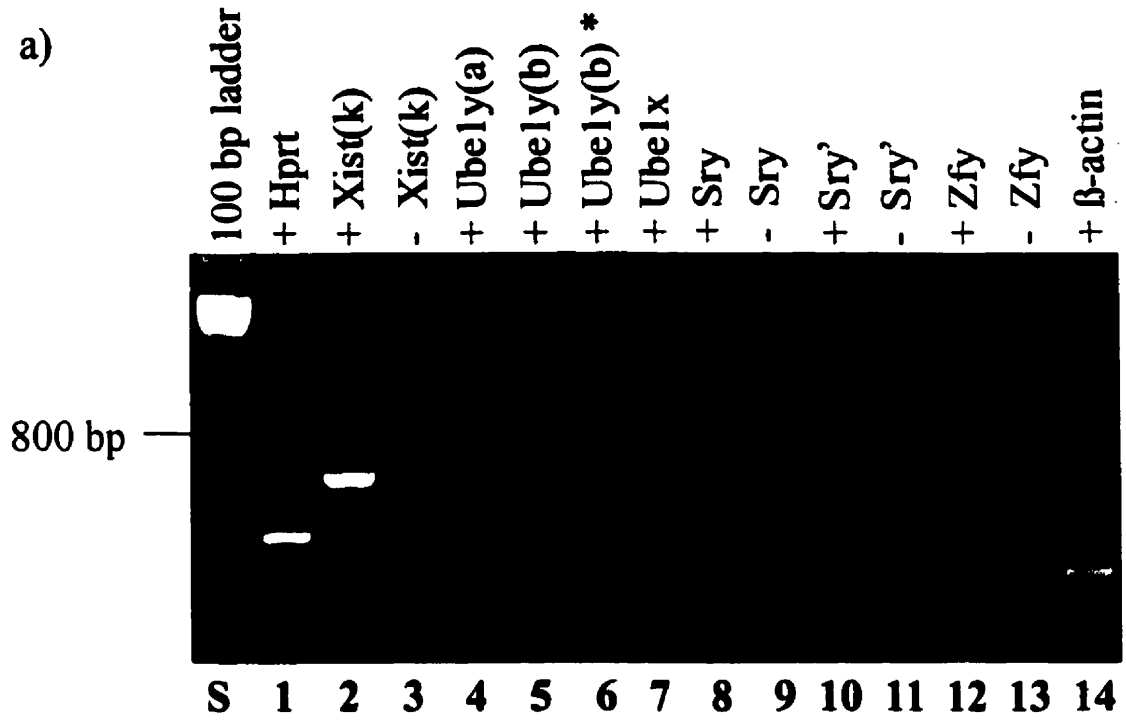

100 bp ladder
+ Zfy
- Zfy
+ Sry
- Sry
+ Sry'
- Sry'
+ Ube1y(a)
+ Ube1y(b)
+ Ube1x
- Ube1x
+ Xist(k)
- Xist(k)
+ Hprt
+ ß-actin
800 bp
400 bp
S 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 9. **RT-PCR results for adult XX ovaries.**
The primers used are indicated above each lane. The "+" and "-" signs indicate whether reverse transcriptase was used or not, respectively, in the cDNA synthesis step. The sample with an "*" were amplified at a Tm of 60°C and contained a final concentration of 2.5 mM $MgCl_2$ in their PCR reaction. Note the high intensity of the *Xist* band in both ovary samples (a, b). *Ube1y(a)* primers amplified a band in some XX ovaries (a) but not others (b). The most intense band observed with the *Ube1x* primers was ~400 bp although a lower molecular weight band of ~300 bp was also at times seen (b). Neither band corresponds to the expected size.

a)


100 bp ladder
+ Hprt
+ Xist(k)
- Xist(k)
+ Ube1y(a)
+ Ube1y(b)
+ Ube1y(b) *
+ Ube1x
+ Sry
- Sry
+ Sry'
- Sry'
+ Zfy
- Zfy
+ ß-actin
800 bp
S 1 2 3 4 5 6 7 8 9 10 11 12 13 14

b)

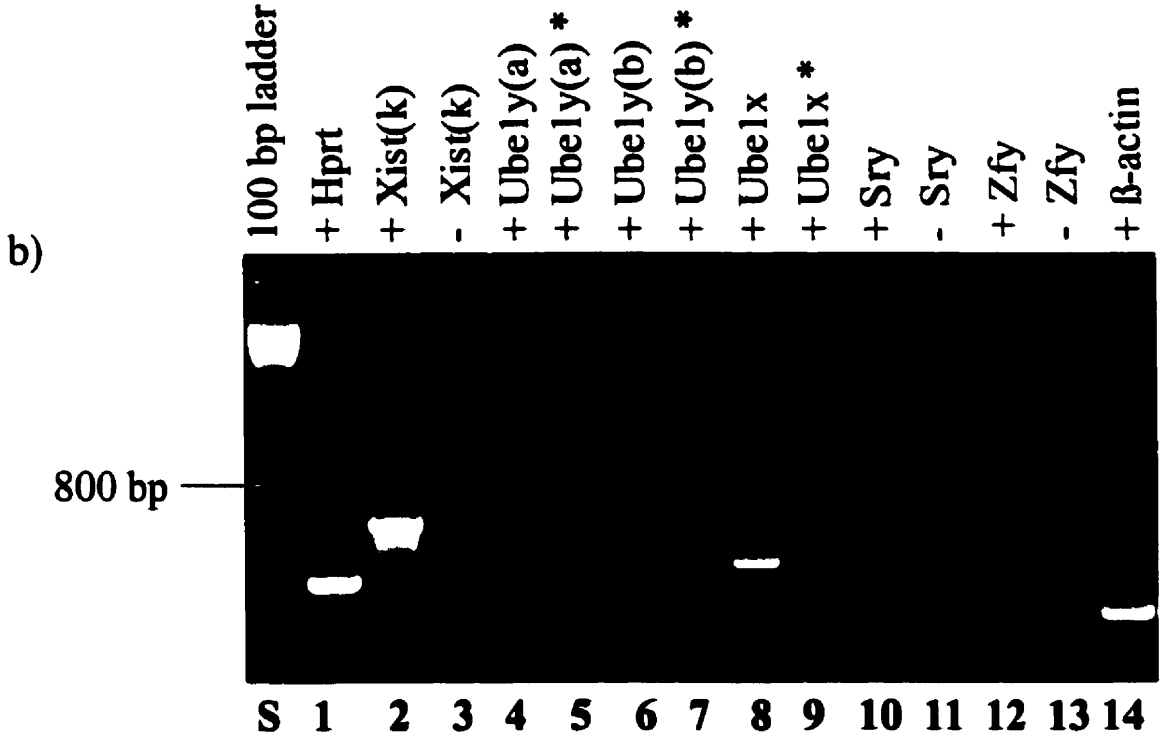

100 bp ladder
+ Hprt
+ Xist(k)
- Xist(k)
+ Ube1y(a)
+ Ube1y(a) *
+ Ube1y(b)
+ Ube1y(b) *
+ Ube1x
+ Ube1x *
+ Sry
- Sry
+ Zfy
- Zfy
+ ß-actin
800 bp
S 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 10. **RT-PCR results from an adult XY ovary.**
The primers used are indicated above each lane. The "+" and "-" signs indicate whether reverse transcriptase was used or not, respectively, in the cDNA synthesis step. The sample with an "*" were amplified at a Tm of 60°C and contained a final concentration of 2.5 mM $MgCl_2$ in their PCR reaction. Note the absence of a *Xist* amplification product and the intensity of the Ube1y amplification products. Once again the most intense band observed with the *Ube1x* primers was ~400 bp.

