# X-INACTIVE SPECIFIC TRANSCRIPT (*XIST*) AND X CHROMOSOME INACTIVATION IN THE BOVINE SPECIES

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of

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by

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**ABSTRACT**

**X -INACTIVE SPECIFIC TRANSCRIPT (*XIST*) AND X CHROMOSOME INACTIVATION IN THE BOVINE SPECIES**

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X-inactive specific transcript (*Xist*) which is thought to be a major factor in regulating X-chromosome inactivation in female mammals, is known to be expressed in male germ cells during spermatogenesis. Expression of *Xist* and the status of the X-linked genes including glucose 6-phosphate dehydrogenase (*G6pd*), hypoxanthine phosphoribosyl transferase (*Hprt*), and zinc finger protein gene (*Zfx*), in gonads and nongonadal tissues of male and female fetuses was compared using the semiquantitative reverse transcription polymerase chain reaction (RT-PCR) to test if the expression of *Xist* leads to the inactivation of these genes in bovine testes of fetuses between 85 to 95 days of gestation. Results showed that the levels of expression of X-linked genes in fetal testes were comparable to those in somatic cells of female fetuses even though *Xist* expression in fetal testis was evident at this stage of development. Tests on the expression pattern of five X-linked genes, including the bovine homologue of the "selected mouse cDNA on the X" (*Smcx*), in cell cultures of chromosomally normal bovine fetuses and X chromosome aneuploids showed that *G6pd*, *Hprt* and *Smcx* are subject to inactivation whereas *Zfx* escapes inactivation in bovine cells. Furthermore, these studies showed that *Xist* expression increases in keeping with the number of X

chromosomes present in the cells, and that it is expressed in normal male (XY) cell cultures. Based on this unexpected finding that male somatic cells which normally do not undergo X inactivation express *Xist* RNA, a strand- specific RT-PCR approach was undertaken to determine whether the amplicon observed in male bovine cells is *Xist* per se or its antisense RNA. The results revealed that the RNA detected in fetal and adult bovine testes and nongonadal tissues and cell cultures of males is exclusively that transcribed by the antisense strand while both sense and antisense RNAs are expressed in bovine female cells. The observation that the transcript detected in fetal and adult bovine testes in this study (and probably in the testes of mouse and man by other investigators in previous studies) represents *Xist* antisense RNA while it is coexpressed with the sense strand of *Xist* in female cells, leads us to hypothesize that *Xist* antisense RNA may have a common role to play in both sexes, and that it may be to preserve one X chromosome in an "uninactivated" state.

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## DECLARATION OF WORK PERFORMED

I declare that with the exception of the items below, all work reported in this thesis was performed by me.

Bovine Fetuses were collected at the local slaughter house by Charles Botchi, Animal Biotechnology and Embryo Laboratory, University of Guelph. Preparations for light and electron microscopy were made by Dr. W. Koykul and photomicrograph and electron microscopic images were provided by Dr P. K. Basrur. Sequencing of PCR products was performed by Mrs. Angela Holliss, Guelph Molecular Supercentre, University of Guelph.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| bp | Base pair |
| cDNA | Complementary DNA |
| CRL | Crown-rump length |
| DEPC | Diethyl pyrocarbonate |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTP | Dinucleotide triphosphate |
| EDTA | Ethylenediamine tetraacetic acid |
| EMEM | Eagle's minimum essential medium |
| ES | Embryonic stem (cells) |
| FCS | Fetal calf serum |
| *G6pd* | Glucose-6phosphate dehydrogenase |
| *Hprt* | Hypoxanthine phosphoribosyl transferase |
| $H_2O_2$ | Hydrogen peroxide |
| LINE | Long interspersed repeat elements |
| KCl | Potassium chloride |
| $MgCl_2$ | Magnesium chloride |
| NADPH | Nicotinamide adenine dinuclotide |
| OD | Optical density |
| Otc | Ornithine transcarbamylase |
| PAR | Pseudoautosomal region |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PPP | Pentose phosphate pathway |
| RNA | Ribonucleic acid |
| *RPS4X* | Ribosomal protein subunit 4, X-linked |
| RT | Reverse transcription |
| RT-PCR | Reverse transcription polymerase chain reacion |
| SEM | Standard error of the mean |
| *Smcx* | Selected cDNA on X, mouse homologue |
| *Sry* | Sex determining region on Y chromosome |
| *STS* | Steroid sulfatase |
| *Tsix* | *Xist* antisense strand [in the mouse] |
| *UBE1* | Ubiquitin-activating enzyme 1 |
| Xic | X chromosome inactivation center |
| *Xist* | X inactive specific transcript |
| Xp | X chromosome, short arm |
| Xq | X chromosome, long arm |
| *Zfx* | Zinc finger protein, X linked |
| *Zfy* | Zinc finger protein, Y-linked |

# INTRODUCTION

Regulation of X-linked gene expression in female mammals is accomplished through a mechanism referred to as "dosage compensation". Different processes have been adopted for dosage compensation in organisms with heteromorphic sex chromosomes (reviewed by Meller, 2000). The inactivation of one of the two X chromosomes of the female somatic cells is the method adopted by mammals (Lyon, 1961; Reviewed by Heard et al., 1997). However, evidence indicates that the X chromosome inactivation is incomplete and some X-linked genes escape inactivation (Disteshe, 1997). Expression profile of X-linked genes escaping inactivation differs between species (Jegalian and Page, 1998), and between developmental stages, tissues, and cell types (Carrel et al., 1996; Sheardown et al., 1996) suggesting that the mechanism of dosage compensation (of X-linked gene products) may have evolved independently in different species and that the differences in the location of the genes on the chromosome, heterochromatin content or other factors may influence the expression of X linked genes (Miller and Willard, 1998).

Difference in the expression status of the X chromosome in male and female germ cells during meiosis has attracted much attention recently. It has been noted that the inactive X chromosome in female germ cells is reactivated when meiosis is initiated in the fetal ovary (Handel et al. 1994) and that the sole X chromosome in male germ cells, in association with the Y chromosome, is condensed during early stages of meiosis to form a structure referred to as the sex body or the XY body (Solari, 1994).

Although meiosis, as a preamble to spermatogenesis, is initiated in the adult testis, fetal male germ cells could enter meiosis under certain conditions (Upadhyay and Zamboni, 1982; Hogg and McLaren, 1988). Factors influencing the onset of meiosis in male germ cells, the relevance of X chromosome inactivation to spermatogenesis, and the mechanism and molecular correlates of X chromosome inactivation in male germ cells remain mainly unknown.

The X inactive specific transcript (*Xist*), which is thought to be the key regulatory factor in the process of X chromosome inactivation in female mammals (reviewed by Brockdorff and Duthei, 1998), is also shown to be expressed in adult testes of various species (McCarry and Dilworth, 1992; Richler et al., 1992; Ayoub et al., 1997). However, the role of *Xist* in X chromosome inactivation in male germ cells remains undefined since studies have also shown that *Xist* deficient mice can accomplish normal spermatogenesis (Marahrens et al., 1997).

Previous studies from this laboratory had shown that *Xist* is expressed in adult bovine testes while it is silent in adult male somatic cells (DeLa Fuente et al., 1999). However, the onset of X inactivation process during the differentiation of bovine testis is not established as yet. The aim of this study was to delineate the expression pattern of *Xist* gene in bovine male germ cells and to investigate its possible role in the process of X chromosome inactivation in the domestic cattle, *Bos taurus*. In order to achieve this goal, the project was approached in different segments with the following specific objectives: (1) to determine the onset of *Xist* expression in fetal bovine testis, (2) to test whether *Xist* expression leads to the inactivation of X-linked genes in bovine fetal

testes, (3) to characterize the pattern of expression of X-linked genes with specific emphasis on testing whether *Xist* expression correlates with the number of X chromosomes present in cell cultures of normal male and female bovine fetuses and sex chromosome aneuploids, and, (4) to identify the RNA transcribed by male gonadal and nongonadal bovine cells. These objectives are dealt with in separate chapters (Chapters 1 to 4).

## LITERATURE REVIEW

### Sexual dimorphism in mammals

Sexual dimorphism is manifested at different periods of development in mammals. A sex dependent difference is expressed at the time of differentiation of the fetal gonad into the testis in males and the ovary in females. Development of male and female reproductive tracts and external genitalia in the direction that is characteristic of male or female follows the gonadal development. Manifestion of the secondary sexual traits during puberty accentuates sexual dimorphism. Regulation of sex dimorphism in mammals has been one of the most fascinating topics in biology through the ages. In 335 BC Aristotle proposed that sex of fetus was determined by the heat of the semen with hot semen leading to the production of male offspring and cold semen producing females. This "environmental" view of sex determination changed drastically when the chromosomal difference between the male and the female was discovered (Bridges, 1916; reviewed by Gilbert, 1997).

Early in mammalian fetal development the gonads are bipotential and the urogenital system contains both the Mullerian and Wolffian ducts. Once the testis is induced the development follows in male direction and in the absence of the testis the female pattern prevails. The endocrine role of the fetal testis in influencing the sexual phenotype was recognized in 1916 independently by Lillie and by Keller and Tandler (cited by Wilson et al., 1995) in their attempt to interpret the origin of the bovine freemartin, a virilized female born twin to a normal male calf. Direct evidence for the

dominant effect of testis on the phenotype was provided by Jost (1953) who demonstrated that the internal and external genitalia in castrated rabbits differentiated in the female pattern. Based on these observations, the fundamental mechanism of mammalian sex differentiation was summarized as the Jost paradigm: the establishment of the genetic sex at conception based on the type of the sex chromosome carried by the male gamete leads to the development of the gonadal sex which, in turn, translates into the somatic sex (Jost, 1953; reviewed by Wilson et al., 1995). Although the Jost principle (1953) generally holds true, recent findings indicate that biological differences between the male and the female begins earlier than the time of gonadal differentiation (Erickson, 1997).

## Sex Chromosomes

Early in the 20th century cytogenetic studies on *Drosophila* revealed that the sex of the fruit fly depends on the ratio of the X chromosome to autosomes. For instance, a diploid fly with one X chromosome develops as a male, while those with two X chromosomes become female ( reviewed by Gilbert, 1997). In this species, the Y chromosome is not involved in testis determination. Progress in karyotyping techniques in the late 1950s and 1960s led to the finding that unlike in *Drosophila*, the Y chromosome in mammals has a profound effect on sex determination (Ford et al., 1959; Jacobs and Strong, 1959). Studies on individuals with sex chromosome abnormalities showed that the Y chromosome was essential for the induction of the testis and for the development of the male phenotype whereas the presence of two X

chromosomes is necessary for the normal development and fertility of females (reviewed by Solari, 1994). In species with this type of chromosomal sex determining mechanism, the gonosomes differ in terms of their size, shape, gene content and chromatin structure (Solari, 1994; Charlesworth, 1991). In most mammals, males carry the heteromorphic sex chromosomes, X and Y, and females carry two homomorphic X chromosomes. In eutherian mammals, X chromosome represents about 5% of the haploid genome, and contains a large number of genes expressed in both sexes (Ohno, 1967). The Y chromosome is thought to carry only a small fraction of the genes on the X chromosome. Ohno (1967) proposed that the X and the Y are derived from an ancestral homomorphic pair of chromosomes carrying a major allelic difference for sex determination. The Y chromosome which is derived from one member of this pair is thought to have progressively lost genes, retaining only genes with male specific functions (reviewed by Graves, 1998).

## X Chromosome Inactivation in Mammals

In organisms with heteromorphic sex chromosomes (XX and XY), the females carry twice as many X linked gene loci as the male. Ohno (1967) had proposed that equalizing the dosage effect of X linked genes is vital under these circumstances since failure to overcome this dosage difference may be lethal. This hypothesis has been proven correct and failed dosage compensation has been shown to be lethal early in development (Tada et al., 1993; Goto and Takagi, 1998 and 1999). Various strategies have evolved for dosage compensation of X linked genes in different organisms

(reviewed by Meller, 2000; Lucchesi, 1998). In the female hermaphrodite *Caenorhabditis elegans*, the transcription of X chromosomes is down- regulated so that each X transcribes only half of what the X chromosome in male generates, whereas in *Drosophila* transcription of the single X chromosome in males is upregulated to the level of the activity of each X chromosome in the female (Lucchesi, 1998). In mammals the dosage compensation is achieved by transcriptional silencing of one of the X chromosomes in female somatic cells early in development as outlined by Lyon (Lyon, 1967). The basic tenets of the Lyon hypothesis are: (1) a single active X chromosome exists in female cells; (2) the other X becomes inactive early in embryogenesis; (3) the inactive state is stable and somatically inherited and (4) the choice of which X is inactivated is random and renders the female mammal a somatic mosaic (Lyon, 1961).

Inactivation of the genes on one of the X chromosomes of female mammals was postulated before the Lyon hypothesis was formulated (Ohno et al., 1959). Ohno et al. (1959) had proposed that the sex chromatin body, reported by Barr and Bertram (1949) was the heterochromatic manifestation of the inactive X in interphase. A mosaic pattern of expression of X linked genes was observed by Beutler et al. (1962) during their studies of the X chromosome linked enzyme, glucose-6-phosphate-dehydrogenase (*G6PD*) in females and males. Since then it has been shown that X inactivation occurs in the early female blastocyst, first in the extraembryonic membranes in which the paternal X undergoes inactivation (Takagi and Sasaki, 1975; West et al., 1977), and later in the embryo proper in which inactivation is random, in that either paternal or

maternal X chromosome becomes inactivated (Epstein, 1978; Monk and Harper, 1979). The inactivated X is recognized by its heterochromatinization, its late replication, and the methylation and acetylation patterns of specific gene loci.

Heterochromatic state and late replication

As stated before, in female somatic cells the inactive X is visible as a heterochromatic body located near the nuclear membrane during interphase (Barr and Carr, 1961; Dyer et al. 1989). When these cells enter the cell cycle, the replication of genes on the inactive X lag behind that of their counterparts on the active X (Schmidit and Migeon, 1990; Boggs and Chinault, 1994; Torchia et al., 1994).

Methylation of CpG islands

Methylation of cytosine residues within the CpG dinucleotides is one of the major modifications contributing to gene regulation during vertebrate development (reviewed by Ng and Bird, 1999; Razin and Shemer, 1995). The silencing effect of methylation is thought to be mediated by proteins which specifically bind to the methylated DNA (Huntriss et al., 1997; Jost and Hofsteenge, 1992). The methylation pattern of X linked housekeeping genes has been shown to differ between the active and the inactive X chromosomes (reviewed by Riggs, 1990; Goto and Monk, 1998). On the inactive X chromosome of man and mouse, the CpG islands of the housekeeping genes are extensively methylated (Norris et al., 1991; Tribioli et al., 1992).

Hypoacetylation of histones

The level of histone acetylation in the metaphase stage differs between active and inactive X chromosomes (reviewed by Keohane et al., 1998). The inactive human and mouse X chromosomes are labeled weakly with antibodies to the acetylated isoforms of H2A, H2B, H3, and H4 histones (Jeppeson and Turner, 1993; Belyaev et al., 1996). Underacetylation of histones, specifically of histone H4, is thought to be involved in gene silencing and it is a general property of heterochromatin. Underacetylation of histone H4 is a characteristic of the inactive X in eutherians and marsupials indicating that it is an ancient component of the mammalian X inactivation process (Wakefield et al., 1997). Using differentiated ES cells as a model Keohane et al (1996) demonstrated that reduced acetylation of histone H4 (which occurs after the early events of inactivation) is involved in the stabilization of the inactive state rather than in the initiation of the silencing process. Furthermore, a specific variant of histone H2, referred to as macroH2A1, is found to be associated with the inactive X (Costanzi and Pehrson, 1998).

**X inactivation center**

A region of the X chromosome referred to as the X inactivation center (Xic) is required for the various steps in X inactivation (described above). This region was defined by cytogenetic studies in X- autosome translocation carriers over 30 years ago (Therman and Sarto, 1983; Russell, 1983; Rastan, 1983). These studies provided evidence that only one of the products of a balanced X- autosome translocation was able

to undergo inactivation. Based on studies on such abnormal chromosomes, the candidate region for Xic was narrowed down to span 600 to 1200 kb in human (Lafreniere et al., 1993) and 460 kb in mouse (Heard et al., 1996). Cytogenetic and transgenic studies suggest that various early events in X chromosome inactivation are directed by the Xic (reviewed in Heard et al., 1997).

Six genes have been assigned to the Xic. These include the X controlling element (*Xce*) in mouse, which is thought to be involved in the choice of which of the two X chromosomes (paternal or maternal) is silenced (Cattanach and Williams, 1972; Simmiler et al., 1993); *BRX/Brx* (for Brain X-linked gene) with a rare transcript preferentially expressed in brain (Simmler et al., 1997); a member of caudal family of homeobox genes, *CDX4/Cdx4*, which is expressed specifically during embryogenesis (Horn and Ashworth, 1995; Gamer and Wright, 1993); a brain specific gene, *BPX/Bpx*, with strong homology to genes encoding nucleosome assembly proteins (Rougeulle and Avner, 1996) and a testis specific gene (*Tsx*), identified in the mouse and the rat (Simmiler et al., 1996), shown to be expressed both in premeiotic germ cells and Sertoli cells and the Sertoli cells of adult testis (Cunningham et al., 1998). In addition a gene referred to as "X inactive specific transcript" *(Xist)* is expressed exclusively from the inactive X and is thought to be involved in various steps of the inactivation process (Brown et al., 1991a; Borsani et al. 1991; Brokdorff et al., 1991). The last addition to the genes mapped to the mouse Xic includes *Tsix* (named with reference to its anti-orientation to *Xist*) is shown to transcribe a 40 kb transcript starting 15 kb downstream to and spanning the entire *Xist* (Lee et l., 1999b; and Mise et al., 1999).

**Features of *Xist***

The *Xist* cDNA was originally cloned during a search for cDNA of the human steroid sulphatase (STS) gene (Brown et al. 1991a). Fine mapping of the gene, using structurally abnormal X chromosomes, led to the mapping of the gene to the smallest candidate XIC interval (Brown et al., 1991b; Leppig et al., 1993). The mouse counterpart was discovered and mapped to the Xic soon after (Borsani et al., 1991; Brockdorff et al., 1991). *Xist* is the first gene found to be expressed exclusively on the inactive X (Lafreniere et al., 1993). Both the mouse and human *Xist* transcripts are large molecules (15-17 kb) which are retained in the nucleus and lack any conserved open reading frame of significant length (Brockdorff et al., 1992; Brown et al., 1992). The human and mouse *Xist* genes consist of eight and six exons, respectively, with conserved repetitive sequences in exon 1 and 6 (Brockdorff et al., 1992; Brown et al., 1992). The most conserved sequences among mammals reside at the 5' end of the first exon of the *Xist*. However, comparison of the human, murine, lepine and bovine *Xist* sequences at this conserved region shows that numerous gaps are required in all species to maintain alignment (Hendrich et al., 1993). The bovine sequence at this conserved region shows the highest homology (76%), while the murine sequence is the most divergent sequence compared to the human sequence (Hendrich et al., 1993). Using flourescent in situ hybridization (FISH) analysis, it was found that *Xist* RNA is localized to the nucleus where it coats the inactive X in mitotic cells and is associated with the Barr body in interphase nucleus (Brown et al., 1992; Clemson et al., 1996). Moreover, in aneuploid cells including 47,XXX, and 49,XXXXX, the *Xist* RNA

hybridization signals correspond with the number of the inactive X chromosomes (Brown et al., 1992).

The expression of *Xist* early in embryogenesis and from the paternal X in preimplantation embryos indicates that *Xist* plays a role in the initiation of X inactivation in the early blastocyst (Kay et al., 1993; Norris et a., 1994). Direct evidence for the involvement of *Xist* in X inactivation came from gene targeting experiments in embryonic stem (ES) cells (Penny et al., 1996; Marahrens et al., 1997). These studies showed that the X chromosome bearing a deleted *Xist* could not undergo X inactivation, while the X chromosome carrying the intact allele was inactivated. Deletion studies have also indicated that the 5' region of *Xist* is essential for the initiation of inactivation (Penny et. al.1996), and that the deletion of the 3' end prevents the process of "counting" the X chromosome for inactivation (Marahrens et al. (1997). Also, studies using transgenes have shown that in *Xist* transfected ES cells, genes on the autosomes carrying the transgene are silenced (Lee and Jaenisch, 1997; Herzing et al., 1997; Lee et al.1999a). More recently, Lee et al.(1999b) have shown that an 80 kb region of the mouse putative Xic containing *Xist*, may be capable of inducing all (counting, choosing and silencing) steps of X chromosome inactivation.

**Activity of the X chromosomes and X linked genes in germ cells**

The expression status of sex chromosomes differs in male and female germ cells. The already inactivated X in female germ cells becomes reactivated at the onset of meiosis, whereas in the male germ cells the sole X chromosome undergoes

inactivation and forms a heterochomatic structure called the sex body or the XY body (reviewed by Solari, 1974). Biochemical assays on the levels of X-linked enzymes (Monk and McLaren, 1981; Tam et al., 1994) have confirmed that the two X chromosomes are active in female germ cells entering meiosis. It has been suggested that the active state of both X chromosomes is required for proper meiotic pairing and recombination in female germ cells. In contrast, quantitative RT-PCR of pyruvate dehydrogenase 1 (*Pdha1*), phosphoglycerate kinase 1 (*Pgk1*), and hypoxanthine phosphoribosyl transferase (*Hprt*) indicate that these genes are silenced during spermatogenesis (Hendricksen et al., 1995; Singer-Sam et al., 1990). The rationale for X chromosome inactivation and the formation of a heterochromatic XY body in male germ cells is not known. However, it has been proposed that X inactivation and XY body formation serve as a device to avoid nonhomologous pairing of heteromorphic sex chromosomes during meiosis (McKee and Handel, 1993). It has been suggested that the inactivation of the sex chromosomes in meiosis occurs to prevent the accumulation of unrepaired chromosome damage due to double strand breaks associated with meiotic recombination (McKee and Handel, 1994). Males carrying X- autosome translocations are known to be sterile in the mouse and *Drosophila* (Lifschytz and Lindsley, 1972), cattle (Basrur et al., 1992) and the pig (Singh et al., 1994). Male sterility in these cases is attributed to the perturbation of X chromosome inactivation and consequent aberrant expression of X linked genes. However, in mouse strains carrying structurally abnormal X chromosomes, the formation of the XY body is unimpaired indicating that the signal for sex chromosome condensation is exogenous to sex chromosomes (Handel

et al., 1994). It is believed that testis specific proteins may be involved in XY body formation, as ectopic germ cells (in adrenal gland) which enter meiosis, do not form an XY body (Zamboni and Upadhyay, 1983). Using in situ reverse transcription polymerase chain reaction (RT-PCR), Ayoub et al.(1997) had demonstrated that the *Xist* RNA is incorporated on the XY body. These investigators also showed that the splicing components of the transcriptional machinary are excluded from the condensed chromatin of the XY body, thus confirming the view that the XY body represents the transcriptionally silenced male sex complements (Richler et al., 1994; Ayoub et al.,1997).

Expression of *Xist* has been detected in the testes of several species of mammals indicating that *Xist* may have a role to play in the inactivation of the X chromosome in male germ cells (McCarry and Dilworth, 1992; Richler et al., 1992; Salido et al., 1992; DeLa Fuente et al., 1999). However, *Xist* deficient male mice show normal spermatogensis while their female counterparts fail to accomplish dosage compensation (Marahrens et al., 1997) indicating that the inactivation process in the two sexes may differ in various points. Some of the features of the inactivated X chromosome in female somatic cells appear to be absent from the inactivated X chromosome of male germ cells. For instance, hypermethylation of X linked gene CpG islands (Driscoll and Meigon, 1990) and hypoacetylation of histones associated with the inactive X in female somatic cells (Armstrong et al., 1997) are not observed in the inactivated X chromosome of male germ cells. Furthermore, the inactivated state is transient in male germ cells in that it is reversed as meiosis progresses whereas the inactivation in female

somatic cells is stable and irreversible (McKee and Handel, 1993).

### Escape from X inactivation

Although the inactivation process leads to the silencing of one entire X chromosome in female somatic cells, not all gene loci on the "inactivated X" are silenced. As a general rule, the gene loci carried on the X chromosome for which there are homologues on the Y chromosome escape. However, the escape is not limited to the genes located in the segment homologous to the X and the Y, referred to as the pseudoautosomal region (PAR), or to other genes outside the PAR with Y chromosome homologues (Disteche, 1995; Graves, 1995). Evidence indicates that genes escaping inactivation and are expressed from both active and inactive X chromosomes, may number in the hundreds (Willard, 1995). Based on the expression pattern, Carrel and Willard (1999) have grouped X linked genes in female somatic cells into four different types: genes subject to inactivation and expressed only from the active X; genes escaping inactivation and expressed from both active and inactive X chromosomes; genes expressed exclusively from the inactive X represented by *Xist;* and genes displaying a heterogenous expression, subject to inactivation in some cells and escaping inactivation in other cells. Using reverse transcription polymerase chain reaction (RT-PCR), Carrel et al. (1999) have found that 34 out of 224 known X linked genes are expressed in the mouse/human somatic cell hybrids containing a normal human inactive X, indicating that these genes escape inactivation. A recent study, using single-cell RT-PCR showed that while the expression of Rab escort protein-1 (*REP1*) gene is

monoallelic in some cell lines (indicating the inactivation of the gene), its expression in others is biallelic (indicating that the same gene escapes inactivation (Carrel and Willard, 1999).

In contrast to the situation with the genes on the human X chromosome, fewer X-linked genes escape inactivation in the mouse (Ashworth et al., 1991). Phenotypic differences between XO genotypes in mouse (Banzai et al., 1995) and humans (Turner's syndrome) are attributed to the effect of genes escaping X inactivation in human (reviewed by Zinn et al., 1993). Such genes, are functionally disomic in normal females and are thought to cause the clinical features associated with X chromosome aneuploidies (Willard, 1995). However, the number of genes studied in the mouse and other mammalian species is low and restricted to the homologues of the human genes escaping inactivation. Genes escaping inactivation reflect either a refractory response to the inactivation signals in early development or instability of the inactive state in specific regions of the X chromosome ( Lyon, 1996). As mentioned above some X-linked genes with no Y homologues, express from both active and inactive X chromosomes indicating that a double dose of these genes may be required for normal development of females.

**Mechanism of X chromosome inactivation**

The exact process by which X chromosome inactivation is accomplished is not clearly known. Various theories including epigenetic processes have been invoked to explain this co-ordinated, multi-step phenomenon. X chromosome inactivation in

mammals was the first described epigenetic alteration of the genome leading to the monoallelic expression of most of the X linked genes (reviewed by Marahrens, 1999). This feature of X chromosome inactivation is also shared by imprinted autosomal genes. The existence of an imprinted form of inactivation in marsupials (Cooper et al., 1971) and in extraembryonic tissues of rodents (Takagi and Sasaki, 1975) reflects a relationship between the two processes. In addition to having monoallelic expression, the imprinted autosomal genes and those on the inactive X, share other features. Both are controlled by *cis*-acting elements; both are associated with hypermethylation (Migeon, 1994); both processes lead to association with hypoacetylated histones and both types show late replication relative to their active partner (reviewed by Jamieson et al., 1996; Hendrich and Willard, 1995). These similarities indicate that X inactivation and autosomal imprinting may be evolutionarily related and may share common mechanisms (Cattanach, 1996). However, the major difference between the two processes is that X chromosome inactivation silences many genes over extremely long distances whereas imprinting of autosomal genes serves to silence individual genes. While some of the features of the inactive X are well defined, the precise molecular mechanism (s) of inactivation remains unclear. The process of X inactivation occurs in several steps which are summarized in the following sections.

Counting

It is believed that a "blocking factor" originating from autosomal genes counts the number of X chromosomes (to remain active or to be inactivated) per diploid

number of autosomes, and that in a normal diploid female cell and cells with X aneuploidy, only one X remains active while in tetraploid cells two active and two inactive X chromosomes are present (reviewed by Lyon, 1991). The developmental cue triggering the initial events in X chromosome inactivation is thought to be associated with the limited quantity of blocking factor produced by the autosomes, which binds to a single X chromosome per diploid cell to protect it from the inactivation process (Lyon, 1994).

The target of the blocking factor (s) is thought to be located in the Xic, as an intact Xic is required for the counting process to occur and the presence of an extra Xic in an abnormal karyotype (or transgenic Xic) can influence the number of active or inactive X chromosomes in the cell (Rastan and Robertson, 1985; Migeon et al., 1999). It has been proposed that "counting" is a constitutive function in male and female, since transgenic male ES cells are able to count and induce inactivation in the presence of an exogenous Xic (Migeon et al., 1999; Lee et al., 1996; Herzing et al., 1997). Recent evidence indicates that the region 3' to *Xist* has a role in the counting mechanism, as deletion of this region leads to inactivation of the X chromosome carrying this deletion (Clerc and Avner, 1998).

Choosing

The mechanism involved in choosing which of the two X chromosomes in the female conceptus becomes inactivated remains unknown. As stated before, in normal diploid cells and in cells with X chromosome aneuploidy only one X remains active. It

is possible that the function of choosing is to determine the X chromosome that is to remain active (Goto and Monk, 1998; Hennig, 1999). In mice, nonrandom choosing occurs because of allelic differences at *Xce* (Cattannach and Williams, 1972; Avner et al., 1998). This locus (*Xce*) was localized downstream to the 3' region of *Xist* and the difference in alleles of *Xce* is thought to be associated with the microsatellite markers characterized by cytosine-adenosine (CA) dinucleotide repeats (Simmler et al., 1993). Brockdorff et al. (1991) have suggested that the level of *Xist* expression is inversely related to the strength of the *Xce* allele, and Simmler et al. (1993) have proposed that different alleles of *Xce* may have different affinities for an " imprint" involved in choosing. Direct evidence for a role for Xic in the choosing process came from *Xist* knockout studies by Marahrens et al.(1997) who showed that the deletion of a 15 kb segment between exon 1 and 5 results in nonrandom inactivation of the intact X chromosome. The skewed inactivation of an X chromosome carrying a 65 kb deletion extending 3' and distal to *Xist* exon 6 (Clerc and Avner, 1998) provides further evidence for the involvement of this region in the choosing process of the active X chromosome.

The results of various experimental data to date are not able to distinguish between the mechanisms involved in counting and choosing, although they have been defined as separate concepts (Carrel and Willard, 1998). However, it is clear that downstream and upstream boundaries of the *Xist* locus play a role in counting and choosing mechanisms, as nucleation sites for binding of regulatory factors (Marahrens et al., 1997; Carrel and Willard, 1998).

Initiation and spreading of the inactive state

Expression of *Xist* is thought to be the first detectable feature of the commitment for X chromosome inactivation (Keohane et al., 1998). Several studies have demonstrated that *Xist* is expressed at low level in mouse ES cells prior to the onset of X chromosome inactivation (Kay et al., 1993; Buzin et al., 1994; Beard et al., 1995). Early expression of *Xist* has also been demonstrated in human (Daniels et al., 1997) and bovine (DeLa Fuente, 1999) preimplantation embryos. The structural association of *Xist* with the inactive X chromosome (Clemson et al., 1996; Lee et al., 1996; Lee and Jaenisch, 1997) and evidence from knockout studies for the requirement of *Xist* for X chromosome inactivation (Penny et al., 1996; Marahrens et al., 1997) indicate that *Xist* RNA is involved in the chromatin changes that occur in the inactive X. In undifferentiated cells and embryos prior to the establishment of the inactive X, a biallelic expression of unstable forms of *Xist* precedes the expression of the stable forms (Panning and Jaenisch, 1996) which, in turn, is followed by the appearance of stable *Xist* only from the inactive X (Sheardown et al., 1997; Panning et al., 1997). The switch to the stable form is thought to be mediated by alternative promoter usage (Johnston et al., 1998) which is controlled by hyperacetylation of the chromatin upstream to the *Xist* (O' Neill et al., 1999).

The *Xist* RNA generated by the Xic of the X destined to be inactivated is not enough to coat the entire inactive X (Buzin et al., 1994). Flourescent in situ hybridization (FISH) has shown that human and mouse *Xist* RNAs appear as large domains consisting of punctuate dots on the inactive X (Clemson et al.,1996; Panning

and Jaenisch, 1996). Duthie et al. (1999) demonstrated a banded localization of *Xist* RNA corresponding to the gene rich G-bands, and that *Xist* is excluded from the constitutive heterochromatin of the metaphase chromosome. These observations suggest that in addition to *Xist* RNA, other factors including DNA elements or proteins are required for propagating the heterochromatic state over the entire inactive X chromosome (Hennig 1999). Lyon (1999) has suggested that the long interspersed repeat elements (LINE), which are abundant on the X chromosome and concentrated in dark G- bands may function as "boosters" or "way stations" at intervals to relay the inactivation signal along the X chromosome. Molecular evidence for Lyon's "repeat hypothesis" (1999) was provided by Bailey et al. (2000) who were able to show that LINE -1 (L1) elements are abundant on the X chromosome especially on human Xq13 (location of XIC) compared to autosomes. This study also demonstrated that the loci escaping inactivation are significantly reduced in L1 content compared to the loci that are subject to inactivation (Bailey et al., 2000).

Maintenance of the inactive state

The inactive state of the X chromosome in female somatic tissues remains stable and is passed through cell divisions. However, it is possible that *Xist* is neither necessary nor sufficient for the maintenance of the inactivated state (Brown et al., 1994; Tinker and Brown, 1998; Hansen et al., 1998, and 1996). Most likely, *Xist* RNA potentiates heterochromatin formation on the inactive X which is manifested by multiple self-perpetuating mechanisms, including late replication, hypermethylation of

CpG islands and underacetylation of histones (Panning and Jaenisch, 1998).

Late replication appears to be the earliest detectable event (prior to the increased expression of *Xist*) associated with X chromosome inactivation in female ES cells (Kaeohane et al., 1998) which brings large chromosomal domains into a transcriptionally silenced state (Hansen et al., 1996). Late assembly of chromatin in S phase may deprive late replicating regions from access to transcription factors required for gene activity (Riggs and Pfeiffer, 1992). This assumption is supported by the fact that genes escaping inactivation do not display asynchronous replication (Boggs and Chinault, 1994). Several studies using different techniques have shown that in adult tissues X linked genes on the inactive X chromosome are extensively methylated at CpG islands (Pfeiffer et al., 1990; Hornstra and Yang, 1994). The extent of CpG methylation of X-linked genes is lower in marsupials (Cooper et al., 1993) and in human extraembryonic tissues (Migeon et al., 1986), in which the inactive state of the X chromosome is not stable. Also, in male germ cells, with reversible X inactivation, CpG islands of several genes are not methylated (Driscoll and Migeon, 1990). Male and female germ cells display distinctive patterns of methylation (Razin and Shemer, 1995), and gamete-specific methylation of *Xist* gene is associated with the imprinted paternal X chromosome inactivation in extraembryonic tissues (Zuccoti and Monk, 1995; Ariel et al., 1995).

Several observations indicate that hyperacetylation of histones are associated with active chromatin. For example, acetylation of lysine residues in core histone is correlated with transcriptional activity (Brownell and Allis, 1996), constitutive

heterochromatin shows low level of histone H4 acetylation (O'Neill and Turner, 1996), and the histone acetylation pattern is heritable through cell division (Wolfe, 1994). In accordance with these observations, the active X chromosome is associated with hyperacetylated forms of H4, whereas the inactive X chromosome lacks the acetylated forms of histone H4 (Jeppeson and Turner, 1993). Furthermore, association of X chromosome inactivation with underactylated histones is also seen in marsupials (reviewed by Keohane et al., 1998).

Other factors associated with *Xist* RNA include two heteronuclear proteins interacting in vitro with the 5' region of *Xist* (Brown and Baldry, 1996) and deposition of a histone like protein named macro H2A1 on the inactive X chromosome (Costanzi and Pehrson, 1998). Deletion of *Xist* disrupts macro H2A1 deposition indicating that *Xist* is required for its localization on the inactive X (Csankovski et al., 1999).

**Bovine sex complements and X-linked genes**

Extensive studies on X chromosome inactivation and X linked genes in the mouse and human have generated ample data on the distribution of homologous loci such that their expression pattern can be compared between these two species. It is apparent that the X chromosome is conserved in gene content but not the order of genes along the X chromosome which may explain the differences in the expression pattern of the X-linked genes among mammalian species. Similar studies in other species may provide valuable information for comparative analysis of different aspects of X chromosome inactivation and X-linked gene expression.

Sex chromosomes in cattle are easily identifiable due to their submetacentric feature compared to the autosomes which are acrocentric. The bovine X chromosome is among the longest chromosomes and contains roughly 5% of the haploid genome, whereas the Y chromosome constitutes about 2% of the total complement length according to the Texas Standard chromosome Nomenclature (Popescu et al., 1996). In cattle, the Y chromsome varies in relative length and centromere index both within and between breeds (Eldridge, 1985). Results derived from cytogenetic maps indicate that even in closely related species such as the goat and cattle, considerable rearrangements have occurred during the evolution of the X chromosome (Gallagher et al.,1999). These evolutionary rearrangements, which change the position and the extent of heterochromatin along the X chromosome, may influence the expression of neighboring genes (Gallagher et al., 1999).

