MAMMALIAN SEX DETERMINATION: APPRAISING THE ROLE OF THE AUTOSOMES

by

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Submitted to the Department of Biology in Partial Fulfillment
of the Requirements for the Degree of

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at the

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ABSTRACT

We took a genetic approach to study the incompatibility between autosomal sex-determining genes in certain inbred *Mus musculus musculus* strains and the Y–chromosome–linked sex determinant of the wild mouse *Mus domesticus poschiavinus*. We present evidence that multiple sex-determining genes are located on mouse autosomes. Although determination of the precise nature of these genes must await their molecular characterization, it is timely to review the theoretical basis of our expectations for autosomal vs. sex chromosome-linked (allosomal) sex-determining genes. The existence of these distinct classes of genes is clearly established in nematodes, fruit flies, mice, and humans. A prevailing notion is that the autosomes and the sex chromosomes cooperate in the determination of sex, co-evolving under the common constraint of producing fertile individuals to reproduce the next generation. Recent results in fruit flies have led to a re-assessment of this notion based on the fundamentally different nature and relative paucity of autosomal sex determining genes. The inference from this invertebrate system is that autosomal and allosomal primary sex determining genes evolve under very different evolutionary constraints by virtue of their disparate chromosomal

locations. Specifically, it is plausible that allosomal sex determining genes are selected during evolution exclusively for their sex-determining function, whereas the functional constraints on autosomal sex-determining genes may pertain to other processes as well as sex-determination. In support of this notion, recent results show that the primary sex determinant in mammals (*Sex-determining region-Y*, or *Sry*) appears to evolve extremely rapidly in several species, suggesting it may be subject to specialized evolutionary pressure in comparison to autosomal sex determining genes. Taken together, these findings suggest the existence of a functional and evolutionary dichotomy between autosomal and allosomal sex determining genes. Therefore, to lend unique insights to this appraisal of the role of the autosomes vs. the sex chromosomes, I will assess current understanding of mammalian sex determination in light of these evolutionary hypotheses.

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DEDICATION

ॐ

कायेन वाचा मानसेनदरडयैरवा
बुद्धयातमना वा प्रकृतेः सवाभवत
करोंम याद्यातसकलम परसमर्ई
नारायणायेती समरपयामी

My body, speech, mind, and senses
intellect, and soul, all arising from innate nature,
Whatever actions I perform with all these
I dedicate to the Lord.

–Sanskrit prayer

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Chapter One

Appraising the Role of the Autosomes in Mammalian Sex Determination

by Jodeane Pringle

Introduction

The long list of thinkers who theorized about the nature and determination of sex in pre-modern times includes Empedocles (*c.* 430 B.C.), Anaxagoras (*c.* 428 B.C.), Democritus (*c.* 370 B.C.), Aristotle (*c.* 322 B.C.), Leeuwenhoek (1677), Drelincourt (who recorded 262 "groundless hypotheses" regarding sex determination *c.* 1685) and finally Geddes and Thomson (1890). Early theories were varied and imaginative, and for the most part centered on the influence of the environment in the determination of sex. Ultimately they proved wrong because the observational or experimental tools available to the early theorists were far too crude to answer the questions they asked. In addition, they lacked the theoretical framework of Mendelian genetics, a crucial piece of the puzzle. We now know that sex determination is fundamentally a genetic process, not influenced by the environment, and yet far more fantastic than the pre-formationists like Leeuwenhoek ever suggested. In this chapter, I pose four questions about sex determination. Some have answers, and some do not; but as they are considered, I hope to demonstrate both the extraordinary amount of progress the century intervening between Geddes and Thomson brought in this field, as well as the scope of the questions which remain today. I will begin by defining terms and describing the development of sexual dimorphism in the embryo and the adult, including the classic experiments of Jost which defined the role of the gonadal hormones in determining the sexual fate of the reproductive tract. Next I will consider the major 20th C paradigm shift in sex determination, the recognition that sex chromosomes and sex-linked genes are sex-determining in mammals. A review of the experiments leading to the isolation of the Y-linked sex determinant, *SRY*,

highlights the importance of making meaningful predictions concerning the function of sex-determining genes. Next I pose the theoretical question of whether all sex-determining genes need reside on the sex chromosomes, and follow with a review of the experimental evidence for sex-determining genes on autosomes. In the next section, I give the topic of meaningful predictions about the functions of sex-determining genes a more formal treatment. At this point, it will be clear that nearly all modern investigations of mammalian sex determination have focused on the sex chromosomes, and it is possible that fundamental differences exist between autosomal and sex-chromosome-linked (allosomal) sex-determining genes. In the Future Directions section, therefore, I will revisit this theme to argue from a theoretical standpoint that autosomal and allosomal sex-determining genes differ in certain key properties, which should serve to focus future investigations.

Section I

What is sex determination?

Some definitions first: what is sex?

The question *what is sex determination?* seems straightforward enough, but the obvious answer–the process by which sex is determined–is not informative for our purposes. But it does lead to the question, *what is sex?*, which is crucial to this analysis. Once again, though, there is no simple answer. Consulting a good dictionary does little by way of clarification:

> *sex* either of the two divisions of organic beings distinguished as male and female respectively; the males or the females...viewed collectively (Oxford English Dictionary 1989, *s.v.* sex).

Obviously we must check further:

> *female* belonging to the sex which bears offspring

(Oxford English Dictionary 1989, *s.v.* female).

male belonging to the sex which begets offspring, or performs the fecundating function of generation (Oxford English Dictionary 1989, *s.v.* male).

This would seem like progress since we now have one characteristic for each of the sexes; that is, the female bears offspring, while the male begets them. Unfortunately, definitions are often circular, for example:

beget to procreate, to generate: usually said of the [male] father...(Oxford English Dictionary 1989, *s.v.* beget).

Perhaps more instructive are the etymologies of these words. For female, the Latin roots are *felare* to suck, and *femina* woman, which is akin to the Old English *delu* nipple and Old High German *tila* female breast (Webster's Third New International Dictionary 1981, *s.v.* female). The etymology for male is somewhat obscure (Oxford English Dictionary 1989, *s.v.*mascle). However, we've learned that females bear offspring and have breasts for them to suckle, whereas males beget the offspring. At the root of these definitions are the features which distinguish male from female, collectively known as sexual dimorphisms. In genetic terms, sex is a phenotype, a collection of forms (male or female) taken by a group of characters (sexually dimorphic features) in a specific individual. Unfortunately for the geneticist, sex in mammals is a rather complex phenotype, with sexually dimorphic features known in many organ systems. Clearly, a cataloguing of all observable characteristics which distinguish male from female for each individual under study would be impossible. For the purposes of studying sex determination, though, dimorphism of the reproductive system is the obvious starting point.

