X- and Y-Chromosomal Gene Expression During Spermatogenesis, Studied in the Context of Separation of X and Y Spermatozoa

X- en Y-Chromosomale Genexpressie Tijdens de Spermatogenese, in Relatie tot Scheiding van X- en Y-Spermatozoa.

PROEFSCHRIFT

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Contents

2-D PAGE	two-dimensional polyacrylamide gel electrophoresis
aa	amino acids
AZF region	Azoospermia factor region
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CHAPS	(3-[(3 cholamidopropyl)-dimethylammonio]-1-propanesulfonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
G6PD	glucose-6-phosphate dehydrogenase
H6PD	hexose-6-phosphate dehydrogenase
HMG	high mobility group
kb	kilo base pairs
kDa	kilo Dalton
mRNA	messenger RNA
MW	molecular weight
PAR	pseudoautosomal region
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pdha	pyruvate dehydrogenase E1α subunit
Pgk	phosphoglycerate kinase
PMSF	phenylmethylsulfonylfluoride
RNA	ribonucleic acid
RT-PCR	reverse transcriptase - polymerase chain reaction
SDS	sodium dodecyl sulfate
Smcy	Selected mouse cDNA on the Y
Spy	spermatogenesis gene on the Y
SRY	sex determining region Y (human)
Sry	sex determining region Y (mouse)
Sxr	sex reversed
Xist	X-inactive specific transcripts

General Introduction and Scope of this Thesis

1.1 Sex predetermination by separation of X and Y chromosome-bearing sperm

1.1.1 Introduction

Mankind has been interested in sex preselection since ancient times. Methods presumed to achieve sex preselection have already been described by Greek philosophers as far as 500 B.C. (reviewed by Betteridge, 1984). Although it took almost 2500 years since then to develop reliable and repeatable methods, sex preselection is now possible and is actually applied in both humans and livestock. It can be achieved either by sexing of early embryos or by separation of X and Y chromosome-bearing spermatozoa (X and Y spermatozoa).

Sexing of embryos is practiced in both humans and livestock, especially cattle, by performing the DNA polymerase chain reaction or *in situ* hybridisation to detect Y-specific DNA, in cells taken from the early embryo (Herr *et al.*, 1990; Levinson *et al.*, 1992). Despite the success of embryo sexing, sperm separation is a more preferred method for sex preselection, because it would avoid the need for embryo manipulation and exclusion. It is evident that the availability of separated X and Y spermatozoa for routine artificial insemination will simplify current procedures in human medicine for the prevention of those X-linked diseases which affect boys but do not, or to a lesser extent, affect girls. In the livestock industry, it will allow a much more efficient use of the reproductive potential of the economically important livestock species. In the swine industry, it will enable the production of multiplier sows without a concomitant production of less valuable boars. It will also enable producers of fattening piglets to produce merely male piglets, which have a more efficient growth and better meat quality. In the dairy industry, it will enable the production of merely female offspring for milk production, whereas male offspring can be produced from breeds more suitable for beef production.

1.1.2 Attempts to separate X and Y spermatozoa based on presumed differences in size and weight

Many attempts have been made to separate X and Y spermatozoa (for reviews see: Amann, 1989; Windsor *et al.*, 1993; Johnson, 1994). Some of the applied methods were based on presumed physical differences resulting from a

difference in size between the X and Y chromosomes. The X chromosome is larger than the Y chromosome, and X spermatozoa contain, therefore, more DNA than Y spermatozoa. This difference in total DNA content ranges from 2.8% in humans to 3.6% in pigs and 3.8% in cattle (Sumner *et al.*, 1971; Johnson, 1992). This led Roberts (1972) to propose that X and Y spermatozoa have a 1% difference in head radius, which would affect swimming and sedimentation velocities. However, other calculations can be made. Since DNA constitutes approximately 18% of the dry mass of spermatozoa, a 3% difference in DNA content results in approximately 0.5% difference in dry weight. Due to the large water and lipid contents of sperm, the difference in weight will be much smaller for viable sperm, and it can be expected that the X to Y sperm difference will be much smaller than the general variation in weight among spermatozoa (Meistrich, 1982; Windsor *et al.*, 1993). Cui and Matthews (1993) exerted excitement by reporting that heads and tails of human X sperm were significantly larger than that of Y sperm. This short 'letter to the editor', however, has not yet been followed by the announced more detailed paper, and, therefore, still awaits confirmation.

In view of assumed differences in swimming velocity and/or density, X and Y sperm separation has been tried using albumin gradients (Ericsson *et al.*, 1973), laminar flow fractionation (Sarkar *et al.*, 1984), Percoll density gradients (Kaneko *et al.*, 1983; Iizuka *et al.*, 1987), sucrose gradients (Rohde *et al.*, 1975), and Sephadex gel filtration (Steeno *et al.*, 1975). Although the reported results were sometimes very spectacular, they could not be confirmed by others (Johnson, 1988; Van Kooij and Van Oost, 1992; Lobel *et al.*, 1993; Vidal *et al.*, 1993; Wang *et al.*, 1994a; Claassens *et al.*, 1995). Moreover, these techniques did not reach the stage of practical application, with exception of the sperm separation method using albumin columns.

This technique is applied in more than 60 human clinical practices in the United States (Beernink *et al.*, 1993; Ericsson 1994), and recently also in a gender clinic in the Netherlands (Gender Behandelcentrum Utrecht). The separation is based on the assumption that Y spermatozoa would be faster than X spermatozoa in swimming down a high density solution. Spermatozoa are allowed to swim down, first through a 10% human serum albumin solution, and subsequently through two layers of 12.5 and 20% human serum albumin (Beernink *et al.*, 1993). The first 0.5 to 10% of the spermatozoa (percentage related to the initial number of motile spermatozoa) that have passed through these layers, would yield 8- to 15-fold more male than female offspring (Pyrzak, 1994). Almost the same procedure is used when female offspring is desired. The main difference is that the spermatozoa are allowed to swim down for a longer time period, resulting in a recovery of more than 10% of the

spermatozoa. A second adjustment of the procedure for yielding female offspring, is that prior to insemination clomiphene citrate is administered to the woman to induce ovulation (Beernink *et al.*, 1993; Pyrzak, 1994). The most recently reported results from the clinical centers about their success rate in achieving either male or female offspring, are 79 and 73%, respectively (Beernink *et al.*, 1993; Ericsson, 1994; Pyrzak, 1994). There are, however, doubts whether these studies were performed in a controlled randomized fashion (Martin, 1994). In the few cases that sperm preparations produced by one of the centers were investigated by others, the claimed enrichment for either X or Y sperm was not confirmed (Brandriff *et al.*, 1986; Flaherty and Mathews, 1994). In addition, the technique did not achieve Y sperm enrichment when applied by others (Wang *et al.*, 1994b; Claassens *et al.*, 1995).

1.1.3 Attempts to separate X and Y spermatozoa based on presumed differences in surface charge or H-Y antigen

Other properties for which X and Y sperm have been proposed to differ, include surface charge and additional surface differences. X and Y spermatozoa have been claimed to be successfully separated by free-flow electrophoresis (Kaneko *et al.*, 1984; Ishijima *et al.*, 1992), "thermal convection counterstreaming sedimentation" (Bhattacharya *et al.*, 1977), or with an ion exchange column (Lang, 1973). Cartwright *et al.* (1993) reported successful separation of X and Y spermatozoa using the "thin-layer countercurrent distribution" technique. For all these techniques, the claimed successes still have to be confirmed by other workers, or were already demonstrated to be incorrect (reviewed by Windsor *et al.*, 1993).

Conflicting results have also been reported about a possible difference between X and Y spermatozoa in expressing a male-specific protein, H-Y antigen. The finding that anti-H-Y antibodies bound to approximately 50% of the spermatozoa in mouse, bull, ram, and human (Goldberg *et al.*, 1971; Ohno and Wachtel, 1978; Bradley, 1989; Iyer *et al.*, 1989), indicated that this might reflect Y sperm-specific binding. This was supported by experiments in which spermatozoa selected for being either negative or positive for H-Y antigen, yielded offspring with altered sex ratio (Bennett and Boyse, 1973; Bryant, 1980; Zavos, 1983), or exhibited an altered ratio of X to Y spermatozoa according to microdensitometry (Ali *et al.*, 1990). However, other authors did not find evidence for a preferential binding of anti-H-Y antibodies to Y spermatozoa (Ohno and Wachtel, 1978; Hoppe and Koo, 1984).

1.1.4 Verification of X and Y sperm enrichment

Part of the apparently premature claims of successful X and Y sperm separation are due to improper analysis of the sperm fractions. In seven of the above cited studies (Ericsson *et al.*, 1973; Rohde *et al.*, 1975; Steeno *et al.*, 1975; Bhattacharya *et al.*, 1977; Kaneko *et al.*, 1983; Kaneko *et al.*, 1984; Iizuka *et al.*, 1987), the F-body test was used to verify X or Y sperm enrichment. This test is based on intense staining of the heterochromatic part of the human Y chromosome by the fluorescent dye quinacrine in metaphase cells, and it was suggested that a fluorescent spot in a fraction of quinacrine-stained spermatozoa also would represent the Y chromosome (Barlow and Vosa, 1970; Vosa, 1970). However, quinacrine staining has been demonstrated to be unreliable for determination of the percentage of Y sperm. The percentage of F-body positive sperm is often lower than 40%, while the incidence of sperm with two or more F-bodies is much higher than the incidence of sperm being disomic for the Y chromosome (Gledhill, 1983; Beckett *et al.*, 1989). Moreover, Van Kooij and Van Oost (1992) confirmed earlier reports that Percoll centrifugation can yield fractions containing 14 to 84% F body-bearing sperm, but also showed that all of these fractions contained equal percentages of X and Y sperm.

In some other studies, presumed X or Y sperm enrichment was verified by determining the sex ratio of offspring. In many studies, however, the number of offspring was too low to produce statistically significant results (reviewed by Amann, 1989).

The development of DNA technology has led to more precise methods to estimate the percentages of X and Y sperm in different fractions (Beckett *et al.*, 1989; Métézeau *et al.*, 1991; Cartwright *et al.*, 1993; Han *et al.*, 1993; Johnson *et al.*, 1993; Lobel *et al.*, 1993; Welch *et al.*, 1995). Among these techniques, fluorescence *in situ* hybridization (FISH) with X- and Y-specific DNA probes provides the most accurate results (Han *et al.*, 1993; Johnson *et al.*, 1993; Vidal *et al.*, 1993; Wang *et al.*, 1994a,b; Claassens *et al.*, 1995). Alternatively, sperm nuclei can be analysed by flow cytometry that involves measurement of DNA content with specifically adapted equipment (Johnson, 1994).

1.1.5 Flow cytometric sorting of X and Y spermatozoa

A well-developed method that is described in this overview, is X and Y sperm separation with a flow cytometer/cell sorter. This method is based on the difference in amount of DNA itself, and not on a possible effect of DNA content on other physical characteristics of X and Y sperm. As mentioned above, the difference in total DNA content ranges from 2.8% in human to 3.6% in pig and 3.8% in cattle, and it even reaches 7.5% in chinchilla and 12.5% in creeping vole (*Microtus oregoni*, which has "0" and Y sperm) (Johnson, 1992). Flow cytometric sperm sorting is based on the use of a fluorescent dye (Hoechst 33342), that can pass the sperm membranes and binds specifically to DNA. Consequently, incubation with this dye results in a slightly brighter fluorescence of X spermatozoa than Y spermatozoa, which enables their separation with a flow cytometer/cell sorter. In this apparatus, the sperm proceed through a laser beam one by one in minute droplets, allowing the fluorescence of individual spermatozoa to be measured. Dependent on the fluorescence intensity, the droplet containing a spermatozoon will be given a positive or negative charge, which will affect its direction when it passes through an electrostatic field (see Figure 1.1) (Johnson, 1994). Flow cytometers are commercially available since the 1970s, and are since then widely applied mainly for the study of blood and tumor cells. The use of this technique for sperm sorting, however, is complicated by the irregular form of the sperm head. The fluorescence of the sperm head differs when the laser beam strikes the edge instead of the flatter side of the spermatozoon. Therefore, proper orientation of the spermatozoa is crucial for optimal fluorescence measurement. The main adjustments to deal with this orientation problem are the use of: 1) a bevelled needle to improve the orientation of the spermatozoa, and 2) a second fluorescence detector to check the orientation of the spermatozoa. In general, 30-50% of the spermatozoa have the proper orientation and are sorted into X and Y sperm populations (Johnson, 1994).

The success of the flow cytometry/cell sorting technique in separating X and Y spermatozoa has been confirmed by the use of flow cytometric reanalysis and DNA techniques in distinct laboratories (Johnson and Clarke, 1988; Métézeau *et al.*, 1991; Johnson *et al.*, 1993, Welch *et al.*, 1995). The enriched X and Y sperm populations can reach purities above 90% (Johnson, 1994). Flow cytometrically sorted sperm have yielded live offspring of expected altered sex ratios in rabbit and pig following surgical insemination (Johnson *et al.*, 1989; Johnson, 1991). A disadvantage of the flow cytometric sorting technique is, that the number of sperm that can be sorted is too low for routine artificial insemination. *In vitro* fertilization, which requires a lower

number of sperm, has been used successfully as an alternative approach to produce offspring with flow cytometrically sorted sperm in cattle (Cran *et al.*, 1993; Cran *et al.*, 1995), and recently in human (Levinson *et al.*, 1995).

There has been discussion about a possible detrimental effect of the DNA binding dye Hoechst 33342 and the excitation of sperm by ultraviolet laser light, concerning development of the resulting embryos (Ashwood-Smith, 1994; Munné, 1994; Johnson and Schulman, 1994). In surgically inseminated rabbits, the use of flow cytometrically sorted sperm has been demonstrated to result in increased embryonic death (McNutt and Johnson, 1996). The results of IVF with flow cytometrically sorted sperm in cattle also suggest a somewhat higher rate of embryonic death or implantation failure (Cran *et al.*, 1995). However, no morphological or reproductive abnormalities have been observed in rabbit, swine, cattle or sheep presently produced with flow cytometrically sorted sperm (Johnson and Schulman, 1994; Cran *et al.*, 1995).

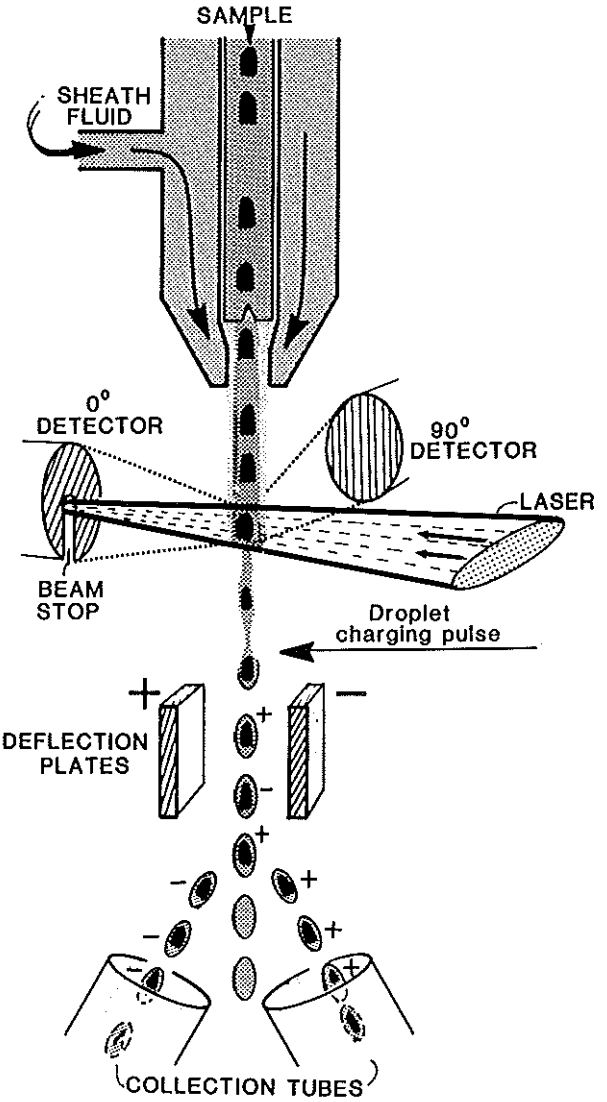

Figure 1.1 *Schematic representation of separation of X and Y chromosome-bearing spermatozoa in a flow cytometer/cell sorter.*

Hoechst 33342-stained spermatozoa proceed one by one through a laser beam. The orientation of the spermatozoa is improved by means of an orienting bevel. Proper orientation is essential, since, due to the irregular form of the sperm head, the fluorescence highly depends on which side of the sperm head and under which angle, the spermatozoon is excitated by the laser beam. The emitted fluorescence is measured by two detectors. The 90° detector checks whether the spermatozoa are properly oriented, and only these spermatozoa are subjected to separation in X and Y sperm fractions. The fluorescence intensity measured by the 0° detector determines the direction of separation. The droplet containing one X or Y spermatozoon is given a positive or negative charge, respectively, dependent on the fluorescence being above or below a certain level. The charge affects the direction of the fluid droplet when it passes through an electric field (Courtesy of Dr L.A. Johnson).

1.2 Scope of this thesis

At present, only one reliable method for separation of X and Y chromosome-bearing spermatozoa exists: flow cytometric cell sorting. Although high purities of X and Y enriched sperm populations can be achieved, a major drawback of this method is the relatively low number of separated sperm that can be produced within a reasonable time period. Therefore, there still is a need for an alternative sperm separation method which can be used to separate a large number of X and Y spermatozoa, and then can be combined with routine artificial insemination.

A logical approach to try to develop such an alternative method is, first to study aspects for which X and Y spermatozoa might differ. At present, the small difference in DNA content due to the larger size of the X chromosome than the Y chromosome is the only known difference.
This brings up two questions:

I Do X and Y spermatozoa differ for other aspects than the difference in DNA content?

II Are gene expression events in X and Y chromosome-bearing spermatids, during spermatogenesis, in concordance with a possible phenotypic difference between X and Y spermatozoa?

I Do X and Y spermatozoa differ for other aspects than the difference in DNA content?

The availability of flow cytometrically sorted X and Y sperm opens the possibility for a direct comparison between these sperm populations. One property which could lead to development of a large-scale separation method, would be the presence of a surface molecule unique to either X or Y spermatozoa. This would allow production of an antibody with specific binding to either X or Y spermatozoa, providing a basis for an immunological separation method.

Two approaches were followed to investigate whether plasma membrane proteins unique to either X or Y spermatozoa might exist.

A Evaluation of a possible difference in H-Y antigen expression between X and Y spermatozoa.

As described under 1.1.3, reported results about a possible preferential presence of H-Y antigen on Y chromosome-bearing sperm were conflicting. It was warranted to perform a more conclusive study. In *Chapter 4*, experiments on the binding of anti-

H-Y monoclonal antibodies to flow cytometrically sorted X and Y spermatozoa of pig and cattle are described.

B Evaluation of a possible difference in plasma membrane protein composition between X and Y spermatozoa.

The aim of this study was to compare as many as possible membrane proteins of X and Y sperm, to investigate whether any protein unique to one of these sperm types might be detected. A powerful technique for analyzing a high number of proteins is 2-dimensional gel electrophoresis. The results of an analysis of plasma membrane proteins of flow cytometrically sorted X and Y spermatozoa of pig, using 2-dimensional gel electrophoresis, are described in *Chapter 5*.

II Are gene expression events in X and Y chromosome-bearing spermatids, during spermatogenesis, in concordance with a possible phenotypic difference between X and Y spermatozoa?

As described under 1.1.2, if the small difference in DNA content between X and Y spermatozoa would result in an effect on swimming velocity and/or sperm density, such an effect will probably be minor compared to the general variation of these properties among spermatozoa. If any other phenotypical difference between X and Y spermatozoa does exist, this has to result from expression of X- and Y-chromosomal genes in spermatids (the post-meiotic haploid germ cells with either an X or Y chromosome; see *Chapter 2*). In other words, phenotypical differences between X and Y spermatozoa can be excluded, when there is no expression of X- and/or Y-chromosomal genes in spermatids. A second prerequisite for the possible occurrence of phenotypical differences between X and Y spermatozoa is, that X- and/or Y-chromosomal gene products should not be equally shared among the X and Y chromosome-bearing spermatids, which are connected through cytoplasmic bridges (see *Chapter 8*).

An overview of spermatogenesis and the behavior of the sex chromosomes during and after meiosis is given in *Chapter 2*. *Chapter 3* gives an overview of the genes presently identified on the mouse and human Y chromosomes. *Chapter 6* describes a study in which it was investigated whether spermatids of the mouse express X- and Y-chromosomal genes. The study described in *Chapter 7* was focussed on expression of the X-chromosomal gene encoding glucose-6-phosphate dehydrogenase, during spermatogenesis. This gene was found to be inactivated in spermatids, but a novel autosomal gene encoding a glucose-6-phosphate

dehydrogenase isoenzyme was identified, which shows spermatid-specific expression.

In addition to the discussion on product sharing among spermatids, *Chapter 8* provides a description of X chromosome inactivation during spermatogenesis, and concluding remarks.

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Behavior of the Sex Chromosomes during Meiosis of Spermatogenesis

2.1 Spermatogenesis

Spermatogenesis is the process of cell division and differentiation, that in the testis starts from undifferentiated spermatogonia and ultimately results in the production of spermatozoa. The undifferentiated spermatogonia are located at the periphery of the testis tubules and undergo mitosis. In the mouse, part of the undifferentiated spermatogonia transform into differentiated spermatogonia, the Type A_1 spermatogonia, while others renew their own population. Through a well-controlled series of mitotic cell divisions, the Type A_1 spermatogonia give rise to the A_2, A_3, A_4, intermediate (In) and Type B spermatogonia. The Type B spermatogonia divide to form meiotic cells, the primary spermatocytes. One primary spermatocyte yields, through the first meiotic division, two secondary spermatocytes, which almost immediately enter the second meiotic division resulting in four round spermatids. The haploid round spermatids do not further divide, but differentiate into elongating spermatids and ultimately into spermatozoa. During this differentiation process, called spermiogenesis, the spermatids undergo a series of morphological events, which involves tail formation, replacement of nuclear proteins, flattening of the nucleus, loss of cytoplasm, and acquirement of other spermatozoon-specific structures, such as the acrosome. During the development from spermatogonium to spermatozoon, the spermatogenic cells proceed from the periphery toward the center of the testis tubule, and will finally be released into the lumen. The blood-testis barrier is passed at zygotene of the first meiotic division. The spermatogenic cells are embedded in Sertoli cells, and remain at the same tubule cross section during their development (Oakberg, 1956; Russell *et al.*, 1990).

Spermatogenesis is highly coordinated. In the mouse, at a certain cross section of a tubule, approximately every 8.6 days a new set of spermatogonia starts spermatogenesis (transformation from undifferentiated spermatogonia into Type A_1 spermatogonia). This time period is also called the length of the spermatogenic cycle. In total, it takes approximately 35 days for differentiated (A_1) spermatogonia to develop into spermatozoa. Therefore, each cross section of a tubule contains, next to the undifferentiated spermatogonia, 4 spermatogenic cell types of more advanced steps in development. A schematic overview of the cell types found at different cross sections of a segment of a testis tubule covering one spermatogenic cycle is provided in Figure 2.1. Based on changes in acrosome and nucleus, the development of spermatids into spermatozoa is divided into 16 steps (see Figure

2.1). The cells from Steps 1 to 8 are the round spermatids, cells in Steps 9 to 11 are known as the elongating spermatids, and cells in the remaining steps are referred to as the elongated spermatids. It takes one spermatogenic cycle for a spermatid to proceed from Step 1 to Step 12. These 12 steps are used to divide the spermatogenic cycle into 12 stages, indicated as Stages I to XII in Figure 2.1 (Oakberg, 1956; Russell *et al.*, 1990).

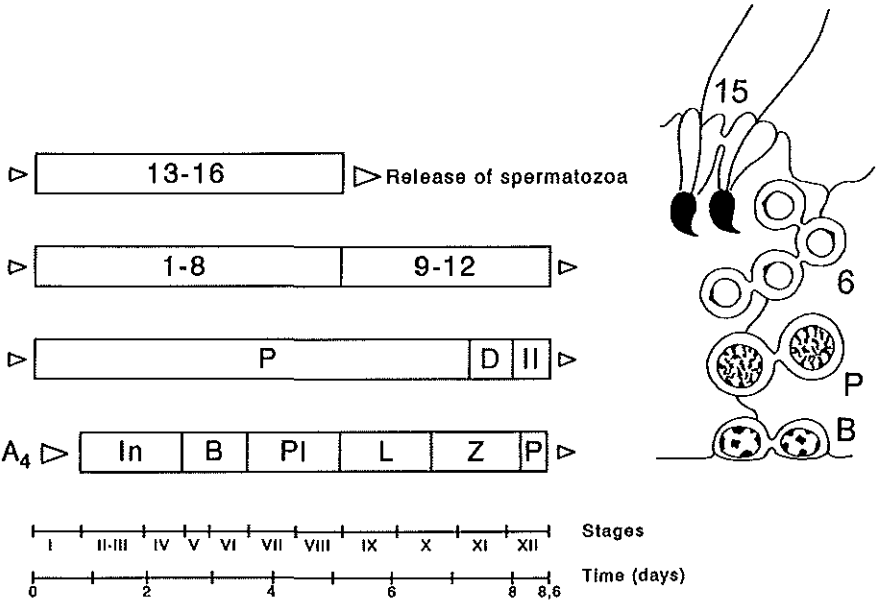

Figure 2.1 *The spermatogenic cycle of the mouse.*

At the left: diagram illustrating the cell types that can be found at subsequent time points within one segment of a testis tubule, covering one spermatogenic cycle in the mouse. The 12 stages of the cycle are indicated by Roman numerals.

At the right: schematic drawing of the cell types situated within Stage VI.

Type A spermatogonia (except A_4) are not included in this figure.

A_4: Type A_4 spermatogonia; In: intermediate type spermatogonia; B: Type B spermatogonia; Pl: preleptotene spermatocytes; L: leptotene spermatocytes; Z: zygotene spermatocytes; P: pachytene spermatocytes; D: diplotene spermatocytes; II: secondary spermatocytes; 1-16: spermatids at successive steps of spermiogenesis (Based on Oakberg, 1956; Russell et al., 1990).

2.2 Meiosis during spermatogenesis

Meiosis starts by DNA replication, that copies each chromosome into two sister chromatids. Meiosis differs from mitosis in three ways: first, meiosis yields haploid instead of diploid daughter cells, and involves two consecutive divisions without intervening DNA replication; second, during the prophase of the first meiotic division (prophase I), the maternal and paternal copies of each chromosome (the homologs) pair to form bivalents, and undergo recombination; third, during first meiotic anaphase, sister centromeres are not separated but function as one unit, enabling the reduction of chromosome number.

The prophase of the first meiotic division consists of 5 stages: leptotene, zygotene, pachytene, diplotene and diakinesis (Alberts *et al.*, 1983).

DNA replication takes place at the preleptotene stage. Subsequently, at leptotene, a proteinaceous longitudinal axial element is formed along each chromosome, through which the individual chromosomes can be made visible. The chromatin becomes organized in loops, that are attached at their base to the protein core. Each chromosome is attached at both of its ends to the nuclear envelope. The homologous chromosomes start pairing, which is defined as the alignment of homologous chromosomes (Miyazaki and Orr-Weaver, 1994).

At zygotene, a third axis (the median central element) and transverse elements are created in the middle of the two chromosome-specific axes. This long ladder-like structure is called the synaptonemal complex, in which the axial elements are then referred to as the lateral axes (Von Wettstein *et al.*, 1984; Dobson *et al.*, 1994). In yeast, double-strand breaks are initiated in the DNA, in the period between the initiation of pairing and synapsis. These double-strand breaks enable cross-overs at a later stage, which are instrumental in meiotic recombination (Hawley and Arbei, 1993). In mammals, the stage of initiation of double-strand breaks is not known.

As soon as synapsis is complete, the cells enter the pachytene stage. During spermatogenesis, pachytene is the longest stage of the meiotic prophase, lasting 8 days in mouse and 16 days in human (Oakberg, 1956; Heller and Clermont, 1963). During pachytene, cross-overs take place between non-sister (maternal and paternal) chromatids within each bivalent. Thus, at these points, later visible as chiasmata, two of the four chromatids have crossed-over. In human, an average of two to three cross-over events occur for each pair of chromosomes.

Desynapsis occurs at the diplotene/diakinesis stage. The synaptonemal complex dissolves, but the two homologous chromosomes remain joined by one or more chiasmata. The chromosomes condense, thicken, become detached from the nuclear envelope, and RNA synthesis ceases.

After prophase I has finished, the bivalents first align during metaphase I, and the homologous chromosomes then segregate into separate daughter nuclei during anaphase I. Although the DNA content of the resulting secondary spermatocytes equals that of diploid cells at G_1 of the cell cycle, the secondary spermatocytes are actually haploid. Different chromosomal segments are either from maternal or paternal origin, and either the X or the Y chromosome is present. During the second meiotic division, the sister chromatids separate, as in normal mitosis, to produce the haploid round spermatids (for reviews see: Alberts *et al.*, 1983; Von Wettstein *et al.*, 1984; Miyazaki and Orr-Weaver, 1994).

2.3 Behavior of the sex chromosomes during meiosis in mouse and human

The sex chromosomes in males differ from the autosomes in having a largely non-homologous pairing partner. While homologous autosomes can pair over their whole length, the X and Y chromosomes differ in length, and sequence homology is restricted to a specific domain, the pseudoautosomal region. In human, the pseudoautosomal region comprises only about 10% of the Y chromosome, although the pairing region may involve more than 50% of the Y chromosome (Ellis and Goodfellow, 1989). Possibly due to this limited pairing, the behavior of the sex chromosomes during meiotic prophase is markedly different from that of the autosomes.

During early-pachytene, the XY chromatin is more condensed than that of the autosomes (Oud *et al.*, 1979; Knibiehler *et al.*, 1981), and from this stage onwards the XY chromatin is also called the sex body (also termed XY body or sex vesicle) (Sachs, 1954; Monesi, 1965; Solari, 1974). The sex body mainly consists of the sex chromosome axes and the surrounding chromatin fibrils (Solari, 1974). During early pachytene, the axes of the sex chromosomes are slightly thicker and more folded than those of the autosomes (Dietrich and Mulder, 1981; Richler *et al.*, 1989). During mid-pachytene, the autosomal and sex chromosomal axes extend, and the nucleus of the spermatocyte expands. At this stage, the XY chromatin has become less condensed than that of the autosomes (Dietrich and Mulder, 1981; Lammers *et al.*, 1995). The XY bivalent then occupies a peripheral and often protruding position (Figure 2.2). During the last part of late-pachytene and the first part of diplotene, the autosomes despiralize, whereas the sex chromosomes contract again (Oud *et al.*, 1979; Lammers *et al.*, 1995). The sex chromosomes remain more condensed than the autosomes until late-diplotene stage, when the autosomes contract as well, before the cells enter metaphase I (Oud *et al.*, 1979).

The sex body fails to incorporate radiolabelled uridine, and therefore, is thought to be transcriptionally inactive (Figure 2.2) (Monesi, 1965; Kierszenbaum and Tres, 1974a; Kierszenbaum and Tres, 1974b; Handel *et al.*, 1994). This spermatogenic X-inactivation is in marked contrast to behavior of the two X chromosomes during oogenesis. In premeiotic oogonia one of the two X chromosomes is inactivated, but both X chromosomes are transcriptionally active during the meiotic prophase (Kratzer and Chapman, 1981; McCarrey and Dilworth, 1992).