Gene mapping studies in farm animals are in part devoted to identifying polymorphic markers in genome associated with loci of economic importance or to comparative evolutionary studies (Ponce De Leon et al., 1996; Robinson et al., 1998; Piumi et al., 1998; Gallagher et al., 1999). In total, 448 coding genes are assigned to bovine chromosomes (Fries and Popescu, 1999). Twenty-six coding sequences have been assigned to the X chromosome. Of these only a small fraction, including Duchenne muscular dystrophy (DMD), clotting factor IX, phosphoglycerate kinase (*PGK*) and *Zfx* are localized on the bovine X chromosome (reviewed by Fries and Popesu, 1998).

In contrast to the situation in man and mouse, expression studies of X linked

genes are limited in cattle. Among the recent molecular studies on cattle are those pertaining to the mRNA of the amelogenin gene, which has an active locus each on the X and the Y chromosome and is expressed during bovine tooth development (Yuan et al., 1996). The expression of *Xist* in relation to X chromosome inactivation has been studied in bovine early embryos (De La Fuente et al., 1999). This study showed that while the expression of *Xist* is evident in bovine embryos at the 8-cell stage, a late replicating X chromosome was not observed until the blastocyst stage (De La Fuente et al., 1999). In another attempt to investigate the expression status of X linked genes prior to X chromosome inactivation, Gutierrez-Adan et al. (2000) have shown that the levels of expression of *G6pd* and *Hprt* in in vitro produced female embryos is higher compared to that of their male counterparts. Bovine *Zfx*, and its Y homologue, the *Zfy*, have been cloned and mapped to Xq34 and Yp13, respectively (Xiao et al., 1998). However, molecular investigation of *Zfx* and *Zfy*, and other genes (Aasen and Medrano, 1990; Bredbacka and Peippo, 1992) have been limited to their application in sexing of bovine embryos.

In spite of the great economic importance of cattle to agricultural industries, information concerning many of the gene loci expected to be on the bovine X chromosome is not currently available. Some of these X-linked genes and their expression pattern could have applications to assisted reproduction technologies in cattle and great relevance to broadening our knowledge base on X linked genes and X chromosome inactivation in mammals in general. The information summarized above clearly points to species specific differences in various aspects of X inactivation even

though comparisons are currently is only based on relevant information gathered from studies on these aspects of human and mouse X chromosomes.

## RATIONALE

As evident from the previous section (LITERATURE REVIEW), much of the information on the mechanisms involved in X chromosome inactivation is based on the studies of two widely divergent mammals: the laboratory mouse and human populations. While the information generated from these sources indicate that various aspects of this phenomenon are common to both, it has also pointed out that they differ from each other in some important aspects, indicating that components of this process in other mammals may differ from those gleaned from these two well studied species. The rationale for choosing the bovine species for the present study is the paucity of information on X chromosome inactivation in this species and the belief that breeders and bovine clinical embryologists eventually stand to benefit from the elucidation of various components of this process addressed by this research.

The recognition of the sex vesicle as a consistent feature of male germ cells in mammals including cattle, predates the discovery of various aspects of X chromosome inactivation in female somatic cells. However, the molecular correlates of X inactivation in male germ cells and its relevance to spermatogenesis remain unknown. Other investigators have shown that the gene (*Xist*) located in the X inactivation centre (XIC) which is thought to play a key role in the initiation of X inactivation in female cells, may also be involved in this process in male germ cells entering meiosis (Richler et al., 1992; McCarry and Dilworth, 1992; Ayoub et al., 1997). However, the age in development or the stage in testicular differentiation at which *Xist* expression is initiated in mammalian male germ cells including that of cattle is not known. One aspect of our

investigation focuses on the onset of *Xist* expression in male fetal bovine germ cells and its possible relevance to the cellular events evident during testicular morphogenesis (Chapter 1). Based on the observation that *Xist* is expressed in the fetal bovine testis long before spermatogenesis is initiated, we examined the expression status of the X linked genes in the testis which expresses *Xist*, using a semi- quantitative reverse transcription polymerase chain reaction (RT-PCR) approach in an attempt to determine whether *Xist* expression is a forerunner of the process that silences X linked genes in male germ cells (Chapter 2).

Since our observations showed that *Xist* expression does not lead to the inactivation of X linked genes in male germ cells, we extended our studies on this locus (*Xist*) to cultured bovine somatic cells derived from normal male and female bovine fetuses and from male and female X chromosome aneuploids . It is well documented that the locus involved in the process of X inactivation (*Xist*) should be expressed for suppressing transcription of other genes carried by the same X chromosome (Brown, et al, 1991; Heard et al, 1998). We speculated that the levels of *Xist* RNA (and other X linked gene specific RNAs) transcribed by male cells will differ from that detected in female cells and that a quantitative difference will be evident in the levels of *Xist* transcribed by normal cells compared to that in cell strains derived from male and female cells carrying extra X chromosomes. Availability of bovine cells from X chromosome aneuploids is also exploited for examining the status of some of the X linked genes which are known to exhibit a species specific (and tissue specific) difference in expression pattern. Thus, our investigation (outlined in Chapter 3) also

focuses on the activity status of four of the the X linked genes in order to test whether they are expressed or silenced in cultured cells of normal and abnormal X chromosome constitution.

Based on our observations that bovine male fetal germ cells and cultured male somatic cells express *Xist* even though its speculated role (X inactivation) is not accomplished in these cells (Chapters 1 to 3), we conjectured that the RNA transcribed by these cells may not be *Xist*, but its antisense. Undifferentiated embryonic stem cells transcribe an *Xist* like RNA which has been shown to be its antisense (Lee et al., 1999; Mise et al., 1999), using a strand specific RT - PCR approach. We utilized this approach on RNA derived from fetal bovine testis, ovaries and non-gonadal organs and cells to identify the RNA transcribed by these cells since this approach offers a unique opportunity not only to understand the mechanism for X inactivation used by male and female bovine cells, but also to shed light on the developmental regulation of gene expression in different cells and organs.

As stated before, information derived from bovine species on these and other basic aspects of X inactivation is sparse in spite of the importance of this species to agricultural industries. The possibility of generating needed and relevant data applicable to assisted reproduction technologies in cattle, strengthens the rationale for undertaking these studies.

# CHAPTER 1

# EXPRESSION OF X INACTIVE SPECIFIC TRANSCRIPT (*XIST*) AND TESTICULAR MORPHOGENESIS IN BOVINE FETUSES

## INTRODUCTION

Transcriptional silencing of X-linked genes through the condensation of one of the two X chromosomes occurs in female mammals early in embryonic development (Lyon, 1996 for Review). The initiation, and the propagation of condensation, are under the control of the X inactivation centre (Xic) whose presence *in cis* is mandatory for X inactivation to occur (Brown et al., 1991). The resident gene in Xic is thought to be the *Xist* which encodes an RNA that remains exclusively associated with the inactive X chromosome (Borsani et al., 1991; Brockdorff and Duthie, 1998; Clemson et al., 1996; Brown et al., 1991). X inactivation has also been shown to occur during spermatogenesis when the only X chromosome of the male germ cells, in association with the Y, becomes condensed in early meiosis I to form a structure referred to as the X-Y body, the sex body or the sex vesicle (Solari, 1994, and Handel et al., 1994, Richler, et al., 1994). This process which is also known to involve the transcription of *Xist*, is thought to occur only in the germ cells entering meiosis (McCarrey and Dilworth, 1992; Ayoub et al., 1997). Previous studies from this laboratory have shown that *Xist* is expressed in adult bovine testes while it is silent in adult male somatic tissues (De La Fuente et al., 1999). However, the onset of the X inactivation process in bovine testes, or its relevance to bovine spermatogenesis, is not established as yet. Since a recent study had indicated that *Xist* expression may not be vital to, or

involved in, spermatogenesis (Marahrens et al., 1997), we examined the status of *Xist* gene in developing (premeiotic) bovine testes for the purpose of determining whether *Xist* plays a role in the differentiation of bovine germ cells.

## MATERIALS AND METHODS

### Collection of fetuses and sex identification

Fetuses retrieved from pregnant uteri of Holstein cows were measured and their age was estimated based on the crown-rump length (CRL) according to Noakes (1997). Gonads and samples of different fetal organs including muscle and lungs, were dissected out, flash frozen and stored at -80°C until use. Sex of the fetuses over 3.5 cm in CRL was identified based on the urogenital features and for younger fetuses (less than 3.5cm in CRL), a PCR amplification procedure based on the difference in the restriction fragment length polymorphism of the *Zfx/Zfy* locus (Aasen and Medrano, 1990), was carried out according to Bredbacka and Peippo (1992).

### RNA extraction, cDNA synthesis and reverse transcription

Total RNA was extracted from fetal gonads and somatic tissues using Trizol reagent according to the manufacturer's instructions (Gibco BRL). To eliminate genomic DNA contamination, 2 μg of RNA samples were treated with DNase I at room temperature for 30 minutes and divided into two lots for reverse transcription with and without reverse transcriptase. cDNA synthesis was carried out in a 20 μl reaction mixture containing 1 μg RNA, 0.25 μg oligo-dT primers and 0.25 μg of random

hexamers, 4 μl of 5X reaction buffer, 10 mM dithiotreitol, 0.25 uM dNTPs, and 200 units of Superscript Reverse Transcriptase. The reaction was carried out at 42°C for 90 minutes. PCR was performed in a 50 μl reaction mixture. For amplifications of the β-actin gene, used as positive control, the reaction mixture contained 2 μl of cDNA, 5 μl of 10X PCR buffer, 0.2 μl of dNTPs, 1 mM of primers and 1 unit of Taq DNA polymerase. To test for *Xist*, primers were designed to amplify a 463-base pair (bp) PCR product from the 5' region of bovine *Xist* described by Hendrich *et al.* (1993). PCR amplification of *Xist* was accomplished using 4 μl of cDNA, 5μl of 10X PCR buffer, 0.3 mM dNTPs, 0.25 mM of primers, 1.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase. PCR conditions and the cycle numbers that proved to be optimum in prior trials, and the primer sequences are illustrated in Table I.

### Histology and Electron Microscopy

One of the testes from each fetus was cut into 1.0 mm pieces and fixed in 5.0% glutaraldehyde for semithin and ultrathin sections. Pieces of testes fixed for 4 hours were post-fixed in 1.0% osmium tetroxide for one hour prior to dehydrating in ascending concentrations of ethanol and embedding in Epon via propylene oxide. Semithin (1.0 μm) sections were cut with a Reichert OMU2 ultramicrotome and stained with 1.0% toluidine blue in 1.0% sodium borate. Stained slides were examined under a Carl-Zeiss photomicroscope and representative areas were photographed using Kodak's Professional T400CN T-MAX black and white film. Selected Epon blocks were trimmed to obtain areas of interest and ultrathin sections (60 to 90 nm) were cut using a Reichert OMU2 ultramicrotome. Sections were picked up on 250 square mesh copper grids, contrasted with

uranyl acetate and lead nitrate and examined under a JEOL JEM 100S transmission electron microscope. Representative areas were photographed using Kodak 4489 sheet film.

## RESULTS

Bovine fetuses collected included 76 males and 66 females ranging in age from 30 to 180 days of gestation. Of these, 48 male fetuses of different age groups were tested for *Xist* expression along with somatic tissues from corresponding age group female fetuses. Only cDNA samples positive for β-actin amplification and negative for genomic DNA contamination, were used for studies on the gonadal expression of *Xist*. In testicular tissues from 16 fetuses which were below 50 days of age, there was no detectable *Xist* expression (Table II). However, a faint but unmistakable band was evident with testicular cDNA from 50 day old fetuses while the somatic tissues of the same fetuses, were negative (Figure 1). *Xist* expression was more evident in the testes of older fetuses even though visually less intense compared to that of female somatic cells (Figure 2). All male fetuses in the age range of 50 to 180 days tested consistently displayed this pattern.

Our histological and ultrastructural studies revealed a few large cells with prominent nuclei and nucleoli, in the seminiferous tubules of 50 day old fetuses (Figure 3) whereas in the younger fetuses such cells were extremely rare. Some of the large germ cells detected in 90 day old fetuses displayed the chromatin characteristics of premeiotic and early meiotic prophase germ cells, even though the tubules at this stage lacked a lumen and mature Sertoli cells. A majority of these intra-tubular cells appeared to undergo eventual degeneration since nuclear fragmentation characteristic of apoptosis was

**Table I.** Sequences of primers used in PCR analysis of *β-actin* and *Xist.*

| Primers | Gene | Sequences (5' to 3') | PCR conditions |
|---|---|---|---|
| 5'<br>3' | *β-actin*[1]<br>*β-actin* | ACTGGGACGACATGGAGAAGAT<br>TGCTCGAAGTCCAAGGCGACGT | 4 min at 94°C; 30 cycles of 1min at 94° C,1min at 54° C, and 1 min at 72° C; final extension at 72° C, 10 min. |
| 5'<br>3' | *Xist*[1]<br>*Xist* | AGCATTGCTTAGCATGGCTC<br>TGGCTGTGACCGATTCTACC | 3 min at 94° C; 40 cycles of 1 min at 94° C, 1 min at 55° C, 90 sec at 72°C; final extension at 72° C, 10 min. |

1. De La Fuente et al., 1999.

**Table II.** Estimated age and number of male fetuses exhibiting *Xist* expressionin testes.

| Estimated Fetal Age* | Number | *Xist* Expression ** |
|---|---|---|
| 30-40 days | 12 | - |
| 45 " | 4 | - |
| 50 " | 5 | + |
| 60 " | 4 | + |
| 70-80 " | 4 | + |
| 90-100 " | 4 | + |
| 120 " | 8 | + |
| 150 " | 6 | + |
| 180 " | 1 | + |

* Based on Noakes, 1997.

** Muscle and lung of male fetuses displayed no *Xist* expression.

Figure 1. RT-PCR products representing *Xist* expression in the testes of bovine fetuses ranging in age from 40 to 50 days of gestation. The amplification products of cDNA using *β-actin* primers (upper panel) serves as controls for the quality of cDNAs used to test for *Xist* expression (lower panel). First lane represents the 100 bp molecular ladder. - and +, respectively, refer to PCR without and with reverse transcriptase. G and S refer to gonadal and somatic tissues, respectively.

Figure 2. *Xist* expression in the testes of bovine fetuses ranging in age from 110 to 140 days of gestation. Total RNA from testes and somatic tissues were reverse transcribed and amplified using specific primers. First lane (L) shows 100bp ladder; second lane (- ) represents negative control, with all components except for the cDNA; third lane (+) represents positive control, a cDNA sample from female somatic tissue. G and S, respectively, refer to gonadal and somatic tissues.

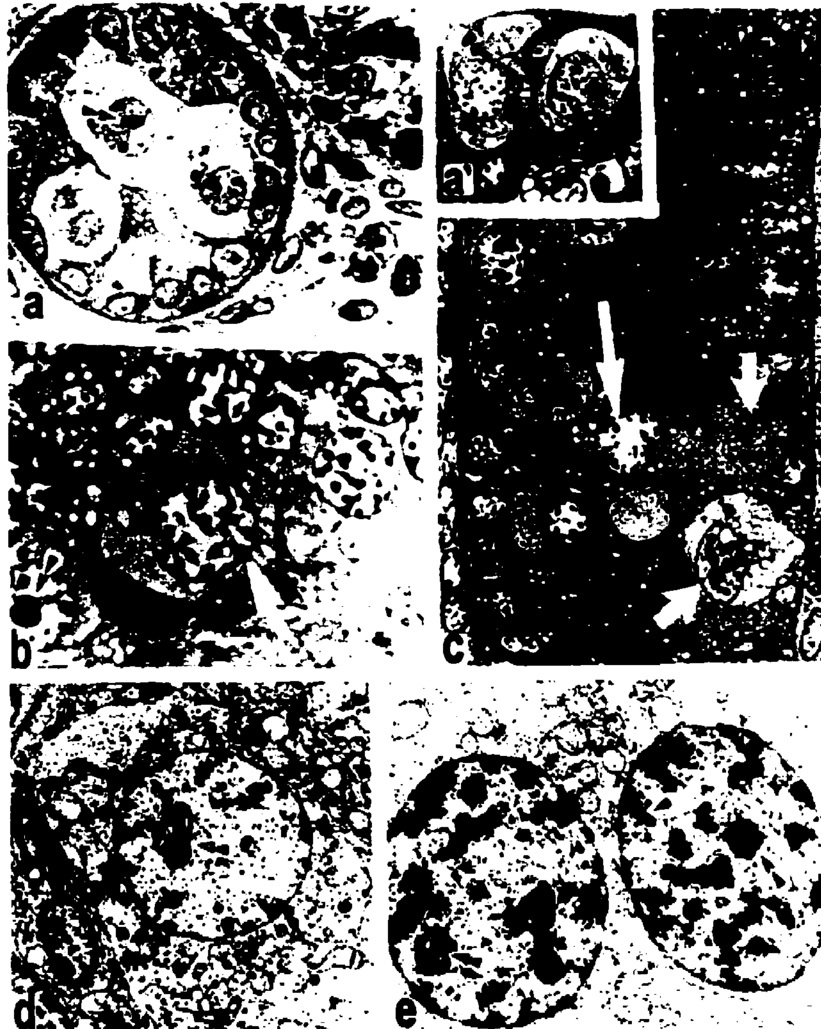



**Figure 3.** Seminiferous tubules of bovine fetuses in semithin section (**a-c**) and ultrathin (**d** and **e**) sections. **a**: Cross section of a tubule from a 50-day-old fetus, showing 3 large cells with one (or more) prominent nucleus and clearer cytoplasm relative to the cells closer to the basement membrane. **a'**: Inset showing the nuclear features of intra-tubular cells from another region of the same testis. **b**: Intra-tubular germ cells of 90-day-old fetus. Note the nuclei displaying chromatin condensation (white arrow) and prominent nucleolus (double arrowhead). **c**: Intra-tubular germ cells showing chromatin condensation (long arrow) and signs of nuclear degeneration (thick arrow). (**a-c**, X800). **d**: Ultrastraucture of the nuclei in large germ cells of a 90-day-old fetus showing active nucleolus (double arrowhead) and nucleolar elements (double arrowhead). (X2000). **e**: Axial elements (single arrowhead) and nucleolar elements (double arrowheads), reminiscent of preleptotene or early leptotene meiocytes, in a 90- day-old fetus. (X3500).

frequently detected (Figure 3). A well defined sex vesicle or an X-Y body characteristic of mid-pachytene meiocytes (Solari, 1994) was not evident in any of the bovine fetal testes we have examined to date although some of these large intra-tubular cells displayed prominent nucleoli and unpaired axial elements reminiscent of the preleptotene or early leptotene meiocytes in their ultrastructural features. These cells which were relatively more abundant in fetuses over 90 days old, continued to be present along with degenerating cells even in the oldest (180 day old) fetus we have examined.

## DISCUSSION

Our RT-PCR data show that *Xist* RNA is expressed in the gonads of male bovine fetuses. Our observations on non-gonadal organs further indicate that *Xist* expression in male fetuses is confined to the testis since their muscle and lung (sampled for fetal somatic tissues), failed to display a band characteristic of *Xist*. The visual demonstration of *Xist* expression in the testes of 50 day old fetuses as a faint band and prominently and consistently in older fetuses thereafter further indicates that the expression of this gene is initiated around 50 days of gestation in bovine fetuses and that it continues to180 days (and probably throughout gestation). If low levels of expression occur in fetal testes prior to 50 days, it may not be detectable with the method used in the present study. Expression of *Xist* has not been demonstrated in the mammalian testes at fetal stage previously. However, a transient *Xist* expression was reported in the somatic cells of the urogenital ridge in the mouse around the time of

gonadal differentiation (10.5- 11.5) coincident with the expression of the *Sry* gene (Jamieson et al., 1997). Our study clearly shows that *Xist* expression in bovine testes is 8 to 10 days later than the gestational age at which bovine testicular induction occurs (Jost et al., 1982). While *Xist* RNA has been demonstrated in human and mouse meiotic germ cells (Ayoub et al., 1997; McCarrey and Dilworth, 1992, and Richler et al., 1992) and its presence by RT-PCR has been ascertained in the adult bovine testes (De La Fuente et al., 1998), its presence in the bovine fetal testis, before germ cells committed to meiosis are thought to be present, is difficult to explain. Possible interpretations of these unexpected findings are: (1) that the *Xist* expression detected in fetal bovine testes is non-specific and unrelated to spermatogenesis, (2) that the primers we used in this study recognised a transcript antisense to *Xist* similar to the *Tsix* RNA expressed in undifferentiated somatic cells (Lee et al., 1999b) or (3) that the transcript detected represents *Xist* per se or a forerunner of *Xist* expression, which, in some way is involved in the mitosis-to-meiosis transition of fetal germ cells and/or in the preleptotene-like chromatin changes in some of these germ cells which enter meiosis in fetal bovine testes.

With regard to the first interpretation, Kay et al., (1993) had demonstrated *Xist* expression in the mouse testis at 3 days postpartum which predates the onset of meiosis in the male mouse by 5 days. Based on this, and the finding that the level of *Xist* RNA generated in the immature testis is 1000 fold lower than that in the somatic tissues of the same species, these investigators postulated that it may represent a non-specific event unrelated to the formation of a sex vesicle or the onset of spermatogenesis (Kay et

al., 1993). However, this explanation is somewhat weakened by the consistent presence of *Xist* RNA in adult bovine testes and in all other mammalian testes examined to date. Since the somatic tissues of the same males do not share this feature, it would appear that *Xist* expression is associated with a testicular function even if it is not related to the formation of a sex vesicle in meiocytes.

The possibility that the transcript detected in fetal bovine testes may be involved in male germ cell differentiation is supported by the cellular events taking place during that time in the developing testes. The presence of one or more relatively large intra-tubular cells with a large nucleus and prominent nucleoli closely resembling the prespermatogonia of postnatal bovine testes (Ertl and Wrobel, 1992) suggest that a degree of germ cell differentiation is already initiated in the seminiferous tubules of bovine fetal testes as early as 50 days of gestation and that a subset of these reach preleptotene-like stage by 90 days when female germ cells in fetal bovine ovary enter meiosis (Koykul and Basrur, 1994). Based on an exhaustive ultrastructural study coupled with morphometric analyses of postnatal germ cells during the prepubertal period in bovine testis, Ertl and Wrobel (1992) suggested that the only detectable postnatal germ cell type prior to the onset of spermatogenesis in the bovine species is the prespermatogonia. The morphological resemblance of the intra-tubular cells of bovine fetuses in the present study to the prespermatogonia (Ertl and Wrobel, 1992) suggests that some of these germ cells are already discernable, albeit in small numbers, in the fetal testes during early gonadal differentiation. Our studies further show that some of these cells may even reach preleptotene stage and that they eventually

degenerate probably due to the absence of a tubular lumen and/ or other cellular and physiological support system for male germ cell differentiation and establishment of normal spermatogenesis at this stage. In this regard, it is interesting that Luciani et al. (1977) had also detected preleptotene condensation in human fetal testes. Furthermore, Zamboni and Upadhyay (1983) had suggested that meiosis may be initiated in male germ cells at the same stage as that in female germ cells and that the main difference between the two genders is that the testicular germ cells do not progress further whereas ovarian germ cells undergo early prophase changes before undergoing a meiotic arrest in diplotene. In the light of these and our present observations, we tend to believe that the transcript detected in this study represents *Xist* or its antisense gene, the *Tsix*, which has recently been proposed to play a regulatory role in the earlier steps of X inactivation (Lee et al., 1999b). Since the method used in this study does not distinguish between *Xist* and *Tsix*, the true identity of the transcript is not known at present. However, based on the fact that a similar PCR product was detected in the adult bull testis (De La Fuente et al., 1999), we believe that the transcript derived from fetal testis may be involved in a function common to both fetal and adult testes. We hypothesize that the transcript detected in fetal bovine testis may be involved in the mitosis-to-meiosis transition of male germ cells in general or the preleptotene chromatin changes taking place in the male germ cells which prematurely enter meiosis in bovine fetuses.

# CHAPTER 2

# SEX-LINKED GENES ARE NOT SILENCED IN FETAL BOVINE TESTES EXPRESSING X-INACTIVE SPECIFIC TRANSCRIPT (*XIST*)

## INTRODUCTION

X-linked gene products are equalized in the two sexes by the inactivation of one of the two X chromosomes of female mammals (Lyon, 1996 for review). The factor responsible for the various steps in the X-inactivation cascade is thought to be the *Xist* gene residing in the X-inactivation centre of the X chromosome which transcribes an RNA that remains associated with the inactive X chromosome (Borsani et al., 1991; Brown, 1991; Brown et al., 1991a; Brockdorff et al., 1992; Clemson et al., 1996; Panning et al., 1997; Brockdorff and Duthie, 1998). X-inactivation has also been shown to occur during spermatogenesis when the only X chromosome present in male germ cells, in association with the Y, becomes condensed to form a structure referred to as the sex vesicle, the sex body or the X-Y body (Sachs, 1954; Handel et al., 1994, Richler et al., 1994; Solari, 1994). This process which is also known to involve the transcription of *Xist*, is thought to occur only in the germ cells entering meiosis (McCarrey and Dilworth, 1992; Richler et al., 1992; Ayoub et al., 1997). Previous studies from this laboratory using reverse transcription polymerase chain reaction (RT-PCR) approach with primers designed to amplify a 463 bp product from a conserved region of the first exon of bovine *Xist* gene (Hendrich et al.,1993) proved that *Xist* is expressed in adult bovine testes (De La Fuente et al., 1999). More recently, however, using the same approach, we detected that *Xist* is expressed in fetal testes of this species

as early as 50 days of gestation and that it continues to be expressed throughout gestation when spermatogenesis (or the prerequisite gonadal and germ cell differentiation to meiosis) is not expected to occur (Farazmand et al., 2000). Morphological studies on fetal testes had revealed the presence of a few large intratubular cells overtly resembling the prespermatogonia at 50 days of gestation coinciding with the initiation of *Xist* expression, and preleptotene-like cells were detected as early as 90 days of gestation by which time *Xist* (or an *Xist*-like RNA) was clearly detectable (Chapter 1, Farazmand et al., 2000). We had hypothesized that the male germ cells in bovine fetuses may enter meiosis at the same time when meiosis is initiated in their female counterparts in fetal ovary as suggested by Zamboni and Upadhyay (1983) and that the expression of the *Xist* gene may have been related to the presence of these large germ cells which prematurely enter meiosis and undergo apoptosis (Farazmand et al.,2000). Since the function of this gonad-specific transcript in males before spermatogenesis is initiated is unknown, we undertook to examine whether the *Xist* gene expression in developing pre-spermatogenic bovine testes is related to transcriptional silencing of X-linked genes.

The X-linked loci chosen for testing their levels of expression in this study, besides *Xist*, are glucose-6-phosphate dehydrogenase (*G6pd*), hypoxanthine phosphoribosyl transferase (*Hprt*) and zinc finger protein locus on the X (*Zfx*). Glucose-6-phosphate-dehydrogenase locus controls the pentose phosphate pathway in all cell types. The protein product (*G6PD*) of this locus is the first, and rate-limiting enzyme for this pathway which generates reduced nicotinamide adenine dinucleotide (NADPH)

needed for the reduction of harmful substances including oxidized glutathione (Vogel and Motulsky, 1997). The *Hprt* locus participates in purine metabolism in cells through its involvement in the "salvage" pathway. The enzyme product (*HPRT*) of this locus converts free purine bases in cells to their corresponding 5'-mononucleotides (Vogel and Motulsky, 1997). The *Zfx* locus, a conserved homologue of the Y chromosome-specific *Zfy* gene, encodes a putative DNA binding protein whose function is not clearly established as yet. However, it is believed that this zinc finger protein of the cysteine-histidine family is involved in transcriptional regulation through nuclear sequence-specific DNA binding, double strand DNA binding and transactivation of genes in response to hormones (Nagamine et al., 1990; Nagamine and Carlisle, 1997; Russell, 1998). The *Zfx* locus is transcribed in all cell types and deletion of this locus leads to reduced size and viability as well as reduced germ cells in the gonads leading to subfertility in males and females (Luoh et al., 1997). Of these, *G6pd* and *Hprt* are the most extensively studied loci for expression status in male and female mammals. The exact locations of *G6pd* and *Hprt* on the bovine X chromosome have not been identified as yet; however, these two loci have been assigned to the long arm of the bovine X, based on comparative gene mapping and syntenic association with other loci (Hendrich et al., 1993; Chowdhary et al., 1996). The *Zfx* locus has recently been mapped to the long arm of the bovine X, at segment Xq34 (Xiao et al., 1998). In human female cells, *Zfx* escapes inactivation whereas it undergoes inactivation in mice (Adler et al., 1991). All these loci are expressed in early bovine embryos (Gutierrez-Adan et al., 2000; Peippo et al., 2000) and thought to be silenced during meiosis when X inactivation

takes place in mammalian testes (McCarrey et al., 1992; Xiao et al., 1998). We hypothesized that these loci will be turned off or subjected to reduced expression, if X inactivation takes place in fetal testes at the time when they transcribe *Xist* RNA. In order to test this, we compared the expression levels of the sex-linked loci mentioned above and of *Xist*, in gonadal and non-gonadal organs of male and female bovine fetuses.

## MATERIALS AND METHODS

### Tissue collection and RNA extraction

Bovine fetuses were retrieved from pregnant uteri collected from a local abattoir within one hour of slaughter. The fetuses were sexed and categorized in age groups based on crown rump length measurement (Noakes, 1997) prior to organ retrieval. Gonads and non-gonadal organs (brain, heart, kidney, liver, lung and muscle) from each fetus were removed, frozen immediately in liquid nitrogen and stored at 80°C until use. Total RNA was extracted from 100 mg tissue from each organ using Trizol (Gibco/BRL), according to the manufacturer's instruction. Extracted RNA samples were dissolved in DEPC treated water and stored at -20°C. The concentration of RNA was estimated using the spectrophotometer (UV-1201, Shimadzu Corp., Japan) using a standard protocol (Sambrook et al., 1989).

### Reverse Transcription

Samples of RNA were treated with DNase to eliminate genomic DNA

contamination, using 1.8 μg of the extracted RNA in a total volume of 12 μl containing 1.2 μl of 10X DNase buffer (Gibco/BRL) and 1.2 units of DNase for 20 minutes (at room temperature). Reverse transcription was carried out in a 20 μl reaction mixture containing 4 μl First Strand Buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, 10 mM DTT), 0.24 mM dNTPs, 250 ng each of random and oligo-dT primers and 200 units of superscript Reverse Transcriptase (Gibco/BRL) at 42°C for 2 hours. The cDNA samples, were incubated at 90°C for 5 minutes to inactivate the reverse transcriptase prior to storing at -20°C.

The method used for the visualization of *Xist* RNA expression in bovine fetuses, was as described elsewhere (Farazmand et al.,2000). For the comparison of *Xist* expression in fetal and postnatal testes, biopsies obtained from adult bulls and newborn and prepubertal bull calves were also processed as described previously (Farazmand et al., 2000). The primers used for the visualization of *G6pd* locus was that of Lequarre et al. 1997); for *Zfx* and *Hprt* loci, the primers used were those of Aasen and Medrano (1990) and Grunig and Antczak (1995) respectively.

### Relative Quantitative Polymerase Chain Reaction (RQ-PCR)

The quantitative approach used in this investigation was according to the Relative Quantitative Reverse Transcription PCR protocol from Ambion. The protocol consisted of several steps including the determination of linear range and optimum ratio of 18 S primers to competimers, before undertaking relative quantitative PCR.

Linear range determination

To determine the linear range of amplification cycles in which the efficiency of PCR is at its maximum level, a master mix of 12 aliquots were removed every two cycles and the products were resolved by electrophoresis on 2% agarose gel. The optical density (OD) values of bands obtained by FMBIO II Image Analysis System (Hitachi Software Ltd.) after staining with SYBR[R] Green I (Molecular Probe Ltd) were plotted against the number of amplification cycle. The amplification cycle from the mid point of the linear range was chosen for subsequent experiments.

Determination of optimum ratio of 18S primers to Competimers

The marker chosen for this study was the 18S ribosomal gene product which is abundant in all cells. RNA transcripts of this gene was used in the semiquantitative assays of specific gene transcripts, as a standard in order to facilitate comparison of gene expression from one experimental run to the next. Since the18S transcript is often high in different tissues, its concentration had to be adjusted to that of the gene of interest. For efficient amplification of both, co-amplification of 18S was carried out with each gene of interest first in ratios of 1:9, 2:8, 3:7 and 4:6 of 18S:Competimers primer by volume. The ratio which gave overtly similar intensity of 18S product to that of the gene specific band, was used as optimum ratio for co-amplification. The primer sequences used, PCR conditions, the cycle numbers and18S:competimer ratios are shown in Table III.

Table III. Amplification conditions for glucose-6-phosphate dehydrogenase (*G6pd*), hypoxanthine phosphoribosyl transferase (*Hprt*), X inactive specific transcript (*Xist*) and zinc finger protein locus on X (*Zfx*).

| Gene locus | Cycle Number | 18S/C Ratio* | Cycling Conditions | Primer Sequences |
|---|---|---|---|---|
| *G6pd* | 29 | 4 : 6 | 90°C, 3 min; 94°C, 1min;<br>56°C,1 min; 72°C, 1 min | 5' CAA GAT GAT GAC CAA GAA GC<br>3' AGC AGT GGT GTG AAG ATA CG |
| *Hprt* | 29 | 4 : 6 | 90°C, 3 min; 94°C, 1 min;<br>54°C, 1 min; 72°C, 1 min | 5' GTA ATG ATC AGT CAA CGG GGG AC<br>3' CCA GCA AGC TTG CAA CCT TAACCA |
| *Xist* | 31 | 4 : 6 | 90°C, 3 min; 95°C, 1 min;<br>55°C, 1 min; 72°C, 75 sec | 5' AGC ATT GCT TAG CAT GGC TC<br>3' TGG CTG TGA CCG ATT CTA CC |
| *Zfx* | 30 | 3 : 7 | 90°C, 3 min; 94°C, 1 min;<br>55°C, 1 min; 72° C, 1 min | 5' ATA ATC ACA TGG AGA GCC ACA AGCT<br>3' GAG CCT CTT TGG TAT CTG AGA AAG T |

*Volume ratio of internal standard (18S RNA) : competimer.
Reaction mixture (50 ml) contained 10X Buffer 5 ml; Mg $Cl_2$, 1.5 mM; Taq polymerase, 1.35 Units; cDNA, 0.2 mg, in addition to 0.2 mM of dNTPs (for all except *Xist* for which 0.3 mM was added) and gene-specific primers (0.8 mM for *G6pd* and *Hprt*; 0.4 mM for *Xist* and 1.0 mM for *Zfx*).

Relative quantitative PCR

The ideal cycle number obtained from linear range and the optimum 18S: Competimer ratio for each gene were used to determine the relative quantitative data for each PCR product. Following PCR, the products were resolved on 2.0 % agarose gel, stained with SYBR[R] Green 1 and scanned using FAMBIO II Image Analysis System (Hitachi Software Ltd.). The ratio (optical density value for each band representing the gene specific product divided by that for 18S co-amplified in the same sample, was recorded as the level of expression of each gene in an organ relative to the expression of 18S in the same tissue. For each of the 3 sex-linked genes, a minimum of 4 runs of RQ RT-PCR were carried out and for *Xist*, 5 runs were carried out using RNA and cDNA samples from male and female fetuses.

Statistical Analysis

Student's paired *t*-test was used to compare the expression level of each gene locus in fetal testes with that in different organs from the same fetus. Unpaired *t*-test was used to compare the levels of expression in testes with those in the somatic organs of male and female fetuses. Difference in values below 5 percent probability ($p < 0.05$) was accepted as significant. Data are presented as mean $\pm$ SEM.

Histology and electron microscopy

One of the gonads of each fetus in the age group of 85 to 95 days was cut into 1.0 mm pieces and fixed in 5.0% glutaraldehyde for semithin and ultrathin sections.

Pieces of testes fixed for 4 hours were post fixed in 1.0% osmium tetroxide for one hour prior to dehydrating in ascending concentrations of ethanol and embedding in Epon via propylene oxide. Semithin (1.0 $\mu m$) sections were cut with a Reichert OMU2 ultramicrotome, stained with 1.0% toluidine blue in 1.0% sodium borate and examined under a Carl-Zeiss photomicroscope. Representative areas were photographed using Kodak's Professional T400CN T-MAX black and white film. Selected Epon blocks were trimmed to obtain areas of interest and ultrathin sections (60 to 90 nm) were cut using a Reichert OMU2 ultramicrotome. Sections were picked up on 250 square mesh copper grids, contrasted with uranyl acetate and lead nitrate and examined under a JEOL JEM 100S transmission electron microscope. Representative areas were photographed using Kodak 4489 sheet film.