The identity of the gonad is the key determinant of sex

In mammals the ovary in the female and the testis in the male exhibit dramatic differences in cellular architecture and identity upon histologic

examination. Both organs have three regions, an inner hilum composed of nerves, blood vessels, and connective tissue; a central medulla; and an outer cortex. Here the similarities end. The adult ovary is a small (4x3x1 cm), pelvic organ with a highly developed cortex and a relatively featureless medulla. There are no tubules or 'cords'(tubules which have not canalized) evident. See **Figure 1–1**. The germ-cell-producing functions of the ovary are located in the cortex, which consists of stromal cells, oocytes enclosed in cellular complexes called follicles, and a thin epithelial covering called the germinal epithelium. Mature follicles lyse at ovulation, releasing the egg from the surface of the ovary. The cell types which uniquely identify the ovary to the histologist are the oocyte, the granulosa cell of the follicle, and the steroidogenic interstitial 'theca interna' cell. (Ross and Schreiber 1986) Starting at 5.5–6 months gestation in humans, oocytes and granulosa cells associate and become surrounded by a membrane or basal lamina, forming the primordial follicles. The granulosa cells of the follicle are somatic derivatives, and they are sometimes referred to as supporting cells because they contact the membrane of the oocyte, forming gap junctions for the passage of nutrients and other material. Immature granulosa cells are spindle-shaped, but assume a cuboidal shape as they differentiate. Once formed, the primordial follicles embark upon a complicated process of growth, maturation, or degeneration, a description of which is beyond the scope of this work (but see **Figure 1–1**). Suffice it to say that follicles present a unique cellular architecture which may be identified by histologic examination of an ovary biopsy. The theca cells are also somatic derivatives, but they are located in the stroma between follicles, outside of the basal lamina. They too change from spindle-shaped to cuboidal as they differentiate, but unlike granulosa cells, they acquire the abundance of smooth endoplasmic reticulum (SER) which is characteristic of cells that produce steroid hormones.

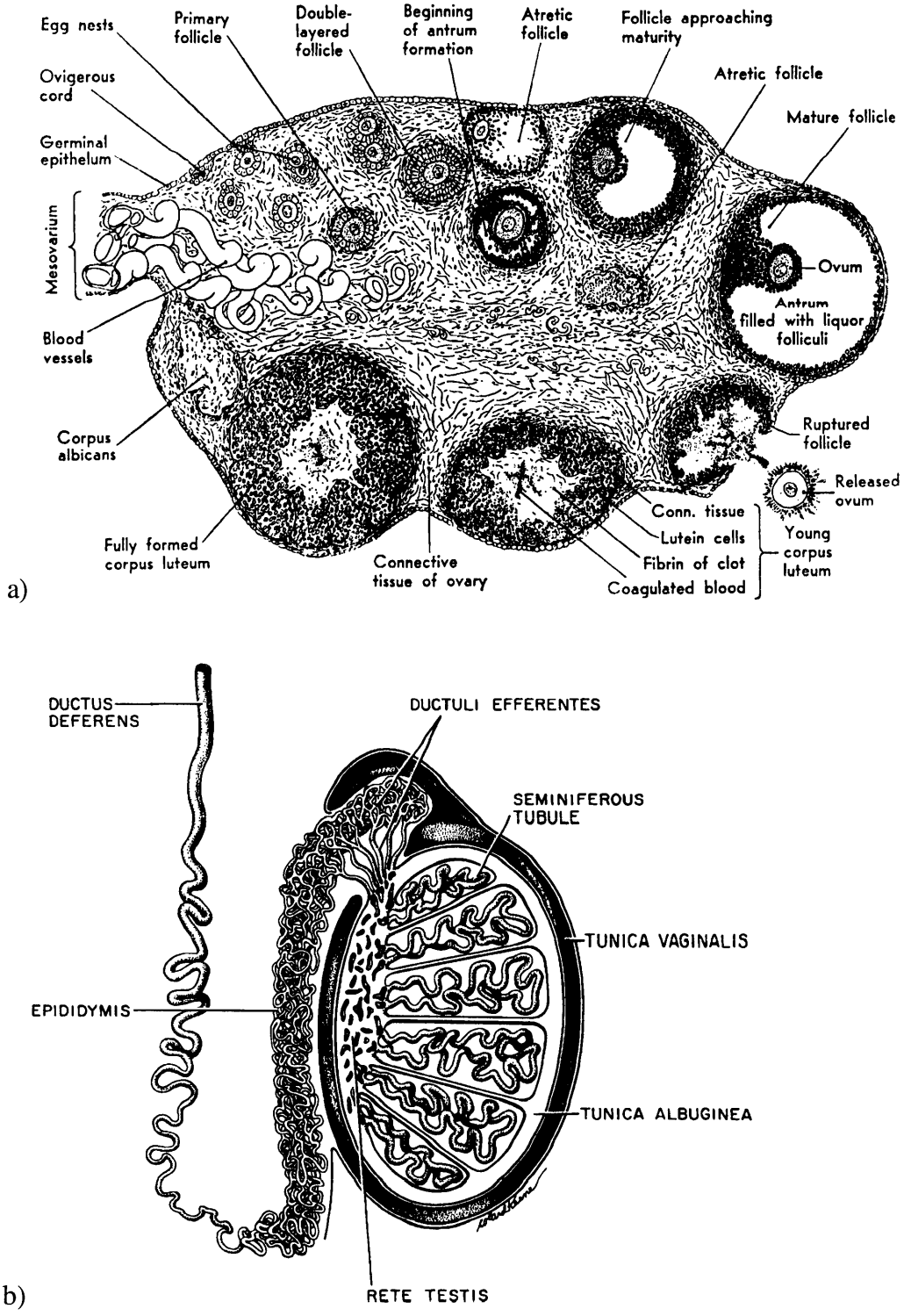

Figure 1–1. Features of the differentiated ovary and testis. a) the ovary b) the testis. Reproduced from *Human Embryology*, 3rd edition, 1968, by B. M. Patten, with permission from McGraw-Hill, Inc.