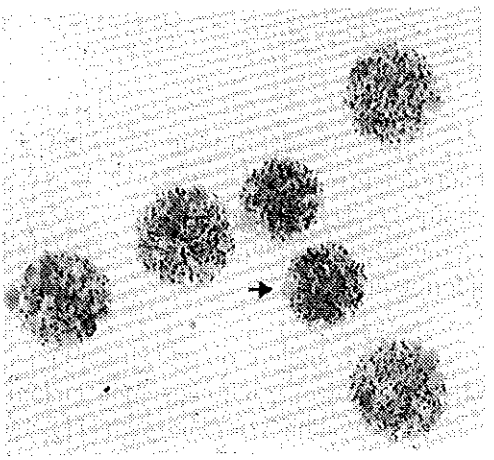

Figure 2.2 *The sex body*

Pachytene spermatocytes cultured in the presence of radio-labelled (^{3}H) uridine, Giemsa stained, and subjected to autoradiography. Arrow points to a sex body. The grains indicate the occurrence of transcription. The distribution of the grains demonstrate that transcriptional activity of the sex body is very low or completely absent (From Grootegoed, 1996).

The exact biochemical composition of the sex body is not known. It contains in addition to the sex chromosomes some surrounding material mainly of unknown nature (Solari, 1974; Pathak and Elder, 1980). The sex body is often closely associated with a nucleolus, but does not contain ribosomal RNA itself (Kierszenbaum and Tres, 1974a; Solari, 1974; Knibiehler *et al.*, 1981).

Sofar, two proteins associated with the sex body have been detected: XMR (*Xlr*-related, meiosis regulated; Xlr: X-linked, lymphocyte regulated) in the mouse (Calenda *et al.*, 1994), and XY40 in the rat (Smith and Benavente, 1992). According to antibody binding, the XMR protein first appears in the nuclei of leptotene spermatocytes, and it is distributed over the whole nucleus of early-pachytene spermatocytes, while in mid-pachytene spermatocytes the protein is present exclusively in the sex body, at a high concentration. Within the sex body, the XMR antibody labeling was highest at the sex chromosome axes, but also the remaining part of the sex body was weakly stained (Calenda *et al.*, 1994). This differs from the location of the XY40 protein in the rat, which is exclusively associated with the X and Y axes and not found in the remaining part of the sex body (Smith and Benavente, 1992). The gene encoding XMR has been identified, and belongs to the *Xlr* gene family, that includes another member encoding a nuclear protein in lymphocytes (Calenda *et al.*, 1994). *Xmr* expression is testis-specific. The C-terminal part of the *Xmr* gene product shows similarity with the *MER2* gene product of the yeast *Saccharomyces cerevisiae* (Calenda *et al.*, 1994). The *MER2* gene product is essential for meiosis and is required for the formation of synaptonemal complexes in *Saccharomyces cerevisiae* (Rockmill *et al.*, 1995).

2.4 Comparison of somatic and spermatogenic X chromosome inactivation

The mechanism of sex body formation is largely unknown, but some information about this process is derived from comparison of the chromatin characteristics of the inactivated X chromosome in spermatocytes and female somatic cells. Additional information is derived from man and mice with chromosomal aberrations.

It is tempting to suggest common steps in the mechanism of inactivation of one of the two X chromosomes in female somatic cells and the mechanism of inactivation of the X chromosome in pachytene spermatocytes. In both situations, the *Xist (X-inactive specific transcripts)* gene is expressed, and the X chromosome becomes condensed. In female somatic cells, the *Xist* gene is transcribed from the X chromosome that will be inactivated, and initiates the inactivation of that X chromosome (Brown *et al.*, 1991; Borsani *et al.*, 1991; Penny *et al.*, 1996). The inactivated X chromosome becomes heterochromatic, and is visible cytologically as the so-called Barr body at the nuclear periphery. The *Xist* gene on the inactivated X chromosome continues to be transcribed.

In spite of these similarities, other indications point to the involvement of different mechanisms in somatic X-inactivation in female cells and in sex body formation in pachytene spermatocytes. It is unlikely that transcription of *Xist* in spermatogenic cells is functionally related to XY condensation in early-pachytene spermatocytes. *Xist* transcription starts in undifferentiated spermatogonia (McCarrey and Dilworth, 1992), which is at a much earlier time point than the onset of X chromosome condensation. Moreover, the expression level of *Xist* in spermatogenic cells is approximately a thousand-fold lower than that in female somatic cells (Kay *et al.*, 1993). In oogonia and oocytes, the time pattern and level of *Xist* expression coincides with the inactivation/activation pattern of the X chromosome (McCarrey and Dilworth, 1992).

Another difference between somatic and spermatogenic X chromosome inactivation concerns the stability of chromatin modification. X chromosome inactivation in pachytene spermatocytes involves less stable chromatin modifications than X chromosome inactivation in female somatic cells. This was demonstrated by Venolia *et al.* (1984), by testing the ability of the inactivated X chromosome from

spermatocytes and female somatic cells to express the X-linked *HPRT* gene after transformation to HPRT-deficient cell lines. HPRT activity was induced by transformation with DNA from pachytene spermatocytes but not by DNA from the inactive X chromosome of female somatic cells. The inactivated X chromosome in pachytene spermatocytes also shows a higher sensitivity to DNase I treatment than the inactive X chromosome of female somatic cells (Chandley and McBeath, 1987; Richler *et al.*, 1989). In general, DNase I sensitivity is considered to reflect a more open chromatin structure, enabling transcription (Weisbrod, 1982).

Stabilization of somatic X chromosome inactivation is thought to be exerted by methylation of CpG islands of genes on the inactivated X chromosome. After initiation by *Xist* expression, methylation is the second step of somatic X chromosome inactivation. This methylation is thought to maintain X-inactivation through mitotic divisions (Grant *et al.*, 1992; Razin and Cedar, 1994). Spermatogenic X chromosome inactivation is not followed by methylation of CpG islands. For several X genes, including *PGK-1* and *G6PD*, the CpG islands are methylated on the inactive somatic X chromosome, but unmethylated on the X chromosome in pachytene spermatocytes as well as in premeiotic oocytes (Driscoll and Migeon, 1990; Grant *et al.*, 1992; Erickson *et al.*, 1993). Both in pachytene spermatocytes and in premeiotic oocytes, this unmethylated state has been correlated with the potency to reactivate the inactivated X chromosome (Driscoll and Migeon, 1990; Grant *et al.*, 1992).

In concordance with its expression from the inactivated X chromosome in female somatic cells, the 5' end of the *Xist* gene on the inactivated X chromosome is completely unmethylated, while it is fully methylated on the active X chromosome (Norris *et al.*, 1994). In spermatogenic cells, the onset of *Xist* expression is correlated with demethylation of several CpG sites at the 5' end of the *Xist* gene, occurring between 18.5 and 21.5 days post coitum (Ariel *et al.*, 1995). This demethylated state is maintained through all following spermatogenic cell stages including spermatozoa, while the same sites are methylated in oocytes. This difference between the paternally and maternally derived *Xist* loci is maintained in the preimplantation embryo (Norris *et al.*, 1994; Ariel *et al.*, 1995; Zuccotti and Monk, 1995). Therefore, the low rate of transcription of *Xist* during spermatogenesis has been suggested to be related to demethylation events enabling its paternal imprinting, rather than to spermatogenic X chromosome inactivation (Norris *et al.*, 1994).

2.5 Chromosomal aberrations and other factors affecting sex body formation

Some information about sex body formation is derived from mice and humans with chromosomal aberrations.

The X chromosome rather than the Y chromosome appears to be instrumental to sex body formation. This can be concluded from the fact that presence of a Y chromosome, as a pairing partner for the X chromosome, is not required for sex body formation. For instance, spermatocytes in X*Sxr*0 mice do still form a sex body (Burgoyne *et al.*, 1986; Handel *et al.*, 1994). Therefore, the X chromosome appears to be the initiator for formation of the sex body. Mouse strains having a fragment of an autosome translocated to the X chromosome, have impairment of spermatogenesis. This impairment is more or less pronounced, dependent on the autosome fragment involved, the location of the breakpoints, and the genetic background (Mahadevaiah *et al.*, 1988). In many cases of X;autosome translocations, the spermatogenic cells do not proceed through the meiotic divisions, but the pachytene spermatocytes do possess a sex body that can include the translocated autosome fragment (Handel *et al.*, 1994).

The structural changes of the X-chromosomal chromatin can extend to the connected translocated autosome fragment (Solari, 1974; Jaafar *et al.*, 1989; Handel *et al.*, 1994). For instance, in mice bearing the reciprocal t(X;16) Searle's translocation, the XY body is enlarged, and part of the translocated autosome fragment adopts the distinctive structure of sex chromatin (Solari, 1974). In mice with an insertion of a fragment of chromosome 7 into the X chromosome (Cattanach's insertion), the inserted fragment behaves as an internal part of the X chromosome, and the sex body remains transcriptionally inactive (Solari, 1974; Handel *et al.*, 1994). Those mice possessing two normal chromosomes 7 next to the translocated fragment are fertile (Meistrich *et al.*, 1979).

In addition to X;autosome translocations, sex body constitution or formation can be affected by the presence of an extra autosomal chromosome or autosomal fragment that lacks a pairing partner. The reason for this is, that unpaired autosomal fragments often associate with the X chromosome (De Boer and Branje, 1979; De Boer *et al.*, 1986). Similarly to translocated fragments, associated autosomal fragments also can take the same morphological characteristics as the X and Y chromosomes, and exhibit reduced transcriptional activity (Guichaoua *et al.*, 1991; Richler *et al.*, 1989; Jaafar *et al.*, 1993). Probably dependent on their size, the

associated autosomal material can be included within the sex body (De Boer and Branje, 1979; Speed, 1986a; Guichaoa *et al.*, 1991), or can prevent the sex chromosomes from moving to a peripheral position and forming the sex body (Richler *et al.*, 1989). Parts of an associated autosome included within the sex body may still be transcriptionally active (Speed, 1986a).

Sex body formation seems to be regulated by extracellular factors. The testicular environment appears to be a prerequisite. Migrating fetal XY germ cells that become located in the adrenal gland instead of the testis, enter meiotic prophase but do not form a sex body (Hogg and McLaren, 1985), whereas testicular fetal XY germ cells, when induced to enter meiosis prematurely, do form a sex body (Whitten *et al.*, 1979). In XY oocytes of sex-reversed mice, the X and Y chromosomes remain univalents and a sex body is not formed (Mahadevaiah *et al.*, 1993).

In summary, the data presented suggest that sex body formation is initiated by a testis-specific signal. The condensation behavior of the X chromosome in spermatocytes is extended to translocated or associated autosome fragments. Sex body formation appears to be prevented when the XY bivalent cannot move to the periphery of the nucleus. This latter finding suggests a role of the nuclear envelope in sex body formation.

2.6 Sex chromosome pairing is essential for progression of spermatogenesis

Successful meiotic sex chromosome pairing and autosome pairing appear to be essential for survival of spermatocytes and oocytes (Miklos, 1974; Burgoyne and Baker, 1984; De Boer and De Jong, 1989; Hale, 1994). Cytogenetic data, both in mouse and human, indicate that diakinesis/metaphase I spermatogenic cells with univalent X and Y chromosomes do not successfully progress beyond the first meiotic division (Burgoyne and Baker, 1984; Gabriel-Robez *et al.*, 1990; Mohandas *et al.*, 1992; Hale, 1994). Examples of mice with male sterility are the X*Sxr*^{a}0 mice, in which the X*Sxr*^{a} chromosome lacks a pairing partner (Mahadevaiah *et al.*, 1988), and the F1 hybrids of *Mus musculus* x *Mus musculus molossinus* or *Mus musculus* x *Mus spretus* mice, in which the pseudoautosomal regions of the sex chromosomes are too distinct to pair efficiently (De Boer and Nijhoff, 1981; Matsuda *et al.*, 1991; Matsuda *et al.*, 1992; Hale *et al.*, 1993).

Spermatogenesis in XY*Sxr*^{a} mice is impaired presumably due to the high incidence of self-pairing (which involves loop formation) of the Y^{Sxr} chromosome (Chandley and Speed, 1987; Mahadevaiah *et al.*, 1988). Nearly all spermatozoa from these mice originate from the minority of spermatocytes in which the X and Y^{Sxr} chromosomes have paired and recombined (Ashley *et al.*, 1994).

Burgoyne *et al.* (1992a) demonstrated that meiosis in X*Sxr*0 mice can be restored by adding a pairing partner. In X*Sxr*0 mice, spermatogenesis is almost completely blocked during meiotic metaphase stage I (Sutcliffe *et al.*, 1991). Burgoyne *et al.* (1992a) produced mice, which possessed, in addition to the X*Sxr* chromosome, a very small chromosome (Y^{*x}) consisting of the pseudoautosomal region and a non-Y centromere, but not containing Y-specific DNA. In the majority of the spermatocytes of this X*Sxr*Y^{*x} mouse, the X*Sxr* chromosome paired with the Y^{*x}, and there was progression of spermatogenesis.

The presence of two X chromosomes in spermatogonia is not compatible with successful spermatogenesis (McLaren, 1981; Mahadevaiah *et al.*, 1988; Handel *et al.*, 1991). In XX*Sxr* male mice, the X chromosomes have a pairing partner and spermatogenesis genes are provided by the *Sxr* fragment, but only a few spermatozoa are formed (Mahadevaiah *et al.*, 1988). The suggestion that this spermatogenic impairment is caused by the presence of two X chromosomes, is

supported by the observation that all pachytene spermatocytes of the XX*Sxr* mice contain only one X chromosome. This indicates that only germ cells that have lost one X chromosome do survive (Mahadevaiah *et al.*, 1988). The reason why spermatogenic cells with two X chromosomes cannot develop, is not known.

Similarly, the mechanism which prevents spermatocytes with unpaired sex chromosomes or autosomes to proceed through meiosis, is unknown. There is discussion whether sex-chromosome pairing without recombination is sufficient for spermatocyte survival (Burgoyne *et al.*, 1992a), or that at least one cross-over is essential (Hale, 1994). Elimination of spermatocytes with unpaired sex chromosomes is not complete. For instance, X*Sxr*0 mice do still produce some spermatozoa (Mahadevaiah *et al.*, 1988). In XYY mice, the elimination mechanism appears to act less efficient or is delayed in older animals (Burgoyne *et al.*, 1992b).

The restrictions for successful meiosis are higher in the male than in the female. For instance, in X0, XY, and XYY female mice (lacking a functional *Sry* gene), the sex chromosomes are unpaired in almost all metaphase I oocytes. In spite of this, X0, XY, and XYY female mice are fertile (Speed, 1986b; Mahadevaiah *et al.*, 1993). Self-pairing of the sex chromosome(s) or non-homologous pairing with autosomal bivalents during meiosis appears to be sufficient for the oocytes to survive (Speed, 1986b; Mahadevaiah *et al.*, 1993; Hale, 1994). Human oogenesis and/or ovary development appears to differ in this aspect, since human X0 females are infertile (Turner, 1938).

2.7 Sex body formation in animals other than mouse and human

Sex body formation takes place in all mammals studied (McKee and Handel, 1993). X-Y recombination, however, is not common for all mammals, since many mammalian species lack a pseudoautosomal region in the X and Y chromosomes. Examples of these animals are the rodents *Baiomys musculus* (southern pygmy mouse; Pathak *et al.*, 1980), *Pitymys duodecimcostatus* (Carnero *et al.*, 1991), *Psammomys obesus* (sand rat; Solari and Ashley, 1977), *Holichilus brasiliensis* (South American marsh rat; Nachman, 1992), and the majority of marsupials (Sharp, 1982). During pachytene stage of spermatogenesis in these species, the X and Y chromosomes are located within the sex body, but do not form a synaptonemal complex.

Sex body formation is absent in birds, reptiles and amphibians. In birds, in which the females are the heterochromatic sex, the behavior of the sex chromosomes (designated Z and W) has been studied for chicken and quail oocytes. The Z chromosome is larger than the W chromosome. During meiosis of oogenesis, the Z axis makes many twists around the W axis, so that the effective lengths of the chromosomes become equal, which enables the chromosomes to join into a synaptonemal complex (Solari, 1992). Homologous pairing and recombination of the Z and W chromosomes appear to be restricted to a small non-twisted part of the synaptonemal complex (Solari, 1992).

In oocytes of the snake *Bothrops jararaca*, the Z and W chromosomes associate end-to-end allowing a part of the Z and W chromosomes to pair; the condensation behavior of this ZW pair is not different from that of the autosomes (Becak and Becak, 1981).

In spermatocytes of the salamander, the X and Y chromosomes synapse at a short fragment. During mid-pachytene stage, the XY bivalent is more condensed than the autosomes (Kezer *et al.*, 1989).

2.8 The function of the sex body

In mammals, sex body formation appears to be essential for pachytene spermatocytes to proceed through meiosis. Several hypotheses have been proposed for the function of the sex body. One suggestion is that the sex body enables and maintains pairing of the heteromorphic X and Y chromosomes. This is consistent with the fact that in oocytes of XY female mice, that do not form a sex body, pairing of the X and Y chromosomes shows a very low efficiency (Mahadevaiah *et al.*, 1993). However, during spermatogenesis, pairing of the sex chromosomes preceeds sex body formation (Richler *et al.*, 1989, Solari, 1989).

Another proposed function is that X chromosomal gene products might be harmful for spermatocytes, which would make inactivation of the X chromosome necessary (Lifschytz and Lindsley, 1972). An increased concentration of X-chromosomal gene products has been reported for primary spermatocytes of sterile mouse strains (Hotta and Chandley, 1982). However, there is no direct evidence for a negative effect of expression of an X-chromosomal gene product in spermatocytes.

A third hypothesis is that the sex chromosomes become heterochromatic to prevent access of enzymes that exert double-strand breaks, which are involved in cross-over between chromatids of autosomes (McKee and Handel, 1993). For the main part of the X and Y chromosomes, such double-strand breaks would be detrimental, because of the lack of a pairing partner.

Another hypothesis is that sex body formation is related to the unability of spermatocytes with univalents to proceed through meiosis. As described under 2.6, the mechanism for elimination of these spermatocytes is unknown. The sex body may play a role in protecting the unpaired parts of the sex chromosomes from being involved in induction of this mechanism.

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The Mouse and Human Y Chromosomes

This chapter gives an overview of the genes, presently identified on the mouse and human Y chromosome. The choice for reviewing the Y chromosome rather than the X chromosome, has been made for the following reasons. For the greater part, Y-chromosomal genes are transcribed in the testis, during embryonic development, and/or postnatally during spermatogenesis. Expression of Y-chromosomal genes in the adult testis is often confined to spermatogenic cells, including postmeiotic spermatids. Several Y-chromosomal loci have been demonstrated to be essential for successful spermatogenesis. In contrast to this, most genes on the X chromosome have no function in testis development and spermatogenesis, that is different from their function in other tissues.

3.1 The mouse Y chromosome

In the mouse, the Y chromosome is the smallest chromosome, representing some 2% of the haploid genome (approximately 50-60 Mb). It is composed of a minute short arm (Yp), a centromeric region, and a long arm (Yq). The distal tip of the long Y arm pairs and recombines with the distal tip of the long X arm, and is therefore called the pseudoautosomal region (Bishop, 1993).

For the greater part, the Y chromosome consists of highly repeated DNA sequences with no obvious function. A large number of DNA probes have been generated which detect sequences that are randomly repeated on the Y chromosome. For instance, the pericentric region of the Y chromosome has a high concentration of simple repeat sequences ([GATA]n/[GACA]n). Several loci on the Y chromosome resulted from viral integration (Bishop, 1993).

Almost all functional Y-chromosomal genes are located on the minute short arm, as has been demonstrated by studies of the so-called sex reversed (*Sxr*) mouse. *Sxr* mice are XX or X0 mice that develop as males, because of the presence of a part of the Y chromosome, the Sxr^a fragment, on an X chromosome. This Sxr^a represents most of the Y short arm (see Figure 3.1). X0Sxr^a mice have testes, containing all stages of spermatogenesis, although the spermatozoa that are produced are few in number and abnormal, which results in infertility (Levy and Burgoyne, 1986). Spermatogenesis is restored to an almost normal sperm output level, when a meiotic pairing partner for the pseudoautosomal region of the XSxr^a chromosome is provided (Burgoyne *et al.*, 1992) (see also *Section 2.5*). Fertility,

however, is not restored, since all spermatozoa maintain an abnormal sperm head (Burgoyne *et al.*, 1992). This indicates that the *Sxr*^{a} fragment contains all genes that are essential for male development and spermatogenesis, and that the long arm of the Y chromosome may contain genes involved in sperm head morphology.

Sxr^b is a variant form of *Sxr*^{a}, that arose by a partial deletion within the *Sxr*^{a} region, as indicated in Figure 3.1 (McLaren *et al.*, 1984; Roberts *et al.*, 1988). X0*Sxr*^{b} mice develop as males, but spermatogenesis stops at the spermatogonia stage (Sutcliffe and Burgoyne, 1989). From these studies, it was concluded that the Y chromosomal region absent in *Sxr*^{b} but present in *Sxr*^{a}, the so-called $_{\Delta}$*Sxr*^{b} region, contains a spermatogenesis gene (*Spy*), while the gene responsible for testis determination, later identified as *Sry* (Koopman *et al.*, 1991a), is located in the region shared by *Sxr*^{a} and *Sxr*^{b}. A strong candidate for the genes on the long arm affecting sperm head morphology is the *Y353/B* gene family (Bishop and Hatat, 1987; Conway *et al.*, 1994).

Figure 3.1 gives an overview of the location of genes on the mouse Y chromosome. The following is a short outline of the functions and characteristics of these genes.

Sry (*sex determining region Y*) gene was demonstrated to be the testis determining gene, since it induced male development in an XX mouse that was transgenic for the *Sry* gene and its flanking sequences (Koopman *et al.*, 1991a). In human, mutations of the homologous gene *SRY* have been found to result in sex reversal to give XY females (Harley *et al.*, 1992; Pontiggia *et al.*, 1994). *Sry* is an intron-less gene, and encodes a protein that contains a DNA-binding domain similar to that found in the family of the high mobility group (HMG) box-containing, DNA binding proteins (Gubbay *et al.*, 1990). The Sry protein binds DNA in a sequence-specific manner, inducing DNA bending (Goodfellow and Lovell-Badge, 1993), which probably is involved in regulation of expression of genes downstream in the testis determining cascade (Harley *et al.*, 1992; Pontiggia *et al.*, 1994). The *Sry* gene is transcribed in the pre-Sertoli cells in the genital ridge at day 10-13 of mouse embryonic development, which is the critical period for the indifferent gonad to differentiate towards testis (Koopman *et al.*, 1990; Jeske *et al.*, 1995).

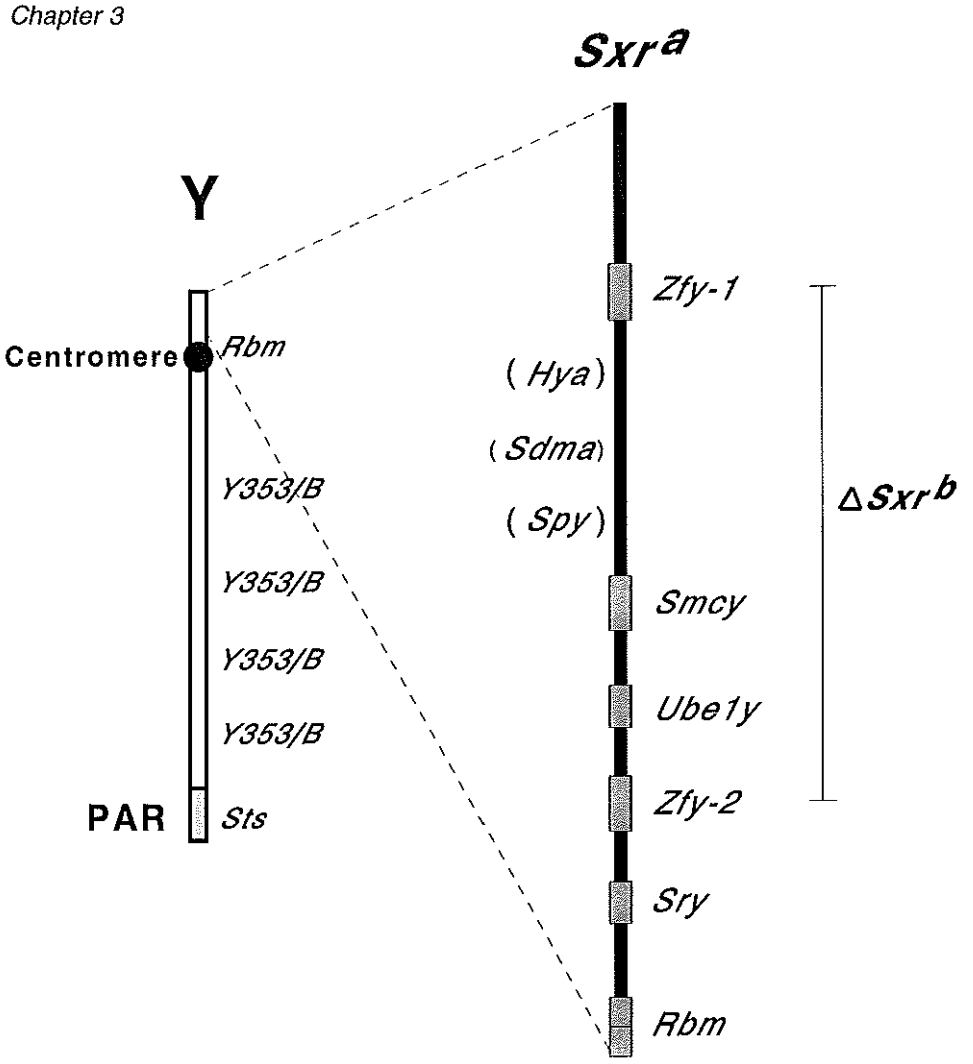

Figure 3.1 *Diagrammatic representation of the mouse Y chromosome and the Sxr (Sex reversed) region.*

The Sxr^a region contains all genes essential for male development and spermatogenesis. ΔSxr^b is a deletion interval within Sxr^a; the remaining sequences form the Sxr^b region. Genes mapping within ΔSxr^b include: Hya, which encodes or controls the expression of H-Y antigen epitopes, as measured by cellular T-cell assays; Sdma, a gene controlling the expression of the serologically defined male antigen (which may be identical to Hya); and Spy, a gene essential for spermatogonial proliferation. Rbm and Y353/B represent gene families, consisting of more copies than presented. The relative position of loci within brackets is not known.

PAR: pseudoautosomal region (Based on Bishop [1994], Conway et al. [1994], and Laval et al. [1995]).

Sry transcription has also been reported to occur in embryos at two-cell and blastocyst stages (Zwingman *et al.*, 1993). Postnatally, *Sry* is transcribed in the adult testis, but this expression is confined to germ cells, mainly spermatids (Rossi *et al.*, 1993; Capel et al, 1993). However, *Sry* mRNA in spermatids for the greater part is circular and probably not translated, while normal linear mRNA is formed in the embryonic genital ridge (Capel *et al.*, 1993; Jeske *et al.*, 1995). In chimeric XY/XY* mice (Y* is a Y chromosome with non-functional *Sry* gene), also spermatids which lack an intact *Sry* gene develop into spermatozoa (Lovell-Badge and Robertson, 1990). *Sry* transcription in spermatids is, therefore, thought to be without specific function.

The *Sxr*^{b} deletion breakpoints have been localized within two homologous genes, the *Zfy-1* and *Zfy-2* genes (Simpson and Page, 1991). *Zfy-1* and *Zfy-2* belong to the zinc finger family of transcription factors. There is a gene homologous to *Zfy-1* and *Zfy-2* on the X chromosome, *Zfx*, and one on chromosome 10, *Zfa* (Koopman *et al.*, 1991b). *Zfa* is an intron-less gene that arose by retroposition of *Zfx* mRNA (Ashworth *et al.*, 1990). *Zfy-2* and *Zfa* are unique for the mouse, and both genes have mutations, as compared to *Zfy-1* and *Zfx*, in the third of the total of 13 zinc fingers (Ashworth *et al.*, 1990; Koopman *et al.*, 1991b). It is not known whether these mutations affect the function of *Zfy-2* and *Zfa*. While *Zfx* is ubiquitously expressed, *Zfy-1*, *Zfy-2*, and *Zfa* are expressed mainly in male germ cells (Koopman *et al.*, 1989; Nagamine *et al.*, 1990; Koopman *et al.*, 1991b), suggesting a role for these three genes in spermatogenesis. In addition, *Zfy-1* is also expressed in embryonic stem cells, in preimplantation embryos, and in some fetal tissues including testis (Koopman *et al.*, 1989; Nagamine *et al.*, 1990; Zwingman *et al.*, 1993). The biological processes in which the genes of the *Zfy* family might have a function, are not known.

Ube1y encodes an E1 ubiquitin-activating enzyme (Kay *et al.*, 1991; Mitchell *et al.*, 1991), which catalyses the first step in ubiquitin-mediated protein degradation or refolding (Jentsch, 1992). The ubiquitin pathway is implicated in many processes, including chromatin remodelling and DNA repair (Jentsch, 1992). A gene homologous to *Ube1y* is located on the X chromosome, *Ube1x* (Kay *et al.*, 1991; Mitchell *et al.*, 1991). While *Ube1x* appears to be expressed in all tissues, *Ube1y* is only expressed significantly in the testis, probably in the germ cells (Kay *et al.*, 1991; Mitchell *et al.*, 1991) (see *Chapter 6*). Although Y homologues of the mouse *Ube1y* gene have been identified in marsupials and all non-primate placental mammals

tested, a Y chromosomal copy is absent in human and chimpanzee (Mitchell *et al.*, 1991; Mitchell *et al.*, 1992).

The $\triangle Sxr^b$ region also contains genes controlling expression of the male-specific transplantation antigen H-Y, and the serological detected male-specific antigen Sdma (McLaren *et al.*, 1984; Goldberg *et al.*, 1991). H-Y antigen is an antigen that causes rejection of male tissue grafts by genotypically identical female mice (Eichwald and Silmser, 1955). It is unknown whether H-Y antigen and Sdma are encoded by the same gene (Wiberg, 1987). Deletion mapping has indicated the presence of two to four H-Y antigen-encoding loci located between *Ube1y* and *Zfy-2* within $\triangle Sxr^b$ (King *et al.*, 1994). One of these genes has been identified: *Smcy* (*Selected mouse cDNA on the Y*) (Agulnik *et al.*, 1994a; Scott *et al.*, 1995). *Smcy* encodes a protein of 321 amino acid residues (Agulnik *et al.*, 1994a). The H-Y epitope, however, is a small peptide of 8 amino acid residues, that is presented with major histocompatibility complex (MHC) molecules at the cell surface (Scott *et al.*, 1995). The mechanisms controlling expression of this protein fragment are not known (Scott *et al.*, 1995). Male cells with different MHC molecules express different H-Y peptides (Falk *et al.*, 1990). The octamer peptide, identified as H-Y antigen peptide by Scott *et al.* (1995), was recognized in combination with the MHC H-$2K^k$ protein. H-Y epitopes presented by other types of MHC molecules may be derived from other parts of the *Smcy*-encoded protein or from other proteins (King *et al.*, 1994; Scott *et al.*, 1995). A gene homologous to *Smcy* is located on the X chromosome, *Smcx* (Agulnik *et al.*, 1994b). The amino-terminal halves of the proteins encoded by *Smcy* and *Smcx* exhibit characteristics of DNA binding proteins, and may, therefore, be involved in regulation of gene transcription (Scott *et al.*, 1995). *Smcy* is expressed in all male tissues, and *Smcx* in all male and female tissues tested (Agulnik *et al.*, 1994a). Its expression starts in preimplantation embryos as early as the 2-cell stage (Agulnik *et al.*, 1994a). According to Southern 'zoo' blot analysis, *Smcy*/*Smcx* homologous genes are also present in other mammals, marsupials, birds, reptiles, and fishes (Agulnik *et al.*, 1994a). Y-specific bands have been detected in human, horse, pig, dog, and kangaroo, but not in rabbit and cattle. Therefore, in rabbit and cattle the X copy of *Smcy*/*Smcx* may have taken over the function of the Y copy (Agulnik *et al.*, 1994a).

Zfy-1 and *-2*, *Ube1y*, and *Smcy* may all be essential for spermatogenesis, and are, therefore, candidates for being the spermatogenesis gene(s) *Spy*, searched for

within $_{\Delta}Sxr^{b}$. Since the $_{\Delta}Sxr^{b}$ region has not been completely characterized yet, the presence of other spermatogenesis genes within this region cannot been excluded.