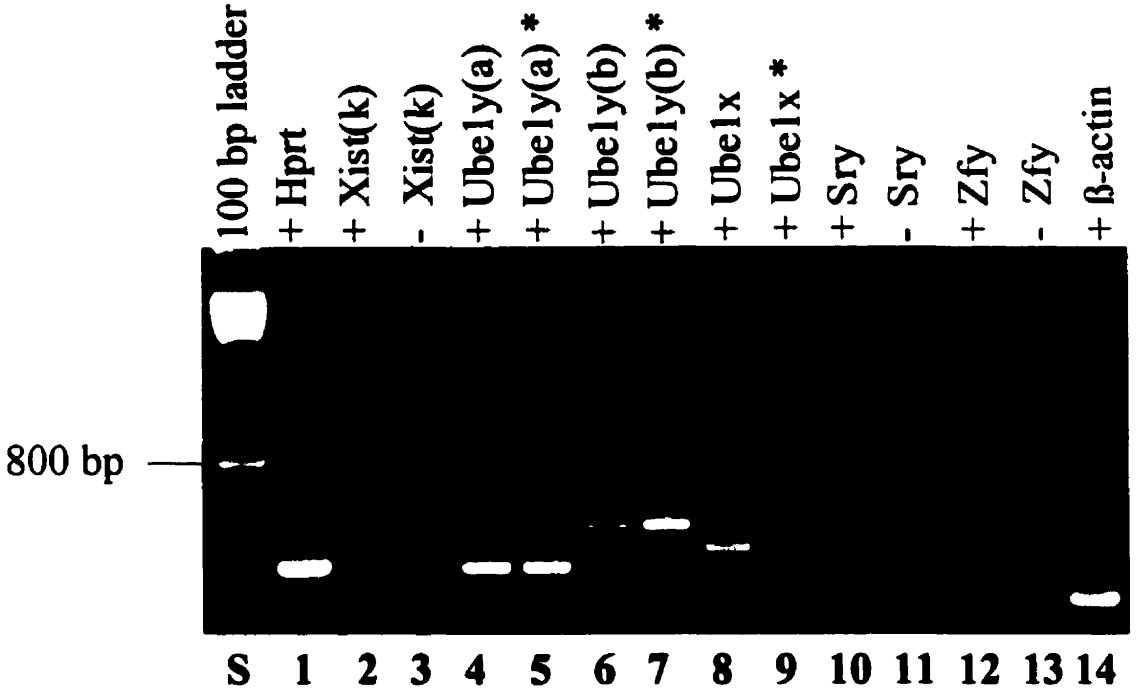

100 bp ladder
+ Hprt
+ Xist(k)
- Xist(k)
+ Ube1y(a)
+ Ube1y(a) *
+ Ube1y(b)
+ Ube1y(b) *
+ Ube1x
+ Ube1x *
+ Sry
- Sry
+ Zfy
- Zfy
+ ß-actin
800 bp
S 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 11. **Restriction enzyme digestion analysis with *ScaI*.**
The 335bp *Ube1y(a)* amplification products from two XY testes and one XX ovarian samples were purified and digested with *ScaI*. The XY samples yielded the expected 279 and 56 bp fragments. The XX samples on the other hand, did not appear to be cut. Fragments of 187 and 148 bp were expected for the XX samples if they were *Ube1x* products.

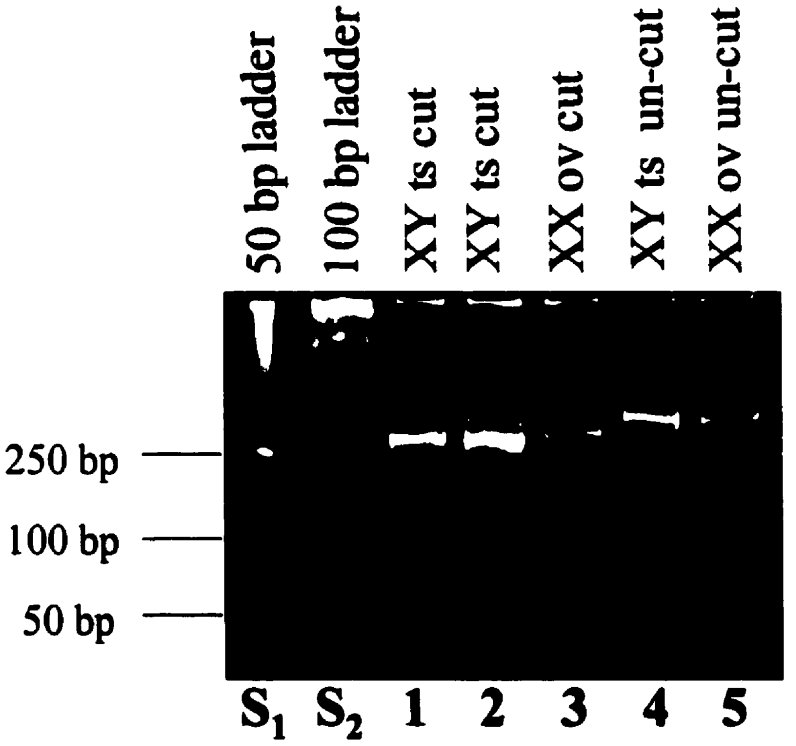
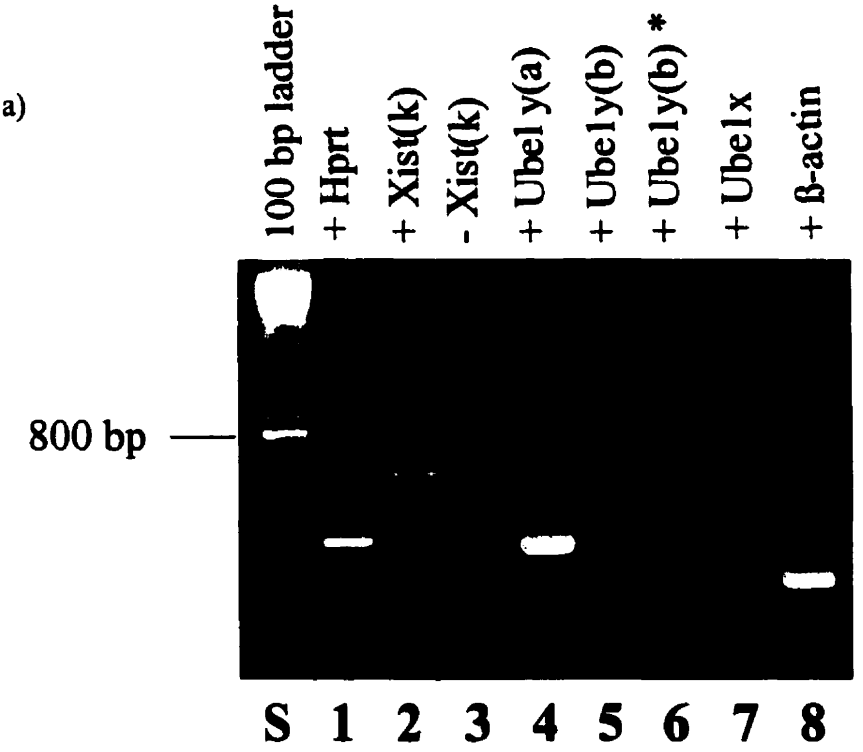

50 bp ladder
100 bp ladder
XY ts cut
XY ts cut
XX ov cut
XY ts un-cut
XX ov un-cut
250 bp
100 bp
50 bp
S1
S2
1
2
3
4
5

Fig. 12. **RT-PCR results from XX oocytes.**
The primers used are indicated above each lane. The "+" and "-" signs indicate whether reverse transcriptase was used or not, respectively, in the cDNA synthesis step. The sample with an "*" were amplified at a Tm of 60°C and contained a final concentration of 2.5 mM $MgCl_2$ in their PCR reaction. Note the absence of a *Xist* amplification product in (b) and the intensity of the *Ube1y* amplification product in (a). The most intense band observed with the *Ube1x* primers was ~400 bp.