## RESULTS

Transcription status of *Xist* in gonadal and non-gonadal organs of bovine fetuses are illustrated along with positive and negative controls in Figure 4a. All of the organs tested from female fetuses showed a distinct band approximating the base pair length (463 bp) for the amplified portion of the bovine *Xist* (De La Fuente et al., 1999) in agarose gel. Of the 7 corresponding organs tested from male fetuses, however, testis was the only organ of male fetuses displaying a similar amplicon (Fig. 4a, lane 4). The expression pattern in adult testis was similar to that in fetal testis (Fig. 4b) and testicular RNA from newborn and prepubertal bull calves also produced similar results (data not shown here).

The expression patterns of *Xist* and the internal standard (18S) in different organs of female fetuses (compared with that of the testis and two non-gonadal organs of a male fetus of corresponding age), are shown in Figure 5. The two non-gonadal organs of male fetuses (kidney and liver) showed no *Xist* expression whereas the testis showed a distinct band resembling *Xist* (Fig. 5). The level of *Xist* expression relative to 18S, was comparable in different non-gonadal organs of female fetuses (Table IV) and the mean expression rate of these organs was not significantly different from that in ovaries which is rich in somatic elements at this stage of fetal development. However, testicular *Xist* expression in male fetuses of corresponding age was significantly (3.43 fold) lower than that in ovaries (Table V).

Expression patterns of the three X-linked loci and 18S are shown in Figure 6 and their relative expression rates are summarized in Table VI. Statistical analysis revealed that the levels of expression in fetal testes were not different from those in somatic organs of female or male fetuses and that the values for somatic organs (with one exception) were not different between male and female fetuses. The exception concerns *G6pd* expression in male brain which was significantly higher than that in female brain ($p < 0.0005$). Comparison of the expression levels of X-linked genes including *Xist* (Fig. 7) in fetal gonads showed that *Xist* and *Zfx* expression levels were higher in ovaries, and that *Hprt* was expressed at similar rate in ovaries and testes. However, *G6pd* expression was higher in fetal testis than in fetal ovary which, in turn, was higher than that in somatic organs. Expression of *G6pd* in testes was also higher than that in all somatic organs except the kidney.

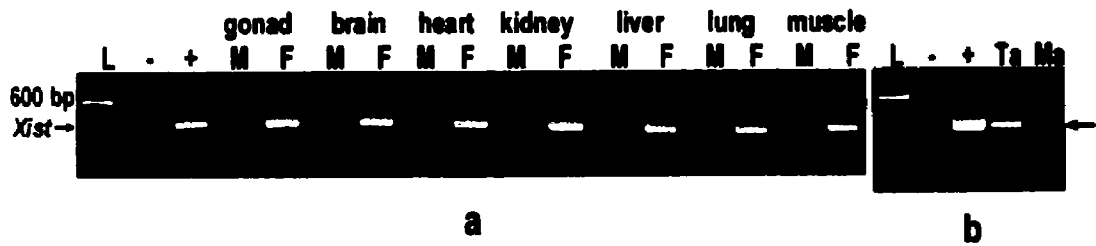



Figure 4. RT-PCR products representing *Xist* RNA expression. a: *Xist* expression in different organs of ~ 90-day -old male (M) and female (F) bovine fetuses. b: *Xist* expression in adult male testis (Ta) and its absence in adult muscle (Ma). L: 100 bp ladder; - : negative control, with all components except the cDNA; +: positive control, cDNA sample from female somatic tissue.

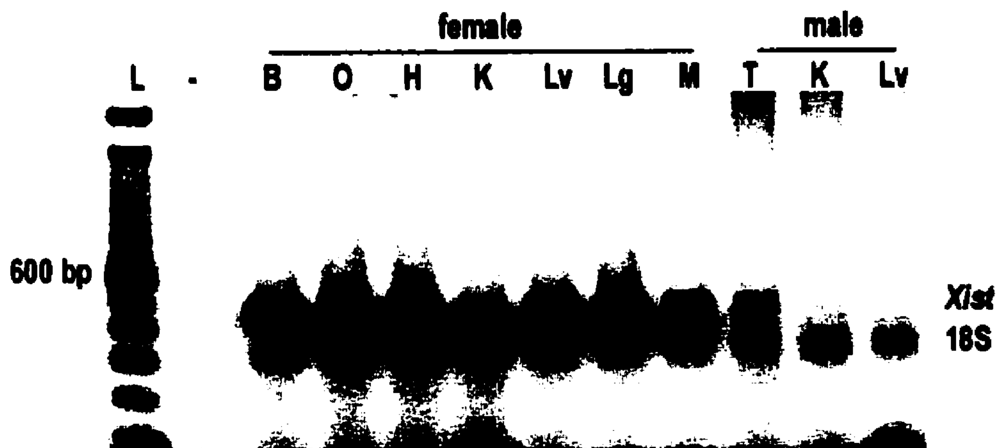



Figure 5. RT-PCR products representing *Xist* and 18S RNA expression in different organs of ~ 90-day-old female bovine fetuses (to the left). L: 100 bp ladder; - : negative control, with all components except the cDNA; B: brain, O: ovary; H: heart; K: kidney; Lv: liver; Lg: lung; M: muscle; T: testis.

**Table IV.** Relative levels of X-inactive specific transcript (*Xist*) expressed* in different organs of female bovine fetuses.

| Fetal organs tested | Trial # | | | | | Mean ± SEM |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| Brain | 1.16 | 1.11 | 1.43 | 1.28 | 1.14 | 1.22 ± 0.06 |
| Heart | 1.25 | 1.18 | 1.48 | 1.38 | 1.13 | 1.28 ± 0.06 |
| Kidney | 1.17 | 1.1 | 1.56 | 1.22 | 1.4 | 1.27 ± 0.10 |
| Liver | 1.11 | 1.14 | 1.19 | 1.2 | 1.37 | 1.20 ± 0.05 |
| Lung | 1.2 | 1.34 | 1.32 | 1.12 | 1.1 | 1.22 ± 0.05 |
| Muscle | 1.14 | 1.15 | 1.6 | 1.24 | 1.2 | 1.27 ± 0.09 |

*Based on the optical density of the band representing *Xist* RNA relative to that of the ribosomal 18S gene co-amplified with each sample.

Table V. Relative levels of X-inactive specific transcript (*Xist*) expressed* in fetal bovine gonads.

| Fetal gonad tested | Trial # | | | | | Mean ± SEM |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | |
| Testis | 0.45 | 0.42 | 0.38 | 0.37 | 0.35 | 0.39* ± 0.02 |
| Ovary | 1.44 | 1.5 | 1.4 | 1.12 | 1.34 | 1.36 ± 0.07 |

*Based on the optical density of the band representing *Xist* RNA relative to that of the ribosomal 18 S gene co-amplified with each sample.

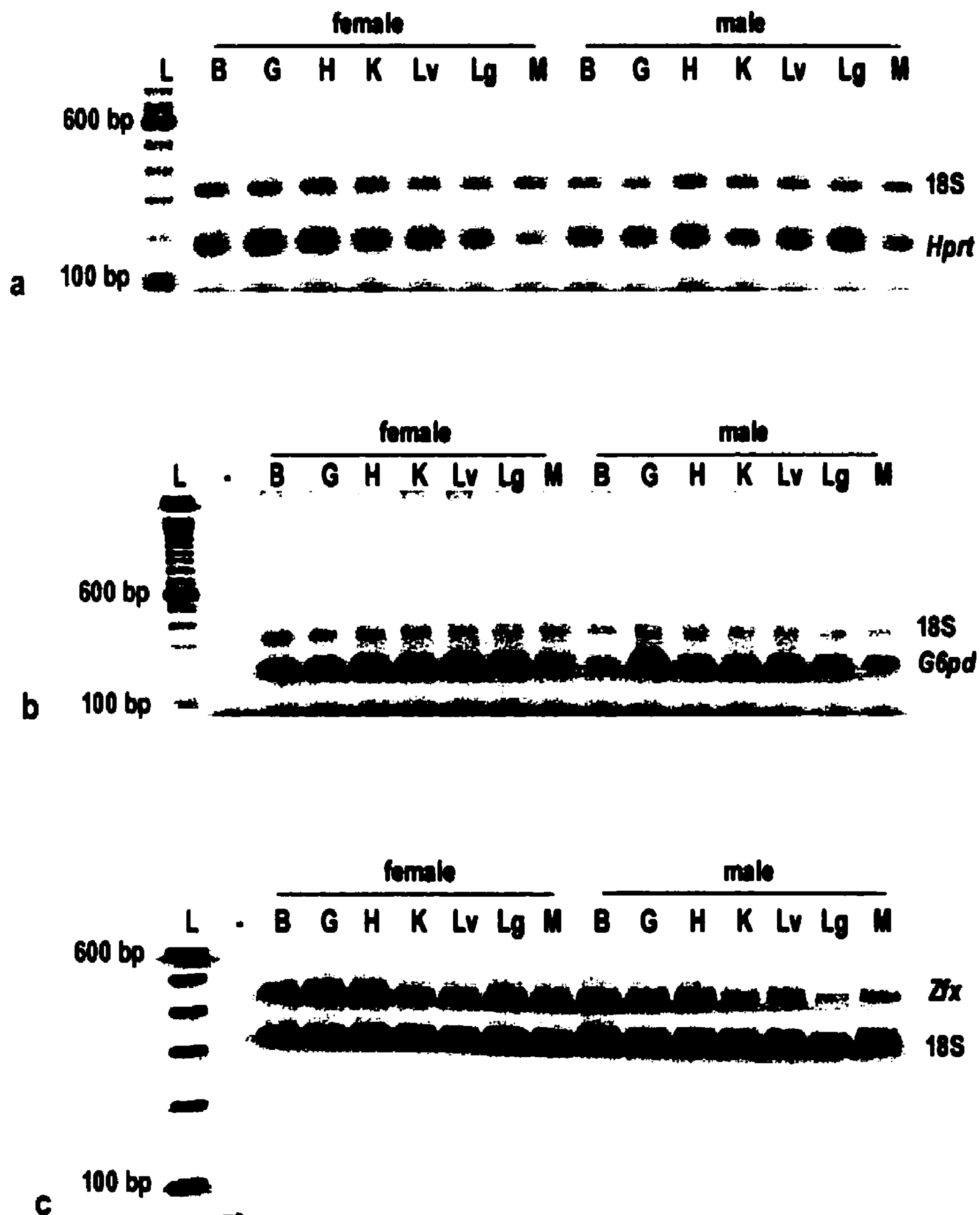



Figure 6. RT-PCR products representing *Hprt* (a), *G6pd* (b) and *Zfx* (c) compared to 18S RNA coamplified with each sample, in different organs of ~ 90-day-old male and female bovine fetuses. L: 100bp ladder; - : negative control with all components except the cDNA; B: brain, G: gonad; H: heart; K: kidney; Lv: liver; Lg: Lung; M: muscle.

Table VI. Relative expression* of three X-linked genes in different organs of female and male bovine fetuses.

| Sex | Organ | *Hprt* | *G6pd* | *Zfx* |
|---|---|---|---|---|
| Female | Brain | 1.24 ± 0.10 | 0.50 ± 0.05 | 1.13 ± 0.24 |
| '' | Heart | 1.20 ± 0.12 | 0.87 ± 0.11 | 1.24 ± 0.20 |
| '' | Kidney | 1.08 ± 0.13 | 1.12 ± 0.20 | 1.10 ± 0.25 |
| '' | Liver | 1.19 ± 0.11 | 0.97 ± 0.14 | 0.82 ± 0.14 |
| '' | Lung | 1.20 ± 0.11 | 0.87 ± 0.14 | 0.98 ± 0.21 |
| '' | Muscle | 1.13 ± 0.10 | 0.68 ± 0.09 | 0.88 ± 0.12 |
| '' | Ovary | 1.26 ± 0.10 | 1.20 ± 0.11 | 1.38 ± 0.20 |
| Male | Brain | 1.15 ± 0.11 | 0.67 ± 0.07 | 0.89 ± 0.12 |
| '' | Heart | 1.19 ± 0.09 | 0.81 ± 0.08 | 0.84 ± 0.10 |
| '' | Kidney | 1.15 ± 0.10 | 0.86 ± 0.06 | 0.76 ± 0.13 |
| '' | Liver | 1.26 ± 0.08 | 0.90 ± 0.08 | 0.74 ± 0.09 |
| '' | Lung | 1.17 ± 0.07 | 0.82 ± 0.09 | 0.69 ± 0.16 |
| '' | Muscle | 1.01 ± 0.09 | 0.67 ± 0.10 | 0.71 ± 0.12 |
| '' | Testis | 1.23 ± 0.11 | 1.49 ± 0.16 | 0.91 ± 0.12 |

*Based on the optical density of the bands representing the RNA transcript of each gene relative to that of the ribosomal 18S gene co-amplified with each sample. *Hprt*: Hypoxanthine phosphoribosyl transferase; *G6pd*: Glucose-6-phosphate dehydrogenase; *Zfx*: Zinc finger protein gene on X. No significant difference between expression levels in fetal testis and non-gonadal organs of male and female fetuses.

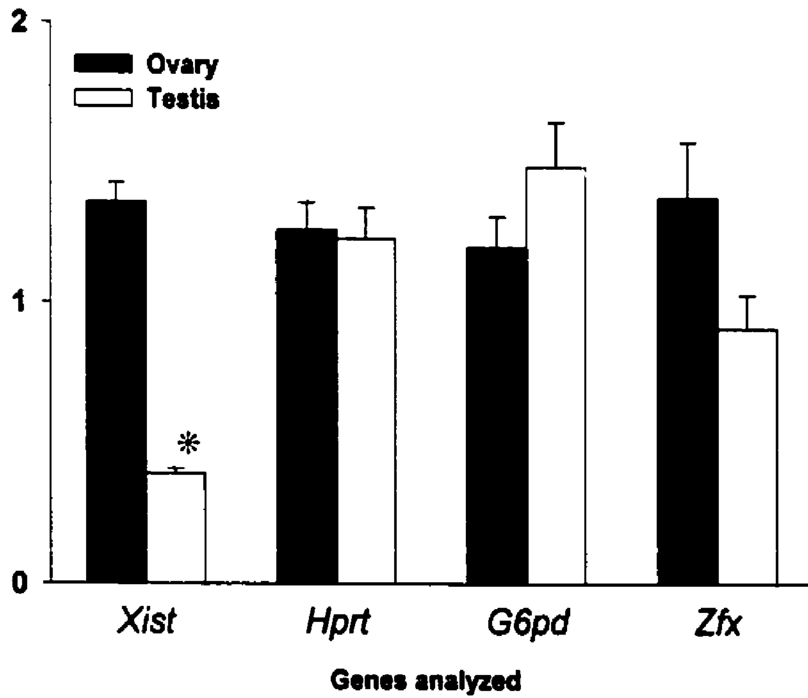



**Figure 7.** Bar chart showing levels of expression of X-linked genes in the gonads of 90-day-old bovine fetuses (mean ± SEM, n=5, * : values for the testis are significantly different from that for the ovary, $p < 0.05$).

Morphological features of fetal testes revealed large cells with prominent nuclei and nucleoli similar to the prespermatogonia of postnatal bovine testes (Ertel and Wrobel, 1992), within the seminiferous tubules of ~ 90-day-old fetuses. Some of these cells were clearly in the process of degeneration (Fig. 8). At the ultrastructural level, the nuclei displayed prominent and electron-dense nucleoli and in some of these cells, evidence of axial element condensation characteristic of degenerating early meiotic cells was noted. However, distinct profiles of synaptonemal complexes similar to those in fetal ovaries (Fig. 9) or sex vesicle characteristic of pachytene stage male germ cells (Ayoub et al., 1997), were not encountered (Fig. 8). In contrast, female fetuses of corresponding age displayed different stages of oogenesis at different regions of the fetal gonads as described previously (Koykul and Basrur, 1994). However, early stages of meiosis and even follicle formation were evident in fetal ovaries, generally closer to the rete ovarii (Fig. 9). Gonads of male and female fetuses were also rich in stroma and other somatic cell contents.

## DISCUSSION

### Expression of X-linked genes

Our data show that an *Xist* transcript similar to that generated by adult bovine testis is also detectable in fetal bovine testes. Although the level of transcription in fetal testis is lower than that at the adult stage, both are similar to the *Xist* amplicons derived from the somatic organs of female fetuses of corresponding age. However, while the expression of *Xist* RNA in somatic tissues of female fetuses could justify its role in

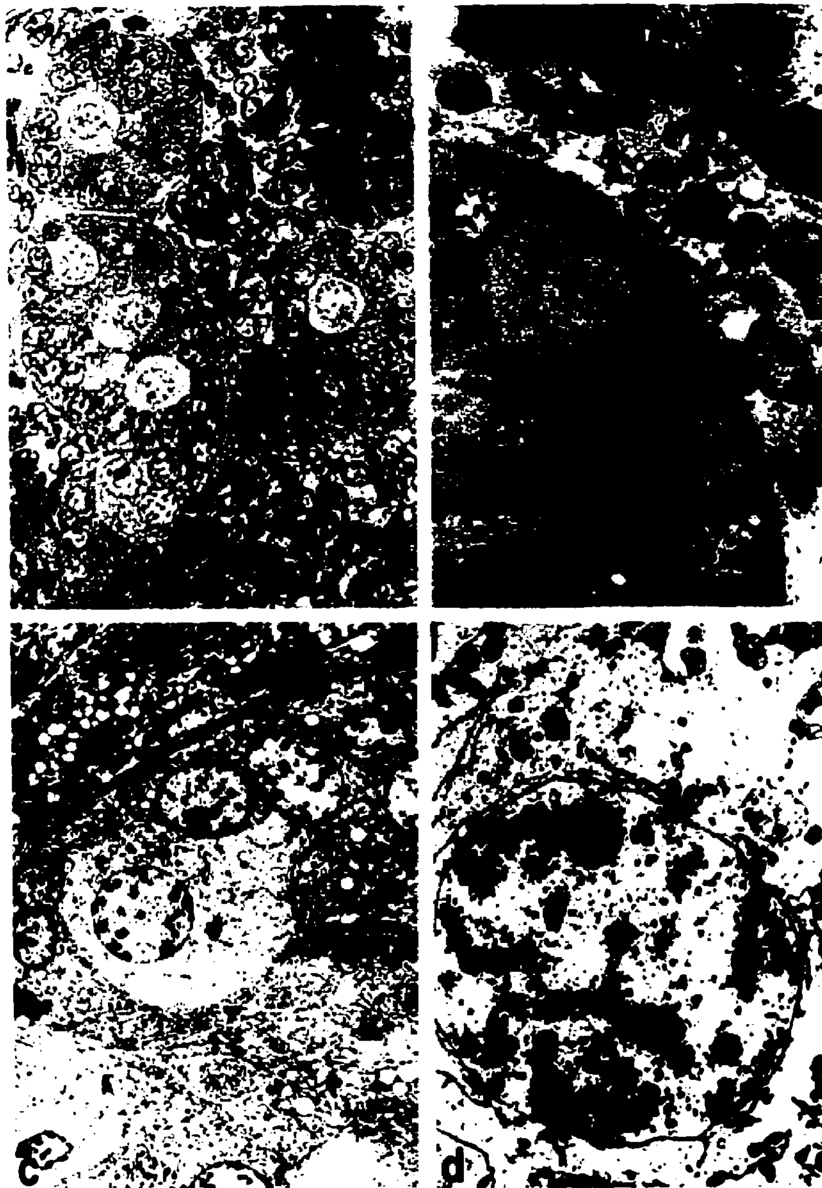


**Figure 8.** Morphological features of testicular cells in ~ 90-day-old bovine fetuses. **a** and **b**: semithin sections showing large male germ cells resembling prespermatogonia within the seminiferous tubules. Condensed chromatin and prominent nucleoli can be seen in the large cells. (X800); **c**: Ultrastructural features of the intratubular cells in seminiferous tubules. (X3000); **d**: Enlarged image of a nucleus showing condensed chromatin characteristic of pre- or early meiotic cells. (X6000).

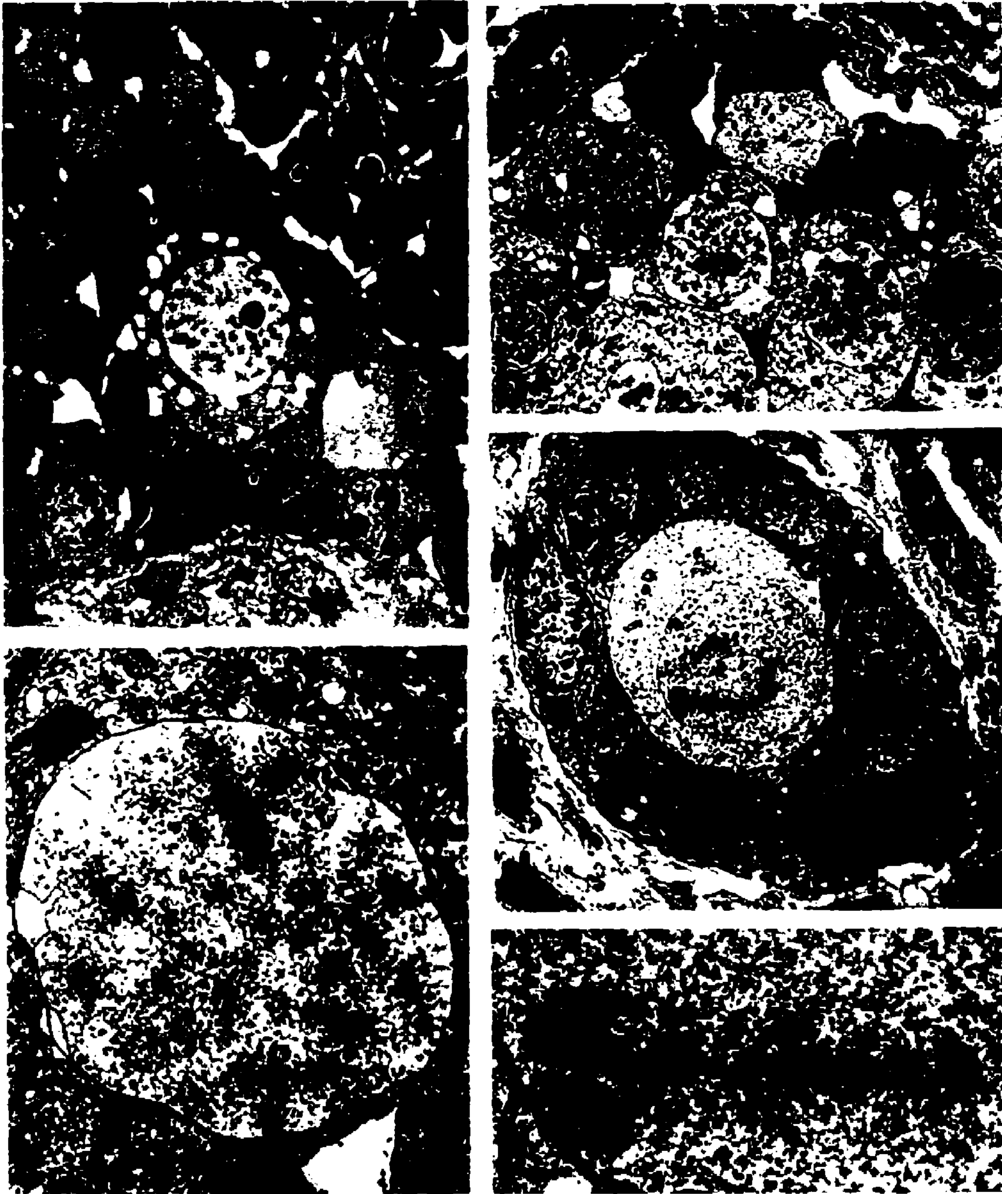


**Figure 9.** Morphological features of ovarian cells at different regions of fetal ovaries at ~ 90 days of gestation, showing female germ cells in different stages of meiosis. **a**: An oocyte showing early prophase nucleus with prominent nucleolus surrounded by germ cells in premeiotic stages. (X400); **b**: Cluster of early meiotic germ cells. (X400); **c**: An oocyte showing profiles of unpaired axial filaments in the nucleus. (X5000); **d**: An oocyte in pachytene from the region closer to the rete ovary. Note the cells around the oocyte resembling follicular cells. (X3000); **e**: Ultra-structural details of a nucleus of the oocyte in figure **9d** showing the tripartite profile of synaptonemal complex in pachytene (X6000).

achieving dosage equivalence with their male counterparts, the transcription of this RNA in fetal testes does not appear to have affected the expression status of the other X-linked genes tested in this study. The relative levels of expression of *G6pd*, *Hprt* and *Zfx* did not differ significantly in the non-gonadal organs (Table VI). Minor variations detected in the mean levels of expression in some of these somatic organs could be due to the escape from inactivation of certain sex-linked genes in different cell types. Regardless of the possible heterogeneous expression of X-linked genes in somatic organs (Carrel and Willard, 1999), it is evident that their levels of expression in male gonads were not lower than that in the somatic organs of either sex.

Of the three X-linked loci tested for expression, *Zfx* was the only locus that appeared to show a difference in favor of ovary relative to testis. While this difference in *Zfx* expression could be interpreted as evidence of reduced transcription of this locus in fetal testes, a more likely explanation concerns the fact that the female germ cells in fetal bovine ovaries have already entered meiosis (Fig. 9). Female germ cells in mammals enter meiosis early in fetal life (Epstein, 1986). In bovine species, meiosis is initiated in fetal ovaries between 81-90 days of gestation (Koykul and Basrur, 1994), and a substantial proportion of germ cells are already in meiotic prophase by 100 days (Koykul et al., 1997). In female mammals the inactive X undergoes a reactivation process prior to their entry into meiosis (Epstein, 1986; Tam et al., 1994). Mroz et al (1999) have shown that X-reactivation occurs when female germ cells enter the genital ridge, long before somatic differentiations are initiated in the female gonad. Although it is not known when the reactivation occurs in fetal bovine ovaries, our morphological

observations confirm the presence of meiotic cells in ~ 90-day-old female fetuses and on that basis, both X chromosomes of meiocytes will be expected to transcribe *Zfx*. Thus, the higher level of *Zfx* expression in fetal ovaries may have been a reflection of meiotic cells in fetal ovaries rather than the reduced transcription of *Zfx* (through X chromosome inactivation) in fetal testes. This view is supported by the similarity in the expression pattern of this locus in fetal testes to that in somatic organs of female fetuses (Table VI). However, a similar increase expected on this basis in the transcription of *Hprt* in fetal ovaries, was not evident in this study. It is possible that the X-reactivation pattern in the bovine species is different from that in mice and that all X-linked genes are not released from inactivation at the same time. The lack of difference in *Hprt* expression between fetal ovary and fetal testis, therefore, may be related to the proportion of somatic cells and premeiotic germ cells which still maintain an inactivated X, in fetal bovine ovaries.

Transcription of *G6pd* was also not elevated in fetal ovaries relative to that in fetal testes although the levels expressed in gonads (Fig. 7) were significantly higher than that in somatic organs (Table VI). The apparent over expression of *G6pd* in fetal testes compared to all other fetal organs could be related to several factors. The activity of *G6pd* enzyme has been known to be high in adult mammalian testes compared to other tissues, and it is higher in germ cells during postnatal testicular maturation (Hitzeman, 1965; Erickson, 1976; Jones and Andrews, 1978). Even though the fetal bovine testes of this study represent stages at which seminiferous tubules are without a lumen, or recognizable (mature) Sertoli cells, they display the presence of large

intratubular cells suggesting that some of the male germ cells within these tubules are in the process of differentiation. Based on the observation that some of these cells exhibit chromatin configuration resembling those of pre-leptotene germ cells, we had hypothesized that these may be germ cells which prematurely entered meiosis and therefore, are forced to undergo degeneration during fetal testicular development (Farazmand et al., 2000). It would appear that the death of these cells and the envisaged cell renewal during the elongation of seminiferous tubules could be related to the elevated expression of *G6pd* in fetal testes. Expression of *G6pd* is generally elevated in such organs as testis and kidney which are subject to cellular damage leading to the release of $H_2O_2$. Under these circumstances, cells resort to the pentose phosphate pathway (PPP) that generates NADPH which is essential for the detoxification through intracellular redox regulation (Hitzeman,1965; Martini et al.,1986). Since *G6pd* is the first and rate limiting enzyme for PPP (Tian et al., 1998), over expression of this locus could be attributed to the dynamic changes taking place in the developing fetal testes.

Another explanation for the apparent increase in *G6pd* expression may be found in the recent report that another type of *G6pd*, an intronless gene referred to as *G6pd-2*, is active in mammalian testes (Hendricksen et al., 1997). This gene which is colinear to the sex-linked *G6pd* (referred to as *G6pd-1*) and with over 90% sequence homology, is thought to be a retroposon that originated by the retroposition of a reverse-transcribed *G6pd-1* mRNA (Hendricksen et al., 1997). Although this gene is expressed only when the sex-linked *G6pd-1* is silent during early stage of meiosis, it is possible that it is expressed in the few germ cells which enter meiosis in bovine fetuses. Thus,

the higher level of *G6pd* expression detected in fetal testes may be attributable to the added effect of the newly identified, testis-specific and functional retroposon (Hendricksen et al., 1997). To summarize our data on the expression status of sex-linked genes in fetal testes, no clear cut evidence of a reduced expression relative to that in fetal somatic organs is detected for any of the loci tested (Table VI). This leads us to question the role of the low level RNA transcript detected in fetal testes and to examine whether it represents *Xist* which is thought to be the key factor in the inactivation of one of the two X chromosomes in female somatic cells (Brown et al., 1991a; Brockdorff and Duthie, 1998).

**X inactivation in male and female cells**

The observation that the *Xist* gene is expressed in mammalian testes (Borsani et al., 1991; McCarrey and Dilworth, 1992; Richler et al., 1992, 1994) while it is silent in other male tissues originally suggested that the X-inactivation process in male germ cells may have resorted to the X inactivation mechanism adopted by female somatic cells (McCarrey and Dilworth, 1992). However, more recent findings have indicated differences in key aspects of the inactivation process in male and female. For example, features of the inactive X chromosome in female somatic cells include expression of *Xist*, chromosome condensation characteristic of heterochromatin, late replication, hypoacetylation of histone, hypermethylation of the promoter regions of gene loci, and reduction or failure of transcription of X-linked genes (Lyon, 1996; Goto and Monk, 1998 for review). Although the molecular mechanisms and biological significance of

X-inactivation in male germ cells still remain obscure, it has long been known that the sex chromosomes set themselves apart spatially and functionally from the autosomes during spermatogenesis. Thus, in nearly all cases of mammals studied to date, when male germ cells enter meiotic prophase, the sex chromosomes are visualized as a distinct entity referred to as the sex vesicle, sex body (Sachs, 1954) or XY body (Solari,1974) depending upon the visual aid used. In addition to their condensed nature, the sex chromosomes in early meiosis are also transcriptionally inactive as evidenced by the absence of uridine incorporation in the sex body (Monesi, 1965; Jaafar et al., 1989, 1993; Richler et al., 1994; Ayoub et al., 1997). However, in contrast to the inactivated X chromosome in female somatic cells, the condensed chromatin of the male germ cells is not made up of hypoacetylated histone, the gene loci are not hypermethylated and the inactivated state (of the sex chromosomes) itself, is reversible (Ayoub et al., 1997). Furthermore, proteins including a 30 - 34 kDa protein referred to as XY40, have been noted to be distributed along the axial elements of the condensed sex chromosomes of the male meiocytes (Smith and Benavente, 1992). Smith and Benavente (1992) suggested that the XY40 protein may be involved in keeping the chromatin material of the sex complement condensed and the gene loci inaccessible for transcription during male meiotic prophase. Noting the similarity in the distribution pattern of XY40 protein and the *Xist* RNA on the inactive X of pachytene stage male germ cells, Ayoub et al. (1997) had postulated that the *Xist* RNA may be specifically bound to these meiosis-specific proteins, and that such a binding process may be a vital link in achieving X-inactivation in spermatogenesis.

### *Xist* expression in male cells

Low level *Xist* expression has been reported in male cells under different circumstances (Goto and Monk, 1998). Kay et al. (1993) detected a low level of *Xist* RNA in the newborn mouse testis in which meiosis has not yet been initiated. These investigators postulated that *Xist* gene may not be involved in the mediation of inactivation of the only X chromosome in male germ cells (Kay et al., 1993). Also, more recently, Marahrens et al. (1997), using the mouse model carrying a targeted deletion in the structural gene, demonstrated that mutant males are fertile while the females carrying the paternally derived mutant allele die during embryogenesis. On the basis of their observation that *Xist* RNA mediated X inactivation is essential for random X inactivation and the survival of the female embryos and that spermatogenesis is normal in mutant males, these investigators (Marahrens et al., 1997) postulated that the production of *Xist* RNA leading to X-inactivation may not be a necessary component of spermatogenesis. However, as stated above, some aspects of X-inactivation including heterochromatinization of the sex complement are universally recognized as integral parts of spermatogenesis. Furthermore, examples of male sterility due to interrupted X-inactivation in structural heterozygotes, are abundant among mammals (Ansari et al., 1993; Jaarfar et al, 1993; Neal et al., 1998). Therefore, the observations of Marahrens et al. (1997) could have meant that X-inactivation is achieved in male and female mammals by different mechanisms.

Low levels of *Xist* expression similar to that displayed by fetal bovine gonads in the present study, were reported in female embryonic stem (ES) cells prior to X-

inactivation when both Xs are active (Penny et al., 1996). Interestingly, these investigators also noted a similar low level of *Xist* expression from the single "active" X of male ES cells. However, *Xist* RNA expression becomes more robust (and confined to the inactive X) as female ES cells become differentiated while in male ES cells *Xist* RNA continues to remain faint and their X chromosome escapes being painted by *Xist* RNA unlike in female ES cells (Clemson et al., 1996). Taken together, these results and our findings on fetal bovine testes, would indicate that *Xist* expression alone does not coincide, or always eventuate, inactivation of the X chromosome.

Expression of *Xist* has not been demonstrated in the mammalian testes at fetal stage previously. While *Xist* RNA has been demonstrated in human and mouse meiotic germ cells (McCarrey and Dilworth, 1992; Richler et al., 1992; Ayoub et al., 1997) and its presence has been ascertained by RT-PCR in the adult bovine testes (De La Fuente et al., 1999), transcription of this RNA before germ cells are committed to spermatogenesis, is difficult to explain. The testicular transcript detected in fetal and adult testes in the present study resemble the low level *Xist* expression reported in adult testes of other species, male ES cells, premeiotic murine testis and the urogenital ridge cells of male mouse fetuses (Kay et al., 1993; Penny et al., 1996; Ayoub et al., 1997; Jamieson et al., 1997). The identity of the transcript detected in low levels in all these undifferentiated cells has attracted much attention recently. In this regard, Lee et al. (1999b) have identified and characterized a transcript from ES cells which shares some of the characteristics of *Xist*. Since it is transcribed from the DNA strand opposite to *Xist*, these investigators named the transcript *Tsix* (Lee et al., 1999b). This antisense RNA expressed in undifferentiated female somatic

cells, becomes progressively less well expressed during cell differentiation prior to its total absence from differentiated cells (Lee et al., 1999b). Based on the findings that *Xist* and *Tsix* are expressed from both X chromosomes of ES cells initially, and both are unstable in undifferentiated ES cells coupled with the fact that only the stable form of *Xist* is transcribed in fully differentiated ES cells, *Tsix* was thought to play a regulatory role in the X chromosome "choosing" step of the inactivation process (Lee et al., 1999b). Since then, using mice carrying a targeted deletion of *Tsix*, Lee and Lu (1999b) have confirmed that this antisense RNA is indeed involved in choosing the X chromosome for inactivation by regulating *Xist* expression from both Xs initially and by its gradual extinction from the active X during differentiation. The exact mechanism by which *Tsix* plays this role is not known as yet. However, Heard et al. (1999a) have suggested that *Tsix* may interact with the *Xist* DNA sequence and interrupt the transcription of the *Xist* locus, form duplex with the *Xist* RNA and cause the degradation of the transcripts, or block the access of RNA transcription factors to *Xist*.