The Y short arm also contains multiple copies of the *RNA-binding motif* (*Rbm*) gene family, which was first detected in human, where it was proposed to be essential for spermatogenesis (see *Section 3.2*). *Rbm* is expressed in mouse testis in a germ cell dependent fashion, peaking around 14 days after birth, but *Rbm* expression is probably not essential for normal fertility (Laval *et al.*, 1995).

Y353/B is a multiple copy gene, from which mRNA species are specifically expressed in round spermatids (Bishop and Hatat, 1987, Prado *et al.*, 1992, Conway *et al.*, 1994). It is estimated that the mouse Y chromosome contains approximately 30 copies of the *Y353/B* sequence and approximately 250 copies of other *Y353/B*-related sequences, which are distributed along the entire length of the Y chromosome (Bishop and Hatat, 1987). The multiple copy gene might be involved in sperm head formation. The presence of multiple copies along the long Y arm agrees with differential effects of partial Y arm deletions. While complete deletion of the long Y arm causes all sperm to have abnormal sperm heads (Burgoyne *et al.*, 1992), partial deletion of the long Y arm causes a part of the spermatozoa to exhibit sperm head abnormalities (Moriwaki *et al.*, 1988; Styrna *et al.*, 1991) (see also *Chapter 8*). Partial deletion of the Y long arm coincides with a decreased level of *Y353/B* related mRNAs in the testis (Conway *et al.*, 1994). Functions of the *Y353/B*-encoded proteins are unknown (Bishop and Hatat, 1987).

The only gene so far mapped to the pseudoautosomal region is *steroid sulfatase* (*Sts*). The enzyme encoded by this gene hydrolyzes several steroid sulfates, especially estrone-sulfate. It is not clear whether or not *Sts* is subject to X inactivation. The published data available is limited to enzyme activity measurements, and these suggest that *Sts* is X-inactivated, resulting in higher STS levels in male than in female mice (Jones *et al.*, 1989).

3.2 The human Y chromosome

As in the mouse, also many human Y-chromosomal genes have been identified by studying Y regions deleted or translocated in individuals with aberrations in sex differentiation or fertility.

The human Y chromosome differs from the mouse Y chromosome in that it possesses two (rather than one) pseudoautosomal regions (see Figure 3.2). The main pairing site, PAR1, is a region of 2.6 Mb at the terminus of the short arm of the Y chromosome (Yp). The most distal Xp-Yp sequences recombine with a frequency of 50%. This recombination rate decreases for the more proximal markers and reaches 0% at the boundary of X- and Y-specific DNA (Ellis and Goodfellow, 1989; Rappold, 1993). At a low frequency, also double cross-overs can occur within the pseudoautosomal region in the human and in the mouse (Rappold *et al.*, 1994; Soriano *et al.*, 1987). Although less frequently, pairing and recombination does also occasionally take place at a 0.32 Mb site, PAR2, at the terminus of the long arm, Yq (Freije *et al.*, 1992; Kvaløy *et al.*, 1994). So far, 7 genes have been identified in PAR1, whereas probably no essential genes are present within PAR2.

The human testis determining gene, *SRY*, is located very near to PAR1 at the tip of the short arm. At a rather high frequency, approximately 1 in 1000, the X and Y chromosomes recombine at a region larger than the pseudoautosomal region, which can result in XY female individuals lacking *SRY*, or XX male individuals containing the *SRY* gene (Vogt, 1992).

A remarkable characteristic of the human Y chromosome is the presence of a large heterochromatic region at the long arm (Vogt, 1992). This heterochromatic region comprises up to 60% of the Y chromosome, but varies in length among individuals. This variation apparently does not affect health or fertility, and even when the heterochromatic region is totally absent, no functional defect is observed (Vogt, 1992). At present, no genes within the heterochromatic region have been identified. The heterochromatic region is mainly composed of two highly repetitive DNA sequences, *DYZ1* and *DYZ2*, occurring in approximately 5000 and 2000 sequence copies, respectively (Smith *et al.*, 1987).

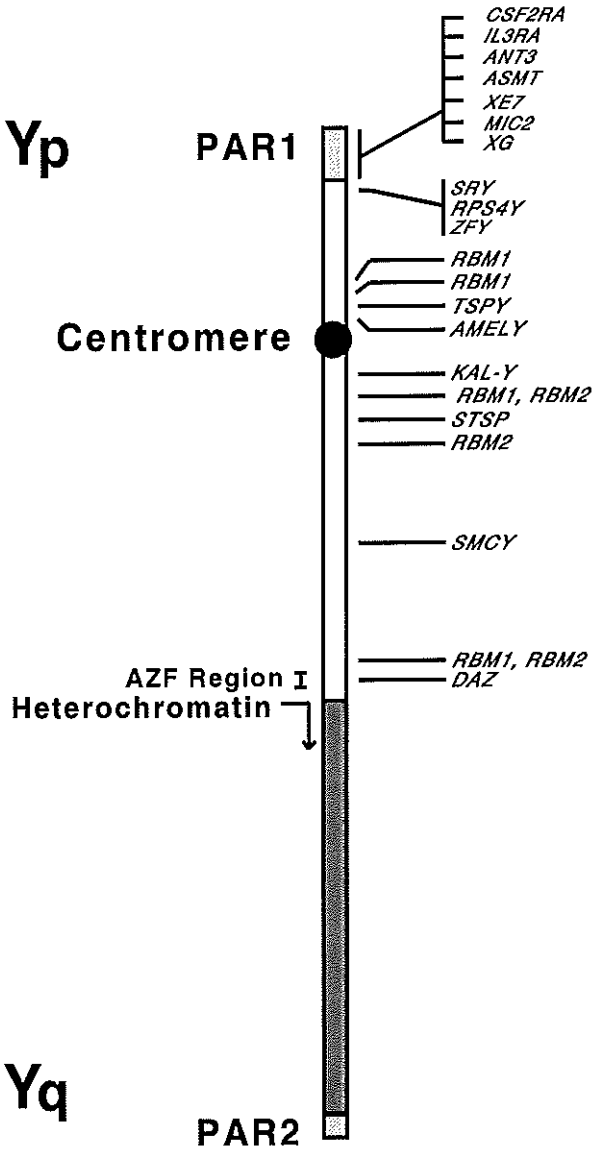

Figure 3.2 *Diagrammatic representation of the human Y chromosome.*

The testis determining gene, SRY, is located very near to the main pseudoautosomal region (PAR1) at the terminus of the short arm (Yp). The DAZ gene is located within the Azoospermia factor region (AZF region), and the DAZ gene product is, therefore, thought be essential for progress of spermatogenesis. RBM1, RBM2, and TSPY represent gene families that consist of more copies than presented (Based on Reijo et al. [1995]).

Terminal deletions of the Y chromosome long arm that include a part of the euchromatin next to the heterochromatin region, as indicated in Figure 3.2, result in azoospermia (no sperm in semen) (Tiepolo and Zuffardi, 1976; Lahn *et al.*, 1994). Azoospermia has also been correlated with deletions that are limited to the euchromatic region located just proximal to the heterochromatin (Vogt, 1992; Ma *et al.*, 1993; Reijo *et al.*, 1995; Stuppia *et al.*, 1996). Studies of genes located within or near this so-called Azoospermia factor region (AZF region) resulted in the identification of the *RBM* gene family and the *DAZ* gene (see *Section 3.2.2*) (Ma *et al.*, 1993; Reijo *et al.*, 1995).

So far, the number of genes identified on the human Y chromosome exceeds that of the mouse Y chromosome. The genes located in the pseudoautosomal region PAR1 are involved in processes that are not related to testis determination or spermatogenesis.

3.2.1 Genes in the human pseudoautosomal region PAR1

This paragraph provides a brief summary of the genes that are located in the pseudoautosomal region PAR1 of the human X and Y chromosomes.

CSF2RA (*colony stimulating factor receptor subunit* α) encodes a growth and differentiation factor that acts on the cells of the monocyte/macrophage lineage (Gough *et al.*, 1990).

IL3RA (*interleukin receptor subunit* α) encodes a cytokine receptor (Kremer *et al.*, 1993). Both *IL3RA* and *CSF2RA* are expressed in hematopoietic cells, and the encoded proteins share the same ß subunit (Rappold, 1993).

ANT3 (*adenine nucleotide translocase*) represents a highly conserved gene from the ADP/ATP translocase family. The encoded protein catalyses the exchange of ATP and ADP across the inner mitochondrial membrane, and thus plays a fundamental role in energy metabolism. *ANT2*, a homologue of *ANT3*, is located on the long arm of the X chromosome and, in contrast to *ANT3*, undergoes X inactivation (Schiebel *et al.*, 1993).

ASMT (*acetylserotonin methyltransferase*) encodes an enzyme that catalyses the final reaction in the synthesis of the hormone melatonin, which is secreted from the pineal gland. It has been suggested to be involved in psychiatric disorders (Yi *et al.*, 1993).

XE7 is a ubiquitously expressed gene with unknown function (Ellison *et al.*, 1993).

MIC2 is located 95 kb distal to the pseudoautosomal boundary. As *XE7*, also *MIC2* is a ubiquitously expressed housekeeping gene. It encodes a cell surface antigen that is involved in cell adhesion processes. A *MIC2* pseudogene (*MIC2P*) is located next to *MIC2* (Goodfellow *et al.*, 1986; Smith *et al.*, 1993).

XG is a polymorphic blood group gene encoding a cell surface antigen on the erythrocyte membrane. *XG* is expressed by hematopoietic tissues and cultured skin fibroblasts. Interestingly, only the promoter region and the first three exons of *XG* are pseudoautosomal, while the remaining seven exons are outside this region and restricted to the X chromosome. Transcription from the *XG* promoter also occurs on the Y gene, although at a low level. The produced RNAs are subject to alternative splicing, which results in hybrid transcripts of *XG* with antisense *SRY* sequences and *XG* with *RPS4Y* sequences. It is unlikely that these transcripts encode a functional protein (Weller *et al.*, 1995).

All pseudoautosomal genes escape X inactivation in somatic XX cells, enabling equal expression of these genes in XX and XY cells. The escape of the *XG* gene from X inactivation leads to a dosage difference between males and females (Weller *et al.*, 1995).

3.2.2 Genes on the Y chromosome outside the pseudoautosomal region

The testis-determining gene *SRY* is encoded by a single exon, located just 5 kb from the pseudoautosomal PAR1 boundary (Sinclair *et al.*, 1990; Foster *et al.*, 1992; Behlke *et al.*, 1993). In contrast to the mouse, human fetal *SRY* expression is widespread rather than restricted to the undifferentiated male gonad (Clépet *et al.*, 1993). Adult expression of *SRY* also is found in testis and several additional tissues (Sinclair *et al.*, 1990; Goodfellow and Lovell-Badge, 1993; Clépet *et al.*, 1993). As indicated in *Section 3.1*, mutation in *SRY*, in particular in the HMG box, results in sex reversal (Harley *et al.*, 1992; Pontiggia *et al.*, 1994). An *SRY* pseudogene is located 2.5 kb from *SRY* and is transcribed in human testis (Behlke *et al.*, 1993).

The *RPS4Y* gene encodes the ribosomal protein small subunit number 4, and is located at approximately 70 kb from the pseudoautosomal boundary (Zinn *et al.*, 1994). A homologous gene, *RPS4X*, is located on the X chromosome, and escapes

X inactivation. Deficiency of *RPS4* protein may contribute to the short stature of XO females, known as the Turner syndrome (Watanabe *et al.*, 1993; Zinn *et al.*, 1994). The mouse has only one *Rps4* gene, which is located on the X chromosome (Zinn *et al.*, 1994).

ZFY is located approximately 200 kb from the pseudoautosomal boundary, thus relatively near to *SRY* and *RPS4Y*. As mentioned in *Section 2.1*, the *ZFY* gene product has characteristics of a transcription factor. Similarly to the mouse, a homologous gene, *ZFX*, is present on the X chromosome. There are also several differences with the mouse. The human Y chromosome contains one instead of two *ZFY* genes, and there is no autosomal homologue. In addition, the human *ZFY* gene is ubiquitously expressed and *ZFX* escapes X inactivation. This suggests that the human *ZFY* and *ZFX* genes, as mouse *Zfx*, have a function in several cell types and tissues (reviewed in Koopman *et al.*, 1991b).

PKX1-Y is a Y-chromosomal homologue of the X-chromosomal *PKX1* gene. *PKX1* encodes a protein kinase. It is not yet known, whether also *PKX1-Y* encodes a functional product. The very precise location of *PKX1-Y* is not known, but the gene resides between *ZFY* and *AMEL1Y* (Klink *et al.*, 1995). The X-chromosomal *PKX1* is located very near to the pseudoautosomal region. Illegitimate recombination occurs relatively frequent between the X- and Y-chromosomal *PKX1* genes, and could be the main cause for Xp/Yp translocations leading to XX males and XY females (Weil *et al.*, 1994; Klink *et al.*, 1995).

The *RNA-Binding Motif* (*RBM*) gene family [formerly called the *Y-located RNA Recognition Motif* (*YRRM*) gene family] was proposed as a candidate for encoding the azoospermia factor, because of the location of *RBM* sequences in or nearby the AZF region and its testis-specific expression (Ma *et al.*, 1993). A more detailed mapping of Y chromosome deletions in men with azoospermia, however, demonstrated the absence of *RBM* sequences and the presence of the *DAZ* gene within the AZF region (see below) (Reijo *et al.*, 1995). The *RBM* gene family has 15 or more members, from which at least two are transcribed. Some of the genes are pseudogenes (Ma *et al.*, 1993). The *RBM* sequences are found dispersed at several locations on the human Y chromosome, but with a clustering on Yq11.23 nearby the interface region between heterochromatin and euchromatin (Ma et al, 1993; Chandley and Cooke, 1994). Expression of the *RBM* gene family is specific for the post-pubertal testis, and is confined to germ cells, predominantly spermatogonia

and/or primary spermatocytes (Ma *et al.*, 1993). According to its sequence, *RBM* belongs to the superfamily of RNA-binding proteins, and may, therefore, have a role in RNA processing, transport, or translational control. Its precise function, however, is unknown. *RBM* sequences are also present in a male-specific fashion in other mammals such as rabbit, bull, and mouse (Ma *et al.*, 1993).

The *DAZ* (*deleted in azoospermia*) gene has been detected by a detailed search for Y chromosome deletions in azoospermic men (Reijo *et al.*, 1995). The *DAZ* gene is located in a region of 500 kb, that was absent in 13% (12 of 89) of azoospermic patients. A recent report shows that deletion of *DAZ* can also result in oligozoospermia (Reijo *et al.*, 1996). Like *RBM*, *DAZ* probably encodes an RNA-binding protein, and is specifically expressed in the testis. Unlike *RBM*, *DAZ* is a single-copy gene in human and chimpanzee (Reijo *et al.*, 1995). The identification of *DAZ* does not exclude the existence of other Y-chromosomal genes involved in male infertility. Approximately 2% of human males are infertile because of severe defects in sperm production (Silber, 1989), while approximately 0.1% are completely azoospermic (Hull *et al.*, 1985). Approximately 10 to 15% of these cases of azoospermia are expected to be caused by a deletion of AZF (Reijo *et al.*, 1995).

The *TSPY* (*testis specific protein Y-encoded*) gene family consists of tandemly repeated, related genes, which are clustered on the short arm of the human Y chromosome (Arnemann *et al.*, 1991; Zhang *et al.*, 1992). The *TSPY* elements reside within each of the 27 to 40 repeat units of approximately 20 kb, that form the so-called *DYZ5* array. The number of *TSPY* elements varies between individuals, from approximately 30 to 60. Transcription of the TSPY elements is testis-specific, and occurs both at fetal and adult stages (Zhang *et al.*, 1992), but not all of the *TSPY* elements are transcribed (Manz *et al.*, 1993). Similar to that of *RBM*, *TSPY* transcription in the adult testis is found to be restricted to spermatogonia and spermatocytes. The function of *TSPY* is unknown, and, so far, there are no data indicating loss of *TSPY* sequences in infertile men (Chandley and Cooke, 1994). The *TSPY* genes appear to have no homologue in mouse, rat, or hamster, but male-specific *TSPY*-related sequences have been found in chimpanzee and macaque, and in pig and cow (Chandley and Cooke, 1994).

The *AMELY* (*amelogenin gene on the Y*) codes for a protein in the enamel of the teeth, and has a homologous gene on the X chromosome. It is not known whether *AMELY* is actively transcribed (Nakahori *et al.*, 1991).

The *KAL-Y* gene is a pseudogene. A functional homologue is located on the X chromosome and belongs to the genes that escape X inactivation. Mutation of the X-encoded *KAL* gene leads to the Kallmann syndrome (Franco *et al.*, 1991; Legouis *et al.*, 1991; Guioli *et al.*, 1992).

STSP, like *KAL-Y*, represents a pseudogene, for which a functional homologue, *STS* (*steroid sulfatase*) resides on the X chromosome and escapes X inactivation (Yen *et al.*, 1987; Yen *et al.*, 1988).

SMCY is the human homologue of the mouse gene encoding H-Y antigen. Similar to the mouse, a homologous gene, *SMCX*, is present on the human X chromosome, that escapes X inactivation (Agulnik *et al.*, 1994b).

Approximately 70 to 80% of the genes that are expected to be present in the human genome still have to be identified. Therefore, the Y chromosome may contain many more genes than those described above. In the context of the human genome project, information about many more genetic loci on the Y chromosome will probably be available within several years from now.

3.3 Evolution of the sex chromosomes

Increasing knowledge about genetic information on the X and Y chromosomes of different species provides indications about the evolution of the sex chromosomes. Most importantly, the existence of several gene pairs with a corresponding location of the two genes on the X and Y chromosomes, indicates that the Y chromosome was once homologous to the X chromosome. The first outline of a prominent hypothesis on evolution of the X and Y chromosomes has been proposed by Ohno (1967), and has since then been extended by others (see Charlesworth, 1991; Graves, 1995), as follows:

The sex chromosomes of all mammals evolved from a pair of autosomes. Due to an unknown event, possibly a single major rearrangement, part of the pro-X and pro-Y chromosomes lost the ability to pair and to recombine. Consequently, the unpaired part of the pro-Y chromosome could evolve independently from the pro-X chromosome, while the unaffected part can be regarded as the ancient pseudoautosomal region. It is inherent to this hypothesis, that the pro-Y chromosome acquired a male-specific function due to a switch-of-function mutation in one or more of its genes. The X chromosome evolved a twofold increase in product output, to compensate for the loss of X-chromosomal genes on the Y chromosome, and a mechanism evolved for inactivation of the extra X chromosome in females. Therefore, most of the Y-chromosomal genes became superfluous, and these genes have undergone gradual genetic deterioration.

After the eutherians (placental mammals) diverged from the marsupials, autosomal regions were added to the eutherian sex chromosomes. Such an autosomal region was probably added to the ancient pseudoautosomal region of one of the two sex chromosomes, and could, therefore, via recombination also become part of the other sex chromosome. After this, the enlarged pseudoautosomal region became also subject to internal rearrangement, and the main part of it was deleted from the Y chromosome. This process is suggested to have occurred several times. Therefore, the X chromosome is thought to have been enlarged in steps, whereas the Y chromosome has gone through cycles of enlargement and attrition. This so-called addition/attrition hypothesis predicts that the pseudoautosomal region is a remnant of the last autosomal addition.

This hypothesis on evolution of the sex chromosomes is based on many indications, and some of these observations will be listed here (see also Charlesworth, 1991; Graves *et al.*, 1995).

a) As described above, several gene pairs are present on the X and Y chromosomes.

b) Genes homologous to genes on the long arm and pericentric region of the human X chromosome map to the X chromosome of marsupials and monotremes (egg-laying mammals) (Graves and Watson, 1991). Marker genes for the short arm of the human X chromosome, however, are autosomal in both marsupials and monotremes (Graves and Watson, 1991). The fact that these short arm markers map to at least three autosomal sites in marsupials and monotremes has led to the idea, that autosomal additions to the mammalian sex chromosomes have occurred several times.

c) The suggestion that the pseudoautosomal region is derived from a relatively recently added autosomal region, is supported by the variety of pseudoautosomal regions among eutherian species. For instance, the two human pseudoautosomal genes *CSFR2A* and *IL3RA* are autosomal in mouse (Disteche *et al.*, 1992; Milatovich *et al.*, 1993).

Acquisition of the male-determining function of the Y chromosome may have been initiated by a Y-specific divergence of a *SOX* gene (*SRY-like HMG box-containing* gene), originally located on the pro-X/Y chromosomes. The X-chromosomal *SOX3* gene is a strong candidate for the gene, from which the Y-chromosomal *SRY* gene has evolved (Foster and Graves, 1994; Collignon *et al.*, 1996).

There are several indications that the Y chromosome has a relatively high freedom for nonfunctional changes during evolution. The Y chromosome contains many retroviral and transposed sequences (Kjellman *et al.*, 1995). In addition, the Y chromosome contains several nonfunctional pseudogenes with functional homologues on the X chromosome. Other Y-chromosomal genes with a X-chromosomal homologue, such as *SRY*, *ZFY*, *SMCY* and *Ube1y*, have acquired a male-specific function. Accumulation of repetitive DNA sequences and loss-of-function mutations on the Y chromosome have been demonstrated to be a consequence of lack of recombination (Steinemann *et al.*, 1993; Rice, 1994).

In birds, the females are heterogametic (ZW), and the males homogametic (ZZ). The bird Z chromosome and the mammalian X chromosome are not homologous (Graves and Foster, 1994). Genes conserved on the mammalian X chromosome are autosomal in birds, while genes on the bird Z chromosome are autosomal in mammals (Graves and Foster, 1994). Therefore, the Z and W chromosomes of birds probably evolved from another pair of autosomes than the mammalian X and Y chromosomes.

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Binding of Anti-H-Y Monoclonal Antibodies to Separated X and Y Chromosome Bearing Porcine and Bovine Sperm

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Abstract

Studies designed to answer the question whether or not H-Y antigen is preferentially expressed on Y chromosome bearing sperm have resulted in conflicting results. This is probably due to the absence of reliable methods for estimating the percentage of X and Y chromosome bearing sperm in fractions, enriched or depleted for H-Y antigen positive sperm.

In recent years a reliable method for separating X and Y chromosome bearing sperm has been published. With this method, separation is achieved by using a flow cytometer/cell sorter, which detects differences in DNA content. This technique provided the first opportunity for testing anti-H-Y antibody binding to fractions enriched for X and Y chromosome bearing sperm, directly.

A total of 7 anti-H-Y monoclonal antibodies were tested using sorted porcine sperm and in one experiment also sorted bovine sperm. All monoclonal antibodies bound only a fraction of the sperm (20 to 50%). However, no difference in binding to the X and Y sperm enriched fractions was found. Therefore, the present experiments do not yield evidence that H-Y antigen is preferentially expressed in Y chromosome bearing sperm.

Introduction

The existence of a male specific antigen, H-Y antigen, was described for the first time in 1955 by Eichwald and Silmser. They discovered that female mice rejected skin transplants derived from syngeneic male mice, while transplants between all other male/female combinations did not result in a rejection. In 1971 the first report (Goldberg *et al.*, 1971) about antibodies directed against H-Y antigen was published. In the early years of H-Y serology, sperm were frequently used for determining the titers of anti-H-Y antibodies. Because of the fact that most anti-H-Y antibodies bound only a fraction of the sperm, it was suggested, that the presence of H-Y antigen is possibly restricted to the Y chromosome bearing sperm (in the following referred to as Y sperm). This was the subject for a multitude of different studies. However, the results of these studies are conflicting. A preference for anti-H-Y antibody binding to Y sperm was found by Bennett and Boyse (1973), Zavos (1983), and Ali *et al.* (1990). However, others did not find any evidence that the presence of H-Y antigen was restricted to Y sperm (Hoppe and Koo, 1984; Ohno and Wachtel, 1978).

Studies designed to answer the question, whether or not anti-H-Y antibodies bind preferentially to Y chromosome bearing sperm, were always hampered by the lack of a reliable method for estimating the percentages of X and Y sperm in different fractions. Methods used for this were: insemination, densitometry or estimation of the percentage of sperm possessing the F-body. The reliability of these methods and/or the statistics used is debatable (see e.g. review of Amann, 1989).

Recently, a method has been described (Johnson *et al.*, 1989), in which relatively small numbers of X and Y sperm can be separated using a flow cytometer/cell sorter. This method makes use of the fact, that X sperm contain more DNA than Y sperm. In livestock species the X chromosome is about 2-3 times the size of the Y chromosome, which is reflected in a difference of total cellular DNA of, depending on the species, about 3.0 to 4.2% (Garner *et al.*, 1983; Johnson, 1986). After treatment of sperm with a DNA-binding fluorescing stain (Hoechst 33342), the X spermatozoa show a slightly brighter fluorescence than the Y spermatozoa, which provides the basis for separation by a modified flow cytometer/cell sorter (Johnson *et al.*, 1987). Besides preparing X and Y sperm enriched fractions, the flow cytometer/cell sorter can also be used for confirming the purity of these fractions.

The reliability of the flow cytometric technique was confirmed in experiments, in which sorted sperm were used to inseminate either rabbits (Johnson *et al.*, 1989) or pigs (Johnson, 1991). The sex ratio of the offspring was very close to the sex ratio as predicted by flow cytometry, varying from 81% males and 94% females in the rabbit, to 68% males and 74% females in the pig for Y and X enriched fractions, respectively.

The objective of this study was to determine the differential binding of anti-H-Y antibodies to enriched X and Y bearing sperm fractions. This was done by immunofluorescence. The availability of enriched X and Y bearing sperm from the boar and bull provided the opportunity to test 1) the percentage of fluorescent X and Y bearing sperm, and 2) the fluorescence patterns of X and Y bearing sperm after anti-H-Y antibody incubation. A total of 7 anti-H-Y MCAs were tested. The gene encoding the protein recognized by one of these anti-H-Y MCAs, MCA 4 VII (Booman *et al.*, 1989a), has been cloned recently (Su *et al.*, 1992). The protein product of this gene (*Male enhanced antigen-2*; *Mea-2*) and a related gene product (*Mea-1*) (Lau *et al.*, 1989) are the only two identified proteins recognized by anti-H-Y antibodies. Interestingly, in the mouse both *Mea-1* and *Mea-2* are expressed during spermatogenesis, reaching the highest levels of expression during post-meiotic

stages (Lau *et al.*, 1989; Su *et al.*, 1992). Another MCA included in our study, MCA 12-49, has been reported to bind preferentially to Y sperm of the bull (Ali *et al.*, 1990).

Materials and Methods

Anti-H-Y Monoclonal Antibodies

Six of the seven tested anti-H-Y monoclonal antibodies were produced in our laboratory. The production and screening of the binding of the anti-H-Y MCAs 5 XI, 4 VII and 5 X have been described by Booman *et al.* (1989a) and that of anti-H-Y MCAs 15 XXI, 21 V and 13 XVIII by Veerhuis *et al.* (1990). One other anti-H-Y MCA, 12-49, was purchased from ATCC (HB9071; Rockville, MD). As a negative control, a MCA directed against an irrelevant epitope (Meuromidase Released Protein of Streptococcus suis) was used (MCA CDI.1), which was kindly provided by Dr. P. v.d. Heijden of the Central Veterinary Institute (CDI-DLO), Lelystad, The Netherlands. All used MCAs have the IgM-isotype. The hybridoma clones were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Gaithersburg, MD) supplemented with 15% fetal calf serum (FCS), 1% w/v penicillin-streptomycin, 0.4% w/v fungizone and 2% w/v glutamine. After a gradual decrease in the percentage of FCS, the DMEM was replaced by serum-free medium (Low Protein Hybridoma Medium, Gibco) prior to harvesting the MCA containing culture medium. The media were concentrated 10 to 20 fold by Amicon filtration (XM300), aliquoted and frozen at -80°C until use. There appeared to be a large difference in MCA production among the several hybridoma clones. Therefore, the IgM-concentrations of the MCAs after concentration varied considerably: CDI.1: 20 µg/ml, 4 VII: 17 µg/ml, 5 XI: 10 µg/ml, 13 XVIII: 67 µg/ml, 12-49: >1000 µg/ml, 21 V: 150 µg/ml, 15 XXI: 71 µg/ml and 5 X: 614 µg/ml. In the fluorescence assay, fluorescence on cells was easier to observe when higher MCA concentrations were used, while the percentage of fluorescent cells was not increased. Based on this observation and on the fact that the comparison of the binding between the X and Y sperm fractions for each individual MCA was more important than the comparison of possible differences in binding among the MCAs themselves, each MCA was tested at the highest possible concentration.

Sperm

Sperm used in the immunofluorescence assays on sorted sperm were collected from mature Landrace, Crossbred or Poland China boars, and a Holstein bull. Sperm of the same animals were also used in the experiments on unsorted sperm together with sperm of Yorkshire boars and an Ayrshire bull. Sperm were used immediately following collection.

Flow Cytometric-Sorting of Sperm

The sorting was performed as previously described (Johnson *et al.*, 1989; Johnson, 1991) with small modifications. Briefly, freshly collected boar sperm were diluted to a cell concentration of $20x10^6$/ml in Beltsville Thawing Solution (BTS) (37.00 g/l D-glucose, 6.00 g/l sodium citrate, 1.250 g/l EDTA, 1.250 g/l sodium bicarbonate, and 0.750 g/l potassium chloride), supplemented with 0.5% Bovine Serum Albumin (BSA). Into each 1 ml sperm suspension, 20 µl Hoechst 33342 (9 mM,

Calbiochem, Inc., LaJolla, CA) was added, followed by incubation for 1 hour at 35°C (Johnson *et al.*, 1989), after which the cells were sorted using a modified (Johnson and Pinkel, 1986) EPICS V flow cytometer/cell sorter (Coulter Corporation, Hialeah, FL). The sorted sperm were collected in previously BSA-coated, 0.5 ml Eppendorf tubes, which contained 50 µl BTS/1%BSA. Owing to the sorting procedure and dilution, cell death occurred rapidly after sorting of the sperm. Therefore, portions of 100,000 sperm were sorted, centrifuged (10 min, 425g), and the supernatant decanted. The sperm were resuspended and stored in the remaining medium (approximately 20µl).

Incubation of bull sperm with Hoechst 33342 was carried out in phosphate-buffered saline (PBS) (136.9 mM NaCl, 2.682 mM KCl, 8.090 mM $Na_2HPO_4.2H_2O$, 1.470 mM KH_2PO_4, and 0.01% NaN_3) supplemented with 0.5% BSA (fraction V). The sorted sperm were collected into 50 µl PBS/1%BSA. Subsequently, they were treated as described for boar sperm.

From each enriched X and Y sperm fraction a sample of about 70,000 cells was used for reanalysis in order to determine the purity of the fraction. Before reanalysis, sperm tails were detached by sonication, since use of sperm heads improves sorting accuracy. The sperm heads were stained again before sorting. Details of the reanalysis method have been described before by Johnson and Clarke (1988) and Johnson *et al.* (1989).

Immunofluorescence Assay

Because of the limited number of viable sperm which could be sorted (about 500,000 of each fraction in 6 hr), an assay was developed for 50,000 sperm per test. In 5 ml polypropylene tubes 20 µl sperm suspension was gently mixed with 80 µl MCA diluted 1:1 in PBS supplemented with 0.1% BSA (PBS/BSA). Unsorted sperm were washed three times in PBS/BSA prior to the first antibody incubation. These washings were not done for sorted sperm, since sorted sperm appeared to be washed efficiently during the sorting procedure, and extra washings strongly reduced viability. After first antibody incubation for 1.5 hr at room temperature, the sperm were washed twice (10 min, 480g) in 4 ml PBS/BSA, after which the supernatant was very carefully aspirated to prevent loss of cells. After the second washing the supernatant was removed down to ±150 µl. The sperm were resuspended in this remaining supernatant and 150 µl Goat anti-Mouse IgM - Phycoerythrin (GAM-PE) (Southern Biotechnology Associates, Inc., Birmingham, AL), prediluted 1/20 in PBS/BSA, was added, incubated for 45 min and washed twice again. Supernatant was removed until 100 µl remained containing about 30,000 to 40,000 cells. Thereafter, the fluorescence of the sperm was observed immediately. Only sperm with intact apical ridges (Pursel *et al.*, 1972) were judged for presence and type of fluorescence. Owing to the limited number of sorted sperm available, in each experiment not more than 5 to 7 of the total set of 8 MCAs could be tested. In some experiments formaldehyde-fixed sperm were used instead of native sperm. The sperm were incubated for 20 min at room temperature in formaldehyde (1% v/v in PBS) and washed twice in PBS.

