THE CELLULAR BASIS OF
SEX DETERMINATION IN THE MOUSE

by

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ABSTRACT

Previous work has shown that in adult XX<->XY chimeras, the XX cell lineage can contribute to all of the testicular somatic cell types except the Sertoli cell population. This led to the hypothesis that the Y chromosome initiates testis formation by cell-autonomously inducing the differentiation of Sertoli cells from their precursors - the supporting cell lineage. In the first project of this thesis, this model was tested by examining the fate of XX cells in the testes of fetal, prepuberal and adult XX<->XY chimeras using in situ hybridization to a transgenic marker. It was found that whilst XX Sertoli cells were strongly selected against, small numbers were found at the fetal and prepuberal stages and very small numbers in adults.

The model was tested further in the second project by examining the fate of XY cells in the gonads of XO/XY mosaics using in situ hybridization with a Y-specific probe. In these circumstances, it was found that XY cells contributed to the follicle cell population (the products of the supporting cell lineage in the ovary) in roughly the same proportions as the other somatic lineages examined. It is argued that in the normal XY individual, the Y acts in a cell-autonomous fashion to induce Sertoli cell formation, but this system is 'leaky', causing some local recruitment of XX cells in chimeras. When insufficient Sertoli cells differentiate for testis cord formation, these cells are susceptible to recruitment into the follicle cell pool.

In the third experiment, the onset of testicular differentiation in mouse fetuses carrying Y chromosomes derived from M.m.domesticus was compared with fetuses carrying a M.m.musculus-derived Y. It was found that fetuses with a M.m.domesticus Y chromosome had a relatively late onset of testicular development and it is suggested that this is due to differing alleles of the testis

determining gene. It is proposed that the late action of this allele forms the basis of the XY sex reversal which occurs in C57BL/6 mice with a <u>M.m.domesticus</u>-derived Y chromosome.

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INTRODUCTION

1. DEVELOPMENT OF THE UROGENITAL RIDGE

The urogenital system is composed of the kidneys, the gonads and a series of interconnecting ducts and excretory pathways. It develops from the nephrogenic cord which is a ridge of the intermediate mesoderm. The functional adult kidney of amniotes develops from the metanephros which is the last of the nephric structures to form. During fetal life, vertebrates first develop a pronephros which is a transient and vestigial organ, followed by development of the mesonephros which is functional in fetuses of reptiles, birds and certain mammalian species and forms the site of gonad development.

1.i. Kidney development

The first signs of nephrogenic differentiation appear in the rat at approximately 9.5 days post coitum (pc) with the beginnings of the Wolffian duct joined to a few narrow canals and funnel-like projections that open into the celomic cavity (Torrey, 1943). This structure is thought to represent the rudimentary pronephros. The Wolffian duct grows caudaly towards the cloaca and, as it does so, induces the formation of the mesonephric tubules. In experiments on chick embryos where the growing tip of the Wolffian duct is destroyed (Boyden, 1927) or a cut is made in the path of the growing Wolffian duct (Wadddington, 1938), the mesonephric tubules either fail to form, or develop very poorly.

Interpretations of sectioned material from rat (Torrey, 1943) and chick (Gruenwald, 1952) fetuses suggest that the mesonephric tubules begin as condensations of cells close to the Wolffian duct which hollow out to form vesicles. These vesicles then send out projections that fuse with, and open out into, the Wolffian duct.

1.ii. Origin of the somatic components of the genital ridge

Precise details of the cellular events involved in the formation of the somatic components of the genital ridge are still unclear, mainly because all are interpreted from sectioned material but also because of differences between species. In the 'classical' cortico-medullary antagonism model (Witschi, 1951), it is proposed that the indifferent gonad is composed of an ovarian cortex derived from the celomic epithelium and a testicular medulla derived from the mesonephros. Under this scheme, a testis or ovary forms by the overgrowth of one or the other components. This hypothesis, derived from histological studies of amphibian gonad development, does not conform to the mammalian picture of development.

At 9 days pc in the mouse, just prior to genital ridge formation, the presumptive region consists of a loose mesenchyme containing the mesonephric tubules, which are close to the surrounding celomic epithelium (Upadhyay et al. 1979; Satoh, 1985). The first sign of genital ridge formation is the absence of a basal lamina underlying the celomic epithelium of the presumptive region (Torrey, 1944; Gruenwald, 1942). The basal laminae surrounding the mesonephric tubules is also lacking or incomplete on the side closest to the celomic epithelium. Mitotic figures are seen, both in the celomic epithelium and in the mesonephric tubules (Merchant, 1975) and epithelial cells appear in the intervening space. These cells comprise the earliest members of the gonadal blastema: the somatic component of the gonads that is thought to give rise to the supporting cell lineage and, perhaps, some of the other somatic lineages. Where the blastemal cells come from is still the subject of debate. Some believe they originate in the celomic epithelium (Torrey, 1944; Gruenwald, 1942), others, solely from the mesonephros (Upadhyay et al. 1979; Upadhyay et al. 1981) and others, from both sources (Wartenberg,

1983; Merchant, 1975; Kanai et al. 1989). Merchant (1975) reports that the majority of cell divisions in the celomic epithelium are at right angles to its surface. Therefore, daughter cells should be displaced towards the forming genital ridge. Wartenberg (1981; 1983) demonstrated the existence of 'dark' and 'light' staining cells within the blastemal mass, suggesting a dual origin. McLaren (1985) argues that much of the confusion can be explained by differences between the various species studied. In particular, the extent to which the mesonephros functions as a kidney during fetal life may affect the formation of the gonadal blastema.

A more important question, as far as this study is concerned is, does the 'indifferent' gonad form in the same way in both sexes? Kanai et al. (1989) showed that XY mouse gonads at the indifferent stage (11.5 days pc) contain some cells which are positive for the periodic acid-schiff (PAS) reaction whilst cells of XX gonads are all PAS-negative. This may indicate that sex differences are established from the beginning of blastemal formation. Alternatively, since early Sertoli cells are also PAS-positive, it may be that the positive cells are the earliest members of the Sertoli cell population prior to their aggregation into cords (Jost et al. 1973; Magre and Jost, 1980). Functional and biochemical evidence (Ross and Lipsett, 1978; Ciccarese and Ohno, 1978; Vigier et al. 1984) suggests that ovarian follicle cells and testicular Sertoli cells which are derived from the blastema, originate from common precursors. The recently cloned testis determining gene Sry, is found to be expressed after formation of the genital ridge has begun (Koopman et al. 1990). When this is considered in conjunction with the information that XX mice may be sex-reversed by the transgenic addition of Sry (Koopman et al. 1991), it must be concluded that the indifferent gonad is truly indifferent prior to Sry expression or, if there are differences, they are of no

consequence to the eventual outcome.

Apart from the supporting cell lineage, the adult gonad also contains other somatic lineages including steroid producing cells and various types of connective tissue cells. When 11.5 day pc genital ridges are separated from their mesonephroi, then reassociated in culture with mesonephroi taken from fetuses containing a transgenic marker, it is possible to identify cells that have migrated into the genital ridge by in situ hybridization to the transgene. In XY gonads treated in this way (Buehr and McLaren, unpublished), none of the Sertoli cells or germ cells were labelled but many of the peritubular-myoid cells and interstitial cells were. This indicates that at least some of the steroid-producing cells and connective tissue cells are derived from the mesonephric region and that this contribution continues beyond 11.5 days pc.

1.iii. Origin of the germ cells

The primordial germ cells (pgc's) begin migration into the genital ridges of mice at 10-11 days pc (McLaren, 1983) and continue until 13 days pc (Upadhyay et al. 1981). These cells enter the genital ridge through the mesonephric region but they originate from an entirely separate source. Chiquoine (1954) identified a population of migratory cells that show a strong positive reaction to alkaline phosphatase staining. The number of such cells is severely reduced in embryos homozygous for the white spotting mutation - W, which deletes germ cells (Mintz, 1959). In the 8 day pc mouse fetus, positive staining cells were found at the base of the allantois (Ozdzenski, 1967). The progenitors of these cells were shown, by dissecting and culturing parts of 7 day pc embryos, to be located at the posterior end of the primitive streak (Snow, 1981). Collected evidence suggests that the pgc's originate in the epiblast (see McLaren 1983; 1985 for reviews) and Ginsburg

<u>et al</u>. (1990) have demonstrated by improved alkaline
phosphatase staining techniques, a cluster of positive
cells in the extra-embryonic mesoderm of 7-7.25 day <u>pc</u>
fetuses, close to the posterior end of the primitive
streak, separated from the embryo by the amniotic fold. At
8.5 days <u>pc</u>, the pgc's are found in the yolk sac endoderm
and from there they migrate through the invaginating hind
gut (9-9.5 days <u>pc</u>), up the dorsal mesentery, around the
celomic angle and into the genital ridges (Chiquoine, 1954;
Mintz, 1959; reviewed by McLaren, 1983 and Eddy <u>et al</u>.
1981). Transfer of the cells to the hind gut may be due to
morphogenetic movements of the developing embryo but
ultrastructural studies of the germ cells in the hind gut
epithelium and at subsequent stages in the migratory
pathway demonstrate morphological and microanatomical
characteristics of amoeboid movement (Zamboni and Merchant,
1973; Spiegelman and Bennet, 1973). Once inside the gonads,
the germ cells continue to divide rapidly in synchronised
clusters (Spiegelman and Bennet, 1973; Hilscher <u>et al</u>.
1974). At this stage the Hilschers refer to the germ cells
as oogonia in the presumptive ovaries and as M-
prospermatogonia in presumptive testes. This distinction is
artificial since there is no evidence to suggest that the
morphology or mitotic kinetics is different between males
and females (Hilscher and Hilscher, 1978).

At 11 days <u>pc</u> the indifferent genital ridge is
composed of rapidly dividing germ cells mixed within a
dense mass of growing somatic epithelial tissue derived
from the gonadal blastema (Upadhyay <u>et al</u>. 1979; Merchant,
1975). The ridge also contains some of the cells required
to form the steroidogenic and connective tissue lineage.
However, organ culture experiments demonstrate that there
is a continuing contribution of these cells from the
mesonephric region that extends beyond 11.5 days <u>pc</u> (Buehr
and McLaren, unpublished)
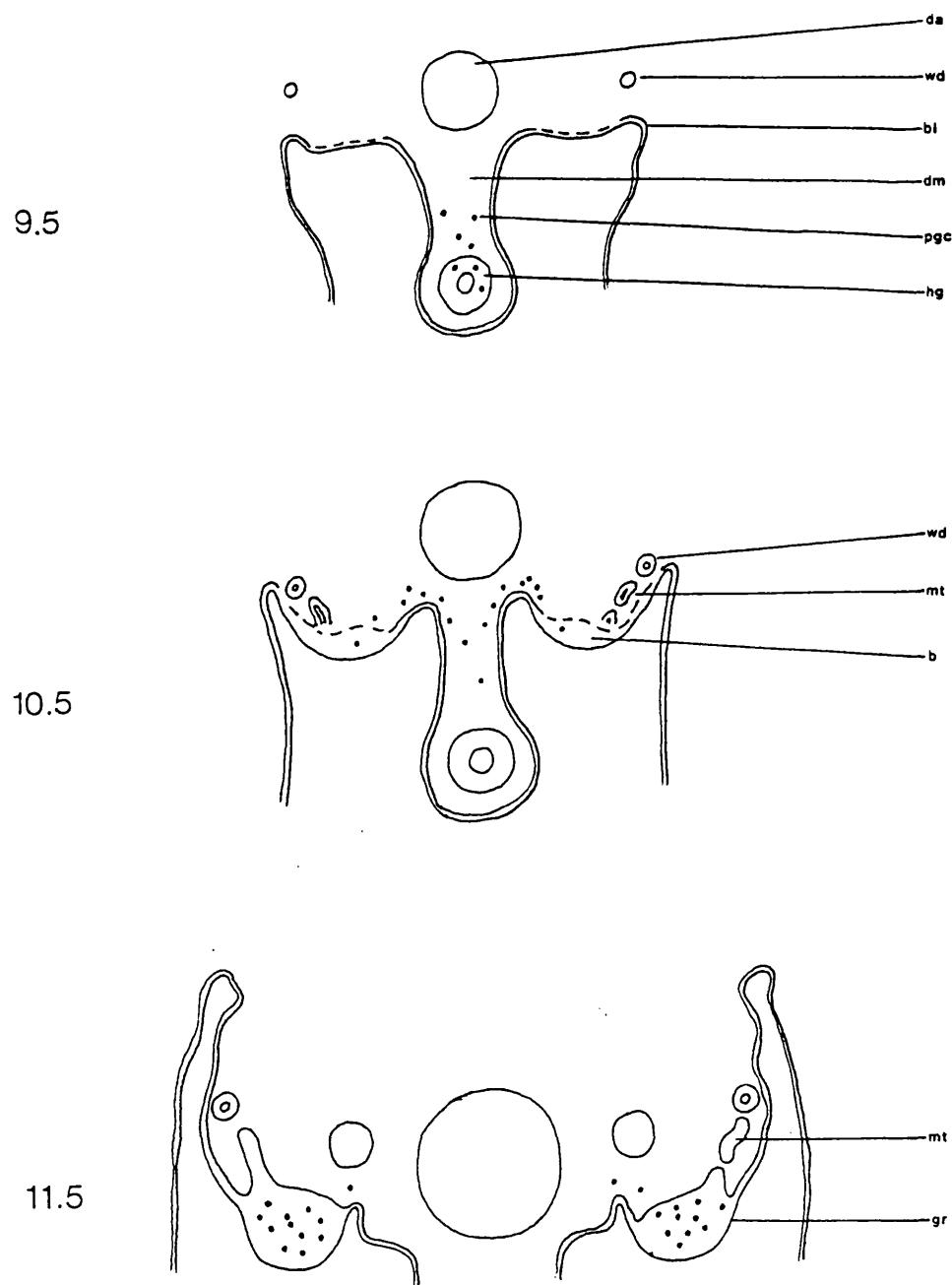
Fig.1. Diagrams of transverse sections through the lower torso of 9.5 to 11.5 day pc fetuses showing the development of the indifferent genital ridge. Da, dorsal aorta; wd, Wolffian duct; bl, basal lamina of the celomic epithelium; dm, dorsal mesentery; pgc, primordial germ cell; hg, hindgut; mt, mesonephric tubule; b, blastema; gr genital ridge. Blastemal cells accumulate between the developing mesonephric tubules and the celomic epithelium. The primordial germ cells migrate from the hindgut, up the dorsal mesentery and into the developing genital ridge.

2. SEXUAL DIFFERENTIATION

2.i. Development of the ovaries

At 12.5 days pc in the mouse, the ovaries show no
gross morphological changes from the indifferent state and
are recognised only by the absence of testicular
differentiation. The ovary is enclosed by an epithelium 1
to 2 cell-layers thick (Odor and Blandau, 1969) and
contains a loosely organised system of cords - the
intraovarian rete (Byskov and Lintern-Moore, 1973; Byskov,
1978), derived from the blastema (Merchant, 1975). A common
basal lamina surrounds the branching system of intraovarian
rete cords and on the inside surface, the rete cells make
direct contact with small groups of oogonia. The position
and ultrastructure of the intraovarian rete cells suggests
that they are the precursors of the follicle cells
(Zamboni, 1974; Byskov, 1978). At the hilus of the ovary,
which is towards the cranial end, the cords make contact
with a loosely defined mass of epithelial cells - the
interconnecting rete which joins to the mesonephric tubules
(extraovarian rete) (Byskov and Lintern-Moore, 1973).

At 13.5 days pc, some of the germ cells in the hilar
region of the ovary have undergone identifiable changes
prior to meiosis (Borum, 1961). By 14.5 days pc, most of
the germ cells have ceased the oogonial phase of mitotic
proliferation and have entered the prophase of meiosis,
thus becoming oocytes (Borum, 1961). The majority are found
as leptotene and zygotene stages, both of which are very
short-lived stages, measured in terms of hours. Entry into
meiotic prophase begins amongst the oogonia in the hilar
region and spreads as a wave towards the periphery
(Upadhyay et al. 1979).

Between 15.5 days pc and birth, the intraovarian
rete cords (ovigerous cords) become more clearly defined
and increase in length, creating a system of twisting

strands that connect at one end to the mesonephric tubules and at the other with the surface epithelium (Upadhyay et al. 1979). The presumptive follicle cells begin to extend cytoplasmic processes that separate groups of oocytes (Odor and Blandau, 1969). Strands of connective tissue and connective tissue cells start to extend into the ovary from the hilar region towards the periphery to form the ovarian septae (Brambell, 1927; Odor and Blandau, 1969; Torrey, 1944).

By 16.5 days pc in the mouse, nearly all of the oocytes have reached the pachytene stage (Borum, 1961), which is a prolonged phase in comparison with leptotene and zygotene. The total number of germ cells reaches a peak at this stage with 70-80,000 germ cells per animal (Jones and Krohn, 1961; Burgoyne and Baker, 1985). In the rat, the germ cell peak is reached 2 days later with approximately 75,000 per animal (Beaumont and Mandl, 1962). Starting at 16.5 days pc in the mouse, and increasingly up to and beyond birth, large numbers of pachytene oocytes degenerate. This process of follicular atresia eventually removes approximately 80% of the germ cells that originally entered meiosis. At 2 days post partum (pp) in the rat, the germ cell number has reduced to 30,000 (Beaumont and Mandl, 1962), at 12 days pp in the mouse, there are approximately 22,000 oocytes and at 200 days pp this has reduced to around 5000 (Burgoyne and Baker, 1981).

At birth, the ovigerous cords have a rosary-like appearance. The oocytes have expanded, particularly those in the central region of the ovary, and the cord tissue between the oocytes has thinned considerably (Uphadyay et al. 1979). As soon as the germ cells reach diplotene they arrest meiotic development in the dictyate phase whilst the accumulation of products required for the post-ovulatory phase occurs.

During the first week of post-natal life the interconnections between individual follicles are lost

(Odor and Blandau, 1969; Upadhyay et al. 1979). The
remnants of the mesonephric tubules lose contact with the
Wolffian duct at one end and the ovigerous cords at the
other and become isolated as a mass of epithelial tissue -
the rete ovarii (Upadhyay et al. 1979).

By two weeks after birth, the ovary consists of many
isolated follicles surrounded by a single layer of follicle
cells and a thick basal lamina. The follicles are embedded
within the ovarian stroma which is composed of connective
tissue cells and cells that differentiate into steroid
producing theca cells in growing follicles.

The initial development of the somatic components of
the ovary does not seem to be dependent upon the presence
of germ cells. In two experiments where germ cell-free
ovaries were studied, in rats treated with busulphan
(Forsberg and Olivecrona, 1966; Merchant, 1975) and in mice
carrying two mutations at the white-spotting locus - $\underline{W}/\underline{W}^V$
(Merchant-Larios and Centeno, 1981), it was found that the
development of the ovigerous cords, connective tissue and
blood vessels, up to 19.5 days pc, is the same as in a
normal ovary. The fine structure of the epithelial cord
cells and stroma cells at 1 day pp is also very similar to
that of an intact ovary (Merchant-Larios and Centeno,
1981). However, without the presence of oocytes, the
epithelial cells remain as ovigerous cords and no steroid
producing theca cells are formed. Corpora lutea are only
found in ovaries in which there is evidence of germ cell
survival.

2.ii. Development of the testes

Under the light microscope, the first signs of
testicular differentiation are the formation of clearly
defined cylindrical cords that appear in the mouse at 12.5
days pc (Simkins, 1923) and in rats at 13.5 days pc
(Torrey, 1944). The testis cords form as two rows of

C-shaped arches, one inside the other, perpendicular to the long axis of the fetal gonad (see fig 2). Except for rare cases where the cords anastomose or end blindly, each of these arches insert at the two ends into the presumptive rete testis (Cleremont and Huckins, 1961).

Ultrastructural studies demonstrate that the differentiation of primordial Sertoli cells precedes the formation of testis cords (Jost et al. 1973; Magre and Jost, 1980). Sertoli cells are identifiable in the rat at 13 days 8 hours pc, in the anterior part of the gonad, close to the mesonephric tubules. Differentiation of fetal Sertoli cells from their precursors appears to spread outwards in a wave towards the periphery (Jost et al. 1973). Under the electron microscope, primordial Sertoli cells are recognised as large clear cells with short rough endoplasmic reticulum and complex junctional specialisations at points of contact with neighbouring Sertoli cells (Magre and Jost, 1980). The formation of testis cords is achieved by the active aggregation of Sertoli cells: a process which is estimated to take 16 hours in the rat (Jost, 1972). Sertoli cells will also aggregate to form cords in cultured fetal testes but at a slower rate. In vitro testis cord formation does not take place, however, if the medium is supplemented with fetal calf serum (Agelopoulou et al. 1984). In these cultures, the Sertoli cells remain isolated as single cells or in small clusters but the ultrastructural morphology of the Sertoli cells appears to be unaffected.

The Mullerian duct inhibiting substance - AMH or MIS, which is secreted by fetal Sertoli cells (Blanchard and Josso, 1974; Tran and Josso, 1982) is detectable in the rat at 13.5 days pc in some Sertoli cells at the cranial end of the fetal testis (Tran et al. 1987). Although AMH production coincides with the aggregation of Sertoli cells into cords (Josso et al. 1985), it is not dependent upon it since AMH production occurs at the same time in cordless

testes cultured in the presence of serum as in control cultures without serum (Magre and Jost, 1984).

At 13.5 days pc in the mouse (which approximates to 14.5 days pc in the rat), the seminiferous cords are tightly packed and occupy most of the gonad except for a small region of disorganised cells that lies between the mesonephric tubules and the newly formed cords (Upadhyay et al. 1981). These cells are assumed to form the rete testis − the ducts that will provide the link from the testis tubules to the excretory channels. Once the cords have formed, the mitotically dividing M-prospermatogonia block at the G1 phase of mitosis and differentiate into T1-prospermatogonia: a state in which they remain until after birth. The first signs of germ cell arrest are detectable at 13.5 days pc in the mouse (McLaren, 1984b; 1985). Sertoli cells can be made to disaggregate in cultured fetal mouse testes by adding cyclic-AMP analogues to the medium. In these cordless gonads, the germ cells still arrest to form T1-prospermatogonia (Taketo et al. 1984a), thus dispelling any suggestion of a connection between enclosure of the germ cells in the seminiferous cords and their arrest.

By 15.5 days pc in the rat, low levels of testosterone can be detected, synthesised from introduced radioactively labelled acetate, indicating the existence of functionally differentiated Leydig cells (Warren et al. 1973). Low levels of testosterone, produced from radioactively labelled pregnenolene, can be detected as early as 13.5 days pc showing that at least some of the necessary enzymes are present at this time (Noumura et al. 1966). Leydig cells only become histologically distinguishable from other interstitial cells at 16.5 days pc in the rat (Roosen-Runge and Anderson, 1959). 16.5 days pc is also the time at which most of the dividing germ cells arrest to form T1-prospermatogonia (Hilscher et al. 1974; Hilscher and Hilscher, 1978) and the time at which

AMH levels reach a peak (Tran et al. 1987).

At 18.5 days pc in the rat, testosterone production reaches a peak, which coincides with the time at which most of the regression of the Wolffian duct occurs in the female. Accordingly, the relative proportion of Leydig cells also reaches a peak during the last days of fetal life (Zirkin and Ewing, 1987). The differentiation of Leydig cells is independent of Sertoli cell aggregation (Patsavoudi et al. 1985).

11.5 day pc XY mouse gonads, isolated from their mesonephroi, develop very poorly in culture and do not form testis cords, whereas control cultures of fetal gonads with intact mesonephroi have good cord development (Buehr and McLaren, unpublished). In the same experiments, it was shown that cells that form the interstitial and peritubular-myoid lineages in the testis continue to migrate into the gonad from the mesonephros after 11.5 days pc. Merchant-Larios and Taketo (1991) argue that the formation of cords in the fetal testis is not primarily caused by Sertoli cell aggregation but is instead due to the invasion of mesenchyme and endothelial cells between the epithelial tissue. This suggests that these lineages play an important role in the formation of the fetal testis. Experiments on isolated prepuberal testicular cells also point to the same conclusion. For example, the in vitro migration of Sertoli cells is greatly enhanced by a fibronectin coating on the substratum (Tung and Fritz, 1986) and fibronectin is only secreted by myoid cells. Secondly, when Sertoli cells and myoid cells are cultured independently, neither secrete detectable amounts of laminin, but when cultured together laminin is produced (Skinner et al. 1985) suggesting that there is a cooperation between the two cell types to form the basal lamina of the testis cord.

Between late fetal life and adulthood, the cytoplasm of the Sertoli cells increases dramatically, completely

enclosing the germ cells and the number of contact
specializations between neighbouring Sertoli cells
multiplies (Flickinger, 1967). The cords increase in length
and twists and folds appear in the arch structure. These
resolve into a regular series of hairpin loops that run
back and forth along the long axis of the testis (Clermont
and Huckins, 1967). By 14 days pp in the mouse, most of the
cords have a small lumen and at 21 days pp, the first signs
of the Sertoli cell tripartite nuclear structure begin to
appear (Flickinger, 1967). AMH production gradually
declines after birth and finally ceases in the rat at 9
days pp (Tran et al. 1987). During the first post-natal
week, the relative proportion of Leydig cells drops
dramatically. This is not due to degeneration of Leydig
cells but a diluting effect caused by the more rapid cell
division of the other testicular lineages (Zirkin and
Ewing, 1987). Leydig cell number then increases once more
and testosterone levels reach a second peak at around four
weeks after birth. The arrested T1-prospermatogonia resume
mitosis on days 4 and 5 pp in the rat (Hilscher et al.
1974) and at 2 days pp in the mouse (Vergouwen et al. 1991)
to produce T2-prospermatogonia. There follows a series of
mitoses which punctuate the differentiation of the
spermatogonial stem cell population, followed by the first
wave of meiosis which begins in the mouse at 1 week after
birth (Bellve et al. 1977).

2.iii. Differentiation of the ducts and external genitalia

In mammals, two sets of duct systems develop during
fetal life: the Wolffian (or mesonephric) ducts and the
Mullerian (or paramesonephric) ducts. In eutherian mammals,
the survival and subsequent differentiation of these ducts
is under the control of gonadal hormones produced after the
initial differentiation of the testes. In XX and XY rabbit
fetuses that have been castrated at 19 days pc, the ducts

develop according to the female pattern: i.e. the Wolffian ducts regress and the Mullerian ducts develop to form the oviducts, uterine horns and upper vagina. This illustrates that the ovaries play no part in the control of the development of accessory structures but the testes must secrete products that prevent the regression of the Wolffian ducts and inhibit the development of the Mullerian ducts. The exogenous introduction of testosterone to XX fetuses stabilizes the Wolffian ducts and promotes their development into the vasa deferentia and induces the formation of the prostate and external genitalia from the urogenital sinus (Jost, 1947). However, testosterone has no effect upon the Mullerian ducts (Jost, 1953). Through studies on the bovine freemartin, it was discovered that the Mullerian ducts are destroyed in the male by the Mullerian inhibitor - AMH or MIS (Jost et al. 1975; Donahoe et al. 1987).

In two marsupial species that have been studied, the Tammar wallaby and the Virginia oppossum, it has been shown that the development of the scrotal rudiments, processus vaginalis and gubernaculum are not under the control of gonadal hormones as in eutherian mammals (Renfree and Short, 1988). Not only do some of these features appear before testis cord formation but also, XO wallabies develop a scrotum and gubernaculum in spite of the presence of ovaries and XXY males develop mammary glands and a pouch (Sharman et al. 1970). Therefore, it is possible that the development of these accessory structures is regulated by the X-autosome ratio.

2.iv. Growth-related sexual differentiation

Differences between males and females in stature, muscle development and a variety of other phenotypic features may be regarded as facets of sexual differentiation but these are largely controlled by the

gonadal hormones. However, differences in growth can also
be detected in embryonic and fetal life before gonadal sex
differentiation and, therefore, before hormone production
begins. XY morulae of the CD1 mouse strain form a
blastocoel cavity at a significantly earlier stage than XX
embryos (Tsunoda et al. 1985) and XY mouse fetuses, removed
at 8.5 days pc, have significantly more somites and are at
a later stage of maturity than their XX littermates (Seller
and Perkins-Cole, 1987). This trend is also observable in
11.5 day pc rat fetuses where the body weight and total
protein content of XY fetuses is significantly higher than
XX siblings. However, there is no evidence for a difference
in the rate of growth since the incorporation of
tritiated-thymidine per unit body mass is equivalent in
males and females (Scott and Holson, 1977). X^PO fetuses
(i.e. carrying a paternally derived X but no maternal sex
chromosome) at 10.5 days pc have been shown to be very
delayed in relation to both XX and XY siblings (Burgoyne et
al. 1983) but mouse fetuses with an X^mO genotype are ahead
of their XX sisters and at an equivalent developmental
stage to their XY brothers (Thornhill and Burgoyne,
unpublished). This demonstrates that the observed
difference in developmental maturity between XX and XY
fetuses is due to a retarding effect of the paternally
imprinted X chromosome.

There is evidence that the XX/XY growth difference
in early embryos of the CD1 mouse strain is caused by the
action of the Y chromosome but embryos carrying an RIII Y
chromosome have no detectable advantage over their XX
littermates (Burgoyne, unpublished).
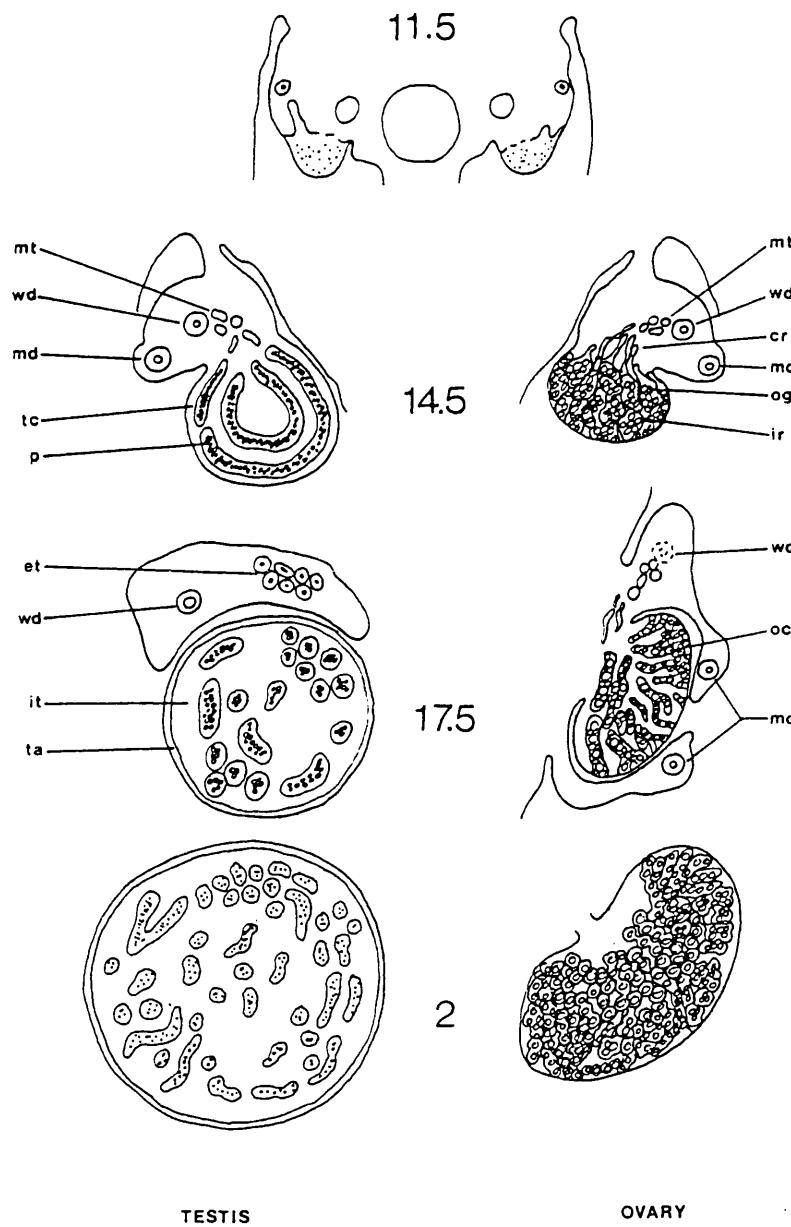
Fig.2. Diagram of sections through mouse gonads showing the development of the testis and ovary from the indifferent genital ridge between 11.5 days pc and 2 days pp. Mt, mesonephric tubules; wd, Wolffian duct; md, Mullerian duct; tc, testis cord; p, T1-prospermatogonia; et, epididymal tubules; it, interstitial tissue; ta, tunica albuginea. Cr, connecting rete; og, oogonia; ir, intraovarian rete; oc, oocytes. At 14.5 days pc, AMH production begins in the testis and some germ cells have entered meiosis in the ovary. At 17.5 days pc, testosterone production reaches a peak in the testis, stabilizing the Wolffian duct: the Mullerian duct has degenerated. In the ovary, atresia of pachytene oocytes has begun and the Wolffian duct is degenerating. At 2 days pp the germ cells resume mitosis in the testis and in the ovary, folliculogenesis is under way.