The adult testis differs from the ovary in many ways. It is larger, resides outside the pelvis in the scrotum, and has a highly developed medulla compared to its cortex. It is enclosed by a dense extracellular matrix or capsule called the tunica albuginea, which separates the surface epithelium from the cortex. The bulk of the organ is composed of convoluted tubules where sperm are produced. These seminiferous tubules lead to the tubules of the rete testis and the efferent ducts, which conduct mature sperm into the vas deferens, and thence out of the body. The differentiated cell types unique to the testis include male germ cells and two kinds of somatic cell derivatives, as for the ovary. In the testis, the germ cells mature into spermatocytes which are nourished by the Sertoli cells (also called supporting or 'sustentacular' cells), while the interstitial Leydig cells specialize to produce androgens. As for the oocytes and granulosa cells, the spermatocytes and Sertoli cells are closely associated, making frequent gap junctional contacts. During development, the primordial germ cells associate with Sertoli cells in the primary testis cords, solid precursors of the seminiferous tubules which canalize during puberty. The Leydig cells are notable for their localization between the testis cords or tubules, and the great abundance of SER in their cytoplasm, which attests to their function as the testosterone-producing cells of the testis. Clearly then, the testis exhibits unique cellular architecture and identity, with differentiated cell types that distinguish it from the ovary.

The internal and external genitals are also dimorphic

The form of the internal and external genitals, as well as the breasts, may also be considered as indicators of sexual phenotype. As we've seen, the gonads are dimorphic in their cellular identity as well as their position within the body. Although the gonads form in the abdomen, by the time of birth, the testis has normally descended into the scrotum, while the ovary is located in the pelvis. The other internal structures which show marked sexual dimorphism are derived from

the reproductive ducts. The male normally has a vas deferens, epididymis, and seminal vesicle on each side. The duct derivatives in the female are the two oviducts, the uterus, cervix and upper vagina. The male also has a prostate and bulbo-urethral glands which both derive from the urethra. Vestigial structures sometimes present are the appendix testis, paradidymis, and appendix epididymis in males, and the epoöphoron, paroöphoron, and Gärtner's cyst in females. These structures are remnants of the embryonic duct system, which includes both male and female components in each embryo. Most of the vestiges result from the incomplete degeneration of the duct appropriate to the opposite sex, and are not considered to be evidence of abnormal sexual development. We shall consider the development and degeneration of the embryonic ducts in some detail later in this section. Externally, the anatomy of the adult male penis and scrotum is strikingly different from the clitoris, labia minora, and labia majora of the female. However, the fusion line which extends from the glans along the posterior aspect of the penis to the anus is evidence of the fusion of the male embryo's urethral folds, which remain separate in the female, leaving the urogenital groove open to form the urethral and vaginal openings. Finally, the breasts and mammary glands depend for their development upon the female hormonal environment, and thus are markedly developed in females after puberty but not in males.

A standard nomenclature is lacking in the field of sex determination

The overall picture which emerges from this brief description is one of striking dimorphism between the sexes. However, abnormalities of sexual development can make sex seem like a continuum rather than a two-state system. What is the definitive feature of sex? This often depends on the purpose of one's investigation. For instance, the form of the external genitals is the critical determinant of sexual phenotype in some situations, such as the birth of a baby. In other situations, the identity of the gonad may be the sole criterion applied to assign

sex. A good example of this is the mouse genetic study that is the topic of the next chapter. To counter such situational definitions of sex, various authors (Langman 1975, Wilson and Goldstein 1975, Polani 1981, Ferguson-Smith 1992) have adopted a 'standard' terminology to describe the sexual phenotypes of humans. The scheme (see below) is practical for the initial categorization of abnormalities of sexual development, *e.g.* in a clinical setting; however, the terminology is cumbersome and has never been universally applied. This is perhaps not surprising given the complex nature of the sexual phenotype, but nonetheless reflects a persistent difficulty in the study of sex determination, which is the lack of a robust terminology. We propose that sex is best defined by the form of the gonad. If the gonad is a testis, the individual is a male. If an ovary, female, and if both tissue types are present, the individual is a hermaphrodite. This is simpler than the older nomenclature which includes both true and psuedohermaphrodites. In this scheme, an individual with mixed gonadal histology is considered a true hermaphrodite. Male or female pseudohermaphrodite individuals are those with normal testes or ovaries, despite some discrepancy between the gonad and another feature of their sexual development. We will consider these individuals males or females, respectively, because their gonadal development is normal. The reason for the preeminence of gonadal type amongst the several criteria which may be applied will become clear shortly when we discuss Alfred Jost's work. But unless otherwise qualified, the terms male, female, and hermaphrodite refer only to the testicular, ovarian, or mixed composition of an individual's gonads.

Each sex has a unique chromosome constitution

One final aspect of sex must be introduced here. In mammals, each sex has a unique chromosome constitution, often depicted as a karyotype. For example, normal human males have a total of 46 chromosomes, two of which are the X and Y chromosomes, which are the only 'heteromorphic' (unlike) pair. See **Figure 1–**

2 (Tjio and Levan 1956) . The standard notation for this karyotype is 46, XY. Normal human females also have 46 chromosomes, including a pair of X chromosomes, but no Y chromosome. Thus the normal female karyotype is denoted 46, XX. A correspondence between sex and karyotype in humans was first postulated by several investigators early in this century. Nonetheless, because human chromosomes were difficult to visualize at best, and mitotic tissue was not readily available until the advent of tissue culture techniques, confirmation of both the chromosome number and that the Y chromosome pairs with the X chromosome during spermatogenesis had to wait until the mid 1950s (Ford and Hamerton 1956, Tjio and Levan 1956), more than thirty years after the Y chromosome was first observed (Painter 1921). Because of the association with sex, the X and the Y chromosomes are called sex chromosomes, to distinguish them from other heteromorphic chromosomes, which may have no relation to sex, and the homomorphic autosomes. I will use the term *allosomes* to refer to the sex chromosomes collectively[1] . The XY male can produce X-bearing or Y-bearing sperm, and is therefore the heterogametic sex. Likewise, the female is said to be the homogametic sex, capable of producing only X-bearing eggs. Subsequent studies of aneuploid individuals offered convincing evidence that sex determination is chromosomal in mammals, and this recognition presaged an exciting search for the sex determining factor(s) of the sex chromosomes, which is the topic of the following section. As we shall see, there are exceptional individuals whose sex and karyotype are discordant; in fact, both XX and XY karyotypes have been reported in males, females and hermaphrodites. Hence the chromosome constitution is not part of the sexual phenotype. It is tempting to consider it part of the genotype, but

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[1]A note about terminology: Strictly speaking, the term *allosomes* denotes heteromorphic chromosomes in general, regardless of an association with sex. I will use this term to refer to the sex chromosomes collectively because they are generally the only heteromorphic chromosomes in the species I am considering, such as mice and humans.