a)


100 bp ladder
+ Hprt
+ Xist(k)
- Xist(k)
+ Ube1 y(a)
+ Ube1y(b)
+ Ube1y(b) *
+ Ube1x
+ ß-actin
800 bp
S 1 2 3 4 5 6 7 8

b)

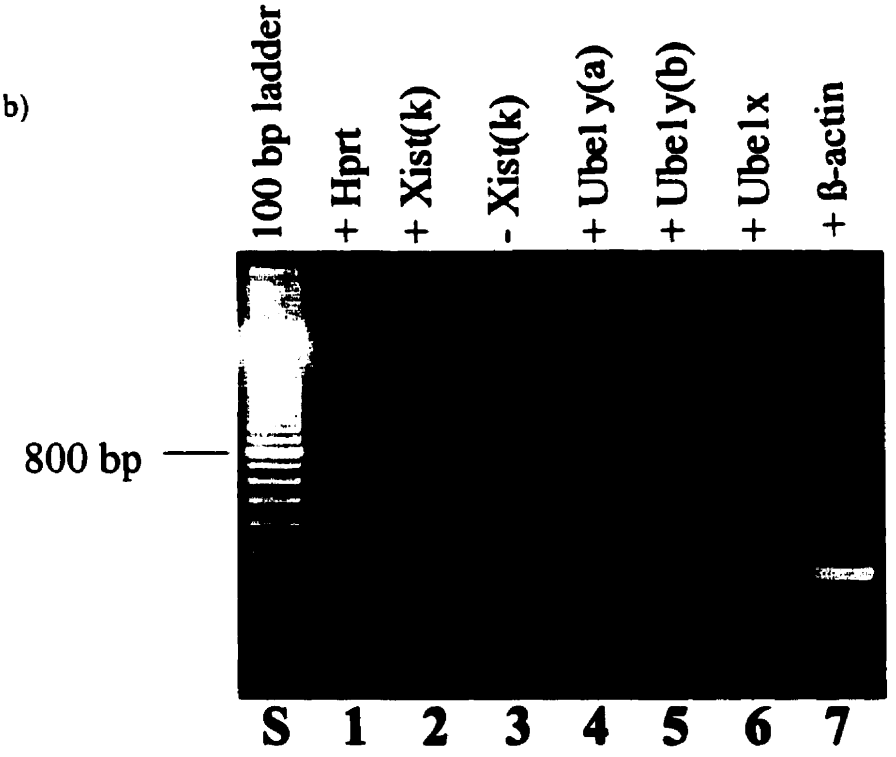

100 bp ladder
+ Hprt
+ Xist(k)
- Xist(k)
+ Ube1 y(a)
+ Ube1y(b)
+ Ube1x
+ ß-actin
800 bp
S 1 2 3 4 5 6 7

Fig. 13. **RT-PCR results from XY oocytes.**
The primers used are indicated above each lane. The "+" and "-" signs indicate whether reverse transcriptase was used or not, respectively, in the cDNA synthesis step. The sample with an "*" were amplified at a Tm of 60°C and contained a final concentration of 2.5 mM $MgCl_2$ in their PCR reaction.

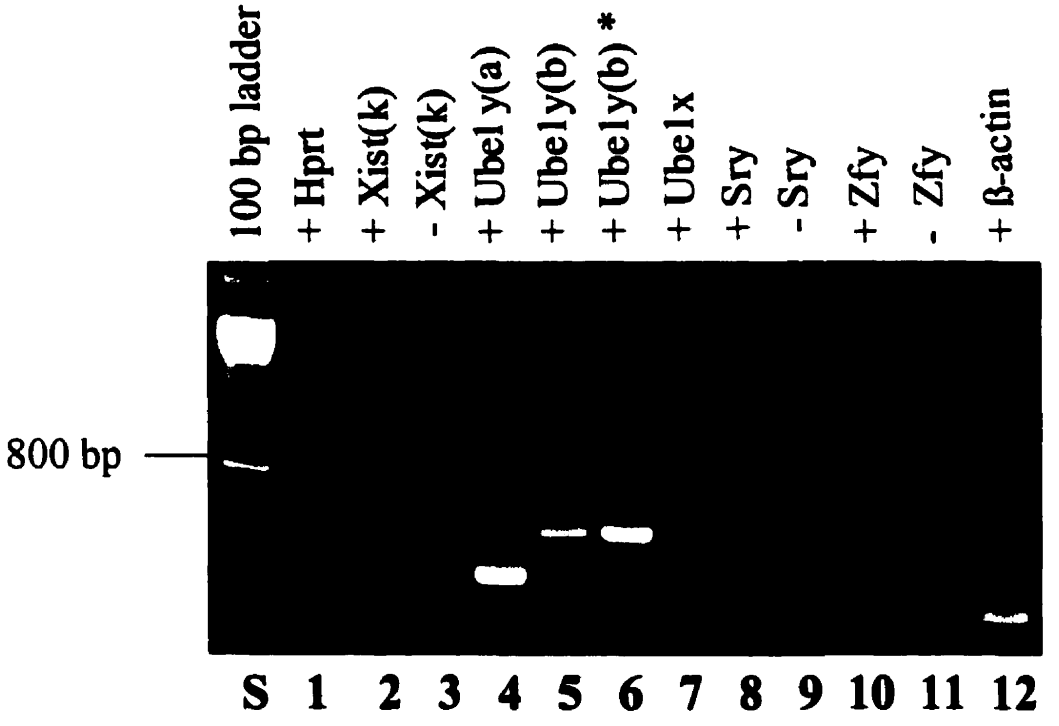

100 bp ladder
+ Hprt
+ Xist(k)
- Xist(k)
+ Ube1 y(a)
+ Ube1y(b)
+ Ube1y(b) *
+ Ube1x
+ Sry
- Sry
+ Zfy
- Zfy
+ ß-actin
800 bp
S 1 2 3 4 5 6 7 8 9 10 11 12

Fig. 14. **RT-PCR results for a XX adult kidney.**
The primers used are indicated above each lane. The "+" and "-" signs indicate whether reverse transcriptase was used or not, respectively, in the cDNA synthesis step. The sample with an "*" were amplified at a Tm of 60°C and contained a final concentration of 2.5 mM $MgCl_2$ in their PCR reaction.