To examine intracellular protein binding, permeabilized or demembranated sperm were used in the immunofluorescence assay as suggested by Jones *et al.* (1983). Permeabilization was performed by freezing (-80°C) and thawing the cells. Demembranated sperm were obtained by incubating $200x10^6$ cells in 0.5 ml Triton X-100 (0.05% w/v in Tris-buffered sucrose, 0.1 M Tris, 0.25 M sucrose, and 1 mM PMSF, pH7.4) for 30 min at 4°C, followed by two washings.

In all experiments the percentage of fluorescent sperm was estimated by judgement of at least 100 cells.

Differential Live/Dead Staining

The percentage of dead cells was estimated using dye exclusion staining. For sperm which nuclei had been stained by Hoechst 33342 (emission wavelength: 450nm) for sorting purpose, differential live/dead staining was carried out using propidium iodide (emission wavelength: 605nm). Five microliters of sperm suspension was gently mixed with 4 µl PBS/BSA and 1 µl propidium iodide (0.5 mg/ml), incubated for 15 min at room temperature, and the percentage of cells with propidium iodide positive fluorescence was determined by microscopic observation. For sperm incubated with anti-H-Y MCAs and GAM-PE (emission wavelength: 625nm), differential live/dead staining was estimated by adding 2 µl 4',6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co., St. Louis, MO; 0.021 mg/ml; emission wavelength: 495 nm) to 10 µl sperm suspension. Dead cells were stained within 5 minutes.

Results

Binding of Anti-H-Y MCAs to Unsorted Sperm

The binding of anti-H-Y MCAs was first tested on unsorted boar sperm. In general, for each MCA used, a portion of the sperm showed fluorescence, which could be divided into two types of fluorescence patterns. Depending on the anti-H-Y MCA used, 14 to 41% of the sperm showed a rather low-intensity fluorescence, visible as small spots, that were mostly located over the entire head region of the sperm. The percentage per MCA, averaged for four tests, were for 4 VII: 41 ± 9%, 5 XI: 37 ± 5%, 13 XVIII: 26 ± 4%, 5 X: 24 ± 5%, 21 V: 21 ± 5%, 15 XXI: 21 ± 6%, and 12-49: 14 ± 4%. The other labelling pattern, a far more intense fluorescence located on the postacrosomal region, was seen in about 10% to 20% of the sperm. This type of fluorescence was observed for all anti-H-Y MCAs except MCA 12-49. Figure 4.1a,d and 4.1b,e illustrates the two types of fluorescence for anti-H-Y MCA 4 VII. Combination of the immunofluorescence assay with differential live/dead staining revealed that the more intense binding to the postacrosomal region was restricted to dead sperm. When all sperm were killed by freezing (-80°C) and thawing, all cells showed this type of fluorescence. This fluorescence was also observed, when sperm were demembranated by Triton X-100 treatment. In addition to the postacrosomal region, tails, and for some anti-H-Y MCAs, acrosomal caps of Triton X-100 treated sperm also showed fluorescence. Therefore, we concluded that the more intense form of fluorescence was due to binding to (an) intracellular protein(s), which was not related to the presence or absence of the Y chromosome. In the following this binding is referred to as "dead sperm binding". In contrast to the other tested anti-H-Y MCAs, MCA 12-49 did not bind more intensely to dead boar sperm. Boars of different breeds did not show interindividual differences in percentage of bound cells nor in binding patterns.

Neither MCA CDI.1, which is directed to an irrelevant antigen and was included in this study as negative control, nor the secondary antibody GAM-PE, bound to living or dead sperm.

Similarly for bull sperm, it was found that anti-H-Y MCAs bound differentially to dead and living sperm. As in boar sperm, the main fluorescence of dead bull sperm was located on the postacrosomal region. In addition to the postacrosomal region, MCA 5 XI bound to the neck and MCA 21 V to the acrosome of dead bull sperm. MCA 4 VII showed very strong binding to the postacrosomal region and midpiece of dead bull sperm (Figure 4.1c,f). In contrast to dead boar sperm, anti-H-Y MCA 12-49 also showed binding to dead bull sperm over the postacrosomal region. The percentage of living cells showing low-intensity fluorescence was in the same range as observed earlier for boar sperm, except for MCAs 4 VII and 15 XXI which bound to a lower percentage of bull sperm (24 ± 4% and 12 ± 3%, respectively) than boar sperm (41 ± 9% and 21 ± 6%, respectively).

The effects of passage of the sperm through the flow cytometer/cell sorter, and of formaldehyde fixation on anti-H-Y MCA binding were estimated. Flow cytometric-sorted sperm showed some decrease in viability. This seemed to be partly due to the flow sorting procedure itself, and partly due to the temporary storage at a very low sperm concentration, in particular directly after sorting. As a result of this, dead sperm fluorescence was somewhat more frequently observed for flow cytometric-sorted sperm than for unsorted sperm. However, there was no difference in binding to the remaining undamaged sperm between unsorted sperm and flow cytometric-sorted sperm. The binding of the anti-H-Y MCAs 4 VII, 5 XI, 12-49, 21 V, and 5 X to formaldehyde-fixed sperm was similar as to non-fixed sperm. Exceptions were MCAs 13 XVIII and 15 XXI which bound to a higher percentage of fixed sperm than native sperm (13 XVIII: 58 ± 6% vs. 23 ± 3%; 15 XXI: 67 ± 8% vs. 26 ± 6%, respectively; averages over two experiments).

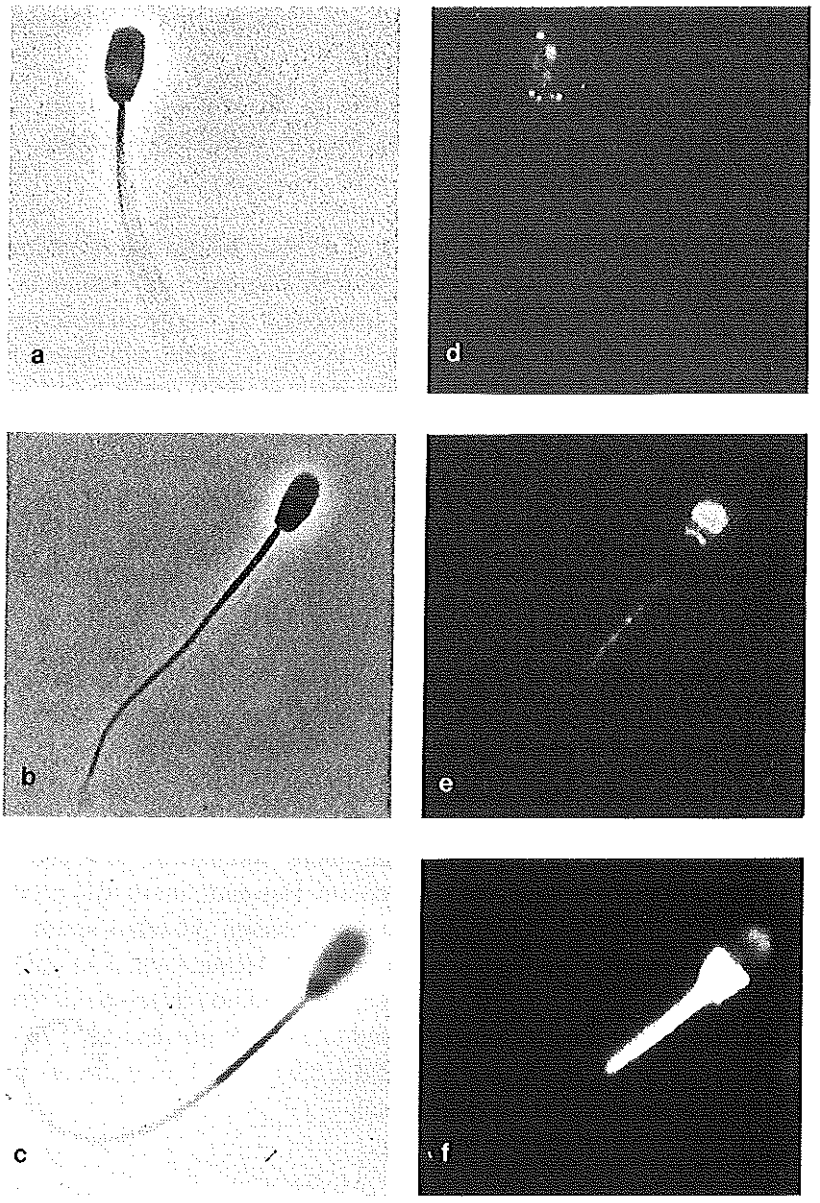

Figure 4.1 *Binding of anti-H-Y MCA 4 VII to boar and bull spermatozoa in an immunofluorescence assay.*

At the left of each panel, the phase contrast image and at the right the fluorescence image is shown. Object lens: x40. ***a,d:*** *Low-intensity fluorescence of boar spermatozoon, as observed on a part of the living sperm.* ***b,e:*** *Strong fluorescence of the postacrosomal region of boar spermatozoon, as observed for dead sperm.* ***c,f:*** *Strong fluorescence of the postacrosomal region and midpiece of bull spermatozoon, as observed for dead sperm.*

Binding of Anti-H-Y MCAs to Sorted Sperm

In total, four experiments with sorted boar sperm were performed. Experiments 1 to 3 used native sperm, while in experiment 4 formaldehyde-fixed sperm were used. Reanalysis of the sorted sperm fractions revealed that in all experiments the purity of the Y fraction was lower than that of the X fraction (see Figure 4.2). The average purity of the X and Y fractions were 90% (range 85-93%) and 61% (range 52-67%), respectively.

For each fraction, approximately 100 cells were judged for fluorescence. Figure 4.3 represents the percentage of fluorescent sperm in the X and Y fraction from the four experiments performed. In experiments 1, 2 and 3 only the percentage of fluorescent cells of the undamaged cell population is given. None of the anti-H-Y MCAs showed a significant difference in percentage of bound cells between the X and Y sperm enriched fractions. Also no difference in binding pattern between the X and Y sperm fractions could be observed. The percentage of sperm showing dead sperm fluorescence was the same in both the X and Y fractions (about 20%).

The use of formaldehyde-fixed sperm in experiment 4 did not reveal any difference in binding between X and Y fractions. In this experiment the total sperm population was judged. This was done because due to an extra washing step prior to fixation, a high percentage of sperm showed some form of membrane damage.

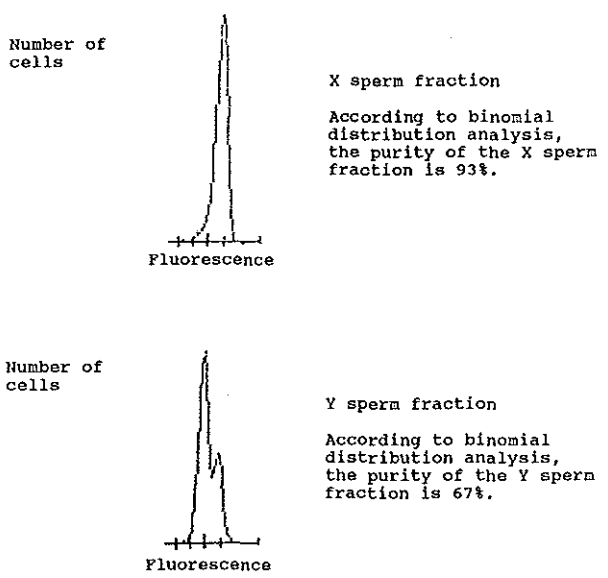

Figure 4.2 *Flow cytometric analyses of sperm nuclei of the X and Y sperm enriched fractions (boar) used in experiment 1. Reanalysis was performed as described in Materials and Methods.*

MCA 12-49 bound a very high percentage of sorted, non-fixed cells (about 80%; Figure 4.3, experiments 2 and 3), while in earlier experiments with unsorted sperm, only a small percentage of the sperm (about 14%) was bound. This increased binding was caused by the presence of a low percentage of BTS in the incubation mixture during the first antibody incubation. This agrees with the results of experiment 4. In this experiment all the BTS was removed during the washing steps prior to and after fixation, and only a very low percentage of each sperm fraction is bound by anti-H-Y MCA 12-49.

One experiment with sorted bull sperm was performed and in this experiment no significant differences in binding to X and Y sperm enriched fractions were found. The percentage of bound cells in the X and Y sperm enriched fractions for the 5 anti-H-Y MCAs tested were 4 VII: 22 vs. 19%, 5 XI: 31 vs. 25%, 15 XXI: 10 vs. 10%, 12-49: 13 vs. 16%, and 21 V: 16 vs. 23%, respectively.

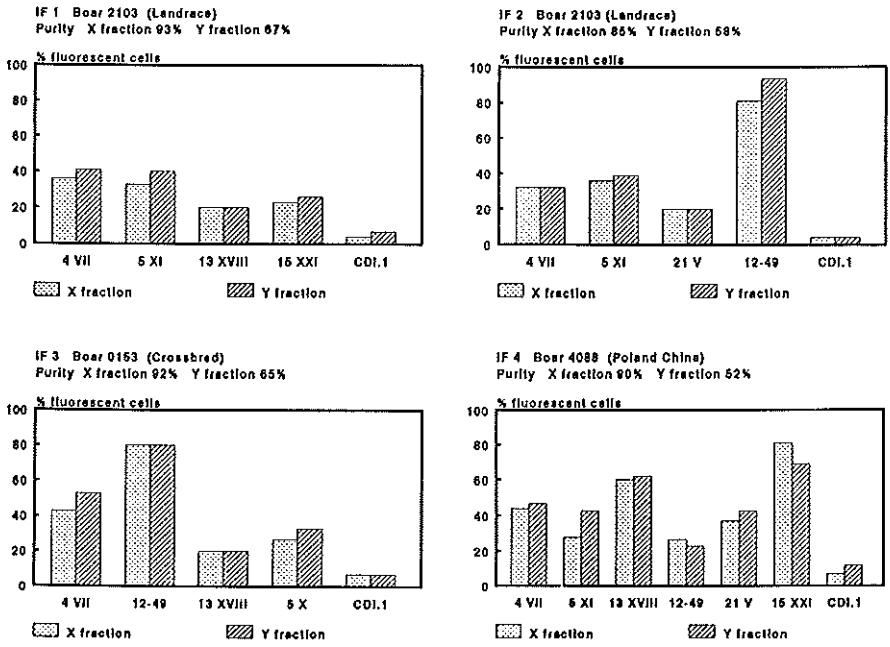

Figure 4.3 *Binding of anti-H-Y monoclonal antibodies to boar sperm fractions, enriched for X and Y chromosome bearing sperm with use of a flow cytometer/cell sorter.*

*In experiments 1-3 (**IF1, IF2,** and **IF3**) only sperm with intact apical ridges were judged for fluorescence. In experiment 4 (**IF4**) sperm were fixed with formaldehyde after sorting. In this experiment all sperm were judged for fluorescence.*

Discussion

The purity of the Y sperm fractions obtained in the present experiments was lower than is desirable. This lower purity is difficult to prevent, and is probably due to a sub-optimal fluorescence of X sperm during the sorting procedure, which then appear as Y sperm. The purity of the sperm fractions, however, was sufficient for detecting MCAs showing Y sperm specificity. The average percentage of Y sperm in the X and Y fraction was 10% and 61%, respectively. This enrichment made no difference to the percentage of cells showing fluorescent binding with any of the anti-H-Y MCAs tested. The labelling levels, both in the X and Y fraction, were the same as those seen in unsorted sperm.

In earlier studies, MCA 5 XI and MCA 4 VII were proven to be partly successful in distinguishing male from female embryos. When using MCA 5 XI, 77% of the tested bovine embryos (Booman *et al.*, 1989b) and 82% of the tested equine embryos (White *et al.*, 1988) were sexed successfully, while MCA 4 VII was able to sex 72% of mouse embryos correctly (Booman *et al.*, 1989b). The male specific antigen, recognized by these antibodies on embryos, seems not to be specifically expressed on Y chromosome bearing sperm. The finding that the expression of H-Y antigen is not related to the presence of the Y chromosome agrees with earlier studies of Hoppe and Koo (1984), and Ohno and Wachtel (1978) with mouse sperm.

The weak binding of the anti-H-Y MCAs to living sperm agrees with earlier published reports, in which anti-H-Y antibodies appeared to have a very low affinity (see, e.g., Wiberg, 1987). In the present experiments the low-intensity fluorescence was located on the entire sperm head. The number of other reports about the distribution of H-Y antigen on sperm are very limited. Koo *et al.* (1973), Zaborski (1979), and Iyer *et al.* (1989) found that the binding of anti-H-Y antibodies to mouse sperm was restricted to the acrosomal cap. Hausman and Palm (1973) described that reactivity of anti-H-Y antibodies with rat sperm was directed primarily to the head. The technique used in their study, mixed hemagglutination, prevented the more precise localization of the anti-H-Y binding. Strikingly, on bull sperm the binding of anti-H-Y antibodies is reported to be directed primarily to either the postacrosomal region (Ali *et al.*, 1990) or both the postacrosomal region and the midpiece (Bradley, 1989). Anti-H-Y binding to the postacrosomal region and midpiece was also found on ram sperm (Bradley *et al.*, 1987). Using the MCA used by Ali *et al.* (1990), anti-H-Y MCA 12-49, we confirmed that the most intense fluorescence of bull sperm was located on the postacrosomal region. However, the suggestion of Ali *et al.* (1990)

that MCA 12-49 binds to about 50% of the sperm, and that the binding is mainly restricted to Y chromosome bearing sperm, could not be confirmed. In our experiments, the postacrosomal binding of MCA 12-49 was related to the condition of the sperm (binding occurred only to dead sperm) and not to the presence of the Y chromosome. A lack of difference in binding of MCA 12-49 to X and Y sperm has also been reported for the mouse (Hoppe and Koo, 1984).

The other anti-H-Y MCAs tested in our study also showed the most intense fluorescence to dead sperm. Since 100% of permeabilized or demembranated sperm showed the same type of fluorescence as dead cells, one can conclude that this type of fluorescence is due to MCA binding to intracellular proteins (Jones *et al.*, 1983). Binding of a polyclonal rat anti-H-Y antibody to the postacrosomal region and midpiece of bull sperm, as described by Bradley (1989), is strikingly similar to the binding of MCA 4 VII to dead bull sperm. In Bradley's study no information is provided about the integrity of the sperm.

In contrast to the binding of MCAs to dead sperm, the low-intensity fluorescence observed on living sperm is probably the result of binding to a membrane protein. The location of this weak fluorescence resembles the fluorescence pattern on mouse sperm described earlier (Koo *et al.*, 1973; Zaborski, 1979).

Confusion exists about the origin of H-Y antigen on spermatozoa. Koo *et al.* (1979) reported that mouse spermatogenic cells show a strong increase of membrane bound H-Y antigen after passing through the meiotic divisions. In contrast to this, Bradley and Heslop (1988) found in the rat that the spermatogonia showed the most intense H-Y staining. It is also suggested (e.g., Bradley and Heslop, 1988) that sperm do not express H-Y antigen themselves but derive H-Y antigen from Sertoli cells. Since the present results suggest that anti-H-Y MCAs bind not only a membrane protein but also react with an intracellular protein, it is tempting to conclude that H-Y antigen is expressed by the sperm themselves. This view is supported by the fact that the *Mea-2* gene, whose product is recognized by anti-H-Y MCA 4 VII, is expressed in spermatogenic cells (Su *et al.*, 1992). Since the binding of anti-H-Y MCAs was only studied on ejaculated spermatozoa and not on spermatogenic cells from earlier stages of spermatogenesis, no conclusion can be drawn about when synthesis of H-Y antigen in spermatogenic cells begins.

In conclusion, the present results show that the presence of H-Y antigen on sperm is not restricted to Y chromosome bearing sperm, as suggested in earlier studies conducted by Zavos (1983), Ali *et al.* (1990), and Bennett and Boyse (1973).

If the antigen is expressed post-meiotically, the presence of the antigen on both X and Y bearing sperm could be due to mRNA and/or protein sharing (Braun *et al.*, 1989). However, an expression in spermatogenic cells independent of the Y chromosome can not be ruled out, since both Mea-1 (Lau, 1987) and Mea-2 (Su *et al.*, 1992) are assigned to autosomal chromosomes (mouse chromosomes 17 and 5, respectively).

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Comparison of Detergent-Solubilized Membrane and Soluble Proteins from Flow Cytometrically Sorted X- and Y-Chromosome Bearing Porcine Spermatozoa by High Resolution 2-D Electrophoresis

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Abstract

The only known and measurable difference between X- and Y-chromosome bearing spermatozoa is the small difference in their DNA content. The X sperm in the human carry 2.8% more DNA than the Y sperm, while in domestic livestock this difference ranges from 3.0 to 4.2%. The only successful sperm separation method, flow cytometric sorting, is based on this difference in DNA content. Using this technique, X and Y sperm populations with purities greater than 90% can be obtained. The number of spermatozoa that can be sorted in a given time period, however, is too low for application of this technique in routine artificial insemination. Therefore, the search for a marker other than DNA to differentiate between X and Y sperm remains of interest in order to develop a method for large scale X and Y sperm separation. The aim of the present study was to investigate whether porcine X and Y sperm contain some difference in their plasma membrane proteins. The flow cytometric sorting of sperm enabled a direct comparison of the proteins of the X and Y sperm populations. High resolution two-dimensional (2-D) electrophoresis was used; however, adaptations were needed to enable its use for analysis of proteins of flow cytometrically sorted sperm, both in the sorting procedure, membrane protein solubilization, and in the 2-D electrophoresis. Up to 1000 protein spots per gel could be detected and quantified. Comparison of the 2-D protein patterns revealed differences in protein spots between sperm of two individual boars. However, no differences in protein spots between the X and Y sperm fractions were found. These results provide additional support for the view that X- and Y-chromosome bearing spermatozoa are phenotypically identical, and cast doubt on the likelihood that a surface marker can provide a base for X and Y sperm separation.

Introduction

Predetermining the sex of offspring is of interest for the prevention of transmission of X-linked diseases in humans and for increasing the rate of genetic progress in livestock. Overall efficiency of animal production is also enhanced through the use of sex preselection. Separation of X- and Y-chromosome bearing sperm for gender preselection is the obvious preference since it places sex determination ahead of fertilization.

Many attempts to separate X and Y sperm have been described. Most of the methods used were based on suggested physical differences between X and Y sperm, such as swimming velocity, density, surface charge, or presence of H-Y

antigen. Separations based on these supposed differences have been claimed, but in all cases the results could not be confirmed by others, and none of the proposed methods have resulted in reliable sperm separation procedures (for reviews see Amann, 1989; Johnson, 1992; Windsor *et al.*, 1993; Johnson, 1994; 1996).

On the other hand, confirmed separation of viable X and Y sperm and the production of sexed offspring have been achieved using flow cytometric cell sorting. This method (Johnson *et al.*, 1989) is based directly on the small difference in DNA content between X and Y sperm. In most mammals, the larger size of the X chromosome compared to the Y chromosome results in a greater amount of DNA in the X compared to the Y sperm. The difference in total DNA content between X and Y sperm ranges from 2.8% in the human to 3.0 - 4.2% in domestic animals (Johnson, 1994). After treatment of sperm with a DNA-binding fluorochrome (Hoechst 33342), the small difference in fluorescence can be detected for separation of X and Y sperm by using a modified flow cytometer/cell sorter (Johnson and Pinkel, 1986). The reliability of this method has been verified by sorted sperm DNA reanalysis and the sex of offspring (Johnson *et al.*, 1989; Johnson, 1991), by fluorescence *in situ* hybridization with X- and Y-chromosome-specific DNA probes in the human (Johnson *et al.*, 1993), and by PCR analysis of single sperm (Welch *et al.*, 1995). The success of the method has also been demonstrated in combination with *in vitro* fertilization in cattle (Cran *et al.*, 1993; Cran *et al.*, 1995) and in humans (Levinson *et al.*, 1995). However, due to its low yield, flow cytometric sorting is not directly applicable for regular artificial insemination and, thus, a procedure to separate large numbers of sperm remains desirable. The presence of an X or Y sperm-specific surface marker (antigenic protein) would be an ideal means for this aim. Such a protein would allow the production of an X or Y sperm-specific antibody and subsequent development of a large scale sperm separation method.

The availability of flow cytometrically sorted nearly pure populations of X and Y sperm enables a direct comparison of proteins between these populations. The goal of the present study was to compare proteins, mainly derived from the plasma membranes, of porcine X and Y sperm using two-dimensional (2-D) electrophoresis. To our knowledge, 2-D electrophoresis studies on plasma membrane proteins of flow cytometrically sorted cells has not been previously reported. Utilization of 2-D electrophoresis for the analysis of plasma membrane proteins of sorted X and Y sperm has been tried before for the bull, but the actual comparison of X and Y sperm proteins was not finalized (Fenner *et al.*, 1992; McNutt and Johnson, 1996). The present report describes our efforts to identify plasma membrane proteins

specific for either X- or Y-chromosome bearing porcine spermatozoa through a 2-D electrophoresis analysis adapted to the use of flow sorted sperm.

Materials and Methods

Sperm

Sperm used for flow cytometric sorting were collected from mature boars of Landrace and Large White breeding using the gloved-hand technique (Johnson *et al.*, 1988). Sperm of a Large White boar were used for Experiments 1 to 4, and sperm of a Landrace boar were used for Experiments 5 and 6 due to unexpected death of the Large White boar.

Flow Cytometric Sorting of Sperm

The sorting was performed as previously described (Johnson *et al.*, 1989; Johnson, 1991; Hendriksen *et al.*, 1993) with some modifications. Briefly, 1.5 ml of freshly collected boar sperm (whole semen) was washed 3 times in 40 ml phosphate-buffered saline containing 1 mM EDTA. The sperm were diluted to a sperm concentration of 15 x 10^6 in 1 ml Beltsville Thawing Solution (BTS; Johnson *et al.*, 1988), supplemented with 0.025% polyvinylalcohol (PVA, hot soluble; Sigma, St Louis, MO), stained with 7 µl of a 0.89 mM Hoechst 33342 solution (6.25 µM final concentration; Calbiochem, Inc., LaJolla, CA), and incubated for 35 min at 32°C. After Hoechst staining, 2 µl of a 0.748 mM propidium iodide solution (1.5 µM final concentration; Calbiochem,Inc.) were added and incubated for 5 min at room temperature (Johnson *et al.*, 1994). Only propidium iodide negative sperm were sorted into X and Y sperm fractions, using modified EPICS V and 750 series flow cytometer/cell sorters (Coulter Corporation, Miami, FL). The stained sperm were excited with the ultraviolet (351, 364 nm) lines of a 5 W 90-5 Innova argon laser (Coherent, Inc., Palo Alto, CA, USA) operating at 175 mW and fluorescence detected through 418 nm long pass filters. A 76-µm jet-in-air flow tip was used. Data were collected on 256 channel histograms. Sheath fluid was phosphate-buffered saline (PBS) containing 1 mM EDTA and 0.01% PVA instead of bovine serum albumin (BSA; McNutt and Johnson, 1996). The sorted sperm were collected in 1.5 ml tubes (Vitech Corp, Columbia, MD), containing 50 µl PBS, 0.01% PVA, 1 mM EDTA and 2 mM phenylmethylsulfonylfluoride (PMSF).

Approximately 150,000 sperm were also sorted into presiliconized 0.5 ml microcentrifuge tubes for sort purity reanalysis by analyzing the sorted sperm for DNA content (Johnson *et al.*, 1987; Johnson *et al.*, 1989).

The X and Y fraction sperm were centrifuged for 30 min at 13,600 g in an Eppendorf microfuge. This long centrifugation time at high speed was required to get tight pellets of the PVA-coated sperm. All supernatant was carefully aspirated and the sperm pellets were frozen and stored at -80°C. All sperm were sorted at Beltsville, MD, USA, and shipped on dry ice to the Netherlands for protein analysis.

Sample Treatment and 2-D Electrophoresis

In total, 6 comparisons of X and Y sperm were performed. In Experiments 1 and 2, four samples of pelleted sorted sperm were used for each analysis. In Experiments 3 to 6, due to the use of larger SDS-PAGE gels, five samples of pelleted sperm per analysis were used. Each sperm pellet

contained 0.8 to 1.0 x 10^6 spermatozoa. Sperm pellets were sequentially solubilized in 50 µl 2% w/v CHAPS (3-[(3 cholamidopropyl)-dimethylammonio]-1-propanesulfonate, Bio-Rad, Richmond, CA) in 1 mM Tris, 25 mM sucrose, 1 mM EDTA and 1 mM PMSF, pH 7.1. After incubating the first sperm pellet for 15 minutes on ice with 50 µl CHAPS solution, the sperm containing solution was transferred to the second vial and this was repeated for the following vials. The last vial was incubated for 60 min. The extracted sperm were centrifuged for 30 min at 13,600 rpm at 4°C. Subsequently, the supernatant was collected and lyophilized. Since next to the proteins also the CHAPS is lyophilized, the lyophilized sample was dissolved in 35 µl sample buffer without detergent (8.5 M urea, 2% v/v Pharmalyte 3-10 (Pharmacia Biotech, Uppsala, Sweden) and 65 mM dithiothreitol (DTT)). This was incubated for 15 min at 30°C, centrifuged for 5 min at 13,500 g, and directly applied on the isoelectric focusing gel.

Both isoelectric focusing and SDS-PAGE were performed on a Multiphor II (Pharmacia) horizontal electrophoresis system. Commercially available immobilized pH gradients (IPG) strips (Pharmacia) were used for isoelectric focusing, according to manufacturer's instructions with minor modifications. The following procedure was used for Experiments 3 to 6 of X and Y sperm. The precast and dried IPG strips (18 cm, pH gradient 3-10 linear) were rehydrated for at least 6 h in 8 M urea, 5% v/v glycerol, 0.5% w/v CHAPS, 1% v/v Pharmalyte 3-10, 10 mM DTT and 0.001% w/v Orange G. The complete sample was applied to the IPG strips and run at 20°C for 35,000 Vh (1 h at 150 V, 4 h at 300 V, 3 h linear raise from 300 V to 3500 V and finally 8 h at 3500 V).

For SDS-PAGE, 0.5 mm thick SDS-PAGE gels were casted, consisting of a 12% T, 3% C resolving gel and a 6% T, 3% C stacking gel, using Bind Silane (Pharmacia) treated glass plates (260 x 200 x 2 mm) as backing. Piperazine diacrylamide (PDA, Bio-Rad) was the cross-linking reagent. The resolving gel contained 0.375 M Tris-HCl, pH 8.8, 0.1% w/v SDS, and 20% v/v glycerol; the stacking gel contained 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS. IPG-strips were incubated for 2 x 15 min with SDS/urea/DTT and SDS/urea/iodoacetamide, respectively, according to manufacturer's instructions, and applied on the SDS-PAGE gel. Next to the IPG-strip, 0.5 µl of molecular weight markers (14 - 94 kDa; Pharmacia) were applied. As source for electrophoresis buffer, ready-made ExcelGel SDS buffer strips (Pharmacia) were used. Electrophoresis was applied at 10°C for 1 h at 20 mA and for the remaining running time (approximately 3 h) at 25 mA, for exactly 1675 Vh. Gels were silver stained using manufacturer's instructions, which are based on the method of Heukeshoven and Dernick (1988).

For Experiments 1 and 2 on X and Y sperm fractions, 18 cm IPG strips were combined with 260 x 120 x 0.5 mm SDS-PAGE gels, since the equipment for casting larger SDS-PAGE gels was not yet available. For some experiments with unsorted sperm, 11 cm IPG strips and 260 x 120 x 0.5 mm SDS-PAGE gels were also used.