3. THE GENETICS OF SEX DETERMINATION

The human X and Y chromosomes were first described by Painter (1923) and at that time, it was thought that sex was determined by the X-autosome ratio, as it is in Drosophila (Bridges, 1916). It was not until aneuploids were found that mammalian sex determination was shown to be regulated by the Y chromosome. XXY mammals develop testes (Jacobs and Strong, 1959) and XO mammals develop ovaries (Ford et al. 1959; Welshons and Russell, 1959). These results clearly demonstrate that, in individuals carrying a functional Y chromosome, the gonad develops as a testis and in its absence an ovary develops, regardless of the number of X chromosomes.

3.i. Sex determination in chimeras and mosaics

The mechanism of sex determination is concerned with how the Y chromosome diverts the cell lineages of the indifferent fetal gonad into the testicular developmental pathway. Some clues to the understanding of this mechanism may be found in the study of opposite sex chimeras (Tarkowski, 1961). Initial work gave conflicting results concerning the sex ratio of chimeric mice (Tarkowski, 1961; Mintz, 1965) but this was resolved when it was shown that the mouse strain combination affects the relative proportions of cells contributing to the animal (Mullen and Whitten, 1971). In unbalanced combinations (i.e. where one component tends to dominate the other), the sex ratio is approximately 50:50, but in balanced combinations the sex ratio is skewed towards males. Data from unkaryotyped chimeras made with balanced strain combinations show approximately 60% males and 40% females (Mullen and Whitten, 1971). If these figures are corrected by removing the expected proportion of chimeras made with embryos of the same genotypic sex, the ratio changes to 70% male: 30%

female. This figure fits well with the sex ratio of karyotyped XX<->XY chimeras combined from several studies (Mystkowska and Tarkowski, 1968; 1970; Milet et al. 1972; Ford et al. 1974; McLaren, 1975; Gearhart and Oster-Granite, 1981; reviewed in McLaren, 1984a) of 71% male: 7% hermaphrodite: 22% female. Mullen and Whitten (1971) found only 2 hermaphrodites in a total of 308 chimeras (1.3% of the assumed XX<->XY chimeras) but it is admitted that not all the mice were examined internally. These findings indicate that the influence of the Y chromosome in testicular differentiation may exceed its numerical representation in the cells of the chimera.

This conclusion is supported by evidence from spontaneously produced sex chromosome mosaics. The Y chromosome of the BALB/cWt inbred mouse strain has a tendency to non-disjoin during mitosis creating mosaic mice with an XO/XY/XYY or XO/XY karyotype (Eicher et al. 1980). BALB/cWt hermaphrodites, found at 14.5-15.5 days pc have a mean XO cell contribution to somatic tissues of approximately 64% ranging from one mouse with 97.8% XO cells and 95% ovarian tissue, to another with 33% XO cells and 2.5% ovarian tissue (Eicher et al. 1980). A clear correlation exists between the proportion of XO cells and the amount of ovarian tissue found in these fetal gonads but again the mean is skewed towards testicular differentiation. An interesting facet of the ovotestes found in these hermaphrodites is the tendency of the testicular tissue to occupy the central and caudal regions of the gonad and the ovarian tissue, the cranial or bipolar regions (Whitten et al. 1979). A similar distribution of tissues is found in fetal XX<->XY chimeras (Bradbury, 1987). A survey of adult males produced from a CXBH x BALB/cWt cross revealed that males with bilateral testes may contain up to 80% XO cells (Levy and Burgoyne, 1986; Palmer et al. 1990). In these males, the mean testis weight is inversely proportional to the percentage of XO cells

found in the bone marrow. Although the level of non-disjunction of the Y chromosome in CXBH/BALB/cWt F_1 mice is generally higher than that found in the pure BALB/c Wt stock (Whitten et al. 1991), the proportion of adult CXBH/BALB/cWt F_1 hermaphrodites (Levy and Burgoyne, 1986 - 3.8%; Palmer et al. 1990 - 3.9%) is much lower than the proportion of fetal BALB/cWt hermaphrodites (Eicher et al. 1980 - 14%). This information, together with the correlation of the proportion of XO cells with testis weight, suggests that fetal ovotestes with a significant amount of testicular tissue resolve into small testes through regression of the ovarian component. Similar conclusions have been drawn from the study of fetal XX<->XY chimeras (Bradbury, 1987).

The fact that lateral hermaphrodites (i.e. a testis on one side and an ovary on the other) are frequently found in opposite sex chimeras and sex chromosome mosaics indicates that the effect of the Y chromosome in the testis determining process is locally restricted. A summary of the numerical data suggests that a threshold of Y-bearing cells must be present in the genital ridge for a testis to form in adult life and that this threshold is probably between 20% and 30%. Presumably, if the proportion of XY cells falls below this threshold, an ovotestis persists into adult life or the gonad resolves into an ovary. Adult females of the genotype T(X;16)/X$\underline{Sxr}$[a] (see section 3.iv) frequently have oocyte-depleted ovaries and haemorrhagic follicles which may indicate the existence of small amounts of testicular tissue in fetal life.

3.ii. Non-heritable XX sex reversal

With the knowledge that there is a quantitative requirement for Y-bearing cells if the genital ridge is to develop as a testis, it is tempting to think in terms of a diffusible testicular morphogen encoded or regulated by a

gene on the Y chromosome. Evidence for a diffusible testicular morphogen was originally thought to exist in the bovine freemartin condition. A freemartin is a genotypically female calf that develops as a twin with a bull calf and is born with sterile or masculinised gonads, abnormal internal ducts and, in very extreme cases, abnormal external genitalia (Lillie, 1917; 1923; Chapin, 1917). It was shown that freemartinism is caused by fusion of the chorionic placentae of male and female co-twins, allowing transfer of a blood borne masculinizing agent through vascular anastomoses (Lillie, 1916; 1917). The majority of freemartins have small ovaries with immature ovigerous cords but a small proportion develop germ cell depleted testis cords (Chapin, 1917). When the development of freemartins was examined in utero, it was found that the first observable effects occurred between 45 and 55 days pc with the degeneration of the upper Mullerian ducts, the cessation of ovarian growth and the prevention of germ cell proliferation (Jost et al. 1972; Jost et al. 1975). This coincides with the time at which regression of the Mullerian ducts occurs in the male co-twin. The first signs of testis cord formation in freemartins may be seen at 93 days pc, approximately 50 days after testis cords form in the bull fetus (Jost et al. 1975). This suggests that the formation of testis cords in the freemartin is not synchronously induced by a diffusible testicular morphogen, rather, it is a secondary event that follows impairment of ovarian development.

The coincidence of the involution of the Mullerian ducts with the first detectable signs of freemartinism suggests that the factor responsible is AMH. In support of this conclusion, it was shown that similar levels of AMH can be detected in the serum of freemartins and their co-twins (Vigier et al. 1982). Furthermore, the freemartin gonad can be simulated by adding AMH to the medium in which fetal rat ovaries are cultured (Vigier et al. 1987). After

5 days in culture, some testis cords may be found and at 10
days testis cords resembling those found in 14 or 15 day pc
male gonads may be seen, except they have few germ cells
and some of the germ cells are in meiotic prophase. XX
mice, transgenic for the AMH gene, also show similar
effects of germ cell depletion and some postnatal
masculinization of the gonads but again this is secondary:
in fetal life, the gonads follow the ovarian pathway of
development (Behringer et al. 1990). On these grounds, AMH
is unlikely to be a primary testicular morphogen.

Freemartins do not occur naturally in rodents but a
similar effect can be induced experimentally by
co-culturing or co-grafting male and female fetal gonads.
Fetal ovaries grafted with fetal testes under the kidney
capsule of adult hosts show the familiar pattern of germ
cell loss and the formation of testis cords (Macintyre,
1956; Macintyre et al. 1960). Turner and Asakawa (1964)
claimed to have found sex-reversed XX germ cells entering
spermatogenesis in such co-grafts but these must have
originated from XY cells since it is now known that XX germ
cells in a testicular environment do not survive beyond the
T1-prospermatogonial stage (Cattanach et al. 1971; West,
1982). The factor responsible for masculinization of the
fetal ovaries is diffusible because the same result is
obtainable in mice when the heterosexual gonad transplants
are separated by a semi-permeable membrane (Ozdzenski et
al. 1976) but it acts very locally (at least in rats) since
separation of the fetal gonads by more than 8 mm abolishes
the inhibition and transformation of the fetal ovaries
(Macintyre et al. 1960). In a study of the early stages of
heterosexual co-grafts in mice, it was found that after 6
days, the fetal ovary shows severe oocyte depletion and the
only sign of masculinization is the formation of a tunica
albuginea around the regressing ovary, but after 10 days,
there is evidence of sterile tubules (Burgoyne et al.
1986a). 14.5 day pc rat ovaries, cultured in vitro with

17.5 day pc rat testes (the time of peak AMH production)
show a sharp fall in the number of germ cells after 3 days.
After 4 days, cells with typical Sertoli cell morphology
are found and after 7 to 10 days, the ovarian remnants have
formed Sertoli cell cords which express
immunohistochemically identifiable AMH (Charpentier and
Magre, 1990). Because the fetal testis is known to secrete
AMH and exactly the same effects described above can be
achieved in culture by adding purified AMH to the medium
(Vigier et al. 1987) it is likely that AMH is the
diffusible factor that causes the loss of oocytes and,
indirectly, the subsequent formation of Sertoli cells in
ovaries co-grafted or co-cultured with fetal testes.

Sertoli cell cords may also form from ovarian tissue
in circumstances where AMH is not present. When fetal
ovaries are grafted into various sites in adult animals, a
certain proportion show masculinization. Examples include
the grafting of fetal rat ovaries into the kidneys (Buyse,
1935) or under the skin (Moore and Price, 1942) of adult
rats. Murine fetal ovaries grafted under the kidney capsule
of adult male and female hosts will also form cords
composed of cells with the typical Sertoli cell morphology
surrounded by putative myoid cells. The grafts also secrete
testosterone, indicating the existence of Leydig cells
(Taketo et al. 1984b; Taketo-Hosotani et al. 1985). In
mice, a variety of parameters affect the frequency of
ovarian grafts that develop Sertoli cell cords, including
the levels of testosterone or 17B-estradiol in the host
circulation and the age of the fetus from which the graft
tissue is removed (Taketo and Merchant-Larios, 1986;
Taketo-Hosotani and Sinclair-Thompson, 1987). Fetal ovaries
grafted with the mesonephros attached show a higher
frequency of masculinization in the kidneys of male hosts
than in female hosts (Taketo and Merchant-Larios, 1986) but
if the mesonephros is removed, the frequency of
masculinization increases and the sex of the host becomes

irrelevant (Taketo-Hosotani and Sinclair-Thompson, 1987).
Encapsulation of the ovarian tissue in a semi-permeable
membrane prior to grafting prevents masculinization except
where the membrane is broken (Taketo-Hosotani, 1987)
indicating that a diffusible macromolecule produced by the
host or contact with host cells stimulates testis cord
formation. However, because transformation of fetal ovaries
into testis-like structures will occur readily in adult
female hosts, the factor responsible cannot be Y-linked and
the relevance of these findings to the normal process of
sex determination is, therefore, limited.

The same is true of the Sertoli cell cords or
"tubular adenomas" that form in ageing or hypophysectomized
rat ovaries (Engle, 1946; Crumeyrolle-Arias and Ascheim,
1981; Crumeyrolle-Arias et al. 1986). Under normal
circumstances of oocyte loss due to ovulation, the follicle
cells luteinize under the control of pituitary hormones. It
is possible that in the absence of stimuli from germ cells
and pituitary hormones, some follicle cells become unstable
and form Sertoli cells. This hypothesis is supported by
grafting studies in mice (Hashimoto et al. 1990). Ovaries
of 12.5 day pc fetuses were disaggregated and the germ
cells removed; then the remaining somatic tissue was
grafted into an adult female. These grafts developed the
familiar sterile testis cords. Control ovaries, which were
disaggregated, then transplanted with the germ cells
intact, developed normal ovaries. If this form of
experimental sex reversal is simply a consequence of oocyte
loss, then the factors that affect the frequency of
transformation of ovaries into testes only gives
information about the ablity of oocytes to survive under
these varying conditions. However, this work does indicate
that the development of the Sertoli, myoid and Leydig cell
phenotypes does not directly require genes on the Y
chromosome. Furthermore, since all three cell types can
develop in these ectopic conditions, inductive interactions

must take place between these testicular lineages.

In view of the lack of evidence for a diffusible testicular morphogen, an alternative explanation may be entertained: the Y-linked testis determining gene may act cell-autonomously within one of the gonadal cell lineages. If this is the case, then the germ cell lineage can be excluded as a potential candidate because testes develop in XY mice homozygous for the white spotting mutation - W which lose their germ cells prior to sex determination (Mintz and Russell, 1957). In an examination of the post natal testes of XX<->XY chimeras, it was found that amongst the somatic cell lineages of the testes, Leydig cells and cells of the tunica albuginea can be either XX or XY. The Sertoli cells however, were predominantly, or perhaps exclusively XY (Singh et al. 1987; Burgoyne et al. 1988a). On the basis of these findings, it was proposed that the function of Tdy is to trigger the differentiation of Sertoli cells from the supporting cell lineage in a cell-autonomous fashion. The differentiation of the other testicular cell lineages could then be regulated by the Sertoli cells without further involvement of the Y chromosome (Burgoyne et al. 1988a; Burgoyne, 1988).

3.iii. Heritable XY sex reversal

When the testis determining process fails in XY individuals, ovarian development occurs. Thus testis determination can be viewed as the diversion of the gonadal cell lineages away from the "default" female pathway and into the male pathway by the action of the Y chromosome (for reviews see Eicher and Washburn, 1986; Eicher, 1988; Burgoyne, 1988, McLaren, 1991). XY sex reversal can be caused by a failure of the Y-linked primary testis determining gene - Tdy, as in the Tdy-negative, XY female mice produced by Lovell-Badge and Robertson (1990). However, there are examples of heritable XY sex reversal

where the mutation shows incomplete penetrance.

The Poschiavinus Y chromosome (Y^{POS}) is derived from wild **M.m.domesticus** mice trapped in Switzerland. When this Y chromosome is repeatedly backcrossed onto the C57BL/6 inbred strain, all of the XY fetuses develop ovaries or ovotestes (Eicher <u>et al</u>. 1982). Breeding studies suggest that this XY sex reversal is caused by a failure of interaction between the Poschiavinus Y chromosome and an autosomal recessive allele (<u>Tda-1</u>) present in the C57BL/6 genome (Eicher and Washburn, 1983). The ovotestes that form in fetal life show a spectrum of ratios of testicular to ovarian tissue and, like opposite sex chimeras and mosaics, the testicular tissue is predominantly found in the equatorial region of the gonad (Nagamine <u>et al</u>. 1987a) suggesting that similar cellular events are occurring. Some of the ovotestes resolve into small testes in adult life through regression of the ovarian component.

Nagamine <u>et al</u>. (1987b) found that F_1 XY fetuses, produced by crossing C57BL/6 males with a **M.m.domesticus**-derived Y chromosome to females of the inbred strains AKR, BALB/c and C3H, also showed "aberrant testicular differentiation" but Eicher and Washburn (1986) found no evidence of abnormalities in XY F_1 adults produced by mating C57BL/6-Y^{POS} males to females of the strains DBA, BALB/c or C58, and used this as evidence that C57BL/6 has a variant <u>Tda-1</u> allele. The autosomal gene - <u>Tda-1</u> has escaped attempts at mapping but another gene - <u>Tda-2</u>, involved in the sex reversal that occurs in XY progeny of C57BL/6-Y^{POS} males crossed with New Zealand Black (NZB) females, has been mapped to chromosome 12 (Eicher, 1988).

A similar sort of incompletely penetrant XY sex reversal occurs in C57BL/6 mice heterozygous for the hairpin tail mutation - $\underline{T}^{hp}$ (a deletion in chromosome 17 that includes the brachyury gene - <u>T</u>: see Bennett, 1975; Herrmann <u>et al</u>. 1990) and carrying the AKR Y chromosome which is, like the Poschiavinus Y, <u>M.m.domesticus</u>-derived

(Washburn and Eicher, 1983). An overlapping deletion at the
$\underline{T}$ locus - $\underline{T}^{orl}$ also causes XY sex reversal when combined
with the AKR Y chromosome on a C57BL/6 background but in
this case, the effect is much stronger so that all XY
fetuses heterozygous for $\underline{T}^{orl}$ develop only ovarian tissue
(Washburn and Eicher, 1989). Again, this effect is due to a
failure of interaction because neither $\underline{T}^{orl}$ nor $\underline{T}^{hp}$ causes
XY sex reversal when combined with the C57BL/6 Y chromosome
and the AKR Y chromosome does not cause sex reversal
without the addition of the $\underline{T}$ alleles (Washburn and Eicher,
1989), although there is some evidence of "abnormalities"
of testicular differentiation in C57BL/6 XY^{AKR} fetuses
(Nagamine et al. 1987b) that Washburn and Eicher (1989)
interpret as delayed cord formation. Washburn and Eicher
proposed that the $\underline{T}$ locus contains a gene (Tas - T-
associated sex reversal) which is deleted in these $\underline{T}$
mutants and the C57BL/6 Tas allele, in a hemizygous state,
interacts improperly with the AKR Y. When this was tested
by crossing C3H/C57BL/6 F_1 females to C57BL/6-Y^{AKR} $\underline{T}^{hp}$/+
males, 50% of the offspring that were hemizygous for the
C3H Tas allele also showed XY sex reversal (Washburn et al.
1990). This means that other genes in the C57BL/6 genome,
possibly Tda-1 and Tda-2, also interact in some way with
this process.

3.iv. Heritable XX sex reversal

Unlike inherited XY sex reversal which results from
a loss or failure of gene function, most of the heritable
forms of XX sex reversal result from a gain of Y chromosome
sequences. Sxr (renamed Sxr^a) is a mutation with an
autosomal dominant inheritance pattern which causes XX and
XO mice to develop as sterile males (Cattanach et al.
1971). Sex reversed XX males were shown to contain Y-
derived DNA sequences (Jones and Singh, 1981) and in situ
hybridization and cytogenetic analysis revealed that XYSxr^a

carrier males possess an altered Y chromosome (Singh and Jones, 1982; Evans et al. 1982). The conclusion was reached that the Sxr^a fragment is transmitted via a Y chromosome that has an extra copy of the sex determining region distal to the pairing and exchange region of the Y, so that Sxr^a may be transferred onto the X during male meiosis (Burgoyne, 1982; Eicher, 1982; Hansmann, 1982).

The Sxr^a fragment clearly contains Tdy but it also contains Hya: the gene that encodes or regulates expression of the minor histocompatibility antigen - H-Y (Bennett et al. 1977; Simpson et al. 1981) that is responsible for the rejection of male skin when grafted onto females (Eichwald and Silmser, 1955). Hya was widely believed to be Tdy (Wachtel et al. 1975) until a variant of Sxr^a was found - Sxr^b which is still male determining but does not carry Hya (McLaren et al. 1984). Adult XXSxr^a males lack germ cells because they degenerate around the time of birth (Cattanach et al. 1971) due to the double X dosage (West, 1982), but in fetal life, the majority of XXSxr^a germ cells enter the prospermatogonial pathway (McLaren, 1981). Some XXSxr^a fetuses develop ovotestes (Mittwoch and Buehr, 1973) in which a proportion of the germ cells enter meiosis precociously as in the female (McLaren, 1981). The oocytes that are occasionally found in the testes of prepuberal XXSxr^a males are presumably derived from these fetal meiotic germ cells (McLaren, 1980). Oocytes have not been observed in the testes of XOSxr^a males (McLaren, 1981), suggesting that their presence in XXSxr^a males is due to a proportion of cells within the gonad failing to express Tdy because of spreading of inactivation into the Sxr region.

The number of cells with an inactivated XSxr^a can be enhanced by mating an XYSxr^a father to a mother carrying the X/autosome translocation - T(X;16)H (Lyon et al. 1964). In the T(X;16)H/XSxr^a offspring, cells that inactivate the T(X;16) die and inactivation of the Sxr^a region is sufficient in the surviving cells to form fertile females,

sterile males and hermaphrodites (McLaren and Monk, 1982;
Cattanach et al. 1982). Hermaphrodites of this genotype
show a complete range of ovotesticular morphology from
testes containing oocytes, through severely abnormal
mixtures of testicular and ovarian tissue, to ovaries with
polyovular and haemorrhagic follicles (Ward et al. 1988).
This is presumably due to variation in the numbers of cells
in the gonad which have the Sxr^a region inactivated. This
situation is akin to chimeras and mosaics that have varying
mixtures of Y-bearing and non-Y-bearing cells.

4. THE MAMMALIAN Y CHROMOSOME

The mammalian sex chromosomes are thought to have evolved from a homologous pair of chromosomes (see Ohno, 1969). In some species of reptiles, the sex chromosomes maintain many of the same alleles, but in mammals the Y chromosome is much reduced, both in relative size and in the number of functional genes that it contains. Dosage compensation by X-inactivation in XX females means theoretically that the only gene that it is necessary for the Y to carry is Tdy. However, recent evidence suggests that the Y may carry homologues of X-linked genes that are not inactivated in the female in order to preserve equivalent dosage between the sexes. The Y chromosome may also serve as a repository for genes with male specific functions that are harmful or unnecessary for the female to carry. The human and mouse Y chromosomes have been studied the most and are described here.

4.i. Repetitive sequences on the human Y chromosome

The human Y can be divided into a heterochromatic and a euchromatic region. The heterochromatic region, which binds the flourescent dye quinacrine, occupies most of the long arm up to the telomere. The length of the human Y is highly variable and this is entirely due to variation in the extent of the heterochromatic region (Bobrow et al. 1971). Southern blots of DNA digested with Hae III reveal that this region contains two repeat units - DYZ1 and DYZ2 (McKay et al. 1978; Cooke et al. 1983). DYZ1 is a 3564 base pair (bp) fragment containing 713 varying pentanucleotide repeats based on a TTCCA motif (Nakahori et al. 1986). DYZ2, which is approximately 2.1 kbp also consists of pentanucleotide repeats but unlike DYZ1, this fragment has autosomal homologues (Prosser et al. 1986).

The euchromatic region can be subdivided into the

euchromatic region of the long arm, the pericentric region and the pseudoautosomal and Y-unique regions of the short arm. It is clear that a great deal of the euchromatic region is also composed of repetitive sequences. One of these, the alphoid repeat, is present in the centromeric region of all human chromosomes. The Y chromosome alphoid DNA is composed of tandomly repeating units approximately 5.7 kbp in length which are made up of 170 bp subunits (Wolfe et al. 1985). These repeats form a single block of 540 kbp that is adjacent to another block of 400 kbp of unknown simple-sequence DNA (Tyler-Smith, 1987). Other non-Y-specific repeats include a considerable number of 2.4 kbp sequences that contain a region with 60% homology to the Alu repeat unit: the major human family of short interspersed elements (SINEs) (Smith et al. 1987). The Y also contains long interspersed elements (LINEs) that have the characteristics of retroposons but these appear to be present at the same frequency as throughout the rest of the genome (Smith et al. 1987).

4.ii. Genes on the human Y chromosome other than TDF

The long-arm euchromatic region contains at least one spermatogenesis gene. This is based on 9 azoospermic patients who have deletions of the heterochromatic and parts of the euchromatic region and 6 infertile patients with Yq;autosome translocations (Tiepolo and Zuffardi, 1976; Davis, 1981). This fertility factor (AZF) has been localized by deletion mapping to interval 6 (according to the deletion map of the human Y, Vergnaud et al. 1986, see fig 3) on Yq (Andersson et al. 1988). By the same type of analysis the gene - HY, which encodes or regulates the production of the H-Y antigen, has been localized to intervals 4B to 7 (Simpson et al. 1987) which places it outside the sex determining region (interval 1 of the short arm, Vergnaud et al. 1986) thus supporting evidence from

the mouse that HY is not the sex determinant.

Two male patients with deletions in Yq show reduced tooth dimensions in comparison with their relatives. Tooth size is thought to be a good measure of overall growth and it is suggested that Yq11 contains a gene (GCY) that affects stature (Alvesalo and de la Chapelle, 1981). Molecular genetic studies have demonstrated the existence of a homologue of the X-linked KALIG-1 gene which is associated with Kallmann's syndrome at Yq11.21 (Franco et al. 1991). A homologue of the X-linked steroid sulphatase gene (STS) resides in deletion interval 6, but it is not transcribed (a pseudogene) and contains several additions, base sustitutions and deletions in comparison with the functional X copy. It is unlikely to have arisen through retroposition because it contains introns (Yen et al. 1988). STS-X is just proximal to the pseudoautosomal boundary but interestingly, it is not dosage compensated. This situation is paralleled by the amelogenin gene (AMG) which is slightly more centromeric but close to STS-X. The Y homologue - AMGL (amelogenin-like) is also near to to STS-Y in a more centromeric position (Lau et al. 1989). Expression studies have not been conducted so far but there is a high degree of conservation between the X and Y copies suggesting that AMGL may be functional (Nakahori et al. 1991). Lau et al. (1989) suggested that AMGL may be a candidate for GCY. The positions of these genes in humans and in a variety of related species provides evidence for a pericentric inversion of the Y chromosome which occured after the radiation of the old world monkeys and the higher primates.

In humans, the pseudoautosomal region is located at the distal tips of the short arms of the X and Y chromosomes. The existence of a homologous region shared by the X and Y which takes part in meiotic pairing and recombination, was proposed by Koller and Darlington (1934). The first gene to be cloned and characterised from

within the human X/Y pseudoautosomal region is MIC2 (Darling et al. 1986) which codes for the E2 antigen (Gelin et al. 1989) recognized by the 12E7 antibody. The level of expression of the E2 protein on the surface of red blood cells is affected by a second pseudoautosomal allele - XGR (Tippett et al. 1986; Goodfellow et al. 1987). A third gene localized to the pseudoautosomal region - GM-R, codes for the granulocyte-macrophage colony stimulating factor (GM-CSF) which is implicated in some forms of acute myeloid leukaemia (Gough et al. 1990). Apart from these characterized functional genes, several anonymous DNA sequences map to this region. Of these, DXYS14 is the most distal and has a recombination frequency with respect to sex of virtually 50%, confirming the view that there is an obligatory exchange between the X and Y during male meiosis (Rouyer et al. 1986; Weissenbach et al. 1987).

In an attempt to elucidate the nature of the boundary between the psedoautosomal region and the Y-specific region of the short arm, flanking markers were isolated (Pritchard et al. 1987), the intervening 110 kbp of DNA was cloned and sequences were isolated which span the X and Y pseudoautosomal boundaries. When the sequence of these clones were compared, it was found that the pseudoautosomal region of the Y ended abruptly with the insertion of an Alu repeat at exactly the point the sequences diverge (Ellis et al. 1989). However, for a further 225 bp there is 77% homology between the X and Y indicating the existence of an ancestral, more proximally located boundary.

Apart from the testis determining gene - TDF, the Y-specific region of the short arm also contains TSPY (testis specific protein): a gene that encodes a mRNA species found in adult testis (Arnemann et al. 1987). ZFY, initially a candidate for TDF also maps to this region and nearby, in interval 1A1B, is RPS4Y which appears to encode an isoform of ribosomal protein S4 (Fisher et al. 1990).

Haploinsufficiency for this gene or its X-linked homologue
RPS4X has been proposed as a possible cause of Turner's
syndrome. ZFX and RPS4X are members of an apparently
growing number of X-linked genes that are not inactivated
in females and have homologues on the Y chromosome that may
produce a functionally interchangeable protein. The normal
dosage for such genes in humans would, therefore, seem to
be two. In mice, Zfx and Rps4X are both subject to X-
inactivation and the Y chromosome does not appear to have a
homologue of Rps4X (Ashworth et al. 1991). The difference
in these forms of gene regulation may explain why, unlike
XO mice, only 1% of human XO embryos survive to term and
those that do are frequently afflicted with Turner's
syndrome.

4.iii. Genes on the mouse Y chromosome other than Tdy

Molecular analysis of the mouse Y chromosome
suggests that, like the human Y, much of it is composed of
non-transcribed repetitive DNA but in the mouse, many of
these sequences are retrovirally derived (Phillips et al.
1982; Eicher and Washburn, 1986). Most of the repetitive
sequences that have been isolated from the mouse Y tend to
show polymorphic RFLP patterns between the Y chromosomes of
differing mouse subspecies and are useful for population
genetics studies (Lamar and Palmer, 1984; Nishioka and
Lamothe, 1986; Bishop et al. 1985). Very few of these
repeats cross-hybridize with DNA from other mammalian
species, including close relatives such as the rat,
indicating that Y-linked repetitive sequences change
rapidly in evolution as might be expected for DNA which may
only function as 'bulk' for the chromosome. One sequence
that is common to both human and mouse Y chromosomes and,
has a sex-specific distribution in many other species, is
the Bkm-related (banded krait minor) repeat (Jones and
Singh, 1981). Bkm-related DNA is found on the W chromosome

of the banded krait, from which it was first isolated, and on the X chromosome of <u>Drosophila</u>. It also hybridizes more strongly to female quail DNA than male (Singh <u>et al</u>. 1981). In humans, <u>Bkm</u>-related DNA is relatively rare but there are minor concentrations on both Yp and Yq (Singh and Jones, 1986; Arnemann <u>et al</u>. 1986). In the mouse, <u>Bkm</u>-related probes identify sequences present in the <u>Sxr</u>^{a} transposed region which is derived from the short arm of the mouse Y (Singh and Jones, 1982).