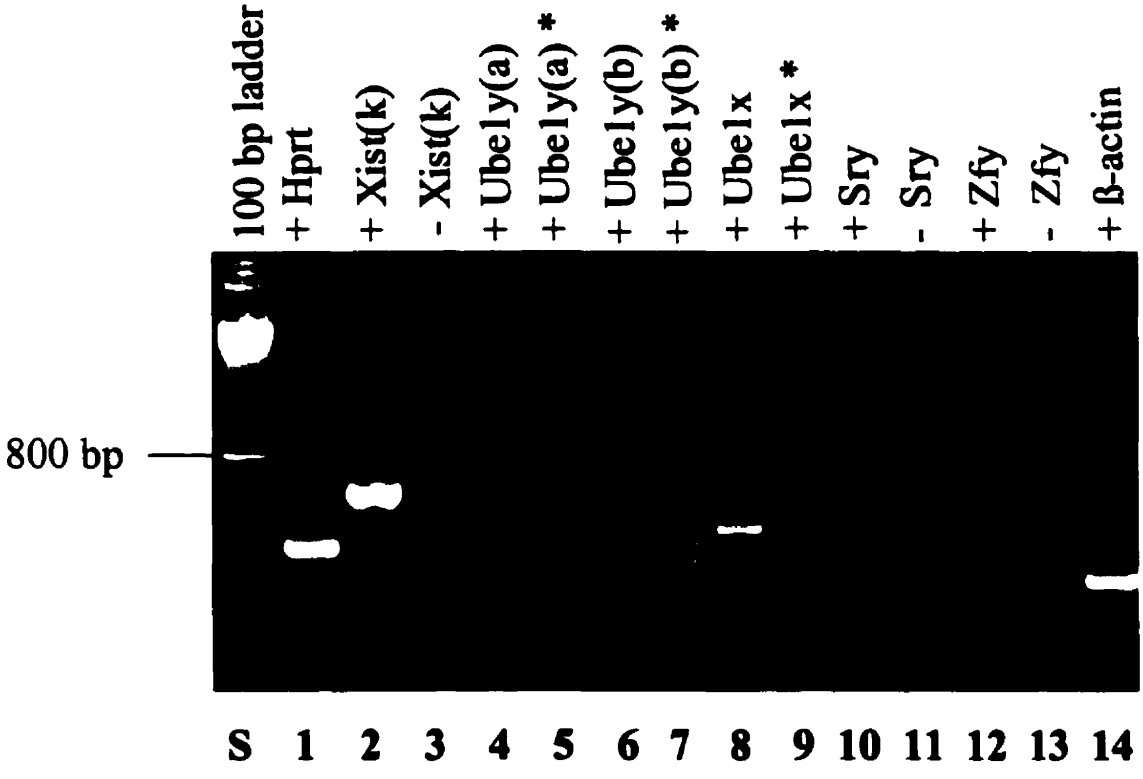

100 bp ladder
+ Hprt
+ Xist(k)
- Xist(k)
+ Ube1y(a)
+ Ube1y(a) *
+ Ube1y(b)
+ Ube1y(b) *
+ Ube1x
+ Ube1x *
+ Sry
- Sry
+ Zfy
- Zfy
+ ß-actin
800 bp
S 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 15. **RT-PCR result for a XY adult kidney.**
The primers used are indicated above each lane. The "+" and "-" signs indicate whether reverse transcriptase was used or not, respectively, in the cDNA synthesis step. The sample with an "*" were amplified at a Tm of 60°C and contained a final concentration of 2.5 mM $MgCl_2$ in their PCR reaction.

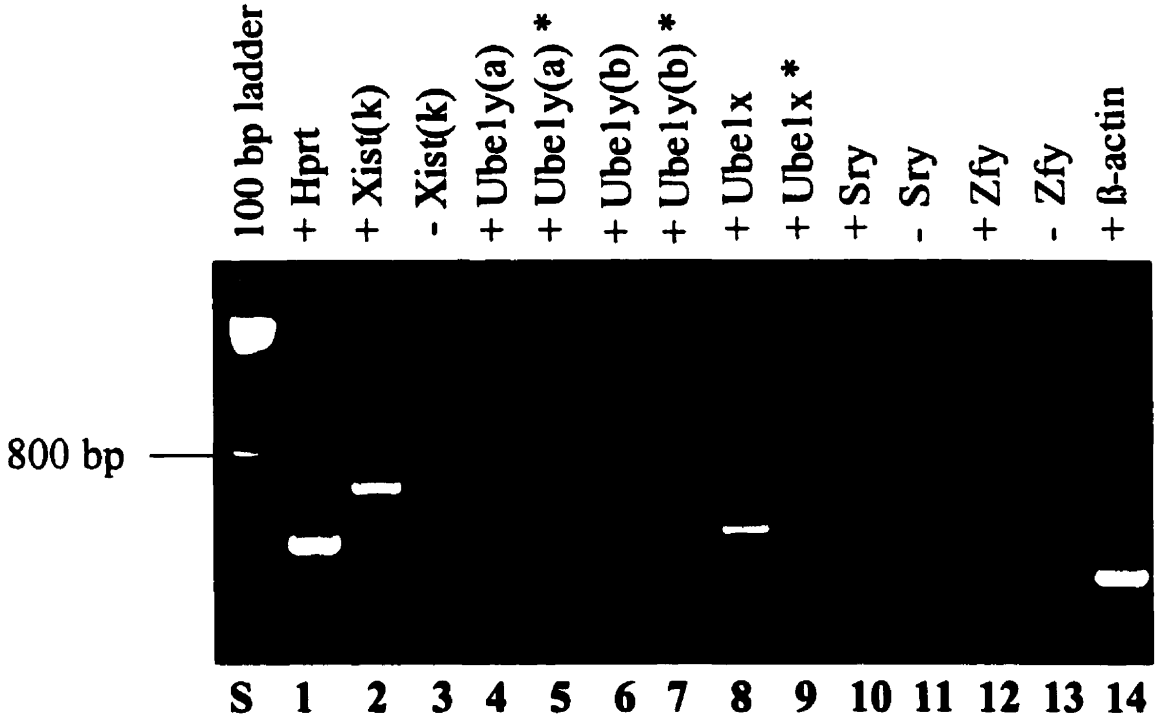
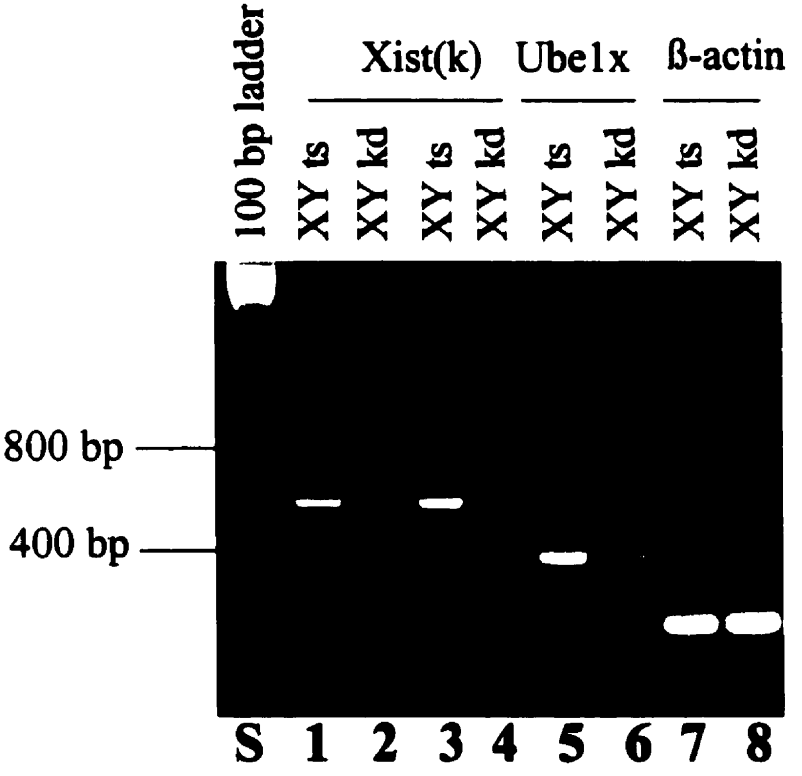

100 bp ladder
+ Hprt
+ Xist(k)
- Xist(k)
+ Ube1y(a)
+ Ube1y(a) *
+ Ube1y(b)
+ Ube1y(b) *
+ Ube1x
+ Ube1x *
+ Sry
- Sry
+ Zfy
- Zfy
+ ß-actin
800 bp
S 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 16. **Comparison of *Xist* band intensity between B6.Y^{TIR} testis and kidney.**
The primers used and the tissue examined are indicated above each lane. 0.5 μg of RNA from each tissue was used per RT-PCR reaction. Negative control reactions, ran on a separate gel, did not amplify a band.