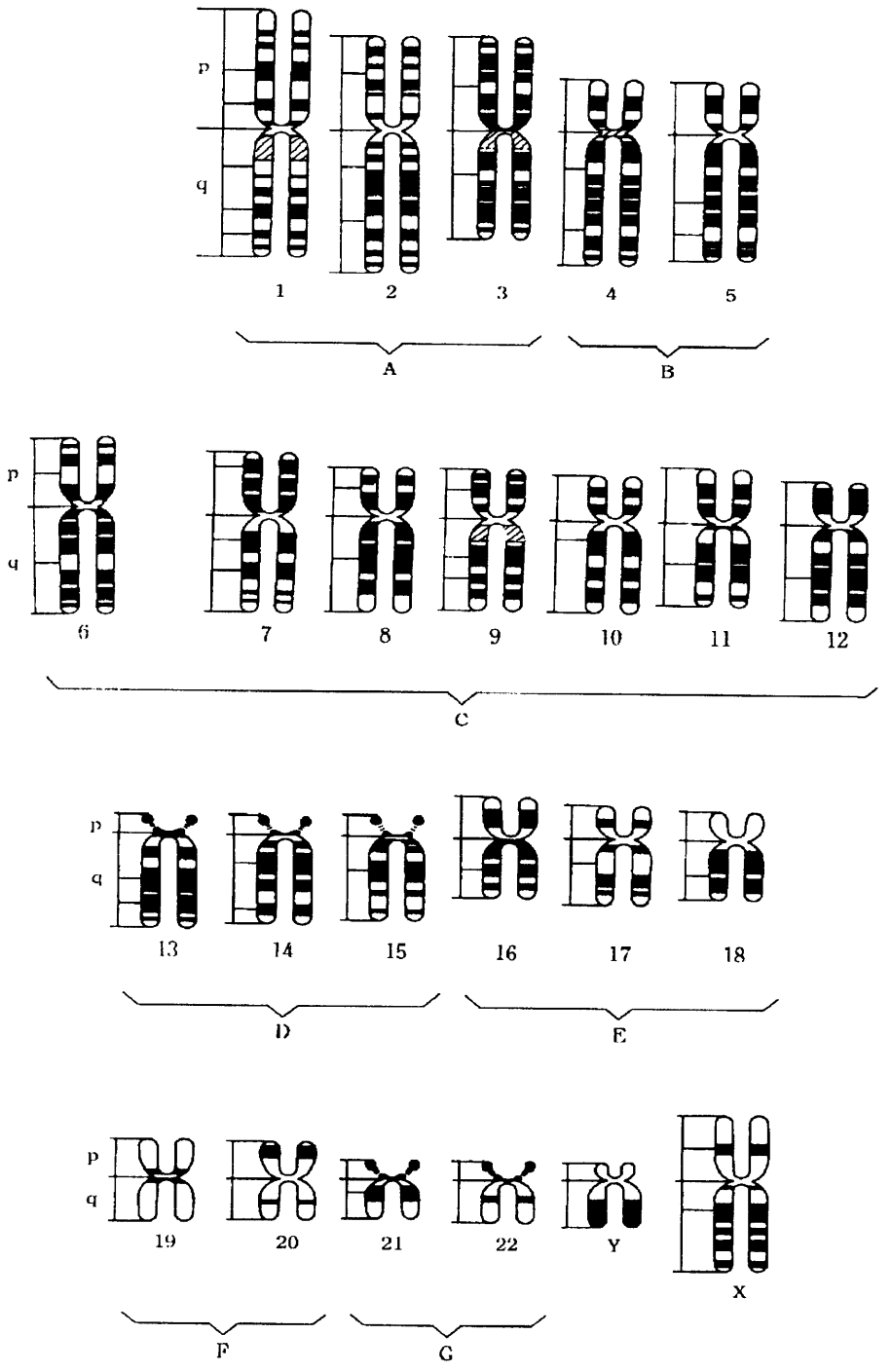


Figure 1–2. The normal human male karyotype is 46, XY. Females have two X chromosomes and lack the Y chromosome (46, XX). Reprinted from *Human Genetics*, 1988, by G. Edlin, with permission from Jones and Bartlett Publishers: Boston.

that becomes problematic when we consider that the sex chromosomes undergo rearrangements relatively frequently. For our purposes here, it is sufficient to state that the sex chromosome constitution, which may be assayed by karyotypic analysis, is a distinctive feature of the sexes, normal males being XY and normal females being XX.

Important caveats concerning methodology

Throughout this dissertation, we will consider individuals with abnormal sexual development. The karyotype, gonadal type, and genital development are all important pieces of the puzzle. But before we can be confident that sex and chromosome constitution are assigned correctly, we must consider two important *caveats* concerning methodology. The first regards gonadal histology. An abnormal gonad may contain patches of ovarian and testicular tissue. If the gonad is not to be removed in its entirety, a small biopsy is taken for histologic examination, and there is always the possibility that the biopsy is not representative of the gonad as a whole. In addition, the other gonad may be quite different in its cellular composition. In experimental animals like mice, this is not generally a problem since both gonads can be harvested and sectioned, or whole fetal gonads can be examined microscopically. When evaluating the case report for a human patient, though, it is important to determine whether the histology of the gonad was tested rigorously, and to remain skeptical of the conclusions if it was not. The second important *caveat* regards excluding the possibility of hidden mosaicism when performing karyotypic analysis. For instance, in studies of hermaphrodite individuals, some 7% are found to have a 46,XY/46,XX chromosome constitution. (Polani 1981) The cause of the hermaphrodite phenotype in this case is simply that the individual has an XY male cell line and an XX female cell line which have both contributed to the development of the gonads. If this patient were not carefully karyotyped, the presence of one cell line or the other might go undetected, in which

case the obvious explanation would be overlooked. To guard against this problem, multiple tissues and multiple cells should be karyotyped whenever possible. Any publication concerning gonadal type or karyotype that lacks careful attention to the considerations stated above can only be considered preliminary.

Some definitions first: what is sex reversal?

Perhaps the most striking anomaly of sexual development in mammals is that of sex reversal. In this rare condition, the karyotype is completely discordant with gonadal development, i. e. testes develop in an XX individual and ovarian tissue in an XY individual. Surprisingly, the phenotypic effect observed may be quite small. For example, somewhere between 1 in 20 000 and 1 in 40 000 human males has a 46,XX karyotype. These males usually appear quite normal during childhood. Later, due to spermatogenic failure, the testes become soft and are much smaller than normal. These individuals are invariably sterile, may have short stature, some breast development (gynecomastia), and are occasionally born with incomplete fusion of the urethral folds, a condition called hypospadias (Polani 1981, Ferguson-Smith 1992) . The histology of the testis can be normal in infants, but has become markedly abnormal by adulthood: small, hyalinized tubules, overgrowth of Leydig cells, and absent spermatogonia are observed. (Polani 1981) Even more rare, females with a 46,XY karyotype have a more extreme phenotype, sometimes described as pure gonadal dysgenesis. These females are sterile, with gonads that have degenerated into a "streak" of ovarian stroma lacking follicles, and may develop an unusual tumor called gonadoblastoma. Secondary sex characteristics are absent after puberty. (Polani 1981, Ferguson-Smith 1992) Since the earliest reports of the phenomenon (de la Chapelle, et al. 1964), a major focus of investigations of sex determination has been to understand the complicated genetics of sex reversal in humans and other mammalian species, such as the mouse and lemming. We will explore this topic in depth throughout this thesis, but most

specifically in sections II, III, and IV of this chapter, and in Chapter 2. For now suffice it to say that these rare, anomalous individuals represent 'the exceptions that proved the rule' that chromosomes determine sex in humans.