XO<u>Sxr</u>^{a} males have relatively normal testis development with the complete cycle of spermatogenesis, but the spermatids produced are abnormally shaped, nonmotile and frequently diploid (Cattanach <u>et al</u>. 1971; Levy and Burgoyne, 1986). This could be taken to suggest that there are genes outside the <u>Sxr</u>^{a} region of the Y involved in spermatid formation, but the issue is clouded by the fact that in these mice, the X chromosome lacks a pairing partner during meiosis which has also been implicated as a cause of spermatogenic impairment (Miklos, 1974). Recent experiments demonstrate that if a pairing partner is provided which is devoid of Y-specific DNA, most of the spermatogenic defects disappear but the sperm head abnormalities remain (Burgoyne <u>et al</u>. unpublished). Therefore, the Y-specific region of the long arm contains a gene (<u>Smy</u>) involved in the development of the sperm head but, it would seem, little else that is vital for normal male structure and function. A repetitive sequence isolated by Bishop <u>et al</u>. (1985) is present on the long arm of the Y, is expressed exclusively in the testis (Bishop and Hatat, 1987) and is, therefore, a potential candidate for <u>Smy</u>. The pseudoautosomal region of the mouse Y chromosome is situated at the tip of the long arm. The only gene localized to this region so far is the steroid sulphatase gene - <u>Sts</u> (Keitges <u>et al</u>. 1985).

The remaining functions of the Y chromosome are restricted to the <u>Sxr</u>^{a} region which contains, amongst other

things, the testis determining gene ($\underline{Tdy}$), and $\underline{Hya}$, the
gene responsible for regulation or production of the minor
histocompatability antigen - H-Y. A deleted form of $\underline{Sxr}^a$ -
$\underline{Sxr}^b$ retains $\underline{Tdy}$ but has lost $\underline{Hya}$ (McLaren $\underline{et\ al}$. 1984).
Studies on the spermatogenesis of XO$\underline{Sxr}^b$ male mice revealed
that germ cells fail to develop beyond the spermatogonial
stage (Sutcliffe and Burgoyne, 1989) implying that there is
a gene ($\underline{Spy}$) within the deleted region that is responsible
for spermatogonial development (Burgoyne $\underline{et\ al}$. 1986b).
Candidates for $\underline{Spy}$ include $\underline{Hya}$ and $\underline{Zfy}$ but perhaps the best
candidate is the recently cloned gene - $\underline{Als9Y}$ that encodes
a ubiquitin activating enzyme which may be involved in the
maintenance of the cell-cycle (Mitchell $\underline{et\ al}$. 1991; Kay $\underline{et}$
$\underline{al}$. 1991).

4.iv. Molecular analysis of the sex determining region in mouse and man

In humans, $\underline{TDF}$ is situated very close to the
pseudoautosomal boundary which is fortuitous for mapping
purposes because exchange occasionally occurs between the
non-homologous regions of the X and Y so that small regions
of the Y containing $\underline{TDF}$ may be transferred onto the X
chromosome. Using DNA from XX male patients carrying such Y
fragments and from a particularly important XY female -
WHT1013, with a Y;autosome translocation and an associated
Y-deletion, the minimum portion of the Y necessary to
induce maleness was assigned to a 140 kbp region - interval
1A2 (see fig 3). A chromosomal walk initiated within this
region provided a series of overlapping phage clones that
were screened for evolutionary conservation. This procedure
identified the gene $\underline{ZFY}$ which has a zinc finger motif
characteristic of a transcription factor (Page $\underline{et\ al}$.
1987). A closely homologous sequence ($\underline{ZFX}$) was found on the
X chromosome (Schneider-Gadicke $\underline{et\ al}$. 1989). $\underline{ZFY}$ was put
forward as a candidate for $\underline{TDF}$ because of its location, its

conservation on the Y chromosome of many mammals and
because of its presumed DNA binding properties: a likely
characteristic for a major developmental switch gene.
However, as the properties of ZFY were explored, it became
increasingly unsatisfactory as a candidate testis
determining gene. In marsupials, which have a Y-dependent
sex determining mechanism, homologous sequences to the
human ZFY were found to be autosomally located (Sinclair et
al. 1988). Secondly, although the expression of the mouse
homologue of ZFY was found to be at an appropriate
developmental stage, it was shown to be germ cell
dependent. Fetuses that have no germ cells, due to
homozygosity for the white spotting mutation, develop
testes in spite of the absence of Zfy expression (Koopman
et al. 1989). Also, Tdy-negative XY female mice
(Lovell-Badge and Robertson, 1990) have normal expression
patterns and normal structure of the mouse ZFY homologues
(Gubbay et al. 1990a). The mouse homologues of ZFY map to
Sxr^a in the form of 2 genes, Zfy-1 and Zfy-2 (Mardon et al.
1989). In Sxr^b, only one copy of Zfy is present. Instead of
this being due to deletion of one of the genes as was first
thought, the copy of Zfy present is a fusion of the 5'
untranslated region of Zfy-2 with the coding sequence of
Zfy-1 (Simpson and Page, 1991). As Sxr^b arose from a male
mouse which carried Sxr^a on both the X and the Y, the
deletion probably occurred through an unequal recombination
event. In normal XY male mice, Zfy-1 and Zfy-2 are both
expressed in the adult testis but Zfy-2 transcripts are
more abundant (Mardon and Page, 1989; Nagamine et al.
1989). However, only Zfy-1 is expressed during the
development of the genital ridge (Koopman et al. 1989).

The final proof that ZFY is not TDF, and the key to
the mapping of the second candidate gene, arose from four
human XX males who are ZFY-negative but have Y-unique
sequences distal to ZFY. The minimum portion of the Y
necessary to induce maleness was redefined as a 60 kbp

stretch, adjacent to the pseudoautosomal boundary (Palmer et al. 1989). Probes from this region mapped the Y-breakpoints of these XX males to approximately 35 kbp from the pseudoautosomal boundary, and a clone was isolated from this region which shared Y-linked conservation with a variety of mammalian species (Sinclair et al. 1989). The clone was found to contain part of the gene now known as SRY (from the sex determining region of the Y). This created a discrepancy with the mapping data of Page et al. (1987) because the new gene is beyond the distal limit of the 1A2 region which is defined by the deletion in the XY female WHT1013. This was resolved when it was discovered that the XY female has an additional deletion that spans from 50 kbp of Y-unique sequence including the SRY gene to at least 600 kbp into the pseudoautosomal region (Page et al. 1990). Evidence that SRY is TDF was provided by the discovery of one XY female with a small internal deletion in SRY that causes a frame shift (Jager et al. 1990) and two XY females with single base sustitutions in the body of the SRY gene (Berta et al. 1990).

The mouse homologue of SRY - Sry, maps to Sxr[b]: a requirement for any potential Tdy candidate, but the proof that Sry is Tdy comes from the mouse in three ways. Firstly, Sry is deleted (Gubbay et al. 1990b) in the Tdy-negative XY females of Lovell-Badge and Robertson (1990). Secondly, Sry is expressed in the genital ridge between 10.5 and 12.5 days pc and is not dependent on the presence of germ cells (Koopman et al. 1990) which is entirely consistent with the expected pattern. Finally, when a 14 kbp mouse genomic DNA fragment containing the Sry gene was microinjected into embryos, some of the XX transgenic individuals developed as apparently normal, but sterile, males (Koopman et al. 1991).
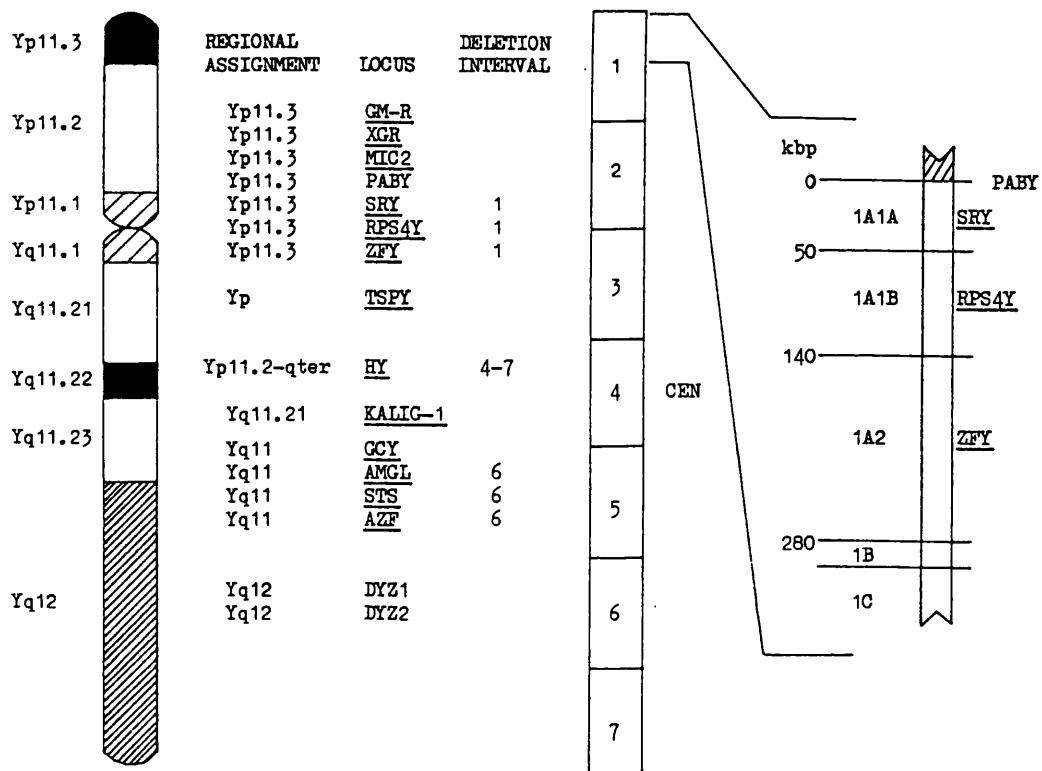
Fig.3A. Cytogenetic map and deletion map of the human Y chromosome. Cen, centromere. The order of the genes is not necessarily correct and no indication of physical distances is intended except for the detail of part of interval 1.
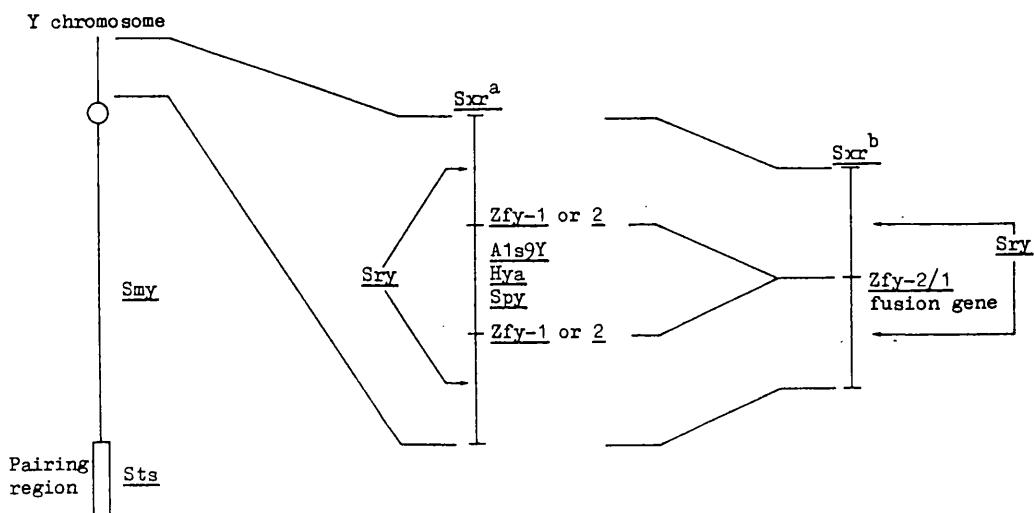
Fig.3B. Map of the mouse Y chromosome and the Y-derived fragments Sxr[a] and Sxr[b]. The genes that have been mapped to the deletion in Sxr[b] are simply in alphabetical order.

5. AIMS OF THE PROJECTS

The first two experiments, described in chapters 2 and 3, were designed to test the hypothesis put forward by Burgoyne et al. (1988a) that Tdy acts cell-autonomously within the supporting cell lineage to bring about Sertoli cell differentiation. In the first experiment, this model was examined by studying the fate of XX or Tdy-negative XY^m cells in the testicular lineages of opposite-sex chimeras. In the second experiment, the fate of XO and Y-bearing cells was examined in the gonads of XO/XY and XO/XY/XYY mosaic hermaphrodites.

The third project, described in chapter 4, was designed to investigate the causes of XY sex reversal in C57BL/6 mice with M.m.domesticus-derived Y chromosomes by examining the effect of different Y chromosomes on the timing of testicular differentiation.

CHAPTER 2

CHIMERIC TESTES STUDIED BY <u>IN SITU</u> HYBRIDIZATION

1. INTRODUCTION

In cellular terms, the process of testis determination involves the diversion of the cell lineages that comprise the gonad into the testicular pathway. The three major cell lineages that are specific to the gonad are the germ cells, the steroidogenic cell lineage and the supporting cell lineage. During fetal life, germ cells enter meiosis in an ovarian environment, but in testes they arrest during the G1 phase of mitotic division and differentiate into T1-prospermatogonia. The steroidogenic cell lineage develops into the theca cells that surround growing follicles in adult females, but in males, this lineage develops into Leydig cells during fetal life. The supporting cell lineage is the precursor of the follicle (or granulosa) cells that surround the developing oocytes in the ovary, but in fetal testes, these cells differentiate into Sertoli cells which aggregate together to form testicular cords. It is assumed that prior to Tdy action, these three gonad-specific lineages are bipotential.

Burgoyne et al. (1988a) examined the relative contribution XX and XY cells made to each of these lineages in the testes of adult and prepuberal XX<->XY chimeras. It was found that Leydig cells and the connective tissue contained both XX and XY cells in similar proportions to the non-gonadal somatic tissues. The germ cells were exclusively XY, which is not surprising since two X chromosomes are incompatible with spermatogonial development (West, 1982). The most interesting part of the results was that the Sertoli cells were predominantly, or perhaps exclusively, XY. Lysates of Sertoli cells, isolated from prepuberal XX<->XY testes, were analysed electrophoretically for the different isozymes of glucose phosphate isomerase (GPI) present in the XX and XY lineages. This revealed a strong GPI band derived from XY

cells but the GPI band from XX cells was barely detectable.
The Sertoli cell isolates were known to be slightly
contaminated by other cell types, which could account for
the minor XX-derived GPI band. To help resolve this
equivocal result, the testes of adult XX<->XY chimeras were
examined by in situ hybridization and no XX Sertoli cells
were found. Singh et al. (1987) had previously reported a
predominance of XY Sertoli cells in the testes of a single
XX<->XY chimera but, because of technical limitations, it
was not possible for them to positively identify XX Sertoli
cells.

The predominance/exclusivity of XY Sertoli cells led
Burgoyne et al. (1988a) to propose that the function of Tdy
is to cell-autonomously induce the differentiation of the
supporting cell lineage into Sertoli cells. The
differentiation of the remaining testicular cell lineages
could then be regulated by the Sertoli cells without
further involvement of the Y chromosome.

The finding that Sertoli cells are predominantly or
exclusively XY in adult XX<->XY chimeric mouse testes does
not necessarily mean that a) this is established at the
time of testis determination or b) that the Y-linked gene
responsible is Tdy. It might be caused by another Y-linked
gene necessary for Sertoli cell differentiation acting at a
later stage; just as in the germ line, the Y is not
necessary for the formation of T1-prospermatogonia but is
vital for spermatogonial proliferation after birth (Levy
and Burgoyne, 1986). The objective of the present study was
to test the hypothesis of Burgoyne et al. (1988a) by
examining the fate of XX cells and Tdy-negative XY^m cells
in the testes of fetal XX<->XY and XY^m<->XY chimeras. In
the light of the results obtained, prepuberal and adult
chimeras were re-examined.

2. MATERIALS AND METHODS

2.1. Mice

The transgenic mouse stock (Line 83) produced by Lo (1986), was used to provide a lineage marker that could be detected by <u>in situ</u> hybridization. This line is homozygous for a single insertion of 1000 copies of the mouse β-major globin gene close to the telomere of chromosome 3. The stock is derived from a mixed CBA, C57 and SJL background and is maintained as a small closed colony but is not formally inbred. (These mice will be referred to as globin transgenics - GT). Chimeras were produced from two different sets of crosses:-

1) CBA-T6,XX x GT,XY<->BALB/c,XX x MF1,XY^{del}

2) GT,XX x MF1,XY^{m}Y^{del}<->(CBAxC57BL/6)F$_1$,XX x CBA-T6,XY^{meta}

The progeny of these crosses contained chromosome markers that allowed the identification of the chromosomal sex, and the parental origin, of both components of the chimeras. These markers include the T6 reciprocal translocation (Ford <u>et al</u>. 1956), the metacentric Y chromosome variant (Y^{meta}, Winking, 1978; Burgoyne <u>et al</u>. 1988b) and the Y^{del} chromosome which is an RIII Y chromosome with a large long arm deletion, maintained on an MF1 (random bred albino) background. The Y^m chromosome (Lovell-Badge and Robertson, 1990) is a 129-derived Y which has a small deletion that includes <u>Sry</u> (Gubbay <u>et al</u>. 1990b) and consequently does not induce male development.

2.ii. Making chimeras

Males and females of the appropriate strains were
caged together and the females were checked each morning
for the presence of a copulatory plug. Pregnant females
were killed at 2.5 days pc by cervical dislocation and the
oviducts and uteri were dissected out and placed into small
drops of M2 culture medium (Fulton and Whittingham, 1978)
on the lid of a petri dish. A 30 gauge stainless-steel
hypodermic needle, which had been rounded-off at the point
to prevent tearing the tissue, was inserted into the
ampulla of the oviduct and approximately 0.5 ml of M2 was
pumped through using a 1 ml syringe. The released 8- to 16-
cell embryos were collected under the dissecting microscope
with a finely drawn hand-pipette and placed into a fresh
drop of M2. All of the embryos were collected together and
sorted according to genotype into two separate drops of M2.
To remove the zona pellucidae, the embryos were transferred
in groups of three or four to a 1 ml drop of acid tyrodes
(see Pratt, 1987) and observed closely under the dissecting
microscope. After about 10-15 seconds, the zona pellucidae
became visibly thin, at which point they were removed and
placed into M2, pipetted up and down several times to
inactivate any acid tyrodes that had been carried over,
then transferred to a second drop of M2. If the process was
judged correctly the zona pellucidae could be removed by
pipetting the embryos up and down. If not, the embryos were
returned to the acid tyrodes for a further few seconds,
then the process repeated.

The zona-free embryos were transferred to M16
(Whittingham, 1971) in a 60-well microtitre plate (Cel-
cult, Sterilin Ltd.), keeping the two genotype groups of
embryos separate. The plate, with M16 in the wells and
covered with a layer of paraffin oil, was prepared at least
two hours in advance and placed into a $37^{\circ}C$ incubator
gassed with 5% CO_2 in air, thus allowing the medium to

reach the correct temperature and pH before the embryos were added. Individual embryos from each genotype group were transferred to new wells to create genotype pairs, then each pair was gently pushed together using the hand pipette so that they were touching. The culture plate was placed back into the incubator and left overnight.

On the following morning, the embryos were checked for successful aggregation. In the afternoon of the same day, the embryos were usually beginning to cavitate. These early blastocysts were transferred into a drop of M2, then transferred to the uterine horns of an anaesthetised 2.5 days pc pseudo-pregnant mother. Pseudo-pregnancy was achieved in the females by mating with either vasectomized or XXSxr sterile males. Before transferring the embryos, the ovaries of each mother were checked for the presence of red corpora lutea: a sign of recent ovulation. If these were not present, a different mother was used. The uterine horn was externalized and the oviduct held gently with a pair of fine forceps. A hole was made at the apex of the uterus with a sterile 25 gauge hypodermic needle that had been dipped in pontamine blue. As the needle was removed a small blue spot of the vital dye was left behind to mark the site of the puncture. The chimera embryos could then be transferred into the uterus through the puncture using a hand pipette, taking care to inject as little M2 as possible.

2.iii. Chimera analysis

For the fetal chimeras, the gonads were dissected out, then fixed and processed according to the protocol for in situ histology (see appendix 2.i). Samples of liver were also removed for cytogenetic preparations (see appendix 1.ii).

Prepuberal and adult chimeras were killed by cervical dislocation and the gonads were removed for in

<u>situ</u> histology. The femurs were removed for bone marrow
preparations (see appendix 1.i). In some cases, samples of
testicular tissue were fixed in 3:1 ethanol:acetic acid for
air-dried testis preparations. These were produced by
transferring some small lengths of fixed testis tubules to
a clean centrifuge tube then applying one or two drops of
60% acetic acid directly onto the tissue. After one minute,
the tubules had dissagregated and the action of the acid
was blocked by the addition of excess 3:1 fixative. The
resulting cell suspension was centrifuged, the fluid
drained off and the cells resuspended in fresh fixative.
After another centrifugation the cells were resuspended in
a few drops of fresh fixative and dropped onto clean glass
slides.

The bone marrow and liver preparations were scored
to establish the genotypes of each component and to provide
an estimate for the percentage contribution of each
component to the somatic tissues. Gonad sections and
air-dried testis preparations were taken through the <u>in</u>
<u>situ</u> procedure (see appendix 2). The probe used was the
entire pMβG2 plasmid which is the vector pBR322 containing
a 7 kbp genomic fragment that spans the mouse β-major
globin gene (Tilghman <u>et al</u>. 1977).

2.iv. Scoring

The proportion of labelled germ cells, myoid cells
and Sertoli cells in sections of the fetal testis were
estimated by scoring all the cells in a series of randomly
selected cross-sections of testis cords. Similarly,
labelled and unlabelled cells were scored in randomly
selected cross-sections of mesonephric tubules. For the
Leydig cells, the proportion of labelled cells was
quantified by scoring all the eosinophilic interstitial
cells that fell inside the area marked out by a randomly
applied gridded eyepiece graticule. To rectify the bias due

to false negative cells, correction factors were produced
by applying the same procedures to testis sections from a
fetus hemizygous for the transgenic insert (i.e. all cells
carry the transgene).

For the air-dried preparations made from adult
testis, the proportion of labelled cells in the various
lineages was estimated by scoring all of the cells
encountered as the slides were scanned until a suitable
total was reached. Because the entire nucleus is present in
air-dried preparations, no correction was required.

3. RESULTS

A total of 67 live offspring were produced from the
two crosses of which 47 were overt chimeras. The remainder
were either non-chimeric or had too low a contribution from
one of the components to be detected in the liver or bone-
marrow (see table 1). Those that were useful to the study
include 14 XX<->XY, 3 XY^m<->XY and 5 XY<->XY chimeras.
These fall into three age categories: fetal (13.5-16.5 days
pc), prepuberal (13-15 days pp), and adult (46-52 days pp).
A close examination of the testis sections of the XX<->XY
chimeras after in situ hybridization revealed that XX
Sertoli cells do in fact exist at all three ages (see figs
2 & 3). Sertoli cells were identified on the basis of their
irregular nuclear morphology, the distribution and density
of heterochromatin and their position in the tubule. The
presence of XX Sertoli cells was confirmed for the adult
chimeras by the in situ analysis of air-dried testis
preparations, in which Sertoli cells are easily recognised
by the characteristic 'triple spots' of a light blue
nucleolus flanked by two heterochromatic blocks. In these
preparations, a small population of such cells was found
with clear hybridization (chimeras 2-4, e.g. fig 3D) or

lacking hybridization (chimera 1) indicating their XX
nature. Hybridization was always found, both in the control
XY<->XY testis preparation and in the experimental
chimeras, closely associated with one of the
heterochromatic blocks. The fact that these regions are
known to contain centromeric sequences (Hsu et al. 1971),
plus the fact that the target transgenic insert is close to
the telomere of chromosome 3, may indicate something about
the organization of chromatin in the Sertoli cell nucleus.

The estimates of the contribution of XX cells to
specific cell types in the fetal testis of XX<->XY chimeras
are presented in table 2A. The chimeras are arranged in
order of increasing XX contributions to liver. With the
exception of the Sertoli cell lineage, there is no
prejudice against XX cells contributing to testicular
cells. Overall, the proportion of XX peritubular myoid
cells (43%) and Leydig cells (48%) is very similar to the
proportion of XX cells in non-testicular tissues (47-48%),
while the overall proportion of XX T_1-prospermatogonia
(65%) is somewhat higher. By contrast, there is a clear
prejudice against XX Sertoli cells in all four chimeras,
with a mean XX contribution of 10%. Comparing the
proportion of XX Sertoli cells in each chimera with the
pooled estimates for all other cell types indicates a
reduction of between 4 and 7-fold. The extent of the
reduction in the contribution of XX cells to the Sertoli
cell lineage is not related either to fetal age or the
strain of the XX component.

The two prepuberal XX<->XY chimeras had XX
contributions to the bone marrow of 29% and 39%. The
labelled Sertoli cells occurred as clonal patches but the
patches were rare, confirming the strong XY bias (fig 3B).
The proportion of XX Sertoli cells has not been estimated
for these two chimeras. The quantitation procedure used for
the sections is inappropriate when the cells occur in rare
patches and, in air-dried preparations of dissociated

testicular tissue, the majority of Sertoli cells from this age lack the typical nuclear morphology seen in the adult. In the four adult chimeras (table 3A), the proportion of XX Sertoli cells was very low (1.6-2.1%) and surprisingly consistent despite a wide range of XX contribution to the bone marrow (10-84%).

Extensive earlier work has demonstrated that XX germ cells do not survive in XX<->XY testes. Of the 2381 pachytene and MI cells scored here, one MI cell lacking the transgene marker was identified in the chimera that had the transgene in the XY component. This probably represents artefactual loss of the bivalent carrying the marker. It was noted that the adult XY<->XY control, in which the XY component lacking the transgene was heterozygous for the T6 translocation, had no unlabelled MI spermatocytes, although this component was well represented in pachytene spermatocytes (13%), Sertoli cells (30%) and bone marrow cells (43%). It has previously been reported that heterozygosity for this translocation causes extensive meiotic failure (Baranov and Dyban, 1968).

In the two fetal XY^m<->XY chimeras, the XY^m component behaved in the same way as the XX component of the fetal XX<->XY chimeras. The proportions of XY^m cells contributing to the mesonephric, myoid, Leydig and germ cell lineages were broadly similar to each other and to the liver tissue. The XY^m cells showed the same under-representation in the Sertoli cell lineage as the XX cells, the relative proportion falling within the 4 to 7 fold reduction observed in the XX<->XY chimeras (table 2B). The same pattern was observed in the XY^m<->XY adult chimera (Table 3B) in which the proportion of XY^m Sertoli cells was within the same range as that estimated for XX Sertoli cells in adult XX<->XY chimeras. Surprisingly, XY^m cells also failed to contribute to the germ cell lineage. This was not just due to a loss of meiotic stages as table 3B might suggest, but a complete absence of XY^m germ cells

since a close examination of testis sections from this
chimera after in situ hybridization revealed that none of
the spermatogonia were labelled either.

4. DISCUSSION

This study demonstrates that some XX Sertoli cells
are present in fetal, prepuberal and adult XX<->XY
chimeras. The presence of XX Sertoli cells in adult XX<->XY
chimeras is contrary to the results of Burgoyne et al.
(1988a). However, the incidence of XX Sertoli cells in the
adults was less than 3% and it is possible that such a low
contribution, occurring as it does in the form of rare
patches, may have been missed in the earlier study. The
presence of XX Sertoli cells in an adult XX/XY male has
previously been inferred by Singh et al. (1987) and is also
reported for a series of adult XX<->XY chimeras by Patek et
al. (1991).

Since XX Sertoli cells are already present in the
13.5 day pc XX<->XY testes, they presumably differentiated
contemporaneously with the XY Sertoli cells, and as a
consequence of Y action. It should be emphasised that,
although the Sertoli cells were not exclusively XY, there
was a strong XY bias that was already established by 13.5
days pc. There was no XY bias in any of the other cell
types in the fetal testes. The fact that the Tdy-negative
XY^m Sertoli cells experience the same prejudice as XX cells
implies that the bias is a function of Tdy activity and is
not due to the activity of other genes on the Y. This
strengthens the conclusion by Burgoyne et al. (1988a) that
Tdy must act by directing cells of the supporting cell
lineage to form Sertoli cells. However, since a small
proportion of fetal XX Sertoli cells were found, at some
point between the expression of Tdy and the formation of

Sertoli cell cords, there must be a step that can locally recruit XX cells. In a normal XY male, this capacity for local recruitment is irrelevant since all cells contain Tdy. The expression of Sry (Tdy) is first seen at 10.5 days pc in the genital ridge and is germ cell independent (Koopman et al. 1990), which is compatible with Sry being expressed in the Sertoli cell precursors. The sequence of Sry suggests that it encodes a DNA-binding protein which would be expected to act cell autonomously. This would imply that the capacity for recruiting some XX cells to form Sertoli cells in XX<->XY testes, is a characteristic of a gene product in the Sry-initiated 'cascade', rather than of Sry itself.

The XX Sertoli cells that form in XX<->XY chimeric gonads should not be equated with those that form in ovarian tissue in a number of situations where there has been germinal failure (reviewed by Burgoyne, 1988 and 1991). Taketo-Hosotani et al. (1985) and Taketo-Hosotani and Sinclair-Thompson (1987), for example, have described the "transdifferentiation" of follicle cells into Sertoli cells, following germ cell loss, when fetal mouse ovaries were grafted under the kidney capsule of adult male, or even adult female, hosts. In the latter instance there was no Y chromosome present in the graft or host, so it can hardly be considered of direct relevance to the mode of Tdy action.

The fact that Sertoli cells are the only cell type to show an XY bias in the fetal testis supports the view of Burgoyne (1988) that the commitment of other testicular cell types to the male pathway is directed by Sertoli cells without further Y involvement. This does not preclude a role for the Y in the subsequent differentiation or function of these other cell types, or indeed, of Sertoli cells. For example, the mouse Y has at least three distinct functions during spermatogenesis (reviewed by Burgoyne, 1991). Patek et al. (1991) suggest that the Y may also be

required for normal Leydig cell function. This suggestion
is based on their finding of an unusually high incidence of
tubules with germinal failure together with a particularly
high proportion of XX Leydig cells in their series of XX<-
>XY chimeras, leading them to propose that the germinal
failure is brought about by XX Leydig cell dysfunction. An
alternative possibility is that this particular chimeric
combination (in which the XY component was always derived
from embryonic stem cells of the 129 inbred strain and the
XX component from C57BL/6 x CBA F_2) not only favours XX
Leydig cells but also favours XX germ cells. The tubules
with germinal failure would then be attributable to the
loss of the XX germ cells around the time of birth.

The absence of XY^m germ cells in the single adult
XY^m<->XY chimera deserves comment. The Y^m mutation was
originally discovered in the progeny of an XX<->XY/XY^m
chimera (Lovell-Badge and Robertson, 1990). This means that
XY^m cells must be capable of transmission through the male
germ line. It is assumed, therefore, that the absence of
XY^m germ cells in this chimera is simply due to chance
exclusion.