We had hypothesized previously that the primers we used in our study recognised either a transcript antisense to *Xist* or another forerunner of *Xist* expression (Farazmand, 2000). If the bovine testicular transcript reported here is antisense to *Xist* such as the murine *Tsix* (Lee and Lu, 1999), it is difficult to assign a role for it in fetal or adult testes. The *Xist* antisense (*Tsix*) thought to be involved in the process of choosing the X chromosome for inactivation in XX female cells would appear to be unnecessary in male cells which do not offer that choice. This problem could be reconciled if we consider the possibility that the source of the inactivation process may

be a homologue of *Xist* (or its antisense) on the Y chromosome. The Y chromosome in male mammals remains heterochromatic and genetically inactive in somatic tissues and makes its impact only in gonads at the time of testis-induction (Ohno, 1967) and during testicular development and spermatogenesis (Chandley and Cooke, 1994). Since the *Xist*-like transcript is detected only in the testes among the male organs tested, we tend to believe that it plays a role in some aspects of male germ cell differentiation which are common to adult, prepubertal and, to a limited extent, fetal testes. The male germ cells that prematurely enter meiosis in fetal testes are relatively few in number and die before they reach pachytene. It is at early pachytene when the X and the Y chromosomes are paired at the pseudo-autosomal region and their unpaired regions become condensed, that a sex vesicle is detected (Solari, 1974, 1994; Ayoub et al., 1997). Absence of RNA transcription indicative of inactivation of the genes on the sex complement also takes place in pachytene (Monesi, 1965; Jaafar et al., 1989). It is probably advantageous to the male germ cells to ensure that their only X chromosome is not inactivated until pachytene. Expression of *Xist* (or its antisense) may be one of the ways to ensure that the X chromosome carrying house keeping genes remains active in those male germ which are in the process of transition from mitosis to meiosis.

# CHAPTER 3

# EXPRESSION PROFILE OF X-LINKED GENES IN BOVINE CELL CULTURES

## INTRODUCTION

Postnatal survival of individuals with X chromosome aneuploidy, although with abnormal phenotype, is relatively common among mammals, compared to those with autosomal aneuploidy which generally leads to embryonic or fetal death (Vogel and Motulsky, 1997). This difference in viability of X chromosome aneuploid mammals is attributed to the difference in expression of X chromosome-linked genes compared to those on autosomes. These differences were evident in the levels of enzymes encoded by X linked genes which were noted to be the same in normal males and females and in individuals who are with supernumery X chromosome (XO, XXY, XXX, and XXXX). Furthermore, females who are heterozygotes for different alleles of an X linked gene (such as glucose 6-phosphate dehydrogenase with A and B variants) express A or B alleles in different tissues resulting in a mosaic pattern of the expression (Beutler, 1962). These observations have helped to formulate the hypothesis that all X chromosomes in excess of one are inactivated in female mammalian cells, and that the normal female mammal is a mosaic resulting from the random inactivation of (one of the two) X chromosomes in different organs (Lyon, 1961). However, the abnormal phenotype associated with X chromosome aneuploidy remains unexplained if all, but one, X chromosomes were to be completely inactivated. The abnormal phenotype of individuals with supernumary X chromosomes, has been attributed to the possibility of

escape from inactivation of specific loci leading to a dosage effect of these genes which may to be expressed from both the active and inactive X chromosomes for normal development (Disteche, 1995).

Genes escaping inactivation on X chromosome have functional homologues on the Y chromosome (Disteche, 1995; Graves, 1995). Genes located in the pseudoautosomal region (PAR), as well as certain genes located outside this region, such as the zinc finger protein locus on the X (*ZFX* ), ribosomal protein subunit 4 (*RPS4X*) , and "selected mouse cDNA homologue on X" (*Smcx*) are among this category (Disteche, 1995; Graves, 1995). However, X-linked genes without functional Y homologues, such as steroid sulfatase (*STS*) or ubiquitin-activating enzyme E1 (*UBE1*) have also been shown to escape inactivation and remain expressed on both X chromosomes of somatic cells (Brown et al., 1997; Jegalian and Page, 1998). While over 30 X-linked genes have been shown to be expressed from the inactive human X chromosome (Carrel et al.,1999), only three X linked genes have so far been known to escape X inactivation in the mouse (Greenfield et al., 1998). The difference in the viability and the phenotype of human and murine XO individuals has been attributed to the relatively large number of genes which escape X chromosome inactivation in humans (Ashworth et al., 1991; Zinn et al., 1993).

Our previous studies have shown that X linked genes are not inactivated in fetal bovine testes even though *Xist* expression is evident in these testes. These observations led us to question whether the *Xist* locus is involved in silencing X linked loci in somatic cells. We hypothesized that male somatic cells of XY constitution which do not

undergo X inactivation will not display *Xist* expression whereas female cells in which one of the two X chromosomes undergoes X inactivation will express *Xist* RNA. Furthermore, if inactivation is triggered by *Xist* expression, male and female bovine cells carrying extra X chromosomes (XXY and XXX) in which all but one X chromosome are inactivated, will display a quantitative difference in the level of *Xist* expressed compared to that of their normal counterparts. We, therefore, undertook to compare the levels of *Xist* RNA transcribed by normal male and female bovine cell cultures with those generated by cell strains of XXX and XXY sex chromosome constitutions. In order to test whether *Xist* expression in these cells is associated with the process of other X linked genes, we extended our studies to the pattern of expression of four other genes which are known to be X-linked in the bovine species (Fries and Popescu, 1999). The genes tested include hypoxantine-phosphorybosil transferase (*Hprt*), glucose 6-phosphate dehydrogenase (*G6pd*), Z finger protein locus on ( *Zfx*), and "selected mouse cDNA on X" (*Smcx*).

## MATERIALS AND METHODS

### Cell cultures

To set up bovine cell cultures of XX and XY sex chromosome constitution, fetuses (approximately three months old) were removed from pregnant uteri collected from a local slaughter house. After identifying the sex of the fetuses as described before (Chapter 1) kidneys were dissected out under sterile conditions and placed in a petri dish containing Eagle's minimum essential medium (EMEM) supplemented with 15%

fetal calf serum (FCS). Tissues were chopped into pieces (approximately 1 mm) and were seeded into culture flasks and incubated at 37° C for 48 hours in EMEM (Butler and Dawson, 1992). Culture medium was replaced 72 hours after the initiation of culture which allowed to grow until the cells reached confluency. Cells were either collected for gene expression studies or they were subcultured to obtain cells for the next passage at this stage. For gene expression studies, the flasks were rinsed with phosphate buffered saline (PBS) and 0.1% trypsin was added and allowed to stand at room temperature for 5-10 minutes to dislodge the cells. After trypsinization, the activity of trypsin was stopped by adding serum-containing medium. Cells were sedimented by centrifugation at 5000xg for 5 minutes, resuspended in PBS and after recentrifugation (at 5000xg for 5 minutes) cell pellets were subjected to snap freezing in liquid nitrogen, and were stored at -80° C until use. For subculture, cells were dislodged and centrifuged as described above and the pellet was suspended in fresh medium. Cell suspension was seeded into tissue culture flasks and incubated at 37° C.

The two cell strains with XXX and XXY sex chromosome constitution used in this study were kindly provided by Dr. Stranzinger (Department of Animal Science, Federal Institute of Technology, Tannenstrasse 1/ ETH-Zentrum, Zurich, Switzerland). The XXY cell strain was established from a Brown Swiss bull with a mosaic XX/XXY karyotype from which the XXY cell type was cloned and frozen (Nett, 1995). The XXX cell strain was derived from a pure bred female Simmental calf born to a cow which was also a carrier of the 1/29 Robertsonian translocation originally described by Gustavsson and Rockborn (1964). Vials of these cell strains were thawed, cultured and sent by air, from

the first passage in alpha medium containing 10% FCS. Upon arrival, cultures were incubated in our laboratory and passaged as described before untill sufficient growth was established. Cells were collected and saved for quantitative gene expression analysis, as described before for XX and XY cells.

### RNA isolation and reverse transcription

Procedures for the isolation of total RNA, DNase treatment of the extracted RNA samples to avoid genomic DNA contamination, and semi-quantitative reverse transcription (RT-PCR), were as described in Chapter 2.

### Primers and PCR conditions

Primer sets used and PCR conditions for *G6pd*, *Hprt*, *Zfx*, and *Xist* were as described in Chapter 2. The primer set for *Smcx*, was designed based on Jegalian and Page (1998), and gives rise to the amplification of a PCR product of 117 bp in length. The linear range for amplification of *Smcx*, and the optimal ratio of 18S:Competomer primers were determined as described for other genes (in Chapter 2). The PCR amplification of *Smcx* was performed in cycling condition of denaturation at 94° C, annealing at 55° C, and extension at 72° C (30 seconds each), for 31 cycles (median cycle number in linear range). A 50 ul reaction mixture for PCR amplification contained 5.0 ul of PCR buffer (Gibco, BRL), 0.25 units of Taq DNA polymerase (Gibco, BRL), 0.2 mM dNTPs, 0.016 uM of each gene-specific primer, 0.04 uM of a 4:6 ratio of 18 S: competomere primers (Ambion patent pending), 30 uM MgCl2, and

2 uL of cDNA.

## Agarose gel electrophoresis and visualization of PCR products

Following PCR amplification, 18 ul of each product was resolved on 2.0 % agarose (Biolab Ltd), and stained with SYBR® Green I (Molecular Probes). The bands were visualized and quantified using the FMBIO II Image Analysis System (Hitachi Software Ltd, Japan) as described in Chapter 2.

## Statistical analysis

The data obtained for optical density (OD) values of gene-specific bands relative to that of the 18 S were compared using one-way analysis of variance (ANOVA) with probability less than 5% being considered significant using Statistix for Windows (Analytical Software, FL,USA). The optical density of individual gene- specific and 18 S bands, after staining with SYBR® Green I (Molecular Probes), was obtained using FMBIO II Image Analysis System (Hitachi Software Ltd, Japan). The relative OD values were calculated as the ratio of OD values of gene-specific bands relative to that of 18 S, co-amplified in the same reaction. The relative OD values of 6 trials of semi-quantitative RT-PCR for each gene were determined for cell cultures carrying different sex complement, and the mean values for each gene were compared among cell cultures using one-way ANOVA ($P < 0.05$). The statistically significant groups were identified using Tukey's test.

## RESULTS

Agarose gels of PCR products representing the expression status of X linked genes in normal male and female (XX and XY) primary cell cultures and cell strains derived from X chromosome aneuploids (XXX and XXY) are shown in Figures 10-15. The OD values for gene-specific bands relative to that of the internal standard (ribosomal *18S* bands, co-amplified with each reaction mixture), and the means of the relative values (± standard deviation) for each gene in different cell cultures are summarized in Tables VII-XI. The intensity of the gene-specific bands relative to that of 18S, is used for comparing the expression of X linked genes in different cell cultures. The expression patterns of *G6pd*, *Hprt*, *Smcx*, *Xist* and *Zfx* genes relative to 18S, in different cell cultures are illustrated in Figures 10-15, respectively. Analysis of variance showed that the levels of expression of X linked genes, *G6pd*, *Hprt*, and *Smcx* were not significantly different in cell cultures with different X chromosome constitution. However, the level of *Xist* expressed was different in these cell cultures. Cultures of trisomy X cells showed the highest level of expression of *Xist* though not proportional to the number of X chromosome in cell cultures (Figure 10b) and the chromosomally normal (XY) male cell cultures showed the lowest (Figures 10a and b). The levels of expression of *Xist* did not differ between normal female cells (XX) and the XXY male cells (Figure 10b). Comparison of *Zfx* expression showed that it was significantly ($P < 0.05$) higher in XXX cells compared to that in all other cell cultures (Figure 14b).

**Table VII.** Relative OD values for *Xist* /18 S representing the expression* of *Xist* among cell cultures carrying different sex complements.

| Cell Line | Trial | | | | | | Mean± SD |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| XY | 0.74 | 0.65 | 0.77 | 0.97 | 0.93 | 0.95 | 0.83± 0.13 |
| XX | 1.4 | 1.32 | 1.62 | 1.52 | 1.29 | 1.49 | 1.44±0.12 |
| XXY | 1.33 | 1.62 | 1.57 | 1.59 | 1.61 | 1.51 | 1.53±0.10 |
| XXX | 1.56 | 1.82 | 1.77 | 1.77 | 1.72 | 1.71 | 1.72±0.09 |

**Table VIII.** Relative OD values for *G6pd* /18 S representing the expression* of *G6pd* among cell cultures carrying different sex complements.

| Cell Line | Trial | | | | | | Mean± SD |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| XY | 1.16 | 1.15 | 1.2 | 1.44 | 1.45 | 1.5 | 1.31± 0.16 |
| XX | 1.22 | 0.99 | 1.23 | 1.56 | 1.13 | 1.53 | 1.27± 0.22 |
| XXY | 1.37 | 1.21 | 1.31 | 1.59 | 1.11 | 1.69 | 1.38± 0.21 |
| XXX | 1.28 | 1.23 | 1.29 | 1.52 | 1.45 | 1.47 | 1.37± 0.12 |

**Table IX.** Relative OD values for *Hprt* /18S representing the expression* of *Hprt* among cell cultures carrying different sex complements.

| Cell Line | Trial | | | | | | Mean± SD |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| XY | 1.4 | 1.11 | 1.35 | 1.15 | 1.29 | 1.54 | 1.30 ± 0.16 |
| XX | 1.34 | 1.32 | 1.35 | 1.12 | 1.28 | 1.4 | 1.30 ± 0.09 |
| XXY | 1.37 | 1.4 | 1.38 | 1.18 | 1.14 | 1.17 | 1.27 ± 0.12 |
| XXX | 1.32 | 1.4 | 1.41 | 1.08 | 1.15 | 1.07 | 1.23 ± 0.15 |

* Based on the optical density of the gene specific bands relative to that of the ribosomal 18 S, co-amplified in each sample.

**Table X.** Relative OD values for *Smcx/ 18S* representing the expression* of *Smcx* among cell cultures carrying different sex complements.

| Cell Line | Trial | | | | | | Mean± SD |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| XY | 1.24 | 1.09 | 1.08 | 1.13 | 1.06 | 1.05 | 1.31± 0.16 |
| XX | 1.09 | 1.07 | 1.08 | 1.13 | 1.05 | 1.02 | 1.27± 0.22 |
| XXY | 1.2 | 1.2 | 1.06 | 1.06 | 1.05 | 1.04 | 1.38± 0.22 |
| XXX | 1.12 | 1.13 | 1.1 | 1.11 | 1.08 | 1.04 | 1.37± 0.12 |

**Table XI.** Relative OD values for *Zfx* / 18 S representing the expression* of *Zfx* among cell cultures carrying different sex complements.

| Cell Line | Trial | | | | | | Mean± SD |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| XY | 0.97 | 0.96 | 0.96 | 1.19 | 1.14 | 1.11 | 1.05± 0.10 |
| XX | 0.9 | 1.02 | 0.94 | 1.14 | 1.3 | 1.09 | 1.06 ± 0.14 |
| XXY | 1.06 | 1.04 | 1.06 | 1.39 | 1.24 | 1.27 | 1.17 ± 0.14 |
| XXX | 1.2 | 1.21 | 1.31 | 1.65 | 1.56 | 1.7 | 1.43± 0.22 |

* Based on the optical density of the gene specific bands relative to that of the ribosomal 18 S, coamplified in each sample.

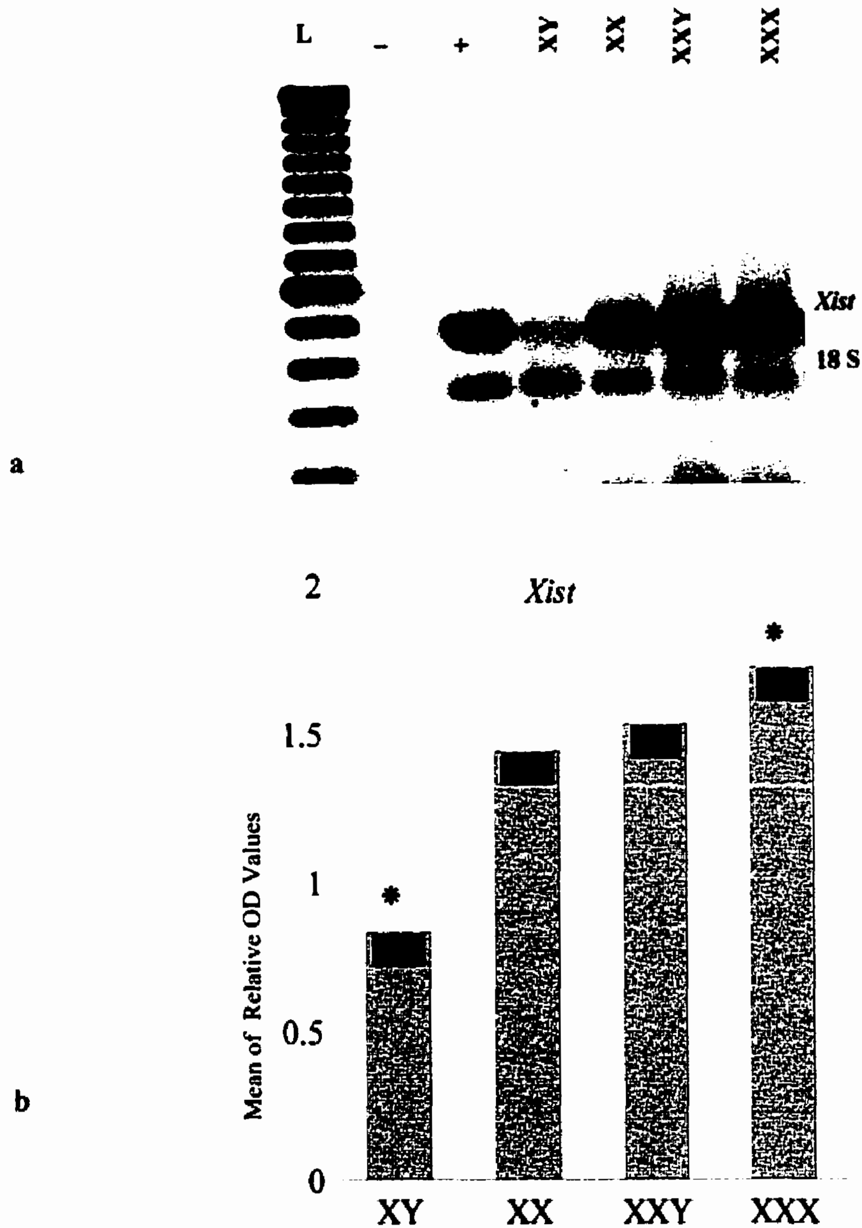



**Figure 10.** Expression of *Xist* in cultures of cells with different sex complements.
a) Agarose gel representing semiquantitative RT-PCR product s of *Xist* in different cell cultures. L, 100 bp ladder; -, negative control, a reaction containing all PCR components except for the template; +, positive control for PCR .
b) Histogram representing the mean OD values of the *Xist* bands relative to that of the18S in cell cultures.

* Significantly different (P <0.05) from each other and the other groups of cell cultures expressing *Xist*.

a

b

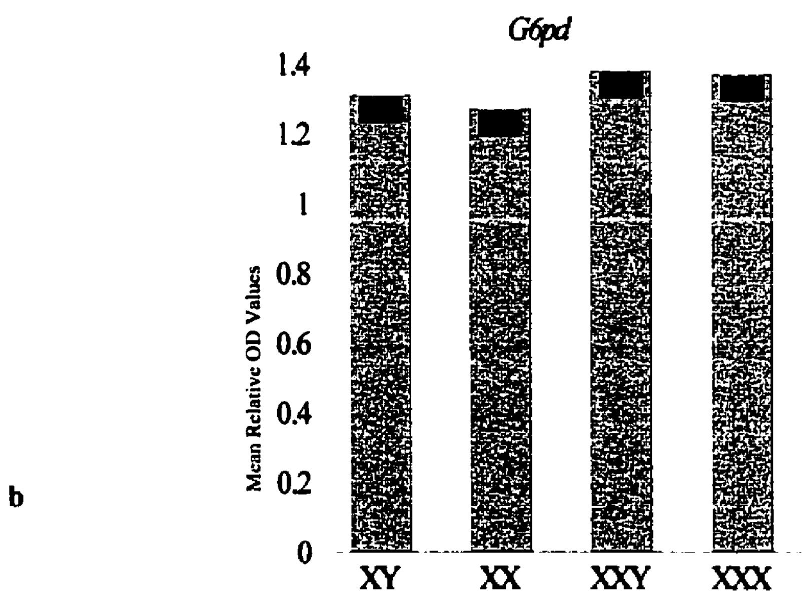
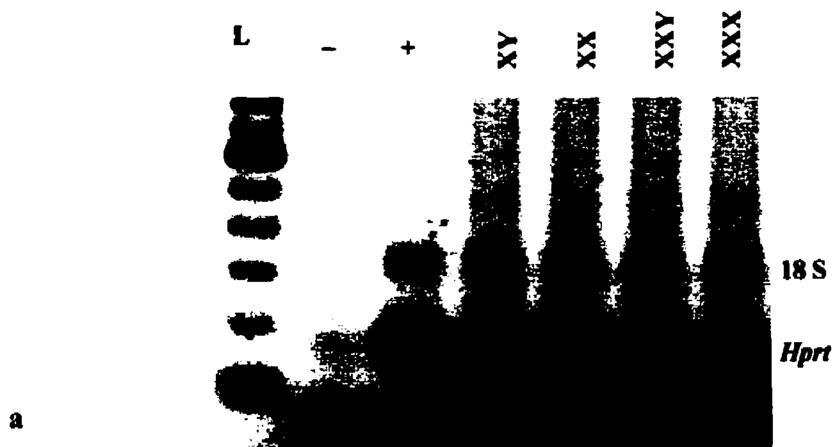



**Figure 11.** Expression pattern of *G6pd* in cultures of cells with different sex complements.
**a)** Agarose gel representing the semiquantitative RT-PCR products of *G6pd* in different cell cultures. L, 100 bp ladder; -, negative control (reaction mixture containing all PCR components except for the template). **b)** Histogram representing the mean OD values of the *G6pd* bands relative to that of the 18S in cell cultures.

a




b

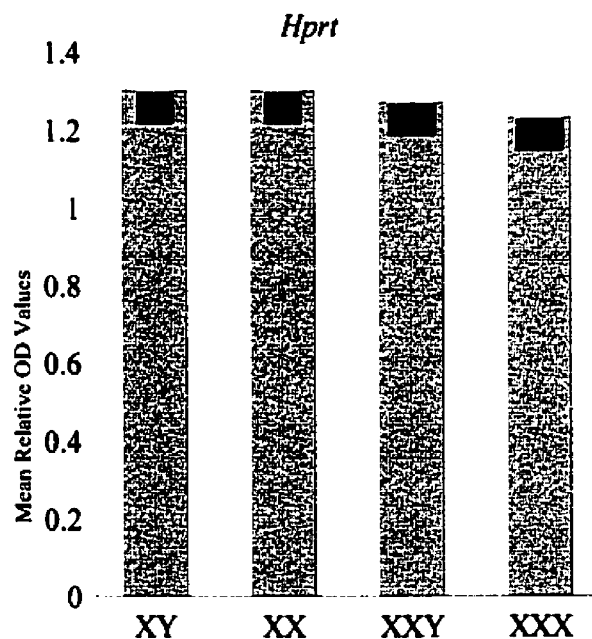



**Figure 12.** Expression of *Hprt* in cultures of cells with different sex complements.
a) Agarose gel representing the semiquantitative RT-PCR products for *Hprt* in different cell cultures. L, 100 bp ladder; -, negative control (reaction mixture containing all PCR components except for the template); +, positive control for PCR.
b) Histogram representing the mean OD values of the *Hprt* bands relative to that of the 18S in cell cultures.

a

b

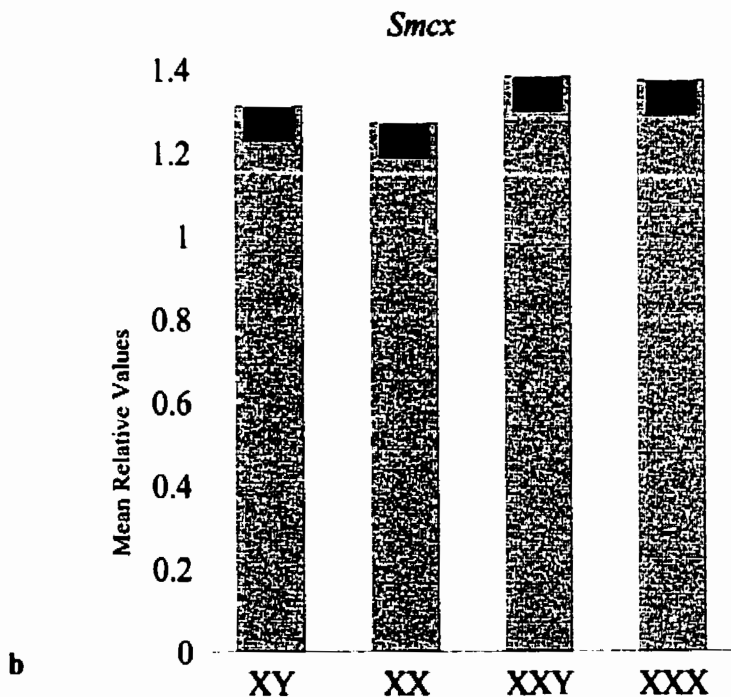
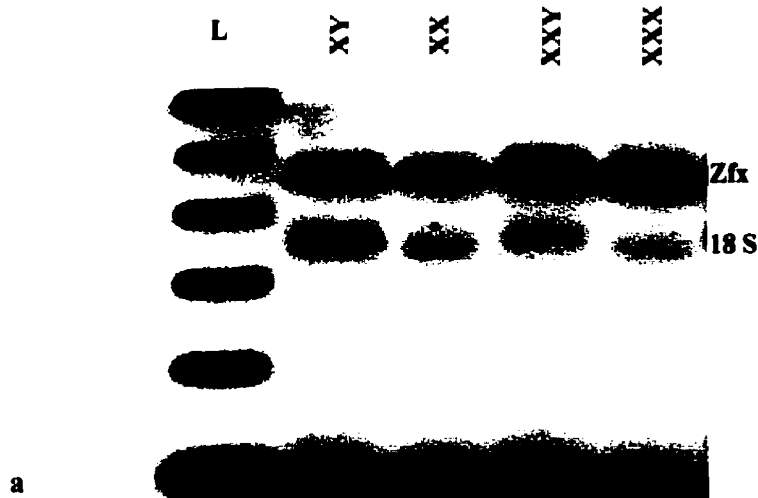



**Figure 13.** Expression pattern of *Smcx* in cultures of cells with different sex complements.
**a)** Agarose gel representing semiquantitative RT-PCR products of *Smcx* in different cell cultures. L, 100 bp ladder; -, negative control (reaction mixture containing all PCR components except for the template). **b)** Histogram representing the mean of OD values of the *Smcx* bands relative to that of the 18S in cell cultures.

a




b

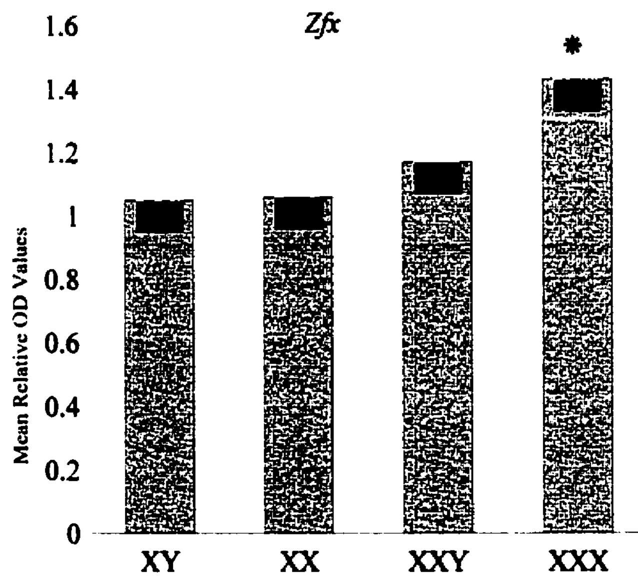



**Figure 14.** Expression pattern of *Zfx* in cultures of cells with different sex complements. **a)** Representative agarose gel of the semiquantitative RT-PCR products of *Zfx* in different cell cultures. L, 100 bp ladder. **b)** Histogram representing the mean OD values of the *Zfx* bands relative to that of the 18S among cell cultures.

* Statistically different ($P < 0.05$) from other groups of cell cultures expressing *Zfx*.

# DISCUSSION

## Expression profile of X-linked genes in bovine cell cultures

Our results showing that normal male bovine (XY) cells display *Xist* expression, was unexpected, even though the level expressed remained the lowest relative to all other cell cultures tested in this investigation. The level of expression was found to be the highest in XXX cell strains and the least in the XY cell cultures (Figure 10b).The elevated expression of the *Xist* in XXX cell strains compared to the two cell cultures carrying two X chromosomes (XX and XXY) is in keeping with its role in the X chromosome inactivation. Since *Xist* RNA is constitutively transcribed only from the inactive X chromosome and spreads in *cis* over the inactive X chromosome (Clemson et al., 1996), the XXX cell strain which carries two inactive X chromosomes conforms to the n-1 rule for the number of X chromosomes to be inactivated in a diploid cell (Vogel and Motulsky, 1997). The increased level of the *Xist* can be attributed to the presence of two active *Xist* loci compared to the one present in XX and XXY cells. However, the expression of *Xist* does not seem to be elevated in proportion to the number of inactive X chromosomes. It is well known that some of the genes that escape X chromosome inactivation do not express at the same level as their partners on the active X chromosome and that most of these genes show only a partial expression (Disteche, 1997). Although *Xist* RNA is a special case as it is expressed only from (and remains spread over) the inactive X chromosome, the lower than expected level of expression from two *Xist* loci in XXX cell strain may be attributable to the partial

expression of one or both *Xist* loci. Thus, the overall results obtained for the level of *Xist* expression in cells with varying number of X chromosomes suggest that our semi-quantitative approach is able to detect overt differences in the relative level of expression of the X inked genes.

The expression levels of *G6pd*, *Hprt*, and *Smcx* genes did not show significant differences between two cell cultures of normal sex chromosome complements showing that these genes are inactivated in female bovine cells. Furthermore, our analysis shows that the expression levels in normal male and female cells do not differ significantly from those of the cell strains with extra X chromosomes (XXX and XXY) (Figure 10-13). It would appear that the levels of expression of these genes are subject to inactivation in the bovine, and expression remains unaffected regardless of the number of the X chromosomes carried by these cells. It is evident that these loci are silenced in the X chromosomes in excess of the one X chromosome in all diploid cells (Disteche, 1995; Brown et al., 1997). Methylation status of the CpG islands of the promoter region of *Smcx* has confirmed that *Smcx* locus on murine and human X chromosomes escapes inactivation (Jegalian and Page, 1998). Our results point out that the bovine species differ from both these species in this regard. Since *Smcx* has a functional Y-linked homologue in man and mouse (Agulnick et al., 1994), its expression from the inactive X chromosome is expected in these species. More recently, Kent-First et al. (1996) had shown that a homologue of *Smcx* is present on the bovine Y chromosome even though Jegalian and Page (1998) claim that there is no Y-linked homoloue for this gene in the cattle as they were not able to get reproducible results for this homolouge

(Jegalian and Page, 1998). Regardless of the presence or absence of a Y homologue, *Smcx* is subject to inactivation in female bovine cells, as the results obtained in current study and that of the Jegalian and Page (1998) concerning the methylation pattern of the CpG island of *Smcx* in bovine female cells support this conclusion.

The *ZFX* gene was originally identified based on its strong homology with *ZFY*, zinc finger protein locus on the Y, once thought to be a candidate for the testis determining factor (Page et al., 1987, Scheider-Gadiche et al., 1989). Since then, Palmer et al. (1990) showed that *ZFX* and *ZFY* are expressed in a wide range of adult and fetal human tissues indicating other functions for these genes. Using gene targeting approach (Luoh et al., 1997) have shown that *Zfx* is important for early embryonic development and germ cell development in male and female mice. Unlike in the human situation, *Zfx* is subject to inactivation in the mouse (Adler et al., 1991) and methylation of the highly conserved CpG islands of the promoter region of *Zfx* has been reported confirming this observation (Luoh et al., 1995). Our semi-quantitative analysis of *Zfx* in male and female tissues (chapter 2) had also indicated that this gene may be subject to inactivation in bovine species, although the female tissues generally displayed higher level of *Zfx* expression. However, methylation studies of the CpG islands of bovine *Zfx* has shown that it escapes inactivation (Jegalian and Page, 1998). The present study on cell cultures demonstrate that the expression of *Zfx* is significantly higher in the XXX cell strain compared to that in other cell cultures although no significant difference was found in *Zfx* expression in cells carrying one (XY) or two X chromosomes (XX and XXY). If the bovine *Zfx* is subject to inactivation, the expression level would have been

similar in all cell types tested in this study. Since our semi-quantitative analysis on the pattern of expression in cell cultures indicated that *Zfx* expression is higher in cells carrying three X chromosomes compared to that in other cell cultures tested, it would appear that the *Zfx* loci on the inactive X chromosomes in XXX cells are partially expressed.

**Evolutionary basis for differences in the expression profile of X linked genes**

Difference in the activity of the X and the Y-linked genes in different species can be explained in the context of the evolution of sex chromosomes and its relation to dosage compensation. Rice (1996) had proposed the inactivation of an X-linked gene is correlated with the lack of a functional Y-linked partner. Comparative mapping of vertebrate sex chromosomes indicates that mammalian heteromorphic sex chromosomes evolved from an ancestral species in which the sex chromosomes were homomorphic with differences only at the allelic level (Grave and Foster, 1994). Homology of the sex chromosomes diminished progressively during the evolution as recombination between the X and the Y was eliminated accompanied by the gradual degradation of the Y chromosome (Rice, 1996; Charlesworth, 1998). In this evolutionary context, the genes located in the pseudoautosomal region of the X chromosome need not be inactivated as they recombine with the Y chromosome and are not subject to degeneration. However, it is hard to explain the lack of dosage compensation for X-linked loci with no Y linked partner, such as *UBE1* in human or the inactivation of the X-linked genes with Y homologues, such as *Zfx*, *Rps4* and *Ube1* in the mouse (Charlesworth, 1996). Mitchell

et al. (1998) have proposed that the inactivation of human *UBE1* may represent a later step in the evolution of the ancestral pair of homologous chromosomes. It is noteworthy that the X chromosome is conserved in gene content but not in the order of genes located on the X chromosome in different species (Graves and Foster, 1994). Therefore, the dosage compensation of X-linked loci may be under the influence of chromosomal domain in which they are located in different species (Miller et al., 1995; Charlesworth, 1996; Miller and Willard, 1998).

In conclusion, the partial expression of *Zfx* evident in cell strains carrying three X chromosomes (including two inactive X chromosomes) may be influenced by its location on the bovine X chromosome regardless of the presence of a Y-linked counterpart. There are examples of X-linked genes which are subject to inactivation (*Rps4x* in the mouse and *Smcx* in cattle) even though their Y homologues in the respective species have not so far been identified. Genes such as *Zfx* that escape inactivation in one species but are subject to inactivation in other species of mammals may represent an intermediate step in the evolution of the genes shared by the X and Y chromosomes in different organisms.

## CHAPTER FOUR

## EXPRESSION OF SENSE AND ANTISENSE STRANDS OF X INACTIVE SPECIFIC TRANSCRIPT(*XIST*) IN BOVINE CELLS

### INTRODUCTION

Inactivation of the single X chromosome in male germ cells is thought to be a prerequisite to prevent the non-homologous pairing and deleterious recombinations in sex chromosomes (Jablonka, 1988; McKee and Handel, 1993). The inactive X in male germ cells is heteropyknotic (Kofman-Alfaro and Chandley, 1970; Odartchenko and Pavillard,1970) and is transcriptionally silent (Monesi, 1965; Richler et al., 1994; Hendricksen et al., 1995). However, unlike in female somatic cells, the X chromosome inactivation in male germ cells is transient and restricted to meiotic prophase (McKee and Handel, 1993). Furthermore, underacetylation of histones and methylation of CpG islands of X linked genes thought to be the maintenance factors for the inactive state of the X chromosome in female somatic cells are not involved in X chromosome inactivation in male germ cell ( Migeon et al., 1989; Armstrong et al., 1997).

The causal role of the X inactive specific transcript (*Xist*) in X chromosome inactivation in female somatic cells is well established as its expression precedes the inactivation process and is expressed only from the inactive X in differentiated somatic cells (reviewed by Brockdorff and Duthie, 1998). Expression of *Xist* has also been demonstrated in the meiotic germ cells of adult testes, suggesting a role for *Xist* in X chromosome inactivation in male germ cells (McCarry and Dilworth, 1992; Richler et al., 1992; Salido et al., 1992; De La Fuente et al., 1999). However, studies on mice

carrying a targeted deletion of exons 1 to 5 of the *Xist* have revealed that spermatogenesis remains unaffected in males while their female counterparts are unable to undergo X inactivation (Marahrens et al., 1997) indicating that *Xist* may not be involved in X chromosome inactivation in male germ cells. Furthermore, *Xist* expression which was presumably noted to be associated with spermatogenesis (McCarry and Dilworth,1992; Richler et al., 1992; Salido et al., 1992) was also detected in fetal bovine testes (described in Chapter 1) as early as 50 days of gestation (Farazmand et al., 2000). Subsequent investigations showed that the expression of *Xist* does not eventuate the inactivation of the X-linked genes in fetal bovine tissues (Farazmand et al., 2001) These observations indicated that the RNA detected in fetal testes may not be *Xist* per se. It has been shown by Lee et al. (1999b) and Mise et al. (1999) that *Xist* antisense strand is transcribed in male and female embryonic stem (ES) cells when they are in undifferentiated state. In order to test wether the RNA expressed in fetal bovine testis is *Xist* antisense, we undertook a strand specific RT-PCR approach to investige the identity of the amplicon detected in fetal and adult gonads and nongonadal tissues.