100 bp ladder
Xist(k)
Ube1x
ß-actin
XY ts
XY kd
XY ts
XY kd
XY ts
XY kd
XY ts
XY kd
800 bp
400 bp
S 1 2 3 4 5 6 7 8

4- Discussion

4.1 The Y Chromosome Is Active in the B6.Y^{TIR} Ovary

Sry

Our lab has previously reported *Sry* transcription in the B6.Y^{TIR} gonad between 10.5 and 14.5 dpc (Lee and Taketo, 1994). In the present study we also detected *Sry* in sexually undifferentiated XY gonads at 12.5 dpc, albeit in a very small portion of the samples. It is known that expression of this gene reaches the maximum at 11.5 dpc and decreases rapidly afterwards. Therefore, the lower frequency of detection in the present study may be partially due to less *Sry* transcripts at this stage. Furthermore, it is possible that lack of detection stems from the inexperience of the experimenter, given that the examination of these gonads were done during the earlier parts of the study. The present finding of *Sry* transcripts in the XY ovary at 14.5 dpc but not in XY ovotestis of the same age is consistent with our previous findings and shows that *Sry* downregulation is slower in the XY ovary than the XY ovotestis.

Zfy

It has previously been reported that both *Zfy-1* and *Zfy -2* are expressed in XY testes. Although different authors have reported slightly different patterns of expression for these two genes, our *Zfy* primers do not differentiate between the two homologues and should amplify a product regardless of the identity of the *Zfy* homologue as long as one or both types are present. We did not detect transcripts for this gene in fetal XY gonads. It is

possible that the transcript levels for this gene are low and that the amount of RNA we used for each reaction was below the threshold of RT-PCR detection. This seems highly plausible given that one of the previous investigators whose primers we used, detected *Zfy* transcripts from RNA isolated from pooled fetal gonads (Nagamine et al., 1990). We avoided pooling, given the fact that the B6.Y^{TIR} gonad may contain both testicular and ovarian tissues. In fact, when higher amounts of RNA were used (approximately one-half of the total RNA from one fetal testis); RT-PCR amplification produced a 618 bp (correct size) band (data not shown). Since other gene transcripts were detectable using lesser amounts of RNA, we concluded that the transcript levels for this gene were very low or negligible.

We, however, did detect a correct size band for this gene in adult XY testes. This confirms that the *Zfy* primers can efficiently amplify an RT-PCR band, if the gene's transcripts are present at a higher concentration. On the other hand, we did not detect an amplification product in the single adult XY ovary or the single preparation of XY oocytes. Although the sample sizes are small, we conclude that the *Zfy* gene is not transcribed in the XY oocytes.

Ubely

Odorisio et al. (1996) have studied the transcription levels of the *Ubely* gene throughout testis development. They reported the presence of *Ubely* transcripts at all stages from 12.5 dpc to 28 dpp. Furthermore, they also detected this gene's transcripts in adult ovaries of the XY(Sry-) females (Lovell-Badge and Robertson, 1990). Hendriksen

et al. (1995) have also detected high mRNA levels in round spermatids and lower levels in pachytene spermatocytes.

Our detection of *Ube1y* transcripts using the *Ube1y(a)* primers in fetal ovotestis and adult testis confirmed the findings of Odorisio et al. (1996). In addition, we detected transcripts for this gene in fetal and adult XY ovaries. As well, growing XY oocytes also expressed this gene. Given Odorisio and her coworkers' findings in the XY(Sry-) ovaries and our finding in the B6.Y^{TIR} ovary, it is possible that *Ube1y* is expressed in the all Y-bearing gonads regardless of the phenotype of the gonad.

Ube1x

Detection of RT-PCR products with the *Ube1y(a)* primers in XX ovaries and oocytes puzzled us. Suspecting that this amplification was due to the presence of *Ube1x* transcripts, we decided to examine the transcriptional activity of this gene. Consequently we designed primers for it based on the available cDNA sequence from the 129/sv/Pas mouse strain. However, further studies revealed that our *Ube1x* primers detected transcripts for the male-enhance antigen-2 (*Mea-2*) (Kondo and Sutou, 1997)

Mea-2 was named as such based on the similarity of its expression pattern to a previously isolated gene called *Mea-1*. Both *Mea-1* and *-2* were isolated from a mouse testis cDNA library via specific antisera against the Serological Histocompatibility-Y (H-Y) antigen (Lau et al., 1989; Su et al., 1992). These two genes are evolutionarily conserved among various mammalian species examined. Furthermore, as seen with Northern blot analysis, both have high expression levels in adult testes but only basal to

very low levels in the ovary and other somatic tissue. More specifically, their transcript levels appear to be the highest at the haploid stages (round spermatids) during spermatogenesis (Lau et al., 1989; Su et al., 1992). In addition, the previous authors have also reported detecting *Mea-2* transcripts in ovaries via RT-PCR (Su et al., 1992). In fact, it appears that *Mea-2* transcripts are present in all somatic and ovarian tissue, albeit at lower levels than that found in the testis. Based on its nucleotide and amino acid sequences, it has been suggested that *Mea-2* encodes a golgi structural protein (Kondo and Sutou, 1997).

The present results therefore indicate that the *Ube1x* primers, designed from the 129/sv/Pas cDNA sequence, most likely do not recognize the *Ube1x* mRNA (cDNA) of the B6 strain. This was unexpected considering the ubiquitous role of *Ube1x* gene products. On the other hand, our *Ube1x* primers annealed to the *Mea-2* cDNA (CD-1 strain) although similar sequences could not be found in the regions flanking the amplified segment. It appears that polymorphisms are common amongst mouse strains. Sequencing of the full *Ube1y* and *Ube1x* cDNAs from the Tirano and B6 strains will help us clarify the problem encountered with the *Ube1y(a)* primers and would help identify the nature of the *pseudo-Ube1y* amplicon.

4.2 The Y Chromosome Is Active in B6.Y^{TIR} Oocytes

Although we only detected transcripts for the *Ube1y* gene in the XY oocytes, and not for the other two Y-encoded genes examined, this finding is sufficient for us to conclude that the Y chromosome is transcriptionally active in these oocytes. It is still

possible that transcripts from the other two genes are also being transcribed in the oocytes but that their levels are below the threshold of RT-PCR detection. Nonetheless, the presence of transcripts for at least one Y-encoded gene opens up the possibility that other Y-linked transcripts may also be present in the XY oocytes. It would be interesting to see whether *Ube1y* transcripts can also be detected in the oocytes from the two strains of XY sex-reversed females that are fertile. If so, then *Ube1y* transcripts per se may not affect the oocyte quality and are not involved in the infertility of the B6.Y^{TIR} female. However, other Y-encoded genes may be involved such that they are transcribed in one strain but not the others. As well, it is also plausible that the transcription of any particular Y-linked gene is not the problem but that a more global structural change of the Y chromosome, due to transcriptional activity, is.

4.3 The X Chromosome Shows Basal Levels of *Xist* Transcripts in the B6.Y^{TIR} Ovary and Is Hence Active

The process of X inactivation is mediated through the RNA product of the *Xist* gene. This RNA presumably associates with and inactivates the X chromosome from which it is transcribed. X inactivation in humans silences most genes on the X chromosome but not all. In mice, even fewer X-linked genes appear to escape X inactivation (Disteche, 1995).

In the present study, we consistently detected *Xist* transcripts in all XX ovaries examined. This finding is in agreement with the idea of one of the two X chromosomes being randomly inactivated in female somatic cells. The ovary being composed of both

somatic and germ cells, would therefore show this pattern of expression.