A puzzling feature of the present data was the
consistency in the proportion of XX Sertoli cells (1.6-
2.1%) in the adult XX<->XY chimeras despite a wide range of
XX contributions to the mice as a whole (10-84%). While the
consistently lower frequency of XX Sertoli cells in the
adult chimeras, as compared to the fetal chimeras, may
indicate selection against XX Sertoli cells subsequent to
their formation, it is hard to imagine a selective
mechanism which acts to produce such a low but invariant
proportion of XX cells.
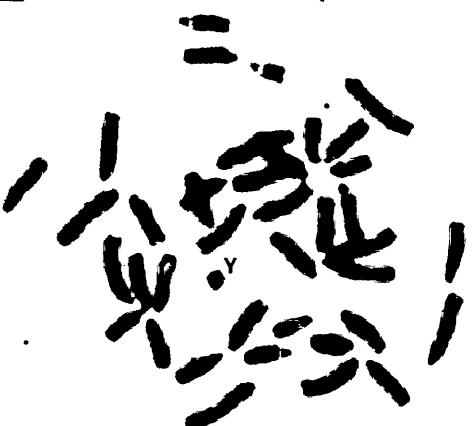
Fig.1. Examples of metaphase plates from the chimeras. (A) XX. (B) XY^m. The Y^m is cytologically indistinguishable from a normal Y chromosome. (C) XXT6/+. (D) XY^{meta}T6/+. (E) XY^{del}. 19, chromosome 19. Y, Y chromosome. T6, marker chromosome 15^{14}: product of the reciprocal translocation - T(14;15)6Ca. Bar, 10 μm
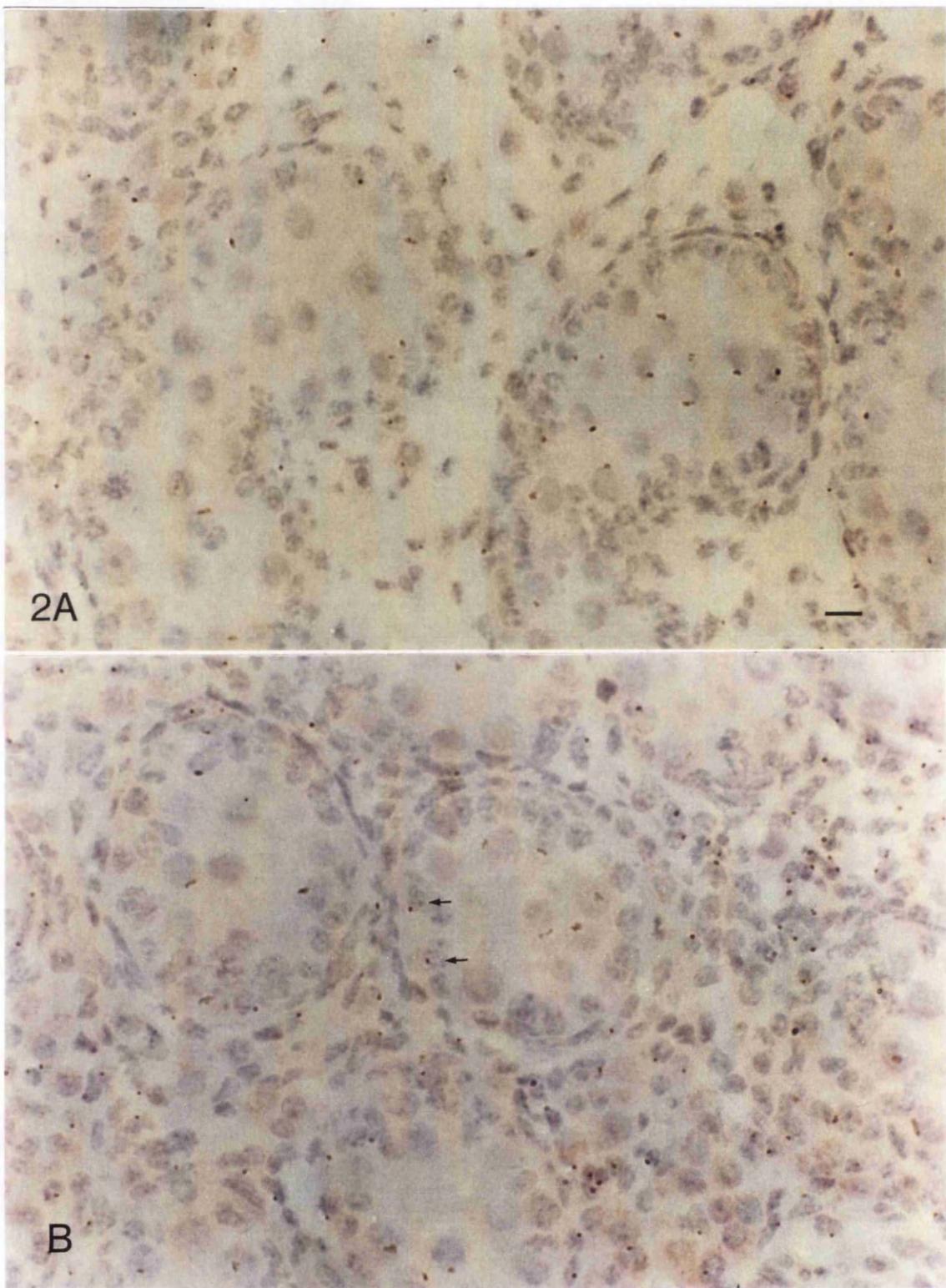
Fig. 2. (A) Section of 16.5 day pc XX<->XY testis showing some labelled XX T1-prospermatogonia inside cords lined with unlabelled XY Sertoli cells. (B) Section of the same testis with two labelled XX cells which, on the basis of position and morphology, are presumed to be Sertoli cells (arrowed). Bar, 10µm.
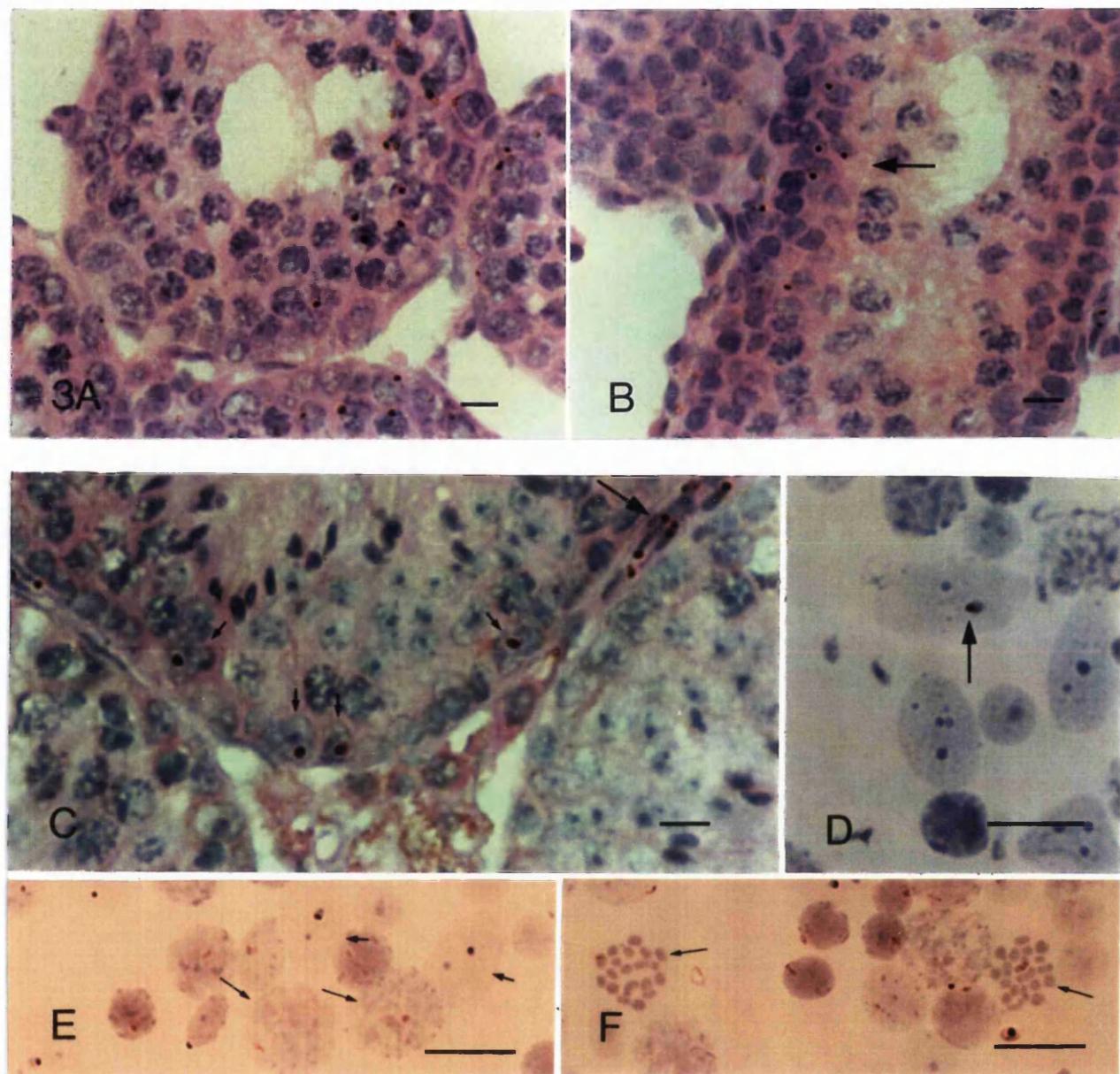
Fig. 3. (A) Prepuberal XY<->XY control testis section
showing the patchy distribution of labelled germ cells and
Sertoli cells. (B) Prepuberal XX<->XY testis section from a
mouse with 29% XX contribution to the bone marrow. A patch
of labelled XX Sertoli cells (arrowed) can be seen in an
otherwise XY dominated testis. (C) Section of testis from
adult XX<->XY chimera 4. Four XX Sertoli cells (small
arrows) can be seen inside a tubule producing condensed
spermatids. The other labelled cells are peritubular myoid
cells (large arrow). (D) Air-dried testis preparation from
chimera 4 showing Sertoli cells with the characteristic
heterochromatic blocks. One of the Sertoli cells has a
hybridisation signal over one of the heterochromatic blocks
(arrow) indicating the XX nature of the cell. (E) and (F)
Air-dried testis preparation from chimera 1 in which the XY
component was labelled, showing, in (E), two labelled
Sertoli cells (small arrows) and two labelled pachytene
spermatocytes (large arrows) and in (F) two labelled MI
spermatocytes (arrowed). Bar,10μm.

Table 1A. Chimeras produced from cross 1

Genotype	Sex	Number killed at each age group			
		Fetal	Prepuberal	Adult	
Non-chimeric	Both	8	3	1	
XX<>XX	Female	6	0	1	
XY<>XY	Male	1	2	0	
XX<>XY	Female	2	0	0	
XX<>XY	Male	5[a]	4	1	
	Total	22	9	3	34

(a) One of the 5 was trisomic for chromosome 19 in the XY component and had a very low XX component so was excluded from the study.

Table 1B. Chimeras produced from cross 2

Genotype	Sex	Number killed at each age group			
		Fetal	Prepuberal	Adult	
Non-chimeric	Both	5	2	1	
XX<>XX	Female	2	2	0	
XX<>XY^{del}	Both	0	0	0	
XX<>XY^m	Female	1	7	2	
XY<>XY^{del}	Male	0	1	1	
XX<>XY	Female	0	1	0	
XX<>XY	Male	0	1	3	
XY<>XY^m	Female	1	0	0	
XY<>XY^m	Male	2	0	1	
	Total	11	14	8	33

Chimeras useful to the study are marked in boxes. Two of the fetal opposite-sex chimeras marked as male contained some ovarian tissue but had enough testicular tissue to be scored.

Table 2A. Estimated proportions of XX cells in cell lineages from fetal XX<->XY chimeras.

| Chimera[a] | Age dpc | Percentage of XX cells (number of cells scored)[b] | | | | | |
| | | Non-testicular cells | | Testicular cells | | | |
		Liver	Mesonephric tubules	Myoid cells	Leydig cells	T1-prosperm- atogonia	Sertoli cells
1 XX*<>XY	16.5	23(13)	8(607)	26(509)	27(502)	54(503)	5(552)
2 XX*<>XY	13.5	32(50)	25(526)	31(54)	41(207)	70(133)[c]	7(158)[c]
3 XX<>XY*	15.5	61(51)	69(560)	56(403)	53(538)	54(317)	8(535)
4 XX<>XY*	15.5	74(50)	90(522)	60(203)	72(491)	82(212)	21(354)
MEAN		47.5	48.0	43.2	48.3	65.0	10.2

Table 2B. Estimated proportions of XY^m cells in cell lineages from fetal XY^m<->XY chimeras

| Chimera[a] | Age dpc | Percentage of XY^m cells (Number of cells scored) | | | | | |
| | | Non-testicular cells | | Testicular cells | | | |
		Liver	Mesonephric tubules	Myoid cells	Leydig cells	T1-prosperm- atogonia	Sertoli cells
XY^{m*}<>XY	14.5	25(36)	9(554)	9(155)	9(379)	21(492)	2(308)
XY^{m*}<>XY	14.5	52(50)	42(351)	24(107)	40(366)	27(428)	7(186)
MEAN		38.5	25.5	16.5	24.5	24	4.5

OVERALL MEAN		44.5	40.5	34.3	40.3	51.3	8.3

(a) The chimeras in table 2A were all from cross 1, and in table 2B from cross 2. The component carrying the transgenic marker is indicated by an asterisk.

(b) For the liver, the counts are based on cytogenetic markers in air-dried mitotic cells. For the other cell types, the estimates are based on the in situ analysis of the transgenic marker, corrected for the incidence of false negatives. The percentage of false negatives by cell type, as estimated in control sections were: mesonephric tubules 29% (344), myoid cells 30% (244), Leydig cells 21% (146), T1-prospermatogonia 43% (249) and Sertoli cells 27% (313)

(c) At 13.5 days pc, many of the cells in the testis cords were in prophase or metaphase of mitosis. Because of the possibility of confusion between dividing Sertoli cells and germ cells, the Sertoli cell counts are based on the unambiguously identifiable, peripherally located interphase cells, and the T1-prospermatogonia counts on centrally located cells whether or not they were dividing.

Table 3A. Estimated proportions of XX cells in the cell lineages of adult XX<->XY chimeras.

Chimera[a]	Cross	Age dpp	Percentage of XX cells (number of cells scored)[b]			
			Bone marrow	Pachytene spermatocytes	MI spermatocytes	Sertoli cells
1 XX<>XY*	1	52	10(50)	0(514)	0.8(124)[c]	1.8(1418)
2 XX*<>XY	2	52	22(50)	0(564)	0(63)	2.1(1078)
3 XX*<>XY	2	46	61(33)	0(500)	0(50)	1.6(1039)
4 XX*<>XY	2	52	84(51)	0(537)	0(29)	1.6(2052)

Table 3B. Estimated proportions of XY^m cells in the cell lineages of an adult XY^m<->XY chimera.

Chimera[a]	Cross	Age dpp	Percentage of XY^m cells (Number of cells scored)			
			Bone marrow	Pachytene spermatocytes	MI spermatocytes	Sertoli cells
XY^m*<>XY	2	52	20(50)	0(572)	0(61)	1.1(1065)

(a) The asterisk indicates the component carrying the transgenic marker.

(b) The bone marrow counts are based on cytogenetic markers in air-dried mitotic cells. For the other cell types, the counts are based on the in situ analysis of the transgenic marker in air-dried testicular cells.

(c) This is based on a single unlabelled cell, which probably lost the marker as a result of metaphase breakage.

CHAPTER 3

THE GONADS OF XO/XY AND XO/XY/XYY MOSAIC HERMAPHRODITES STUDIED BY IN SITU HYBRIDIZATION

1. INTRODUCTION

Gonadal sex differentiation can first be recognized in mice at 12.5 days pc with the appearance of testicular cords in the gonads of XY fetuses. Female gonads show no gross morphological change from the indifferent state until 13.5 days pc when the germ cells first enter meiosis (McLaren, 1984b). Studies on fetal rat testes by light and electron microscopy have revealed that the Sertoli cells can be recognized as a distinct cell type prior to their aggregation into cords (Magre and Jost, 1980). These cells are the first testicular cell type to appear. It is widely accepted that Sertoli cells differentiate from a pool of bipotential cells (the supporting cell lineage) which in females forms the follicle cells of the ovary. The derivation of Sertoli cells and follicle cells from a common lineage is supported, in part, by the fact that they share certain biochemical properties, including production of the Mullerian inhibitor - AMH or MIS (Vigier et al. 1984; Donahoe et al. 1987). They also both share a lineage-specific cell surface antigen, as detected by cytotoxic T cell assay (Ciccarese and Ohno, 1978). Follicle cells have the ability to 'transdifferentiate' into Sertoli cells under special circumstances, as in the ovaries of ageing or hypophysectomized female rats (Crumeyrolle-Arias et al. 1986) and in tissue derived from fetal ovaries grafted under the kidney capsule of adult mice (Taketo-Hosotani et al. 1985; Taketo-Hosotani and Sinclair-Thompson, 1987). Examination of ovotestis sections from adult XO/XY mosaic hermaphrodites (see fig 1) and of T16/XSxr hermaphrodites (Ward et al. 1988), has shown testis tubules which connect smoothly with follicles (which may contain oocytes) all bounded by a continuous basement membrane.

Burgoyne et al. (1988a) proposed that the primary function of the testis-determining gene (Tdy) is the

cell-autonomous diversion of the supporting cell lineage to form Sertoli cells and that the remainder of testis development is directed by these Sertoli cells. In the absence of a Y (and thus Tdy), the supporting cell lineage differentiates into follicle cells. This "cell-autonomous action of Tdy" model of sex determination, in its simplest form, predicts that follicle cells should be exclusively XX in XX<->XY female chimeras. This is because all supporting cells containing a Y chromosome are expected to be diverted into the Sertoli cell pathway. XY follicle cells have, however, been shown to exist in three XX<->XY female chimeras (Ford et al. 1974; Burgoyne et al. 1988b). In the two female chimeras of Ford et al., the proportion of XY follicle cells was found to be 97% in one and 33% in the other. Similar proportions of XY cells were scored in other somatic cell lineages. If sex determination was operating normally in these chimeras, it is hard to imagine how they failed to develop as males. Accumulated evidence shows that a contribution of around 25% XY cells to a chimera or mosaic is usually sufficient to induce testicular development. It was suggested by Burgoyne et al. (1988b) that these XY follicle cells could be formed by a 'timing mismatch' mechanism in which Tdy was pre-empted by the ovarian determination process initiated by the XX component of the chimera. In chimeras, there is clearly the potential for a developmental mismatch between the two components and, in Ford et al.'s XX<->XY females, the XY component was AKR, which other evidence suggests has a late-acting Y (Eicher and Washburn, 1986; see chapter 4). The mismatch hypothesis is discussed in chapter 4.

The aim of this study was to test whether XY follicle cells can be formed in a situation where 'mismatch' can be ruled out: that is, in the ovaries of XO/XY and XO/XY/XYY mosaic hermaphrodites that occur in crosses involving BALB/cWt males (Eicher et al. 1980). These mosaics arise through mitotic non-disjunction of the

Y, so there are no strain differences between the components. Furthermore, although XO fetuses with a paternal X are developmentally retarded (Burgoyne et al. 1983), XO fetuses with a maternal X develop as fast as their XY sibs (Thornhill and Burgoyne, unpublished data); so, on these additional grounds, we expect the XO and XY cell lines of these mosaics to be developmentally balanced.

2. MATERIALS AND METHODS

CXBH/By females were mated to BALB/cWt males to produce XO/XY and XO/XY/XYY mosaics. The mice were killed 8-11 days after birth and examined internally for signs of hermaphroditism. A sample of bone marrow was removed from the hermaphrodites for cytogenetic analysis (see appendix 1.i) and the gonads were fixed and prepared for in situ histology (see appendix 2.i).

In situ hybridization was carried out using the probe pY353B (Bishop et al. 1985), which hybridizes specifically to a series of repetitive sequences on the Y chromosome.

Estimates of Y-bearing and non-Y-bearing cells in the gonads were achieved by scoring all the cells in randomly selected tubule and follicle cross-sections or, in the case of interstitial regions, using a gridded eyepiece graticule. These estimates of relative cell numbers are subject to bias due to the amount of false negative cells that occur in tissue sections. This happens when the microtome blade passes through nuclei leaving one part with, and the other part without, the target DNA sequence. To rectify this bias, correction factors were produced by scoring control sections from ovaries in which all nuclei were Y-bearing. These ovaries were from a 2 week-old XXY^m

female mouse carrying the mutant 129 Y of Lovell-Badge and
Robertson (1990), which has lost Tdy (Gubbay et al. 1990b).

3. RESULTS

60 litters were produced from the CXBH/By x
BALB/cWt cross comprising a total of 424 mice. Of these,
300 were female, 118 were male and 6 were hermaphrodites.
In addition to these six, two other hermaphrodites were
found at weaning in the BALB/cWt stock. Because of
technical problems with the in situ hybridization,
quantitative data are presented for only five of the six
CXBH/BALB hermaphrodites.

Sections of ovaries from these five CXBH/BALB
hermaphrodites show a number of follicle cell nuclei with
positive hybridization to the Y chromosome (fig 2B). XY
follicle cells are clearly able to proliferate since they
appear as discrete patches in the multilayered follicles of
the 3-week-old BALB/cWt hermaphrodites (fig 2C). The
overall estimate of the proportion of labelled follicle
cells is almost identical to the estimated proportion of XY
cells in bone marrow, and is also close to that for ovarian
stroma cells (table 1). The "interstitial" cells in the
contralateral ovotestis or testis also have a similar
proportion of XY cells to the bone marrow but the Sertoli
cells show a very strong bias towards the XY component
(table 1 and fig 2D).

4. DISCUSSION

These results demonstrate that XY follicle cells
can form in the ovaries of XO/XY and XO/XY/XYY mosaic

hermaphrodites. Since there are no grounds for expecting the XY component to be delayed relative to the XO component, we conclude that XY follicle cells can form even without a 'mismatch' between Tdy action and ovary determination. However, 'mismatch' remains the most reasonable explanation for the Ford et al. (1974) XX<->XY female chimeras, particularly the one in which XY cells predominated and 97% of the presumed follicle cells were XY. Similar conclusions have been reached by Patek et al. (1991) based on an in situ analysis of a series of XX<->XY chimeric ovaries.

How do we account for the formation of XY follicle cells in these mosaics? In chapter 2 it is shown that the "cell-autonomous action of Tdy" model is incorrect: XX Sertoli cells do occur in fetal, prepuberal and adult XX<->XY testes, albeit at a low frequency. The strong bias in favour of XY Sertoli cells, even in fetal XX<->XY testes (and also in the ovotestes and testes analysed here) confirms the original conclusion of Burgoyne et al. (1988a) that Tdy acts in the lineage that forms Sertoli cells, but the fact that some XX Sertoli cells are formed means that, at some point between Tdy expression and the formation of fetal Sertoli cell cords, there is a step that is able to recruit a few XX cells. If some XX cells can be recruited by XY cells to form Sertoli cells, could some XY cells fail to be recruited when their numbers fall below a certain threshold? If the source of the 'recruiting factor' is the XY supporting cells, it seems implausible that they fail to recruit themselves. An alternative possibility is that the XY supporting cells are triggered to form fetal Sertoli cells by the action of Tdy but subsequently 'transdifferentiate' into follicle cells under the influence of ovarian factors. It may be a characteristic of Sertoli cells and follicle cells that they retain the ability to transdifferentiate into the reciprocal cell type. Burgoyne (1988, 1991) has argued that

the XX Sertoli cell cords that form in ovarian tissue in various situations (in all cases preceded by oocyte loss) are the result of transdifferentiation of follicle cells. Transdifferentiation of Sertoli cells to follicle cells through contact with oocytes has recently been suggested as an explanation for the occurrence of oocytes surrounded by 'granulosa-like' cells in some T16/XSxr testes (McLaren, 1991).

Fig.1. Sections of an ovotestis from an XO/XY mosaic
hermaphrodite. (A) to (H), series of sections,
approximately 25µm apart, showing the spatial
transformation from a follicle containing an oocyte with a
small patch of Sertoli cells (A), to a testis tubule
connected to a larger tubule containing dividing germ cells
(H). (I) High power of the cells in tubule showed in (H)
some of which have the 'triple-spot' nuclear morphology
typical of Sertoli cells (arrows). Bar, 20µm.
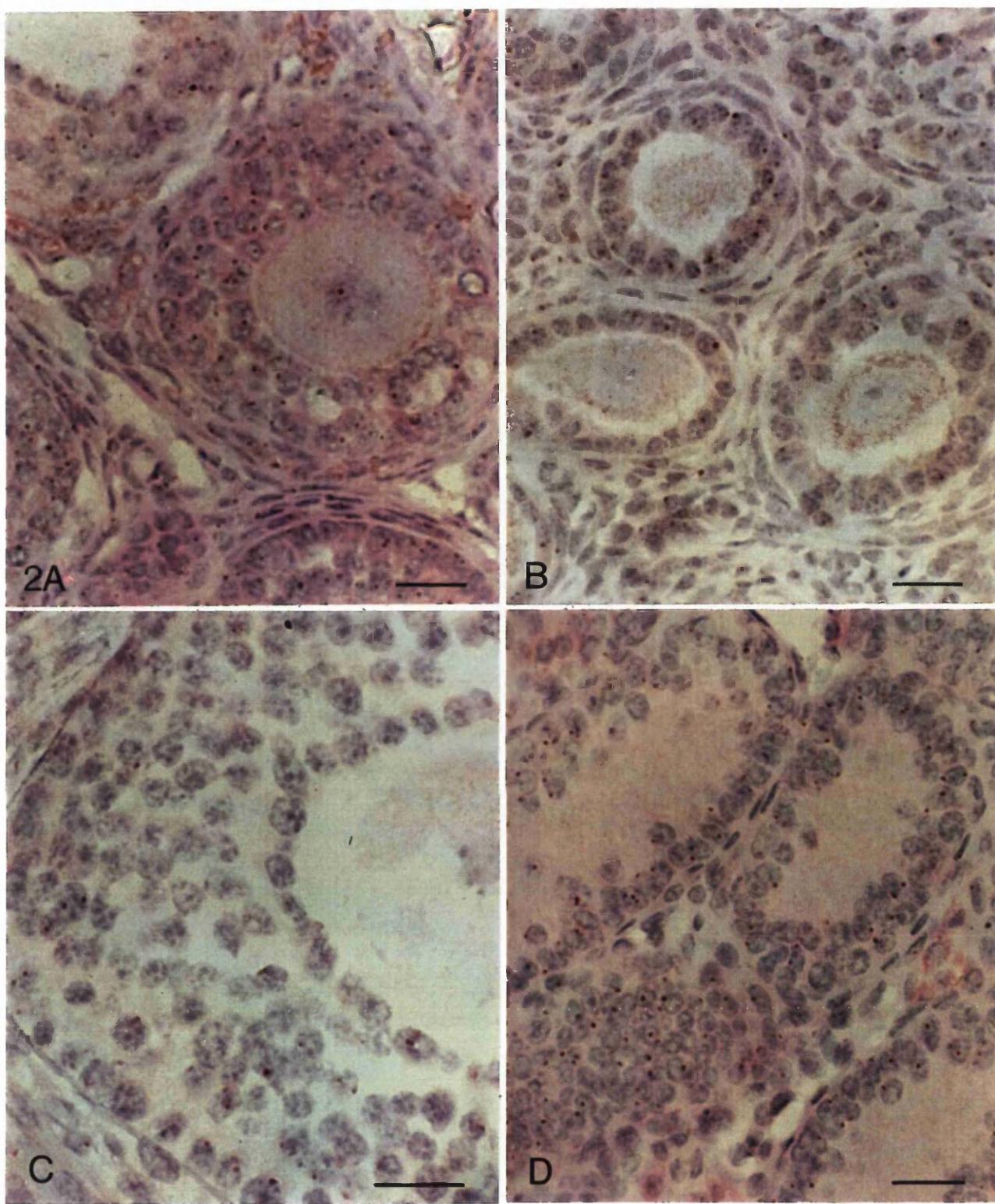
Fig. 2. (A) Section of ovary from a 2-week-old XXY^m female. Counts of labelled and unlabelled cells from these sections produced the correction factors for the mosaic ovaries. (B) Section of ovary from mosaic 5 showing three single-layered follicles, which include labelled XY follicle cells. (C) Multilayered follicle of a 3-week-old BALB/cWt mosaic ovary showing a patch of labelled XY follicle cells. (D) Testis section from mosaic 5,the contralateral gonad to the ovary shown in B. This testis had virtually no germ cells due to the high XO contribution but the Sertoli cells are nearly all XY. The patch of labelled cells that appear to be outside a tubule (bottom left) are Sertoli cells from a tangentially cut tubule. Bar, 20μm.

Table 1. The proportion of Y-bearing cells in bone marrow and gonadal cell lineages of XO/XY and XO/XY/XYY mosaics.

Mosaic	Age (dpp)	Gonads[a] L	Gonads[a] R	Bone marrow	Ovary Stroma	Ovary Granulosa	Testis or Ovotestis Interstitial	Testis or Ovotestis Sertoli
				Percentage of Y-bearing cells (number of cells scored)				
1	8	OT	O	20(30)	17(558)	22(558)	24(517)	99(587)
2	11	O	OT	20(30)	11(552)	19(633)	25(551)	90(535)
3	8	O	T	11(28)	15(554)	15(549)	--b	--b
4	11	O	OT	20(30)	22(510)	17(640)	--b	--b
5	8	T	O	29(28)c	21(560)	25(654)	20(502)	84(735)
Mean+/-SE				20.0+/-2.9	17.2+/-2.0	19.6+/-1.78	23.0+/-1.53	91.0+/-4.4

(a) O,ovary; T,testis; OT,ovotestis.

(b) Not recorded due to technical failure.

(c) 11% of the Y-bearing cells were XYY.

CHAPTER 4

A COMPARISON OF <u>MUS MUSCULUS MUSCULUS</u> AND
<u>MUS MUSCULUS DOMESTICUS</u> Y CHROMOSOMES WITH RESPECT TO THEIR
EFFECT ON THE TIME OF ONSET OF TESTICULAR DIFFERENTIATION

1. INTRODUCTION

In 1982, Eicher et al. described a case of XY sex
reversal in mice, resulting from backcrossing the Mus
musculus domesticus-derived Poschiavinus Y chromosome
(Y^{POS}) onto a C57BL6/J background. When fully backcrossed,
adult mice with an XY karyotype display a range of sexual
phenotypes (Nagamine et al. 1987a) including fully
sex-reversed XY females, true hermaphrodites (i.e. they
have both ovarian and testicular tissue) and males. The
males that are produced always have small testes and are of
poor fertility. Observations on fetal litters from this
cross have revealed that the XY individuals at 16.5 days pc
have either ovaries, or ovotestes, with a varying ratio of
testicular to ovarian tissue. It seems clear that most
ovotestes go on to form testes in adult life, the
regression of the ovarian component accounting for the
reduced testicular size.

Further studies have shown that a number of other
(but not all) M.m.domesticus-derived Y chromosomes generate
some XY females when placed on the C57BL/6 background
(Eicher et al. 1982; Nagamine et al. 1987a; Biddle &
Nishioka, 1988). Eicher & Washburn (1986) have suggested
that the M.m domesticus Y carries a Tdy allele which is
later-acting than that on the M.m.musculus Y of the C57BL/6
strain and that this delay, in conjunction with an element
of the C57BL/6 background, causes a breakdown in the normal
sequence of events that lead to testis formation.