2-D Gel Scanning and Data Analysis

The 2-D gels were scanned using a Desk Top Densitometer model DNA35 (Pharmacia); digitized images were analyzed with the PDQuest 4.1 software package developed by PDI (Pharmacia). Gel spots were automatically detected and visually reviewed by using the image detector. After applying more than 100 landmarks within each image, the images were matched and the match was extensively checked and corrected manually.

To detect possible spots of interest, quantitative and qualitative comparisons were made using the analysis programs included in the software of the scanning equipment, in particular by

creating quantitative, qualitative, and statistical user sets. The parameters for making these user sets are indicated in the results section. An additional comparison was made by determining the ratio of the quantity of the individual spots between the X and Y gel for each of the six experiments.
The average Y/X ratio was calculated with the formula:

$$\ln (Y/X)_{average} = (\sum_{n=1}^{6} \ln Yn/Xn) / n.$$

Yn and Xn stand for the quantity of a spot in the Y and X gel, respectively, of experiment n. The value of n was smaller than 6 for those spots which were absent in one or more gels. A correction on spot quantities within each experiment was applied to take account of the variation in summed intensity of all spots per gel.

Results

Adaptations necessary to enable 2-D electrophoresis of flow cytometrically sorted sperm

To minimize the number of sperm and the sorting time required for each 2-D analysis, it was important to use a 2-D system with high sensitivity. Earlier reports on bull sperm, using conventional 2-D equipment with rod gels for first dimension and 1.5 mm slab gels for second dimension, showed that a minimum of 50 µg solubilized protein is required when proteins are detected by silver staining (Fenner *et al.*, 1992). In the present study, the required amount of protein for one analysis could be reduced to 20 µg (solubilized from 3.5×10^6 boar sperm), mainly by decreasing the thickness of the gels to 0.4-0.5 mm. Approximately 3.0×10^5 X and 3.0×10^5 Y sperm are collected per hour of sorting. At this rate 12 hours of sorting was required per gel. Another advantage of the use of thin gels and a small amount of protein sample is an improvement of the resolution. Immobilized pH gradients were used for isoelectric focusing, which considerably improved the reproducibility.

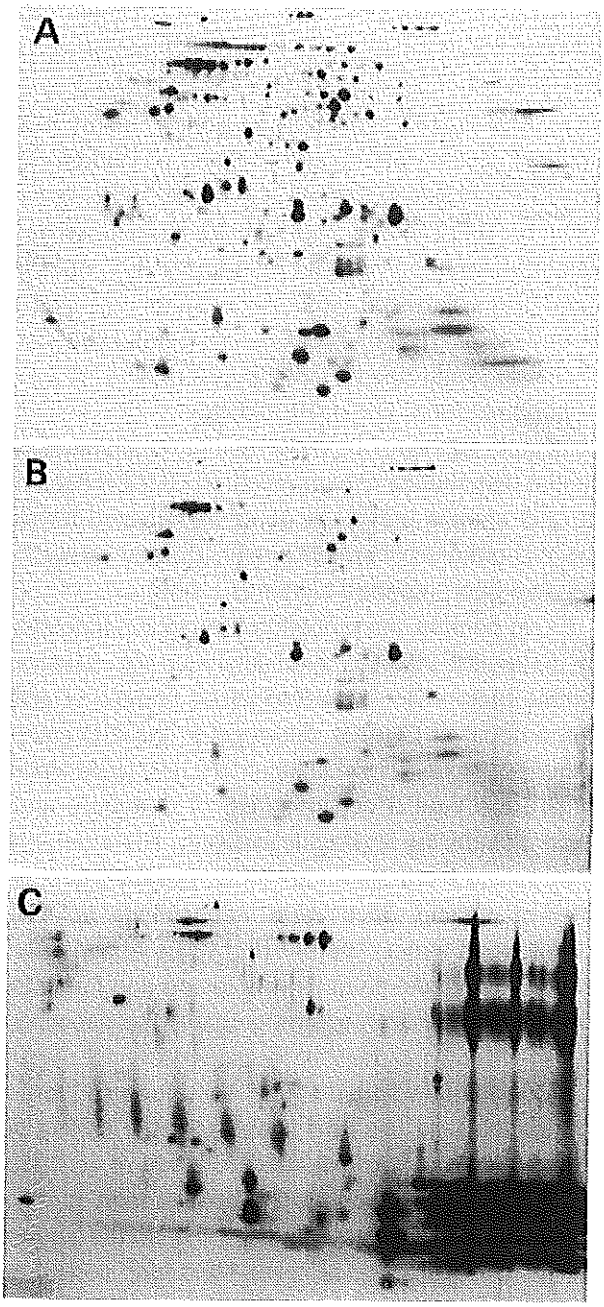

Figure 5.1 *Loss of sperm membrane proteins during flow cytometric sorting when no proteins or polymers were added to the staining fluid, sheath fluid and collection fluid.*

Shown are the 2-D patterns of proteins of: ***A)*** *untreated sperm,* ***B)*** *sperm after undergoing the cell sorting procedure without X/Y selection, and* ***C)*** *seminal plasma. For* ***A*** *and* ***B****, 4 frozen-thawed pellets of 800,000 sperm each were incubated with the same 50 µl of 2% CHAPS, and analyzed as described in Materials and Methods. The 2-D pattern of seminal plasma proteins indicates that the proteins lost during cell sorting (present in* ***A****, absent in* ***B****) are not seminal plasma proteins which are adhered to the spermatozoa. Isoelectric focusing was performed in horizontal direction, SDS-PAGE in vertical direction.*

The limited availability of sorted sperm does not allow the application of a standard plasma membrane isolation procedure. The procedure for solubilizing the membrane proteins, therefore, involved incubation of the sperm directly in detergent solution. This method has been demonstrated to be very efficient in solubilizing plasma membrane proteins both for bovine, boar and rat sperm, while keeping the amount of solubilized intracellular proteins limited (Klint *et al.*, 1985; Jones, 1986; McNutt *et al.*, 1992). Klint *et al.* (1985) used deoxycholate as the detergent for solubilizing boar sperm membrane proteins. We replaced deoxycholate by CHAPS for three reasons: 1) CHAPS did improve the resolution of the isoelectric focusing, 2) isoelectric focusing allows a higher concentration of CHAPS than deoxycholate without disturbance of separation, 3) CHAPS does not require additional detergent in the 2-D sample buffer, as generally advised for deoxycholate solubilized proteins. The 2-D patterns of proteins solubilized by deoxycholate or CHAPS were similar, as has also been reported by Jones (1986).

In general, the procedure was to incubate sperm in CHAPS solution, lyophilize the extracted proteins, and subsequently dissolve the lyophilized sample in 2-D sample buffer. Since the lyophilized sample of proteins also contained a relatively high amount of CHAPS, detergent in the 2-D sample buffer was omitted. The total amount of solubilization solution still had to be limited to 50 µl, since larger volumes caused too high a concentration of detergent and salt in the lyophilized sample, which disturbs the isoelectric focusing. Therefore, prior to solubilization, sorted sperm were pelleted and stored at -80°C. On the day of 2-D analysis, the sperm pellets of different sorting days were sequentially solubilized with the same 50 µl of solubilization buffer. The effect of using frozen sperm pellets instead of fresh sperm on the 2-D protein patterns was tested and found to be very limited with some extra spots from the frozen sperm, probably representing additional intracellular proteins.

When flow sorted sperm are to be used for fertilization, both the staining fluid and sheath fluid used for flow cytometric sorting contain BSA, and the sperm are collected in Test-yolk extender (Johnson *et al.*, 1989), since proteins coat the sperm and improve their condition. These added proteins, however, dominate the 2-D sperm protein patterns. Washing the sorted sperm with PBS was not effective in removing these added proteins. Moreover, sorting of sperm in the absence of added proteins resulted in a high loss of sperm membrane proteins (Figure 5.1A and 5.1B). This may be due to the hydrodynamic and atmospheric forces applied to the sperm during the sorting procedure. In addition, the sorted sperm are subjected to high dilution in the sorting process, and it is known that sperm viability reduces rapidly at

high dilution of the semen (Harrison *et al.*, 1982). Proteins lost during the flow cytometric sorting procedure were not derived from the seminal plasma proteins (Figure 5.1C).

In the search for a compound that could prevent this loss of membrane proteins, a number of compounds were tested: polyvinylpyrrolidone, polyvinylalcohol (PVA, both cold and hot soluble), polyethyleneglycol, Ficoll-400, sodiumhyaluronate, dextran (MW 60-90 kDa), polymerized amino acids (MW < 1 kDa), and glycine. Among these compounds, PVA (hot soluble) was found to be very efficient in preventing protein loss and maintaining a high viability of the sperm without affecting the 2-D pattern.

Another improvement of the quality of the sorted sperm and the relative amount of solubilized proteins was achieved by combining the Hoechst staining with propidium iodide staining. This enabled the sorting of only the viable (propidium iodide negative) sperm into X and Y sperm fractions as previously reported (Johnson *et al.*, 1994).

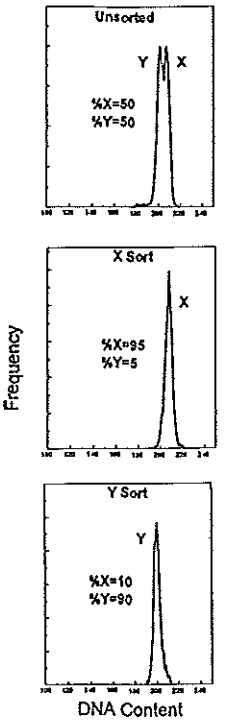

Figure 5.2 *Representative flow cytometric analyses of unsorted, X sorted and Y sorted boar sperm, used in one of the experiments.*

The histograms were fitted with a double Gaussian routine to calculate the proportion of X and Y sperm (Johnson et al., 1987).

Comparison of membrane proteins of X and Y boar sperm

In total, six comparisons of X and Y sorted sperm have been performed. Sperm were derived from two boars, one for Experiments 1 to 4, the other for Experiments 5 and 6. The purities of the sorted X and Y fractions were 94% (range 91-98%) and 88% (range 83-94%), respectively (see Figure 5.2). Figure 5.3 shows the 2-D patterns of proteins of one of the analyses of X and Y sperm. The flow cytometric sorting procedure and excitation by the laser beam did not exert major effects on the 2-D patterns. Care was taken to keep the number of sperm within a set equal, though some variation in number and intensity of spots between the two gels within a set could not be avoided. This was probably caused by a small loss of sperm or sperm membrane proteins in the sperm handling and solubilization procedure, and minor gel to gel variation of the silver staining procedure.

Sophisticated scanning and matching equipment was used to analyze the gels. The number of protein spots per gel ranged from 655 to 823 for Experiments 1 and 2 and from 856 to 1131 for Experiments 3 to 6. This difference is due to the use of larger SDS-PAGE gels and a 25% higher protein load in Experiments 3 to 6. The spots of the X and Y gels were matched to each other. A total of 568 spots could be matched for all 12 gels; 643 spots could be matched for 8 or more gels. The computer-assisted gel analysis was first directed to detect proteins unique for either X or Y sperm. For this, a qualitative analysis was performed to detect spots unique for either the X or Y sperm fractions. In addition, a quantitative analysis was performed to search for spots with at least a five-fold quantitative difference between X and Y fractions. The latter was done, to take into account the possible presence of contaminating X and Y sperm-specific spots on 2-D gels of the Y and X sperm fractions, respectively, that could have been caused by minor impurities of the sorted sperm fractions. A second analysis was performed to search for proteins present on X and Y sperm but with a different level of expression. For this, a quantitative analysis was performed to search for spots with at least a two-fold quantitative difference between X and Y fractions. In both quantitative comparisons, a correction was applied for the variation in summed intensity of all spots per gel.

The qualitative analysis of the X and Y sperm fractions did not result in the detection of unique protein spots. Within one set of X and Y gels, specific protein spots were detected, but these were mainly due to unequal protein loading. Other spots, uniquely present on an X or Y gel within one set, could not be confirmed by the X and Y gels of other sets. These spots probably resulted from some variation in protein solubilization efficiency or electrophoretic migration.

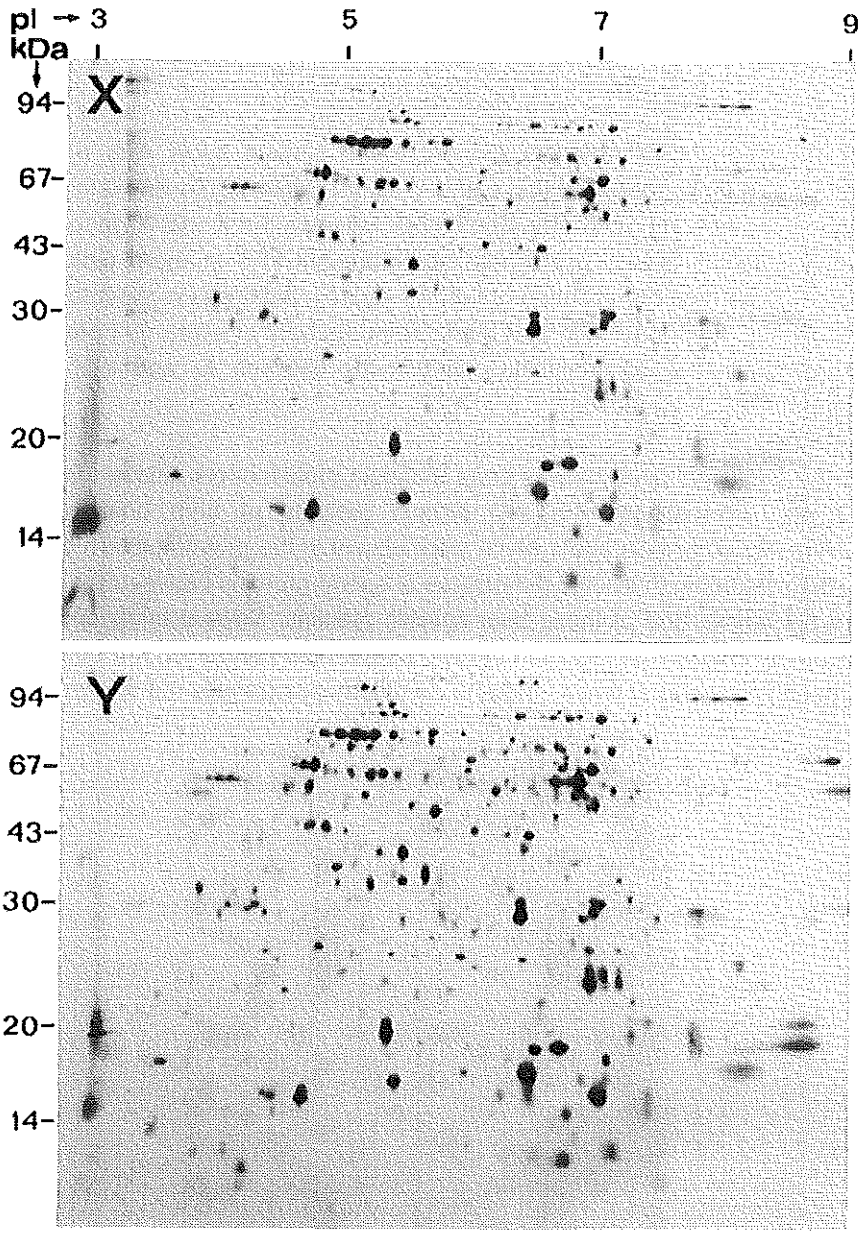

Figure 5.3 *Two-D patterns of membrane proteins from flow cytometrically sorted X and Y sperm fractions.*

The position of the molecular weight markers and the range of pI are indicated in the margins. Five frozen-thawed pellets of approximately 800,000 sperm each were incubated with 2% CHAPS, and analyzed as described in Materials and Methods. X: X sperm fraction, Y: Y sperm fraction.

The quantitative analyses did not detect protein spots with a significant difference in quantity between the X and Y sperm fractions. To illustrate this, Table 5.1 shows the average ratio of the quantities between the X and Y gel of 50 spots, randomly selected from a set of 643 spots present in 8 or more gels. As to be expected, in general the variation of the Y/X quantity ratio was highest for the spots of low density. It is concluded that the present analysis of some 700 proteins does not show qualitative or quantitative differences between X- and Y-chromosome bearing porcine spermatozoa.

An additional analysis was done to detect protein spots unique for one of the two boars. Interestingly, the 2-D protein patterns of both boars possessed approximately 20 protein spots which were absent in the protein pattern of the other boar, demonstrating individual differences of sperm proteins between the two boars. Figure 5.4 shows one of these boar-specific proteins. Several other spots differed considerably in quantitity between the two boars. As a control, sperm from one boar was reanalyzed several times, showing no differences (results not shown).

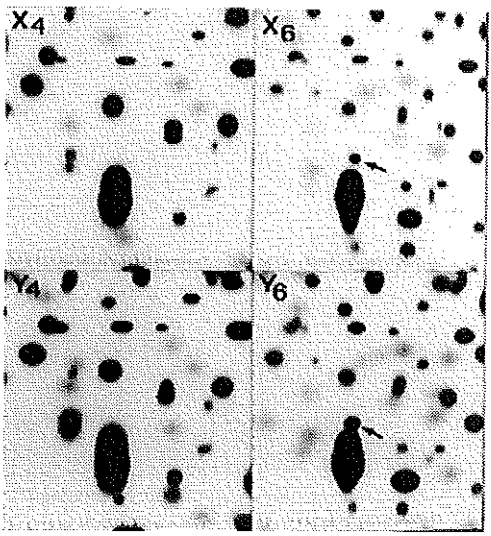

Figure 5.4 *Detection of boar-specific sperm membrane proteins.*

Shown are digitized images of an identical part (pI ranging from approximately 5 to 6, molecular weight ranging from approximately 17 to 28 kDa) of four 2-D gels. X/Y 4 and 6 indicate gels of X and Y sperm fractions of Experiments 4 and 6, respectively. Sperm used in Experiments 4 and 6 were derived from two different boars. The arrows indicate a spot unique for sperm of the boar used in Experiment 6. Several other spots differ in quantity.

TABLE 5.1 Average Y/X Ratio of the Quantity and average Quantity for 50 randomly selected Spots[a]

Spot No (SSP)[a]	Y/X Density Ratio[b] Average (%)	Range (%)	Average density[c] ± SD	
0104	102	83 - 110	115 ± 35	
0206	86	33 - 177	31 ± 20	
0310	89	67 - 114	131 ± 53	
0312	79	52 - 97	45 ± 22	
1010	93	62 - 116	140 ± 53	
1016	110	87 - 146	333 ± 74	
1107	118	87 - 240	13 ± 5	
1303	104	67 - 144	12 ± 5	[1]
1315	77	46 - 108	97 ± 27	
1408	93	58 - 155	19 ± 8	
1703	115	52 - 306	24 ± 12	[2]
2411	100	68 - 173	34 ± 21	
2501	85	50 - 140	13 ± 5	
3001	100	72 - 121	364 ± 126	
3104	113	82 - 166	245 ± 118	
3208	94	40 - 143	18 ± 12	[3]
3306	86	44 - 129	12 ± 3	
3501	97	80 - 109	191 ± 57	
3603	91	43 - 172	27 ± 11	
3826	93	38 - 323	14 ± 8	
4103	97	77 - 118	105 ± 55	
4112	94	54 - 177	17 ± 9	
4113	73	38 - 100	14 ± 8	
4406	92	42 - 200	45 ± 21	
4512	92	56 - 112	251 ± 66	
4713	111	84 - 203	175 ± 67	
4801	91	35 - 200	19 ± 7	
5103	86	61 - 100	310 ± 115	
5402	110	60 - 170	26 ± 12	
5803	79	35 - 129	43 ± 13	
6103	110	103 - 121	609 ± 183	
6205	83	64 - 95	43 ± 17	
6402	83	60 - 115	128 ± 48	
6505	112	84 - 134	579 ± 128	
6602	75	31 - 136	101 ± 60	
6606	108	71 - 208	419 ± 124	
6610	89	30 - 238	27 ± 13	
6805	109	23 - 283	7 ± 3	[2]
6806	110	100 - 133	9 ± 6	[4]
7104	95	80 - 115	45 ± 15	
7108	110	95 - 137	91 ± 53	
7504	98	79 - 112	163 ± 41	
7602	104	73 - 136	172 ± 56	
7703	107	72 - 220	29 ± 8	
7706	94	69 - 117	139 ± 35	
7714	141	94 - 309	45 ± 17	[5]
8124	83	33 - 194	66 ± 38	
8303	99	69 - 147	38 ± 30	
8503	132	79 - 227	31 ± 15	
8803	87	47 - 121	105 ± 38	

[a]For reasons of illustration, 50 spots were randomly selected from a set of 643 spots, which were present in 8 or more gels.

[a]SSP stands for standard spot number; the first two digits correspond to the horizontal and vertical position of the spot, respectively. [b]For each experiment the ratio of the quantity on the Y gel versus the X gel was taken, and the average of this ratio was calculated for the 6 experiments (see Materials and Methods). [c]The quantity is given in arbitrary units reflecting the optical density.

[1]: Absent in gel Y3; [2]: absent in gels X1, Y1, X2 and Y2; [3]: absent in gel Y2; [4]: absent in gels X1, X5 and Y6; [5]: absent in gels Y1, X4 and Y4.

Discussion

The present report is the first to describe a direct comparison of membrane proteins from specifically separated X- and Y-chromosome bearing spermatozoa. Inherent to the flow cytometric sorting method is the fact that only a limited number of sorted sperm can be produced in a reasonable period of time. Therefore, several adaptations had to be made. This is reflected in our choice of plasma membrane protein solubilization and 2-D electrophoresis procedures. In the protein solubilization procedure, some intracellular proteins were solubilized with the plasma membrane proteins. Although a potential difference of intracellular proteins would not be the basis for an immunological separation method of viable sperm, the study of these proteins is still interesting to the general question of whether any phenotypical protein differences between X and Y sperm do exist. In total, six comparisons of porcine X and Y sperm proteins were performed. Among the analyzed proteins, no proteins were unique for either X or Y sperm, or showed a statistically significant difference in quantity between X and Y sperm. Therefore, this study did not provide evidence for the existence of differences in CHAPS-extracted proteins between porcine X and Y sperm. However, approximately 20 proteins were detected which were unique for sperm of either of the two boars tested. This finding deserves further investigation.

The present results do not exclude differences between X and Y sperm for: 1) membrane proteins below the present detection level, 2) intracellular not-solubilized proteins, 3) carbohydrate moieties that do not affect the migration during 2-D electrophoresis. The present results, however, provide additional basis for the hypothesis that X and Y spermatozoa might be phenotypically identical. In spite of many research efforts, confirmed evidence for phenotypical differences between X and Y sperm has not been provided. In many studies in which X and Y sperm separation was claimed, the percentage X and Y sperm were estimated using the F-body test which has been demonstrated to yield inconclusive results (Ueda and Yanagimachi, 1987; van Kooij and van Oost, 1992). A differential expression of H-Y antigen by X and Y sperm has been reported (Bennett and Boyse, 1973; Ali et al, 1990), but was not confirmed in experiments on the binding of anti-H-Y monoclonal antibodies to flow cytometrically sorted X and Y sperm populations (Hendriksen *et al.*, 1993). A difference between X and Y sperm in swimming velocity has been proposed by Roberts (1972), and this knowledge was used to develop the albumin column technique (Ericsson *et al.*, 1973). The reported successes of this method in clinical human practice (Beernink *et al.*, 1993), however, were not confirmed by

others using fluorescence *in situ* hybridization (FISH) to verify the proposed X and Y sperm enrichment (Wang *et al.*, 1994; Claassens *et al.*, 1995). In addition, X and Y sperm appear not to differ in swimming-up properties (Han *et al.*, 1993).

Theoretically, the protein composition of X and Y sperm can only be different when after meiosis X- and/or Y-chromosomal genes are still expressed and when the gene products would be confined to the individual haploid spermatids. Several X-chromosomal genes are found to be inactivated during and after meiosis, e.g. *Pgk-1*, *Pdha-1*, *Phka*, and *Zfx* (Dahl *et al.*, 1990; McCarrey *et al.* 1992). However, post-meiotic expression of the X-chromosomal genes *Ube1x*, *MHR6A* and possibly *Hprt* indicates that after completion of the meiotic divisions the X chromosome is not transcriptionally inactive (Shannon and Handel, 1993; Hendriksen *et al.*, 1995). Furthermore, all Y-chromosomal genes presently studied in the mouse, are post-meiotically expressed in spermatogenic cells (Nagamine *et al.*, 1990; Capel *et al.*, 1993; Conway *et al.*, 1994; Hendriksen *et al.*, 1995).

Post-meiotic expression of sex chromosomal genes, however, does not necessarily result in phenotypical differences between X and Y spermatogenic cells, since gene products can be exchanged among sister spermatids via the intercellular cytoplasmic bridges (Braun *et al.*, 1989; De Boer *et al.*, 1990; Caldwell and Handel, 1991). This exchange, however, does not occur for all gene products. This is illustrated most clearly by a differential fertilizing ability among sperm segregants of male mice heterozygous for mutations within the *t* complex (Olds-Clarke, 1988). Another indication for inequal sharing of gene products among spermatids comes from studies on male Chinese hamsters that are heterozygous for two reciprocal translocations (Sonta *et al.*, 1991). Furthermore, a difference in fertilizing ability has been reported for X- and Y-chromosome bearing spermatozoa, in mice heterozygous for either a Rb(6.15) or Rb(6.16) translocation (Aranha and Martin-DeLeon, 1992; Chayko and Martin-DeLeon, 1992; Aranha and Martin-DeLeon, 1995), and in mice carrying a partial deletion of the long arm of the Y chromosome (Moriwaki *et al.*, 1988; Conway *et al.*, 1994). Hence, in these mutant mouse strains, unequal sharing appears to occur for X- and Y-chromosomal gene products.

It can be speculated that differences in protein composition between X- and Y-chromosome bearing spermatozoa might occur for certain gene products, for which the mRNAs are rapidly translated, and not subject to the translational control that occurs for a number of mRNAs in spermatids, and which encode proteins that are rapidly transferred to membrane systems with limited cell-to-cell dynamics.

Therefore, from a theoretical point of view, phenotypical differences between X- and Y-chromosome bearing sperm cannot be excluded, although our 2-D electrophoresis analysis of the more abundant boar sperm cell surface proteins has not revealed such a difference.

In conclusion, the results of the present study indicate that alternative approaches to sperm separation cannot be based on a relatively abundantly expressed sperm plasma membrane protein, that would lend itself to antibody development. Consequently, we are still left with only one marker by which to differentiate X and Y sperm, that being DNA content.

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Postmeiotic Transcription of X and Y Chromosomal Genes during Spermatogenesis in the Mouse

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Abstract

During the meiotic prophase of spermatogenesis, the X and Y chromosomes form the heterochromatic sex body, showing little transcriptional activity. It has been suggested that transcription of the *Xist* gene is involved in this inactivation. After completion of the meiotic divisions in haploid spermatids, at least two Y chromosomal genes, *Zfy* and *Sry*, are transcribed. In contrast, postmeiotic transcription of X chromosomal genes has not been demonstrated. Using highly purified preparations of mouse pachytene spermatocytes, round spermatids, and cytoplasmic fragments from elongated spermatids, the present experiments show differential postmeiotic expression of the Y chromosomal genes *Ube1y* and *Sry*, with highest mRNA levels in round spermatids and cytoplasmic fragments, respectively. Postmeiotic transcription of the X chromosomal gene *Ube1x* is indicated by an increased level of *Ube1x* mRNA in round spermatids and cytoplasmic fragments. The X chromosomal gene *MHR6A* shows a marked temporary postmeiotic expression in round spermatids.

This postmeiotic activity of the X chromosome is a novel finding, which may have implications for our understanding of X chromosome inactivation during spermatogenesis and paternal genome imprinting.

Introduction

In meiotic prophase oocytes, the two X chromosomes are actively transcribed. In marked contrast, in pachytene spermatocytes both the X and Y chromosomes are heterochromatic, forming the so-called sex body. Incorporation studies using [^{3}H]uridine revealed very low, if any, transcriptional activity of sex body chromatin (reviewed by McKee and Handel, 1993). The long list of genes reported to be expressed postmeiotically in haploid spermatids does not include X chromosomal genes. In fact, it is known that the X chromosomal genes *phosphoglycerate kinase* (*Pgk-1*) and *pyruvate dehydrogenase E1α subunit* (*Pdha-1*) are silenced in spermatocytes and spermatids. The inactivation of these genes is followed by germ cell-specific expression of the autosomal genes *Pgk-2* (McCarrey *et al.*, 1992a) and *Pdha-2* (Dahl *et al.*, 1990). In contrast to X chromosomal genes, clear postmeiotic transcription has been reported for two Y chromosomal genes, *Zfy* (Nagamine *et al.*, 1990) and *Sry* (Capel *et al.*, 1993). In the present study we have analysed the postmeiotic expression of five X chromosomal genes, *Ube1x*, *MHR6A*, *Fmr1*, *Pdha-1*, and *Xist*, and two Y chromosomal genes, *Ube1y and Sry*.

Results

Spermatogenic cells were isolated from testes of mature Swiss CD-1 mice and highly purified using sedimentation at unit gravity (Staput procedure) followed by density gradient centrifugation (Percoll gradients) (Grootegoed and Den Boer, 1989, and references therein). The selected cell types were the meiotic prophase pachytene spermatocytes, postmeiotic round spermatids, and cytoplasmic fragments from elongated spermatids (these fragments are released during the cell isolation procedure and are different from residual bodies). RNA was isolated using the LiCl/urea procedure. Gene expression was studied by reverse transcriptase/polymerase chain reaction (RT-PCR) assay, and *MHR6A* was also studied by Northern blotting. For the RT-PCR assay, cDNA was synthesized using random hexamers. The nucleotide sequences of the primers used in the PCR are presented in Table 6.1. RT-PCR conditions were optimized to obtain semiquantitative results (Figure 6.1). Three separate isolations of spermatogenic cells resulted in identical RT-PCR results.

TABLE 6.1

PRIMERS USED IN THE RT-PCR ASSAYS

Gene	Primers used	Product Size	
		cDNA	Genomic DNA
FSH receptor	GAATC CGTGG AGGTT TTCG CACCT TGCTA TCTTG GCAG	171 bp	171 bp
Activin receptor	CAGGG AACTG GATAT CTAGA GAGAA CTTCC TGGTC CTGGG TCTCG AGTAG GAACA AGTAC	596 bp	—
ß-Actin	GCGGA CTGTT ACTGA GCTGC GT GAAGC AATGC TGTCA CCTTC CC	453 bp	453 bp
Ube1x	TGTCC ACACC CACTT ACT GCACT CTGCA ACTCC TGG	210 bp	340 bp
MHR6A	GACTG CTGAG CCCGC TAAAG GACTC CAACG GTTCT CGAAG	317 bp	— [a]
Fmr1	CTGGA GGTGC CAGAA GATTT ACG CTAGC TTGCT GAATA TTAGC ACC	344 bp	—
Pdha-1	CAAGT GTTGA AGAAT TAAAG TTCAA GCCTT TTTGT TGTCT G	286 bp	—
Xist	ACTGC CAGCA GCCTA TACAG GTTGA TCCTC GGGTC ATTTA	578 bp	—
Ube1y	GACCC CAAGT TCATG GAG CCTCC TAGTC CGTAT GTC	330 bp	1000 bp
Sry	GTGGT CCCGT GGTGA GAG TTTTG TTGAG GCAAC TGCAG	250 bp	250 bp

[a] Unpublished results. The nucleotide sequences were chosen to allow for amplification of X-encoded *MHR6A* mRNAs, but not of autosomally-encoded *MHR6B* mRNAs.

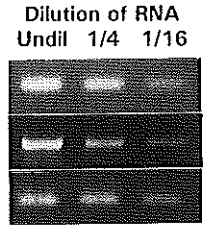

Figure 6.1 *The RT-PCR assay was optimized to yield semiquantitative results.*

The RT-PCR products of dilutions of total testis RNA are shown. Undil: undiluted.

As a control, RT-PCR was performed for the *follicle-stimulating hormone (FSH) receptor* gene, which is known to be specifically expressed in Sertoli cells. *FSH receptor* mRNA was detected in RNA from total testis (which contains approximately 5% Sertoli cells), but of the three spermatogenic cell preparations only the pachytene spermatocytes (containing less than 0.5% Sertoli cells) yielded a weak signal. RT-PCR results of two autosomal genes, *activin type II receptor* and *ß-actin*, demonstrate the integrity of the RNA preparations (Figure 6.2A).