However, an amplified *Xist* product was also sometimes detected in the XY fetal ovaries and ovotestes, albeit at lower intensity than that observed with XX ovaries. It is our belief that these corresponded to the basal levels of *Xist* transcription from the active X chromosome. Further support for this idea comes from XY kidney samples, where *Xist* transcripts were also detected (Fig. 15, lane 2). Panning et al. (1997) have shown that X chromosome inactivation is mediated through stabilization of the *Xist* RNA. As such, the active X chromosome in male (XY) ES cells show low levels of *Xist* expression. This observed level is due to ongoing transcription for this gene. These transcripts are however, not stabilized as they are in the female somatic cells and hence, the single X in the XY somatic cells remains active. In contrast, we detected higher levels of *Xist* in the XY adult testis as compared to XY adult kidneys. This finding is consistent with the idea that the single X chromosome is transcriptionally inactive during spermatogenesis and that this X chromosome inactivation is most likely mediated through *Xist* (Richler et al, 1992; Salido et al., 1992) .

4.4 The X Chromosome Is Active in Both XX and B6.Y^{TIR} Oocytes

In our early experiments, we detected *Xist* transcripts in isolated XX and XY oocytes. However, as our collection technique improved (mostly due to the conversion from lysozyme to trypsin in the ovary digestion step) we no longer detected *Xist* in most oocyte samples tested. We believe that contamination with somatic cells was the reason why we detected *Xist* RT-PCR product in some of our oocyte samples. Our latter findings

are consistent with the idea that both sex chromosomes are active in female germ cells undergoing meiosis. McCarrey and Dilworth (1991) have previously examined the presence of *Xist* in female germ cells. They have detected *Xist* transcripts in oogonia prior to 12.5 dpc but noted that transcripts disappeared between 12.5 and 13.5 dpc, the alleged time of entry into meiosis. It has generally been assumed that both X chromosomes remain active in oocytes via the same mechanism. Since we did not detect *Xist* transcripts in most of our XX oocyte samples, it would appear that the lack of expression of this gene plays a role in allowing the second X chromosome to (become and) remain active.

In addition, the absence of *Xist* transcripts in XY oocytes suggests that the single X chromosome is also active in these oocytes. As such, X chromosome reactivation in oocytes may be a general characteristic of oogenesis.

4.5 Cause of Infertility

In the B6.Y^{TIR} strain, the testis determination pathway is partially impaired, resulting in development of either fertile or infertile XY males and hermaphrodites and exclusively infertile XY females at a 1:1:2 ratio. It is unlikely that the infertility of the B6.Y^{TIR} female is entirely due to the presence of a Y chromosome given that other XY sex-reversed females are fertile (Lovell-Badge and Robertson, 1990; Capel et al., 1993). One important difference in the infertility between the XY(Sry-), XY^{del} and XY^{TIR} females may involve the activity of the *Sry* gene. The *Sry* gene is absent in the first strain, and not transcribed in the second strain due to a deletion elsewhere on the Y chromosome. In contrast, there are no known mutations or deletions on the Y^{TIR}

chromosome. Furthermore, the *Sry* gene is transcribed at the proper time in fetal life in the XY^{TIR} mouse (Lee and Taketo, 1994). Thus, it is possible that the presence of *Sry* transcripts, if present in the oocyte, affects their competence for postfertilization development. Although, in the present study, we did not detect any *Sry* transcripts in the growing oocytes we examined, this does not rule out the possibility of it being transcribed in the oocyte at earlier stages of development. The expression of *Sry* may have a secondary, but persistent, effect on the expression pattern of other genes involved in oogenesis. Isolation of oocytes from fetal ovaries, although technically demanding, is feasible. As such, we have tried isolating oocytes from fetal XY ovaries at 14.5 dpc. We were however not successful in detecting any gene product (including *β-actin*) by RT-PCR in these oocytes. It is possible that the number of oocytes collected (20 or less per reaction) were not sufficient or that transcripts from these genes in fetal oocytes are at levels below the threshold of RT-PCR sensitivity. However, it is also possible that we still need to optimise the conditions for collection of these "younger" oocytes as well as the conditions for RT-PCR when using this source as template.

A haplodeficiency of X-encoded products can also be ruled out as the sole cause of infertility, given that both types of XY sex-reversed females mentioned above are as stated fertile despite having only one X chromosome. Furthermore, the XO female mouse is also fully fertile despite having fewer progeny per litter and a shorter reproductive life span (Lyon and Hawker, 1973; Burgoyne and Baker, 1981; 1985). However, it is possible that X chromosome haplodeficiency combined with Y chromosomal gene expression worsens the situation. For instance, the transcriptionally active Y chromosome

may reduce the efficiency of X chromosome gene expression. The activity of the Y chromosome in the other XY females needs to be examined in order to assess this possibility. In addition, quantitative analysis of X-encoded gene products in isolated oocytes would also shed some light on this question.

4.6 Behaviour of Sex Chromosomes During the First Meiotic Prophase

During prophase of meiosis I, homologous chromosomes pair, synapse and exchange genetic material. The advantages of this process are two fold: 1- It results in genetic variability in the next generation. 2- It insures proper disjunction (separation) of the homologous chromosomes during anaphase I.

The sex chromosomes also pair and recombine. In XX primary oocytes, which are at this point both transcriptionally active, the X chromosomes pair and have the potential for chiasma formation along their entire length. In XY primary spermatocytes, the X and Y chromosomes also pair and recombine, but given their limited homology, this event takes place only in the small PAR at the distal end of the X and Y chromosomes. Furthermore, the X and Y chromosomes assume a condensed chromatin configuration (the sex body), whereas the X chromosome pair in the female germ cells remain decondensed as are the autosomes. It has been hypothesised that the purpose of the sex body is to bring the X and Y chromosomes together and thus facilitate pairing between the two.

McKee and Handel (1993) have suggested that condensation of sex chromosomes during spermatogenesis is a meiotic adaptation which prevents " the initiation of

potentially damaging recombination events in nonhomologous regions of the X and Y chromosomes." As such, transcriptional inactivity during spermatogenesis is a consequence of chromatin condensation.

Dr. Taketo's lab has examined the behaviour of X and Y chromosomes during meiotic prophase in the B6.Y^{TIR} oocytes. In the XY oocyte, the X and Y chromosomes are not sequestered into the sex body; they do not appear to pair and recombine as they are seen to do in the XY spermatocyte. Transcriptional activity of the X and Y chromosomes in these oocytes may be in part responsible for the X-Y pairing failure.

Furthermore, as a result of this pairing failure, it can be inferred that proper segregation of the sex chromosomes does not always occur at the end of meiotic prophase I. In fact, in their review article, Eicher and Washburn (1986) briefly mentioned that upon examining B6.Y^{POS} oocytes at the first meiotic metaphase, they noted that the X and Y chromosomes were present as univalents. Hunt and LeMaire (1992) who have also examined B6.Y^{POS} oocytes, reported the presence of univalent sex chromosomes at metaphase II. Therefore, based on these findings, it is possible that a major cause of infertility in these females is aneuploidy. This, however, cannot explain the death of all embryos since random segregation of the sex chromosomes can still result in the production of some normal karyotypic progeny as well as some XO progeny. Therefore, some other element(s) must also be involved in the defect of the XY oocytes. Transcription and/or translation of Y-encoded genes is a likely candidate. This idea is highly favoured because of the findings with nuclear transplantation experiments. In these experiments, it was shown that both the nuclear and cytoplasmic components of the XY

oocytes are incompetent in supporting embryonic development (Amleh et al., in preparation).

Therefore, it is possible that Y-encoded gene transcripts and/or products in the oocyte's cytoplasm create an unfavourable environment for post-fertilization development of zygotes derived from B6.Y^{TIR} females.