An indication of the element in the C57BL/6
background that causes this incompatability is apparent
from the pedigree of the Y^{POS} backcross. When males of
several recombinant inbred lines produced from a NMRI x
M.m.poschiavinus cross were mated to C57BL6/J females, all
the F_1 XY progeny developed as normal males. However, when
the F_1 males were backcrossed to C57BL6/J females, half of
the XY progeny showed signs of partial or complete sex

reversal and the other half were normal males with normal testes (Eicher and Washburn, 1983). When the males were backcrossed further, the proportion of sex reversed individuals increased until all XY offspring were affected. This inheritance pattern is indicative of an autosomal recessive allele ($\underline{Tda-1}^b$) present in the C57BL6/J background (Eicher and Washburn, 1983). Presence of the dominant M.m.domesticus allele ($\underline{Tda-1}^{do}$) allows normal testicular development, but homozygosity for $\underline{Tda-1}^b$ causes sex reversal.

There are two possible models for how Tda-1 is involved in the process of sex determination (Eicher & Washburn, 1983). Firstly this autosomal gene may be one element in the Tdy-initiated cascade of genes involved in testicular differentiation. A second interpretation supported by Burgoyne (1988), proposes that it is an ovary-determining gene, with the C57BL/6 allele acting earlier than those of other inbred strains. When this is brought together with the late-acting M.m.domesticus Tdy allele, a 'timing mismatch' may occur such that ovary determination may pre-empt testis determination. It is not hard to imagine how minor differences in the timing of expression of these two key genes could lead to the range of gonadal phenotypes observed in these mice.

A similar sort of incompletely penetrant XY sex reversal occurs in C57BL/6 mice heterozygous for the hairpin tail mutation $\underline{T}^{hp}$, and carrying the AKR Y chromosome, which is, like the Poschiavinus Y, M.m.domesticus-derived (Washburn and Eicher, 1983). Heterozygosity for an overlapping deletion at the $\underline{T}$ locus - $\underline{T}^{Orl}$ also causes XY sex reversal when combined with the AKR Y on a C57BL/6 background, but in this case, all the XY fetuses develop ovarian tissue only (Washburn and Eicher, 1989). The similarities between these two forms of sex reversal and that found in the C57BL/6-Y^{POS} mice, indicate that they may be caused by the same mechanism.

The aim of the present study was to test directly the hypothesis that the Poschiavinus Y chromosome and the AKR Y chromosome are later acting than the C57BL/6 Y. Since C57BL/6 XY^{POS} fetuses develop ovaries or ovotestes rather than testes, the effect of the Poschiavinus Y on the timing of testicular development was assessed in F_1 hybrids in which the paternal parent was C57BL/6 or C57BL/6-Y^{POS}. In these F_1 hybrids all the XY fetuses develop testes. Although C57BL/6 XY^{AKR} adults show no signs of sex reversal, there is some evidence for testicular "abnormalities" at the fetal stage (Nagamine et al. 1987b). For this reason, and also so that data from fetuses carrying the AKR Y could be compared with data from Poschiavinus Y-bearing fetuses on as similar genetic backgrounds as possible, the effect of the AKR Y on the timing of testicular development was also assessed in F_1 hybrids.

2. MATERIALS AND METHODS

2.i. Experiment 1

C57BL/6Mcl-Y^{POS} stud males were produced by backcrossing C57BL/6J-Y^{POS} males to C57BL/6Mcl females for more than 10 generations. Inbred C3H/Bi females were mated to stud C57BL/6Mcl-Y^{C57} and to C57BL/6Mcl-Y^{POS} males in a room in which the dark period was 7pm to 5am. Mating was assumed to have taken place at midnight. Pregnant mothers were killed at 12 days 15 hours pc and the fetuses placed into Hepes-buffered Eagle's minimum essential medium (EMEM, ICN Flow Ltd). Amniotic membranes were removed for sex chromatin analysis (see appendix 1.3). Fetuses were killed by decapitation, the hind limbs staged (see below) and the gonads removed. The gonads were photographed under EMEM

using a Wild M400 Photomakroskop. Photographic prints of male and female gonads from both crosses were randomized and scored blind for the presence of testicular cords.

The hind limbs were staged according to the developmental series of McLaren and Buehr (1990) which covers the period spanning sexual differentiation in the mouse. This scheme was subdivided to provide maximum sensitivity and extended to cover the later stages found at this time point and the stages of the subsequent two days studied in experiment 2 (see fig 1).

2.ii. Experiment 2

Consomic C57BL/6Mcl-Y^{C57} and C57BL/6Mcl-Y^{POS} stud males were mated to females of the inbred strain CXBH/By and to females of the random-bred albino stock MF1/Ola. Once again the dark period was 7pm to 5am and mating was assumed to have taken place at 12.00 midnight. In order to study the gonadal growth rates in the fetuses produced by these crosses, pregnant females were killed at time points between 11.5 and 14.5 days pc. Amniotic membranes were fixed for sexing and the hind limbs were scored as before.

Gonads were dissected out and scored under the dissecting microscope for the presence of testicular cords. The length and breadth of each gonad was measured to provide continuous variables to study the relative growth rates. However, it was found that the length measurements were highly variable at the earlier stages due to difficulties in delineating the ends of the gonads. The breadth was better defined and showed a relatively smooth exponential growth curve. This was therefore chosen as the representative parameter for gonadal growth. Breadth was measured by gently holding the mesonephros with forceps so that the gonad was uppermost. The width across the widest part was measured using a graticule fitted in the eyepiece of the dissecting microscope.

2.iii. Experiment 3

Consomic C57BL/6J-Y^{C57} and C57BL/6J-Y^{AKR} stud males were mated to females of the inbred strain CXBH/By, in a room in which the dark period was 7pm to 5am. Pregnant mothers were killed at 12 days 15 hours pc. Amniotic membranes were removed from the fetuses for sex chromatin analysis and the hind limbs were staged. The gonads were removed and photographed as in experiment 1. Photographic prints of male and female gonads from both crosses were randomized and scored blind for the presence of testicular cords.

3. RESULTS

3.i. Experiment 1
Poschiavinus Y versus C57BL/6 Y: transverse study

9 litters of fetuses produced from the cross C3H x C57BL/6-Y^{C57} were found to contain 19 individuals typed as sex-chromatin negative. In the C3H x C57BL/6-Y^{POS} cross, a total of 8 litters yielded 15 sex-chromatin-negative individuals. These sex-chromatin-negative fetuses are assumed to be XY although in rare instances they could be XO. All but 2 of the XY^{C57} males were scored as having visible testis cords whereas none of the XY^{POS} fetuses showed any sign of testicular differentiation at this time point. The probability of this occurring by chance (χ^2 = 26.8, 1 degree of freedom) is P= 0.00014.

When these data are plotted against hind limb stage (see fig 2), individuals that are at the same stage of limb development show a clear difference in testicular development dependent upon the source of the Y chromosome. A comparison of the distributions of the hind limb stages

demonstrates that, as far as the sensitivity of this measure will allow, there is no significant difference between the two male populations with respect to overall fetal development. (χ^2 = 3.01 4df. P= 0.56)

3.ii. Experiment 2
Poschiavinus Y versus C57BL/6 Y: longitudinal study

The delay in the onset of testicular differentiation is also found in the XY fetuses produced from crosses of the consomic C57BL/6-Y^{C57} and C57BL/6-Y^{POS} males with CXBH/By or MF1/Ola females (see fig 3). These data also show that all the XY^{POS} fetuses in both crosses develop testes from late 12.5 days pc onwards and no signs of XY sex reversal were found in a total of 47 sex-chromatin-negative individuals between 13.5 and 14.5 days pc.

The gonadal growth rate data are shown in figure 4. The mean gonad breadth measurements are log-transformed to improve the linearity of the resulting regression lines, decreasing the residual variance. Regression lines and their associated errors were computed using litter means of gonadal breadth for each sex, weighted according to the number of females or males in the litter. For the males, only the data between 12.5 - 14.5 days pc are included to be as certain as possible that the points fall during the phase of increased growth that is characteristic of testes. Some outliers have been removed using a test for outliers in regression analysis. Regression lines of gonadal growth in XY^{C57} and XY^{POS} fetuses were compared for both the CXBH and the MF1 outcross using an analysis of covariance. This test compares separately the variance from the mean due to the elevation, the slope and the residual error.

The regression lines for the XX fetuses produced by the two consomic fathers were also compared for each outcross. These data (see figure 4 legend) demonstrate that, for both the MF1 cross and the CXBH cross, the

females produced by the two consomic males show no
significant difference in the residual variance, the
variance due to the slope or the variance due to the
elevations. This is to be expected since there is no
genetic difference between the F_1 females produced by each
paired cross. It is therefore justified to pool these two
populations within each outcross.

A comparison of the regression lines for the males
(XY^{C57} versus XY^{POS}) on the other hand, although revealing
no significant difference in residual variance or slope,
shows a highly significant difference between elevations in
both crosses (see fig 4 legend). This supports the
conclusions of experiment 1 and shows that the late onset
of testis cord formation in XY^{POS} fetuses is correlated
with a delayed onset of increased growth.

3.iii. Experiment 3
AKR Y versus C57BL/6 Y: transverse study

The cross CXBH x C57BL/6J-Y^{C57} produced 5 litters of
fetuses containing 29 individuals typed as sex-chromatin
negative. In the CXBH x C57BL/6J-Y^{AKR} cross, a total of 6
litters yielded 20 sex-chromatin negative individuals. 25
of the 29 XY^{C57} fetuses were scored as having visible
testis cords but only 7 of the 20 XY^{AKR} fetuses had visible
testis cords. The probability of this occuring by chance
(χ^2=11.5, 1 degree of freedom) is P=0.00084.

A comparison of the distributions of hind limb
stages found in the two male groups reveals that, according
to this measure, there is no significant difference in
overall fetal development (χ^2=0.47, 3df. P=0.92). These
data demonstrate that presence of the AKR Y chromosome
causes a delay in testicular differentiation relative to
fetuses carrying the C57BL/6 Y (see fig 5).

4. DISCUSSION

The experiments demonstrate that the Poschiavinus Y chromosome and the AKR Y chromosome cause a later onset of testicular development than the C57BL/6 Y chromosome. The simplest explanation for this disparity is a difference between the Tdy alleles. This may be caused by differences in the regulatory elements or the structural sequence of Tdy.

These findings provide an explanation for the results of Nagamine et al. (1987b) who found that when C57BL/6 males with a M.m.domesticus-derived Y chromosome are outcrossed to various inbred females, a proportion of the F_1 male fetuses produced show "abnormalities" of testicular development. In the light of the results presented here it seems likely that these "abnormalities" are due to a delay in testicular cord formation caused by a late acting Tdy allele.

From the growth rate data of experiment 2, it is possible to model the growth kinetics of male and female gonads and thereby estimate the timing difference between the action of the Y^{POS} and Y^{C57} chromosomes (see fig 6). If Tdy is the first gene to influence male and female gonads differentially, it is logical that prior to Tdy action the growth rates of the indifferent XX and XY gonads should be the same. After Tdy expression however, the gonadal growth of XY fetuses increases dramatically but the gonads of XX fetuses continue to grow at a slower rate. The gonads of XY fetuses with delayed Y action would be expected to continue growing at the same rate as female gonads until expression of the delayed Tdy has occurred. Therefore, to estimate the timing difference it is necessary to find where the XY^{C57} and XY^{POS} testicular growth curves depart from the XX curve. Calculations based on this model show that the points of intersection for the CXBH outcross are: XY^{C57} fetuses, 269 hours pc; XY^{POS} fetuses, 283 hours pc; and for

the MF1 outcross XY^{C57}, 270 hours pc; XY^{POS}, 284 hours pc.
The estimate for the timing difference between the action
of these two Y chromosomes is therefore 14 hours. The
estimated points of intersection should be regarded with
caution since they result from extrapolations which may
misrepresent the true shape of the curves. In spite of this
caution, the timing for the C57BL/6 Y (early on the 12th
day) fits well with the onset of Sry expression, which
begins during the 11th day, peaks on the 12th and declines
during the 13th day (Gubbay et al. 1990b; Koopman et al.
1990).

The AKR Y chromosome is clearly delayed relative to
the C57BL/6 Y in terms of the onset of testicular
differentiation, but a condition which must be met if the
mismatch model is correct is that it is not delayed by as
much as the Poschiavinus Y. This is because the AKR Y,
unlike the Poschiavinus Y, does not cause XY sex reversal
on a C57BL/6 background without additional factors, such as
heterozygosity for mutations at the T locus. Comparing the
data presented in figure 3A with that in figure 5, which
are derived from fetuses of similar genetic background, it
is not clear whether the AKR Y chromosome causes less of a
delay than the Poschiavinus Y. A larger data set would be
required to settle this question.

XY fetuses have been shown to have an overall
developmental advantage over their XX siblings (Seller &
Perkins-Cole, 1987) males being about 1½ hours ahead of
females (Burgoyne et al. unpublished). This developmental
advantage is now known to be a consequence of a retarding
effect of the paternally-imprinted X chromosome in XX
fetuses, rather than an accelerating effect of the Y
chromosome (Thornhill & Burgoyne, unpublished). Because it
is not Y-linked, this developmental advantage of XY fetuses
should not affect the estimate for the difference in timing
of Y^{POS} and Y^{C57} action. However, it does complicate the
interpretation of XX v XY gonadal size differences.

Measurements of gonadal volume in rat fetuses have shown that XY gonads are larger than XX gonads prior to the formation of testis cords (Lindh, 1961; Mittwoch et al. 1969). This led Mittwoch (1969, 1989) to propose that an early growth advantage of XY gonads, rather than a single gene 'switch', forms the basis of the sex-determining mechanism in mammals. However, there is no previous evidence to show that these 'within litter' differences between XX and XY gonadal volume are not simply a manifestation of the fact that males are ahead of females. Data presented here, on the other hand, indicate that the increased growth, characteristic of testes, precedes testis cord formation. Nevertheless, this increased growth is after the onset of Sry expression so it remains a moot point whether the increased gonadal size is simply a consequence of Sry expression, perhaps caused by the differentiation and growth of Sertoli cell precursors (Jost et al. 1973; Magre and Jost, 1980), or whether it is a requirement for determining the gonad as a testis.

The results presented here appear to be contradicted by a recent study on the onset of Mullerian inhibitor - AMH (or MIS) production in fetal testes (Taketo et al. 1991). The authors compared the onset of AMH production in SJL XY^{dom} fetuses with control SJL XY^{C57} fetuses and found no difference. It is possible that the assay system used and time intervals of 24 hours, were not sensitive enough to detect a timing difference of only 14 hours.

The delay in action of the Poschiavinus Y chromosome supports, in part, the hypothesis that sex-reversal in C57BL/6-Y^{POS} individuals is caused by a developmental mismatch involving a late-acting Tdy allele in conjunction with an early acting ovarian program. The delay in action of the AKR Y chromosome allows the use of the same hypothesis to explain the sex reversal displayed in XY^{AKR} mice heterozygous for mutations at the T locus.
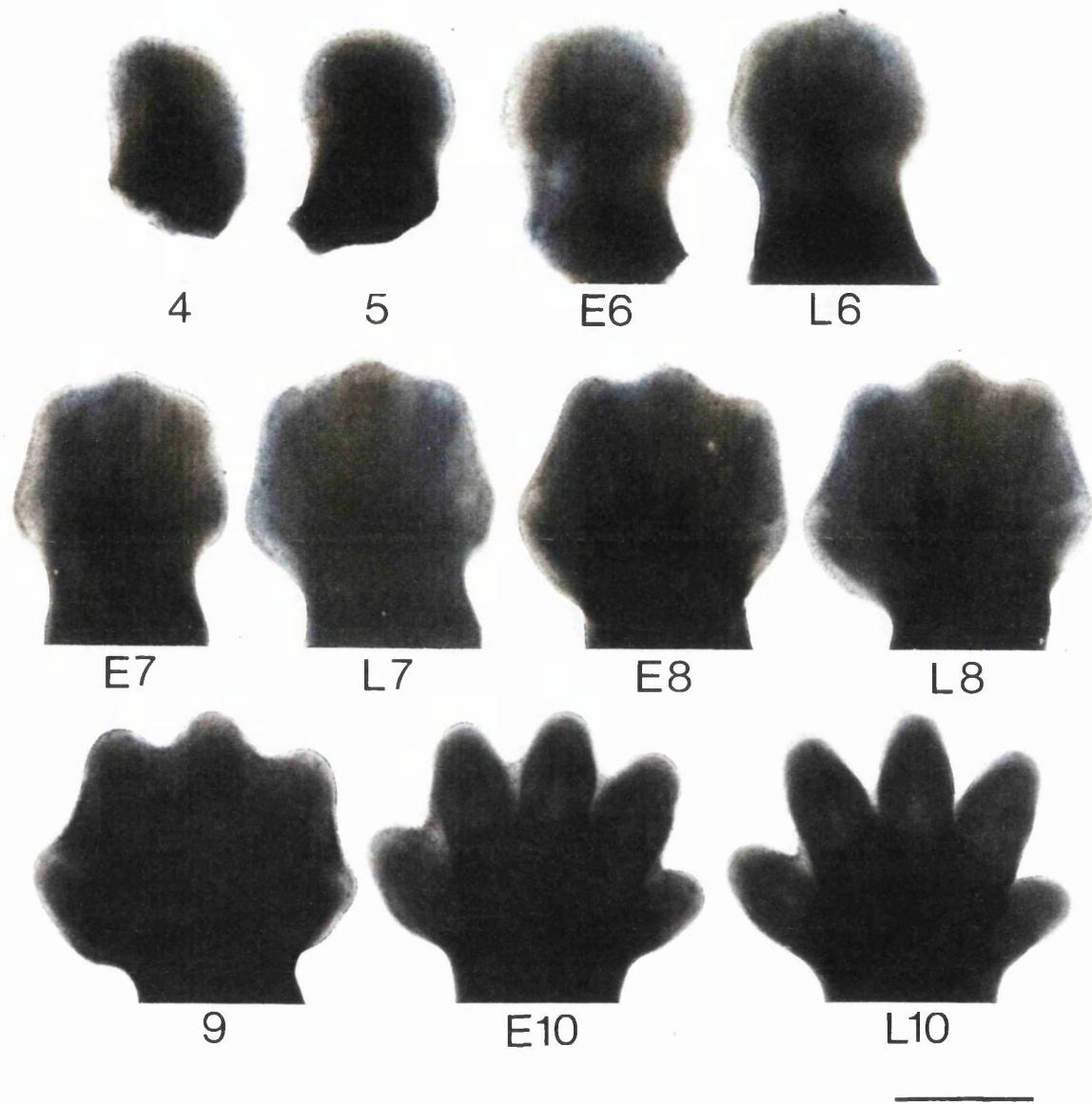
Fig.1. Hind limb stages of fetuses between 11.5 and 14.5 days post coitum. E, early; L, late. In general, stages 4 and 5 were found during the 12th day of pregnancy, 5 to L7 during the 13th, L7 to 9 during the 14th and 9 to L10 during the 15th. Bar, 1mm.
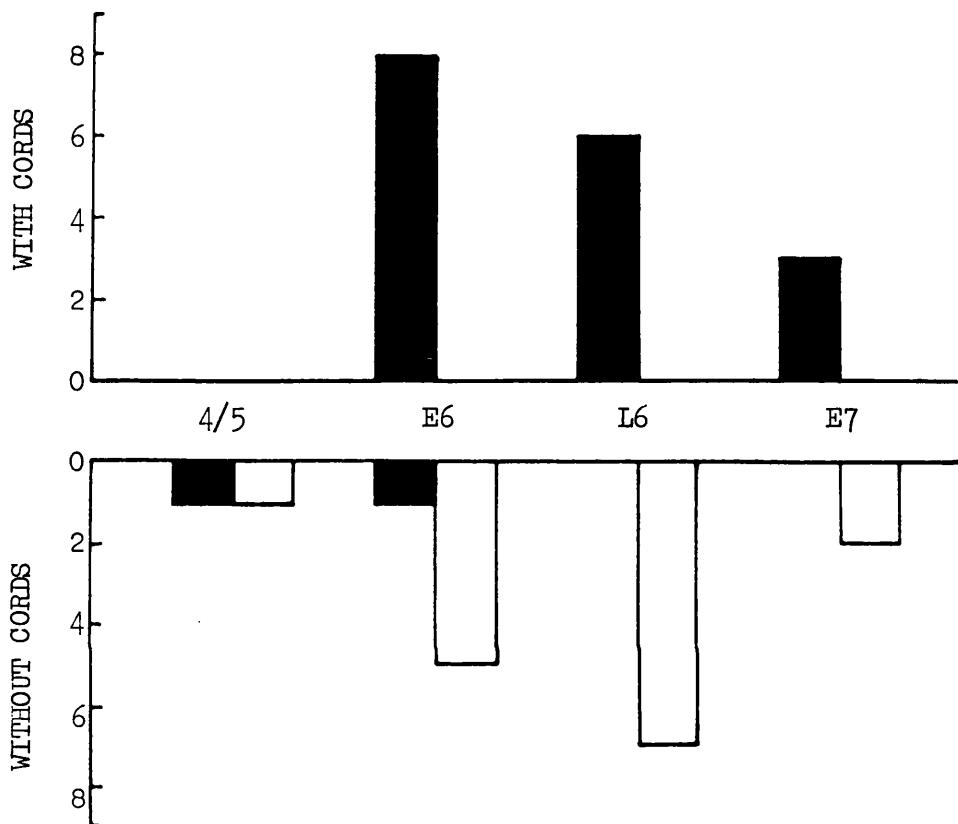
Fig.2. Frequency of sex-chromatin-negative individuals with and without visible testis cords at 12 days 15 hours post coitum, plotted against hind limb stage. Solid bars, C3H x C57BL/6-Y^{C57} F$_1$; open bars, C3H x C57BL/6-Y^{POS} F$_1$.
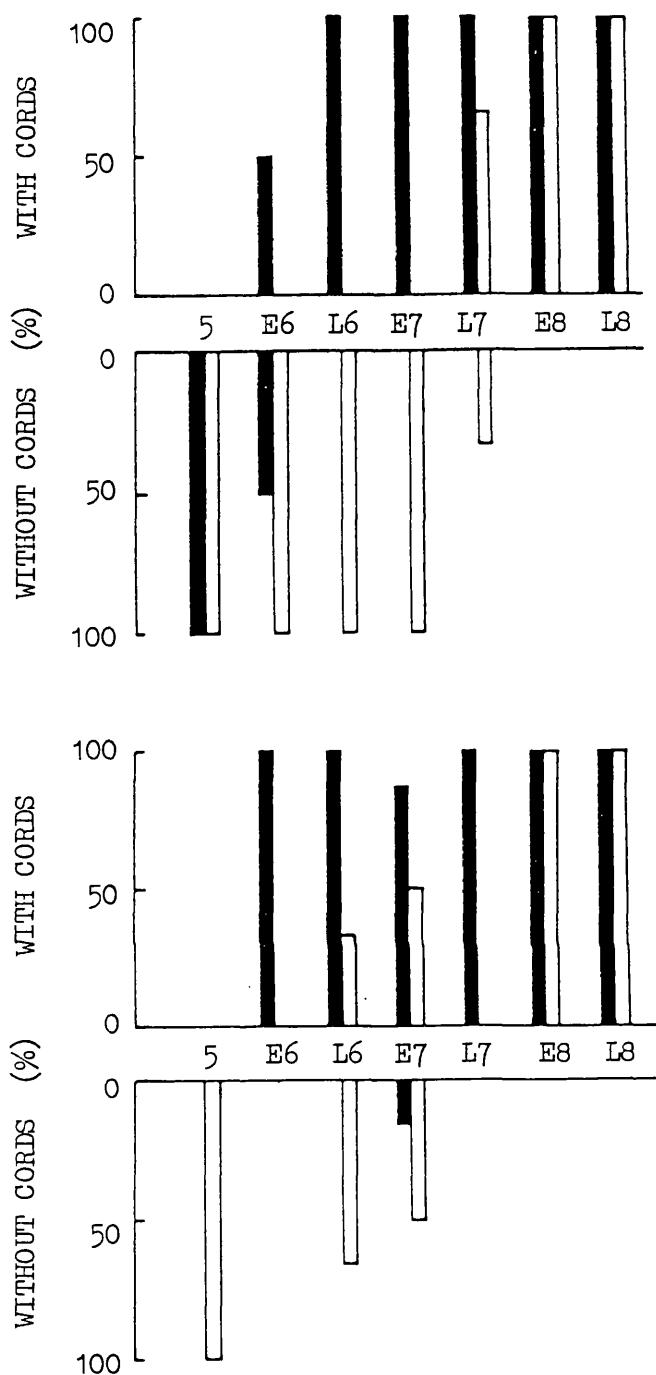
Fig.3. Percentage of sex-chromatin-negative individuals
with and without visible testicular differentiation at a
range of hind limb stages. Solid bars, XY^{C57} fetuses; open
bars, XY^{POS} fetuses. (A) F_1 males produced from CXBH
outcross. (B) F_1 males from MF1 outcross. Missing bars
indicate that no sex-chromatin-negative individuals were
found with that hind limb stage. The dip in XY^{C57} data at
E7 in the MF1 cross is caused by a single individual. It is
possible that this fetus was mistyped or may have been a
rare XO.

Fig.4. Regression lines of gonadal growth in males and females between 11.5 and 14.5 days post coitum. Closed circles, litters fathered by C57BL/6-YC57 males; open circles, litters fathered by C57BL/6-YPOS males. Each point is the litter mean (for that sex) of the individual gonadal breadth means. Regression data are compared in each case with respect to the Y-chromosome-type of the father using an analysis of covariance. The residual variances are not significantly different in any of the four tests.
(A) Females produced from CXBH outcross. Difference between slopes, P=0.59; between elevations, P=0.86. Data are pooled to produce one regression line.
(B) Males produced from CXBH outcross. Difference between slopes, P=0.67; between elevations, P=0.00009. The combined slope is used in plotting the two regression lines.
(C) Females produced from MF1 outcross. Difference between slopes, P=0.321; between elevations, P=0.286. Data are pooled to produce one regression line.
(D) Males produced from MF1 outcross. Difference between slopes, P=0.203; between elevations, P=0.00126. The combined slope is used in plotting the two regression lines.

Fig.5. Frequency of sex-chromatin-negative individuals with and without visible testis cords at 12 days 15 hours post coitum, plotted against hind limb stage. Solid bars, CXBH x C57BL/6-Y^{C57} F$_1$; open bars, CXBH x C57BL/6-Y^{AKR} F$_1$.
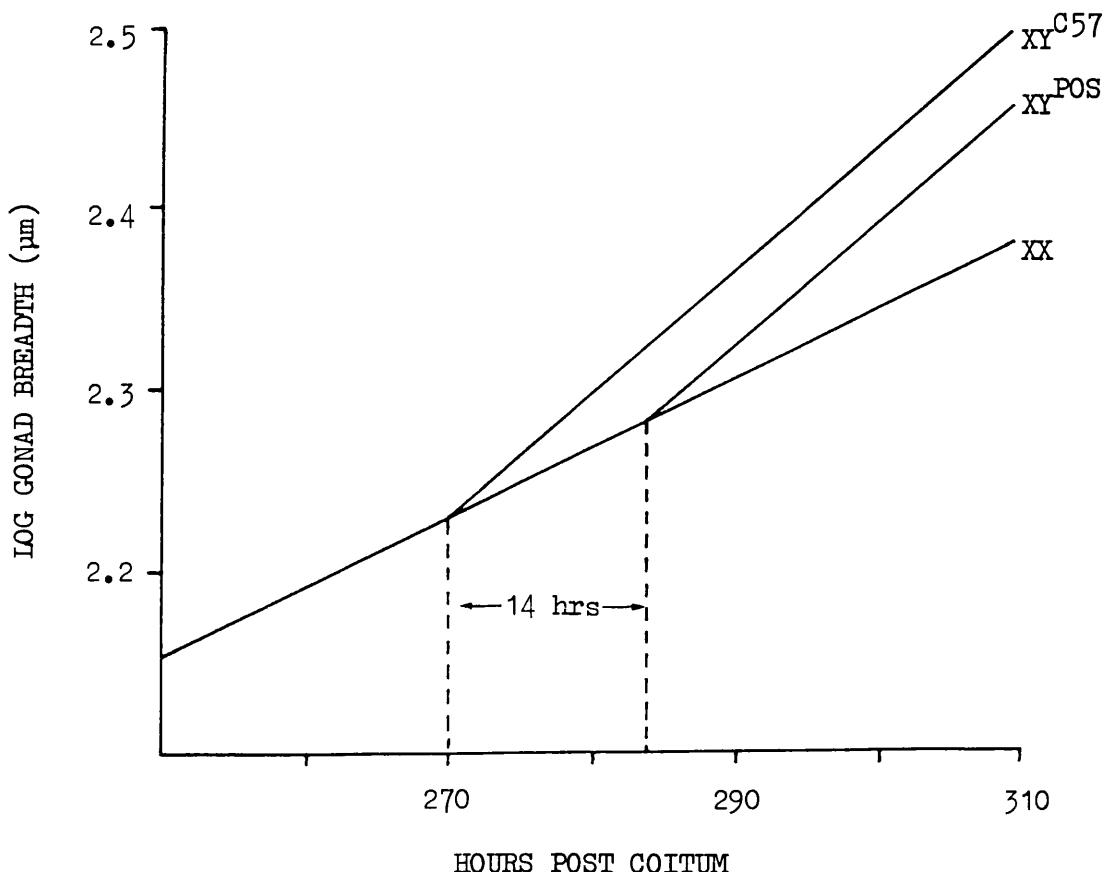
Fig.6. Model of gonadal growth in females and in males carrying either the Y^{C57} or the Y^{POS}. The initial single line represents the growth rate of the indifferent XX or XY gonads. After expression of either of the two Tdy alleles the testicular growth rate increases and departs from that of the female gonad. The testicular growth rate is the same in Y^{C57}-bearing and Y^{POS}-bearing fetuses, only the time of departure differs. The points of intersection are calculated from the regression lines shown in Fig.4. For two lines with slopes m_1 and m_2 and elevations c_1 and c_2, at the point of intersection
$x=(c_2-c_1) - (m_1-m_2)$.

CHAPTER 5

DISCUSSION

It is now established that the recently cloned gene
- Sry is Tdy (Koopman et al. 1991) and that this is the
only Y-linked gene necessary for testis determination
(Gubbay et al. 1992). In mouse fetuses, Sry expression is
restricted to the genital ridge, in a time window from 10.5
to 12.5 days pc and is not dependent upon the presence of
germ cells (Koopman et al. 1990). The sequence of the gene
suggests that it encodes a DNA-binding protein (Sinclair et
al. 1989; Gubbay et al. 1990b). It may be assumed,
therefore, that Sry initiates the expression of a suite of
testis-forming genes within the cells, or a subset of
cells, in the somatic component of the genital ridge.
Continued expression of Sry does not seem to be required to
maintain the fetal testis, as expression ceases after 12.5
days pc. The results presented in this thesis provide
further insight into the cellular events that follow Sry
expression and their importance to the process of testis
determination.