The mRNAs transcribed from the X chromosomal genes *Fmr1* and *Ube1x* were found in all germ cell types, but postmeiotic transcription of the *Ube1x* gene is indicated by an increased level of *Ube1x* mRNA in round spermatids and cytoplasmic fragments (Figure 6.2B). The X chromosomal gene *MHR6A* shows a marked temporary postmeiotic expression in round spermatids (Figure 6.2B). In contrast to *Ube1x* and *MHR6A*, postmeiotic transcription of *Fmr1* is not certain. *Fmr1* has been reported to be highly expressed in type A_1 spermatogonia (Bächner et al., 1993), and it cannot be excluded that the *Fmr1* mRNA in spermatocytes and spermatids is derived from spermatogonia.

The X chromosomal genes *Pdha-1* and *Xist*, for which testicular expression has been published (McCarrey et al., 1992b; McCarrey and Dilworth, 1992), were included in the present experiments. mRNA representing *Pdha-1* was detected in total testis, and only a very low amount was detected in the pachytene spermatocyte preparation, but not in round spermatids and cytoplasmic fragments (Figure 6.2B). The level of *Xist* mRNA found in round spermatids was lower than that in pachytene spermatocytes, while no *Xist* mRNA was detected in the cytoplasmic fragments (Figure 6.2B). These results are in concordance with earlier published data (McCarrey et al., 1992b; McCarrey and Dilworth, 1992). In addition, the *Pdha-1* mRNA expression data confirm the purity of the spermatocyte and spermatid preparations.

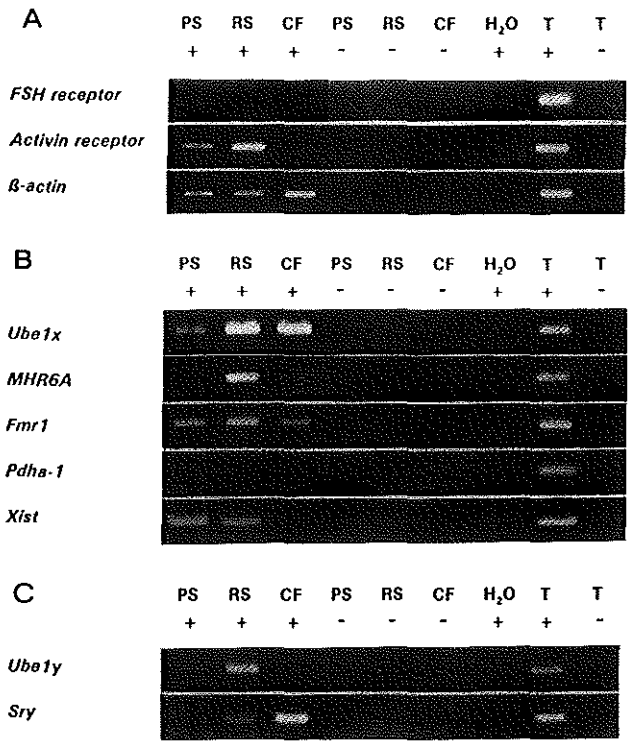

Figure 6.2 RT-PCR analysis of mRNA expression in spermatogenic cell preparations.

(A) Autosomal genes included as a control;
(B) X chromosomal genes; and
(C) Y chromosomal genes. The photomicrographs of ethidium bromide-stained gels are shown. PS, pachytene spermatocytes; RS, round spermatids; CF, cytoplasmic fragments from elongated spermatids; T, total testis; +/-, presence or absence of reverse transcriptase during cDNA synthesis.

The postmeiotic expression of *MHR6A* as detected using RT-PCR was confirmed by Northern blotting. Using a mouse *MHR6A* cDNA probe, a mRNA with a size of approximately 0.8 kb was detected in round spermatids but not in pachytene spermatocytes (Figure 6.3). A mRNA of that size can encode the MHR6A protein (Koken et al., 1991).

As with *Ube1x* and *MHR6A*, the two Y chromosomal genes *Ube1y* and *Sry* also are expressed at higher mRNA levels in round spermatids than in pachytene spermatocytes (Figure 6.2C). A remarkable accumulation of *Sry* mRNA was found at a late stage of spermatogenesis, in cytoplasmic fragments from elongated spermatids (Figure 6.2C).

Figure 6.3 Northern blot analysis of total RNA from pachytene spermatocytes (PS) and round spermatids (RS).

Twenty micrograms of total RNA from each cell preparation was used. Blots were hybridized with a mouse MHR6A cDNA probe and, after washing, rehybridized with a mouse ß-actin probe as a control for the integrity and amount of the RNAs.

Discussion

In spite of abundant postmeiotic transcription of the testis-determining gene *Sry*, this gene may not exert an essential function in spermatogenic cells (discussed in Capel *et al.*, 1993). *Sry* mRNA in adult mouse testes are circular molecules and probably are not translated (Capel *et al.*, 1993). The accumulation of *Sry* mRNA in elongated spermatids, as observed in the present experiments, may be a consequence of this lack of translation, since translation can lead to degradation of mRNA.

Mutation of the human *Fmr1* gene is involved in the fragile X syndrome, which is characterized by various symptoms including mental retardation and macroorchidism (Bakker et al., 1994). Male transgenic mice in which the *Fmr1* gene is not functional are fertile (Bakker et al., 1994). Therefore, the observed *Fmr1* expression in spermatogenic cells appears not to be essential for completion of spermatogenesis.

The protein products of *Ube1x* and *Ube1y* (Kay *et al.*, 1991) and *MHR6A* (Koken *et al.*, 1992) are all involved in ubiquitination; Ube1x and Ube1y are ubiquitin-activating enzymes, and MHR6A is a ubiquitin-conjugating enzyme. Ubiquitination might play a role in the turnover of histones (Jentsch, 1992). Therefore, it is tempting to suggest a role for *Ube1x*, *Ube1y*, and *MHR6A* gene products in the replacement of histone proteins by transition proteins and protamines during spermatogenesis.

The present results indicate that inactivation of both the X and Y chromosomes during spermatogenesis is limited to the period of meiosis. The main function of sex body formation in spermatogenic cells may be to prevent the initiation of potentially damaging recombination events between the sex chromosomes (McKee and Handel, 1993). This places the function of *Xist* expression in spermatogenic cells in another perspective. The level of *Xist* mRNA in testis is 1000- to 2000-fold lower than in female somatic tissues (Kay *et al.*, 1993), and *Xist* mRNA has been detected in developing germ cells from type A spermatogonia up to spermatids (McCarrey and Dilworth, 1992). Hence, the presence of *Xist* mRNA in spermatogenic cells might be unrelated to X chromosome inactivation. X and Y chromosome inactivation in spermatocytes might involve a mechanism other than X chromosome inactivation in female somatic cells. Possibly, *Xist* expression in spermatogenic cells is related to demethylation of the *Xist* gene during spermatogenesis, which recently was proposed to result in paternal imprinting of the X chromosome (Norris *et al.*, 1994).

The postmeiotic reactivation of the X chromosome raises the question of why the gene products of the X chromosomal genes *Pgk-1* and *Pdha-1* are replaced during spermatogenesis by isoenzymes encoded by the autosomal genes *Pgk-2* and *Pdha-2*. The cessation of *Pgk-1* transcription may involve the activity of a testis-specific transcription inhibitor, TIN-1 (Goto *et al.*, 1991), and might not be caused by a general X chromosome inactivation mechanism. Furthermore, during spermatogenesis, there is not only a switch from X-encoded genes to testis-specific autosomal genes, but also a switch from autosomal genes to testis-specific autosomal genes. Such testis-specific autosomal genes include *glyceraldehyde 3-phosphate dehydrogenase*$_S$, *lactate dehydrogenase C*, and *cytochrome* c_T genes (for a review, see Grootegoed and Den Boer, 1989, and Mori *et al.*, 1992). Therefore, the switch from X-encoded *Pgk-1* and *Pdha-1* to *Pgk-2* and *Pdha-1* may not be related to X chromosome inactivation, but may reflect a requirement of spermatozoa for specific isoenzymes.

Postmeiotic expression of X and Y chromosomal genes probably does not cause an inequality between X and Y chromosome bearing spermatids, because sister spermatids can exchange gene products (Braun *et al.*, 1989). All Y chromosomal genes studied until now, *Sry*, *Zfy-1*, *Zfy-2*, and *Ube1y*, have been found to be transcribed in postmeiotic spermatogenic cells. Transcription of X chromosomal genes after meiosis shows differential regulation.

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Testis-Specific expression of a functional retroposon encoding glucose-6-phosphate dehydrogenase in the mouse

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Submitted

Abstract

The X-chromosomal *glucose-6-phosphate dehydrogenase* (*G6pd*) gene is known to be expressed in most cell types of mammalian species. In the mouse, we have detected a gene encoding a G6PD isoenzyme, that is uniquely expressed in post-meiotic spermatogenic cells in which the X-encoded *G6pd* gene is not transcribed. This novel gene, designated *G6pd-2*, does not contain introns, and appears to represent a retroposed gene. Expression of the *G6pd-2* sequence in a bacterial system showed that the encoded product is an active enzyme. Zymogramic analysis demonstrated that recombinant G6PD-2, but not recombinant G6PD-1 (the X chromosome-encoded G6PD), formed tetramers under reducing conditions. Using the same conditions, G6PD tetramers were also found in extracts of spermatids and spermatozoa. This indicates that *G6pd-2* encoded isoenzyme is present in these cell types.

G6pd-2 is one of the very few known expressed retroposons encoding a functional protein. Remarkably, next to *G6pd-2*, three previously reported functional retroposons also originated from an X-chromosomal encoded mRNA transcript, and are also uniquely transcribed in spermatogenic cells.

Introduction

The behavior of the X chromosome during and after completion of the meiotic divisions in spermatogenic cells evokes many intriguing questions.

During the early-pachytene stage of the meiotic prophase of spermatogenesis, the X and Y chromosomes separate from the autosomes and form the so-called sex body, in which the sex chromosomes are condensed (Sachs, 1954; Solari, 1974) and transcriptionally inactive (Monesi, 1965). The function of the sex body is still unknown. After completion of the meiotic divisions, the X and Y chromosomes regain their ability to express genes, but we do not yet know to what extent (Nagamine *et al.*, 1990; Capel *et al.*, 1993; Conway *et al.*, 1994; Hendriksen *et al.*, 1995). Certain important X-chromosomal genes remain transcriptionally silent in spermatids (after completion of the meiotic divisions). Two examples of ubiquitously expressed X-chromosomal genes that are not transcribed in spermatids are *phosphoglycerate kinase-1* (*Pgk-1*) and the *E1α-subunit of pyruvate dehydrogenase* (*Pdha-1*) (Kramer, 1981; McCarrey *et al.*, 1992b; Takakubo and Dahl, 1992). In contrast, two other X-chromosomal genes, *Ube1x* and *MHR6A* (encoding ubiquiting-activating and

ubiquitin-conjugating enzymes, respectively), are examples of post-meiotically transcribed genes.

The inactivation of *Pgk-1* and *Pdha-1* in spermatocytes and spermatids is compensated by testis-specific expression of the autosomal genes *Pgk-2* (McCarrey *et al.*, 1992b) and *Pdha-2* (Takakubo & Dahl, 1992), which encode isoenzymes. Interestingly, *Pgk-2* and *Pdha-2* are intron-less retroposons, which have originated from reversed transcribed mRNA molecules (Boer *et al.*, 1987; McCarrey & Thomas, 1987; Dahl *et al.*, 1990). This is remarkable, since retroposons are very rarely found to be functionally expressed (Vanin, 1985; Nouvel, 1994). *Pgk-2* and *Pdha-2* were found to be present both in mouse and human (Boer *et al.*, 1987; McCarrey & Thomas, 1987; Brown *et al.*, 1990). The autosomal gene *Zfa*, that encodes a protein of unknown function, represents another retroposon that shows testis-specific expression, and that thus far has been found only in the mouse. *Zfa* is homologous to *Zfx*, *Zfy-1* and *Zfy-2* (Ashworth *et al.*, 1990; Erickson *et al.*, 1993). While the Y-chromosomal genes *Zfy-1* and *Zfy-2* are transcribed in spermatids (Nagamine *et al.*, 1990), their X-chromosomal homologue *Zfx* is expressed in all cell types except meiotic and post-meiotic spermatogenic cells (McCarrey *et al.*, 1992a).

We started out to study the expression of another X-chromosomal gene, *glucose-6-phosphate dehydrogenase* (*G6pd*), during mouse spermatogenesis. G6PD is the first enzyme of the pentose-phosphate pathway, which produces reduced coenzyme NADPH and pentoses. G6PD is found in most if not all cell types and organisms, and can be regarded as a housekeeping enzyme (Luzzato and Mehta, 1995). In the present experiments, another gene was identified, encoding a functional G6PD isoenzyme. This novel gene, designated *G6pd-2*, is specifically expressed in spermatids. Nucleotide sequence comparison indicated that *G6pd-2* is a retroposon that has originated from an X-encoded *G6pd-1* mRNA.

Materials and Methods

Mice and purified spermatogenic cell preparations

For all experiments, unless otherwise indicated, Swiss CD-1 mice were used. G6PD-deficient mice were bred on a Swiss background; the original G6PD-deficient mice were kindly provided by Dr W. Pretsch, Institut für Säugetiergenetik, Neuherberg, Germany. Sterile T(11;19)42H mice, with spermatogenesis blocked at meiotic pachytene (De Boer *et al.*, 1986), were kindly provided by Dr L. Vizor, Medical Research Council, Radiobiology Unit, Oxon, U.K. Pachytene spermatocytes and round spermatids were highly purified from Swiss CD-1 testes using sedimentation at unit gravity (Staput

procedure), followed by density gradient centrifugation (Percoll gradients) (Grootegoed and Den Boer, 1989). Epididymal spermatozoa were isolated by cutting the epididymal cauda in small pieces in phosphate-buffered saline solution in a 35mm Petri dish. Spermatozoa were allowed to swim up for 20 min at 35°C. The supernatant was centrifuged for 5 min at 6xg, to pellet larger particles, and the resulting supernatant was centrifuged for 5 min at 800xg, to pellet the spermatozoa.

DNA sequencing

PCR products, obtained from genomic DNA and cDNA and containing biotinylated reverse primers, were purified by Dynabeads (Hultman *et al.*, 1989). Subsequently, these products were subjected to direct sequencing, using FITC-labelled oligonucleotides and the A.L.F. DNA Sequencer system (Pharmacia, Uppsala, Sweden), as described before (Zimmermann *et al.*, 1990). Each sequence was determined at least twice using separately produced PCR products, and most of the ORF was sequenced in both orientations.

RT-PCR and Northern blotting

RNA was isolated using the LiCl/urea precipitation protocol (Auffray and Rougeon, 1980), and treated with RNase-free DNase I (Stratagene, La Jolla, CA) according to the manufacturer's instructions. cDNA was synthesized using random hexamers. The oligonucleotides used as primers for PCR were 5'-ACAGATCTGTGAACGTGTTTGG-3' and 5'-TGCACCCATGATGATAAATATG-3'. These primers enabled amplification of both *G6pd-1* and *G6pd-2* gene products. A proportion of the reaction product was digested with Hae III (Boehringer, Mannheim, Germany) according to the manufacter's instructions, then run on a 3% agarose gel, stained with ethidium bromide, and photographed under UV light.

Northern blotting was performed on poly(A)$^+$ RNA according to standard procedure, using a *G6pd-2* PCR product (nt -22 to 1564; Figure 7.1) as probe. Twenty micrograms of total RNA from each tissue or cell preparation was used.

***In vitro* expression**

PCR product containing the *G6pd-2* open reading frame was obtained from genomic DNA using the primers 5'-ACAGATCTGTGAACGTGTTTGG-3' and 5'-GAAGTTCCAGGGCTCAGTGC-3'. The product was cloned within the T-cloning site of pT7Blue vector (Novagen Inc., Madison, WI), and the nucleotide sequence of the clone was verified. On nucleotide position 907, for which the original template was heterozygous (C and T), the clone had a C-residue, resulting in an additional amino acid difference between the *G6pd-1* and *G6pd-2* products (amino acid 295: Cys $\rightarrow$ Arg). A 1.52 kb Bsu36 I-Sma I (partial)fragment of the *G6pd-2* clone was inserted into the IPTF-inducible expression vector pKK233-2 (Bautista *et al.*, 1992), containing the mouse *G6pd-1* gene, which had been cleaved with Bsu36 I and Pvu II. This *mG6pd-1* clone was kindly provided by Dr P.J. Mason, Royal Postgraduate Medical School, Hammersmith Hospital, London, U.K. The cloning strategy resulted in a protein product containing all G6PD-2 amino acids except amino acid residue 6, which was alanine (as in G6PD-1) instead of threonine. Because of the very poor conservation of the first 20 amino acids of G6PD (Jeffery *et al.*, 1993), this mutation is not expected to affect G6PD function. For expression of mouse *G6pd-1* and *G6pd-2*, the *E. coli* strain DR61212 (Fraenkel, 1968; Rowley and Wolf, 1991) was used, which is devoid of endogenous G6PD activity. Induction of G6PD expression and lysis of the cells were performed essentially as described by Town *et al.* (1992) and Bautista *et al.* (1992), in which for the extraction buffer 0.02% ß-mercaptoethanol was replaced by 5 mM DTT.

Zymograms

Tissue proteins were solubilized according to Ninfali and Palma (1990), with some modifications. Tissues were frozen in liquid nitrogen and stored at -80°C for maximal one month. The frozen tissues were cut into small pieces and pottered on ice in lysis buffer, containing 5 mM sodium/potassium phosphate buffer pH 7.1, 0.25 M saccharose, 0.2% (v/v) Triton X-100, 5 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM EDTA. After 1 h on ice, the suspension was centrifuged for 15 min at 15,000xg at 4°C. Protein concentration of the supernatant was determined with the Bio-Rad protein assay (Bio-Rad, Richmond, CA), using bovine serum albumin in lysis buffer as standard. For native PAGE, 0.5mm thick gels (25 x 12 cm) attached to a 1 mm thick glass plate were used, existing of a stacking gel of 6%T/3%C polyacrylamide, 0.125 M Tris-HCl pH6.8, and a running gel of 6%T/3%C polyacrylamide, 0.375 M Tris-HCl pH 8.8, and 20% w/v glycerol. A horizontal electrophoresis system was used (Multiphor system, Pharmacia, Uppsala, Sweden). Electrophoresis buffer strips were prepared, which consisted of 12%T/3%C polyacrylamide, 0.15 M Tris and 1.14 M glycine pH 8.3. Samples containing 20 µg protein were applied and electrophoresed at constant current (30mA) at 10°C. Gels were stained for G6PD activity, during 30 min at 37°C in the dark, with a mixture containing 0.1 M Tris-HCl pH 7.5, 5.7 mM D-glucose-6-phosphate, 0.34 mM $NADP^+$, 0.057 mM phenazine methosulfate, and 0.21 mM nitroblue tetrazolium (all chemicals from Sigma, St. Louis, MO).

Identification of G6PD dimers and tetramers

The molecular weight (MW) of the protein bands with G6PD activity were estimated using the method of Hedrick and Smith (1968). For this, samples were run on native PAGE gels with polyacrylamide concentrations of 4, 6, 8, and 10%, which did not contain a stacking gel. The retardation of the migration of the G6PD bands was compared to that of co-runned proteins (ovalbumin, 43 k; bovine serum albumin, 67 k; aldolase, 158 k; catalase, 232 k; and ferritin, 440 k), that were visualized by silver staining. The MWs of the faster and slower migrating G6PD proteins were estimated on approximately 140 k and 270 k, respectively. This corresponds with these proteins being dimers and tetramers of 60 k subunits, respectively. When DTT was omitted during the procedures, other tissues than testis and epididymis did also yield a tetramer band (although weak). G6PD monomers do not exhibit enzymatic activity (Grigor, 1984). The MW of hexose-6-phosphate dehydrogenase was estimated on approximately 200 k.

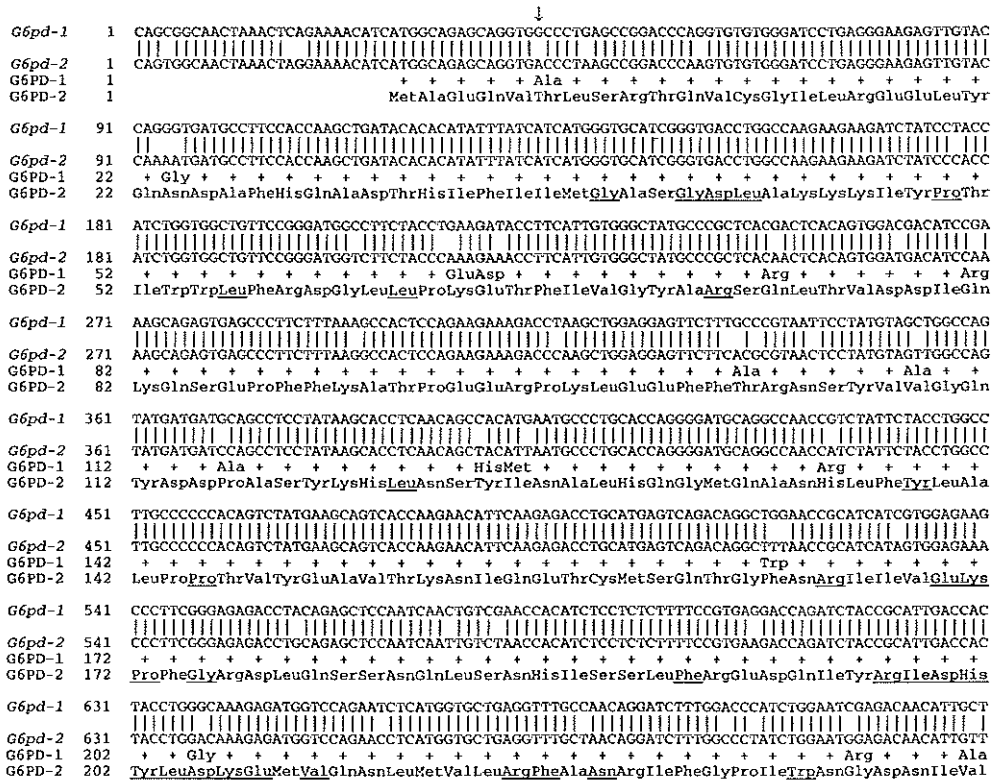
Construction of phylogenetic tree of rat and mouse *G6pd* genes

The tree was constructed according to Fitch and Margoliash (1967). A few nucleotides have been mutated twice, causing uncertainty about the time periods of these mutations. These uncertainties were dealt with in the following way. Nucleotides 462 and 1224 were assumed to have been mutated both in rat and mouse *G6pd* (prior to the origin of the retroposons), but one mutation might have taken place before rather than after radiation of rat and mouse. In the rat, nucleotide 1450 was assumed to have been mutated prior to the origin of the pseudogene, but this mutation might also have been taken place after the origin of the pseudogene. In the mouse, nucleotide 219 was assumed to have been mutated before, and nucleotide 1468 was assumed to have been mutated after the origin of *G6pd-2*, while both nucleotides could have been mutated before or after the origin of *G6pd-2*. The numbering of the nucleotides is according to Figure 7.1.

Results

Characterization of the Intron-less *G6pd-2* Gene

PCR experiments using oligonucleotides spanning different exons (not shown), indicated that the mouse genome contains an intron-less *G6pd* gene, besides the X-chromosomal *G6pd* gene that contains introns. The nucleotide sequence of the open reading frame of the intron-less *G6pd* gene was determined from PCR products of genomic DNA and cDNA. The intron-less *G6pd* gene has an intact open reading frame, that is co-linear with the X-chromosomal *G6pd* gene, encoding a protein of 515 amino acids. It contains one deletion of 6 nucleotides at the 3' end, resulting in the absence of amino acid residues 510 and 511 (Figure 7.1). According to the nomenclature for *Pgk* and *Pdha* genes, the X-chromosomal and the intron-less *G6pd* genes will further be referred to as *G6pd-1* and *G6pd-2*, respectively.



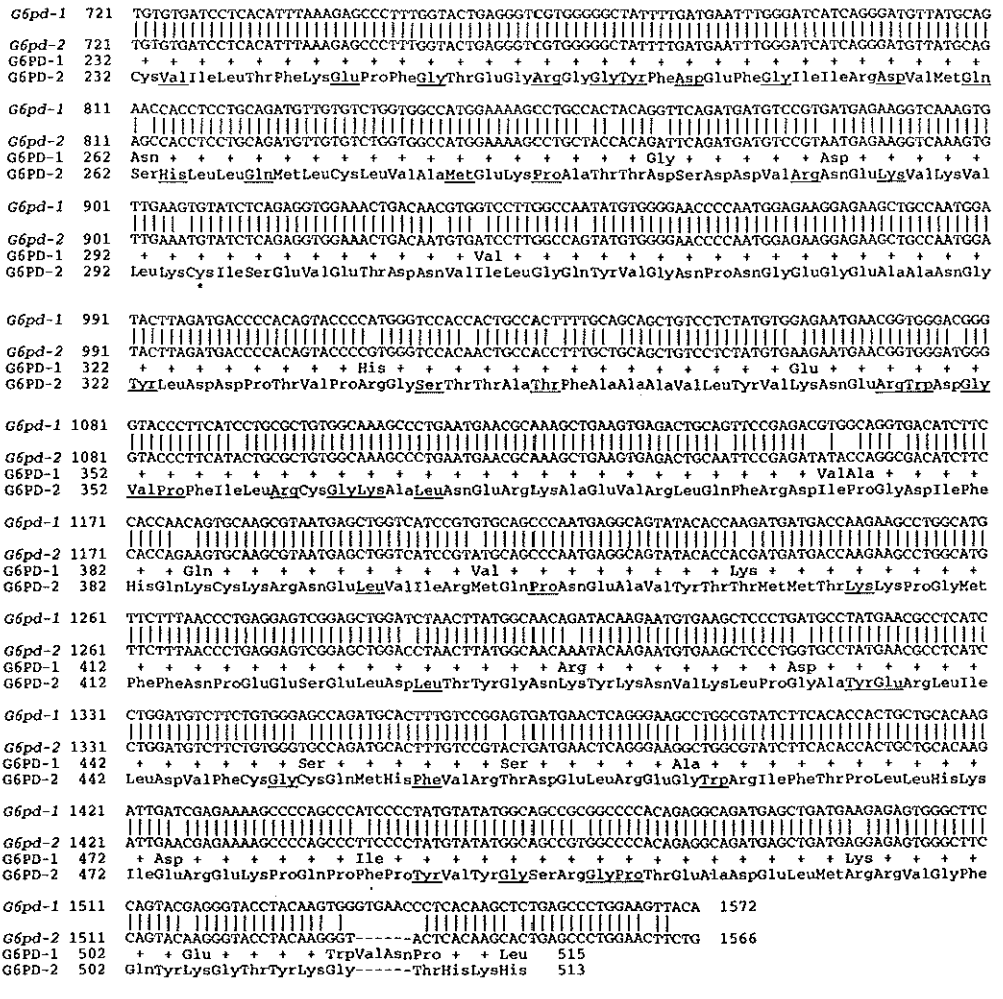


Figure 7.1. *Nucleotide sequence and derived amino acid sequence of mouse G6pd-2 compared to that of X-chromosomal G6pd-1 (Zollo et al., 1993).*

Only those amino acids in which G6pd-1 differs from G6pd-2 are given. Underlined are 72 amino acids, which are conserved among human, rat, mouse (G6pd-1), Drosophila melanogaster, Japanese puffer fish (Fugu rubripes), Saccharomyces cerevisiae, Escherichia coli, Leuconostoc mesenteroides, Zymomonas mobilis and Pichia jadinii (Jeffery et al., 1993, Zollo et al., 1993; Mason et al., 1995).

↓: site that was used to specifically digest PCR products derived from G6pd-1 mRNA.

**: The template from which the G6pd-2 nucleotide sequence was obtained exhibited heterozygosity for nucleotide 907, that repeatedly was determined as both T and C. This mutation causes a change of an additional amino acid (residue 294: Cys → Arg) in one of the two G6pd-2 genes present in the mouse from which the DNA was obtained.*

Sequence identity of the *G6pd-1* and *G6pd-2* open reading frames is 94.3% at the nucleotide level and 92.0% at the amino acid level. A heterozygosity was found for one nucleotide in *G6pd-2*, resulting in an additional amino acid mutation (residue 294: Cys → Arg) in the other allele. Six of the 41 missing or mutated amino acid residues are located in a stretch of 12 amino acids at the carboxy terminus. A total of 72 amino acids of G6PD (underlined in Figure 7.1) are conserved in human, rat, mouse (*G6pd-1*), *Drosophila melanogaster*, Japanese puffer fish (*Fugu rubripes*), *Saccharomyces cerevisiae*, *Escherichia coli*, *Leuconostoc mesenteroides*, *Zymomonas mobilis*, and *Pichia jadinii* (Jeffery *et al.*, 1993, Zollo *et al.*, 1993; Mason *et al.*, 1995). From these 72 amino acids, 71 are also conserved in mouse *G6pd-2*. The exception is amino acid residue 204, where a glycine residue is replaced by aspartic acid (Figure 7.1). Amino acid 204 is located next to lysine 205, that is probably involved in glucose-6-phosphate binding (Camardella *et al.*, 1988) and is essential for G6PD activity (Mason *et al.*, 1988).

Using primers that amplified both *G6pd-1* and *G6pd-2* sequences, the *G6pd-1*/*G6pd-2* PCR product ratio was found to be higher for female DNA than for male DNA (not shown). This indicated autosomal location of the *G6pd-2* gene.

Expression of mRNA

Expression of *G6pd-1* and *G6pd-2* mRNAs in different tissues was studied using RT-PCR. The selected oligonucleotides enabled the amplification of a stretch of 166bp (nt -22 to +144; forward primer located just upstream of the *G6pd-2* sequence shown in Figure 7.1). An Hae III restriction site is present in the RT-PCR product of *G6pd-1*, but absent in that of *G6pd-2*. This was used to discriminate between the two RT-PCR products. As shown in Figure 7.2A, RT-PCR products of lung, brain, epididymis, spleen, liver, embryo, and ovary were all completely digested by Hae III, indicating expression of *G6pd-1*. Other tissues found to contain *G6pd-1* mRNA but not *G6pd-2* mRNA, were epididymal fat, skeletal muscle, bone marrow, kidney, heart, and mammary gland (not shown). In testis, most of the RT-PCR product remained undigested, indicating a relatively high level of *G6pd-2* mRNA.

RT-PCR was also performed on testis RNA from mice of different ages, to study *G6pd-2* mRNA expression during initiation of spermatogenesis. It was found, that *G6pd-2* transcription starts between days 22 and 26 of postnatal testis development, when round spermatids become a prominent cell type (Figure 7.2B). RT-PCR on RNA from purified spermatogenic cell preparations demonstrated a very

low amount of *G6pd-1* mRNA in pachytene spermatocytes (prophase of the first meiotic division), and no detectable *G6pd-1* mRNA in round and elongating spermatids (post-meiotic cells). In contrast, a high level of *G6pd-2* mRNA was found in round spermatids, followed by a lower *G6pd-2* mRNA concentration in cytoplasmic fragments derived from elongating spermatids (Figure 7.2C).