4.7 Conclusion

The B6.Y^{TIR} female is most likely infertile due to a number of reasons. The major cause of infertility, however, appears to be due to a defect within the XY oocyte. This defect is probably due to the presence of a X and a Y chromosome in an oocyte; both haplodeficiency of X-encoded gene products and the expression of Y-encoded gene products may render the oocyte incompetent for post-fertilization development. In addition, transcriptional activity of the X and Y chromosomes may impair proper pairing and segregation. This, in turn, could further reduce the fertility of the B6.Y^{TIR} female. The present study provides evidence that both sex chromosomes are transcriptionally active in the oocytes in the B6.Y^{TIR} female.

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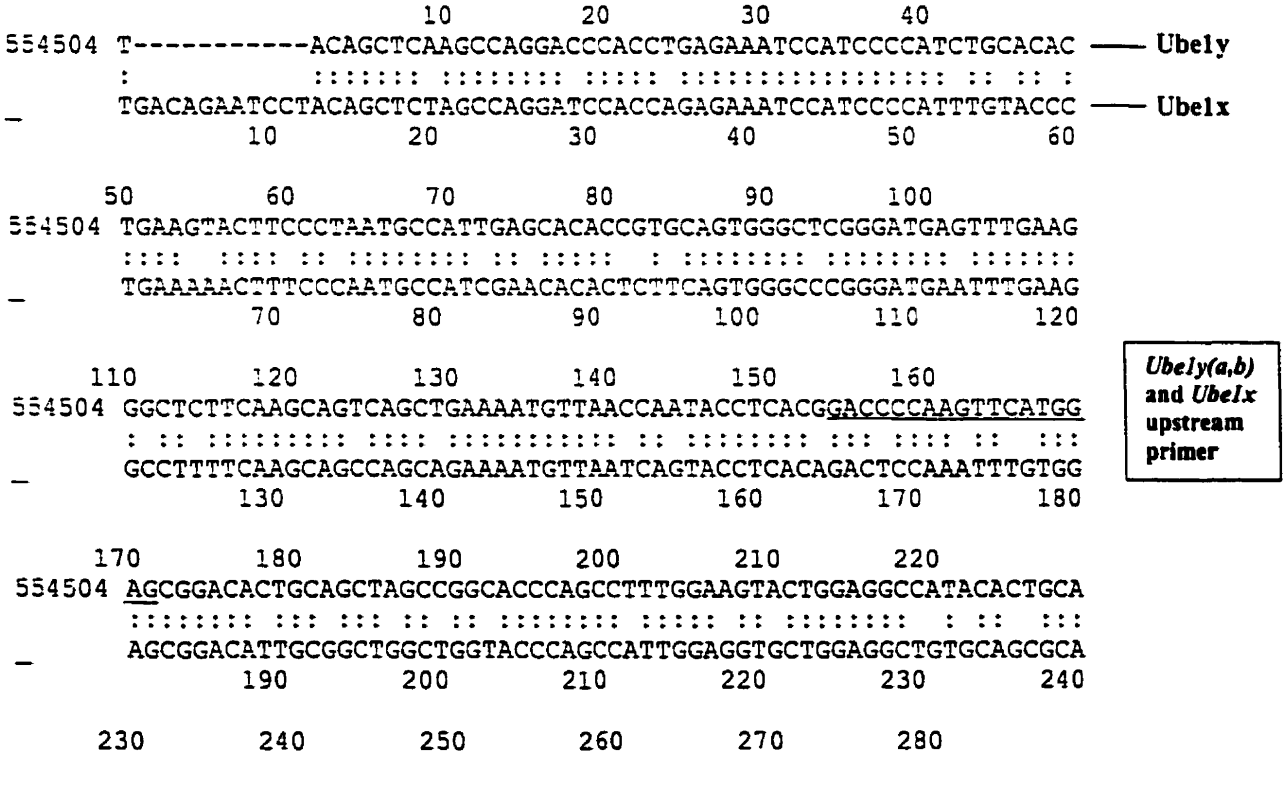
Appendix A

Ube1y RT-PCR product sequenced from pooled XY fetal ovary samples. Negative strand.

5'AAACAGGTTGGCAGCAGNNATCACATAATCCAGATGCAGGGGATTGTTTAT
GTCAAAGGTGAGAGGATGTGGACAGCGTTTTGGTCCTGACCAAAAAAGTGYT
CCAGAGCTCGTAAGCTGTGCTGGAGGGAAGTTGTGCAGCAACTGCTGGATGT
TGTGAGATACTGGGTNNNN3'

Appendix B

Alignment results comparing the similarity between the *Ubely* and the *Ubelx* cDNA sequences.



```
554504 GCCTGGTCCTGCAGAGGCCACAGACTTGGGCCGACTGTGTGACTTGGGCCTACCAGCACT
       :::::::  ::::: : ::::::::::::  :::::::::: ::::::: ::: ::::
_      GCCTGGTGTTGCAGCGACCACAGACTTGGGGAGACTGTGTGACCTGGGCCTGCCACCACT
              250       260       270       280       290       300

     290       300       310       320       330       340
554504 GGCACACCCAGTATTCTCACAACATCCAGCAGTTGCTGCACAACTTCCCTCCAGCACAGC
       :::::::::::::: : : ::::::::: :::  :::::::::::::: ::::: :  ::::
_      GGCACACCCAGTACTGTAACAACATCCGGCAACTGCTGCACAACTTTCCTCCTGACCAGC
              310       320       330       340       350       360

     350       360       370       380       390       400
554504 TTACGAGCTCTGGAGCACTTTTTTGGTCAGGACCAAAACGCTGTCCACATCCTCTCACCT
       : :: ::::: :: :: : ::: ::::: ::::: :::::::::::::: :: :: :: :
_      TCACCAGCTCAGGGGCCCCTTTCTGGTCTGGACCCAAACGCTGTCCACACCCACTTACTT
              370       380       390       400       410       420

     410       420       430       440       450       460
554504 TTGACATAAAACAATCCCCTGCATCTGGATTATGTGATGGCTGCTGCCAACCTGTTTGCTC
       ::::  : :::::: :  :::::::::::::::::::::::::::::::::: ::::: :
_      TTGATGTTAACAATACATTGCATCTGGATTATGTGATGGCTGCTGCCAACCTTTTTGCCC
              430       440       450       460       470       480

     470       480       490       500       510       520
554504 AGACATACGGACTAGGAGGGTCCCAGGACTGTGCTGTGGTGGCCAAACTCCTGCAGTCTC
       :::: :: ::  :    :: ::::: ::: : :::: ::::::  ::::::::::::
_      AGACCTATGGGTTGACTGGATCCCAAGACCGAGCTGCTGTGGCCTCACTCCTGCAGTCAG
              490       500       510       520       530       540

     530       540       550       560       570       580
554504 TGCCAGTCCCCAAGTTTGCTCCCAAGTCTGGCATCAGGATCCATGTTTCTGAGCAGGAGC
       : : ::::::  ::::  : ::::::::::::  ::: ::: ::::::::::: ::::::
_      TACAAGTCCCAGAGTTCACCCCCAAGTCTGGTGTCAAGATTCATGTTTCTGACCAGGAGT
              550       560       570       580       590       600

     590       600       610       620       630       640
554504 TGCAGAGTACCAGTGCCACCACCATTGATGACAGCCACCTGGAGGAACTCAAGACTGCAC
       :::::::: ::: :::: :      :::::::::::::  :: ::::: :::::  :  ::
_      TGCAGAGTGCCAATGCCTCTG---TTGATGACAGCCGTCTTGAGGAGCTCAAAGCCACAT
              610       620          630       640       650

     650       660       670       680       690       700
554504 TTCCTACTCCAGACAAGCTGCTTGGATTCAAGATGTACCCCATTGACTTTGAGAAGGATG
       : :: :  :::::::::: : :  ::::: :::::::::::::::::: ::::::::::::
_      TGCCCAGCCCAGACAAGTTACCCGGATTTAAGATGTACCCCATTGATTTTGAGAAGGATG
       660       670       680       690       700       710

     710       720       730       740       750       760
554504 ACGACAGCAACTTTCACATGGATTTCATTGTGGCAGCATCCAACCTCCGAGCAGAAAACT
       : :::::::: :: :::::::::::::::::::: :::::::: :: :: :: :::::::
_      ATGACAGCAATTTCCACATGGATTTCATTGTGGCTGCATCCAATCTTCGGGCCGAAAACT
       720       730       740       750       760       770

     770       780       790       800       810       820
554504 ATGGCATTTCCCCAGCAGACCGGCATAAGAGCAAACTGATTGCAGGCAAGATCATCCCAG
```