Burgoyne et al. (1988a) proposed that Tdy acts
cell-autonomously within the supporting cell lineage to
bring about Sertoli cell differentiation. This was based on
the finding that Sertoli cells, in prepuberal and adult
XX<->XY chimeras, were predominantly or exclusively XY,
whilst all the other somatic cell lineages showed no bias.
Similar results had previously been reported by Singh et
al. (1987) in a single XX<->XY chimera. Whilst this result
demonstrated that there is a requirement for the Y
chromosome in the Sertoli cell lineage, it did not prove
that this was due to the cell-autonomous expression of Tdy.
In chapter 2, the "cell-autonomous action of Tdy" model was
tested by examining the fate of XX and XY cells within the
gonads of fetal XX<->XY chimeras by in situ hybridization.
It was found that mesonephric tubule cells, peritubular
myoid cells, Leydig cells and germ cells all contained an
unbiased contribution from the XX component. The fetal
Sertoli cells, however, were predominantly, but not

exclusively XY, having on average, a 10% XX contribution. The XX Sertoli cells that were found showed no morphological difference from the XY Sertoli cells but because no markers were available to identify fetal Sertoli cells, the possibility existed that these were simply epithelial cells that had become entrapped during the formation of testis cords. The fate of XX cells in the testes of post-natal XX<->XY chimeras was re-examined and small scattered patches of XX Sertoli cells were found in prepuberal chimeras, and in adult chimeras between 1 and 2% of the Sertoli cells were XX. In sections of adult XX<->XY testes, XX Sertoli cells could be found inside apparently normal tubules and in air-dried preparations, they showed the normal adult nuclear phenotype, characteristic of mature Sertoli cells. On these grounds, it seems likely that the fetal XX Sertoli cells are functional since at least a proportion survived through puberty and into adulthood.

Examination of the fetal testes of XY^m<->XY chimeras, in which the Y^m chromosome has a deletion that removes Sry (Gubbay et al. 1990b), revealed that the XY^m cells experienced the same prejudice as XX cells. Assuming that the Y^m chromosome is not compromised in any way other than the 11 kbp deletion removing Sry (Gubbay et al. 1992), these results demonstrate that the paucity of XX Sertoli cells in XX<->XY chimeras is specifically due to the absence of Tdy. This must be taken as overwhelming support for the conclusion of Burgoyne et al. (1988a) that Tdy acts within the cells that give rise to the Sertoli cells. It is less easy to explain why a small number of XX Sertoli cells form in the fetal testis that subsequently decrease in number, leaving a consistent proportion of 1 to 2% in the adult testis. One possibility is that these XX Sertoli cells are formed in the same way as those found in ovarian tissue that has undergone transformation when grafted into various ectopic sites, or cocultured or cografted with

fetal testes (see chapter 1, 3.ii). This seems unlikely, however, as those experiments show that masculinization of the XX tissue takes several days and always follows destruction of the oocytes. It is more likely that these XX Sertoli cells form contemporaneously with the XY Sertoli cells and as a consequence of Tdy activity. If this is the case, then one may speculate that although Tdy action is cell-autonomous (based on the knowledge that Sry encodes a DNA-binding protein), one of the subsequent steps leading to differentiation of the fetal Sertoli cell is not, and some minor local recruitment of XX cells may occur. In normal XY males where every cell contains a Y chromosome, this would be unnecessary.

It is puzzling to find that the proportion of XX Sertoli cells falls between fetal and adult life. This might indicate that in the pathway to the formation of mature Sertoli cells, expression of a Y-linked gene is required. On the basis of the single adult $XY^m<->XY$ chimera in which the proportion of XY^m Sertoli cells fell within the same range as XX Sertoli cells in the adult $XX<->XY$ chimeras, the gene required would have to be Sry (assuming again that the Y^m chromosome is not compromised in any way other than the 11 kbp deletion that removes Sry). This could correspond with the expression of Sry in post natal testes, although it is known that this is germ cell dependent since Sry transcripts are not found in adult XXSxr testes (Koopman et al. 1990). However, the absence of spermatogenesis could affect the behaviour of the Sertoli cells and expression of Sry by Sertoli cells in the adult testis cannot yet be ruled out. An alternative possibility is that whilst the XX cells found in the cords of the fetal testis may be competent to perform the role of Sertoli cells at this stage, their phenotype is in some way flawed or incomplete such that when challenged with a function that is subsequently required they are unable to respond and are selectively removed. In either scenario, it is hard

to imagine why a small but consistent number of XX Sertoli cells should escape a second round of selection.

The "cell-autonomous action of Tdy" model predicts that all the XY supporting cells in an XX<->XY chimera become Sertoli cells. Chapter 2 demonstrated that a few XX supporting cells may also become Sertoli cells, although there are doubts concerning the functional competance of these XX Sertoli cells. In the experiment described in chapter 3, an attempt was made to address the question of what happens to the XX and XY supporting cell lineages when the gonad develops as an ovary due to an insufficient number of XY cells. A complicating factor to this question is the possibility that ovarian development may occur in XX<->XY chimeras, not because there are insufficient XY cells, but as a result of the type of XY sex reversal seen in C57BL/6 mice carrying M.m.domesticus Y chromosomes where the action of the Y fails to pre-empt the default ovarian pathway. To avoid this possibility, the gonads of XO/XY and XO/XY/XYY hermaphrodites were studied instead. The reasons for this are explained in the introduction of chapter 3.

The result that was predicted on the basis of the hypothesis was that the follicle cells should be exclusively XO. This is because, if the Y-bearing supporting cells all differentiate into Sertoli cells but there are an insufficient number of Sertoli cells to produce a testis, then only the non-Y-bearing supporting cells will remain to form follicle cells. The results presented in chapter 3 show that this prediction is not fulfilled. XY follicle cells were found in the ovarian tissue of the mosaic hermaphrodites in roughly the same proportions as the XY contribution to the other gonadal and non-gonadal somatic lineages, except for the Sertoli cells in the testicular tissue which showed a high XY bias. However, the numbers of XY follicle cells were consistently at or below the estimated 'threshold' amount required to form a testis (see chapter 1, 3.ii). The existence of XY

follicle cells could imply that follicle cells are derived
from a separate lineage to that which produces Sertoli
cells: a hypothesis that is supported by Patek et al.
(1991). However, the weight of evidence suggests that this
is not the case (see chapter 3, introduction). An
alternative explanation is that the XY supporting cells
differentiate as Sertoli cells initially, but subsequently
'transdifferentiate' into follicle cells in response to the
ovary-forming stimulus. This would suggest that the ovary-
forming stimulus is a diffusible or cell-contact mediated
factor.

In 1982, Eicher et al. described a form of XY sex
reversal that occurs in C57BL/6 mice carrying the
M.m.domesticus-derived Poschiavinus Y chromosome. In 1983,
Washburn and Eicher described a second case of XY sex
reversal, caused by the presence of the M.m.domesticus-
derived AKR Y chromosome in C57BL/6 mice heterozygous for
T^{hp}. XY sex reversal will only occur, in both of these
forms, if the Y chromosome involved is M.m.domesticus in
origin and the genetic background is C57BL/6. This suggests
two things: (a) the two forms of sex reversal are probably
caused by the same mechanism and (b) the failure in testis
determination is caused by some sort of breakdown in
interaction between the Y chromosome and an allele or
alleles present in the C57BL/6 genome. There are two
possibile explanations for this breakdown in interaction.
Firstly, it could be caused by a partial failure, at the
biochemical level, for molecules to recognize and bind to
each other. Secondly, the M.m.domesticus-derived Y
chromosome could act at an inappropriate time, such that it
is unable to interact, in the usual way, with other
elements of the sex determining process. Burgoyne (1988)
has argued that the most parsimonious version of this
second model is that the failure is caused by a timing
'mismatch', in which the M.m.domesticus Tdy allele acts too
late to be able to pre-empt the ovary determining program,

initiated by an autosomal allele that acts particularly early in the C57BL/6 strain.

In chapter 4, the <u>M.m.domesticus</u>-derived Poschiavinus and AKR Y chromosomes were shown to cause a later onset of testicular differentiation than the <u>M.m.musculus</u>-derived C57BL/6 Y chromosome, the Poschiavinus Y acting approximately 14 hours later than the C57BL/6 Y. This finding fits the 'mismatch' hypothesis for C57BL/6-Y^{dom} sex reversal but, since the <u>domesticus</u> and <u>musculus</u> substrains have diverged considerably, functional variations are to be expected and it does not prove that this delay is the cause of the sex reversal. What further evidence can be provided to support this hypothesis? Unlike C57BL/6-Y^{POS} mice, C57BL/6-Y^{AKR} mice show no sign of post natal sex reversal without additional factors, such as heterozygosity for mutations at the <u>T</u> locus. If the mismatch model is correct, it must be concluded that the AKR Y chromosome is slightly less delayed in its action than the Poschiavinus Y. Unfortunately, a much larger data set than that presented in chapter 4 would be required to demonstrate whether a relatively minor difference exists in the time of onset of testicular differentiation in Y^{POS}-bearing and Y^{AKR}-bearing fetuses.

Evidence to support the mismatch hypothesis is available, however, from the study of the 'additional factors', referred to above, that are necessary to produce XY sex reversal in C57BL/6-Y^{AKR} mice. Approximately 50% of C57BL/6-Y^{AKR} $\underline{T}^{hp}$/+ individuals are born as females whereas all C57BL/6-Y^{AKR} $\underline{T}^{Orl}$/+ individuals develop a female phenotype. All C57BL/6-Y^{AKR} $\underline{W}^{19}$/+ mice also develop as females (Cattanach, 1987). Therefore, the proportion of XY individuals that develop ovaries varies according to the 'strength' of the mutation. When the steel-Dickie mutation - $\underline{Sl}^d$, is introduced into the C57BL/6-Y^{POS} stock, the proportion of XY individuals that are born as females can be increased from the basic level of 14% in mice that don't

contain the mutation to approximately 55% in mice that are heterozygous $\underline{Sl}^d$. Furthermore, by producing C57BL/6-Y^{POS} mice that are homozygous $\underline{Sl}^d$, the proportion of XY females increases again to around 83%. The effect of the $\underline{W}^e$ mutation appears to be much stronger, in that approximately 82% of C57BL/6-Y^{POS} mice heterozygous for $\underline{W}^e$ develop as females and the few males that are produced are of such low fertility that it is extremely difficult to produce C57BL/6-Y^{POS} mice homozygous for $\underline{W}^e$ (Burgoyne and Palmer, 1991). Under the mismatch model, it is proposed that these factors independently influence the time of onset of the testis determining or ovary determining pathways and thereby alter the degree of sex reversal.

If C57BL/6-Y^{dom} sex reversal is caused by a failure of interaction at the biochemical level, it would have to be assumed that the various mutations that affect the degree of sex reversal are at testis determining loci. If the 'mismatch' hypothesis is accepted, these mutations could simply affect the relative rates of growth or development of different cell lineages that influence the timing of the testicular or ovarian programs. In the case of the mutations at the $\underline{T}$ locus - $\underline{T}^{hp}$ and $\underline{T}^{Orl}$, these are both null mutants of the $\underline{T}$ gene that have abnormalities of primitive streak formation which affects the establishment of the mesodermal lineage (Herrmann et al. 1990). The $\underline{T}$ gene is expressed in those lineages that are affected in $\underline{T}$ mutants and it is thought that haploinsufficiency for the $\underline{T}$ product causes the malformations of the tail in $\underline{T}$ heterozygotes. Since the somatic components of the gonad are formed from mesodermal derivatives, it is quite feasible that haploinsufficiency for the $\underline{T}$ product also causes a delay in testis formation that exacerbates the degree of sex reversal. Under the mismatch model, it would have to be assumed that the ovary determining process is less affected by the $\underline{T}$ mutations. Nevertheless, $\underline{T}^{hp}$ and $\underline{T}^{Orl}$ are both caused by large deletions of chromosome 17

(Herrmann et al. 1990) that could have removed genes that are specifically involved in testis determination.

The mutations W^{19}, W^e and Sl^d affect the proliferation and migration of haematopoietic cells, melanocytes and primordial germ cells (Mintz and Russell, 1957; Bennett, 1956). The steel locus encodes a stem cell growth factor (Anderson et al. 1990; Huang et al. 1990; Martin et al. 1990) that binds to c-kit receptors (encoded at the W locus; Chabot et al. 1988; Geissler et al. 1988) that are present on the cell surface of the haematopoietic cells, melanocytes and primordial germ cells. The Sl^d mutation is caused by a small deletion in the kit-ligand (KL) gene that removes the transmembrane and part of the intracellular domain of the cell-surface-bound product (Flanagan et al. 1991). The secreted form of the stem cell growth factor has normal activity. The W^{19} mutation is caused by a large deletion that removes c-kit and several other genes (Chabot et al. 1988). The nature of the W^e mutation has not yet been characterized. The c-kit gene is expressed cell-autonomously within the three lineages affected by the W mutations (Orr-Urtreger et al. 1990). In the W and Sl mutants, the only grossly affected lineage that seems to be relevant to the process of sex determination is the germ cells but as was discussed earlier (chapter 1, 3.ii) germ cells are not required in the process of testis determination. Therefore, W and Sl are unlikely to have direct roles in the process of testis determination and, in the case of Sl^d, no other genes can be implicated because the deletion only affects the steel product. In the light of these findings, the hypothesis that C57BL/6-Y^{dom} sex reversal is caused by a partial failure in the chain of biochemical interactions involved in testis determination seems untenable. It is not unreasonable, however, to suggest that the altered behaviour of the germ cells in W and Sl mutants could independently influence the developmental timing of the

testicular or ovarian pathways such that the degree of sex reversal is exacerbated.

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APPENDICES

1. CYTOGENETIC METHODS

1.i. Air-dried preparations from bone marrow.

Femurs were removed from the mice and a 25 gauge hypodermic needle attached to a 2 ml syringe was inserted into the knee end of the bone. Approximately 1 ml of Hepes-buffered Eagle's minimum essential medium (EMEM, ICN Flow Ltd.) containing 0.08 µg/ml colcemid (N-deacetyl-N-methylcolchicine) was used to flush out the bone marrow and the fluid was collected in a round-bottomed centrifuge tube.

The cells were incubated in this medium for 15 to 30 minutes at 31°C, then pelleted by centrifugation at 1000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 0.56% pottasium choride in deionized water. After 25 minutes at room temperature, the cells were centrifuged again at 1000 rpm for 5 minutes, the supernatant discarded and the tubes inverted on a paper towel to drain as much fluid as possible.

Cells were fixed by running about 0.5 ml of freshly prepared 3:1 methanol: glacial acetic acid down the side of the tube, allowing the fixative to wash over the pellet, then tipping it away. This was repeated two more times before resuspending the cells in 2 ml of fixative. The fixative was changed immediately by centrifuging at 1000 rpm for 5 minutes, discarding the supernatant and resuspending. The fixative was changed a further 3 or 4 times and after the final spin, slightly less fixative was added so that a milky-white suspension of the cells was formed.

3 drops of the cell suspension were applied to a glass slide that had been pre-soaked in 1% hydrochloric acid in 70% ethanol and wiped dry with a paper towel. Once the cells had air-dried, the slides were stained in a 2% solution of Giemsa (Gurr, BDH Ltd.) in pH 6.8 buffer (made

with pH 6.8 buffer tablets, Gurr, BDH Ltd.) for 20 minutes, rinsed with deionized water and allowed to dry.

1.ii. Air-dried preparations from fetal liver tissue.

A small sample of liver tissue was removed from the fetus and dropped into a round bottomed centrifuge tube containing 1 ml of EMEM containing 0.08 µg/ml colcemid. The cells were dispersed by pipetting the tissue in and out of a glass pasteur. Slides were prepared by the same process as described for bone marrow cells.

1.iii. Air-dried preparations from amniotic membranes for sex-chromatin analysis.

The amniotic membranes were processed using an adaptation of the method of Evans et al. (1972) for yolk sac preparations (Burgoyne et al. 1983). Each membrane was placed near the top of a conical-bottomed centrifuge tube and flushed down into the tube with 3:1 methanol:glacial acetic acid fixative. Tubes containing the amniotic membranes were centrifuged briefly at 1000 rpm and excess fixative decanted off (being careful to retain the amnion). The tubes were inverted on a paper towel and tapped on the bench to bring the amnion 1 or 2 cm away from the base of the tube. A single drop of 60% glacial acetic acid in distilled water was applied directly onto the amnion in order to dissociate the cells. After 60 seconds the action of the 60% acetic acid was blocked by the addition of approximately 1 ml of fixative. The cells were then centrifuged at 1000 rpm for 5 minutes. Excess fix was decanted off and the tube inverted for a few seconds to allow the tube to drain, without letting the cells dry completely. When the tube was righted again, the cells were resuspended in the small amount of fixative that ran down to the bottom from the sides of the tube. If this process

was judged correctly, the cells could be concentrated into a single drop of fluid. This single drop was spotted onto a clean glass slide. When the cells had air-dried, one drop of 1% aqueous toluidine blue was spotted onto the cells and a coverslip pressed on. These preparations were scored immediately for the presence or absence of the sex-chromatin (see fig 1).
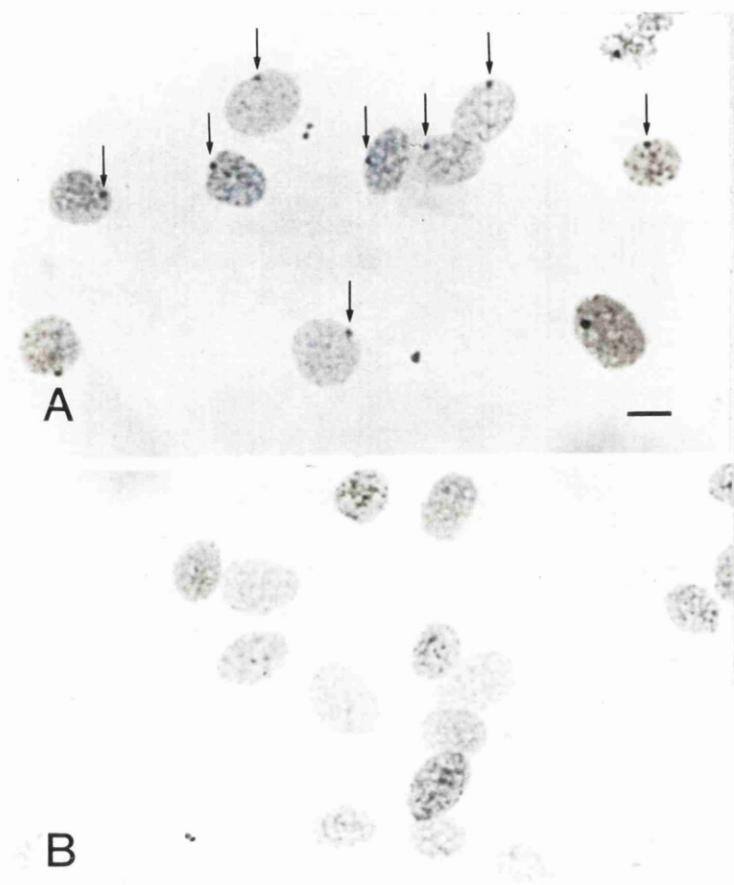
Fig.1. Dissaggregated amnion cells (A) positive and (B) negative for sex chromatin (arrows). Bar, 10μm.

2. IN SITU HYBRIDIZATION TO DNA IN TISSUE SECTIONS AND AIR-DRIED MATERIAL USING BIOTINYLATED DNA PROBES.

(Based on the method of Rossant et al. 1986)

The solutions were prepared in deionized water and the incubations were at room temperature unless otherwise stated.

2.i. Preparation of histological sections.

Samples of tissue were fixed overnight in 3:1 ethanol: glacial acetic acid at 4°C. The fixed samples were washed in two changes of absolute ethanol for 30 minutes each, immersed in two changes of cedarwood oil for 1 hour each and then left in a final change of cedarwood oil overnight. The cleared samples were taken through three 45 minute immersions in fresh changes of molten parrafin wax (melting point - 58°C) kept in a 60°C oven. The samples were embedded in fresh molten wax and left to solidify at room temperature. Sections were cut at 5μm, the ribbons were floated on a thin film of deionized water covering a glycerin/albumen coated slide, and placed onto a hot plate set for 50°C. Once the sections had expanded, excess water was removed with a pipette and the sections were left overnight in a 42°C oven to dry and adhere to the slides.

2.ii. Biotin labelling of probe DNA

DNA was labelled using the BRL (Bethesda Research Laboratories) Nick Translation System and BRL Biotin-11-dUTP according to the manufacturers instructions. Unincorporated nucleotides were removed from the reaction mix by exclusion chromatography through a Sephadex G50 (Pharmacia Ltd.) spun column equilibrated with 1xSSC, 0.1% SDS (see Maniatis et al. 1982 for method and solutions). A 1 μl sample of the labelled DNA was diluted to

concentrations of 250 pg/µl and 100 pg/µl, then 2 µl of each of these dilutions, together with an appropriate control, were spotted onto a nitrocellulose membrane that had been pre-washed in deionized water and dried. The membrane was baked at 80°C under vacuum for 1 hour, then soaked in 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS, see list of solutions) for 10 minutes. The membrane was then placed into a polythene bag into which 1 ml of streptavidin-horse radish peroxidase (streptavidin-HRP) working solution (see list of solutions) was pipetted and the opening heat-sealed. After 30 minutes the membrane was cut out of the bag and washed twice for 5 minutes each in pre-detection buffer (see list of solutions). Finally, the membrane was placed into a petri dish and 5 ml of diaminobenzidine working solution (see list of solutions) was pipetted onto the surface. If the labelling reaction was successful, brown spots appeared after 5 minutes and the level of incorporation of biotinylated nucleotides could be estimated from the intensity of the colour reaction in comparison with the control.

2.iii. Hybridization.

Sectioned material was dewaxed by immersion in two changes of histoclear for 10 minutes each, followed by a 2 minute wash in absolute ethanol. For air-dried material, these steps were ommited. Endogenous peroxidases were inactivated by immersing the slides in a 3% solution of hydrogen peroxide in methanol for 10 minutes. The slides were then rinsed in absolute ethanol for 2 minutes and left to air-dry. DNA in the sectioned or air-dried material was denatured by submerging the slides in 0.07M sodium hydroxide in 70% ethanol for 5 minutes at room temperature, then dehydrated through an alcohol series of 70%, 95% and 2 changes of 100% ethanol for two minutes in each and finally

left to air-dry.

Appropriate quantities of labelled probe DNA, sheared salmon sperm DNA and deionized water were mixed together in an eppendorf (see hybridization mix, list of solutions) and the eppendorf was placed into boiling water for 5 minutes to denature the DNA. The mix was quenched on ice for a few minutes, condensation was brought down from the lid and the sides of the tube by centrifuging at 15,000 rpm in a microfuge for 2 seconds, then the SSPE and dextran sulphate were added (see list of solutions) and the tube was returned to ice.

50 ul of the hybridization mix was applied to each of the slides and a 22x50 mm coverslip (prewashed in 1% hydrochloric acid in 70% ethanol and wiped with a tissue) was gently lowered on, so that a thin film of the fluid covered the slide. The slides were loaded into a metal slide-rack with the handle removed to keep them horizontal. This was placed inside an air-tight plastic box containing a sheet of tissue soaked in 2xSSC (see Maniatis et al. 1982) to maintain the humidity. The box was placed in a 60°C oven overnight.

2.iv. Post-hybridization washes and detection

On the following morning, the slides were rinsed in 2xSSC, 0.1% Triton X-100 (octyl phenoxy polyethoxy ethanol, Sigma Ltd.) to remove the coverslips, then washed with agitation in fresh 2xSSC, 0.1% Triton for 5 minutes. After a further 5 minutes agitation in 0.1xSSC, 0.1% Triton, the slides were immersed in the stringent wash - 0.1xSSC, 0.1% Triton for 10 minutes at 50°C. The slides were transferred to a solution of 5% BSA in 0.1xSSC, 0.1% Triton for 5 minutes then, without letting the tissue dry, 90 µl of streptavidin-HRP working solution (see list of solutions) was pipetted onto the surface of each slide and a 22x50 mm coverslip lowered on. The slides were loaded into the metal

rack, placed inside the humid chamber and transferred to a
37°C oven for at least 1 hour.

At the end of the incubation, the coverslips were
gently teased off and the slides washed in 0.1xSSC, 0.1%
Triton for 5 minutes followed by immersion in 5% BSA in
0.1xSSC, 0.1% Triton for a further 5 minutes. The slides
were then transferred into diaminobenzidine/hydrogen
peroxide working solution (see list of solutions), which
was prepared just before use, and incubated in the dark for
30 to 50 minutes. Finally, the slides were washed in
running tap-water for 5 minutes and checked for signs of
hybridization signal.

Sectioned material was subsequently stained with
haemotoxylin and eosin by standard histological methods,
dehydrated through an alcohol series, cleared in histoclear
and mounted in DPX. Staining of the air-dried material in
Giemsa obscured the hybridization signal, so these were
stained in haemotoxylin for 15 minutes, washed in tap-water
and left to dry before mounting in DPX.

2.v. Modifications of the in situ procedure

For the projects presented in this thesis, it was
important to obtain both clear in situ hybridization and
good histology. A major problem was the damage inflicted on
the material during the processing for in situ. The most
significant steps were found to be the fixation of the
tissue and the denaturation of the DNA in the tissue. The
protocol shown above is the final version after several
modifications which are listed below.

1. Material to be sectioned was fixed in 3:1
ethanol: glacial acetic acid. This is a crude histological
fixative and does not give good histology under normal
conditions. Aldehyde-based cross-linking fixatives give
better histology but the in situ did not work without the
use of proteinases which cause considerable damage. This is

presumably due to difficulties in penetration of the probe DNA.

2. In the method of Rossant et al. (1986), tissue was embedded in ester wax 1960 (BDH Ltd.). Because this wax is partially water soluble, it is difficult to float out the sections, and fetal material, which is delicate, has a tendency to break up without a more solid support. Embedding in parrafin wax caused a slight reduction in the intensity of the signal but the integrity of the sections was maintained.

3. Denaturation of the DNA in the tissue was originally acheived by a 5 minute incubation in 70% deionized formamide in 2xSSC at 70°C (Rossant et al. 1986). It was found that the use of 0.07M sodium hydroxide in 70% ethanol (J.Crolla, personal communication) caused less damage to the tissue. A slight problem with this method is that it has a tendency to cause variability in the intensity of the signal in different regions of the slide.

2.vi. List of solutions.

Phosphate buffered saline (PBS) 10x.

1.30M sodium chloride (NaCl)
0.07M dibasic sodium phosphate (Na_2HPO_4)
0.03M monobasic sodium phosphate (NaH_2PO_4)

Pre-detection buffer.

2xSSC
0.1% BSA
0.05% Triton X-100
1mM EDTA pH 8.0 (see Maniatis et al. 1982)

Streptavidin-horse raddish peroxidase working solution.

1xPBS
5mM EDTA pH 8.0
1% BSA
5 µl of a 1 mg/ml solution of streptavidin-HRP (Amersham Ltd.) was added per 1 ml of total, just before use.

Diaminobenzidine/ hydrogen peroxide working solution.

50mM sodium phosphate buffer pH 7.4
0.1% Triton X-100
0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB)
+15 µl of 30% hydrogen peroxide (BDH Ltd) in 50 ml of total.

Hybridization mix.

5xSSPE (see Maniatis et al. 1982)
10% dextran sulphate
0.8 ng/µl probe DNA
500 ng/µl sheared salmon sperm DNA (see Maniatis et al. 1982)

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In situ analysis of fetal, prepuberal and adult XX↔XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY

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Summary

The testes of fetal, prepuberal and adult XX↔XY chimaeras were examined using *in situ* hybridisation to identify the β-globin transgenic marker contained in one component of each chimaera. This enabled the proportion of XX and XY cells contributing to the major cell lineages of the testis to be estimated from sectioned and air-dried material. A few XX Sertoli cells were found in all three age groups, but the XX contribution was always much lower than in other somatic cell types. Significantly, in fetal XX↔XY testes, Sertoli cells were the only cell type to show a bias in favour of the XY component. This strengthens the view that *Tdy* acts solely in the lineage that gives rise to Sertoli cells. However, the finding of some fetal XX Sertoli cells means that one of the steps in the *Tdy*-initiated process of Sertoli cell determination is capable of locally recruiting XX cells.

Key words: testis determination, XX↔XY chimaeras, XX Sertoli cells, mouse testes.

Introduction

In order to divert the indifferent fetal gonad away from ovarian development into the testicular pathway, the Y chromosomal testis determinant *Tdy* must alter the fate of at least three gonad-specific cell lineages – the germ cell lineage, the steroid cell lineage and the supporting cell lineage (see Burgoyne, 1988, for a review). It is assumed that these three lineages are bipotential prior to *Tdy* action.

Burgoyne *et al.* (1988*a*) examined the contribution of XX and XY cells to these lineages in prepuberal and adult XX↔XY male mouse chimaeras. The germ cells, not surprisingly, were exclusively XY because two X chromosomes are incompatible with spermatogonial development after birth (see West, 1982). However, the Leydig cells (steroid cell lineage) had XX and XY contributions in similar proportions to non-gonadal tissues. Importantly, the Sertoli cells (supporting cell lineage) were found to be predominantly, or perhaps exclusively, XY. Specifically, the analysis of Sertoli cells from prepuberal XX↔XY chimaeras using isozymes of GPI-1 as lineage markers revealed an intense XY band and a barely detectable XX band, while an examination of adult testicular material from XX↔XY chimaeras by *in situ* hybridisation detected no XX Sertoli cells. It was concluded that the minor XX band from prepuberal testes was probably due to contamination of the Sertoli cell fraction with peritubular myoid cells. These results led Burgoyne (1988) to propose that *Tdy* acts to bring about testis determination by triggering, cell-autonomously, the differentiation of the supporting cell lineage into Sertoli cells, and that the commitment of the other components of the testis to the male pathway is directed by the Sertoli cells without further *Tdy* involvement.

If Sertoli cells are exclusively XY in adult XX↔XY testes, this need not necessarily reflect the situation in fetal XX↔XY gonads when Sertoli cells first form. The objective of the present study was to extend the *in situ* analysis of XX↔XY testes to cover a range of stages from fetus to adult.

Materials and methods

Mice

A transgenic mouse stock, (line 83) produced by Lo (1986), was used to mark cells of one component of each chimaera. This line is homozygous for a single insertion of 1000 copies of the β-major globin gene close to the telomere of chromosome 3. The stock is derived from a mixed CBA, C57BL/6, SJL background and is maintained as a small closed colony but is not formally inbred. (These mice will be referred to as GT). Chimaeras were produced from two different sets of crosses:

1) CBA-T6,XX×GT,XY↔BALB/c,XX×MF1,XY[del]
2) GT,XX×MF1,XYY[del]↔(CBA×C57BL/6)F₁,XX×CBA-T6,XY[meta]

The chromosome markers include: the T6 translocation (Ford *et al.* 1956), the metacentric Y chromosome variant (Y[meta], Winking, 1978; Burgoyne *et al.* 1988*b*) and the Y[del] chromosome, which is an RIII Y chromosome with a large long arm deletion, maintained on an MF1 (random bred albino) background. These markers allow the positive

identification of all possible combinations in cytogenetic preparations. The Ȳ chromosome is the *Tdy*-negative mutant Y of Lovell-Badge and Robertson (1990). This cross was set up for a different project but four of the resulting chimaeras that lacked the mutant Y chromosome are included here.

Pairs of embryos were aggregated at the 8- to 16-cell stage, then transferred on the following day to pseudopregnant F_1 (CBA×C57BL/6) mothers. The age of the fetal chimaeras is taken from the plug date of the female recipients.

Cytogenetic analysis

Air-dried metaphase spreads were prepared from bone marrow of adult and prepuberal chimaeras by standard methods. Fetal chimaeras were karyotyped from samples of liver disaggregated with a glass Pasteur pipette and then processed in the same way as the bone marrow.

In situ *hybridisation*

All tissues for *in situ* histology were fixed in 3:1 ethanol/ glacial acetic acid, rinsed in ethanol, cleared in cedarwood oil and blocked out in 58°C m.p. paraffin wax. Sections were cut at 5μm and mounted on glycerine/albumin coated slides. Air-dried adult testis preparations were made by a modification of Meredith's method (Levy and Burgoyne, 1986).