What is sex determination?

Now that we have considered the meaning of sex and even sex reversal, we can return to the original question, what is sex determination? Sex determination has broad and narrow definitions, both of which will be useful. Bull's definition is all-encompassing:

> "The inheritance of sex may be influenced by three measurable effects, (i) major sex factors (ii) minor sex factors, and (iii) environmental differences, and the study of sex determination is one of quantifying the relative magnitudes of these effects as well as their evolutionary consequences" (Bull 1983).

A corollary of this definition is that of sex factors, which are "the segregating units that provide the inherited basis of differences in sex determination" (Bull 1983). Bull needs such broad terms because he discusses a wide variety of organisms and sex determining systems. The sex factors are often sex chromosomes, but Bull prefers "to regard sex factors as the genes... responsible for controlling the inheritance of sex, and to differentiate them from genes which are incidentally co-inherited..." (Bull 1983). In these broadest of terms, then, this dissertation concerns the identification of major sex factors, or sex-determining genes, in mice and humans, with some insights from lemmings and the invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans*. Certainly, a more focused definition for sex determination will also be required. As we have already exhausted the definition of sex, we need only consider that of the term *determination*. In the narrowest sense, determination is the commitment of a developing cell to a particular fate. This process is theoretically distinct from differentiation, the development of specialized cell types from the single fertilized egg (Gilbert 1991). For our purposes, sex determination is the special case of an embryo committing to

a particular sexual fate during development. Of course, in terms of genetic potential, the sexual fate of a mammalian embryo is decided at fertilization; however, sexually dimorphic structures do not appear until much later. This window in development after the embryo receives its genetic instructions and before the sexual program is executed has quite rightly become the primary focus of modern investigations in sex determination.

The sexual program is executed during embryonic development

Sexual development of the early mammalian embryo hinges on the differentiation of the primordial gonad. This structure is unique amongst the organ primordia in that it is truly bipotential, giving rise to an ovary or a testis depending on the genetic makeup of the embryo. **Figure 1–3** shows the landmarks in genital system development in humans and mice, the sequence of steps in the sexual program being virtually identical in the two species. In the following description, I shall refer to human gestational age, which can be converted to mouse gestational age by referring to the figure. The genital system develops in close association with the urinary system. A proliferation of intermediate mesoderm forms two ridges on either side of the hindgut at about four weeks gestation. These urogenital ridges will give rise to three nephric systems, two of which are transitory, as well as the gonad and associated ducts. For several weeks after the onset of urogenital development, no differences are detected between male and female embryos, so that the embryo is traditionally described as sexually 'indifferent'. The urogenital ridge differentiates to form the forekidney or pronephros, which degenerates rapidly, contributing only a few ducts to the midkidney or mesonephros. This structure develops tubules, glomeruli, and the mesonephric or Wolffian duct, which contacts the urogenital sinus by late in the fourth week. At this time, the gonadal primordium appears on the surface of the mesonephros, forming from two cell