## MATERIALS AND METHODS

### Collection of samples

Tissue samples including gonad, brain, liver, muscle, and heart were obtained from bovine fetuses retrieved from pregnant uteri collected from a local slaughter house. Samples were subject to snap freezing in liquid nitrogen and were stored at -80° C until

use. Samples from the testes and muscle of adult bulls and ovary and oviduct samples from adult cows also were collected, snap-frozen and saved at -80° C for future use.

**RNA extraction, DNase treatment and reverse transcription**

Isolation of RNA from tissues, and the DNase treatment of RNA (1.8 ug of total RNA) were accomplished as described in Chapter two. The DNase treated samples were divided into three aliquots, two with 5ul in volume, and one with 3 ul. The first two aliquots were used for strand specific reverse transcription, whereas the third one was used as control for testing possible contamination with genomic DNA. Only samples devoid of genomic DNA contamination were used for the following experiments.

Reverse transcription was carried out in a 20 ul reaction mixture as described in Chapter two, with the exception of the primers used for reverse transcription. For strand-specific cDNA synthesis, instead of using a mixture of random/ oligo-dT primers (which are potentially able to prime the cDNA synthesis of any transcript presented in total RNA), 400 ng each of strand specific primer was used in separate reactions: the upstream primer (5', p133) to prime the synthesis of cDNAs complementary to the antisense strand, and downstream primer (3', p596) to prime the synthesis of cDNAs complementary to the sense strand. The reaction mixtures were incubated at 42° C for 2 hours. The cDNA samples were incubated at 90° C for 5 minutes to inactivate the reverse transcriptase, and stored at -20° C until use.

Principles of strand-specific RT-PCR approach used in this Chapter, are outlined in Figure 15. In brief, either strand of a DNA molecule may serve as template.

Conventionally, the DNA strand that serves as the template for the transcription of mRNA is called the antisense strand, and the transcribed RNA (with sequence homology to the "sense" strand of the DNA) is referred to as the "sense transcript". However, there are cases including *Xist* locus in which the same segment of the double helix contains information for functional RNAs on both strands since either or both sense and antisense strands of DNA can act as the template for transcription. The RNA molecule which is transcribed from the "sense" strand of DNA is referred to as the "antisense" transcript.

**Cell culture and cell strains**

Primary cultures were set up using kidney cells derived from male and female bovine fetuses to obtain XY and XX cells. The cell strains of XXX and XXY sex chromosome constitution were described and passaged as outlined in Chapter 3.

**PCR amplification of cDNAs**

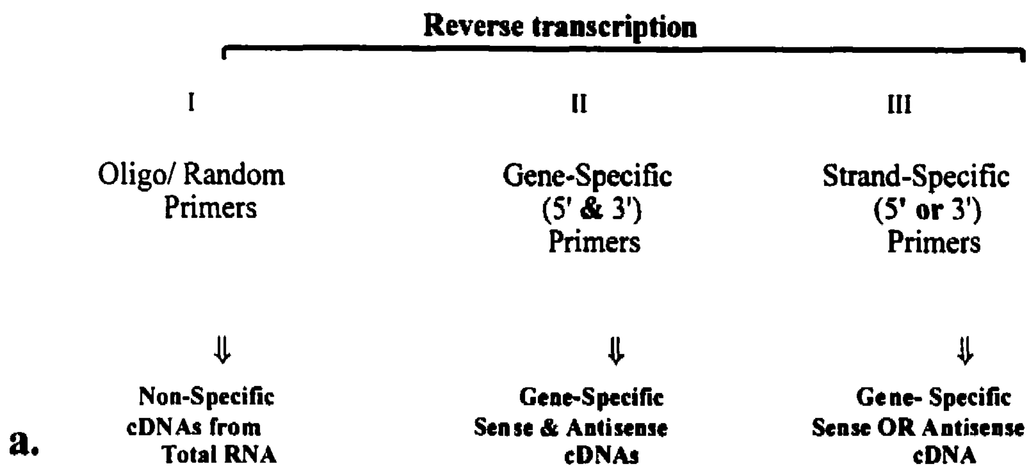
For PCR, 4.0 ul of prepared cDNA samples was subjected to amplification in a 50 ul volume containing 0.4 uM of each downstream (3') and upstream (5') primers, 0.3 mM dNTPs, 1.5 mM MgCl2, 5ul of 10X buffer, and 1 unit Taq DNA polymerase (Gibco/BRL). The PCR products were resolved on 2% agarose (Biolab) gel by electrophoresis in 1X TAE (Tris Acetate) containing 0.5 ug per ml ethidium bromide. The PCR products were visualized under UV light using GelPrint 2000i BioPhotonic (Bio/CanScientific).

Figure 15. Reverse transcription (RT) approaches.

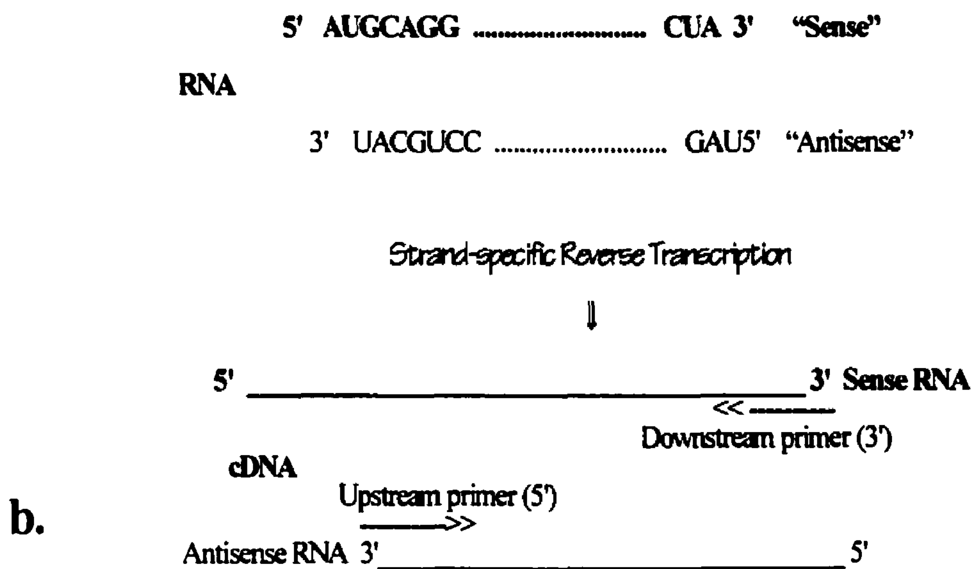
a) Strand-specific RT and conventional RT. In conventional method (I) reverse transcription is accomplished with a mixture of oligo-dT and random primers which potentially leads to the synthesis of cDNAs from all possible messages presented in total RNA. In gene-specific approache, reverse transcription is accomplished using a pair (II) of gene-specific primers leading to only the synthesis of specific cDNAs of the same gene. In strand-specific RT, reverse transcription is carried out using either the upstream (5') or downstream (3') primers in separate reactions. In this case cDNAs complimentary to the antisense or sense strands is made in separate reactions representing the expression of the respective transcript. The PCR amplification of cDNAs made in different approaches by gene-specific primers will reveal the expression profile of the gene in different samples.

b) Depending on which DNA strand serves as template for transcription, sense or antisense RNA is produced. Complimentary sense RNA is primed by downstream (3') and that for the antisense RNA is primed by upstream (5') primers.

## Reverse Transcription ( RT) Approaches




a.




b.

## RESULTS

### Expression of *Xist* antisense strand in male tissues

Strand specific reverse transcription PCR (SS-RT-PCR) showed that the RNA detected in the fetal testis is exclusively transcribed from the antisense strand (cDNA samples primed by 5', p133, primer) whereas no PCR product was detected using cDNAs prepared with the 3' (p596) primer which primes the synthesis of the sense strand (Figure 16a). This pattern was noted in all male somatic tissues including brain, heart, liver, and muscle (Figure 16a). The results obtained for testes and somatic tissues of adult bulls were similar to that of fetal testis and non-gonadal tissues (Figure 17) with the exception that in adult testis (and some male somatic tissues) the 5' band was less distinct and extra bands were also visible (Figure 17). The pattern described for the fetal tissues (Figure 16a) was consistently noted in the fetuses tested (with two replicates of strand specific RT-PCR for each fetus). The results for adult testes and somatic tissues of adult bulls are shown in Figure 17.

### Expression of *Xist* sense and antisense strands in female tissues

Sense and antisense strands of *Xist* were noted to be expressed in female fetal somatic tissues, including the brain, heart, kidney, liver, and muscle (Figure 16b). Similar pattern of expression was also noted for *Xist* sense and antisense strands in adult ovary and oviduct (Figure 17). The cDNA prepared from female tissues using upstream primer (5', p133) representing the expression of the antisense strand revealed a band of expected size (463 bp) comparable to that of the male gonad and somatic tissues.

However, cDNA generated using the downstream primer (3', p596) and representing the expression of the *Xist* sense strand, revealed two products after amplification by PCR. One of these two corresponds to the expected size (463 bp) and a second band (approximately 120 bp) similarly appeared in female fetal tissues tested (Figure 16b) and adult female tissues tested (Figure 17).

**Sense and antisense strands of *Xist* in cell cultures and cell strains**

Tests for *Xist* sense and antisense strand transcription in the two bovine cell strains derived from X chromosome aneuploids (XXY and XXX) and cell cultures of normal male and female bovine fetuses revealed that 5' and 3' strands are transcribed in cells of female (XX) cells (Figure 18, lanes 8 and 9) whereas only 5'strand is transcribed in male (XY) cells (Figure 18, lanes 5 and 6). The 5' and 3' strands were also transcribed in cell strains of X chromosome aneuploids XXY and XXX cells (Figure 18, lanes 11 and 12, and 14 and 15 respectively). The amplicons detected using 5' primer for cDNA synthesis was of the size (463 bp) similar to that noted in bovine fetal testes (Figure 17, lane 15). The cDNAs prepared with 3' or 5' primers, from cell cultures showed that in the primary cultures from male kidney cells only the 5' strand is transcribed (Figure 18, lane 5) whereas those derived from female kidney and the cell strains of the two X chromosome aneuploids (XXY and XXX) displayed transcription of both 5' and 3' strands (Figure 18, lane 8 and 9, 11 and 12, and 14 and 15) representing the expression of sense and antisense strands. The amplification of sense cDNAs of cell strains of XXY and XXX constitution, occasionally revealed two PCR products, one of

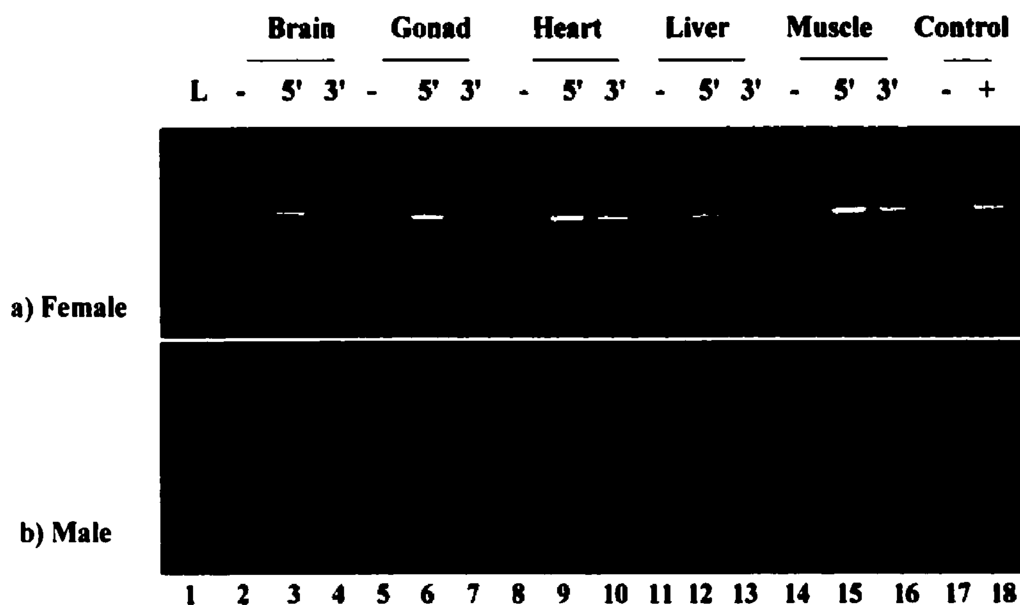



**Figure 16.** Representative gels for strand-specific RT-PCR products amplified from male and female fetal tissues.

a) A representative gel of the expression of the sense and antisense strands in gonad and somatic tissues of female fetus. L: 100bp ladder; - : negative control (without reverse transcriptase ($RT^-$); 5' lane: RT-PCR product originated from cDNA samples primed by upstream (5') primer (indicating the presence of the antisense strand in total RNA); 3' lane: RT-PCR product originated from cDNA sample primed by downstream (3') primer (indicating the presence of the sense strand in total RNA); + : positive control (cDNA) sample from a female somatic tissue.

b) A representative gel of the expression of the sense and antisense stands in gonad and somatic tissues of female fetus. Abbreviations are as described for figure **16a.**

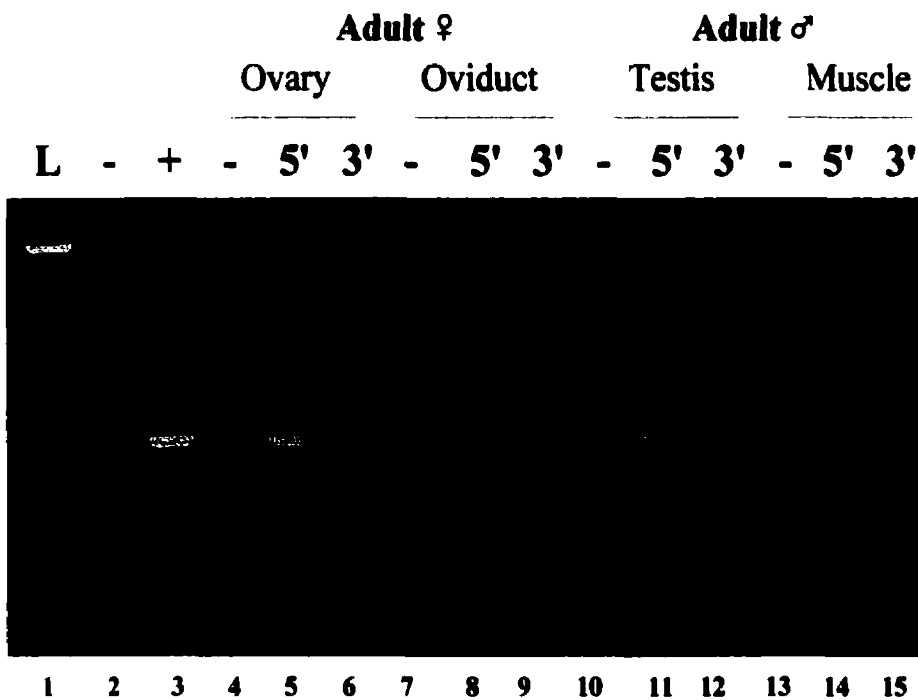



**Figure 17.** Representative gels for strand-specific RT-PCR products amplified from adult male and female tissues. L: 100bp ladder; - : negative control (without reverse transcriptase (RT⁻); + : positive control (cDNA sample from a female somatic tissue). - lane for each individual sample: samples without reverse transcriptase (RT⁻) as control for possible genomic DNA contamination; 5' lane: RT-PCR product originated from cDNA samples primed by upstream (5') primer indicating the presence of the antisense strand in total RNA; 3' lane: RT-PCR product originated from cDNA samples primed by downstream (3') primer indicating the presence of the sense strand in total RNA.

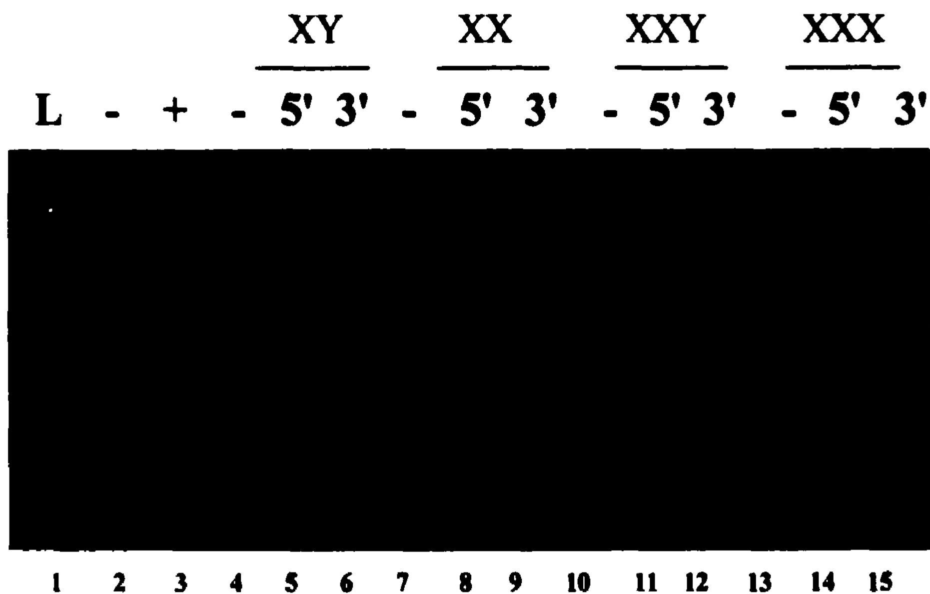



**Figure 18.** A representative gel of strand-specific RT-PCR products amplified from cDNA samples of cell cultures. L: 100bp ladder; -: negative control (reaction mixture with all components except the template); + : positive control (cDNA) sample from a female somatic tissue. - lane for each individual sample: negative control, without reverse transcriptase ($RT^-$) . 5' lane : RT-PCR product of cDNA sample primed by upstream (5') primer (indicating the presence of antisense strand in total RNA); 3': RT-PCR product of cDNA sample primed by downstream (3') primer (indicating the presence of the sense strand in total RNA).

463bp and a smaller band similar in size to that obtained in amplification of cDNA samples of female somatic tissues prepared by downstream (3') primer (Figure 16b, lanes 10, 13, and 16).

### Sequence analysis of PCR products

Strand-specific RT-PCR, using downstream primer (3'), reflecting the expression of the sense strand in female somatic tissues led to the amplification of two products, one of the expected (463 bp) size, and the other of smaller size (approximately 120 bp). Alignment of the sequence of the larger band using BLAST search, confirmed that the PCR product has significant homology with the *Xist* DNA sequence described for *Bos taurus* (99%), and 97% sequence homology with that of bison ( Altschul et al, 1997). Results of BLAST search for the smaller (120 bp) band revealed that a major part (94 bp) of this sequence in 5' direction (representing the template for sense strand) also has significant homology with part of the *Xist* gene characteristics of several species, including *Bos taurus Xist* (100% homology), and bison (98% homology). Data concerning these sequences and the BLAST search results for the sequences of RT-PCR products originated from fetal testis and ovary are summarized in Appendix II.

## DISCUSSION

Strand specific RT-PCR tests in the present study has clearly shown that the RNA detected in fetal bovine testes and nongonadal tissues and cell cultures of male bovine fetuses is that transcribed from 5' strand while an amplification product of 3'

strand is consistently absent in all these tissues. Based on the results of our previous studies (Chapters 1 and 2) we had speculated that the RNA transcript detected in bovine fetal testes thought to be *Xist* may not have been *Xist* but its antisense RNA (Farazmand et al., 2000). Other investigators have shown that *Xist* antisense strand is expressed in male and female ES cells and murine cells in early stages of embryogenesis (Lee et al., 1999b; Mise et al., 1999). Our results using strand specific RT-PCR, confirmed that it is the *Xist* antisense strand that is expressed in the male gonad (Figure 15a). Furthermore, *Xist* antisense transcript was also detected in all male somatic tissues tested in this study while none of these showed the *Xist* (sense) transcript (Figure 15a). On the other hand, all female tissues tested revealed both sense and antisense transcripts (Figure 15b).

Our findings also suggest that it may be the *Xist* antisense RNA that is reported as *Xist* in the male gonad of other mammals. This observation on bovine testis, if proven to be applicable to all other mammalian testes provides an explanation for the contradictory data concerning the involvement of the "*Xist*" in X chromosome inactivation during spermatogenesis. Previous observation that *Xist*-deficient mice can undergo normal spermatogenesis has weakened the assumption that *Xist* is involved in X chromosome inactivation in male germ cells (Marahrens et al., 1997). The expression of the *Xist* antisense strand in bovine fetal and adult testes and the failure to detect evidence of *Xist* sense strand transcription in fetal and adult testes call for a revision of the current view on the role of *Xist* in X chromosome inactivation in male germ cells. It is important to note that *Xist* antisense RNA is expressed in male and female fetal and adult bovine somatic tissues in the present study (Figures 15 and 16), and in differentiated ES

cells and adult tissues of other mammals (Debrand et al., 1999). The low level expression of "*Xist*" in male heart and liver cells has been suggested to be the result of "illegitimate transcription" of a tissue specific gene in non-specific cell types (Richler et al.,1992). The expression of "*Xist*" also has been reported in prepubertal mice (Kay et al., 1993; Richler et al., 1992), bovine fetal and prepubertal testes (Farazmand et al., 2000), and in the genital ridge of mice around the time of testis differentiation (Jamieson et al.,1997). Since *Xist* RT-PCR studies prior to the discovery of the *Xist* antisense transcript in mice (Lee et al., 1999b; Mise et al., 1999) were accomplished by the use of random primers which do not discriminate between sense and antisense strands during cDNAs synthesis, we believe that the "illegitimate" or "immature" expression of "*Xist*" reported (Richler et al., 1992; Kay et al., 1993; Farazmand et al., 2000) could well have been the expression of the antisense strand.

Despite intensive study during the past ten years, the precise function of *Xist* RNA in the regulation of X chromosome inactivation remains unknown. Therefore, before speculating on the possible function of *Xist* antisense RNA it will be helpful to review some aspects of the potential involvement of *Xist* in X chromosome inactivation. Although *Xist* RNA has been shown to remain in the nucleus intimately associated with the inactivated X chromosome the quantity of *Xist* RNA generated is not enough to coat the entire X chromosome (Buzin et al., 1994; Clemson et al., 1996). Hence, other factors have been invoked to bring about the inactivation of the X chromosome. It has been suggested that these factors are present and function during embryogenesis since the induction of *Xist* expression in somatic cell hybrids (a differentiated state) does not

lead to culmination of X chromosome inactivation (Clemson et al., 1999).

Several investigators including Clemson et al. (1999) have shown that *Xist* is not necessary for the maintenance of the inactive state of X chromosome (Brown and Willard, 1994; Rack et al., 1994). Hypermethylation of the CpG islands of the X linked genes (Norris et al., 1991; Tribioli et al., 1992) and histone deacetylation of the inactive X chromosome (Keohane et al., 1998) are among the known features involved in the maintenance of the inactive state. Other possible factors involved in this process include heteronuclear proteins interacting with the 5' region of the *Xist* (Brown and Baldry, 1996) and the deposition of the histone macro- H2A1 molecules on the inactive X chromosome (Constanzi and Pehrson, 1998). Interestingly, *Xist* deletion disrupts macro H2A1 deposition without leading to the reactivation of the inactive X chromosome (Csankovski et al., 1999).

Our strand specific RT-PCR tests show that both sense and antisense strands are co-expressed in (differentiated) female somatic cells of oviduct and ovaries (Figure 2). These observations confirm that the RNA expressed in female bovine tissues and female cell cultures is *Xist*, while that expressed in male gonads and non-gonadal tissues is exclusively the *Xist* antisense RNA. The major question is why is antisense strand expressed in male somatic cells in which there is no X chromosome inactivation? The observations that antisense strand is expressed in all bovine male and female somatic tissues (present study) and that it is expressed in differentiated ES cells and somatic tissues in the mouse (Debrand et al., 1999), suggest that the transcription of the antisense RNA may have a common role in ensuring that an X chromosome remains

active in male and female cells. Hennig (1999) has proposed that it is the active X chromosome which is determined (marked) first in both sexes since, regardless of the number of the X chromosomes present, one X chromosome is noted to remain active in male and female cells (Lyon, 1996). The choice as to which of the two X chromosomes needs to remain active is thought to be a default process (Marahrens et al., 1998; Lee et al., 1999). We propose that the expression of the *Xist* antisense RNA may be one of the factors involved in choosing the active X chromosome based on our own and previous observations. For instance, the expression profile of the *Xist* antisense RNA during in vitro differentiation of ES cells indicates a dynamic relationship between *Xist* and its antisense strand the *Tsix* (Heard et al., 1999). The sense and antisense transcripts show biallelic low level expression in undifferentiated ES cells and, during differentiation, the antisense strand transcription gradually ceases while a high level of the *Xist* sense RNA begins to accumulate as the X chromosome becomes inactive (Lee et al., 1999; Debrand et al., 1999). In contrast, the other X chromosome which retains a low level of antisense expression, becomes the active X chromosome upon differentiation (Lee et al., 1999; Debrand et al., 1999). The expression of the *Xist* antisense strand was also found in early mouse embryos prior to X chromosome inactivation (Mise et al., 1999; Debrand et al., 1999). In male ES cells Lee et al. (1999) observed three patterns of sense and antisense strand expression: undifferentiated form (expressing both sense and antisense), a transient form expressing only antisense, and somatic form expressing neither. However, Debrand et al. (1999) using strand specific RT-PCR, have detected a low level of *Xist* antisense expression in differentiated ES cells and adult male and female

somatic cells. It has been postulated that the antisense transcript may be involved in the regulation of *Xist* expression or its stability, by blocking its action on the X chromosome (Heard et al., 1999; Lee et al., 1999). Unlike *Xist*, the antisense strand does not coat the X chromosome but it is localized at Xic , indicating that the antisense RNA may not be involved in the process of heterochromatinization during X inactivation (Lee et al., 1999).

In conclusion, the absence of the sense transcript in the bovine testis supports the idea that *Xist* is not involved in X chromosome inactivation in male germ cells. The expression of the *Xist* antisense RNA in male gonadal and nongonadal tissues would appear to be related to the function of preserving the active state of the X chromosome. Testing the antisense strand expression in female meiotic germ cells in which both X chromosomes are thought to be in active state, may provide proof for this hypothesis.

## GENERAL DISCUSSION

The observation that *Xist*, a key factor in the inactivation of one of the X chromosomes in female somatic cells (Brown et al., 1991a; Borsani et al., 1991), is expressed in the testes of several mammalian species indicated that *Xist* may have a similar role in X inactivation in male germ cells (McCarrey and Dilworth,1992; Richler et al., 1992; Ayoub et al., 1997; DeLa Fuente et al., 1999). However, normal spermatogenesis in *Xist*-deficient mouse (Marahrens et al., 1997) and premature expression of *Xist* in male germ cells (Kay et al., 1993) indicates that *Xist* expression may not be associated with the inactivation of X linked genes in male germ cells (Chapter 2). Unlike the process of X chromosome inactivation in female somatic cells which has been studied extensively in the past four decades, the mechanism and function of X chromosome inactivation in male germ cells remains undefined.

This study was conducted to examine the expression of *Xist* and its possible role in the activity of X linked genes in male germ cells of bovine species. The finding that "*Xist*" is expressed in fetal bovine testes was surprising since unlike in female fetuses there is no functional gametogenesis at this stage in male mammals (Chapter 1). Furthermore, semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) of X linked genes demonstrated that *Xist* expression in the fetal testis does not affect the expression of X linked genes (Chapter 2). Although these observations would suggest that Xist expression may not be relevant to the process of X inactivation, our subsequent studies on X chromosome aneuploid cell strains and normal male and female

bovine cell cultures (Chapter 3) clearly showed that *Xist* expression increases in keeping with the increased number of X chromosomes which, in turn, is correlated with the number of inactive X chromosomes in these aneuploid cells. However, studies on these cell cultures also showed that *Xist* expression is evident in cultures of male bovine fetuses, even though the level of expression is lower than that in cells carrying more than one X chromosome. This observation, coupled with our own findings on fetal bovine testes (Chapters 1 and 2) and those of Mise et al. (1999) and Lee et al., (1999b) which showed that *Xist* antisense strand is expressed in both male and female cells provided the impetus for us to investigate the identity of the transcript found in fetal and adult male gonads. Using the strand specific RT-PCR approach (Chapter 4) we were able to demonstrate that it is the *Xist* antisense and not the sense strand which is transcribed in fetal and adult bovine testes. Furthermore, the same approach revealed that the antisense strand is expressed in male somatic tissues as well, while the gonads and somatic tissues of female fetuses express both sense and antisense *Xist* RNAs. Extending this approach to normal fetal cell cultures and cell strains carrying different sex complements we noted that in XY cells only the antisense strand is transcribed, whereas in cells with more than one X chromosome (XX, XXY and XXX) both strands are expressed. Expression exclusively of the antisense strand in fetal and adult bovine gonads and somatic tissues of males and the co-expression of both strands in female tissues indicate that *Xist* may not have a role in X chromosome inactivation in male germ cells and that the antisense transcript may be involved in a major function that is common to both sexes. We believe that this function may be related to ensuring that one

X chromosome in both sexes remains "uninactivated".

## Role of *Xist* antisense in the regulation of X chromosome inactivation

Natural antisense RNAs, originally described in prokaryotes, play a role in the regulation of gene expression in eukaryotes (Kumar and Carmichael, 1998; Vanhee-Brossollet and Vaquero, 1998). Antisense-mediated regulation of sense RNA transcription may occur through different mechanisms. They may have similar structural features and therefore, may compete for binding sites for interacting proteins or they may function through the formation of sense-antisense duplex. In the latter case, RNA duplexes may prevent sense strand from interacting with cellular components required for the normal expression of sense RNA or they may undergo degradation by double-strand specific enzymes (Vanhee-Brossollet and Vaquero, 1998). Although several examples indicate that the accumulation of sense and antisense strands are inversely related, persistent co-expression of both transcripts has been shown in different organisms (Vanhee-Brossollet and Vaquero, 1998).

The molecular correlates of early events in X inactivation, including the counting of the number of X chromosomes and choosing the one to become inactive (or to remain active) are not known. The Xic through which the X chromosome inactivation is initiated is a large complex domain with a number of genes, including *Xist* sense and antisense loci, *Xce* and several other genes (Heard et al., 1997). Based on the fact that Xic deletion has never been observed on the active or the inactive X chromosome Willard et al. (1993) have proposed that Xic may have a function in maintaining the X

chromosome integrity or in the viability and early development of *both* male and female conceptuses. Of importance to this prediction is the fact that a member of homeobox genes, *Cdx4*, is localized within the Xic, mapped distal to the mouse and the human *Xists* (Horn and Ashworth, 1995). This gene which is subject to inactivation in the mouse, is expressed during embryogenesis, and later expressed in all cells over the posterior half of the primitive streak (Gamer and Wright, 1993). The *Cdx4* belongs to the caudal class of homeobox transcription factors. It is expressed in early embryogenesis and is involved in the specification of anterio-posterior axial polarity (Gamer and wright, 1993). The location of *Cdx4*, distal to *Xist* and overlapping *Xce*, has led to the suggestion that it may be the candidate gene for *Xce* (Horn and Ashworth, 1995). It is interesting that *Xist* antisense strand locus in mouse also spans this region (Lee et al., 1999b; Mise et al., 1999). The functional relations between these genes and their respective roles in the activity of X chromosome in early embryos remain to be clarified.

The observation that the expression of the *Xist* antisense RNA in early embryonic development and in undifferentiated embryonic stem cells precedes the expression of the sense strand (Lee et al., 1999b; Mise et al., 1999) and our own finding that the antisense strand is expressed in male and female somatic tissues, leads us to suggest that *Xist* antisense RNA may be one of the factors involved in choosing the active X chromosome. In the course of X chromosome inactivation in female cells, the expression of *Xist* antisense ceases and *Xist* sense RNA accumulates and spreads over the inactivated X, while the other X (which is destined to remain active) shows a low level

of antisense expression (Lee et al., 1999; Debrand et al.,1999). Co-expression of both strands at comparable levels in bovine fetal and adult female tissues (Chapter 4) indicates that the function of antisense strand is not restricted to early embryos and undifferentiated cells. The selection of the X chromosome to remain active may be accomplished by the interference of antisense RNA with the expression and/or the accumulation of *Xist* sense strand, and by simultaneously recruiting factors (e.g. acetyl transferase activity) involved in maintaining the active structure of chromatin. Developmental changes in chromatin structure potentiate the expression or suppression of genes (determination) which occur long before the overt expression of this potential (differentiation), and are maintained through many cell divisions (Singh, 1994). In this context *Xist* sense and antisense transcripts (in association with other epigenetic imprints) may play a role in the determination of the inactive and the active states, respectively. Although the mechanism by which the non-coding RNAs modulate chromatin structure is unclear, an effect on transcription itself or association with other participants in chromatin structure, are among the proposed modes for their action (Panning and Jaenisch, 1998).

Recently, Hennig (1999) has proposed that it is the active X chromosome which is first determined in both sexes since, regardless of the number of the X chromosomes, one X chromosome needs to remain active in somatic cells of both sexes. The choice of the X chromosome to remain active has also been proposed as an active (positive) process by others (Marahrens et al., 1998; Lee et al., 1999b; Debrand et al., 1999). Henning (1999) has hypothesized that a small number of X activation regulators (XARs)

are produced early in development and that they bind to a regulatory site, such as Xic, to choose the X chromosome to remain active. These XARs may function by inactivating a regulatory gene, such as *Xist*. Since the quantity of XARs is limited, all remaining X chromosomes become inactive (Henning, 1999). The expression of *Xist* antisense strand during early development could be the most critical event in the determination of the active state of the X chromosome, since the antisense strand can target the expression and/or accumulation of the sense strand. On the other hand, the antisense strand expression in differentiated cells (Current study and Debrand et al., 1999) may be a backup device to preserve the active state of one X chromosome in somatic tissues of both sexes. In this context, the persistent expression of antisense strand may be required to suppress the sense strand expression or to present its function as a nucleation site for the accumulation of factors in order to preserve the active state of chromatin.

It has been proposed that the *Xist* antisense strand, by regulating the expression of *Xist*, may function as the target of the X-counting mechanism (Meller, 2000). The "counting process" seems to be a constitutive function, as in male ES cells carrying *Xist* transgenes and in clinical cases of XXY individuals, X chromosome inactivation occurs (Migeon et al., 1999). Based on the observation that the antisense strand deletion does not lead to the accumulation of *Xist* on the mutant X chromosome, Lee and Lu, 1999 suggested that other factors, in addition to *Xist*, are required for the initiation of the X chromosome inactivation. These authors proposed that the factors involved may be X linked since the autosomal complement is identical in the male and the female (Lee and Lu, 1999).

**Strategies of dosage compensation**

Three different systems of dosage compensation are known among organisms with heteromorphic sex complements (Panning and Jaenisch, 1998; Meller, 2000). In *Caenorhabditis elegans* hermaphrodites, transcription from the X chromosomes is halved, in *Drosophila* males, transcription from the X is doubled, and in mammalian females one X chromosome is silenced. While the molecules used to achieve dosage compensation in *Caenorhabditis elegans* comprise a group of proteins that bind to both X chromosomes of hermaphrodites, in *Drosophila* males and mammalian females dosage compensation is mediated by non-coding RNAs (Meller, 2000). Despite the profound difference between the mechanisms, all these three approaches use chromatin based modulation of gene regulation.