The transgenic insert of the GT mice was identified using the probe pMβG2 (Tilghman *et al.* 1977), which contains a 7 kb insert spanning the β-major globin sequence. The entire plasmid was biotin labelled using the BRL (Bethesda Research Laboratories) nick translation kit and BRL biotin-11-dUTP. *In situ* hybridisation was carried out according to the method of Rossant *et al.* (1986) and bound biotin was detected using horseradish peroxidase conjugated with streptavidin, followed by incubation in a solution of diamino-benzidine. Positive hybridisation is recognised by a discrete area of brown precipitate in the nucleus.

The proportion of labelled cells in the different lineages of the fetal testis was estimated from the sections either by scoring all the cells in a series of randomly selected tubules, or in the interstitial regions by using a gridded eyepiece graticule. To rectify the bias due to false negative cells, correction factors were produced by applying the same procedure to testis sections from a fetus hemizygous for the transgenic insert (i.e. all cells carry the transgene).

Results

The results of the *in situ* analysis of 12 chimaeric males are presented here: 10 with an XX↔XY genotype and 2 XY↔XY controls. These 12 fall into three age categories: fetal (13.5–16.5 days *post coitum*, dpc), prepuberal (13–15 days pp), and adult (46–52 days pp). A close examination of the testis sections after *in situ* hybridisation revealed that XX Sertoli cells do in fact exist at all three ages (see Figs 1 and 2). Sertoli cells were identified on the basis of their irregular nuclear morphology, the distribution and density of hetero-chromatin and their position in the tubule. The presence of XX Sertoli cells was confirmed for the adult chimaeras by the *in situ* analysis of air-dried testis preparations, in which Sertoli cells are easily recognised by the characteristic 'triple spots' of a light blue nucleolus flanked by two heterochromatic blocks. In these preparations, a small population of such cells were found with clear hybridisation (chimaeras 2–4, e.g. Fig. 2D) or lacking hybridisation (chimaera 1) indicating their XX nature.

Hybridisation was always found, both in the control XY↔XY testis preparation and in the experimental chimaeras, closely associated with one of the hetero-chromatic blocks. The fact that these regions are known to contain centromeric sequences (Hsu *et al.* 1971), plus the fact that the target transgenic insert is close to the telomere of chromosome 3, may indicate something about the organisation of chromatin in the Sertoli cell nucleus.

The estimates of the contribution of XX cells to specific cell types in the fetal testis are presented in Table 1. The chimaeras are arranged in order of increasing XX contributions to liver. With the exception of the Sertoli cell lineage, there is no prejudice against XX cells contributing to testicular cells. Overall, the proportion of XX peritubular myoid cells (43 %) and Leydig cells (48 %) is very similar to the proportion of XX cells in non-testicular tissues (47–48 %), while the overall proportion of XX T_1-prospermatogonia

Table 1. *Estimated proportions of XX cells in cell lineages from fetal XX↔XY chimaeras*

		Percentage of XX cells (number of cells scored)†					
		Non-testicular cells		Testicular cells			
Chimaera*	Age dpc	Liver	Mesonephric tubules	Myoid cells	Leydig cells	T1-prosperm-atogonia	Sertoli cells
1 XX*↔XY	16.5	23(13)	8(607)	26(509)	27(502)	54(503)	5(552)
2 XX*↔XY	13.5	32(50)	25(526)	31(54)	41(207)	70(133)‡	7(158)‡
3 XX↔XY*	15.5	61(51)	69(560)	56(403)	53(538)	54(317)	8(535)
4 XX↔XY*	15.5	74(50)	90(522)	60(203)	72(491)	82(212)	21(354)
	MEAN	47.5	48.0	43.2	48.3	65.0	10.2

*The chimaeras were all from cross 1. The component carrying the transgenic marker is indicated by an asterisk.

†For the liver, the counts are based on cytogenetic markers in air-dried mitotic cells. For the other cell types, the estimates are based on the *in situ* analysis of the transgenic marker, corrected for the incidence of false negatives. The percentage of false negatives by cell type, as estimated in control sections were: mesonephric tubules 29 % (344), myoid cells 30 % (244), Leydig cells 21 % (146), T1-prospermatogonia 43 % (249) and Sertoli cells 27 % (313).

‡At 13.5 *dpc*, many of the cells in the testis cords were in prophase or metaphase of mitosis. Because of the possibility of confusion between dividing Sertoli cells and germ cells, the Sertoli cell counts are based on the unambiguously identifiable, peripherally located interphase cells, and the T1-prospermatogonia counts on centrally located cells whether or not they were dividing.
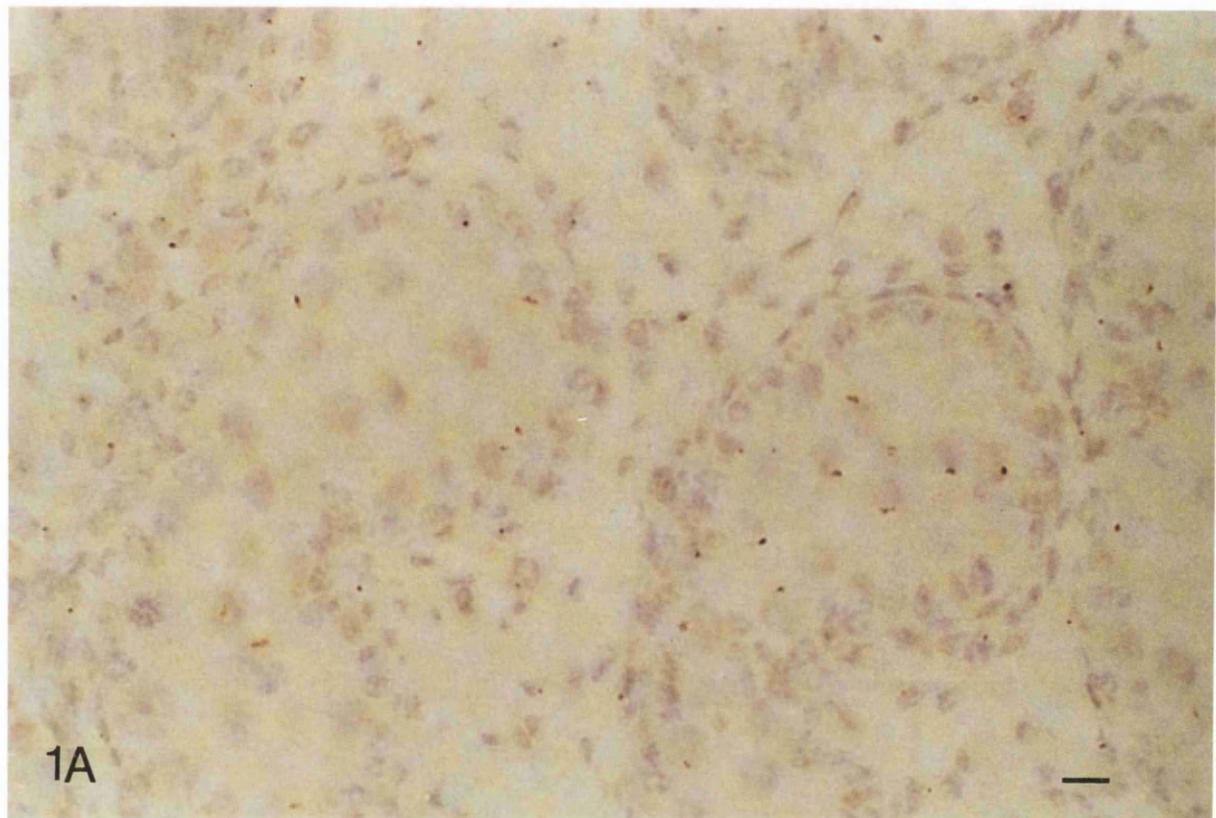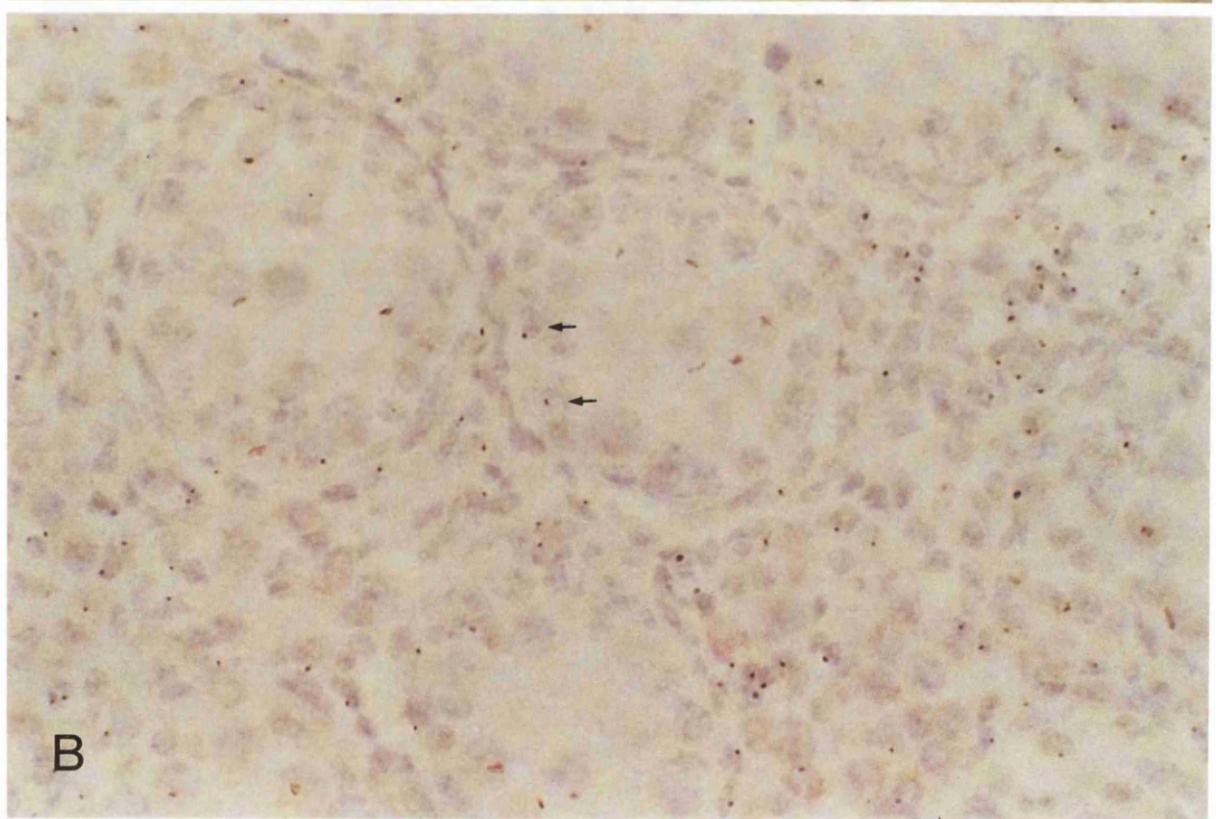
Fig. 1. (A) Section of 16.5 dpc XX↔XY testis showing some labelled XX T1 prospematogonia inside cords lined with unlabelled XY Sertoli cells. (B) Section of the same testis with two labelled XX cells which, on the basis of position and morphology, are presumed to be Sertoli cells (arrowed). Bar. 10 μm.
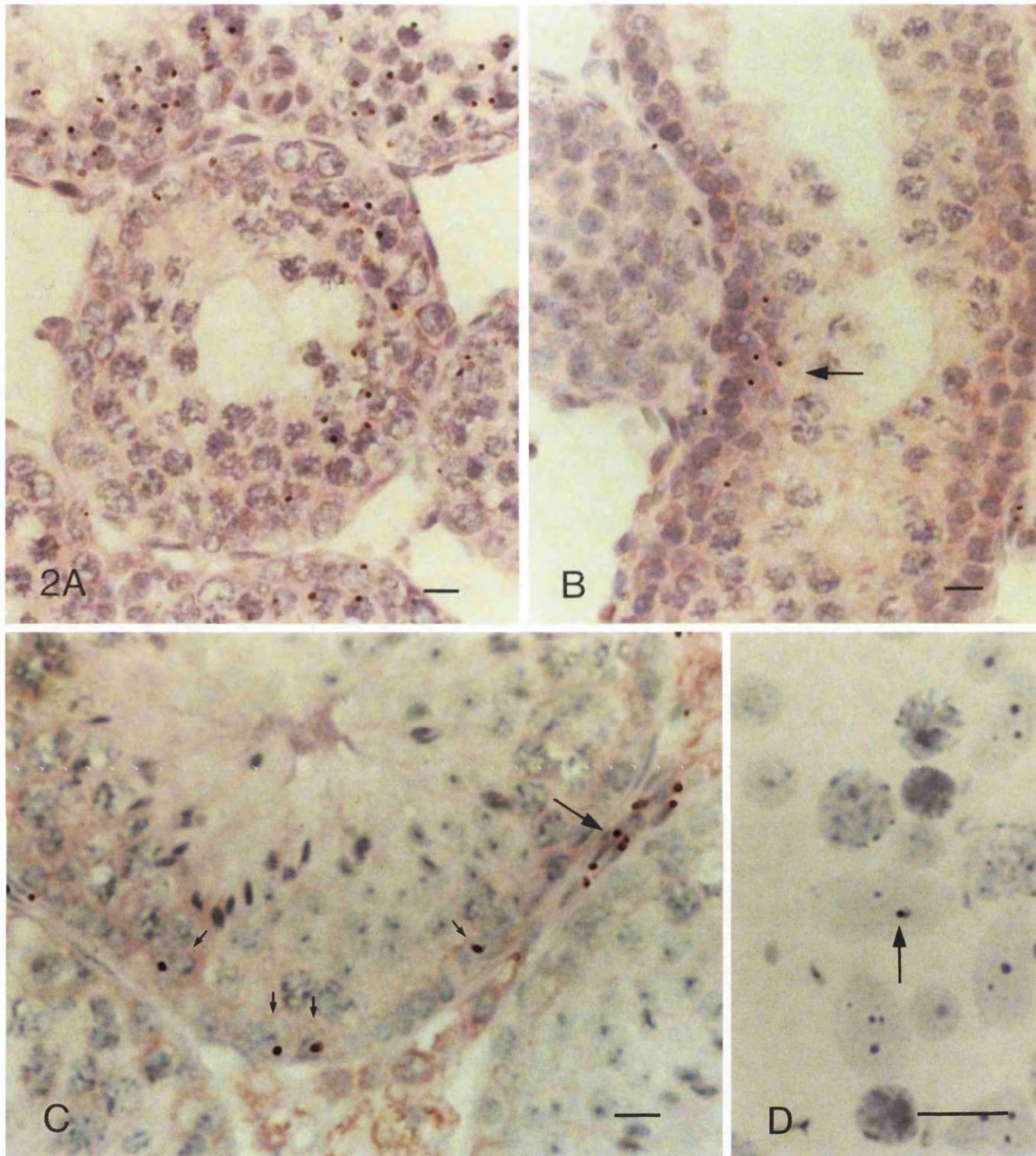
Fig. 2. (A) Prepuberal XY↔XY control testis section showing the patchy distribution of labelled germ cells and Sertoli cells. (B) Prepuberal XX↔XY testis section from a mouse with 29% XX contribution to the bone marrow. A patch of labelled XX Sertoli cells (arrowed) can be seen in an otherwise XY dominated testis. (C) Section of testis from adult XX↔XY chimaera 4. Four XX Sertoli cells (small arrows) can be seen inside a tubule producing condensed spermatids. The other labelled cells are peritubular myoid cells (large arrow). (D) Air-dried testis preparation from chimaera 4 showing Sertoli cells with the characteristic heterochromatic blocks. One of the Sertoli cells has a hybridisation signal over one of the heterochromatic blocks (arrow) indicating the XX nature of the cell. Bar, 10 μm.

Table 2. *Estimated proportions of XX cells in the cell lineages of adult XX↔XY chimaeras*

Chimaera	Cross	Age dpp	Percentage of XX cells (number of cells scored)†			
			Bone marrow	Pachytene spermatocytes	MI spermatocytes	Sertoli cells
1 XX↔XY*	1	52	10(50)	0(514)	0.8(124)‡	1.8(1418)
2 XX*↔XY	2	52	22(50)	0(564)	0(63)	2.1(1078)
3 XX*↔XY	2	46	61(33)	0(500)	0(50)	1.6(1039)
4 XX*↔XY	2	52	84(51)	0(537)	0(29)	1.6(2052)

*The component carrying the transgenic marker.

†The bone marrow counts are based on cytogenic markers in air-dried mitotic cells. For the other cell types, the counts are based on the *in situ* analysis of the transgenic marker in air-dried testicular cells.

‡This is based on a single unlabelled cell, which probably lost the marker as a result of metaphase breakage.

(65 %) is somewhat higher. By contrast, there is a clear prejudice against XX Sertoli cells in all four chimaeras, with a mean XX contribution of 10 %. Comparing the proportion of XX Sertoli cells in each chimaera with the pooled estimates for all other cell types indicates a reduction of between 4- and 7-fold. The extent of the reduction in the contribution of XX cells to the Sertoli cell lineage is not related either to fetal age or the strain of the XX component.

The two prepuberal XX↔XY chimaeras had XX contributions to the bone marrow of 29 % and 39 %. The labelled Sertoli cells occurred as clonal patches but the patches were rare, confirming the strong XY bias (Fig. 2B). The proportion of XX Sertoli cells has not been estimated for these two chimaeras. The quantitation procedure used for the sections is inappropriate when the cells occur in rare patches and, in air-dried preparations of dissociated testicular tissue, the majority of Sertoli cells from this age lack the typical nuclear morphology seen in the adult. In the four adult chimaeras (Table 2), the proportion of XX Sertoli cells was very low (1.6–2.1 %) and surprisingly consistent despite a wide range of XX contribution to the bone marrow (10–84 %).

Extensive earlier work has demonstrated that XX germ cells do not survive in XX↔XY testes. Of the 2381 pachytene and MI cells scored here, one MI cell lacking the transgene marker was identified in the chimaera that had the transgene in the XY component. This probably represents artefactual loss of the bivalent carrying the marker. It was noted that the adult XY↔XY control, in which the XY component lacking the transgene was heterozygous for the T6 translocation, has no unlabelled MI spermatocytes, although this component was well represented in pachytene spermatocytes (13 %), Sertoli cells (30 %) and bone marrow cells (43 %). It has previously been reported that heterozygosity for this translocation causes extensive meiotic failure (Baranov and Dyban, 1968).

Discussion

This study demonstrates that some XX Sertoli cells are present in fetal, prepuberal and adult XX↔XY chimaeras. The presence of XX Sertoli cells in adult XX↔XY chimaeras is contrary to the results of

Burgoyne *et al.* (1988*a*). However, the incidence of XX Sertoli cells in the adults was less than 3 % and it is possible that such a low contribution, occurring as it does in the form of rare patches, may have been missed in the earlier study. The presence of XX Sertoli cells in an adult XX/XY male has previously been inferred by Singh *et al.* (1987) and is also reported for a series of adult XX↔XY chimaeras by Patek *et al.* (1991).

Since XX Sertoli cells are already present in the 13.5 *dpc* XX↔XY testes, they presumably differentiated contemporaneously with the XY Sertoli cells, and as a consequence of *Tdy* action. What does this tell us about the mode of *Tdy* action? It should be emphasised that, although the Sertoli cells were not exclusively XY, there was a strong XY bias that was already established by 13.5 *dpc*. There was no XY bias in any of the other cell types in the fetal testes. This strengthens the conclusion by Burgoyne *et al.* (1988*a*) that *Tdy* must act by directing cells of the supporting cell lineage to form Sertoli cells. However, since a small proportion of fetal XX Sertoli cells were found, at some point between the expression of *Tdy* and the formation of Sertoli cell cords, there must be a step that can locally recruit XX cells. In a normal XY male, this capacity for local recruitment is irrelevant since all cells contain *Tdy*. Recently, a new gene (*Sry*) has been identified on the mouse Y chromosome (Gubbay *et al.* 1990), which is almost certainly *Tdy*. The expression of *Sry* is first seen at 10½ *dpc* in the genital ridge and is germ cell independent (Koopman *et al.* 1990), which is compatible with *Sry* being expressed in the Sertoli cell precursors. The sequence of *Sry* suggests that it encodes a DNA-binding protein which would be expected to act cell autonomously. This would imply that the capacity for recruiting some XX cells to form Sertoli cells in XX↔XY testes is a characteristic of a gene product in the *Sry*-initiated 'cascade', rather than of *Sry* itself.

The XX Sertoli cells that form in XX↔XY chimaeric gonads should not be equated with those that form in ovarian tissue in a number of situations where there has been germinal failure (reviewed by Burgoyne, 1988 and 1991). Taketo-Hosotani *et al.* (1985) and Taketo-Hosotani and Sinclair-Thompson (1987); for example, have described the 'transdifferentiation' of follicle cells into Sertoli cells, following germ cell loss, when fetal mouse ovaries were grafted under the kidney capsule of

adult male, or even adult female, hosts. In the latter instance, there was no Y chromosome present in the graft or host, so it can hardly be considered of direct relevance to the mode of *Tdy* action.

The fact that Sertoli cells are the only cell type to show an XY bias in the fetal testis supports the view of Burgoyne (1988) that the commitment of other testicular cell types to the male pathway is directed by Sertoli cells without further Y involvement. This does not preclude a role for the Y in the subsequent differentiation or function of these other cell types, or indeed, of Sertoli cells. For example, the mouse Y has at least three distinct functions during spermatogenesis (reviewed by Burgoyne, 1991). Patek *et al.* (1991) suggest that the Y may also be required for normal Leydig cell functions. This suggestion is based on their finding of an unusually high incidence of tubules with germinal failure together with a particularly high proportion of XX Leydig cells in their series of XX↔XY chimaeras, leading them to propose that the germinal failure is brought about by XX Leydig cell dysfunction. An alternative possibility is that this particular chimaeric combination (in which the XY component was always derived from embryonic stem cells of the 129 inbred strain and the XY component from C57BL/6×CBA F$_2$) not only favours XX Leydig cells but also favours XX germ cells. The tubules with germinal failure would then be attributable to the loss of the XX germ cells around the time of birth. We therefore prefer to reserve judgement on a possible role for the Y in Leydig cells until information on the XX contribution to the germ line is available for fetal XX↔XY testes of this particular chimaeric combination.

A puzzling feature of the present data was the consistency in the proportion of XX Sertoli cells (1.6–2.1 %) in the adult chimaeras despite a wide range of XX contributions to the mice as a whole (10–84 %). While the consistently lower frequency of XX Sertoli cells in the adult chimaeras, as compared to the fetal chimaeras, may indicate selection against XX Sertoli cells subsequent to their formation, it is hard to imagine a selective mechanism that acts to produce such a low but invariant proportion of XX cells.

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XY follicle cells in the ovaries of XO/XY and XO/XY/XYY mosaic mice

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Summary

XO/XY and XO/XY/XYY mosaic hermaphrodites were generated from crosses involving BALB/cWt males. The distribution of Y-bearing cells in the gonads of these mice was studied by *in situ* hybridisation using the Y-specific probe pY353B. XY cells were found to contribute to all cell lineages of the ovary including follicle cells. The proportion of XY follicle cells was not significantly different from the XY contribution to other gonadal or non-gonadal cell lineages. However, this proportion was consistently low, all the hermaphrodites having a low XY contribution to the animal as a whole.

Because the XO- and Y-bearing cell lineages are developmentally balanced, the XY follicle cells cannot have formed as a result of a 'mismatch' in which the Y-directed testis determination process is pre-empted by an early acting programme of ovarian development. These results are discussed with respect to the hypothesis that *Tdy* acts in the supporting cell lineage, the lineage from which Sertoli cells and follicle cells are believed to be derived.

Key words: mosaic, hermaphrodite, XY follicle cells.

Introduction

Gonadal sex differentiation can first be recognised in mice at 12.5 days *post coitum* with the appearance of testicular cords in the gonads of XY fetuses. Female gonads show no gross morphological change from the indifferent state until 13.5 days *post coitum* when the germ cells first enter meiosis (McLaren, 1984). Studies on fetal rat testes by light and electron microscopy have revealed that the Sertoli cells can be recognised as a distinct cell type prior to their aggregation into cords (Magre and Jost, 1980). These cells are the first testicular cell type to appear. It is widely accepted that Sertoli cells differentiate from a pool of bipotential cells (the supporting cell lineage) which in females forms the follicle cells of the ovary. The derivation of Sertoli cells and follicle cells from a common lineage is supported in part by the fact that they share certain biochemical properties including production of the Mullerian inhibitor – AMH or MIS (Vigier *et al.* 1984; Donahoe *et al.* 1987). They also both share a lineage-specific cell surface antigen, as detected by cytotoxic T cell assay (Ciccarese and Ohno, 1978). Follicle cells have the ability to 'transdifferentiate' into Sertoli cells under special circumstances, as in the ovaries of ageing or hypophysectomised female rats (Crumeyrolle-Arias *et al.* 1986) and in tissue derived from fetal ovaries grafted under the kidney capsule of adult mice (Taketo-Hosotani *et al.* 1985; Taketo-Hosotani and Sinclair-Thompson, 1987). Examination of ovotestis sections from adult XO/XY mosaic hermaphrodites (Burgoyne and Palmer, unpublished data) and of T16/X*Sxr* hermaphrodites (Ward *et al.* 1988), has shown testis

tubules which connect smoothly with follicles (which may contain oocytes) all bounded by a continuous basement membrane.

Burgoyne *et al.* (1988*a*) proposed that the primary function of the testis-determining gene (*Tdy*) is the cell-autonomous diversion of the supporting cell lineage to form Sertoli cells and that the remainder of testis development is directed by these Sertoli cells. In the absence of a Y (and thus *Tdy*), the supporting cell lineage differentiates into follicle cells. This 'cell-autonomous action of *Tdy*' model of sex determination, in its simplest form, predicts that follicle cells should be exclusively XX in XX↔XY female chimaeras. This is because all supporting cells containing a Y chromosome are expected to be diverted into the Sertoli cell pathway. XY follicle cells have, however, been shown to exist in three XX↔XY female chimaeras (Ford *et al.* 1974; Burgoyne *et al.* 1988*b*). The latter authors suggested that these XY follicle cells could be formed by a 'timing mismatch' mechanism in which *Tdy* was pre-empted by the ovarian determination process initiated by the XX component of the chimaera. In chimaeras, there is clearly the potential for a developmental mismatch between the two components and, in Ford *et al.*'s XX↔XY females, the XY component was AKR, which other evidence suggests has a late-acting Y (Eicher and Washburn, 1986).

In this paper, we ask whether XY follicle cells can be formed in a situation where 'mismatch' can be ruled out: this is, in the ovaries of XO/XY and XO/XY/XYY mosaic hermaphrodites that occur in crosses involving BALB/cWt males (Eicher *et al.* 1980). These mosaics arise through mitotic non-disjunction of the Y,

Table 1. *The proportion of Y-bearing cells in bone marrow and gonadal cell lineages of XO/XY and XO/XY/XYY mosaics*

| | | Gonads* | | | Ovary | | Testis or ovotestis | |
	Age (dpp)	L	R	Bone marrow	Stroma	Granulosa	Interstitial	Sertoli
Mosaic								
1	8	OT	O	20 (30)	17 (558)	22 (558)	24 (517)	99 (587)
2	11	O	OT	20 (30)	11 (552)	19 (633)	25 (551)	90 (535)
3	8	O	T	11 (28)	15 (554)	15 (549)	−†	−†
4	11	O	OT	20 (30)	22 (510)	17 (640)	−†	−†
5	8	T	O	29 (28)‡	21 (560)	25 (654)	20 (502)	84 (735)
		Mean±s.e.		20.0±2.9	17.2±2.0	19.6±1.78	23.0±1.53	91.0±4.4

Percentage of Y-bearing cells (number of cells scored)

* O, ovary; T, testis; OT, ovotestis.
† Not recorded due to technical failure.
‡ 11% of the Y-bearing cells were XYY.

so there are no strain differences between the components. Furthermore, although XO fetuses with a paternal X are developmentally retarded (Burgoyne *et al.* 1983), XO fetuses with a maternal X develop as fast as their XY sibs (Thornhill and Burgoyne, unpublished data); so, on these additional grounds, we expect the XO and XY cell lines of these mosaics to be developmentally balanced.

Materials and methods

CXBH/By females were mated to BALB/cWt males to produce XO/XY and XO/XY/XYY mosaics. The mice were killed 8–11 days after birth and examined internally for signs of hermaphroditism. A sample of bone marrow was removed from the hermaphrodites and air-dried metaphase spreads were prepared by standard methods. The gonads were fixed in 3:1 ethanol:glacial acetic acid at 4°C overnight, washed in two changes of absolute ethanol and cleared in three changes of cedarwood oil. After three changes of 58°C MP paraffin wax, they were embedded in fresh molten wax. Sections were cut at 5 μm and mounted on glycerine/albumin-coated slides.

In situ hybridisation was carried out according to the method of Rossant *et al.* (1986) using the probe pY353B (Bishop *et al.* 1985), which hybridises specifically to a series of repetitive sequences on the Y chromosome. The entire plasmid was labelled using the BRL (Bethesda Research Laboratories) nick translation kit and BRL Biotin-11-dUTP. Biotin residues linked to hybridised DNA was detected using streptavidin/horse radish peroxidase followed by incubation in a solution of diaminobenzidine. Positive hybridisation, identifying Y-bearing cells, was recognised by a small, discrete area of brown precipitate in the nucleus.

Estimates of Y-bearing and non-Y-bearing cells in the gonads were achieved by scoring all the cells in randomly selected tubule and follicle cross-sections or, in the case of interstitial regions, using a gridded eyepiece graticule. These estimates of relative cell numbers are subject to bias due to the amount of false negative cells that occur in tissue sections. This happens when the microtome blade passes through nuclei leaving one part with, and the other part without, the target DNA sequence. To rectify this bias, correction factors were produced by scoring control sections from ovaries in which all nuclei were Y-bearing. These ovaries were from a 2-week-old XXY female mouse carrying the mutant 129 Y of

Lovell-Badge and Robertson (1990), which has lost *Tdy* (Gubbay *et al.* 1990).

Results

60 litters were produced from the CXBH/By× BALB/cWt cross comprising a total of 424 mice. Of these, 300 were female, 118 were male and 6 were hermaphrodites. In addition to these six, two other hermaphrodites were found at weaning in the BALB/cWt stock. Because of technical problems with the *in situ* hybridisation, quantitative data are presented for only five of the six CXBH/BALB hermaphrodites.

Sections of ovaries from these five CXBH/BALB hermaphrodites show a number of follicle cell nuclei with positive hybridisation to the Y chromosome (Fig. 1B). XY follicle cells are clearly able to proliferate since they appear as discrete patches in the multilayered follicles of the 3-week-old BALB/cWt hermaphrodites (Fig. 1C). The overall estimate of the proportion of labelled follicle cells is almost identical to the estimated proportion of XY cells in bone marrow, and is also close to that for ovarian stroma cells (Table 1). The 'interstitial' cells in the contralateral ovotestis or testis also have a similar proportion of XY cells to the bone marrow but the Sertoli cells show a very strong bias towards the XY component (Table 1 and Fig. 1D).