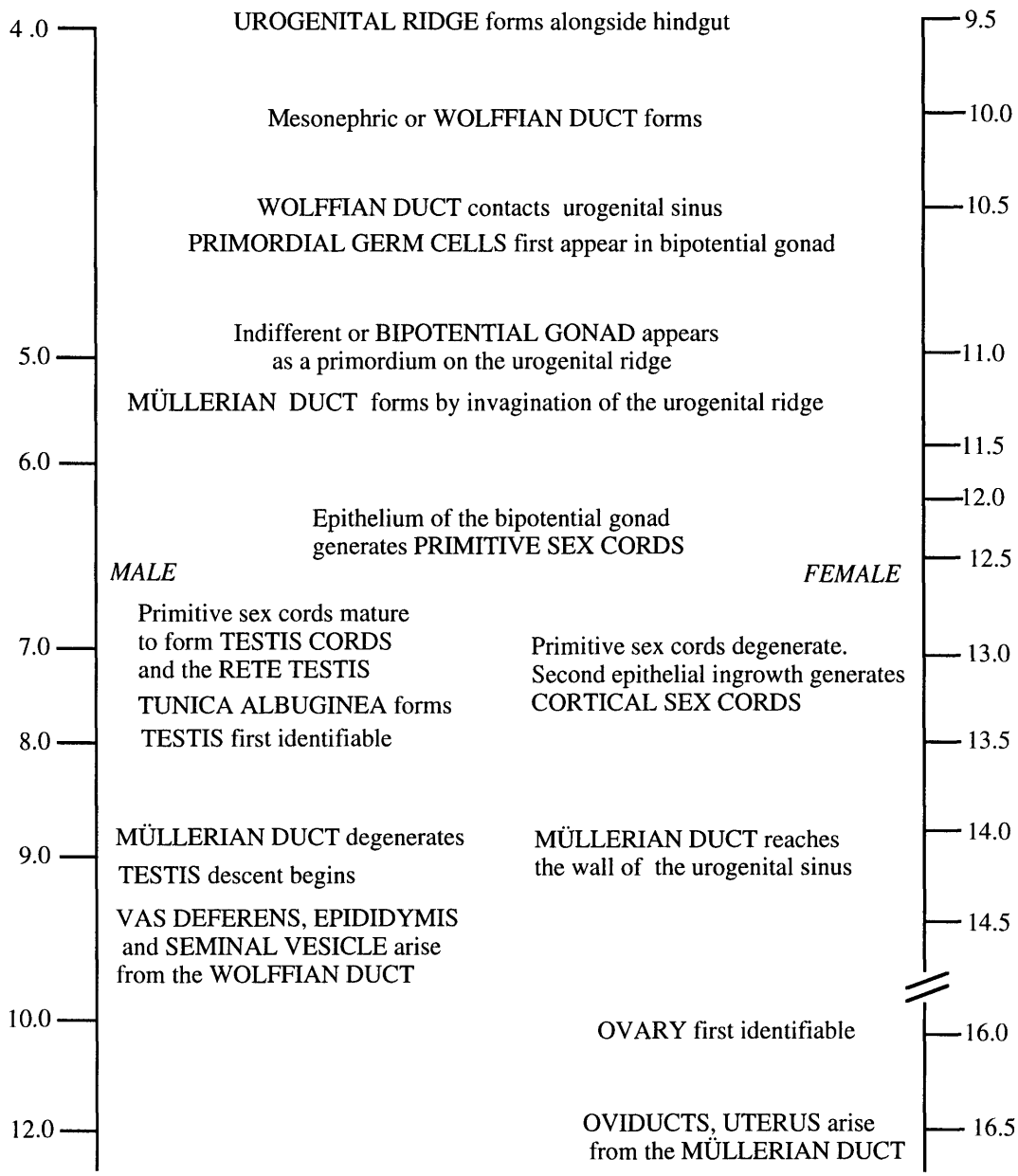


Figure 1–3 . Landmarks in genital system development in human and mouse.[1] The sexually indifferent period extends to 7 weeks gestation in human, 12.5–13.0 d*pc* in mouse. Human gestational age is actual weeks as opposed to menstrual weeks.

[1]Compiled from numerous sources: (Otis and Brent 1954, van Wagenen and Simpson 1965, Langman 1975, Moore 1988, Kaufman 1992)

types, mesodermal epithelium and mesenchyme. Another duct, the paramesonephric, or Müllerian duct, forms during the fifth week from an invagination of the urogenital ridge, close to the Wolffian duct. The Müllerian duct will form the oviducts and uterus in the female, whereas the Wolffian duct forms the vas deferens, epididymis, and seminal vesicle in the male. Despite their sexually dimorphic fates, both duct systems form in every embryo. An early derivative of the Wolffian duct in both sexes is the ureteric bud, which combines with the metanephric region of the urogenital ridge to form the metanephros (permanent kidney) and ureter beginning in the fifth week. Between the fourth and fifth week a third cell type, the primordial germ cell (PGC), appears in the embryonic gonad. These cells are not derived from the gonadal epithelium as was once thought. The PGCs originate in the yolk sac and migrate along the hindgut by ameboid movement to reach the gonadal primordium. There they associate with true epithelial derivatives, the primitive sex cords, which are solid ingrowths of proliferating cells. The PGCs invade the bipotential gonad about four to five weeks' gestation. The situation at the end of the sexually indifferent period (late sixth week) is depicted in **Figure 1–4**. The external genitals are also clearly undifferentiated with respect to sex at this time.

The sexually bipotential stage ends early in gestation

It is at this stage that differences between male and female embryos are first detected (seventh week of gestation). In males, the primitive sex cords mature and proliferate to form the testis cords and the rete testis, a network of interconnected cords in the hilar region of the organ. In contrast, the primitive sex cords of the female degenerate, and the presumptive ovary remains relatively featureless until 12 weeks gestation. However, one exception to this generalization is that in the ovary, a second ingrowth of epithelium forms cortical sex cords, which are not observed in the testis. In fact, the cortex of the developing testis is markedly underdeveloped

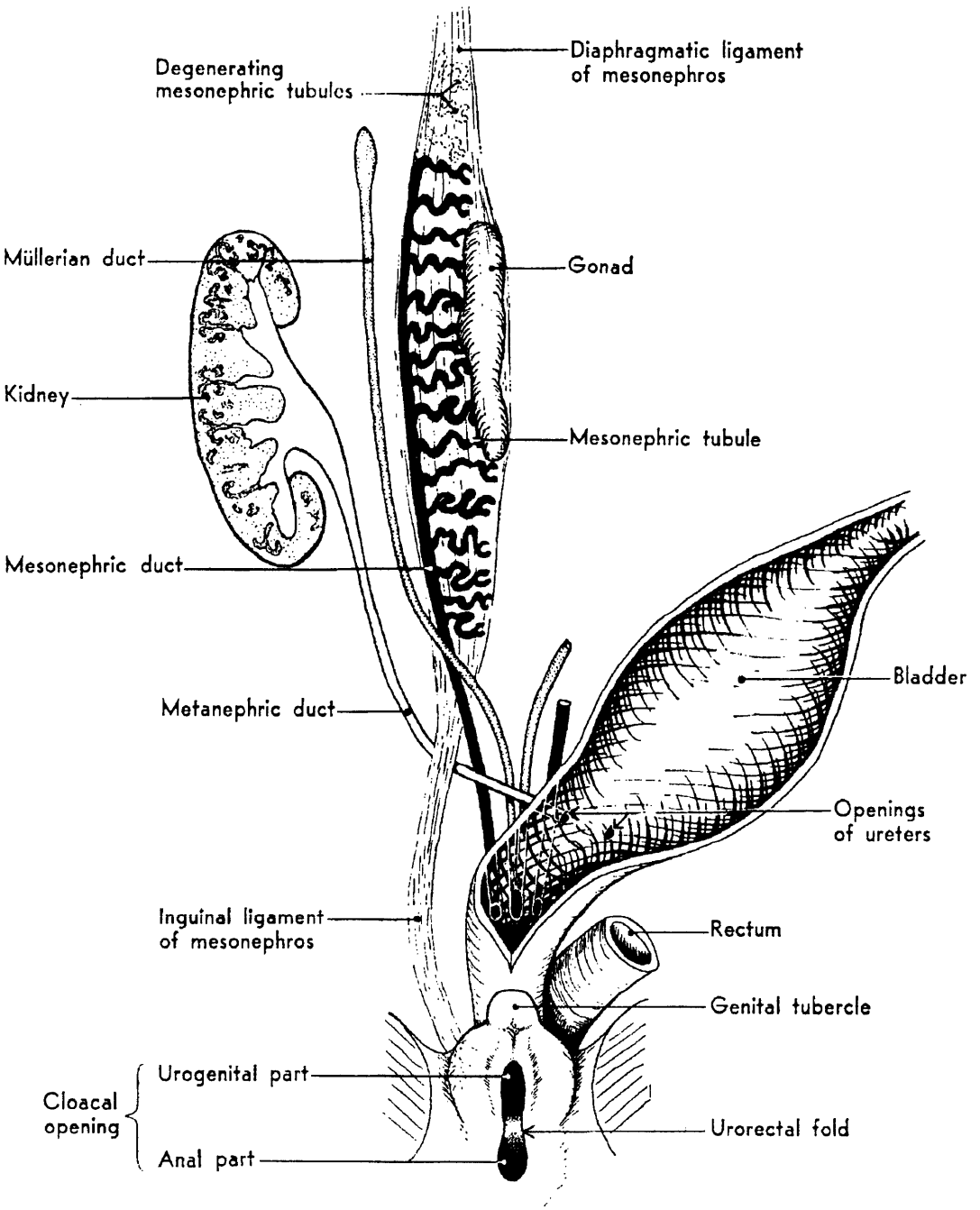


Figure 1–4. Unilateral view of the urogenital system before the onset of sexual differentiation. Reproduced from *Human Embryology*, 3rd edition, 1968, by B. M. Patten, with permission from McGraw-Hill, Inc.