Complementary to the *Ubely(a)* downstream primer

Complementary to the *Ubelx* downstream primer

Complementary to the *Ubely(b)* downstream primer

```
       ::: ::::::::: ::::::::: :: ::::::::: :::::::::: ::::::::::::
_      ATGATATTTCCCCTGCAGACCGACACAAGAGCAAGCTGATTGCAGGGAAGATCATCCCAG
       780        790        800        810        820        830

     830        840        850        860        870        880
554504 CCATTGCAACCACCACATCTGCTATAGTTGGCCTTGTGTGTCTGGAGCTGTACAAGGTGG
       ::::::: :: :::::: ::::: : ::::::::::::::::::::::: :::::::: :
_      CCATTGCCACAACCACAGCTGCTGTGGTTGGCCTTGTGTGTCTGGAGCTCTACAAGGTAG
       840        850        860        870        880        890

     890        900        910        920        930        940
554504 TTCAGGGCCACCAACAACTGGAGTCTTATAAAAACAGTTTTATCAACTTGGCTCTGCCTT
       :::: :: :::::::: :: :: :: :: ::::: :::: : :::::::: ::::: :
_      TTCAAGGGCACCAACAGCTCGATTCCTACAAAAATGGTTTCCTGAACTTGGCCCTGCCCT
       900        910        920        930        940        950

     950        960        970        980        990       1000
554504 TGTTTAGCTTTTCTGCACCTCTGGCTCCAGAGTGTCATCAGTTCTATGATCAAGAGTGGA
       : ::: : :::::::: :::::: ::: ::    :::: :::: :::: ::::::::::::
_      TCTTTGGGTTTTCTGAACCTCTTGCTGCACCTCGTCACCAGTACTATAATCAAGAGTGGA
       960        970        980        990       1000       1010

    1010       1020       1030       1040       1050       1060
554504 CACTGTGGGATCGCTTTGATGTGCAAGGACTGCAACCTAGCGGTGAGGAGATGACCTTGA
       :: ::::::::::::::::: :: :::::  : :: ::::  ::::::::::::::: : :
_      CATTGTGGGATCGCTTTGAAGTACAAGGGGTCCAGCCTAATGGTGAGGAGATGACCCTCA
      1020       1030       1040       1050       1060       1070

    1070       1080       1090       1100       1110       1120
554504 AGCAGTTTCTAGACTACTTTAAGACAGAGCACAAGCTGGAAGTCATCATGCTGTCCCAGG
       ::::::: :: :: ::::::::::::::::::::  ::::  ::: ::::::::::::::
_      AGCAGTTCCTTGATTACTTTAAGACAGAGCACAAATTGGAGATCACCATGCTGTCCCAGG
      1080       1090       1100       1110       1120       1130

    1130       1140       1150       1160       1170       1180
554504 GTGTGTCCATGCTCTATTCTGTCTTTATGCCAGCCAGCAAGCTCAAAGAACGGTTGGATC
       : :::::::::::::::::: :::: ::::::::    :::::::: ::::: :::::::
_      GCGTGTCCATGCTCTATTCTTTCTTCATGCCAGCTGCTAAGCTCAAGGAACGATTGGATC
      1140       1150       1160       1170       1180       1190

    1190       1200       1210       1220       1230       1240
554504 AGCCGATGACAGAGATTGTGAGCTGTGTGTCAAAGCAAAAACTGGGCCATCATGTGAAGT
       :::::::::::::::::::::::: : :::::::::  ::: :::::::  ::::::  :
_      AGCCGATGACAGAGATTGTGAGCCGAGTGTCAAAGAGAAAGCTGGGCCGCCATGTGC-GG
      1200       1210       1220       1230       1240       1250

    1250       1260       1270       1280       1290       1300
554504 CACTTGTGTTTGAGCTGTGCTGCAACAGTGACAGTGGAGACGACATAGAGGTTCCTTATG
       :::: ::: :::::::::::::::::  ::: :: :: :: ::: : ::::: :::::::
_      CACTGGTGCTTGAGCTGTGCTGCAACGATGAAAGCGGCGAGGACGTCGAGGTCCCTTATG
       1260       1270       1280       1290       1300       1310

    1310       1320       1330            1340       1350       1360
554504 TACGATACATCATCCGCTGATCTC------TTTCTTACTGACTCCGTTCTTTCAGATCTC
       : ::::: : ::: ::::::::::      :: :     : : : :: ::  :   :: :
```

```
_       TCCGATATACCATTCGCTGATCTCCGTGCATTCCCCTAAGCCCCAGTCCTCCC---TC-C
        1320       1330       1340       1350       1360          1370

           1370       1380          1390                        1400
554504  TCAGTCTATATCAAAACCT---TTCTAGTTA---TGAGGAA-----------ACCTCAGT
        ::::     : :: :: :::    ::: : ::     : ::: :            : :   ::
_       TCAGATCCTTTCCAATCCTGGTTTCCATTTGGCCTCAGGCAGTGGTCCATGTAGCCAAGC
            1380       1390       1400       1410       1420       1430

        1410       1420                  1430       1440       1450
554504  TTGAACCTCCTTGTT---------CCT-AAGAACTTTTTAGTGCTGTTTT-TACATTGTT
         ::   : ::: : ::         ::: :::: : : : :  :::: ::: ::: :::::
_       CTGGCCTTCCCTATTACCCAGCAACCTCAAGACCCTCTCACCGCTGCTTTGTACCTTGTT
            1440       1450       1460       1470       1480       1490

         1460         1470         1480       1490       1500       1510
554504  AAGAACTGAAATTC--ATAATA--ATAAATTTTCAGCCTGTGAAAAAAAAAAAAAAAAAA
          ::::  :::: :  :::: :  :::: :   :::       ::::::::::::::::::
_       TGGAACCTAAATCCCAATAAAAGGATAATTACCCAGAAAAAAAAAAAAAAAAAAAAAAAA
            1500       1510       1520       1530       1540       1550


554504  ----------------------------------------------------

_       AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
            1560       1570       1580       1590       1600

Elapsed time:  0:00:00
```

Appendix C

Ube1x RT-PCR product from an XX adult ovary. Positive strand.

5'CCTCTACACGGTTATCAGTCTCAGATGATGGGCTAACCTCAGACACAACTGT
AAGTGCTACTTCTACTTTGTTTCAGGGKAACTTCCAACAGCATCAGCAGTAGA
ACCAAGGCCACCAGAGTCACTTGGATCTTCAGTTCTATAGTCAGCCAGAGAC
CGAATCTTGCTTGATTTGGAGTTCTTTTTCTCTTTAGAATGTGCCAGCAGCCCT
AAGCTGCCTACCTTGGGCCCTCGAGGAACACTGGTACGCAGGAAGGAATATT
CTTTTGTCACAGCTACAGTACTGGCCCTTGGCAAATTTTCTGGATTTAACATG
AGCTCAGGATCAAGTGTACTAAAAAGTTCATTTTAGTTGCAGGTTGACTGGAT
CCAAAAAAAAANN3'