Although the exact mechanism of action of *Xist* antisense RNA is not defined, comparison of the mechanism involved in keeping the X chromosome in active state in mammals with the mechanism of dosage compensation in *Drosophila* may prove useful. In *Drosophila,* the transcriptional upregulation of the single X chromosome in males is mediated by non-coding RNAs in close association with a number of proteins (Meller, 2000). Similar to the *Xist* sense and antisense RNAs, both *roX* (RNA on X chromosome) RNAs, lack open reading frames (ORF) and their loci are located on the X chromosome (Meller, 2000). In *Drosophila*, the association of *roX*s and MSL (male specific lethal) proteins as nucleoprotein complexes (termed compensosome) leads to changes in the acetylation of the histone H4 isoforms (H4Ac16) and hypertranscription of the X linked genes on the sole X chromosome in the males (Franke and Baker, 2000). These modifications are similar to the hyperacetylation of histones leading to increased

transcriptional activity of X linked genes (Turner, 1998; Gilbert and Sharp, 1999). The male-specific expression of *roX* RNAs, their localization within the nucleus and their binding to the X chromosome of males indicate that *rox* RNAs play a crucial role in dosage compensation in *Drosophila* (Frank and Baker, 1999; Meller, 2000). Similarly, *Xist* RNA (and possibly its antisense strand), are able to mark and direct the effectors involved in the long range chromatin modifications along the X chromosome. Thus *Xist* antisense RNA possibly preserves the active state of the X chromosome in mammals by a mechanism similar to the hypertranscription of X linked genes on the X chromosome of male *Drosophila*. Hyperexpression of X linked genes from the single active X chromosome compared to that of the autosomal gene expression was hypothesized as the first evolutionary step in the accommodation of X linked genes to their hemizygous situation in males (Ohno, 1967). Experimental data for the expression of the chloride chanel gene, *Clc4*, in *Mus spretus* is consistent with this prediction (Adler et al., 1997). This gene, which is autosomal in the laboratory mouse, but X linked and subject to inactivation in *Mus spretus*, provided a unique opportunity to test Ohno's prediction (Ellis, 1995). Quantitative measurements of *Clc4* expression in interspecific hybrids of *Mus spretus* x laboratory strain crosses revealed that each autosomal locus express half the level of *Clc4* compared to that from the single active X-linked locus (Adler et al., 1997).

**Evolutionary conservation of activity status of the sex chromosome in two sexes**

The observation that "*Xist*" RNA is expressed in male germ cells and that it binds to the XY body (McCarry and Dilworth, 1992; Richler et al., 1992; Ayoub et al., 1997;

De La Fuente et al., 1999) have been taken as evidence to conclude that X chromosome inactivation in male meiocytes and female somatic cells is brought about by similar mechanisms (Charlesworth, 1996; Ayoub et al.,1997). It has been hypothesized that the X chromosome inactivation in females may have evolved by imprinting the *Xist* mediated mechanism of X inactivation first established in males (Charlesworth, 1996; Ayoub et al.,1997). According to this view difference in the features of the inactive X in the two sexes is attributable to the epigenetic modifications superimposed on the fundamental *Xist* RNA-directed mechanism (Ayoub et al., 1997). Since *Xist* sense and antisense strands were not distinguished from each other in previous experiments which used double stranded DNA probes in flourescent in situ hybridization (FISH) or random primers for RT-PCR, the hypothesis of Ayoub et al. (1997) needs to be revised. Instead, we may speculate that *Xist* locus, through the expression of its antisense strand in male and females may serve another important role. We speculate that: 1) *Xist*-mediated inactivation of the X chromosome is restricted to female mammals for achieving dosage compensation; and, that 2) the antisense strand is involved in preserving the active state of one X chromosome in both sexes since it is co-expressed (with *Xist* sense RNA) in differentiated female somatic cells.

In any speculation on the differential activity of sex chromosomes in the two sexes one has to consider the fact that prior to the evolution of the heteromorphic sex chromosome their homologous ancestors had non-gender functions. The evolution of the primitive sex chromosome from the ancestral pair of autosome is closely associated with the evolution of genetically determined sexes (Charlesworth, 1991; Marin and Baker,

1998). Attempts to find possible similarities in the mechanisms of X chromosome inactivation in female somatic cells and male germ cells have not been totally successful. Even if we accept that X inactivation in female somatic cells is the consequence of the evolution of heteromorphic sex chromosomes, this process does not necessarily have to be through the inactivation of the X chromosome in male germ cells. It is evident that the purpose of the inactivation of the X chromosome in the two sexes is different, as in male germ cells the need for gene dosage constraints does not exit as it does in female. Furthermore, the inactivation of X chromosome in male germ cells is transient and restricted to the pachytene stage of meiosis (Solari, 1994). Comparison of the common denominator in these two processes (the active state of the sole X chromosome in male cells and the active state of the one of the X chromosomes female cells) would lead to the suggestion that a mechanism for keeping one copy of the X-linked genes in active state had to be developed. Our studies indicate that this goal may have been achieved, in part, through the antisense *Xist* RNA during the evolution of the heteromorphic sex chromosomes.

There is compelling evidence to support the belief that the organization of the X chromosome per se is distinctly different from that of the autosomes (Lyon, 1998). In mammals these distinctive features are reflected in the organization of the X inactivation center (Xic) which is a complex region carrying several genes (Heard et al., 1997) and a concentration (enrichment) of long interspersed elements (LINE-1) (Lyon, 1998; Bailey et al., 2000). Cytogenetic, transgenic, and molecular studies suggest that Xic is critical for the inactivation of the mouse and the human X chromosomes (Migeon, 1994; Heard

et al., 1997). However, so far investigations on Xic have been focused on discerning to test for its function in the inactivation of the X chromosome in female somatic cells. Differences in the organization of the X chromosome (compared to that of the autosomes) reflect the evolution of the X chromosome in a way that promotes the recognition and binding of the molecules involved in dosage compensation (Meller, 2000). Factors thought to be involved in the maintenance of the silent state of the X chromosome in female somatic cells, including methylation of CpG islands and chromatin hypoacetylation, are absent in meiotic male germ cells (Driscoll and Migeon, 1990; Armstrong et al., 1997). It has been shown that the products of certain X-linked genes are maintained in male germ cell during meiosis and some of the genes, apparently inactive in pachytene stage, are reactivated in post meiotic cells (Singer-Sam et al., 1990; Shannon and Handel, 1993; Hendriksen et al., 1995). These data indicate that in spite of the apparent "X inactivation", male germ cells use different strategies to maintain X chromosome related housekeeping functions during spermatogenesis (Shannon and Handel, 1993). Furthermore, *Xist*, once thought to be a common correlates in the two process of X inactivation, is not expressed in male germ cells (current study). Instead, the *Xist* antisense strand, with a negative regulatory control on *Xist* expression (and hence on X chromosome inactivation) seems to be expressed persistently in male gonads and nongonadal tissues in the bovine species.

The molecular correlates of X chromosome inactivation in male germ cells could well be among the testis and XY-body specific proteins. Recently, histone macroH2A1 was noted to be concentrated on the XY body at the pachytene stage of spermatogenesis

(Hoyer-Fender et al., 2000; Richler et al., 2000). This finding is interesting in that it represents the first common epigenetic modification to be associated with the inactive X in both sexes, especially since our studies indicate that *Xist* is not involved in X chromosome inactivation in male germ cells. Several other proteins have been identified which are shown to be localized to the XY body (Smith and Benavente, 1995; Alshemir et al., 1997; Kralewski and Benavente, 1997; Motzkus et al., 1999). Although the exact function and importance of these proteins in XY body formation are not known, some of them display interesting features. For instance, M31 protein shows a dynamic pattern of spreading from the tip of the XY body to its entire length at the pachytene stage (Motzkus et al., 1999). Since the spreading pattern of M31 is reminiscent of the "quasi-cis" fashion of spreading of "*Xist*" along the Y chromosome in the XY body (Ayoub et al., 1997), it has been suggested that an M31 containing complex is involved in the hetreochromatinization of the X and the Y chromosomes in male germ cells (Motzkus et al., 1999). Another protein to be mentioned in this regard is a nuclear orphan receptor, designated GSNF (germ cell nuclear factor), which is expressed in spermatocytes of adult mouse and accumulates in the XY body of the late pachytene spermatocytes (Bauer et al., 1998). Since nuclear receptors are involved in the regulation of transcription by interfering with chromatin remodeling, Bauer et al., 1998 have suggested that GCNF may have a role in establishing the transcriptionally inactive state of the sex chromosomes constituting the XY body.

In conclusion, the molecular mechanism controlling the XY body formation in male meiosis remains an enigma despite the progress made recently in finding candidate

genes and proteins for various components of spermatogenesis. Perhaps some of the proteins associated with the XY body are among the "trans acting" factors expressed by autosomes, or they may be components of pathways responding to extrinsic signals (originating from outside of the meiotic germ cells) as proposed by Handel et al. (1994). That the sex vesicle formation may be dependent on the environment of meiotic germ cells was proposed by Hogg and McLaren (1988) who noticed that ectopic XY germ cells which enter meiosis lack the XY body. The specific organization of the sex chromosomes as the XY body during male meiosis perhaps represents a male specific coping device to protect the integrity of nonrecombining parts of the sex chromosomes during meiosis (McKee and Handel, 1993. Solari, 1994). How this is accomplished is not known. In addition to preserving the active state of the X chromosome, it would appear that the function of the *Xist* antisense RNA may be to attract and bind specific proteins to the nonhomologous regions of the sex chromosomes in male germ cells of mammals.

## SUMMARY AND CONCLUSIONS

The role of the X-inactive specific transcript (*Xist*) in the process of X chromosome inactivation in male germ cells of the domestic cattle, *Bos taurus*, was examined using reverse transcription polymerase chain reaction (RT-PCR) technique on fetal bovine gonadal and nongonadal tissues. Tests for *Xist* RNA in fetal bovine testes revealed that *Xist* expression begins to be evident in fetuses as early as 50 days of gestation and that it is unequivocally detectable thereafter (Chapter 1). Examination of the cellular events in fetal testes which express *Xist* indicated that some of the intra tubular male germ cells enter meiosis early in fetal life (before 90 days) although they are eliminated by apoptosis.

In order to investigate whether *Xist* expression leads to the inactivation of X-linked genes in fetal testes a semi-quantitative RT-PCR approach was used to test the levels of the expression of hypoxanthine phosphoribosyl transferase (*Hprt*), glucose 6-phosphate dehydrogenase (*G6pd*), and zinc finger protein gene on the X (*Zfx*) in gonad and nongonadal tissues of male and female bovine fetuses. The results of this study (Chapter 2) demonstrated that the expression levels of these X-linked genes in the bovine testis were not significantly different from that of the somatic tissues of males and females tested in this study, indicating that *Xist* expression is not accompanied by X linked gene silencing in fetal bovine testis.

Extension of the semiquantitative RT-PCR approach to test the expression profile of X-linked genes in cultured bovine cells of normal sex chromosome make up (XX and XY) and cell strains of sex chromosome aneuploids (XXX and XXY) revealed that *Xist*

expression in XXX cell strain was the highest although it was also expressed (at a low level) in male (XY) cells. These results indicated that the expression of *Xist* is correlated with the number of the X chromosomes in these cells, and that the expression levels of three out of the few X-linked genes tested were comparable in normal cell cultures and in cell strains with excessive X chromosomes, while the expression of *Zfx* locus was higher in XXX cell strain compared to all other bovine cell cultures tested in this study. These results indicate that in the bovine species, as in human, *Zfx* escapes inactivation, whereas the bovine *Smcx* locus, unlike that of mouse and man is subject to inactivation.

Since the "premature" expression of *Xist* in fetal testes, and its "unexpected" expression in normal male cells cannot be explained in the context of X chromosome inactivation, a strand-specific RT-PCR approach was undertaken to determine whether the amplicon referred to a as "*Xist*" represents the *Xist* RNA or its antisense RNA. The results demonstrated that the RNA expressed in fetal and adult bovine testes is exclusively the *Xist* antisense RNA and that the expression of antisense RNA in males is not restricted to the testis. Our studies on gonadal and nongonadal tissues of females indicated that the antisense strand is co-expressed with the sense strand in all female tissues, and in cell cultures and strains of normal and aneuploid X chromosome constitutions.

Results of these studies indicate that the RT-PCR product referred to as "*Xist*" in males represents the antisense *Xist* RNA; and that it is co-expressed along with the sense strand in female cells. We hypothesize that the role of *Xist* antisense RNA may be to preserve the active state of one of the X chromosomes in male and female cells.

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## APPENDIX I

### Preliminary steps in semi-quantitative RT-PCR approach

Semi-quantitative polymerase chain reaction (RT-PCR) used in this study comprises several steps, including determining the *Linear Range* for optical density for choosing the number of PCR cycle used in the study and the determination of *Optimum Ratio* of 18 S Primers to Competimers for the delineation of the levels of expression.

Determination Linear Range

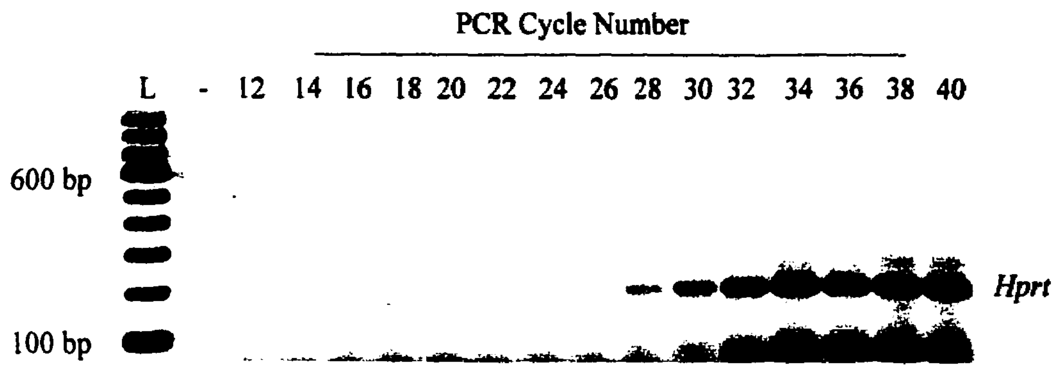
Aliquots of an identical mixture of PCR components, including cDNA sample and gene specific primers, were subjected to amplification. Aliquots were removed at two cycle intervals , and products were resolved on a 2% agarose gel (Figures 1-5, a). The optical density (OD) values of bands obtained by FMBIO II Image Analysis System (Hitachi Software Ltd), after staining with SYBR[R] Green I (Molecular Probe Ltd), were plotted against the PCR cycle numbers (Figures 1-5, b). The amplification cycle from the mid point of the linear range was chosen for subsequent semiquantitative analysis for each of the genes in different samples, since this cycle gives a range on both sides of the linear range for comparison of the relative gene expression in different tissues and cell cultures.

Determination of the Optimal ratio of 18 S: Competimers

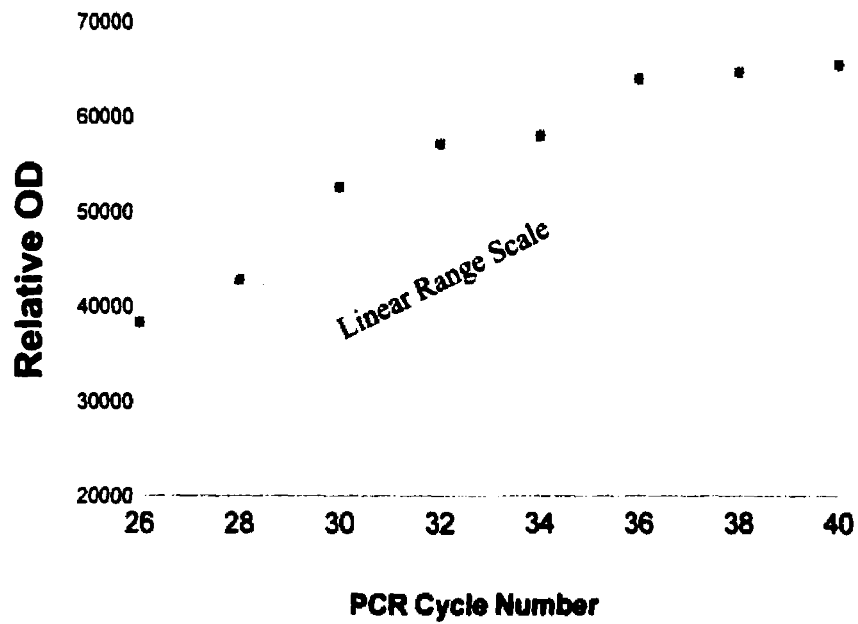
The ribosomal 18S RNA used as the internal control (Quantum RNA 18S

Internal Standards) for the semiquantitative PCR is expressed abundantly in animal tissues. To equalize the co amplification of 18S and gene-specific RNA in each reaction a set of primers ("Competimers" which compete with 18S primers) was used in the ratio providing the most acceptable (similar) yields of products. Using the number of cycles determined in the linear range determination, PCR products of the gene-of -interest and 18 S ribosomal RNA were assessed by agarose gel electrophoresis (panel c in Figures 1-5).

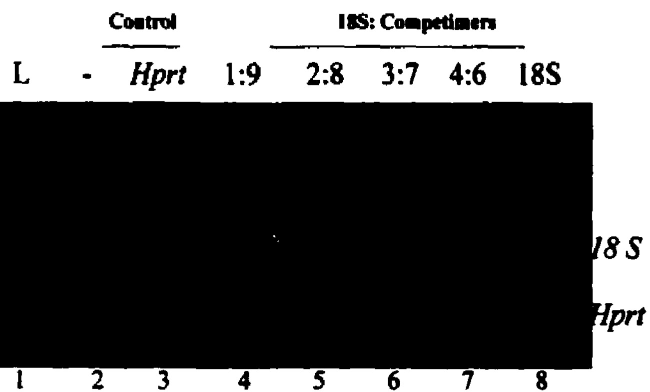
Figure 1. a) An agarose gel representing the PCR products of *Hprt*, removed from thermocycler at the indicated cycle number. b) Optical density (OD) values of bands plotted against the cycle number. The straight line represents the linear range of the amplification. c) An agarose gel representing *Hprt*-specific bands with and without the internal standard (18 S), coamplified in the same reaction using different ratios of 18S to Competimers primers. Note that the yield of two products are more similar when the 18 S to Competimers ratio is set at 4:6 (lane 7) compared to those with lower concentration of 18 S (lanes 4, 5, and 6).




a

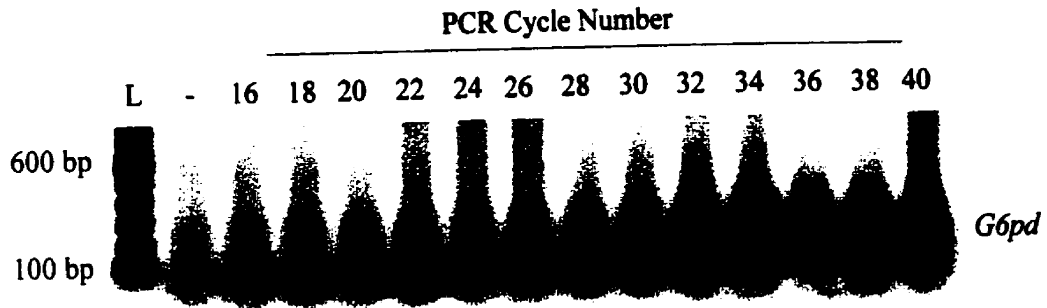



b

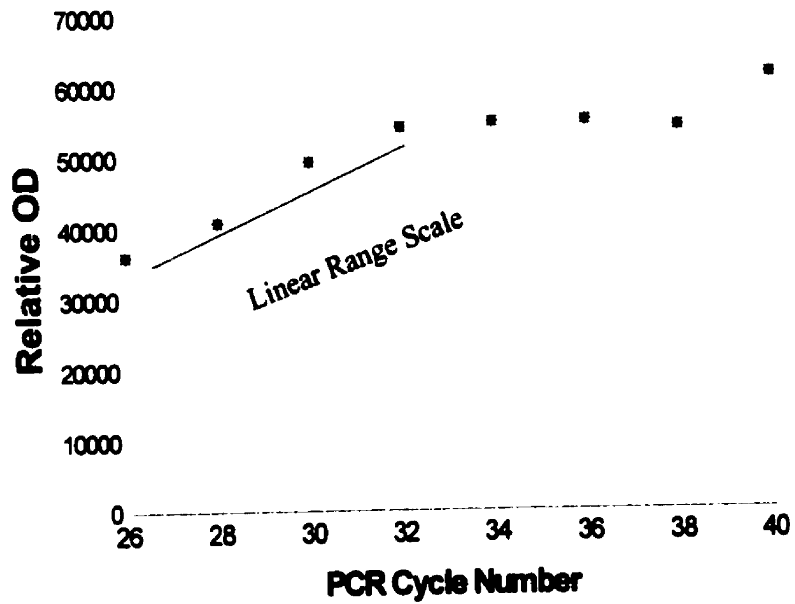



c

Figure 2. a) An agarose gel representing the PCR products of *G6pd*, removed from thermocycler at the indicated cycle number. b) Optical density (OD) values of bands plotted against the cycle number. The straight line represents the linear range of the amplification. c) An agarose gel representing *G6pd*-specific bands with and without the internal standard (18 S), coamplified in the same reaction using different ratios of 18S to Competimers primers. Note that the yield of two products are more similar when the 18 S to Competimers ratio is set at 4:6 (lane7) compared to those with lower concentration of 18 S (lanes 4, 5, and 6).



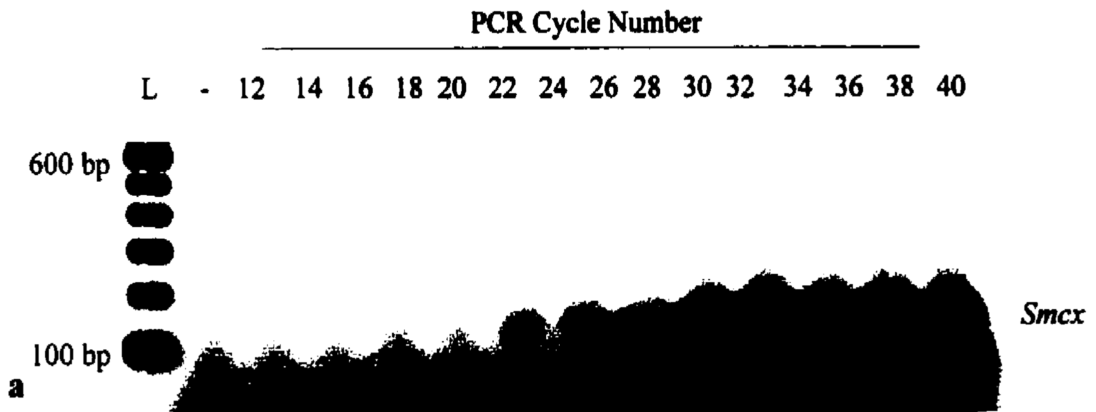

a



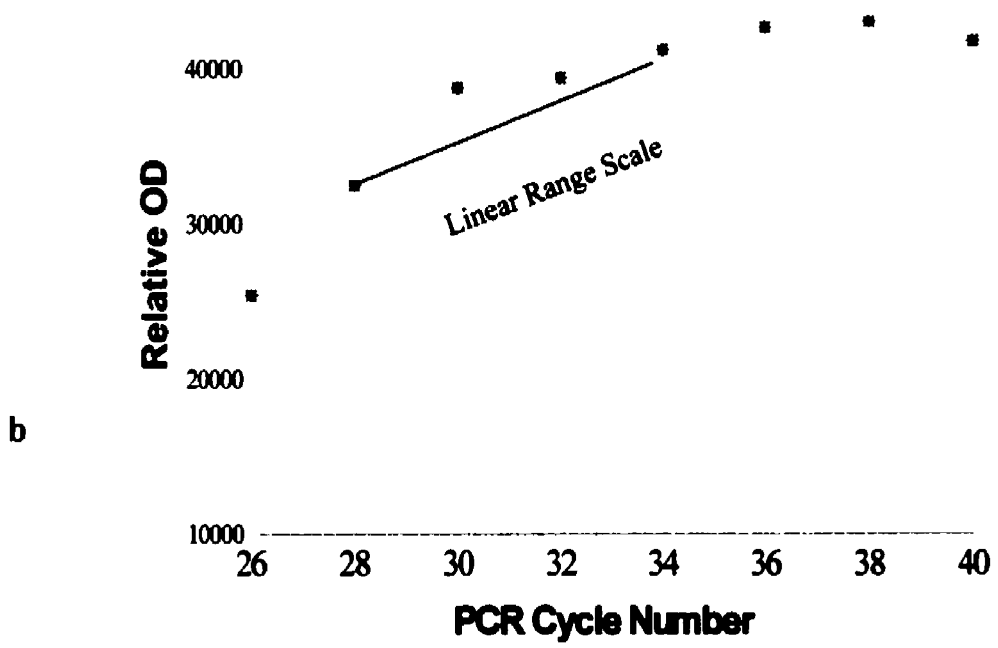

b

c

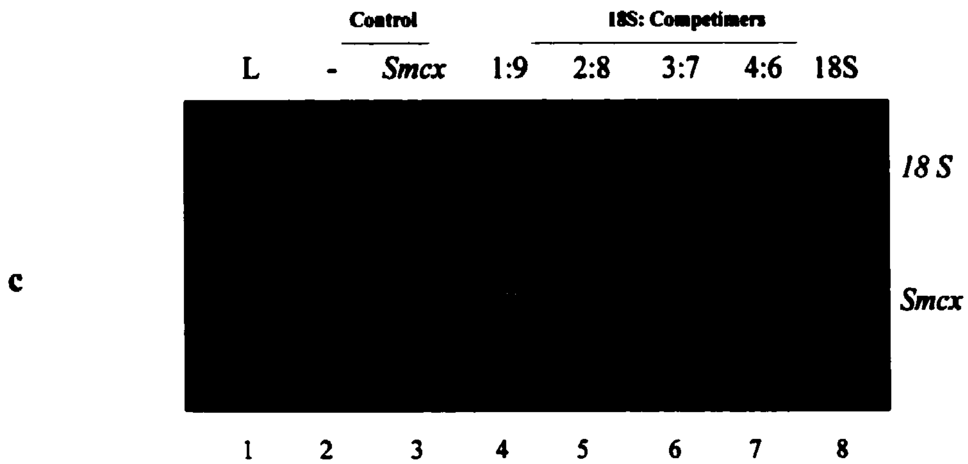
Figure 3. a) An agarose gel representing the PCR products of *Smcx*, removed from thermocycler at the indicated cycle number. **b)** Optical density (OD) values of bands plotted against the cycle number. The straight line represents the linear range of the amplification. **c)** An agarose gel representing *Smcx*-specific bands with and without the internal standard (18 S), coamplified in the same reaction using different ratios of 18S to Competimers primers. Note that the yield of two products are more similar when the 18 S to Competimers ratio is set at 4:6 (lane7) compared to those with lower concentration of 18 S (lanes 4, 5, and 6).



PCR Cycle Number
L
-
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
600 bp
100 bp
Smcx
a

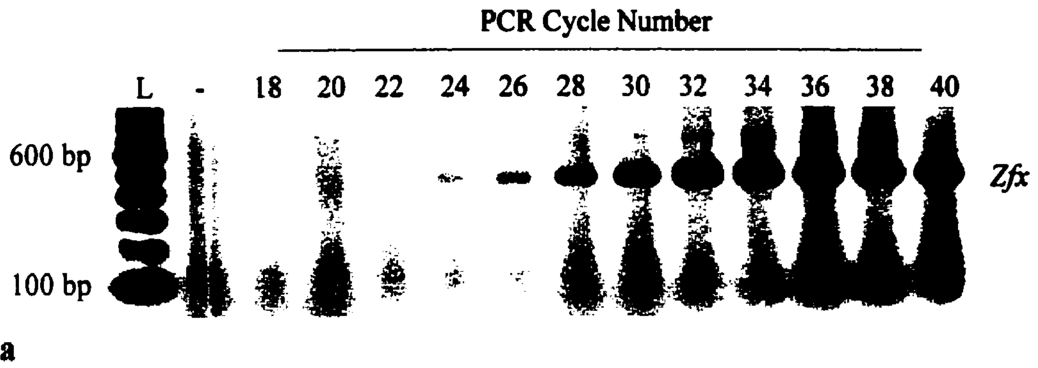



40000
30000
20000
10000
Relative OD
Linear Range Scale
b
26
28
30
32
34
36
38
40
PCR Cycle Number

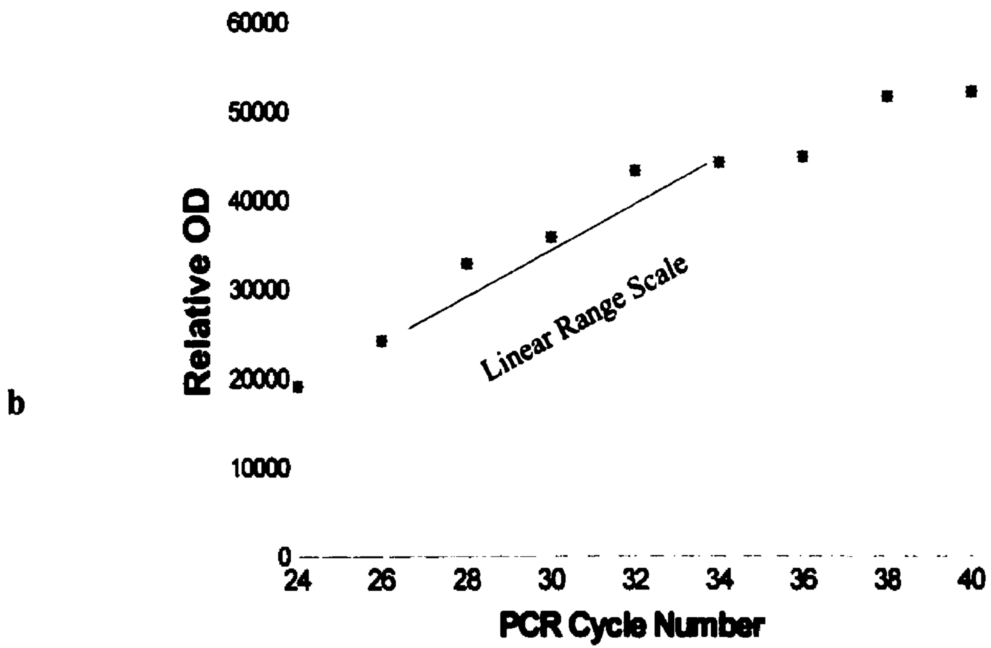



Control
18S: Competimers
L
-
Smcx
1:9
2:8
3:7
4:6
18S
18 S
Smcx
c
1
2
3
4
5
6
7
8

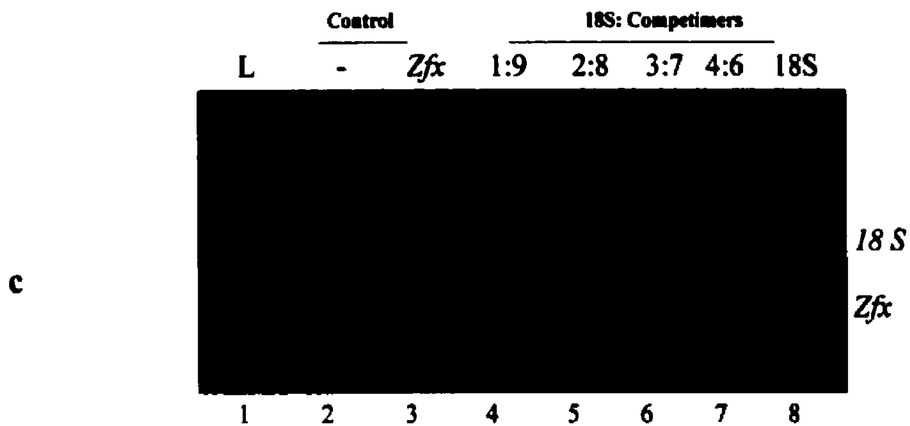
Figure 4. a) An agarose gel representing the PCR products of *Zfx*, removed from thermocycler at the indicated cycle number. b) Optical density (OD) values of bands plotted against the cycle number. The straight line represents the linear range of the amplification. c) An agarose gel representing *Zfx*-specific bands with and without the internal standard (18 S), coamplified in the same reaction using different ratios of 18S to Competimers primers. Note that the yield of two products are more similar when the 18 S to Competimers ratio is set at 3:7 (lane 6) compared to those with other ratios (lanes 4, 5, and 7).




a




b




c

Figure 5. a) An agarose gel representing the PCR products of *Xist*, removed from thermocycler at the indicated cycle number. b) Optical density (OD) values of bands plotted against the cycle number. The straight line represents the linear range of the amplification. c) An agarose gel representing *Xist*-specific bands with and without the internal standard (18 S), coamplified in the same reaction using different ratios of 18S to Competimers primers. Note that the yield of two products are more similar when the 18 S to Competimers ratio is set at 4:6 (lane 6) compared to those with lower concentration of 18S (lanes 4, 5).

PCR Cycle Number

L - 14 16 18 20 22 24 26 28 30 32 34 36 38 40

600 bp

100 bp

*Xist*

a

20000

15000

10000

5000

0

Relative OD

Linear Range Scale

26 28 30 32 34 36 38 40

PCR Cycle Number

b

Control 18S: Competimers

L - *Xist* 2:8 3:7 4:6 18S

600 bp

*Xist* (463 bp)

18 S (324 bp)

1 2 3 4 5 6 7

c

# APPENDIX II

## Results of the sequence alignment (BLAST search) for strand-specific RT-PCR products obtained in CHAPTER 4

Sequense 1. Results of the BLAST search for strand-specific RT-PCR product (larger band in 3' lanes in figure 16a), obtained from female fetal lung.