compared to its medulla, as we observed for the adult organ. During the seventh week, a thick fibrous capsule called the tunica albuginea forms around the testis. This sheath of white connective tissue is a characteristic and diagnostic feature of testis development, making the testis easily identifiable at this stage. However, in the mouse, it has been shown that examination of fetal gonads to distinguish testes, ovaries, and ovotestes is more reliable if postponed until 14.5–16 dpc (9 or 10 weeks gestation in humans). At this time even small patches of ovarian or testicular tissue are distinguishable. (Eicher, et al. 1980, Eicher, et al. 1982) As the gonad develops, cells of the mesenchyme between the testis cords become Leydig cells, which secrete testosterone. This steroid hormone and its metabolite dihydrotestosterone have a profound effect on the developing genital tract, stimulating the development of the Wolffian duct derivatives (vas deferens, epididymis, and seminal vesicle), and masculinizing the external genitals. The sustentacular or Sertoli cells of the testis cords also produce a hormone, in this case a peptide hormone known as Müllerian inhibiting substance (MIS; alternatively, anti-Müllerian hormone, AMH). This hormone is known to cause the regression of the Müllerian duct in male embryos, with the possible exception of the cranial end, which forms the vestigial appendix testis if it persists. As the testis matures and signals the Wolffian duct via testosterone secretion, the cords of the rete testis establish connections with ducts of the degenerating mesonephros. These efferent ductules are contiguous with the region of the Wolffian duct destined to become the epididymis. This structure may exhibit two vestiges, the paradidymis and the appendix epididymis, the ducts of which do not contact the rete testis. Two other accessory glands, the prostate and the bulbo-urethral glands are derivatives of the urethra which are well-developed at birth. **Figure 1-5** gives an overview of the development of the internal genital tract in males, including the descent of the testis into the scrotum.

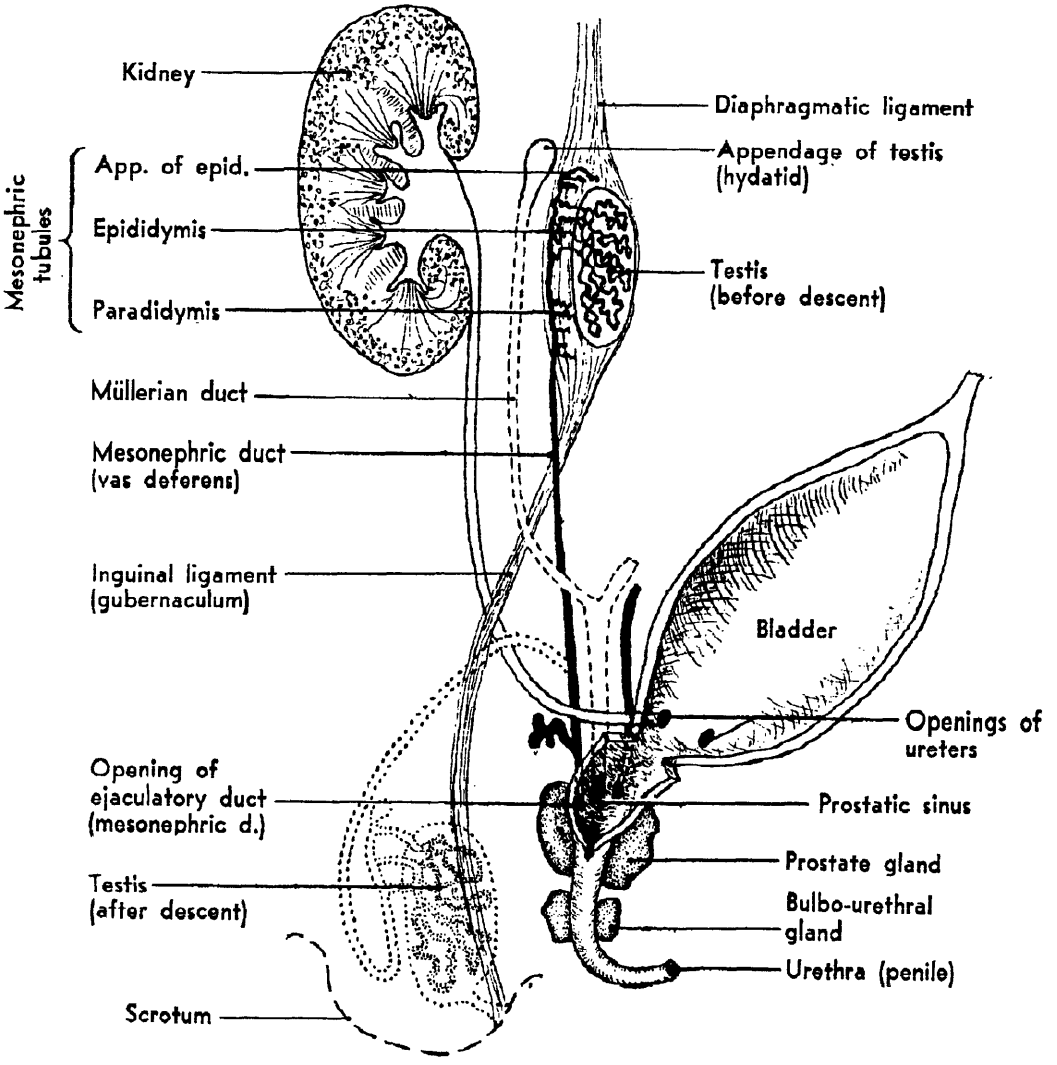


Figure 1–5. Unilateral view of the urogenital system in a male after sexual differentiation occurs. Reproduced from *Human Embryology*, 3rd edition, 1968, by B. M. Patten, with permission from McGraw-Hill, Inc.