Discussion

These results demonstrate that XY follicle cells can form in the ovaries of XO/XY and XO/XY/XYY mosaic hermaphrodites. Since there are no grounds for expecting the XY component to be delayed relative to the XO component, we conclude that XY follicle cells can form even without a 'mismatch' between *Tdy* action and ovary determination. However, 'mismatch' remains the most reasonable explanation for the Ford *et al.* (1974) XX↔XY female chimaeras, particularly the one in which XY cells predominated and 97% of the presumed follicle cells were XY. Similar conclusions have been reached by Patek *et al.* (1991) based on an *in situ* analysis of a series of XX↔XY chimaeric ovaries.
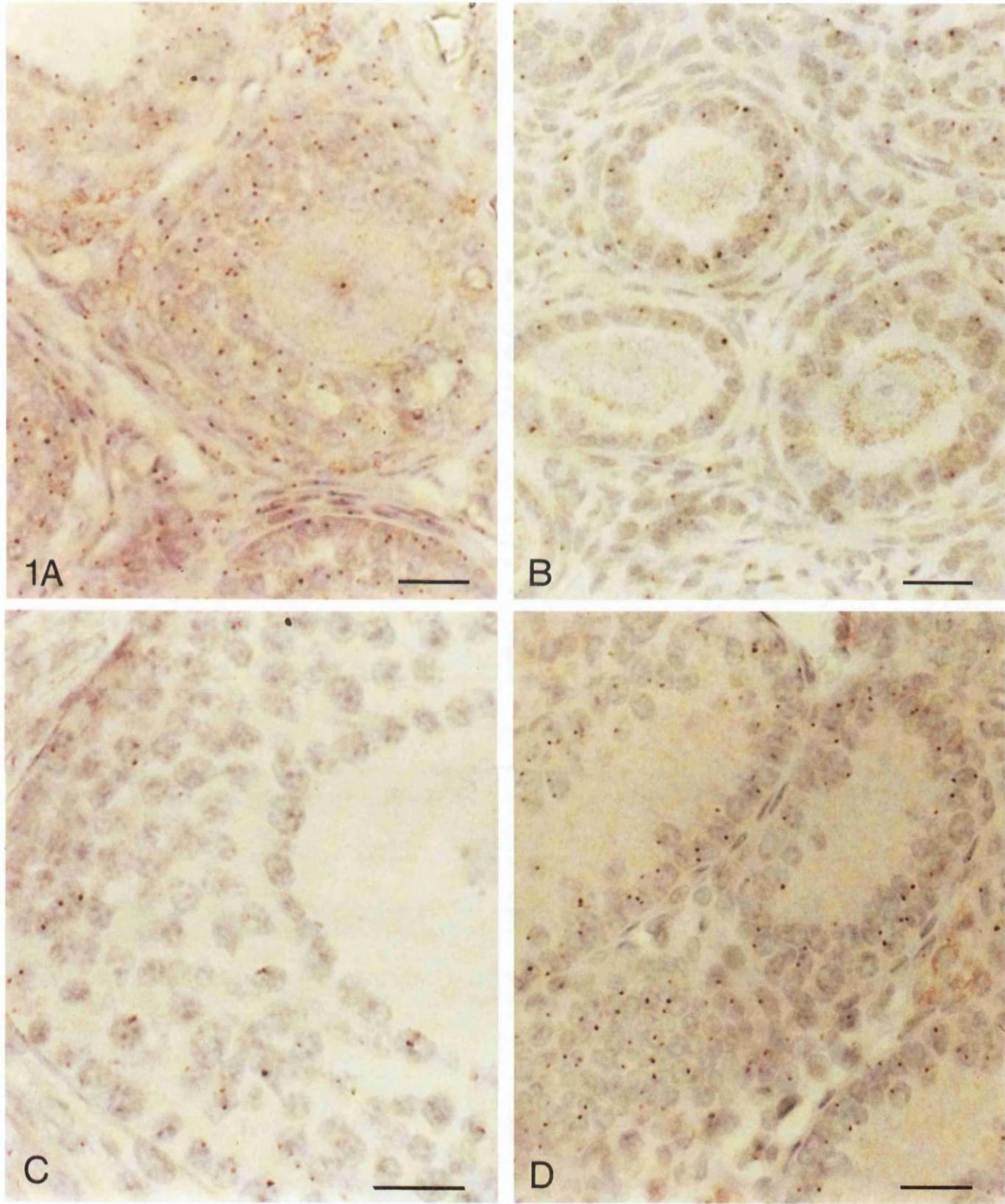
Fig. 1. (A) Section of ovary from a 2-week-old XXY female. Counts of labelled and unlabelled cells from these sections produced the correction factors for the mosaic ovaries. (B) Section of ovary from mosaic 5 showing three single-layered follicles, which include labelled XY follicle cells. (C) Multilayered follicle of a 3-week-old BALB/cWt mosaic ovary showing a patch of labelled XY follicle cells. (D) Testis section from mosaic 5, the contralateral gonad to the ovary shown in B. This testis had virtually no germ cells due to the high XO contribution but the Sertoli cells are nearly all XY. The patch of labelled cells that appear to be outside a tubule (bottom left) are Sertoli cells from a tangentially cut tubule. Bar, 20 μm.

How do we account for the formation of XY follicle cells in these mosaics? In an accompanying paper, we have shown that the 'cell-autonomous action of *Tdy*' model is incorrect: XX Sertoli cells do occur in fetal, prepuberal and adult XX↔XY testes, albeit at a low frequency (Palmer and Burgoyne, 1991). The strong bias in favour of XY Sertoli cells, even in fetal XX↔XY testes (and also in the ovotestes and testes analysed here) confirms the original conclusion of Burgoyne *et al.* (1988*a*) that *Tdy* acts in the lineage that forms Sertoli cells, but the fact that some XX Sertoli cells are formed means that, at some point between *Tdy* expression and the formation of fetal Sertoli cell cords, there is a step that is able to recruit a few XX cells. If some XX cells can be recruited by XY cells to form Sertoli cells, could some XY cells fail to be recruited when their numbers fall below a certain threshold? If the source of the 'recruiting factor' is the XY supporting cells, it seems implausible that they fail to recruit themselves. An alternative possibility is that the XY supporting cells are triggered to form fetal Sertoli cells by the action of *Tdy* but subsequently 'transdifferentiate' into follicle cells under the influence of ovarian factors. It may be a characteristic of Sertoli cells and follicle cells that they retain the ability to transdifferentiate into the reciprocal cell type. Burgoyne (1988, 1991) has argued that the XX Sertoli cell cords that form in ovarian tissue in various situations (in all cases preceded by oocyte loss) are the result of transdifferentiation of follicle cells. Transdifferentiation of Sertoli cells to follicle cells through contact with oocytes has recently been suggested as an explanation for the occurrence of oocytes surrounded by 'granulosa-like' cells in some T16/X*Sxr* testes (McLaren, 1991). An *in situ* Y-probe analysis of fetal XO/XY gonads might help to resolve this question.

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The *Mus musculus domesticus Tdy* allele acts later than the *Mus musculus musculus Tdy* allele: a basis for XY sex-reversal in C57BL/6-Y^{POS} mice

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Summary

Consomic C57BL/6 males, carrying either the *Mus musculus musculus*-derived C57BL/6 Y chromosome or the *Mus musculus domesticus*-derived Poschiavinus Y chromosome, were outcrossed to females of the inbred strains C3H/Bi and CXBH/By and to females of the random bred strain MF1/Ola. In a study at 12.5 days *post coitum*, gonads of XY^{C57} and XY^{POS} fetuses were assessed for the presence of testicular cords. It was found that XY^{POS} fetuses had a later onset of testicular development than XY^{C57} fetuses. Limb development, which was monitored as a measure of overall development, was unaffected by the strain of Y present. These data were supported by a longitudinal study in which the increased growth rate of the testes relative to undifferentiated gonads, was also shown to be delayed in XY^{POS} fetuses. The extent of the delay was estimated to be approximately 14 h. It is concluded that this delay in the onset of testicular differentiation must be caused by differences between the two Y-chromosome types, most probably allelic differences in the testis determinant *Tdy*.

Key words: Sex-reversal, testis determination, Poschiavinus, *Tda-1*, mouse embryo.

Introduction

In 1982, Eicher *et al.* described a case of XY sex reversal in mice, resulting from backcrossing the *Mus musculus domesticus*-derived Poschiavinus Y chromosome onto a C57BL6/J background. Adult C57BL/6-Y^{POS} mice show a range of sexual phenotypes, including fully sex-reversed XY females, true hermaphrodites (i.e. they have both ovarian and testicular tissue) and males; although the males have small testes and are of poor fertility. Observations on fetal litters from this cross have revealed that the XY individuals at 16.5 days *post coitum* (dpc) have either ovaries, or ovotestes with a varying ratio of ovarian to testicular tissue. It seems clear that most ovotestes go on to form testes in adult life, the regression of the ovarian component accounting for the reduced testicular size.

Further studies have shown that a number of other (but not all) *domesticus*-derived Y chromosomes generate some XY females when placed on the C57BL/6 background (Eicher *et al.* 1982; Nagamine *et al.* 1987a; Biddle and Nishioka, 1988). Eicher and Washburn (1986) have suggested that the '*domesticus*' Y carries a *Tdy* allele which is later-acting than that on the '*musculus*'-derived Y chromosome of the C57BL/6 inbred strain and that this delay sometimes enables the process of ovary determination to pre-empt Y action. To explain the requirement for a C57BL/6 background, Eicher and Washburn (1983, 1986) proposed that

C57BL/6 carries a recessive autosomal allele (*Tda-1^b*) which is in some way incompatible with the '*domesticus*' Y chromosome.

There are two possible models for how *Tda-1* is involved in this scheme (Eicher and Washburn, 1983). (1) This autosomal gene may be one element in the *Tdy*-initiated cascade of genes involved in testicular differentiation. (2) A second interpretation supported by Burgoyne (1988), proposes that it is an ovary-determining gene, with the C57BL/6 allele acting earlier than those of other inbred strains. When this is brought together with the late-acting '*domesticus*' *Tdy* allele, a 'timing-mismatch' may occur such that ovary determination may pre-empt testis determination. It is not hard to imagine how minor differences in the timing of expression of these two key genes could lead to the range of gonadal phenotypes observed in these mice. The 'timing mismatch' concept is discussed more fully in Burgoyne and Palmer (1991).

The aim of the present study was to test directly the hypothesis that the Poschiavinus Y chromosome is later acting than the C57BL/6 Y. Since C57BL/6 XY^{POS} fetuses develop ovaries or ovotestes, rather than testes, the effect of the Poschiavinus Y on the timing of testicular development was assessed in F$_1$ hybrids in which the paternal parent was C57BL/6 or C57BL/6-Y^{POS}. In these F$_1$ hybrids all the XY fetuses develop testes.

Materials and methods

Experiment 1

C57BL/6Mcl-Y^{POS} stud males were produced by backcrossing C57BL/6J-Y^{POS} males to C57BL/6Mcl females for more than 10 generations. Inbred C3H/Bi females were mated to stud C57BL/6Mcl-Y^{C57} and to C57BL/6Mcl-Y^{POS} males in a room in which the dark period was 7pm to 5am. Pregnant mothers were killed at 12 days 15 h *post coitum* (*pc*) (mating was assumed to have taken place at 12:00 midnight) and the fetuses placed into Hepes-buffered Eagle's minimum essential medium (EMEM, ICN Flow Ltd). Amniotic membranes were removed for sex chromatin analysis. Each membrane was placed near the top of a conical-bottomed centrifuge tube and flushed down into the tube with 3:1 methanol:glacial acetic acid fixative. Fetuses were killed by decapitation, the hind limbs staged (see below) and the gonads removed. The gonads were photographed under EMEM using a Wild M400 Photomakroskop. Photographic prints of male and female gonads from both crosses were randomized and scored blind for the presence of testicular cords.

The hind limbs were staged according to the developmental series of McLaren and Buehr (1990), which covers the period spanning sexual differentiation in the mouse. This scheme was subdivided to provide maximum sensitivity and extended to cover the later stages found at this time point and the stages of the subsequent two days studied in experiment 2 (see Fig. 1).

The amniotic membranes were processed using an adaptation of the method of Evans *et al.* (1972) for yolk sac preparations (Burgoyne *et al.* 1983). Tubes containing the amniotic membranes were centrifuged briefly at 1000 revs min^{-1} and excess fixative decanted off (being careful to retain the amnion). The tubes were inverted on a paper towel and tapped on the bench to bring the amnion 1 or 2 cm away from the base of the tube. A single drop of 60 % glacial acetic acid in distilled water was applied directly onto the amnion in
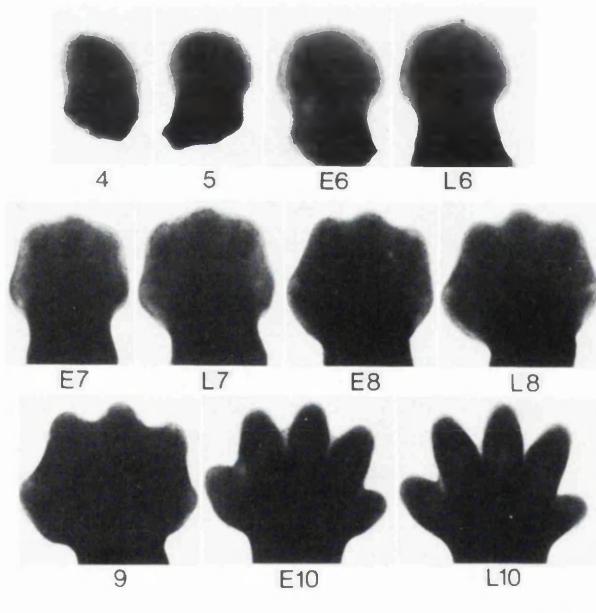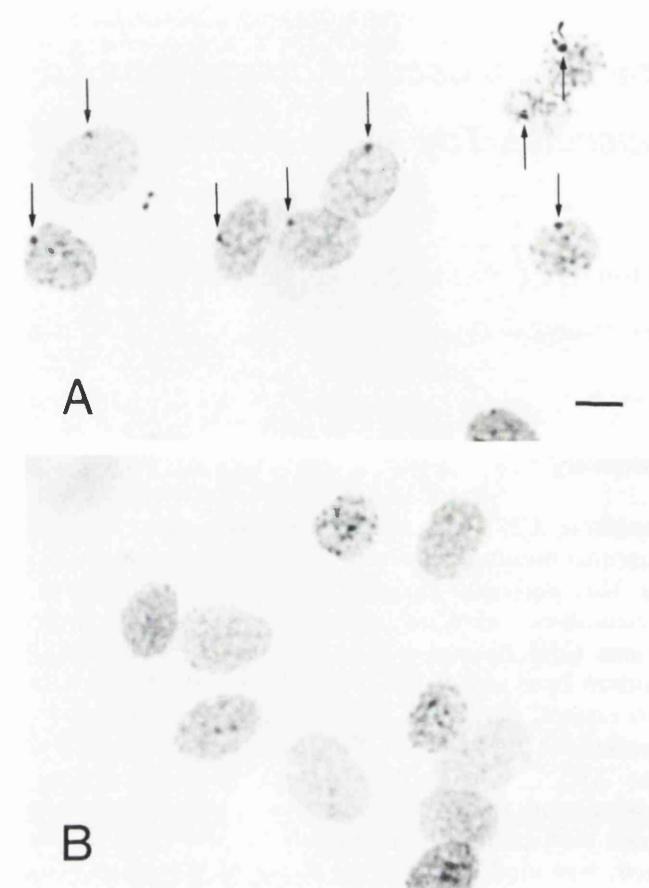

Fig. 2. Disaggregated amnion cells (A) positive and (B) negative for sex chromatin (arrows). Bar, 10 μm.

order to dissociate the cells. After 60 s, the action of the 60 % acetic acid was blocked by the addition of approximately 1 ml of fixative. The cells were then centrifuged at 1000 revs min^{-1} for 5 min. Excess fix was decanted off and the tube inverted for a few seconds to allow the tube to drain, without letting the cells dry completely. When the tube was righted again, the cells were resuspended in the small amount of fixative that ran down to the bottom from the sides of the tube. If this process was judged correctly, the cells could be concentrated into a single drop of fluid. This single drop was spotted onto a clean glass slide. When the cells had air-dried, one drop of 1 % aqueous toluidine blue was spotted onto the cells and a coverslip pressed on. These preparations were scored immediately for the presence or absence of the sex chromatin (see Fig. 2).

Experiment 2

Consomic C57BL/6Mcl-Y^{C57} and C57BL/6Mcl-Y^{POS} stud males were mated to females of the inbred strain CXBH/By and to females of the random-bred albino stock MF1/Ola. Once again the dark period was 7pm to 5am and mating was assumed to have taken place at 12:00 midnight. In order to study the gonadal growth rates in the fetuses produced by these crosses, pregnant females were killed at time points between 11.5 and 14.5 d*pc*. Amniotic membranes were fixed for sexing and the hind limbs were scored as before.

Gonads were dissected out and scored under the dissecting microscope for the presence of testicular cords. The length and breadth of each gonad was measured to provide continuous variables to study the relative growth rates. However, we found that the length measurements were highly

Fig. 1. Hind limb stages of fetuses between 11.5 and 14.5 days *post coitum*. E, early; L, late. In general, stages 4 and 5 were found during the 12th day of pregnancy, 5 to L7 during the 13th, L7 to 9 during the 14th and 9 to L10 during the 15th. Bar, 1 mm.

variable at the earlier stages due to difficulties in delineating the ends of the gonads. The breadth was better defined and showed a relatively smooth exponential growth curve. This was therefore chosen as the representative parameter for gonadal growth. Breadth was measured by gently holding the mesonephros with forceps so that the gonad was uppermost. The width across the widest part was measured using a graticule fitted in the eyepiece of the dissecting microscope.

Results

Experiment 1. Transverse study: fetal litters examined at 12 days 15 h pc

9 litters of fetuses produced from the cross C3H× C57BL/6-Y^{C57} were found to contain 19 individuals typed as sex-chromatin negative. In the C3H×C57BL/ 6-Y^{POS} cross, a total of 8 litters yielded 15 sex-chromatin-negative individuals. These sex-chromatin-negative fetuses are assumed to be XY although in rare instances they could be XO. All but 2 of the XY^{C57} males were scored as having visible testis cords whereas none of the XY^{POS} fetuses showed any sign of testicular differentiation at this time point. The probability of this occurring by chance ($\chi^2=26.8$, 1 degree of freedom) is $P=0.00014$.

When these data are plotted against hind limb stage (see Fig. 3), individuals that are at the same stage of limb development show a clear difference in testicular development dependent upon the source of the Y chromosome. A comparison of the distributions of the hind limb stages demonstrates that, as far as the sensitivity of this measure will allow, there is no significant difference between the two male populations with respect to overall fetal development ($\chi^2=3.01$ 4df. $P=0.56$).

Experiment 2. Longitudinal study

The delay in the onset of testicular differentiation is also found in the XY fetuses produced from crosses of the consomic C57BL/6-Y^{C57} and C57BL/6-Y^{POS} males
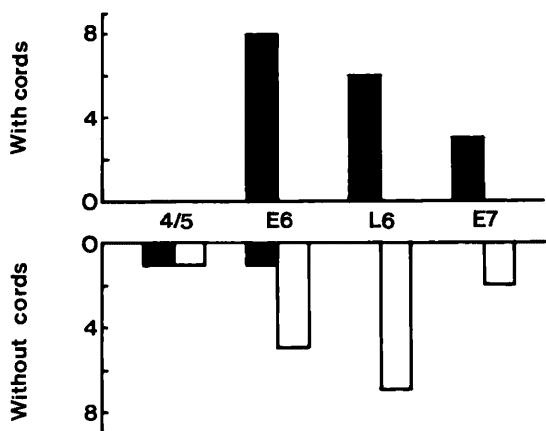

Fig. 3. Frequency of sex-chromatin-negative individuals with and without visible testis cords at 12 days 15 h *post coitum*, plotted against hind limb stage. Solid bars, C3H× C57BL/6-Y^{C57} F_1; open bars, C3H×C57Bl/6-Y^{POS} F_1.
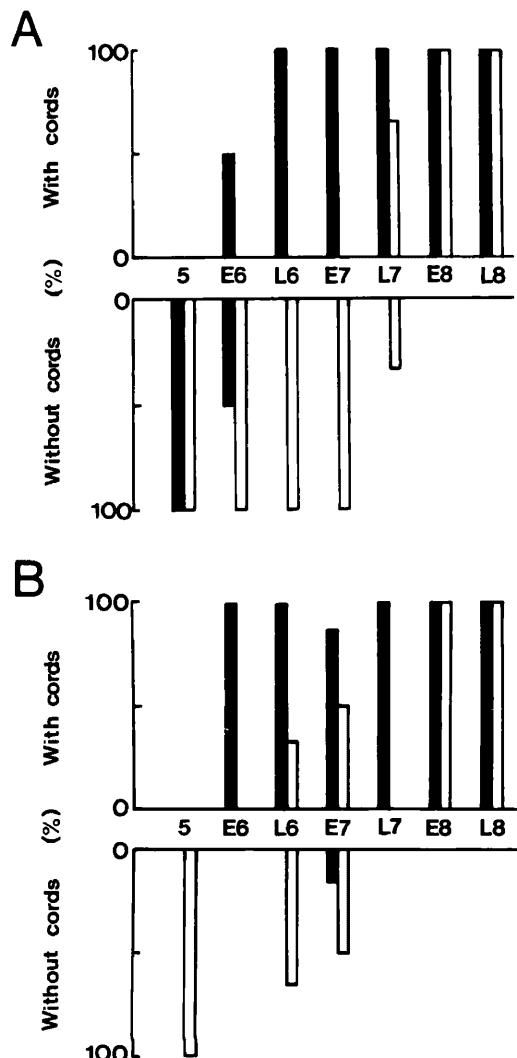

Fig. 4. Percentage of sex-chromatin-negative individuals with and without visible testicular differentiation at a range of hind limb stages. Solid bars, XY^{C57} fetuses; open bars, XY^{POS} fetuses. (A) F_1 males produced from CXBH outcross; 33 XY^{POS} and 28 XY^{C57} fetuses. (B) F_1 males from MF1 outcross; 28 XY^{POS} and 56 XY^{C57} fetuses. Missing bars indicate that no sex-chromatin-negative individuals were found with that hind limb stage. The dip in the XY^{C57} data at E7 in the MF1 cross is caused by a single individual. It is possible that this fetus was mistyped or may have been a rare XO.

with CXBH/By or MF1/Ola females (see Fig. 4). These data also show that all the XY^{POS} fetuses in both crosses develop testes from late 12.5 dpc onwards and no signs of XY sex reversal were found in a total of 47 sex-chromatin-negative individuals between 13.5 and 14.5 dpc.

The gonadal growth rate data are shown in Fig. 5. The mean gonad breadth measurements are log-transformed to improve the linearity of the resulting regression lines, decreasing the residual variance. Regression lines and their associated errors were computed using litter means of gonadal breadth for each sex, weighted according to the number of females or males in the litter. For the males, only the data between 12.5 and 14.5 dpc are included to be as certain

Fig. 5. Regression lines of gonadal growth in males and females between 11.5 and 14.5 days *post coitum*. Closed circles, litters fathered by C57BL/6-Y^{C57} males; open circles, litters fathered by C57BL/6-Y^{POS} males. Each point is the litter mean (for that sex) of the individual gonadal breadth means. Regression data are compared in each case with respect to the Y-chromosome-type of the father using an analysis of covariance. The residual variances are not significantly different in any of the four tests. (A) Females produced from CXBH outcross. Difference between slopes, $P=0.59$; between elevations, $P=0.86$. Data are pooled to produce one regression line. (B) Males produced from CXBH outcross. Difference between slopes, $P=0.67$; between elevations, $P=0.00009$. The combined slope is used in plotting the two regression lines. (C) Females produced from MF1 outcross. Difference between slopes, $P=0.321$; between elevations, $P=0.286$. Data are pooled to produce one regression line. (D) Males produced from MF1 outcross. Difference between slopes, $P=0.203$; between elevations, $P=0.00126$. The combined slope is used in plotting the two regression lines.

as possible that the points fall during the phase of increased growth that is characteristic of testes. Some outliers have been removed using a test for outliers in regression analysis. Regression lines of gonadal growth in XY^{C57} and XY^{POS} fetuses were compared for both the CXBH and the MF1 outcross using an analysis of covariance. This test compares separately the variance from the mean due to the elevation, the slope and the residual error.

The regression lines for the XX fetuses produced by the two consomic fathers were also compared for each outcross. These data (see Fig. 5 legend) demonstrate that, for both the MF1 cross and the CXBH cross, the females produced by the two consomic males show no significant difference in the residual variance, the variance due to the slope or the variance due to the elevations. This is to be expected since there is no genetic difference between the F$_1$ females produced by

each paired cross. It is therefore justified to pool these two populations within each outcross.

A comparison of the regression lines for the males (XY^{C57} *versus* XY^{POS}), on the other hand, although revealing no significant difference in residual variance or slope, shows a highly significant difference between elevations in both crosses (see Fig. 5 legend). This supports the conclusions of experiment 1 and shows that the late onset of testis cord formation in XY^{POS} fetuses is correlated with a delayed onset of increased growth.

Discussion

Both experiments demonstrate that the Poschiavinus Y chromosome causes a later onset of testicular development than the C57BL/6 Y chromosome. The simplest explanation for this disparity is a difference between the two *Tdy* alleles. This may be caused by differences in the regulatory elements or the structural sequence of *Tdy*. This is currently being tested by examining the structure and expression of the Poschiavinus *Sry* since it is now established that *Sry* is *Tdy* (Koopman *et al.* 1991).

These findings provide an explanation for the results of Nagamine *et al.* (1987*b*) who found that when C57BL/6 males with a *domesticus*-derived Y chromosome are outcrossed to various inbred females, a proportion of the F_1 male fetuses produced show 'abnormalities' of testicular development. In the light of results presented here, it seems likely that these 'abnormalities' are due to a delay in testicular cord formation caused by a late-acting *Tdy* allele.

From the growth rate data, it is possible to model the growth kinetics of male and female gonads and thereby estimate the timing difference between the action of the two Y chromosomes (see Fig. 6). If *Tdy* is the first gene to influence male and female gonads differentially, it is logical that prior to *Tdy* action the growth rates of the indifferent XX and XY gonads should be the same. After *Tdy* expression, however, the gonadal growth of XY fetuses increases dramatically but the gonads of XX fetuses continue to grow at a slower rate. The gonads of XY fetuses with delayed Y action would be expected to continue growing at the same rate as female gonads until expression of the delayed *Tdy* has occurred. Therefore, to estimate the timing difference it is necessary to find where the XY^{C57} and XY^{POS} testicular growth curves depart from the XX curve. Calculations based on this model show that the points of intersection for the CXBH outcross are: XY^{C57} fetuses, 269 h *post coitum* (h*pc*); XY^{POS} fetuses, 283 h*pc*; and for the MF1 outcross XY^{C57}, 270 h*pc*; XY^{POS}, 284 h*pc*. The estimate for the timing difference between the action of the two Y chromosomes is therefore 14 h. The estimated points of intersection should be regarded with caution since they result from extrapolations that may misrepresent the true shape of the curves. In spite of this caution, the timing for the C57BL/6 Y (early on the 12th day) fits well with the timing of expression of *Sry* which begins to
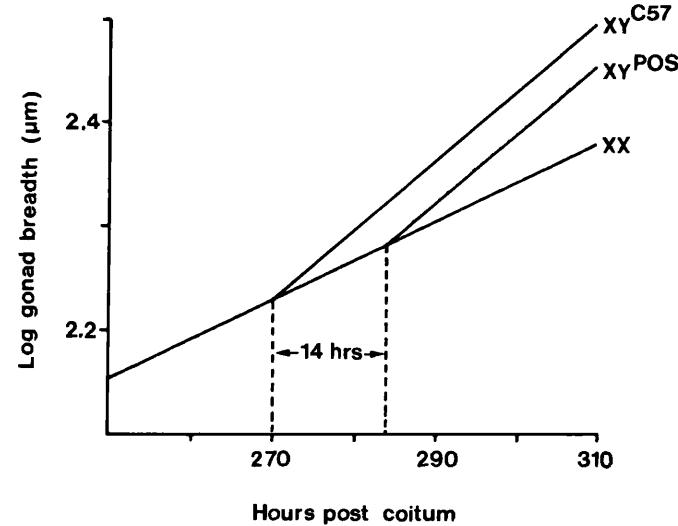

Fig. 6. Model of gonadal growth in females and in males carrying either the Y^{C57} or the Y^{POS} chromosome. The initial single line represents the growth rate of the indifferent XX or XY gonads. After expression of either of the two *Tdy* alleles the testicular growth rate increases and departs from that of the female gonad. The testicular growth rate is the same in Y^{C57}-bearing and Y^{POS}-bearing fetuses, only the time of departure differs. The points of intersection are calculated from the regression lines shown in Fig. 5. For two lines with slopes m_1 and m_2 and elevations c_1 and c_2, at the point of intersection $X = (c_2-c_1) \div (m_1-m_2)$.

be expressed during the 11th day, peaks on the 12th and declines during the 13th day (Gubbay *et al.* 1990; Koopman *et al.* 1990).

XY fetuses have been shown to have an overall developmental advantage over their XX siblings (Seller and Perkins-Cole, 1987), males being about $1\frac{1}{2}$ hours ahead of females (Burgoyne *et al.* unpublished). This developmental advantage is now known to be a consequence of a retarding effect of the paternally imprinted X chromosome in XX fetuses, rather than an accelerating effect of the Y chromosome (Thornhill and Burgoyne, unpublished). Because it is not Y-linked, this developmental advantage of XY fetuses should not affect the estimate for the difference in timing of Y^{POS} and Y^{C57} action. However, it does complicate the interpretation of XX *versus* XY gonadal size differences.

Measurements of gonadal volume in rat fetuses have shown that XY gonads are larger than XX gonads prior to the formation of testis cords (Lindh, 1961; Mittwoch *et al.* 1969). This led Mittwoch (1969, 1989) to propose that an early growth advantage of XY gonads, rather than a single gene 'switch', forms the basis of the sex-determining mechanism in mammals. However, there is no previous evidence to show that these 'within litter' differences between XX and XY gonadal volume are not simply a manifestation of the fact that males are ahead of females. Our data, on the other hand, indicate that the increased growth, characteristic of testes, precedes testis cord formation. Nevertheless, this increased growth is after the onset of *Sry* expression so

it remains a moot point whether the increased gonadal size is simply a consequence of *Sry* expression, perhaps caused by the differentiation and growth of Sertoli cell precursors (Jost *et al.* 1973; Magre and Jost, 1980), or whether it is a requirement for determining the gonad as a testis.

The results presented here appear to be contradicted by a recent study on the onset of Mullerian inhibitor, AMH (or MIS), production in fetal testes (Taketo *et al.* 1991). The authors compared the onset of AMH production in SJL XY^{dom} fetuses with control SJL XY^{C57} fetuses and found no difference. It is possible that the assay system used and time intervals of 24 h, were not sensitive enough to detect a timing difference of only 14 h.

The delay in action of the Poschiavinus Y chromosome supports, in part, the hypothesis that sex-reversal in C57BL/6-Y^{POS} individuals is caused by a developmental mismatch involving a late-acting *Tdy* allele in conjunction with an early-acting ovarian program. It remains to be seen whether the other component of C57BL/6-Y^{POS} sex reversal, the recessive C57BL/6 allele of *Tda-1*, is a relatively early-acting gene in the ovary-determining pathway.

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