PCR product:

```
TNNNTTTGATGTCCCGTTNAGTGTTCAANATGGCGCGATCCATTTTGCCGCAGTGTTCCAATGGCGGGAAGCCACATCA
TGGGTGTCTTTGTTCTAGTGTGCAGCATGGCGGTAGAAATATTCTGTTACATAGTAAAAGATGGCCGCTTAAGTACTTG
CCGCAGTCTAAAACATGGCGGGCTTTTGTCTCTGCCGTGTGCATTTCCTGATAGGTTTTGCTGCAGGGACAATATGGCT
GACCTTGTCATGTGGATATCATGGCAGTTTGTCACGTGGATATCGTGGCAGGGGTGTTTGACCGTTACATTCTTGGCGG
GCTTTGCATCAGGAGGGCCTGCCGCATTGTTAAAGATGGCGTGCTTTGCCGCGGACAAAGTGAAAGGAGGGATTGGCAA
TGTTAGATTGCCGCGTGTCCCACCCAATCAGAAAGGGTGGTAGAATCGGTCACCAGCAANNNNNNNNNNNNNNNC
```

| Sequences producing significant alignments: | Score (bits) | E Value |
|---|---|---|
| gb\|AF104906.5\|AF104906 Bos taurus X-inactive specific trans... | 829 | 0.0 |
| gb\|L10727.1\|BISXISTAA Bison (clone pCow1) Xist DNA sequence | 757 | 0.0 |
| gb\|U50911.1\|ECU50911 Equus caballus Xist gene, promoter reg... | 373 | e-101 |
| gb\|M97168.1\|HUMXIST Homo sapiens X (inactive)-specific tran... | 101 | 3e-19 |
| gb\|L10732.1\|RABXISTAA Rabbit (clone pRab1) DNA sequence | 100 | 1e-18 |

Alignments:

Bos taurus XIST gene, partial sequence (Length = 2182). Score = 829 bits(418), Expect = 0.0, Identities = 427/429(99%), Gaps =1/429 (0%),Strand = Plus / Plus

```
Query: 20   agtgttcaanatggcgcgatccattttgccgcagtgttccaatggcgggaagccacatca 79
            ||||||||| |||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1278 agtgttcaaaatggcg-gatccattttgccgcagtgttccaatggcgggaagccacatca 1336

Query: 80   tgggtgtctttgttctagtgtgcagcatggcggtagaaatattctgttacatagtaaaag 139
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1337 tgggtgtctttgttctagtgtgcagcatggcggtagaaatattctgttacatagtaaaag 1396

Query: 140  atggccgcttaagtacttgccgcagtctaaaacatggcgggcttttgtctctgccgtgtg 199
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1397 atggccgcttaagtacttgccgcagtctaaaacatggcgggcttttgtctctgccgtgtg 1456

Query: 200  catttcctgataggttttgctgcagggacaatatggctgaccttgtcatgtggatatcat 259
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1457 catttcctgataggttttgctgcagggacaatatggctgaccttgtcatgtggatatcat 1516

Query: 260  ggcagtttgtcacgtggatatcgtggcaggggtgtttgaccgttacattcttggcgggct 319
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1517 ggcagtttgtcacgtggatatcgtggcaggggtgtttgaccgttacattcttggcgggct 1576

Query: 320  ttgcatcaggagggcctgccgcattgttaaagatggcgtgctttgccgcggacaaagtga 379
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1577 ttgcatcaggagggcctgccgcattgttaaagatggcgtgctttgccgcggacaaagtga 1636

Query: 380  aaggagggattggcaatgttagattgccgcgtgtcccacccaatcagaaagggtggtaga 439
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1637 aaggagggattggcaatgttagattgccgcgtgtcccacccaatcagaaagggtggtaga 1696

Query: 440  atcggtcac 448
            |||||||||
Sbjct: 1697 atcggtcac 1705
```

Appendix II, Sequence 1. Continue

Bison (clone pCowl) DNA sequence, Length = 650
Score = 757 bits (382), Expect = 0.0, Identities = 420/429 (97%),
Gaps = 3/429 (0%), Strand = Plus / Plus

```
Query: 20  agtgttcaanatggcgcgatccatttgccgcagtgttccaatggcgggaagccacatca 79
           ||||||||| |||||| ||||||||||||||||||||||||||||||||||| ||||||
Sbjct: 166 agtgttcaaaatggcg-gatccatttgccgcagtgttccaatggcgggaagc-acatca 223

Query: 80  tgggtgtctttgttctagtgtgcagcatggcggtagaaatattctgttacatagtaaaag 139
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 224 tgggtgtctttgttctagtgtgcagcatggcggtagaaatattctgttacatagtaaaag 283

Query: 140 atggccgcttaagtacttgccgcagtctaaaacatggcgggcttttgtctctgccgtgtg 199
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 284 atggccgcttaagtacttgccgcagtctaaaacatggcgggcttttgtctctgccgtgtg 343

Query: 200 catttcctgataggttttgctgcagggacaatatggctgaccttgtcatgtggatatcat 259
           ||||||||||||||    |||||||| |||||||||||||||||||||||||||||||||
Sbjct: 344 catttcctgataggaaaagctgcagg-acaatatggctgaccttgtcatgtggatatcat 402

Query: 260 ggcagtttgtcacgtggatatcgtggcaggggtgtttgaccgttacattcttggcgggct 319
           ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 403 ggcagtttgtcacgtggatatcgtggcagggttgtttgaccgttacattcttggcgggct 462

Query: 320 ttgcatcaggagggcctgccgcattgttaaagatggcgtgctttgccgcggacaaagtga 379
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 463 ttgcatcaggagggcctgccgcattgttaaagatggcgtgctttgccgcggacaaagtga 522

Query: 380 aaggagggattggcaatgttagattgccgcgtgtcccacccaatcagaaagggtggtaga 439
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 523 aaggagggattggcaatgttagattgccgcgtgtcccacccaatcagaaagggtggtaga 582

Query: 440 atcggtcac 448
           |||||||||
Sbjct: 583 atcggtcac 591
```

Equus caballus Xist gene, promoter region and exon 1, Length = 6055

Score = 373 bits (188), Expect = e-101, Identities = 289/319 (90%),
Gaps = 4/319 (1%), Strand = Plus / Plus

```
Query: 43   ttttgccgcagtgttccaatggcgggaag-ccacatcatgg--gtgtctttgttctagtg 99
            |||||||||||||||| | |||||||||| |||||||||||  |||||||||||||||||
Sbjct: 5005 ttttgccgcagtgttcaagtggcgggaaggccacatcatggtggtgtctttgttctagtg 5064

Query: 100  tgcagcatggcggtagaaatattctgttacatagtaaaagatggccgcttaagtacttgc 159
             ||||||||||||| ||||||||||||||||| | |||||||||| ||| ||||||||||
Sbjct: 5065 cgcagcatggcggtggaaatattctgttacatggcaaaagatggcggctgaagtacttgc 5124

Query: 160  cgcagtctaaaacatggcgggcttttgtctctgccgtgtgcatttcctgataggttttgc 219
            |||||| ||||| ||||||||||||||| | ||||||||||||||||||| |||||||||
Sbjct: 5125 cgcagtataaaatatggcgggcttttgtgtttgccgtgtgcatttcctgacaggttttgc 5184
```

Appendix II. Sequence1- Continue.

```
Query: 220  tgcagggacaatatggctgaccttgtcatgtggatatcatggcagtttgtcacgtggata 279
             |||||||||||||||| ||||||||||||||||||||||||||||| ||||||||||
Sbjct: 5185 cgcagggacaatatggcagaccttgtcatgtggatatcatggcagttcgtcacgtggacg 5244

Query: 280  tcgtggc-aggggtgtttgaccgttacattcttggcgggctttgcatcaggagggcctgc 338
            || ||||  ||||  |||| ||| |||||||||||||||||||||| |||| ||||||||
Sbjct: 5245 tcatggcgggggggatttgcccggtacattcttggcgggctttgcaccaggtgggcctgc 5304

Query: 339  cgcattgttaaagatggcg 357
            |||||||||||||||||||
Sbjct: 5305 cgcattgttaaagatggcg 5323
```

Human (Homo sapiens) XIST. Complete exon, Length= 16481, Score= 101 bits(51),
Expect =3e-19, Identities=130/154(84%),Gaps= 3/154(1%), Strand= Plus/Plus

```
Query: 135  aaaagatggccgcttaagtacttgccgcagtctaaaacatggcgggctt--ttgtctctg 192
            |||||||||| ||| |||  ||||||||||| ||||||||||||||| |  |||||| ||
Sbjct: 1366 aaaagatggcggctgaaggtcttgccgcagtgtaaaacatggcgggcctctttgtctttg 1425

Query: 193  ccgtgtgcatttcctgataggttttgctgcagggacaatatggctgaccttgtcatgtgg 252
            | |||||| |||| || | |||||||| |||||||||||||||| | | ||||||| ||
Sbjct: 1426 ctgtgtgcttttcgtgttgggttttgccgcagggacaatatggcaggcgttgtcatatgt 1485

Query: 253  atatcatggcagtttgtcacgtggatatcgtggc 286

            ||||||||||  ||||||||||||| ||| ||||
Sbjct: 1486 atatcatggc-ttttgtcacgtggacatcatggc 1518
```

Score = 52.0 bits (26), Expect = 2e-04,Identities = 35/38 (92%)
Strand = Plus / Plus

```
Query: 331  gggcctgccgcattgttaaagatggcgtgctttgccgc 368
            |||| |||||||||||||||||||||| | ||||||||
Sbjct: 1519 gggcttgccgcattgttaaagatggcgggttttgccgc 1556
```

Rabbit (clone pRab1) Xist DNA sequence, Length = 570
Score = 99.6 bits (50), Expect = 1e-18, Identities = 107/125 (85%),
Gaps = 2/125 (1%), Strand = Plus / Plus

```
Query: 155 cttgccgcagtctaaaacatggcgggctt--ttgtctctgccgtgtgcatttcctgatag 212
           ||||||||||| ||||| ||||||||| |  |||||| ||||||||||||||| ||   |
Sbjct: 313 cttgccgcagtgtaaaatatggcgggcgtctttgtctttgccgtgtgcatttcgtggcgg 372

Query: 213 gttttgctgcagggacaatatggctgaccttgtcatgtggatatcatggcagtttgtcac 272
           ||||||| ||||||||||||||||||||   | |||||||| ||||||||   ||||||||
Sbjct: 373 gttttgccgcagggacaatatggctgacgatatcatgtggttatcatggagatttgtcac 432

Query: 273 gtgga 277
           |||||
Sbjct: 433 gtgga 437
```

Score = 44.1 bits (22), Expect = 0.059, Identities = 28/30 (93%),
Strand = Plus / Plus

```
Query: 339 cgcattgttaaagatggcgtgctttgccgc 368
           ||||||||||||||||||| || |||||||
Sbjct: 493 cgcattgttaaagatggcgggcgttgccgc 522
```

Appendix II. Sequence 1 (Reverse).

The results of the BLAST search for strand-specific RT-PCR products (larger band in 3' lanes in figure 16a), obtained from female fetal lung.

PCR product:

```
NTTTGAAAGACCNTTGGNAANNTTCGACCCTTCCTGTNTGGGTGGGACACGCGGCAATCTAACATTGCCAATCCCTCCT
TTCACTTTGTCCGCGGCAAAGCACGCCATCTTTAACAATGCGGCAGGCCCTCCTGATGCAAAGCCCGCCAAGAATGTAA
CGGTCAAACACCCCTGCCACGATATCCACGTGACAAACTGCCATGATATCCACATGACAAGGTCAGCCATATTGTCCCT
GCAGCAAAACCTATCAGGAAATGCACACGGCAGAGACAAAAGCCCGCCATGTTTTAGACTGCGGCAAGTACTTAAGCGG
CCATCTTTTACTATGTAACAGAATATTTCTACCGCCATGCTGCACACTAGAACAAAGACACCCATGATGTGGCTTCCCG
CCATTGGAACACTGCGGCAAAATGGATCCGCCATTTTGAACACTCTAACAAAGCAGAGAGCCATGCTAAGCAATGCTAN
```

| Sequences producing significant alignments: | Score (bits) | E Value |
|---|---|---|
| gb\|AF104906.5\|AF104906 Bos taurus X-inactive specific trans... | 862 | 0.0 |
| gb\|L10727.1\|BISXISTAA Bison (clone pCowl) Xist DNA sequence | 791 | 0.0 |
| gb\|U50911.1\|ECU50911 Equus caballus Xist gene, promoter reg... | 394 | e-107 |
| gb\|M97168.1\|HUMXIST Homo sapiens X (inactive)-specific tran... | 101 | 3e-19 |
| gb\|L10732.1\|RABXISTAA Rabbit (clone pRabl) DNA sequence | 100 | 1e-18 |

Alignments:

Bos taurus XIST, partial sequence, Length = 2182, Score = 862 bits (435),
Expect = 0.0, Identities = 443/446 (99%), Strand = Plus / Minus

```
Query: 42   acccttcctgtntgggtgggacacgcggcaatctaacattgccaatccctcctttcactt 101
            |||||| |||  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1690 accctttctgattgggtgggacacgcggcaatctaacattgccaatccctcctttcactt 1631

Query: 102  tgtccgcggcaaagcacgccatctttaacaatgcggcaggccctcctgatgcaaagcccg 161
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1630 tgtccgcggcaaagcacgccatctttaacaatgcggcaggccctcctgatgcaaagcccg 1571

Query: 162  ccaagaatgtaacggtcaaacacccctgccacgatatccacgtgacaaactgccatgata 221
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1570 ccaagaatgtaacggtcaaacacccctgccacgatatccacgtgacaaactgccatgata 1511

Query: 222  tccacatgacaaggtcagccatattgtccctgcagcaaaacctatcaggaaatgcacacg 281
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1510 tccacatgacaaggtcagccatattgtccctgcagcaaaacctatcaggaaatgcacacg 1451

Query: 282  gcagagacaaaagcccgccatgttttagactgcggcaagtacttaagcggccatctttta 341
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1450 gcagagacaaaagcccgccatgttttagactgcggcaagtacttaagcggccatctttta 1391

Query: 342  ctatgtaacagaatatttctaccgccatgctgcacactagaacaaagacacccatgatgt 401
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1390 ctatgtaacagaatatttctaccgccatgctgcacactagaacaaagacacccatgatgt 1331

Query: 402  ggcttcccgccattggaacactgcggcaaaatggatccgccattttgaacactctaacaa 461
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1330 ggcttcccgccattggaacactgcggcaaaatggatccgccattttgaacactctaacaa 1271

Query: 462  agcagagagccatgctaagcaatgct 487
            ||||||||||||||||||||||||||
Sbjct: 1270 agcagagagccatgctaagcaatgct 1245
```

Appendix II. Sequence 1 (Reverse)-Continue.

Bison (clone pCowl) Xist DNA sequence, Length =650, Score =791 bits(399), Expect = 0.0, Identities= 436/446(97%), Gaps= 2/446(0%), Strand= Plus/Minus

```
Query: 42   acccttcctgtntgggtgggacacgcggcaatctaacattgccaatccctcctttcactt 101
            |||||| |||  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 576  accctttctgattgggtgggacacgcggcaatctaacattgccaatccctcctttcactt 517

Query: 102  tgtccgcggcaaagcacgccatctttaacaatgcggcaggccctcctgatgcaaagcccg 161
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 516  tgtccgcggcaaagcacgccatctttaacaatgcggcaggccctcctgatgcaaagcccg 457

Query: 162  ccaagaatgtaacggtcaaacacccctgccacgatatccacgtgacaaactgccatgata 221
            |||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct: 456  ccaagaatgtaacggtcaaacaaccctgccacgatatccacgtgacaaactgccatgata 397

Query: 222  tccacatgacaaggtcagccatattgtccctgcagcaaaacctatcaggaaatgcacacg 281
            ||||||||||||||||||||||||||| ||||||||    ||||||||||||||||||||
Sbjct: 396  tccacatgacaaggtcagccatattgt-cctgcagcttttcctatcaggaaatgcacacg 338

Query: 282  gcagagacaaaagcccgccatgttttagactgcggcaagtacttaagcggccatctttta 341
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 337  gcagagacaaaagcccgccatgttttagactgcggcaagtacttaagcggccatctttta 278

Query: 342  ctatgtaacagaatatttctaccgccatgctgcacactagaacaaagacacccatgatgt 401
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 277  ctatgtaacagaatatttctaccgccatgctgcacactagaacaaagacacccatgatgt 218

Query: 402  ggcttcccgccattggaacactgcggcaaaatggatccgccattttgaacactctaacaa 461
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 217  -gcttcccgccattggaacactgcggcaaaatggatccgccattttgaacactctaacaa 159

Query: 462  agcagagagccatgctaagcaatgct 487
            ||||||||||||||||||||||||||
Sbjct: 158  agcagagagccatgctaagcaatgct 133
```

Equus caballus Xist gene, promoter region and exon 1, Length =6055, Score= 394 bits (199), Expect =e-107, Identities =334/373(89%), Gaps =7/373 (1%), Strand = Plus/Minus

```
Query: 118   cgccatctttaacaatgcggcaggccctcctgatgcaaagcccgccaagaatgtaacggt 177
             ||||||||||||||||||||||||||| |||| |||||||||||||||||||||| ||| 
Sbjct: 5323  cgccatctttaacaatgcggcaggcccacctggtgcaaagcccgccaagaatgtaccggg 5264

Query: 178   caaacacccct-gccacgatatccacgtgacaaactgccatgatatccacatgacaaggt 236
             ||||  ||||  |||| ||  |||||||||| ||||||||||||||||||||||||||||
Sbjct: 5263  caaaatccccccgccatgacgtccacgtgacgaactgccatgatatccacatgacaaggt 5204

Query: 237   cagccatattgtccctgcagcaaaacctatcaggaaatgcacacggcagagacaaaagcc 296
             | |||||||||||||||| ||||||||| ||||||||||||||||||| | |||||||||
Sbjct: 5203  ctgccatattgtccctgcggcaaaacctgtcaggaaatgcacacggcaaacacaaaagcc 5144
```

Appendix II. Sequence 1 (Reverse)-Continue.

```
Query: 297  cgccatgttttagactgcggcaagtacttaagcggccatcttttactatgtaacagaata 356
            |||||| ||||| |||||||||||||||| ||| |||||||||| | |||||||||||||
Sbjct: 5143 cgccatattttatactgcggcaagtacttcagccgccatcttttgccatgtaacagaata 5084

Query: 357  tttctaccgccatgctgcacactagaacaaagacac--ccatgatgtgg-cttcccgcca 413
            |||| ||||||||||||| |||||||||||||||||  ||||||||||| ||||||||||
Sbjct: 5083 tttccaccgccatgctgcgcactagaacaaagacaccaccatgatgtggccttcccgcca 5024

Query: 414  ttggaacactgcggcaaaa-tggatccgccattttgaacactctaacaaagcagagagcc 472
             | |||||||||||||||| ||  |||||||||||| |||  ||||||||||||  ||||
Sbjct: 5023 cttgaacactgcggcaaaactgtgtccgccattttggaca--ctaacaaagcaggcagcc 4966

Query: 473  atgctaagcaatg 485
            ||||| |||||||
Sbjct: 4965 atgctgagcaatg 4953


Homo sapiens XIST. complete exon, Length = 16481
 Score =  101 bits (51), Expect = 3e-19, Identities = 130/154 (84%),
 Gaps = 3/154 (1%), Strand = Plus / Minus

Query: 189  gccacgatatccacgtgacaaactgccatgatatccacatgacaaggtcagccatattgt 248
            |||| ||| ||||||||||||| |||||||||| || ||||||| | | ||||||||||
Sbjct: 1518 gccatgatgtccacgtgacaaa-gccatgatatacatatgacaacgcctgccatattgt 1460

Query: 249  ccctgcagcaaaacctatcaggaaatgcacacggcagagacaa--aagcccgccatgttt 306
            |||||| |||||||| | || |||| |||||| ||| ||||||  |.|||||||||||||
Sbjct: 1459 ccctgcggcaaaacccaacacgaaaagcacacagcaaagacaaagaggcccgccatgttt 1400

Query: 307  tagactgcggcaagtacttaagcggccatctttt 340
            || |||||||||||  ||| ||| ||||||||||
Sbjct: 1399 tacactgcggcaagaccttcagccgccatctttt 1366


 Score = 60.0 bits (30), Expect = 1e-06, Identities = 65/74 (87%),
 Gaps = 2/74 (2%), Strand = Plus / Minus

Query: 394  catgatgtggc-ttcccgccattggaacactgcggcaaaa-tggatccgccattttgaac 451
            ||||||||||| ||||||||| | |||||||||| || || |||||||||||||||| ||
Sbjct: 1308 catgatgtggccttcccgccacttgaacactgcgacagaactggatccgccattttggac 1249

Query: 452  actctaacaaagca 465
            |  |||||||||||
Sbjct: 1248 aacctaacaaagca 1235


 Score = 52.0 bits (26), Expect = 3e-04, Identities = 35/38 (92%)
 Strand = Plus / Minus

Query: 107  gcggcaaagcacgccatctttaacaatgcggcaggccc 144
            |||||||| | |||||||||||||||||||||| ||||
Sbjct: 1556 gcggcaaaacccgccatctttaacaatgcggcaagccc 1519
```

Appendix II. Sequence 1 (Reverse)- Continue.

Rabbit (clone pRab1) Xist DNA sequence, Length = 570

```
 Score = 99.6 bits (50), Expect = 1e-18,
 Identities = 107/125 (85%),
 Gaps = 2/125 (1%), Strand = Plus / Minus


Query: 198 tccacgtgacaaactgccatgatatccacatgacaaggtcagccatattgtccctgcagc 257
           |||||||||||||   |||||||| |||||||| |  |||||||||||||||||||| ||
Sbjct: 437 tccacgtgacaaatctccatgataaccacatgatatcgtcagccatattgtccctgcggc 378

Query: 258 aaaacctatcaggaaatgcacacggcagagacaa--aagcccgccatgtttagactgcg 315
           ||||||   || ||||||||||||||| ||||||  | ||||||||| ||||| ||||||
Sbjct: 377 aaaacccgccacgaaatgcacacggcaaagacaaagacgcccgccatattttacactgcg 318

Query: 316 gcaag 320
           |||||
Sbjct: 317 gcaag 313


 Score = 44.1 bits (22), Expect = 0.063
 Identities = 28/30 (93%)
 Strand = Plus / Minus

Query: 107 gcggcaaagcacgccatctttaacaatgcg 136
           ||||||| || ||||||||||||||||||
Sbjct: 522 gcggcaacgcccgccatctttaacaatgcg 493
```

Appendix II. Sequense 2.

The results of the BLAST search for srtand-specific RT-PCR product, representing the smaller band in 3' lanes in figure 16 a), obtained from female fetal lung.

PCR product:

```
TTNNTTTGAATGTCCGTTNAAGTTCACCATGGCNGATNCATTTTGCCGCAGTGTNCCAATGGCGGGAAGCCACATCATGGG
TGTCTTTGTTCTAGTGTGCAGCATGGCGGTAGAATCGGTCACAGCCAAGCNTGGCTNTGANNAANNTTACNCNGCCANANT
NNGCANAGCCACTTNGCTNTGNTNAACTTTACCCAGCCNAANTTNTNAANACTNTATCGNNNTGTNCNNCNTTGCTCANCN
AANTTANNTNNANTGNNTTTNNTTNNNTNNNNNNTTTNCNNNCNNNNNNNNTNNNNNNNC
```

| Sequences producing significant alignments: | Score (bits) | E Value |
|---|---|---|
| gb\|AF104906.5\|AF104906 Bos taurus X-inactive specific trans... | 155 | 1e-35 |
| gb\|L10727.1\|BISXISTAA Bison (clone pCowl) Xist DNA sequence | 139 | 8e-31 |
| gb\|U50911.1\|ECU50911 Equus caballus Xist gene, promoter reg... | 80 | 7e-13 |

Alignments:

Bos taurus XIST gene, partial sequence, Length = 2182, Score = 155 bits (78), Expect = 1e-35, Identities = 84/87 (96%), Strand = Plus / Plus

```
Query: 29   atggcngatncatttgccgcagtgtnccaatggcgggaagccacatcatgggtgtcttt 88
            ||||| ||| ||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct: 1288 atggcggatccatttgccgcagtgttccaatggcgggaagccacatcatgggtgtcttt 1347

Query: 89   gttctagtgtgcagcatggcggtagaa 115
            |||||||||||||||||||||||||||
Sbjct: 1348 gttctagtgtgcagcatggcggtagaa 1374
```

Score = 40.1 bits (20), Expect = 0.58, Identities = 20/20 (100%), Strand = Plus/Plu s

```
Query: 109  ggtagaatcggtcacagcca 128
            ||||||||||||||||||||
Sbjct: 1691 ggtagaatcggtcacagcca 1710
```

Bison (clone pCowl) Xist DNA sequence, Length = 650, Score = 139 bits (70), Expect = 8e-31,
Identities = 83/87 (95%), Gaps = 1/87 (1%), Strand = Plus / Plus

```
Query: 29  atggcngatncatttgccgcagtgtnccaatggcgggaagccacatcatgggtgtcttt 88
           ||||| ||| ||||||||||||||| ||||||||||||||| |||||||||||||||||
Sbjct: 176 atggcggatccatttgccgcagtgttccaatggcgggaagc-acatcatgggtgtcttt 234

Query: 89  gttctagtgtgcagcatggcggtagaa 115
           |||||||||||||||||||||||||||
Sbjct: 235 gttctagtgtgcagcatggcggtagaa 261
```

Appendix II. Sequense 2-Continue.

```
 Score = 40.1 bits (20), Expect = 0.58
 Identities = 20/20 (100%)
 Strand = Plus / Plus


Query: 109 ggtagaatcggtcacagcca 128
           ||||||||||||||||||||
Sbjct: 577 ggtagaatcggtcacagcca 596
```

Equus caballus Xist gene, promoter region and exon 1, Length = 6055

```
 Score = 79.8 bits (40), Expect = 7e-13
 Identities = 67/74 (90%), Gaps = 3/74 (4%)
 Strand = Plus / Plus


Query: 41    ttttgccgcagtgtnccaatggcgggaag-ccacatcatgg--gtgtctttgttctagtg 97
             |||||||||||||| | | ||||||||| ||||||||||  ||||||||||||||||
Sbjct: 5005  ttttgccgcagtgttcaagtggcgggaaggccacatcatggtggtgtctttgttctagtg 5064

Query: 98    tgcagcatggcggt 111
              |||||||||||||
Sbjct: 5065  cgcagcatggcggt 5078
```

Appendix II. Sequence 2 (Reverse).

The result of the BLAST search for strand-specific RT-PCR product, (representing the smaller band in 3' lanes in figure 16 a), obtained from female fetal lung sequensed by reverse primer.

The sequence of PCR product:

TTGAAAGNCCTTTGGNANNNTTTGGANTGCCGTTCACTANGAACANATANACTNCATGATGTGGCTTCCCGCCATTGG
AACACTGCGGCAAAATGGATCCGCCATTTTGAACACTCTAACAAAGCAGAGAGCCATGCTAAGCAATGCTAACNTGGC
TGTGANNAANATNNCNCGGNCNAANNNGGCANAGCCANTTNGCATTGCTTANCATGGCTCGGGNGAAANTNNCNNAGA
CNNTTNNNNTTNNTTTNNNTGGGTTNNNNGNAANNNNNNNNNNNNNNNNNNNNNNNNTNNNNNNNNNNNNNNNNNNNC

| Sequences producing significant alignments: | Score (bits) | E Value |
|---|---|---|
| gb\|AF104906.5\|AF104906 Bos taurus X-inactive specific trans... | 186 | 4e-45 |
| gb\|L10727.1\|BISXISTAA Bison (clone pCow1) Xist DNA sequence | 170 | 2e-40 |
| gb\|U50911.1\|ECU50911 Equus caballus Xist gene, promoter reg... | 70 | 7e-10 |
| gb\|M97168.1\|HUMXIST Homo sapiens X (inactive)-specific tran... | 60 | 6e-07 |

Alignments:

Bos taurus XIST gene, partial sequence (Length = 2182).

Score = 186 bits (94), Expect = 4e-45, Identities = 94/94 (100%)
Strand = Plus / Minus

```
Query: 55   catgatgtggcttcccgccattggaacactgcggcaaaatggatccgccattttgaacac 114
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1338 catgatgtggcttcccgccattggaacactgcggcaaaatggatccgccattttgaacac 1279

Query: 115  tctaacaaagcagagagccatgctaagcaatgct 148
            ||||||||||||||||||||||||||||||||||
Sbjct: 1278 tctaacaaagcagagagccatgctaagcaatgct 1245
```

Bison (clone pCow1) Xist DNA sequence, Length = 650

Score = 170 bits (86), Expect = 2e-40
Identities = 93/94 (98%), Gaps = 1/94 (1%), Strand = Plus / Minus

```
Query: 55  catgatgtggcttcccgccattggaacactgcggcaaaatggatccgccattttgaacac 114
           ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 225 catgatgtg-cttcccgccattggaacactgcggcaaaatggatccgccattttgaacac 167

Query: 115 tctaacaaagcagagagccatgctaagcaatgct 148
           ||||||||||||||||||||||||||||||||||
Sbjct: 166 tctaacaaagcagagagccatgctaagcaatgct 133
```

Equus caballus Xist gene, promoter region and exon 1, Length = 6055.

Score = 69.9 bits (35), Expect = 7e-10
Identities = 82/94 (87%), Gaps = 4/94 (4%)
Strand = Plus / Minus

```
Query: 55   catgatgtggc-ttcccgccattggaacactgcggcaaaa-tggatccgccattttgaac 112
            ||||||||||| ||||||||| | |||||||||||||||| ||  ||||||||||| ||
Sbjct: 5044 catgatgtggccttcccgccacttgaacactgcggcaaaactgtgtccgccattttggac 4985
```

Appendix II. Sequence 2 (Reverse)-Continue.

```
Query: 113  actctaacaaagcagagagccatgctaagcaatg 146
            |  ||||||||||||  ||||||||| |||||||
Sbjct: 4984 a--ctaacaaagcaggcagccatgctgagcaatg 4953
```

Homo sapiens X (inactive)-specific transcript (XIST) complete exon, Length = 16481

```
 Score = 60.0 bits (30), Expect = 6e-07
 Identities = 65/74 (87%), Gaps = 2/74 (2%)
 Strand = Plus / Minus

Query: 55   catgatgtggc-ttcccgccattggaacactgcggcaaaa-tggatccgccattttgaac 112
            ||||||||||| ||||||||| | |||||||||| || || |||||||||||||||| ||
Sbjct: 1308 catgatgtggccttcccgccacttgaacactgcgacagaactggatccgccattttggac 1249

Query: 113  actctaacaaagca 126
            |  |||||||||||
Sbjct: 1248 aacctaacaaagca 1235
```

Appendix II. Sequence 3.

The results of the BLAST search for strand-specific RT-PCR product, primed by upstream primer (133, 5') representing the expression of antisense strand in bovine fetal ovary.

PCR product:

```
AAACCNNNNTGGNANATGGGNGNGACCAACATTNCCANCCCTCCTTTCACTTTGTCCGCGGCAAANCACGCCATCTTT
AACAATGCGGCAGGCCCTCCTGATGCAAAGCCNGCCAAGAATGTAACGGTCAAACACCCCTGCCACGATATCCACGTG
ACAAACTGCCATGATATCCACATGACAAGGTCAGCCATATTGTCCCTGCAGCAAAACCTATCAGGAAATGCACACGGC
AGAGACAAAAGCCCGCCATGTTTTAGACTGCGGCAAGTACTTAAGCGGCCATCTTTTACTATGTAACAGAATATTTCT
ACCGCCATGCTGCACACTAGAACAAAGACACCCATGATGTGGCTTCCCGCCATTGGAACACTGCGGCAAAATGGATCC
GCCATTTTGAACACTCTAACAAAGCAGAGAGCCATGCTAAGCAATGCTANTTTNTNTNGNTNNANTTGTTTNCTNGNT
TNNNNNNTAT
```

| Sequences producing significant alignments: | (bits) | Value |
|---|---|---|
| gb\|AF104906.5\|AF104906 Bos taurus X-inactive specific trans... | 781 | 0.0 |
| gb\|L10727.1\|BISXISTAA Bison (clone pCow1) Xist DNA sequence | 710 | 0.0 |
| gb\|U50911.1\|ECU50911 Equus caballus Xist gene, promoter reg... | 391 | e-106 |
| gb\|M97168.1\|HUMXIST Homo sapiens X (inactive)-specific tran... | 101 | 3e-19 |
| gb\|L10732.1\|RABXISTAA Rabbit (clone pRab1) DNA sequence | 100 | 1e-18 |

Alignments:

Bos taurus XIST gene, partial sequence (Length = 2182)

```
 Score =  781 bits (394), Expect = 0.0
 Identities = 398/400 (99%)
 Strand = Plus / Minus

Query: 39   ccctcctttcactttgtccgcggcaaancacgccatctttaacaatgcggcaggccctcc 98
            |||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct: 1644 ccctcctttcactttgtccgcggcaaagcacgccatctttaacaatgcggcaggccctcc 1585

Query: 99   tgatgcaaagccngccaagaatgtaacggtcaaacacccctgccacgatatccacgtgac 158
            |||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1584 tgatgcaaagcccgccaagaatgtaacggtcaaacacccctgccacgatatccacgtgac 1525

Query: 159  aaactgccatgatatccacatgacaaggtcagccatattgtccctgcagcaaaacctatc 218
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1524 aaactgccatgatatccacatgacaaggtcagccatattgtccctgcagcaaaacctatc 1465

Query: 219  aggaaatgcacacggcagagacaaaagcccgccatgttttagactgcggcaagtacttaa 278
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1464 aggaaatgcacacggcagagacaaaagcccgccatgttttagactgcggcaagtacttaa 1405

Query: 279  gcggccatcttttactatgtaacagaatatttctaccgccatgctgcacactagaacaaa 338
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1404 gcggccatcttttactatgtaacagaatatttctaccgccatgctgcacactagaacaaa 1345

Query: 339  gacacccatgatgtggcttcccgccattggaacactgcggcaaaatggatccgccatttt 398
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1344 gacacccatgatgtggcttcccgccattggaacactgcggcaaaatggatccgccatttt 1285

Query: 399  gaacactctaacaaagcagagagccatgctaagcaatgct 438
            ||||||||||||||||||||||||||||||||||||||||
Sbjct: 1284 gaacactctaacaaagcagagagccatgctaagcaatgct 1245
```

Appendix II. Sequence 3-Continue.

Bison Xist (clone pCow1) DNA sequence, Length = 650

```
 Score =  710 bits (358), Expect = 0.0
 Identities = 391/400 (97%), Gaps = 2/400 (0%)
 Strand = Plus / Minus

Query: 39  ccctcctttcactttgtccgcggcaaancacgccatctttaacaatgcggcaggccctcc 98
           ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct: 530 ccctcctttcactttgtccgcggcaaagcacgccatctttaacaatgcggcaggccctcc 471

Query: 99  tgatgcaaagccngccaagaatgtaacggtcaaacacccctgccacgatatccacgtgac 158
           |||||||||||| ||||||||||||||||||||||| |||||||||||||||||||||||
Sbjct: 470 tgatgcaaagcccgccaagaatgtaacggtcaaacaaccctgccacgatatccacgtgac 411

Query: 159 aaactgccatgatatccacatgacaaggtcagccatattgtccctgcagcaaaacctatc 218
           ||||||||||||||||||||||||||||||||||||||||| ||||||||    ||||||
Sbjct: 410 aaactgccatgatatccacatgacaaggtcagccatattgt-cctgcagcttttcctatc 352

Query: 219 aggaaatgcacacggcagagacaaaagcccgccatgttttagactgcggcaagtacttaa 278
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 351 aggaaatgcacacggcagagacaaaagcccgccatgttttagactgcggcaagtacttaa 292

Query: 279 gcggccatcttttactatgtaacagaatatttctaccgccatgctgcacactagaacaaa 338
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 291 gcggccatcttttactatgtaacagaatatttctaccgccatgctgcacactagaacaaa 232

Query: 339 gacacccatgatgtggcttcccgccattggaacactgcggcaaaatggatccgccatttt 398
           |||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 231 gacacccatgatgt-gcttcccgccattggaacactgcggcaaaatggatccgccatttt 173

Query: 399 gaacactctaacaaagcagagagccatgctaagcaatgct 438
           ||||||||||||||||||||||||||||||||||||||||
Sbjct: 172 gaacactctaacaaagcagagagccatgctaagcaatgct 133
```

Equus caballus Xist gene, promoter region and exon 1, Length = 6055

```
 Score =  391 bits (197), Expect = e-106
 Identities = 342/385 (88%), Gaps = 7/385 (1%)
 Strand = Plus / Minus

Query: 57   cgcggcaaancacgccatctttaacaatgcggcaggccctcctgatgcaaagccngccaa 116
            |||| |||| | ||||||||||||||||||||||||||| |||| ||||||||| |||||
Sbjct: 5335 cgcgccaaaccccgccatctttaacaatgcggcaggcccacctggtgcaaagcccgccaa 5276

Query: 117  gaatgtaacggtcaaacacccct-gccacgatatccacgtgacaaactgccatgatatcc 175
            ||||||| ||| ||||  ||||  |||| ||  |||||||||| ||||||||||||||||
Sbjct: 5275 gaatgtaccgggcaaaatccccccgccatgacgtccacgtgacgaactgccatgatatcc 5216

Query: 176  acatgacaaggtcagccatattgtccctgcagcaaaacctatcaggaaatgcacacggca 235
            ||||||||||||| |||||||||||||||| ||||||||| |||||||||||||||||||
Sbjct: 5215 acatgacaaggtctgccatattgtccctgcggcaaaacctgtcaggaaatgcacacggca 5156

Query: 236  gagacaaaagcccgccatgttttagactgcggcaagtacttaagcggccatcttttacta 295
             | ||||||||||||||| ||||| |||||||||||||||| ||| |||||||||| | |
Sbjct: 5155 aacacaaaagcccgccatattttatactgcggcaagtacttcagccgccatcttttgcca 5096
```

Appendix II. Sequence 3. Continue.

```
Query: 296  tgtaacagaatatttctaccgccatgctgcacactagaacaaagacac--ccatgatgtg 353
            |||||||||||||||| ||||||||||||| |||||||||||||||||  ||||||||||
Sbjct: 5095 tgtaacagaatatttccaccgccatgctgcgcactagaacaaagacaccaccatgatgtg 5036

Query: 354  g-cttcccgccattggaacactgcggcaaaa-tggatccgccatttgaacactctaaca 411
            | |||||||||| | |||||||||||||||| ||  ||||||||||| |||  ||||||
Sbjct: 5035 gccttcccgccacttgaacactgcggcaaaactgtgtccgccatttggaca--ctaaca 4978

Query: 412  aagcagagagccatgctaagcaatg 436
            ||||||  ||||||||| |||||||
Sbjct: 4977 aagcaggcagccatgctgagcaatg 4953
```

Homo sapiens XIST complete exon Length = 16481

```
 Score =  101 bits (51), Expect = 3e-19
 Identities = 130/154 (84%), Gaps = 3/154 (1%)
 Strand = Plus / Minus

Query: 140  gccacgatatccacgtgacaaactgccatgatatccacatgacaaggtcagccatattgt 199
            |||| ||| |||||||||||||  |||||||||| || ||||||| | | ||||||||||
Sbjct: 1518 gccatgatgtccacgtgacaaaa-gccatgatatacatatgacaacgcctgccatattgt 1460

Query: 200  ccctgcagcaaaacctatcaggaaatgcacacggcagagacaa--aagcccgccatgttt 257
            |||||| |||||||| | || |||| |||||| ||| ||||||  | |||||||||||||
Sbjct: 1459 ccctgcggcaaaacccaacacgaaaagcacacagcaaagacaaagaggcccgccatgttt 1400

Query: 258  tagactgcggcaagtacttaagcggccatctttt 291
            || |||||||||||  ||| ||| ||||||||||
Sbjct: 1399 tacactgcggcaagaccttcagccgccatctttt 1366


 Score = 60.0 bits (30), Expect = 1e-06
 Identities = 65/74 (87%), Gaps = 2/74 (2%)
 Strand = Plus / Minus

Query: 345  catgatgtggc-ttcccgccattggaacactgcggcaaaa-tggatccgccatttgaac 402
            ||||||||||| ||||||||| | |||||||||| || || |||||||||||||||| ||
Sbjct: 1308 catgatgtggccttcccgccacttgaacactgcgacagaactggatccgccattttggac 1249

Query: 403  actctaacaaagca 416
            |  |||||||||||
Sbjct: 1248 aacctaacaaagca 1235


 Score = 54.0 bits (27), Expect = 6e-05
 Identities = 35/38 (92%)
 Strand = Plus / Minus

Query: 58   gcggcaaancacgccatctttaacaatgcggcaggccc 95
            |||||||| | |||||||||||||||||||||| ||||
Sbjct: 1556 gcggcaaaacccgccatctttaacaatgcggcaagccc 1519
```

Appendix II. Sequence 3. Continue.