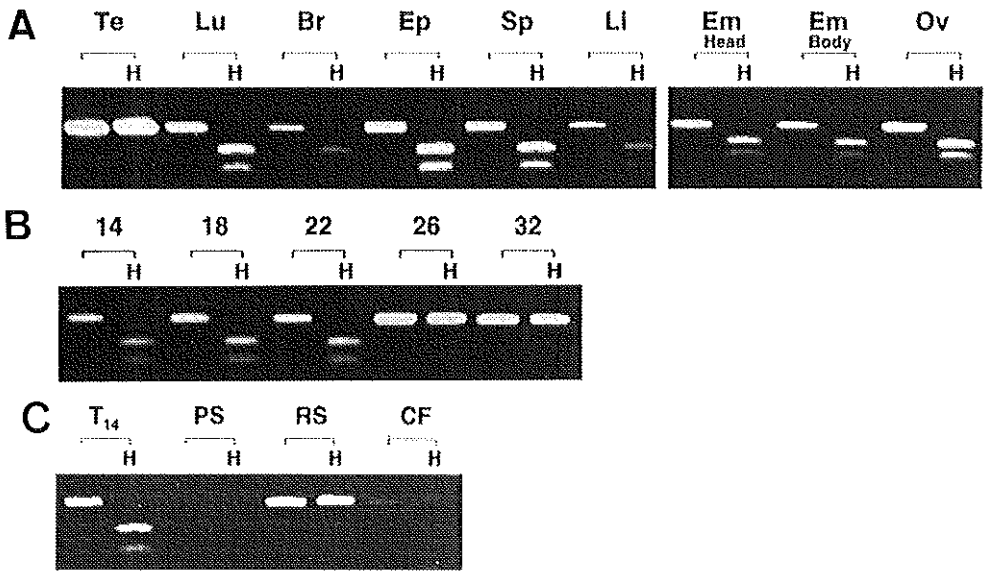


Figure 7.2 *RT-PCR analysis of G6pd mRNA expression in mouse tissues.*

The 166 bp amplification band of G6pd-1, but not that of G6pd-2, is digested by Hae III into 100 bp and 66 bp fragments.

A) *PCR products (untreated and after Hae III digestion **[H]**), obtained from different tissues. The results demonstrate that G6pd-2 is transcribed in adult testis **(Te)**, but not in adult lung **(Lu)**, brain **(Br)**, epididymis **(Ep)**, spleen **(Sp)**, and liver **(Li)**. G6pd-2 transcription does also not occur in the head and body halves of male 14 day p.c. embryos **(Em)**, and in adult ovary **(Ov)**. G6pd-1 transcripts are present in all tissues tested.*

B) *Results obtained for testes at postnatal days 14, 18, 22, 26, and 32. The digestion pattern indicates that G6pd-2 transcription starts between 22 and 26 days of postnatal development.*

C) *Results obtained for purified spermatogenic cell preparations, demonstrating a very low amount of G6pd-1 mRNA in pachytene spermatocytes **(PS)**, a high amount of G6pd-2 mRNA in round spermatids **(RS)**, and a reduced amount of G6pd-2 mRNA in cytoplasmic fragments **(CF;** these cytoplasmic fragments are released from elongating spermatids during the cell isolation procedure and are different from residual bodies). RNA from testes at postnatal day 14 **(T_{14})** was included as a control.*

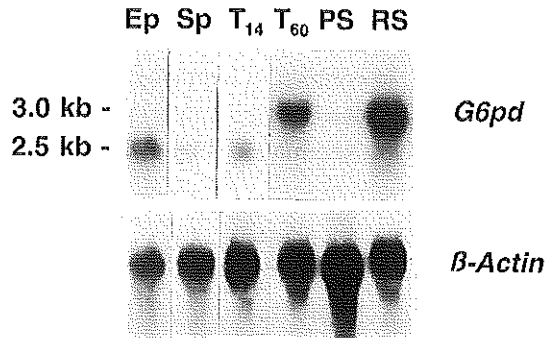


Figure 7.3 Northern blotting analysis of *G6pd* mRNA expression in mouse tissues.

The results demonstrate the presence of a relatively large mRNA species (~3.0 kb) in adult testis (T_{60}) and round spermatids (**RS**), compared to the *G6pd* mRNA species of ~2.5 kb in adult epididymis (**Ep**) and spleen (**Sp**), and in testis at postnatal day 14 (T_{14}). The 3.0 kb and 2.5 kb mRNA species presumably represent *G6pd-2* and *G6pd-1* mRNAs, respectively. Twenty micrograms of total RNA from each cell preparation or tissue was used. Blots were hybridized with a mouse *G6pd-2* probe, and rehybridized with a mouse *ß-Actin* probe as a control for integrity and amount of RNA.

Northern blotting showed the presence of a unique *G6pd* mRNA species in adult testis and round spermatids (Figure 7.3). This mRNA species of approximately 3.0 kb is somewhat larger than the *G6pd-1* mRNA of approximately 2.5 kb that is found in prepuberal testis, epididymis, and spleen (Figure 7.3), and most likely represents *G6pd-2.*

G6pd-2 encodes a Functional Enzyme in Post-meiotic Spermatogenic Cells

G6pd-2 was tested for its ability to encode a functional product, by expression of a *G6pd-2* DNA construct in an *E. coli* strain that does not produce endogenous G6PD (Fraenkel, 1968). G6PD activity was analyzed using zymograms, which are native PAGEs in which the position of G6PD activity is visualized by precipitation of nitroblue tetrazolium. Expression of a *G6pd-2* construct resulted in two bands with G6PD activity (Figure 7.4), demonstrating that the *G6pd-2* gene encodes a protein with enzymatic activity. For comparison, a mouse *G6pd-1* DNA construct was expressed in the same *E. coli* system, which yielded one band with G6PD activity, that comigrated with the faster migrating G6PD-2 band. Additional experiments (see *Materials and Methods*) showed that the lower band consisted of G6PD dimers, while the higher band consisted of G6PD tetramers. Apparently, under the present

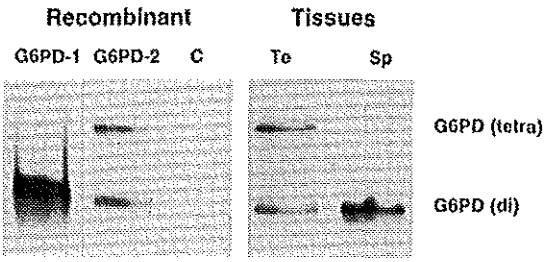


Figure 7.4 *Zymogram of glucose-6-phosphate dehydrogenase (G6PD) activity of recombinant G6PD-1 and G6PD-2.*

Proteins were solubilized from E. coli (lacking endogenous G6PD) transformed with expression vectors containing the open reading frame of either mouse G6pd-1 or mouse G6pd-2 (indicated ***G6pd-1*** *and* ***G6pd-2****), or no insert (indicated* ***C*** *for control), and from mouse tissues. The lower, faster migrating band represents G6PD dimer* ***(di)****, while the upper band represents G6PD tetramer* ***(tetra)****. Both bands are present in E. coli expressing G6pd-2 and in adult mouse testis* ***(Te)****, while only the dimer band is present in E. coli expressing G6pd-1 and in mouse spleen* ***(Sp)****.*

conditions, G6PD-2 tetramers were more resistent to reduction by dithiothreitol (DTT) than G6PD-1 tetramers. The property of G6PD to form tetramers has been reported before, for instance for the rat (Grigor, 1984; Martins *et al.*, 1985). Similarly to the present finding for mouse G6PD-1, rat G6PD tetramers dissociate into dimers by treatment with DTT (Grigor, 1984; Martins *et al.*, 1985). The apparent difference in sensitivity of G6PD-1 and G6PD-2 tetramers for DTT was used to analyze the presence of G6PD-2 enzyme in mouse tissues.

Zymograms of solubilized proteins from mouse tissues showed G6PD dimers and tetramers, co-migrating with dimers and tetramers produced by expression of *G6pd-1* and *G6pd-2* sequences in *E. coli* (Figure 7.4; shown are testis and spleen). Dimers were present in all tissues tested, while tetramers were unique for testis and epididymal spermatozoa (Figure 7.5A).

For comparison, zymograms were also performed for tissues of a mouse strain with X-linked G6PD-deficiency; G6PD activity in these mice is 15 to 40% of that in wild type mice (Pretsch *et al.*, 1988; Neifer *et al.*, 1991). For all tissues examined, the dimer band from the G6PD-deficient mice was of lower intensity than that from the wild type mouse, while the tetramer band for testis and spermatozoa from both mice showed approximately equal intensity (Figure 7.5B; shown are spleen and spermatozoa). This confirmed that the tetramers, as observed under the present conditions, are not encoded by the X-chromosomal *G6pd-1* gene. The tetramer form of G6PD, most likely encoded by the *G6pd-2* gene, contributes the greater part of spermatozoal total G6PD (Figure 7.5). Some tissues gave an additional band with

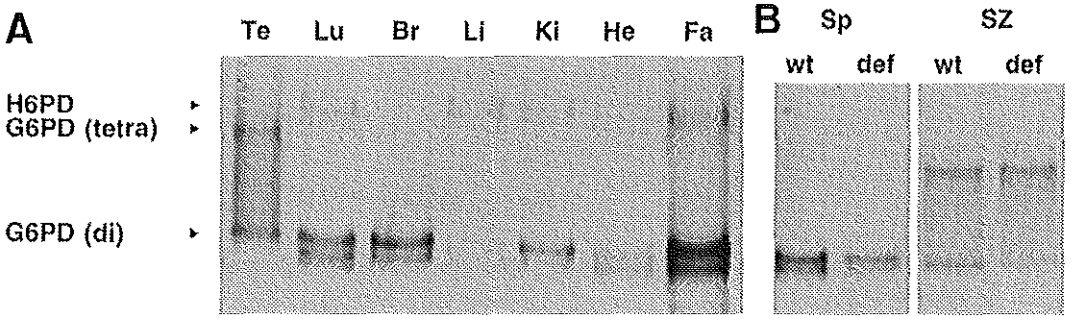


Figure 7.5 *G6PD zymogram of proteins from mouse tissues and spermatozoa.*

A) *Adult testis **(Te)** and epididymal spermatozoa **(SZ)** contain both dimeric **(di)** and tetrameric **(tetra)** G6PD, whereas all other tissues tested contain only dimeric G6PD. A third band with G6PD activity is due to another enzyme, hexose-6-phosphate dehydrogenase **(H6PD)**.*

B) *Spleen **(Sp)** and spermatozoa **(SZ)** from a G6PD-deficient mouse **(def)** show a relatively low enzymatic activity of dimeric G6PD but not of tetrameric G6PD, as compared to wild type mice **(wt)**.*

From all tissues, 20 µg of protein was applied per lane.

Lu: *lung;* ***Br:*** *brain;* ***Li:*** *liver;* ***Ki:*** *kidney;* ***He:*** *heart;* ***Fa:*** *fat tissue.*

G6PD-activity (Figure 7.5), which has been described before and was attributed to autosomally encoded hexose-6-phosphate dehydrogenase, an enzyme with equal affinities for glucose-6-phosphate and galactose-6-phoshate (Ohno *et al.*, 1966; Shaw, 1966; Bonsignore *et al.*, 1971).

Tetrameric G6PD was found to be absent in the testis at days 18 and 22 of postnatal development, weakly present at day 26, and present with higher intensity at day 32 (Figure 7.6A). Furthermore, the tetramer was absent in pachytene spermatocytes, and present in round spermatids (Figure 7.6A). Tetrameric G6PD was not found in testis and epididymis of a sterile mouse with spermatogenesis blocked at meiotic pachytene (De Boer et al., 1986) (Figure 7.6B). G6PD tetramers were not found in rat testis (not shown), indicating that formation of tetramers as observed for mouse testis and spermatogenic cells, is not due to a testis-specific factor.

The presence of G6PD tetramers in developing testis and spermatogenic cells, therefore, is in perfect concordance with *G6pd-2* mRNA expression.

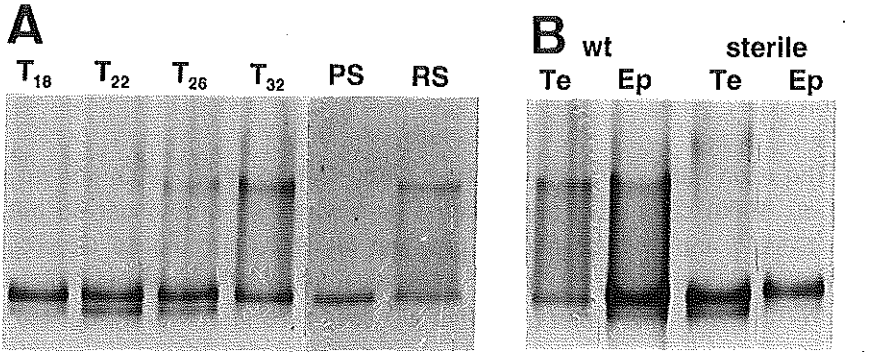


Figure 7.6. *G6PD zymogram of proteins from mouse testis, spermatogenic cell types, and epididymis.*
A) *Tetrameric G6PD becomes first apparent in mouse testis between postnatal days 22 and 26, and is present in round spermatids* ***(RS)*** *but absent in pachytene spermatocytes* ***(PS)****.*
B) *Lack of tetrameric G6PD in testis* ***(Te)*** *and epididymis* ***(Ep)*** *from adult sterile mice (spermatogenesis blocked at meiosis) confirms that the presence of tetrameric G6PD in testis and epididymis corresponds with the presence of post-meiotic spermatogenic cells.*
From all tissues, 20 µg of protein was applied per lane. $T_{18, 22, 26, \text{and } 32}$*: testis from 18-, 22-, 26-, and 32-day-old mice (postnatal ages).*

Discussion

The present results concern the identification of a novel intron-less gene in the mouse, that encodes active glucose-6-phosphate dehydrogenase (G6PD), and that is specifically expressed in post-meiotic spermatogenic cells. This gene is designated *G6pd-2*, whereas the X-chromosomal gene encoding G6PD is referred to as *G6pd-1*.

Since the *G6pd-2* gene lacks introns and shows 94% sequence identity with the coding sequences of *G6pd-1*, it is very likely that *G6pd-2* represents a retroposon that originated by retroposition of a reverse transcribed *G6pd-1* mRNA molecule. Almost all retroposons in the genome of mouse and other species are nonfunctional (Nouvel, 1994). It is remarkable, therefore, that *G6pd-2* is the fourth example of a retroposon that is functionally expressed in spermatogenic cells, next to *Pgk-2*, *Pdha-2*, and *Zfa*. The genes *Pgk-2* and *Pdha-2* encode functional enzymes (Boer *et al.*, 1987; Dahl *et al.*, 1990), and functionality of the *Zfa* gene product is expected but awaits evidence (Ashworth *et al.*, 1990; Koopman *et al.*, 1991). It has been suggested that expression of the retroposons *Pgk-2*, *Pdha-2*, and *Zfa* in spermatogenic cells might be important to compensate for loss of expression of the

X-chromosomal genes *Pgk-1*, *Pdha-1*, and *Zfx*, encoding the corresponding isotypic proteins (Boer *et al.*, 1987; McCarrey and Thomas, 1987; Takakubo & Dahl, 1992; Ashworth *et al.*, 1990).

The open reading frames of mouse *G6pd-1* (Zollo *et al.*, 1993) and rat *G6pd* (Ho *et al.*, 1988) differ in 62 nucleotides, from which 43 are conserved between mouse *G6pd-1* and *G6pd-2*, and 17 between rat *G6pd* and mouse *G6pd-2*. This indicates that mouse *G6pd-2* arose after divergence of mouse and rat during evolution. An intron-less *G6pd* gene has also been found in the rat, but the open reading frame of that gene contains several deletions, insertions, and termination codons, precluding it from being functional (Fritz *et al.*, 1989). This pseudogene must have originated independently from mouse *G6pd-2*. In human, the *G6pd* gene has been studied thoroughly by others (Luzzato and Mehta, 1995), but no human intron-less *G6pd* gene has been reported.

Nucleotide sequence comparison using the principle of maximum parsimony (Fitch and Margoliash, 1967) was used to construct a phylogenetic tree for mouse and rat *G6pd* genes (Figure 7.7). This figure gives the number of mutations and the percentage of non-silent mutations. When *G6pd-2* is compared to *G6pd-1*, it appears that the *G6pd-2* gene has undergone mutations at a relatively high rate.

There is a marked difference in the evolutionary origin of the four functional retroposons that are presently known to be specifically expressed in the testis. *Pgk-2* is present in different mammalian and marsupial species, and is expected to have arisen prior to radiation of marsupials from the eutherians (Boer *et al.*, 1987; McCarrey and Thomas, 1987). *Pdha-2* is present in mouse and human, where it may have evolved independently (Dahl *et al.*, 1990; Brown *et al.*, 1990); to our knowledge its presence has not been investigated for other species.

The retroposed genes *Zfa* (Ashworth *et al.*, 1990) and *G6pd-2* are unique for the mouse, indicating that these genes arose some 100 million years later than *Pgk-2*. In concordance with the different evolutionary origin, the nucleotide sequence identity, compared to the X chromosomal homologues, of the coding region of *Zfa* (97%) and *G6pd-2* (94%) is higher than that of *Pgk-2* (80%) and *Pdha-2* (76%) (Ashworth *et al.*, 1990; Boer *et al.*, 1987; Fitzgerald *et al.*, 1992). However, the mutation rate of *G6pd-2* is probably very high, relative to that of *Pgk-2* and *Pdha-2*. The latter two genes show a mutation rate that is only slightly higher than that of the X-chromosomal homologues (McCarrey, 1990; Fitzgerald *et al.*, 1992).

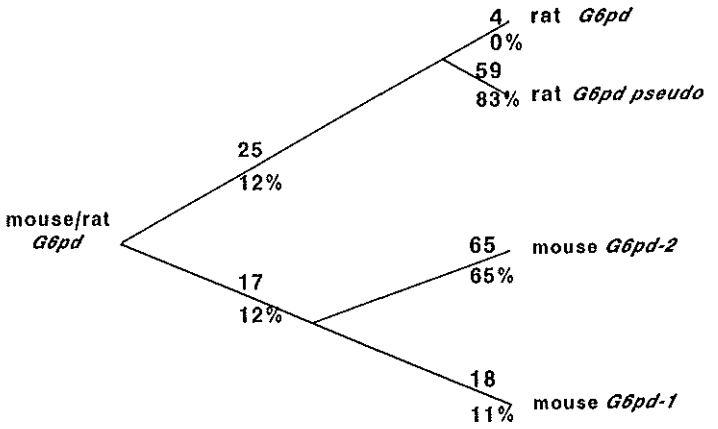


Figure 7.7 *Phylogenetic tree of rat and mouse G6pd genes.*

The tree was constructed according to Fitch and Margoliash (1967), and shows the number of nucleotide mutations and the percentage of these mutations that are non-silent (causing an amino acid change). The net number of 65 nucleotide mutations in the mouse G6pd-2 gene is much higher than the net 18 nucleotide mutations that have occurred in the same time period in mouse G6pd-1. Furthermore, the percentage of non-silent nucleotide mutations (65%) in mouse G6pd-2 is much higher than that in mouse G6pd-1 (11%). This indicates that the G6pd-2 gene has mutated at a relatively high rate. In addition to the indicated nucleotide mutations, the mouse G6pd-2 gene has one deletion of 6 nucleotides, whereas the rat G6pd pseudogene has five deletions, containing a total of 100 nucleotides. Furthermore, the rat G6pd pseudogene has two insertions of 1 and 8 nucleotides, respectively.

Most retroposons that are transcribed, originated from aberrantly transcribed mRNA molecules that included promoter sequences (Soares *et al.*, 1985; Vanin, 1985; Chakrabarti *et al.*, 1995). This has also been demonstrated for *Pgk-2* (McCarrey, 1990). Preferential expression of *Zfa* and *G6pd-2* in spermatogenic cells may reflect transcription from a fragmentary promoter, that somehow is sufficient for testis-specific transcription. However, this does not imply total loss of control and specificity. During spermatogenesis, *G6pd-2* transcription starts later than that of *Pgk-2*, *Pdha-2*, and *Zfa*. Transcription of *Pgk-2* and *Pdha-2* starts in spermatocytes (Goto *et al.*, 1990; Iannello and Dahl, 1992; Fitzgerald *et al.*, 1994). Likewise, *Zfa* is first transcribed in 14-day-old testis, when spermatocytes are being formed but spermatids are still absent (Erickson *et al.*, 1993). In contrast, *G6pd-2* mRNA is first detected after completion of the meiotic divisions, in spermatids. Expression of *Pgk-2* and *Pdha-2* is possibly regulated by a very similar set of transcription factors (Iannello *et al.*, 1994), but regulation of the expression of *G6pd-2* may involve another set of transcription factors and/or is related to some aspect of chromosomal location of the gene and chromatin structure.

G6pd-1, *Pgk-1*, *Pdha-1*, and *Zfx*, are X-chromosomal genes found to remain transcriptionally inactive after completion of the meiotic divisions. The inactivity of these genes probably is not due to lack of proper transcription factors, since the *Pgk-1* promoter drives a very high transcription of autosomally located transgenes in spermatocytes and spermatids (McBurney *et al.*, 1994). Hence, lack of post-meiotic transcription of these four X-chromosomal genes may result from maintenance of the inactivated state of the greater part of the X chromosome, that is initiated with the formation of the sex body during meiotic prophase. Post-meiotic transcription of the X-chromosomal genes *Ube1x* and *MHR6A* (Hendriksen *et al.*, 1995), and possibly also *Hprt* (Shannon and Handel, 1993), indicates that some regions of the X chromosome regain the ability to transcribe genes after completion of the meiotic divisions.

During spermatogenesis, several autosomally encoded gene products are known to be replaced or complemented by isotypic proteins (Grootegoed and den Boer, 1989; Welch *et al.*, 1992; Kalab *et al.*, 1994; Eddy, 1995; Fulcher *et al.*, 1995). None of these testis-specific isotypic proteins, however, is encoded by retroposons. Therefore, the finding of *G6pd-2* provides additional support for an evolutionary mechanism favoring spermatogenic cell-specific expression of retroposons derived from ubiquitously expressed X-chromosomal genes. Spermatogenic X chromosome inactivation (sex body formation) is the most likely driving force behind this mechanism.

It has been hypothesized that, immediately after its origin, *Pgk-2* was first widely expressed in most tissues. Since expression of *Pgk-2* in somatic cells next to *Pgk-1* was redundant, the promoter region of *Pgk-2* was free to evolve into a promoter with spermatogenic cell-specific expression (McCarrey, 1990). Some animal species do still express *Pgk-2* in both spermatogenic and somatic cells (McCarrey, 1990). The origin of *Pgk-2* may have preceded spermatogenic X inactivation, and it can be postulated that PGK-2 has made sex body formation possible, by providing post-meiotic spermatogenic cells with an isoenzyme of an essential X-encoded glycolytic enzyme. The same may apply to *Pdha-2*. However, mouse *Zfa* and *G6pd-2* arose relatively recently, when sex body formation was already established. Other species than the mouse apparently do not require these genes. Possibly, expression of *Zfa* and *G6pd-2* during mouse spermatogenesis results in some kind of advantage. For G6PD activity, such an advantage might involve production of reduced coenzyme NADPH in the pentose phosphate pathway, which could

contribute to protection of the spermatozoa against reactive oxygen species (Aitken and Fisher, 1994).

A high mutation rate of mouse *G6pd-2* as compared to *G6pd-1* indicates that changes in properties of G6PD-2 are compatible with the function of this enzyme in spermatozoa, or that G6PD-2 is not of decisive importance for survival of the species.

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General Discussion

8.1 Introduction

Part of the research described in the previous chapters was directed to the question whether X and Y spermatozoa show differences resulting from X- and Y-chromosomal gene expression.

A conclusive positive answer to this question would have been provided, when X- and Y-spermatozoa would have been found to differ in protein composition. However, as described in *Chapters 4* and *5*, no qualitative or quantitative differences in H-Y antigen expression or in any other plasma membrane protein between porcine X- and Y-chromosome bearing spermatozoa were detected.

A conclusive negative answer to the question indicated above would have been provided, when the sex chromosomes would have been found to be transcriptionally inactive in post-meiotic stages of spermatogenesis. However, as described in *Chapter 6*, mouse spermatids do express X- and Y-chromosomal genes.

Next to post-meiotic X- and Y-chromosomal gene expression, a second prerequisite for the occurrence of possible differences between X and Y spermatozoa is, that the encoded gene products are not equally shared among X and Y spermatids. *Section 8.3* will give an overview about: 1) evidence for the occurrence of gene product sharing among spermatids, and 2) examples showing that this sharing appears to be incomplete or absent for some gene products. Therefore, differences between X and Y spermatozoa cannot be excluded.

The present results also provide additional information about the process of X chromosome inactivation during spermatogenesis. It is generally accepted that the sex chromosomes (sex body) show little or no transcriptional activity during the pachytene stage of the meiotic prophase. All X-chromosomal genes studied before by others, were found to remain transcriptionally silent after completion of the meiotic divisions. In the case of the X-chromosomal genes *Pgk-1*, *Pdha-1*, and *Zfx*, meiotic and post-meiotic inactivity in the mouse appeared to be compensated by expression of the homologous autosomal genes *Pgk-2*, *Pdha-2*, and *Zfa*.

Chapter 7 describes the finding of a fourth example of such a switch in expression from an X-chromosomal gene (*G6pd-1*) to a homologous, presumably autosomal, gene (*G6pd-2*) during mouse spermatogenesis. However, two other X-chromosomal genes were found to be transcribed in spermatids (*Chapter 6*), and there may be many more.

A possible explanation for post-meiotic inactivation of several X-chromosomal genes *versus* transcription of other X-chromosomal genes will be provided in *Section 8.2.* That section will also include a comparison of spermatogenic X chromosome inactivation with somatic X chromosome inactivation.

8.2 X chromosome inactivation

Knowledge about the process of inactivation of one of the two X chromosomes in female somatic cells has increased rapidly in recent years. An important role is played by the *Xist* gene. Transcription of the *Xist* gene initiates inactivation of the one X chromosome from which *Xist* is transcribed. Targeted inactivation of *Xist* on one of the X chromosomes results in inability of that X chromosome to become inactivated (Penny *et al.*, 1996). The mechanism of *Xist* action is still unknown. The *Xist* RNAs are not translated, and *in situ* hybridization demonstrated that the RNA is associated with the inactive X chromosome (Brown *et al.*, 1992).

It is known that X chromosome inactivation progresses in two directions, starting at the so-called X chromosome inactivation centre (Xic) (Lyon, 1993). *Xist* maps to this Xic region. In individuals in which, due to a reciprocal translocation, part of the X chromosome is associated to part of an autosome, only the part of the X chromosome that carries the Xic region becomes inactivated. The inactivation extends into the translocated autosomal portion, although the degree of spreading is highly variable (Lyon, 1993; Disteche, 1995).

Although *Xist* is transcribed in all female tissues, ongoing transcription is not required for maintenance of the inactive state of the X chromosome (Brown and Willard, 1994). Stabilization of X chromosome inactivation is probably achieved by methylation of CpG islands located at the 5' end of X-chromosomal housekeeping genes (Migeon, 1994). This methylation may also be the major mechanism for transmission of the inactive state of the inactivated X chromosome through mitosis. The time point of first methylation of CpG islands during female embryogenesis, when X chromosome inactivation is initiated, varies among X-chromosomal genes. In the mouse, *Pgk* is methylated at 3.5 days post-coitum (d.p.c.), *G6pd* at 5.5 d.p.c., and *Hprt* at 6.5 d.p.c. (Lock *et al.*, 1987; Grant *et al.*, 1992). Since this corresponds with the location of *Pgk* closer to Xic than *G6pd* and *Hprt*, there might be a general mechanism in which methylation takes place at later time points for genes situated further away from the *Xist* locus (Grant et al., 1992). Hence, methylation appears to follow the spreading process for X chromosome inactivation.

Methylation does not take place on the inactivated X of fetal oogonia (Driscoll and Migeon, 1990). This is probably related to the reactivation of the inactivated X chromosome in oocytes during meiotic prophase.

The inactivated X chromosome of female somatic cells is visible cytologically as the so-called Barr body, or sex chromatin mass; the chromosome is heterochromatic, and forms a loop structure (Migeon, 1994; Walker *et al.*, 1991). In the mouse, first appearance of sex chromatin occurs at the blastocyst stage at about the time of implantation (3.5 d.p.c.). At this stage, the occurrence of sex chromatin is limited to the extraembryonic cells, and it is always the paternally derived X chromosome that is inactivated. In mouse embryonic tissues, sex chromatin formation starts at 6.5 d.p.c., and randomly involves either the maternal or paternal X chromosome (Monk and Harper, 1979; Takagi *et al.*, 1982; Migeon, 1994). Based on expression of an X-linked transgene, it takes until 11.5 d.p.c. before X chromosome inactivation is complete in all embryonic tissues (Tan *et al.*, 1993).

Exclusive inactivation of the paternal X chromosome in extraembryonic tissue is not seen in human, although the paternal X chromosome might be inactivated slightly more often than the maternal homologue (Lyon, 1993; Migeon, 1994).

The DNA of the inactive X chromosome, and also of heterochromatic segments of autosomes, is replicated late in S phase of the cell cycle. A shift in timing of DNA replication precedes the first formation of sex chromatin. In the early mouse embryo, the first sex chromatin formation in embryonic tissues is preceded by late replication, but in the extraembryonic tissues it is preceded by early replication (Issa *et al.*, 1969; Takagi *et al.*, 1982).

In concordance with an initiating function, *Xist* transcription precedes the formation of heterochromatin of the inactivated X chromosome by at least one day (Kay *et al.*, 1993; Kay *et al.*, 1994). *Xist* is first transcribed at the 4-cell stage, exclusively from the paternal allele (Kay *et al.*, 1994). Transcription of the maternal *Xist* allele starts just prior to gastrulation (Kay *et al.*, 1993).

The mechanism of formation of heterochromatin is unknown. One suggestion is that, starting from the X inactivation center, an enzyme would 'reel in' DNA by bringing together scaffold attachment regions for chromosome loops (Riggs and Pfeifer, 1992). The resulting closer packaging of the nucleosomes might be facilitated by electrostatic interactions resulting from an altered charge of the histones, which might be exerted by differential acetylation. The inactive X chromosome differs from the active X chromosome by a lack of acetylation of histone H4 (Jeppesen and Turner, 1993). Next to the X chromosome, major heterochromatic domains of several autosomes are also underacetylated (Jeppesen and Turner, 1993; Loidl, 1994).

In the human, several X-chromosomal genes have been found to escape X chromosome inactivation. These include genes located at or near the pseudoautosomal region, but also more proximally located genes which are interspersed with inactivated genes (Disteche, 1995). It is not known whether these genes escape inactivation from the onset of X inactivation, or are initially inactivated and become reactivated later. In the mouse, no X-chromosomal genes are known to escape X chromosome inactivation, with exception of the pseudoautosomal gene *Sts* (Penny et al., 1996). Escape of human X-chromosomal genes to inactivation might be regulated at the level of relatively large chromatin domains rather than on the level of individual genes (Disteche, 1995). The boundaries between chromatin loops that are attached at scaffold elements might be involved in the inactivation process (Disteche, 1995). Genes that escape X inactivation co-localize with regions within the inactivated X chromosome containing acetylated histone H4 (Jeppesen and Turner, 1993).

One of the remarkable features of spermatogenic X chromosome inactivation, is the lack of an obvious relationship between the heterochromatinized state of the X chromosome and transcriptional silence of X-chromosomal genes. The X chromosome is condensed during early pachytene of meiotic prophase, but not in leptotene spermatocytes, mid/late-pachytene spermatocytes, and spermatids (Oud *et al.*, 1979). In spite of this, transcription of the X-chromosomal *Pgk-1* and *Pdha-1* genes stops at the preleptotene stage, thus prior to sex body formation, and the inactivity of these genes is maintained in spermatids (Singer-Sam *et al.*, 1990; Takakubo and Dahl, 1992). This silencing, however, is not due to the absence of proper transcription factors, since the *Pgk-1* promoter drives a very high rate of transcription of autosomally located transgenes in these cell types (McBurney *et al.*, 1994).

The apparent lack of concurrence between transcriptional inactivation and heterochromatinization of the X chromosome is in contrast to the situation in female somatic cells. However, it should be noted that X chromosome condensation in spermatogenic and somatic cells probably involves two different processes. During spermatogenesis, condensation of the sex chromosomes is concurrent with that of the autosomes at zygotene of the meiotic prophase. This condensation is due to the formation of the axial cores, which later, after synapsis, are called the lateral elements of the synaptonemal complexes. The first difference observed between autosomes and sex chromosomes is thought to be due to an increased accumulation of axial materials at the sex chromosomes, in particular at the unpaired regions

(Solari, 1989). Therefore, sex body chromatin in spermatocytes can be regarded as a consequence of differential behavior of the X and Y meiotic axial cores, while the somatically inactivated X chromosome is heterochromatic during interphase of the mitotic cell cycle when axial cores are lacking.