Rabbit (clone pRab1) Xist DNA sequence, Length = 570

```
 Score = 99.6 bits (50), Expect = 1e-18
 Identities = 107/125 (85%), Gaps = 2/125 (1%)
 Strand = Plus / Minus

Query: 149 tccacgtgacaaactgccatgatatccacatgacaaggtcagccatattgtccctgcagc 208
           |||||||||||||   |||||||| |||||||| |  |||||||||||||||||||| ||
Sbjct: 437 tccacgtgacaaatctccatgataaccacatgatatcgtcagccatattgtccctgcggc 378

Query: 209 aaaacctatcaggaaatgcacacggcagagacaa--aagcccgccatgttttagactgcg 266
           ||||||   || ||||||||||||||| ||||||  | ||||||||| ||||| ||||||
Sbjct: 377 aaaacccgccacgaaatgcacacggcaaagacaaagacgcccgccatattttacactgcg 318

Query: 267 gcaag 271
           |||||
Sbjct: 317 gcaag 313


 Score = 38.2 bits (19), Expect = 3.7
 Identities = 19/19 (100%)
 Strand = Plus / Minus

Query: 69  cgccatctttaacaatgcg 87
           |||||||||||||||||||
Sbjct: 511 cgccatctttaacaatgcg 493
```

Appendix II. Sequence 4

The result of the BLAST search for RT-PCR product of Xist in bovine fetal testis, sequenced by using a pair of nested primers (P495, and p190).

PCR product:

```
CGGGAACCCATCATGGGTGTCTTTGTTCTAGTGTGCAGCATGGCGGTAGAAATATTCTGTTACATAGTAAAA
GATGGCCGCTTAAGTACTTGCCGCAGTCTAAAACATGGCGGGCTTTTGTCTCTGCCGTGTGCATTTCCTGAT
AGGTTTTGCTGCAGGGACAATATGGCTGACCTTGTCATGTGGATATCATGGCAGTTTGTCACGTGGATATCG
TGGCAGGGGTGTTTGACCGTTACATTCTTNGGGNNNNNNNNNNN
```

| Sequences producing significant alignments: | | Score (bits) | E Value |
|---|---|---|---|
| gb\|AF104906.5\|AF104906 | Bos taurus X-inactive specific trans... | 470 | e-130 |
| gb\|L10727.1\|BISXISTAA | Bison (clone pCowl) Xist DNA sequence | 414 | e-114 |
| gb\|U50911.1\|ECU50911 | Equus caballus Xist gene, promoter reg... | 278 | 1e-72 |
| gb\|M97168.1\|HUMXIST | Homo sapiens X (inactive)-specific tran... | 101 | 2e-19 |
| gb\|L10732.1\|RABXISTAA | Rabbit (clone pRabl) Xist DNA sequence | 100 | 6e-19 |

Alignments:

Bos taurus XIST gene, partial sequence, Length = 2182.

Score = 470 bits (237), Expect = e-130, Identities = 237/237 (100%)
Strand = Plus / Plus

```
Query: 9    catcatgggtgtctttgttctagtgtgcagcatggcggtagaaatattctgttacatagt 68
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1332 catcatgggtgtctttgttctagtgtgcagcatggcggtagaaatattctgttacatagt 1391

Query: 69   aaaagatggccgcttaagtacttgccgcagtctaaaacatggcgggcttttgtctctgcc 128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1392 aaaagatggccgcttaagtacttgccgcagtctaaaacatggcgggcttttgtctctgcc 1451

Query: 129  gtgtgcatttcctgataggttttgctgcagggacaatatggctgaccttgtcatgtggat 188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1452 gtgtgcatttcctgataggttttgctgcagggacaatatggctgaccttgtcatgtggat 1511

Query: 189  atcatggcagtttgtcacgtggatatcgtggcaggggtgtttgaccgttacattctt 245
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1512 atcatggcagtttgtcacgtggatatcgtggcaggggtgtttgaccgttacattctt 1568
```

Bison (clone pCowl) DNA sequence, Length = 650

Score = 414 bits (209), Expect = e-114, Identities = 231/237 (97%),
Gaps = 1/237 (0%), Strand = Plus / Plus

```
Query: 9   catcatgggtgtctttgttctagtgtgcagcatggcggtagaaatattctgttacatagt 68
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 219 catcatgggtgtctttgttctagtgtgcagcatggcggtagaaatattctgttacatagt 278

Query: 69  aaaagatggccgcttaagtacttgccgcagtctaaaacatggcgggcttttgtctctgcc 128
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 279 aaaagatggccgcttaagtacttgccgcagtctaaaacatggcgggcttttgtctctgcc 338

Query: 129 gtgtgcatttcctgataggttttgctgcagggacaatatggctgaccttgtcatgtggat 188
           |||||||||||||||||||    |||||| ||||||||||||||||||||||||||||||
Sbjct: 339 gtgtgcatttcctgataggaaaagctgca-ggacaatatggctgaccttgtcatgtggat 397
```

Appendix II. Sequence 4- Continue.

```
Query: 189 atcatggcagtttgtcacgtggatatcgtggcaggggtgtttgaccgttacattctt 245
           |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 398 atcatggcagtttgtcacgtggatatcgtggcagggttgtttgaccgttacattctt 454
```

Equus caballus Xist gene, promoter region and exon 1, Length = 6055

Score = 278 bits (140), Expect = 1e-72, Identities = 182/196 (92%)
Strand = Plus / Plus

```
Query: 16   ggtgtctttgttctagtgtgcagcatggcggtagaaatattctgttacatagtaaaagat 75
            |||||||||||||||||| ||||||||||||| ||||||||||||||||| | |||||||
Sbjct: 5047 ggtgtctttgttctagtgcgcagcatggcggtggaaatattctgttacatggcaaaagat 5106

Query: 76   ggccgcttaagtacttgccgcagtctaaaacatggcgggcttttgtctctgccgtgtgca 135
            ||| ||| |||||||||||||||| ||||| ||||||||||||||| | |||||||||||
Sbjct: 5107 ggcggctgaagtacttgccgcagtataaaatatggcgggcttttgtgtttgccgtgtgca 5166

Query: 136  tttcctgataggttttgctgcagggacaatatggctgaccttgtcatgtggatatcatgg 195
            |||||||| ||||||||| |||||||||||||||| ||||||||||||||||||||||||
Sbjct: 5167 tttcctgacaggttttgccgcagggacaatatggcagaccttgtcatgtggatatcatgg 5226

Query: 196  cagtttgtcacgtgga 211
            ||||| ||||||||||
Sbjct: 5227 cagttcgtcacgtgga 5242
```

Human (Homo sapiens) XIST complete exon, Length = 16481.

Score = 101 bits (51), Expect = 2e-19, Identities = 130/154 (84%),
Gaps = 3/154 (1%), Strand = Plus / Plus

```
Query: 69   aaaagatggccgcttaagtacttgccgcagtctaaaacatggcgggctt--ttgtctctg 126
            |||||||||| ||| |||  ||||||||||| ||||||||||||||| |  |||||| ||
Sbjct: 1366 aaaagatggcggctgaaggtcttgccgcagtgtaaaacatggcgggcctctttgtctttg 1425

Query: 127  ccgtgtgcatttcctgataggttttgctgcagggacaatatggctgaccttgtcatgtgg 186
            | |||||| |||| || | |||||||| |||||||||||||||| | | ||||||| ||
Sbjct: 1426 ctgtgtgcttttcgtgttgggttttgccgcagggacaatatggcaggcgttgtcatatgt 1485

Query: 187  atatcatggcagtttgtcacgtggatatcgtggc 220
            ||||||||||  ||||||||||||| ||| ||||
Sbjct: 1486 atatcatggc-ttttgtcacgtggacatcatggc 1518
```

Rabbit (clone pRab1) Xist DNA sequence, Length = 570

Score = 99.6 bits (50), Expect = 6e-19, Identities = 107/125 (85%),
Gaps = 2/125 (1%), Strand = Plus / Plus

```
Query: 89  cttgccgcagtctaaaacatggcgggctt--ttgtctctgccgtgtgcatttcctgatag 146
           ||||||||||| ||||| ||||||||| |  |||||| ||||||||||||||| ||   |
Sbjct: 313 cttgccgcagtgtaaaatatggcgggcgtctttgtctttgccgtgtgcatttcgtggcgg 372
```

Appendix II. Sequence 4- Continue.

```
Query: 147 gttttgctgcagggacaatatggctgaccttgtcatgtggatatcatggcagtttgtcac 206
           ||||||| ||||||||||||||||||||  | |||||||| ||||||||   ||||||||
Sbjct: 373 gttttgccgcagggacaatatggctgacgatatcatgtggttatcatggagatttgtcac 432

Query: 207 gtgga 211
           |||||
Sbjct: 433 gtgga 437
```

Appendix II. Sequence 4 (Reverse)- Continue.

The results of the BLAST search for PCR product of Xist in bovine fetal testis, sequenced by using the p190 primer.

PCR products:

```
TGCCCGATATCCCGTGACAAACTGCCTGATATCCACATGACAAGGTCAGCCATATTGTCCCTGCAGCAAAA
CCTATCAGGAAATGCACACGGCAGAGACAAAAGCCCGCCATGTTTTAGACTGCGGCAAGTACTTAAGCGGC
CATCTTTTACTATGTAACAGAATATTTCTACCGCCATGCTGCACACTAGAACAAAGACACCCATGATGTGG
CTTCCCGCCATTGGAACACTGCGGNANNNNNNNNNN
```

| Sequences producing significant alignments: | Score (bits) | E Value |
|---|---|---|
| gb\|AF104906.5\|AF104906 Bos taurus X-inactive specific trans... | 434 | e-120 |
| gb\|L10727.1\|BISXISTAA Bison (clone pCow1) Xist DNA sequence | 371 | e-100 |
| gb\|U50911.1\|ECU50911 Equus caballus Xist gene, promoter reg... | 274 | 2e-71 |
| gb\|M97168.1\|HUMXIST Homo sapiens X (inactive)-specific tran... | 86 | 9e-15 |
| gb\|L10732.1\|RABXISTAA Rabbit (clone pRab1) DNA sequence | 86 | 9e-15 |

Alignments:

Bos taurus XIST gene, partial sequence, Length = 2182.

Score = 434 bits (219), Expect = e-120, Identities = 233/235 (99%),
Gaps = 2/235 (0%), Strand = Plus / Minus

```
Query: 5    cgatatcc-cgtgacaaactgcc-tgatatccacatgacaaggtcagccatattgtccct 62
            |||||||| |||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 1539 cgatatccacgtgacaaactgccatgatatccacatgacaaggtcagccatattgtccct 1480

Query: 63   gcagcaaaacctatcaggaaatgcacacggcagagacaaaagcccgccatgttttagact 122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1479 gcagcaaaacctatcaggaaatgcacacggcagagacaaaagcccgccatgttttagact 1420

Query: 123  gcggcaagtacttaagcggccatcttttactatgtaacagaatatttctaccgccatgct 182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1419 gcggcaagtacttaagcggccatcttttactatgtaacagaatatttctaccgccatgct 1360

Query: 183  gcacactagaacaaagacacccatgatgtggcttcccgccattggaacactgcgg 237
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1359 gcacactagaacaaagacacccatgatgtggcttcccgccattggaacactgcgg 1305
```

Bison (clone pCow1) Xist DNA sequence, Length = 650.

Score = 371 bits (187), Expect = e-100, Identities = 227/235 (96%),
Gaps = 4/235 (1%), Strand = Plus / Minus

```
Query: 5   cgatatcc-cgtgacaaactgcc-tgatatccacatgacaaggtcagccatattgtccct 62
           |||||||| |||||||||||||| |||||||||||||||||||||||||||||||||| |
Sbjct: 425 cgatatccacgtgacaaactgccatgatatccacatgacaaggtcagccatattgtcc-t 367

Query: 63  gcagcaaaacctatcaggaaatgcacacggcagagacaaaagcccgccatgttttagact 122
           |||||    |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 366 gcagcttttcctatcaggaaatgcacacggcagagacaaaagcccgccatgttttagact 307
```

Appendix II. Sequence 4 (Reverse)-Continue.

```
Query: 123 gcggcaagtacttaagcggccatcttttactatgtaacagaatatttctaccgccatgct 182
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 306 gcggcaagtacttaagcggccatcttttactatgtaacagaatatttctaccgccatgct 247

Query: 183 gcacactagaacaaagacacccatgatgtggcttcccgccattggaacactgcgg 237
           ||||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct: 246 gcacactagaacaaagacacccatgatgt-gcttcccgccattggaacactgcgg 193
```

Equus caballus Xist gene, promoter region and exon 1, Length = 6055

```
 Score =  274 bits (138), Expect = 2e-71
 Identities = 209/229 (91%), Gaps = 4/229 (1%)
 Strand = Plus / Minus

Query: 13   cgtgacaaactgcc-tgatatccacatgacaaggtcagccatattgtccctgcagcaaaa 71
            |||||| ||||||| ||||||||||||||||||||| |||||||||||||||| ||||||
Sbjct: 5238 cgtgacgaactgccatgatatccacatgacaaggtctgccatattgtccctgcggcaaaa 5179

Query: 72   cctatcaggaaatgcacacggcagagacaaaagcccgccatgttttagactgcggcaagt 131
            ||| ||||||||||||||||||| | ||||||||||||||| ||||| ||||||||||||
Sbjct: 5178 cctgtcaggaaatgcacacggcaaacacaaaagcccgccatattttatactgcggcaagt 5119

Query: 132  acttaagcggccatcttttactatgtaacagaatatttctaccgccatgctgcacactag 191
            |||| ||| |||||||||| | ||||||||||||||||| ||||||||||||| ||||||
Sbjct: 5118 acttcagccgccatcttttgccatgtaacagaatatttccaccgccatgctgcgcactag 5059

Query: 192  aacaaagacac--ccatgatgtgg-cttcccgccattggaacactgcgg 237
            |||||||||||  ||||||||||| |||||||||| | |||||||||||
Sbjct: 5058 aacaaagacaccaccatgatgtggccttcccgccacttgaacactgcgg 5010

Query: 87   acacggcagagacaa--aagcccgccatgttttagactgcggcaag 130
            |||||||| ||||||  | ||||||||| ||||| |||||||||||
Sbjct: 2260 acacggcaaagacaaagacgcccgccatattttacactgcggcaag 2215
```

Human (Homo sapiens) XIST complete exon, Length = 16481.

```
 Score = 85.7 bits (43), Expect = 9e-15
 Identities = 106/126 (84%), Gaps = 2/126 (1%)
 Strand = Plus / Minus

Query: 27   tgatatccacatgacaaggtcagccatattgtccctgcagcaaaacctatcaggaaatgc 86
            |||||| || ||||||| | | |||||||||||||||| |||||||| | || |||| ||
Sbjct: 1491 tgatatacatatgacaacgcctgccatattgtccctgcggcaaaacccaacacgaaaagc 1432

Query: 87   acacggcagagacaa--aagcccgccatgttttagactgcggcaagtacttaagcggcca 144
            |||| ||| ||||||  | ||||||||||||||| |||||||||||  ||| ||| ||||
Sbjct: 1431 acacagcaaagacaaagaggcccgccatgttttacactgcggcaagaccttcagccgcca 1372

Query: 145  tctttt 150
            ||||||
Sbjct: 1371 tctttt 1366
```

Appendix II. Sequence 4 (Reverse)-Continue.

Rabbit (clone pRab1) DNA sequence, Length = 570.

```
 Score = 85.7 bits (43), Expect = 9e-15
 Identities = 91/106 (85%), Gaps = 2/106 (1%)
 Strand = Plus / Minus

Query: 27  tgatatccacatgacaaggtcagccatattgtccctgcagcaaaacctatcaggaaatgc 86
           ||||| |||||||| |  |||||||||||||||||||| ||||||||   || |||||||
Sbjct: 418 tgataaccacatgatatcgtcagccatattgtccctgcggcaaaacccgccacgaaatgc 359

Query: 87  acacggcagagacaa--aagcccgccatgttttagactgcggcaag 130
           |||||||| ||||||  | ||||||||| ||||| |||||||||||
Sbjct: 358 acacggcaaagacaaagacgcccgccatattttacactgcggcaag 313
```

## APPENDIX III. Collection of bovine fetuses, their estimated ages and collected fetal tissues.

| Fetus # | Date | Sex | CRL* (cm) | ~Age** (days) | Tissues collected*** | Remarks |
|---|---|---|---|---|---|---|
| F1 | 20/03/98 | F | 9 | 70 | Gd, Kd, Lv, Lg, ML | |
| F2 | 20/03/98 | F | 10.5 | 80 | Gd, Kd, Lv, Lg,Ml | |
| F3 | 20/04/98 | M | 26.5 | 120 | Gd, Ht,Kd, Lv, Lg,Ml | |
| F4 | 35904 | F | 8.5 | 70 | Gd, Ht,Kd, Lv, Lg,Ml | |
| F5 | 5/05/98 | M | 28 | 120 | Gd, Ht, Kd, Lg, Ml | |
| F6 | 5/05/98 | F | 8.5 | 70 | Gd, Kd, Lg, Ml | |
| F7 | 5/05/98 | F | 8.3 | 70 | Gd, Kd, Lg, Ml | |
| F8 | 5/05/98 | M | 26 | 120 | Gd, Ht, Kd, lg, Lv, Ml | |
| F9 | 6/05/98 | F | 6 | 60 | Gd, Ht, kd, Lg, Lv, Ml | |
| F10 | 6/05/98 | F | 5.8 | 60 | Gd, ht, Lg, Lv, Ml | |
| F11 | 6/05/98 | M | 9.3 | 70 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F12 | 8/05/98 | M | 15.5 | 90 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F13 | 8/05/98 | F | 10.8 | 80 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F14 | 11/05/98 | F | 11.5 | 80 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F15 | 11/05/98 | M | 5.5 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F16 | 11/05/98 | F | 5 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F17 | 13/05/98 | F | 13.5 | 90 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F18 | 13/05/98 | M | 10 | 70 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F19 | 15/05/98 | M | 27 | 120 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F20 | 15/05/98 | M | 24 | 112.5 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F21 | 15/05/98 | M | 25.5 | 116 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F22 | 20/05/98 | M | 19 | 100 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F23 | 20/05/98 | M | 25.5 | 116 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F24 | 21/05/98 | M | 25.5 | 116 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F25 | 21/05/98 | M | 25 | 115 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F26 | 25/05/98 | M | 26 | 117.5 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F27 | 25/05/98 | M | 26.5 | 119 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F28 | 27/05/98 | F | 31 | 131 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F29 | 27/05/98 | F | 8 | 70 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F30 | 29/05/98 | F | 7.8 | 70 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F31) |
| F31 | 29/05/98 | M | 8 | 70 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F30) |
| F32 | 29/05/98 | M | 28 | 122.5 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F33) |
| F33 | 29/05/98 | F | 26 | 119 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F32) |
| F34 | 29/05/98 | M | 50 | 178 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F35 | 1/06/98 | F | 25 | 115 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F36) |
| F36 | 1/06/98 | M | 26 | 117.5 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F35) |
| F37 | 1/06/98 | M | 30 | 127.5 | Gd, Ht, Kd, Lg, Lv, Ml | |

## APPENDIX III- Continue.

| Fetus # | Date | Sex | CRL* (cm) | ~Age** (days) | Tissues collected*** | Remarks |
|---|---|---|---|---|---|---|
| F38 | 3/06/98 | M | 31 | 131 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F39 | 35948 | F | 29 | 125 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F40 | 3/06/98 | F | 29 | 125 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F41 | 5/06/98 | M | 35 | 140 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F42 | 5/06/98 | M | 34 | 137.5 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F43 | 5/06/98 | M | 5 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F44) |
| F44 | 5/06/98 | M | 5.2 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F43) |
| F45 | 8/06/98 | M | 13.5 | 90 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F46) |
| F46 | 8/06/98 | M | 14 | 90 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F45) |
| F47 | 8/06/98 | M | 11 | 80 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F48) |
| F48 | 8/06/98 | F | 10.5 | 80 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F47) |
| F49 | 8/06/98 | F | 32.5 | 136 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F50 | 8/06/98 | M | 29.5 | 126 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F51 | 10/06/98 | M | 40 | 152.5 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F52 | 10/06/98 | F | 37 | 150 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F53 | 12/06/98 | F | 39 | 150 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F54 | 12/06/98 | F | 37 | 150 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F55 | 15/06/98 | M | 30 | 120 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F56 | 15/06/98 | M | 33 | 150 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F57 | 17/06/98 | M | 8.5 | 70 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F58 | 17/06/98 | F | 4.5 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F59 | 22/06/98 | F | 4.8 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F60 | 22/06/98 | F | 5.2 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F61) |
| F61 | 22/06/98 | M | 5.3 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | Twin (with F60) |
| F62 | 22/06/98 | M | 14 | 90 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F63 | 22/06/98 | M | 19.5 | 100 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F64 | 22/06/98 | F | 4 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F65 | 29/06/98 | M | 15 | 90 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F66 | 29/06/98 | M | 4.5 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F67 | 29/06/98 | M | 7 | 70 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F68 | 29/06/98 | F | 6.2 | 60 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F69 | 6/07/98 | M | 16 | 90 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F70 | 6/07/98 | M | 4.8 | 50 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F71 | 6/07/98 | - | - | - | - | - |
| F72 | 6/07/98 | - | 6 | 60 | - | - |
| F73 | 6/07/98 | F | 2.8 | 45 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F74 | 6/07/98 | M | 21.5 | 110 | Gd, Ht, Kd, Lg, Lv, Ml | |

## APPENDIX III- Continue.

| Fetus # | Date | Sex | CRL* (cm) | -Age** (days) | Tissues collected*** | Remarks |
|---|---|---|---|---|---|---|
| F75 | 6/07/98 | F | 20.5 | 110 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F76 | 10/07/98 | F | 2.8 | 40 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F77 | 10/07/98 | F | 6.5 | 60 | Gd, Ht, Kd, Lg, Lv, Ml | |
| F78 | 36466 | M | 20 | 110 | Gd, Kd,Lv, Ml | |
| F79 | 03/11/99 | M | 18.5 | 100 | Gd, kd, Ml, Liv | |
| F80 | 03/11/99 | M | 15 | 90 | Gd, kd, Ml, Liv | |
| F81 | 03/11/99 | F | 15.5 | 90 | Gd, kd, Ml, Liv | |
| F82 | 05/11/99 | M | 9 | 75 | Gd, kd, Ml, Liv | |
| F83 | 05/11/99 | M | 7 | 70 | Gd, kd, Ml, Liv | |
| F84 | 05/11/99 | M | 7 | 70 | Gd, kd, Ml, Liv | |
| F85 | 05/11/99 | M | 12.5 | 80 | Gd, kd, Ml, Liv | |
| F86 | 05/11/99 | M | 15 | 90 | Gd, kd, Ml, Liv | |
| F87 | 05/11/99 | M | 3.5 | 45 | Gd, Liv, Ml | |
| F88 | 12/11/99 | F | 7 | 70 | Gd, Liv, Ml | |
| F89 | 12/11/99 | M | 10 | 80 | Gd, Liv, Ml | |
| F90 | 12/11/99 | F | 10 | 80 | Gd, Liv, Ml | |
| F91 | 12/11/99 | F | 7.5 | 70 | Gd, Liv, Ml | |
| F92 | 26/11/99 | F | 7 | 70 | Gd, Liv, Ml | |
| F93 | 26/11/99 | M | 6 | 60 | Gd, Liv, Ml | |
| F94 | 26/11/99 | M | 4.5 | 50 | Gd, Liv, Ml | |
| F95 | 26/11/99 | M | 7 | 70 | Gd, Liv, Ml | |
| F96 | 26/11/99 | F | 4.5 | 50 | Gd, Liv, Ml, Lg | |
| F97 | 03/12/99 | F | 5.5 | 50 | Gd, Liv, Ml, Lg | |
| F98 | 03/12/99 | M | 6.5 | 60 | Gd, Liv, Ml, Lg | |
| F99 | 03/12/99 | - | 7 | 70 | Gd, Liv, Ml, Ms | |
| F100 | 03/12/99 | M | 3.5 | 45 | Gd, Liv, Ml, Ms | |
| F101 | 13/01/99 | M | 5.8 | 60 | Gd, Liv, Ml | |
| F102 | 14/01/99 | F | 2.5 | 40 | Gd, Liv, Ml | |
| F103 | 18/01/99 | - | 1.5 | 30 | Gd, Liv, Ml | Not sure for Gd* |
| F104 | 20/0199 | F | 1.5 | 30 | Gd, Liv, Ml | |
| F105 | 20/01/99 | F | 2.5 | 40 | Gd, Liv, Ml | |
| F106 | 20/01/99 | F | 1.5 | 30 | Gd, Liv, Ml | |
| F107 | 20/01/99 | F | 5.5 | 50 | Gd, Liv, Ml | |
| F108 | 20/01/99 | M | 1.5 | 30 | Gd, Liv, Ml | |
| F109 | 21/01/99 | - | 1.5 | 30 | Gd, Liv, Ml | Not sure for Gd* |
| F110 | 21/01/99 | M | 2 | 40 | Gd, Liv, Ml | |
| F111 | 21/01/99 | M | 5 | 50 | Gd, Liv, Ml | |

**APPENDIX III- Continue.**

| Fetus # | Date | Sex | CRL* (cm) | ~Age** (days) | Tissues collected*** | Remarks |
|---|---|---|---|---|---|---|
| F112 | 21/01/99 | F | 5 | 50 | Gd, Liv, Ml | |
| F113 | 22/01/99 | F | 2 | 40 | Gd, Liv, Ml | |
| F114 | 22/01/99 | M | 3.2 | 45 | Gd, Liv, Ml | Twin with 115 |
| F115 | 22/01/99 | F | 3 | 45 | Gd, Liv, Ml | Twin with 114 |
| F116 | 22/01/99 | F | 3 | 45 | Gd, Liv, Ml | |
| F117 | 36181 | F | 1.5 | 30 | Gd, Liv, Ml | |
| F118 | 28/01/99 | F? | 4 | 50 | Gd, Liv, Ml | |
| F119 | 28/01/99 | F | 5 | 50 | Gd, Liv, Ml | |
| F120 | 28/01/99 | F | 1.5 | 30 | Gd, Liv, Ml | |
| F121 | 01/01/99 | F | ~1.5 | 30 | Gd, Liv, Ml | |
| F122 | 01/01/99 | M | ~1.5 | 30 | Gd, Liv, Ml | |
| F123 | 01/01/99 | F | 2 | 30 | Gd, Liv, Ml | |
| F124 | 02/2/99 | F | 1.5 | 30 | Gd, Liv, Ml | |
| F125 | 04/02/99 | F | 1.5 | 30 | Gd, Liv, Ml | |
| F126 | 04/02/99 | M | 1.5 | 30 | Gd, Liv, Ml | |
| F127 | 07/04/99 | M | 2.7 | 40 | Gd, Liv, Ml | |
| F128 | 07/04/99 | M | 5.5 | 50 | Gd, Liv, Ml | |
| F129 | 12/04/99 | M | 3.2 | 45 | Gd, Liv, Ml | |
| F130 | 15/04/99 | M | 2.6 | 40 | Gd, Liv, Ml | |
| F131 | 15/04/99 | M | 2.2 | 40 | Gd, Liv, Ml | |
| F132 | 15/04/99 | F | 3.2 | 45 | Gd, Liv, Ml, kid | |
| F133 | 15/04/99 | F | 7 | 70 | Gd, Liv, Ml | |
| F134 | 19/04/99 | M | 2.2 | 40 | Gd, Liv, Ml | |
| F135 | 19/04/99 | M | 2.2 | 40 | Gd, Liv, Ml | |
| F136 | 03/05/99 | ? | 2.5 | 40 | Gd, Liv, Ml | |
| F137 | 03/05/99 | M | 16 | 90 | Gd, Ml | |
| F138 | 03/05/99 | M | 16 | 90 | Gd, Liv, Ml | |
| F139 | 13/05/99 | M | 19.5 | 100 | Gd, Liv, Ml | |
| F140 | 13/05/99 | M | 37 | 150 | Gd, Liv, Ml | |
| 141 | 13/05/99 | F | 2.3 | 40 | Gd, Liv | |
| 142 | 14/06/99 | F | 1.5 | 30 | Gd, Li, Ml, Ms | |
| 143 | 16/06/99 | F | 1.7 | 30 | Gd, Li, Ml, Ms | |
| 144 | 16/06/99 | M? | 2.2 | 40 | Gd, Li, Ml, Ms | |
| 145 | 16/06/99 | F | 1.5 | 30 | Gd, Li, Ml, Ms | |
| 146 | 16/06/99 | M | 1.0 | 30 | Gd, Li, Ml, Ms | |
| 147 | 16/06/99 | F | 2.7 | 40 | Gd, Li, Ml, Ms | |
| 148 | 21/06/99 | M | 3.5 | 45 | Gd, Li, Ml, Ms | |

**APPENDIX III- Continue.**

| Fetus # | Date | Sex | CRL* (cm) | -Age** (days) | Tissues collected*** | Remarks |
|---|---|---|---|---|---|---|
| 149 | 21/06/99 | M | 1.6 | 30 | Gd, Li, Ml, Ms | |
| 150 | 21/06/99 | M | 3.1 | 45 | Gd, Li, Ml, Ms | |
| 151 | 23/06/99 | F | 1.3 | 30 | Gd, Li, Ml, Ms | |
| 152 | 05/07/99 | M | 2.7 | 40 | Gd, Li, Ml, Ms | |
| 153 | 05/07/99 | F | 1.3 | 30 | Gd, Li, Ml, Ms | |
| 154 | 05/07/99 | M | 1.3 | 30 | Gd, Li, Ml | |
| 155 | 12/07/99 | F | 2.0 | 40 | Gd, Li, Ml | |
| 156 | 12/07/99 | F | 3.0 | 45 | Gd, Li, Ml | |
| 157 | 14/07/99 | M | 2.5 | 40 | Gd, Li, Ml | |
| 158 | 14/07/99 | F | 1.5 | 30 | Gd, Li, Ml | |
| 159 | 14/07/99 | M | 2.6 | 40 | Gd, Li, Ml | |
| 160 | 23/07/99 | M | 1.4 | 30 | Gd, Li, Ml | |
| 161 | 26/07/99 | - | 1.6 | 30 | Gd, Li, Ml | Not sexed |
| 162 | 26/07/99 | - | 1.2 | 30 | Gd, Li, Ml | Not sexed |
| 163 | 26/07/99 | - | 2.7 | 40 | Gd, Li, Ml | Not sexed |
| 164 | 26/07/99 | - | 1.7 | 30 | Gd, Li, Ml | Not sexed |
| 165 | 02/08/99 | - | 2.2 | 40 | Gd, Li, Ml | Not sexed |
| 166 | 06/08/99 | F | 2.7 | 40 | Gd, Li, Ml | |
| 167 | 06/08/99 | M | 2.7 | 40 | Gd, Li, Ml | |
| 168 | 16/08/99 | M | 2.5 | 40 | Gd, Li, Ml | |
| 169 | 16/08/99 | M | 2.3 | 40 | Gd, Li, Ml | |
| 170 | 20/08/99 | F | 1.9 | 40 | Gd, Li, Ml | |
| 171 | 20/08/99 | M | 1.4 | 30 | Gd, Li, Ml | |
| 172 | 20/08/99 | F | 1.8 | 40 | Gd, Li, Ml | |
| 173 | 20/08/99 | F | 2.5 | 40 | Gd, Li, Ml | |
| 174 | 20/08/99 | M | 1.2 | 30 | Gd, Li, Ml | |
| 175 | 02/09/99 | F? | 3.7 | 50 | B,Gd, H, K, Li, Lg, Ml | |
| 176 | 02/09/99 | M | 5.2 | 50 | B,Gd, H, K, Li, Lg, Ml | |
| 177 | 02/09/99 | F | 19.5 | 100 | B,Gd, H, K, Li, Lg, Ml | |
| 178 | 02/09/99 | M | 20 | 110 | B,Gd, H, K, Li, Lg, Ml | |
| 179 | 09/09/99 | M | 15 | 90 | B,Gd, H, K, Li, Lg, Ml | |
| 180 | 09/09/99 | M | 19.5 | 100 | B,Gd, H, K, Li, Lg, Ml | |
| 181 | 09/09/99 | F | 19 | 100 | B,Gd, H, K, Li, Lg, Ml | |
| 182 | 28/09/99 | M | 13 | 80 | B,Gd, H, K, Li, Lg, Ml | |
| 183 | 28/09/99 | F | 15.5 | 90 | B,Gd, H, K, Li, Lg, Ml | |
| 184 | 28/10/99 | M? | 5.2 | 50 | B,Gd, H, K, Li, Lg, Ml | |
| 185 | 12/10/99 | F | 5.3 | 50 | B,Gd, H, K, Li, Lg, Ml | |

**APPENDIX III- Continue.**

| Fetus # | Date | Sex | CRL* (cm) | ˉAge** (days) | Tissues collected*** | Remarks |
|---|---|---|---|---|---|---|
| 186 | 36444 | M? | 6 | 60 | B,Gd, H, K, Li, Lg, Ml | |
| 187 | 14/10/99 | M | 8 | 70 | B,Gd, H, K, Li, Lg, Ml | |
| 188 | 14/10/99 | M | 30 | 120 | B,Gd, H, K, Li, Lg, Ml | |
| 189 | 28/10/99 | M | 29 | 120 | B,Gd, H, K, Li, Lg, Ml | |
| 190 | 28/10/99 | F | 19.5 | 100 | B,Gd, H, K, Li, Lg, Ml | |

* Crown-Rump Length.

** Estimated age based on the CRL (Noakes, 1997).

*** Abbreviations for collected tissues: B, Brain; Gd, Gonad; H, Heart; K, Kidney; Li, Liver; Lg, Lung; Ml, Muscle; Ms, Mesonephros.

† Fetus with unspecified sex.

## APPENDIX IV

### Sources of Supplies and Materials

| Material | Source |
|---|---|
| Agarose | Biolab |
| Chloroform | Fisher Scientific |
| Diethylpyrocarbonate | ICN Biomedical Inc. |
| DNA ladder (100 bp) | Gibco BRL, Life Technologies |
| DNA low mass lader | Gibco BRL, Life Technologies |
| Dnase | Gibco BRL, Life Technologies |
| dNTPs | Pharmacia Biotech Inc. |
| EDTA disodium salt | Fisher Scientific |
| EMEM | Pathobiology, University of Guelph |
| Ethidium bromide | Boehringer-Mannheim |
| Fetal calf serum | ICN Biomedical Inc. |
| FIMBIO II Image Analysis System | Hitachi Software Ltd. |
| Formaldehyde | Fisher Scientific |
| Isopropanol alcohol (2-Propanol) | Fisher Scientific |
| Oligo dT primers | Gibco BRL, Life Technologies |
| PBS | Pathobiology, University of Guelph |
| Penicillin/Streptomicin antibiotic | Pathobiology, University of Guelph |
| PstI | Gibco BRL, Life Technologies |
| QIA quick PCR purification kit | Qiagen Inc. |

**APPENDIX IV- Continue.**

| | |
|---|---|
| Quantum RNA 18S Internal Standard Kit | Ambion |
| Random hexamers | Gibco BRL, Life Technologies |
| Superscript (Reverse Transcriptase) | Gibco BRL, Life Technologies |
| SYBR Green I | Molecular Probe (Ltd) |
| Taq DNA polymerase | Gibco BRL, Life Technologies |
| Tissue culture flasks | Fisher Scientific |
| TRIzol reagent | Gibco BRL, Life Technologies |
| Trypsin | Pathobiology, University of Guelph |

# APPENDIX V

## Primer sequences used for PCR amplification of respective genes

| Gene locus | Primer Sequences |
|---|---|
| *G6pd*[1] | 5'* CAA GAT GAT GAC CAA GAA GC<br>3'** AGC AGT GGT GTG AAG ATA CG |
| *Hprt*[2] | 5' GTA ATG ATC AGT CAA CGG GGG AC<br>3' CCA GCA AGC TTG CAA CCT TAACCA |
| *Smcx*[3] | 5' CCT CGG GCC CAC CAT GGA<br>3' CTG ATT TTC GCG ATG TAG CC |
| *β-actin*[4] | 5' ACTGGGACGACATGGAGAAGAT<br>3' TGCTCGAAGTCCAAGGCGACGT |
| *Xist*[4] | 5' AGC ATT GCT TAG CAT GGC TC<br>3' TGG CTG TGA CCG ATT CTA CC |
| *Zfx*[5] | 5' ATA ATC ACA TGG AGA GCC ACA AGCT<br>3' GAG CCT CTT TGG TAT CTG AGA AAG T |

* All primers starting with 5' refer to the upstream (sense) primers.
** All primers starting with 3' refer to the downstream (antisense) primers.

1. Lequarre et al., 1997.
2. Grunig and Antczak, 1995.
3. Jegalian and Page, 1998.
4. De LA Fuente et al., 1999.
5. Aaesen and Medrano, 1990.