Other findings supporting this difference in condensation mechanism concern methylation of CpG islands (Driscoll and Migeon, 1990; Grant *et al.*, 1992; Erickson *et al.*, 1993), acetylation of histone H4 (Jeppesen and Turner, 1994; Moens, 1995), and DNase I sensitivity (Chandley and McBeath, 1987). The lateral elements do not prevent transcription of autosomes during meiotic prophase, but transcription of the X and Y chromosomes within the sex body might be prevented by the altered state of the axial cores/lateral elements and by sex body-specific proteins (Calenda *et al.*, 1994; Smith and Benavente, 1992).

Expression of the *Xist* gene might play some role with respect to the duration of spermatogenic X chromosome inactivation. During spermatogenesis, *Xist* RNA is first detected in spermatogonia, reaches it highest level in spermatocytes, and declines in round spermatids. Similarly to somatic cells (Brown *et al.*, 1992), *Xist* RNA might be associated to the X chromosome in spermatogenic cells. An intriguing question is whether *Xist* RNA association is sufficient for transcriptional silencing of the X chromosome without further requirement for heterochromatinization. If so, genes located relatively close to the *Xist* locus might be the first to become inactivated at meiotic prophase, and this might precede the onset of heterochromatinization. In addition, genes located at a relatively large distance from the *Xist* locus might be the last genes to become inactivated, and the first to be reactivated in round spermatids.

The period of inactivation of a few X-chromosomal genes during spermatogenesis gives some support for this idea. *Hprt* is located at a larger distance from the *Xist* locus than *Pgk-1* and *Pdha-1*, and is, in contrast to *Pgk-1* and *Pdha-1*, still transcribed in leptotene/zygotene spermatocytes and might be reactivated in round spermatids (Shannon and Handel, 1993). The post-meiotically expressed X-chromosomal gene *Ube1x* is located more distally than *Hprt*. The location of *MHR6A* is unknown, so that the post-meiotic expression of this gene cannot be related to its position on the X chromosome.

In summary, sex body formation and transcriptional silencing of the X chromosome during spermatogenesis might be two independent processes, the first process being regulated by axial cores and sex body-specific proteins, and the

second by *Xist* RNA. Post-meiotic transcription of the X-chromosomal genes *Ube1x*, *MHR6A*, and possibly *Hprt*, might be related to a relatively large distance of these genes from the *Xist* locus. This hypothesis was not given in *Chapter 6*.

8.3 Sharing of gene products among spermatids

It is hypothesized that X and Y chromosome-bearing spermatozoa can differ phenotypically, based on differential gene expression, when: 1) X- and/or Y-chromosomal genes are expressed post-meiotically, and 2) the encoded X and/or Y gene products are not equally shared among the haploid spermatids. At this point, it is of interest to note that the first spermatogenic cell type that is haploid and contains either the X or Y chromosome, is the secondary spermatocyte. However, in view of the relatively short duration of this step, it is anticipated that X- and Y-chromosomal gene expression may not cause a significant difference between secondary spermatocytes.

This section will describe evidence for the occurrence of sharing of gene products among spermatids. The two next sections describe certain mouse strains in which sharing of gene products appears to be absent or incomplete.

During the mitotic and meiotic divisions of spermatogenesis, cytokinesis is not complete. Cells that are at the same step of spermatogenesis form a syncytium, with intercellular bridges of approximately 1 μm in diameter (Dym and Fawcett, 1971). Such a syncytium can consist of more than 50 spermatids, which remain connected until the end of spermatogenesis.

Evidence for passage of gene products through these intercellular bridges is provided by several studies. These studies involved mice with a heterozygous genetic defect, so that part of the spermatids lacked a certain gene.

Willison *et al.* (1988) studied T^{hp}/+ mice. While half of the spermatids of these mice lack the *Tcp-1* gene, the TCP-1 protein was found to be present in all spermatids.

Braun *et al.* (1989) developed mice transgenic for human growth hormone encoding sequences. The transgene was transcribed post-meiotically, since it was linked to promoter sequences of the mouse *protamine 1* gene. In mice that were hemizygous for the transgene, the human growth hormone protein was found to be present in nearly all spermatozoa.

The products of the two *protamine* genes have also been demonstrated to be shared among mouse spermatids. De Boer *et al.* (1990) studied the protamine content of spermatozoa from a mouse strain with aberrant chromosomes, that

produced spermatozoa of which a part were nullisomic or disomic for chromosome 16. Both *protamine* genes are located on this chromosome and are transcribed post-meiotically. In spite of the aneuploidy, all spermatozoa were found to contain approximately the same amount of protamine (De Boer *et al.*, 1990). The results of these three reports indicate sharing of mRNAs and/or proteins.

Caldwell and Handel (1991) demonstrated that sharing can occur at the level of mRNA. They also studied a mutant mouse strain in which many spermatozoa were either nullisomic or disomic for chromosome 16. Using *in situ* hybridization, *protamine 1* mRNAs were found to be equally present in all spermatids.

The intercellular bridges become disrupted at the very end of spermiogenesis in the mouse, when the step 16 spermatids are released into the lumen of the tubules (Russell, 1980). Using electron microscopy to detect nuclear incorporation of ^{3}H-uridine, RNA synthesis was reported to be arrested at step 12 of mouse spermiogenesis (Kierszenbaum and Tres, 1975). In the rat, *in situ* hybridization demonstrated that *de novo* synthesis of poly(A)$^{+}$ RNA stops when spermatids reach step 9 (Morales and Hecht, 1994). Therefore, gene transcription in spermatids after disruption of the intercellular bridges can be ruled out. However, mRNAs remain present in elongating spermatids until step 14 in the mouse and step 17 in the rat (Penttilä *et al.*, 1995). Indeed, high mRNA stability and translational control are involved in regulation of expression of many genes during spermatogenesis (Hecht, 1990; Eddy *et al.*, 1993; Braun *et al.*, 1995).

8.4 An example of incomplete sharing of gene products: the *t* complex

This and the following section give examples of mouse strains with aberrant chromosome constitution, from which the sperm segregants (spermatozoa that originated from the same spermatocyte) differ in fertilizing ability. It can be anticipated that this difference in fertilizing ability among sperm segregants is due to unequal sharing of a gene product. In most cases, the gene product involved has not been identified.

The *t* complex is a variant form of a 30,000-kb region located in the proximal third of chromosome 17, representing 0.5-1% of the mouse genome (reviewed by Silver, 1985). So far, the *t* complex is known to contain around 100 genes. The principal variation between the *t* complex and the corresponding region of the normal chromosome 17 concerns two large and two small inversions encompassing nearly the entire length of the *t* complex, as indicated in Figure 8.1 (Hammer *et al.*, 1989). These inversions suppress recombination of the *t* complex with the wild type form, so that the entire *t* haplotype is transmitted as a single unit.

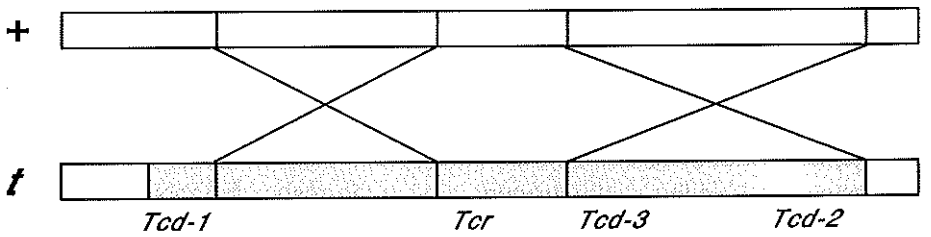

Figure 8.1 *Diagrammatic representation of the genetic structure of the t complex.*

Normal chromatin is shown white and t chromatin hatched. Only the two major inversions by which normal chromatin and the t complex differ are shown. The extent of t chromatin is not drawn to scale, but merely indicates the relative positions of the three t complex distorter genes (Tcd-1, -2, and -3) and the t complex responder gene (Tcr) (Based on Lyon [1990]).

Embryos that are homozygous for the *t* complex die *in utero*, due to gene mutations within the *t* complex. Therefore, the *t* complex would normally be expected to be eliminated by selection during evolution, but, surprisingly, the *t* complex is found with a relatively high frequency (approximately 20%) in wild mouse populations. This is due to so-called male transmission ratio distortion (TRD), which involves the following. Male mice heterozygous for a wild-type form of the *t* complex and a *t* haplotype (+/*t*) transmit the *t* complex to 90-99% of their offspring. In contrast, female +/*t* mice transmit the *t* complex according to normal Mendelian segregation to 50% of the offspring.

The fertilizing capacity of the total sperm population of +/*t* mice is less than that of sperm from +/+ mice. Sperm defects of +/*t* mice include impairment of calcium-dependent flagellar function (Olds-Clarke and Johnson, 1993) and sperm-egg fusion (Johnson et al., 1995; Olds-Clarke et al., 1996). The +/*t* males produce equal numbers of spermatozoa carrying the wild type locus (+ sperm) or the *t* locus (*t* sperm). Taken together, these data indicate that factors encoded by the *t* complex are harmful for sperm function. TRD appears to be caused by a relatively higher resistance of *t* sperm, compared to + sperm, to these harmful factors (Klein and Hammerberg, 1977; Frischauf, 1985; Silver, 1985; Lyon, 1986).

Many mouse strains have been identified or developed, that carry only part of the *t* complex. Using such strains, mice were bred carrying two different *t* haplotypes. Studies of such mouse strains revealed that at least 4 loci in the *t* complex are involved in TRD (see Figure 8.1). One of these loci, called the responder, is absolutely required for any deviation of the normal transmission ratio. Abnormal transmission arises from cumulative action of at least three distorter loci. When the responder is present but distorter loci are absent, the responder is transmitted at a frequency of less than 20%. However, when present, the distorters act additively to raise transmission of the spermatozoon carrying the responder above 90% (Silver, 1985; Lyon, 1984, 1990). Male mice homozygous for one of the distorters are sterile (Lyon, 1986). This has led to a model, that the distorter gene products are harmful for spermatogenic cells, and that spermatozoa with the *t* complex form of the responder gene are relatively resistent to this harmful action. The distorter genes are *trans*-acting, while the responder gene is always *cis*-acting (Lyon and Zenthon, 1987; Lyon, 1990). Therefore, the responder gene is the main candidate for the gene encoding a product that is not (completely) shared by spermatids.

The responder locus maps to a region which is embedded within a series of 3 large duplicated tracts of DNA known as "T66 loci" (Bullard and Schimenti, 1990). The responder locus was mapped to the middle of these tracts, T66B, and within this region only one gene has been discovered that is expressed in the testis, *t complex protein-10b* (*Tcp-10b*, formerly *T66B-a*) (Schimenti *et al.*, 1988; Rosen *et al.*, 1990; Cebra-Thomas *et al.*, 1991). The two loci flanking the T66B locus, T66A and T66C, contain two genes homologous to *Tcp-10b*, called *Tcp-10a* and *Tcp-10c*, which are also uniquely transcribed in male germ cells at roughly equal levels as *Tcp-10b* (Schimenti *et al.*, 1988; Bullard and Schimenti, 1990). These genes are present both in wild type and in *t* haplotype forms of chromosome 17 (Bullard and Schimenti, 1991; Pilder *et al.*, 1992). In pairwise comparison, the minimum nucleotide identity of the open reading frames among all *Tcp-10* genes of wild-type and *t* haplotype is strikingly high: 98.6% (Pilder *et al.*, 1992). A *homologous Tcp-10* gene family does also exist in human (Islam *et al.*, 1993). The functions of the *Tcp-10* gene products are unknown, but sequence data suggest that the *Tcp-10* gene family might encode a novel set of cytoskeletal elements (Davies and Willison, 1991; Islam *et al.*, 1993).

An important question for the origin of TRD is, how the product of *Tcp-10b* in the *t* complex (*Tcp-10b*^{t}) differs from that of the wild type gene (*Tcp-10b*$^+$). Both *Tcp-10b*^{t} and *Tcp-10b*$^+$ are transcribed in meiotic and post-meiotic spermatogenic cells. The deduced amino acid sequence of the *Tcp-10b*^{t} gene product differs in only one of the amino acids that are conserved among the *Tcp-10a/b/c*$^+$, *Tcp-10a*^{t}, and *Tcp-10c*^{t} gene products, and this difference is not expected to have a strong effect on its function (Pilder *et al.*, 1992). In addition, transcription and translation of *Tcp-10b*^{t} starts at the spermatocyte stage, which precludes specific haploid expression (Ewulonu *et al.*, 1993). In round and elongating spermatids, however, the mRNA of *Tcp-10b*^{t}, and not the *Tcp-10b*$^+$ mRNA, is alternatively spliced, and translation of this alternatively spliced mRNA results in a variant polypeptide (Cebra-Thomas *et al.*, 1991; Snyder and Silver, 1992).

Support for *Tcp-10b*^{t} being the *t* complex responder gene has been provided by studies on transgenic mice in which the *Tcp-10b*^{t} gene was introduced. The effect of the transgene on TRD, however, was much less pronounced than expected (Bullard *et al.*, 1992; Snyder and Silver, 1992). The precise mechanism causing TRD of the *t* locus, therefore, is still unclear. The nature of the distorter gene products and the mechanism of their interaction with *Tcp-10b*^{t} and *Tcp-10b*$^+$ gene products remains to be resolved.

8.5 Incomplete sharing of X- and Y-chromosomal gene products

There are two other examples of transmission ratio distortion in the mouse, which may point to unequal sharing of X- and Y-chromosomal gene products.

The first example concerns mice heterozygous for one of the Robertsonian translocations 6.16 or 6.15 (more precisely Rb(6.16)24Lub and Rb(6.15)1Ald) (Aranha and Martin-Deleon, 1992; 1995; Chayko and Martin-DeLeon, 1992). A Robertsonian translocation is a translocation which leaves the two participating chromosomes intact or nearly intact. Mice with the balanced Rb(6.16) translocation possess one normal chromosome 6, one normal chromosome 16, and the 6.16 translocation. These mice are fertile, and most of the spermatozoa have a normal chromosome content, thus either one normal chromosome 6 and one normal chromosome 16 (type I), or the 6.16 translocation (type II).

When these males mate at 3-day intervals, the normal type I spermatozoa yield 2.7- to 3.6-fold more fertilizations than the type II spermatozoa carrying the translocation (Aranha and Martin-Deleon, 1992; 1995; Chayko and Martin-DeLeon, 1992). In addition, the Y chromosome-bearing type II spermatozoa yield 2.0-fold more fertilizations than the X chromosome-bearing type II spermatozoa. However, type I and II spermatozoa fertilize equally well during *in vitro* fertilization (Chayko and Martin-DeLeon, 1992).

Furthermore, the transmission distortion did not take place when males mated 6 to 14 days after surgical ligation of the corpus epididymis (Aranha and Martin-Deleon, 1992). This indicates that the difference in fertilizing ability between the type I and type II spermatozoa, and between the X or Y chromosome-bearing type II spermatozoa, is related to a difference in epididymal maturation (Aranha and Martin-Deleon, 1992).

Similar observations have been made for male mice heterozygous for the Rb(6.15) translocation, suggesting a role of chromosome 6 in maturation-dependent distortion effects (Aranha and Martin-Deleon, 1995).

It is not clear why X-chromosomal spermatozoa appear to be more affected by the translocations than Y-chromosomal spermatozoa. The findings suggest that factors on chromosome 6 and on the X or Y chromosomes additively affect sperm function, and that these factors appear to be not completely shared among developing spermatids (Aranha and Martin-Deleon, 1995).

In mice carrying the Rb(11.14) translocation, translocation-bearing spermatozoa also have a lower fertilizing ability than the normal sperm segregants, while the sex ratio is not affected (Corkery and Martin-DeLeon, 1996). In this case, *in vitro* fertilization increases the transmission ratio distortion, compared to *in vivo* fertilization. A possible explanation for this phenomenon might be a defect in capacitation of translocation-bearing sperm, that would become more apparent during *in vitro* capacitation (Corkery and Martin-DeLeon, 1996).

Differential fertilizing ability of sperm segregants is not unique for the mouse, but has also been reported for Chinese hamster strains that are heterozygous for a reciprocal translocation (Sonta et al., 1991). A reciprocal translocation involves exchange of chromosomal segments. In these Chinese hamster strains, spermatozoa which lacked a certain autosomal segment had a decreased fertilization rate (Sonta et el., 1991).

The second example of transmission ratio distortion due to possible unequal sharing of X- and Y-chromosomal gene products, concerns mice with a partial deletion of the long arm of the Y chromosome. Spermatozoa of these mice have an increased frequency of abnormal sperm heads, and produce offspring with a small distortion of the sex ratio in favor of females (38-46% males), which is not due to increased male embryo death (Moriwaki *et al.*, 1988; Conway *et al.*, 1994). The increased frequency of abnormal sperm heads might be caused by reduction of *Y353/B* gene copies and a corresponding reduced level of *Y353/B*-related transcripts (see *Section 3.1*) (Conway *et al.*, 1994).

If product sharing is incomplete, a shortage of essential Y-chromosomally encoded gene products would have more severe effects on X chromosome-bearing spermatozoa than on Y chromosome-bearing spermatozoa. The sex ratio of the offspring is, however, just opposite to this expectation, which suggests a more complicated relationship between gene products of the Y long arm and the X chromosome.

These examples of chromosomally aberrant mice with differential fertilizing ability of X and Y spermatozoa, confirm that sex chromosomal genes are expressed in spermatids. Moreover, in these examples, at least one X- and/or Y-chromosomal gene product is not equally shared among spermatids. Since the gene products involved are unknown, it remains unclear whether these genes are also expressed,

and unequally shared, in post-meiotic spermatogenic cells of mice with normal chromosome constitution.

The occurrence of X- and Y-chromosomal gene expression in spermatids (*Chapter 6*), and data supporting unequal sharing of X- and Y-chromosomal gene products among spermatids (this section), keep open the possibility that X and Y spermatozoa do differ with respect to qualitative and/or quantitative expression of some gene products.

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Separation of X and Y chromosome-bearing spermatozoa would be of high commercial benefit in the breeding of agriculturally important animals. In human, it would offer a possibility to prevent transmission of X-linked diseases which affect boys but not girls.

At present, the only difference between X and Y spermatozoa known, concerns the difference in size between the X and Y chromosomes. The X chromosome is larger than the Y chromosome, and, therefore, in most mammals, X spermatozoa contain 2.8 to 4 % more DNA than Y spermatozoa. The only sperm separation method that has been proven to be reliable, flow-cytometric sorting, is based on this difference in DNA content. This method involves staining of sperm DNA with a fluorescent dye. Then, the slightly brighter fluorescing X spermatozoa can be separated from the Y spermatozoa by use of a flow cytometer/cell sorter.

Theoretically, the difference in DNA content between X and Y spermatozoa might result in a small difference in weight and size between X and Y sperm heads. This difference, however, is expected to be marginal, compared to the general variation in weight and size among spermatozoa. The lack of success of sperm separation methods which were based on this assumed difference, confirms this expectation.

Other differences between X and Y spermatozoa might result from expression of X- and Y-chromosomal genes during spermatogenesis. The present thesis concerns evaluation of several aspects of X- and Y-chromosomal gene expression during spermatogenesis, studied in the context of X and Y sperm separation.

An overview of various methods that have been attempted to separate X and Y spermatozoa, is given in *Chapter 1*. The behavior of the sex chromosomes during spermatogenesis is described in *Chapter 2*, and the genes that have been identified on the mouse and human Y chromosomes are described in *Chapter 3*.

Practical application of flow cytometric sperm sorting is hampered by the relatively low number of spermatozoa that can be sorted per time period. On the other hand, the availability of separated X and Y sperm fractions enabled for the first time a direct comparison between X and Y spermatozoa.

Chapter 4 describes a comparison between flow-cytometrically separated porcine and bovine X and Y spermatozoa, for the presence of serologically detectable H-Y antigen. The H-Y antigen is an antigenic epitope present on the plasma membrane of male cells, but absent on female cells. Previous reports gave

conflicting data about preferential expression of H-Y antigen by Y chromosome-bearing spermatozoa. In the present study, the binding of anti-H-Y monoclonal antibodies to flow-cytometrically separated X and Y spermatozoa was investigated using immunofluorescence microscopy. The antibody binding was found to be identical for the X and Y sperm fractions, indicating no difference in serologically detectable H-Y antigen between these two sperm types.

Chapter 5 describes a comparison between flow-cytometrically separated porcine X and Y spermatozoa for a large number of membrane proteins. For this study, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was used, which enabled analysis of the electrophoretic properties of up to a thousand proteins. Comparison of proteins from X and Y spermatozoa did not result in detection of proteins unique for either X or Y spermatozoa. The analyzed proteins of X and Y spermatozoa also did not significantly differ in quantity between the two cell preparations.

Chapter 6 describes a study of X- and Y-chromosomal gene expression during spermatogenesis in the mouse. Special emphasis was directed to the question whether the sex chromosomes are transcriptionally active after completion of the meiotic divisions, in haploid spermatids. Using RT-PCR and Northern blotting on purified spermatogenic cell preparations, two X-chromosomal and two Y-chromosomal genes were found to be transcribed in spermatids. Post-meiotic transcription of X-chromosomal genes was a new finding, since earlier reports pointed to an inactive state of the X chromosome during spermatogenesis, from meiotic prophase onwards.

Chapter 7 describes a study on expression of *glucose-6-phosphate dehydrogenase* (*G6pd*) genes during spermatogenesis in the mouse. The enzyme glucose-6-phosphate dehydrogenase is known to be encoded by an X-chromosomal gene, and is present in almost all cell types. In *Chapter 7*, the finding of a second *G6pd* gene, designated *G6pd-2*, is described. *G6pd-2* is specifically expressed in mouse spermatids, whereas the X-chromosomal *G6pd* gene is not transcribed in spermatocytes and spermatids. *G6pd-2* is an intron-less gene, and it is expected that this gene finds its origin in retroposition of a mRNA species of the X-chromosomal *G6pd* gene. Such a retroposed gene is called a retroposon. Most often, retroposons undergo many mutations, leading to a nonfunctional pseudogene. Functional retroposons are very rare. *G6pd-2*, however, encodes a functional G6PD isoenzyme. *Chapter 7* includes a discussion on the biological significance of this finding.

It is discussed in *Chapter 8*, that the results of the studies on X- and Y-chromosomal gene expression during spermatogenesis have implications for our understanding of the process of X chromosome inactivation during spermatogenesis.

The results of the experiments described in this thesis do not provide a conclusive answer to the question whether X- and Y-chromosomal gene expression can result in some phenotypic difference between X and Y spermatozoa. As discussed in *Chapter 8*, gene products are shared among spermatids, by passage through intercellular bridges. However, not all gene products appear to be completely shared, and a phenotypic difference between X and Y spermatozoa cannot be excluded.

Het kunnen scheiden van X- en Y-chromosoomdragende spermatozoa zou grote economische voordelen opleveren voor de fokkerij van landbouwhuisdieren. Voor klinische toepassing bij mensen zou het de mogelijkheid bieden om overdracht te voorkomen van X chromosoom gebonden ziekten, die bij jongens maar niet bij meisjes tot uiting komen.

Het enige verschil dat momenteel bekend is tussen X- en Y-chromosoomdragende spermatozoa betreft het verschil in grootte tussen het X en het Y chromosoom. Het X chromosoom is groter dan het Y chromosoom, en daarom bevatten, bij de meeste zoogdieren, de X spermatozoa 2.8 % tot 4 % meer DNA dan de Y spermatozoa. De enige spermacel-scheidingsmethode waarvan de betrouwbaarheid bewezen is, flowcytometrische celscheiding, is gebaseerd op dit verschil in DNA inhoud. Bij deze methode wordt het DNA van de spermatozoa met een fluorescerende kleurstof gekleurd. Vervolgens kunnen de iets meer intens fluorescerende X spermatozoa worden gescheiden van de Y spermatozoa, met behulp van een flowcytometer/celscheider.

Het verschil in hoeveelheid DNA tussen X en Y spermatozoa zou theoretisch kunnen leiden tot een klein verschil in gewicht en grootte tussen de X en Y spermacelkoppen. Het wordt echter aangenomen dat dit verschil marginaal is ten opzichte van de algemene variatie in gewicht en grootte tussen spermatozoa. Deze aanname wordt bevestigd door het gebrek aan succes van spermacel-scheidingsmethoden, die op dit veronderstelde verschil gebaseerd waren.

Andere mogelijke verschillen tussen X en Y spermatozoa zouden het gevolg kunnen zijn van expressie van X- en Y-chromosomale genen tijdens de spermatogenese. Dit proefschrift behandelt verschillende aspecten van X- en Y-chromosomale genexpressie tijdens de spermatogenese, in relatie tot X en Y spermacel-scheiding.

Een overzicht van verschillende methoden, waarmee geprobeerd is X en Y spermatozoa te scheiden, wordt gegeven in *Hoofdstuk 1*. Het gedrag van de geslachtschromosomen tijdens de spermatogenese wordt beschreven in *Hoofdstuk 2*; de genen die op het muize en humane Y chromosoom geïdentificeerd zijn, worden beschreven in *Hoofdstuk 3*.

De praktische toepassing van flowcytometrische spermacel-scheiding wordt bemoeilijkt door het relatief klein aantal spermatozoa dat per tijdsperiode kan worden gescheiden. Daarentegen biedt de beschikbaarheid van gescheiden X en Y

spermacelfrakties voor het eerst de mogelijkheid om X en Y spermatozoa rechtstreeks met elkaar te vergelijken.

Hoofdstuk 4 beschrijft een vergelijking tussen flowcytometrisch gescheiden varkens en runder X en Y spermatozoa voor de aanwezigheid van serologisch detecteerbaar H-Y antigeen. Het H-Y antigeen is een epitoop dat aanwezig is op het plasmamembraan van mannelijke cellen, maar afwezig op vrouwelijke cellen. Eerdere artikelen leverden tegenstrijdige resultaten over de mogelijke preferentiële expressie van het H-Y antigeen door Y-chromosoomdragende spermatozoa. In de huidige studie werd de binding van anti-H-Y monoclonale antistoffen aan gescheiden X en Y spermatozoa onderzocht met behulp van immunofluorescentie-microscopie. De antistofbinding was identiek voor de X en Y spermacelfrakties, hetgeen aangeeft dat deze twee spermaceltypen niet verschillen in serologisch detecteerbaar H-Y antigeen.

Hoofdstuk 5 beschrijft een vergelijking tussen flowcytometrisch gescheiden varkens X en Y spermatozoa voor een groot aantal plasmamembraaneiwitten. Voor deze studie werd van de twee-dimensionale polyacrylamide gel electroforese (2-D PAGE) techniek gebruik gemaakt, die analyse van tot zo'n 1000 eiwitten mogelijk maakte. Vergelijking van de eiwitten van X en Y spermatozoa resulteerde niet in detectie van eiwitten die uniek waren voor X of Y spermatozoa. Daarnaast verschilden de geanalyseerde eiwitten van de X en Y spermatozoa ook niet in kwantiteit tussen de twee celpopulaties.

Hoofdstuk 6 beschrijft een studie naar X- en Y-chromosomale genexpressie tijdens de spermatogenese in de muis. Hierbij was vooral de vraag van belang of de geslachtschromosomen nog steeds genen tot expressie brengen na voltooiing van de meiotische delingen, in de haploïde spermatiden. RT-PCR en Northern blotting op RNA van geïsoleerde spermatogene celtypen toonden aan, dat twee X-chromosomale en twee Y-chromosomale genen in spermatiden worden getranscribeerd. De postmeiotische transcriptie van X-chromosomale genen was een nieuwe vondst; eerdere artikelen wezen juist op een blijvende inactiviteit van het X chromosoom vanaf de profase van de meiose.

Hoofdstuk 7 beschrijft een studie naar de expressie van *glucose-6-fosfaatdehydrogenase* (*G6pd*) tijdens spermatogenese in de muis. Het was bekend dat het enzym glucose-6-fosfaatdehydrogenase door een X-chromosomaal gen gecodeerd wordt, en dat dit enzym in bijna alle typen cellen aanwezig is. In *Hoofdstuk 7* wordt de ontdekking van een tweede *G6pd* gen (*G6pd-2*) beschreven, dat specifiek in muize spermatiden tot expressie komt. Het X-chromosomale *G6pd*

gen komt niet in spermatocyten en spermatiden tot expressie. *G6pd-2* is een intronloos gen, en is zeer waarschijnlijk ontstaan door retropositie van een mRNA molecuul van het X-chromosomale *G6pd-1* gen. Een gen dat via retropositie ontstaan is, wordt een retroposon genoemd. In het algemeen ondergaan retroposons veel mutaties, waardoor een functieloos pseudogen ontstaat. Functionele retroposons zijn zeldzaam; *G6pd-2* codeert echter voor een functioneel G6PD isoenzym. Deze ontdekking wordt in *Hoofdstuk 7* nader besproken, in het kader van testis-specifieke expressie van andere retroposons.

In *Hoofdstuk 8* wordt bediscussieerd, dat de resultaten van de studies naar X- en Y-chromosomale genexpressie tijdens spermatogenese een ander licht werpen op het proces van X chromosoom inactivatie tijdens spermatogenese.

De resultaten van de in dit proefschrift beschreven experimenten geven geen eenduidig antwoord op de vraag of de waargenomen X- en Y-chromosomale genexpressie kan resulteren in een verschil in phenotype tussen X en Y spermatozoa. Zoals in *Hoofdstuk 8* besproken wordt, kunnen genprodukten tussen spermatiden worden uitgewisseld via passage door de intercellulaire bruggen. Deze uitwisseling is echter niet volledig voor alle genprodukten, waardoor het bestaan van een fenotypisch verschil tussen X en Y spermatozoa niet kan worden uitgesloten.

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Peter Jozef Maria Hendriksen werd geboren op 24 augustus 1959 te Arnhem. In 1977 behaalde hij het HAVO diploma aan de Thorbecke Scholengemeenschap te Arnhem. Vervolgens werd in 1980 het diploma HBO-A Histologie aan de Opleiding Laboratoriumpersoneel Arnhem Nijmegen (OLAN) te Arnhem en in 1982 het diploma HBO-B Biochemie aan de Hogere en Middelbare Laboratorium School (HMLS) te Oss behaald. In 1982 begon hij de studie Scheikunde, specialisatie Biochemie, aan de Katholieke Universiteit Nijmegen (KUN). Tijdens deze studie werd een bijvakstage uitgevoerd bij de Afdeling Nierziekten van de KUN, waarbij de rol van interleukine-1 bij de activatie van humane T-lymfocyten werd onderzocht (dr. W.J. Tax). Een hoofdvakstage werd uitgevoerd bij de Afdeling Biochemie, Sectie Moleculaire Oncologie, van de KUN, waarbij ondermeer de promotor van het proto-oncogen *sis*/PDGF-2 werd gekarakteriseerd (prof. dr. H.P. Bloemers). In augustus 1987 werd het doctoraalexamen behaald.

Van januari 1988 tot en met december 1990 was hij wetenschappelijk medewerker bij het DLO-Instituut voor Veeteeltkundig Onderzoek (IVO-DLO) in Zeist. Hierbij werden anti-H-Y monoclonale antistoffen gekarakteriseerd, en de invloed van H-Y antigeen op de ontwikkeling van de embryonale kippengonade onderzocht.

In januari 1991 werd hij aangesteld bij de Vakgroep Endocrinologie & Voortplanting van de Erasmus Universiteit Rotterdam. Het in dit proefschrift beschreven onderzoek werd deels bij deze vakgroep en deels bij het IVO-DLO te Zeist (later DLO-Instituut voor Dierhouderij en Diergezondheid, ID-DLO) uitgevoerd. Daarnaast werd voor dit projekt 3 maanden onderzoek verricht bij het Germplasm and Gamete Physiology Laboratory, Beltsville Agricultural Research Center (BARC) te Beltsville, Verenigde Staten.

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