In the female, ovary development is significantly delayed with respect to testis development. For several weeks after the ingrowth of the cortical sex cords, the ovary is recognizable more for the lack of testis-specific structures than for any remarkable features of its own. It may be referred to as the presumptive ovary at this time. By about 12 weeks gestation, the cortical sex cords have displaced the primitive sex cords, giving the gonad the beginnings of its mature identity. Primordial germ cells are not incorporated into cortical sex cords until about 16 weeks gestation, when the cords break up and form primordial follicles. In each of these cell clusters, one PGC develops into an oögonium which enters mitosis. Many of the oögonia degenerate before birth, but several million persist and enlarge, forming the primary oocytes. These cells together with the somatic derivatives which surround them are the primary follicles, which remain dormant until puberty. Beginning at about the same time (12 weeks gestation), the genital ducts take on the female form. Without testosterone, the Wolffian duct degenerates, and the Müllerian ducts fuse ventrally as they grow towards the wall of the urogenital sinus. The unfused portions form the oviducts, whereas the joined ducts will generate the uterus and the upper vagina. Once the ducts contact the urogenital sinus, a proliferation of endodermal tissue forms the solid vaginal plate. The inner cells of this structure degenerate later, leaving behind the vaginal lumen. See **Figure 1–6**. The female develops auxiliary genital glands that derive from the urethra. The urethral and paraurethral glands of Skene, and the greater vestibular glands of Bartholin correspond to the male's prostate and bulbo-urethral glands, respectively. Note that the embryonic sexual development of the female is independent of hormone production, in marked contrast to that of the male.

The final components of the genital system, the external genitals, follow the same basic developmental plan as the gonad and ducts. The initial structures are bipotential, but they subsequently take on male or female forms. In the case of the

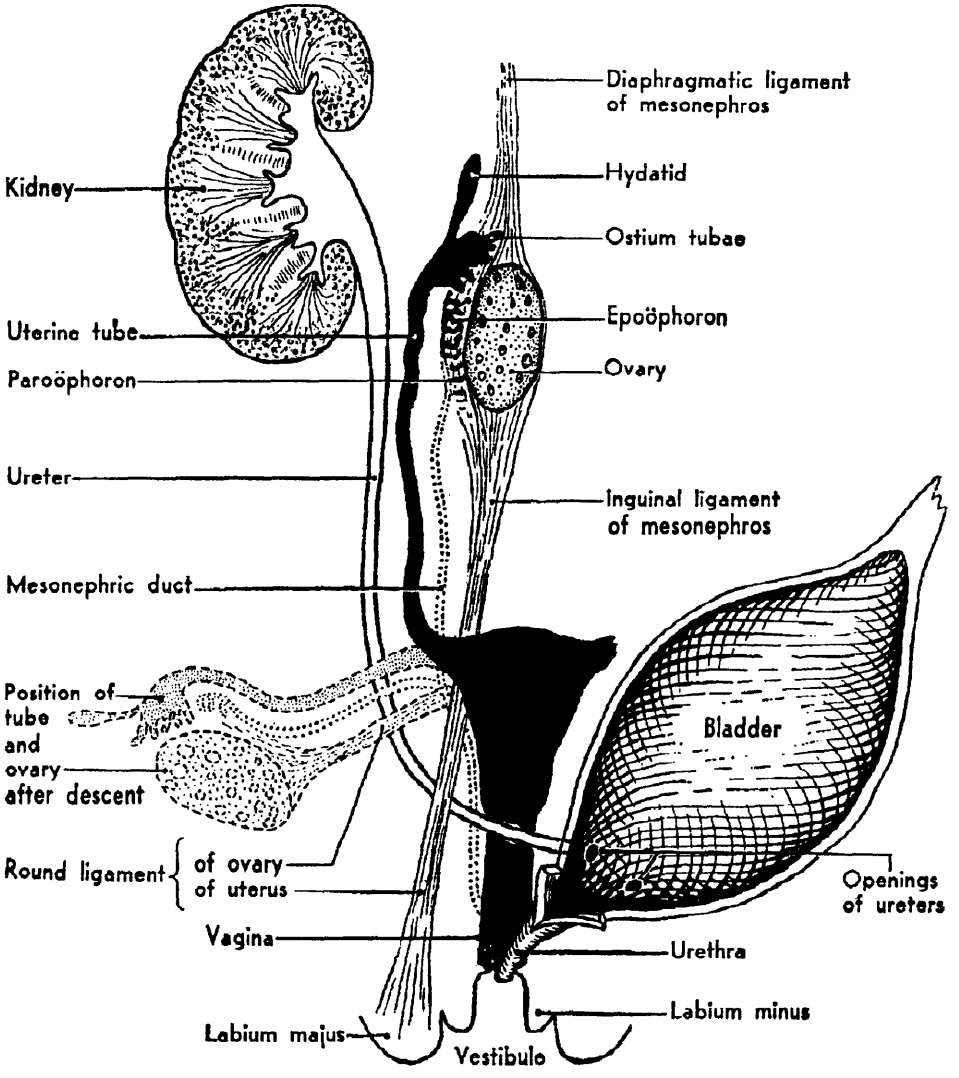


Figure 1–6. Unilateral view of the urogenital system in a female after sexual differentiation occurs. Reproduced from *Human Embryology*, 3rd edition, 1968, by B. M. Patten, with permission from McGraw-Hill, Inc.

external genitals, the bipotential structures are the genital tubercle, genital swellings, and urogenital folds. In the female, these form the clitoris, labia majora, and labia minora, respectively. In the male, their derivatives are the penis, scrotum, and midline raphe (the ridge that forms as the folds fuse). **Figure 1-7** illustrates the transformations of the external genitals between 7 weeks' and 6 months' gestation. To conclude this summary of embryonic sexual development, a list of the major bipotential elements of the genital system along with their derivatives in the developed male or female is given in Table 1-1.

Table 1-1
Female and Male Derivatives of Sexually Bipotential Embryonic Structures.

Structure in the Female	*Structure in the Embryo*	*Structure in the Male*
Ovary	Bipotential gonad	Testis
Disappear	Primitive sex cords	Seminiferous tubules
Disappear*	Mesonephric tubules	Epididymis
Disappears*	Mesonephric duct	Vas deferens
Oviducts, uterus	Müllerian duct	Disappears
Vestibule	Urogenital sinus	Urethra
Clitoris	Genital tubercle	Penis
Labia minora	Urogenital folds	Midline raphe
Labia majora	Genital swellings	Scrotum

*Except for vestigial remnants. Modified from *Human Reproduction and Development*, 1983, by C. T. Grabowski, with permission from Holt, Rinehart, & Winston Publishers.

Sex determination is distinct from sex differentiation

Knowledge of the embryology of sexual development was fairly well advanced in the mid 1940s when a series of remarkable investigations by Alfred Jost galvanized the field of sex determination as it moved from the descriptive to the experimental stage. Some years earlier, a theory of hormonal control of embryonic sexual development had been proposed to explain the freemartin effect in cattle (Lillie 1917). A freemartin is a female whose reproductive system has become almost completely masculinized as a result of an interchange of the fetal blood supply with a male twin. The hormonal theory supposes that male genital tract development is controlled from the beginning by hormones circulating

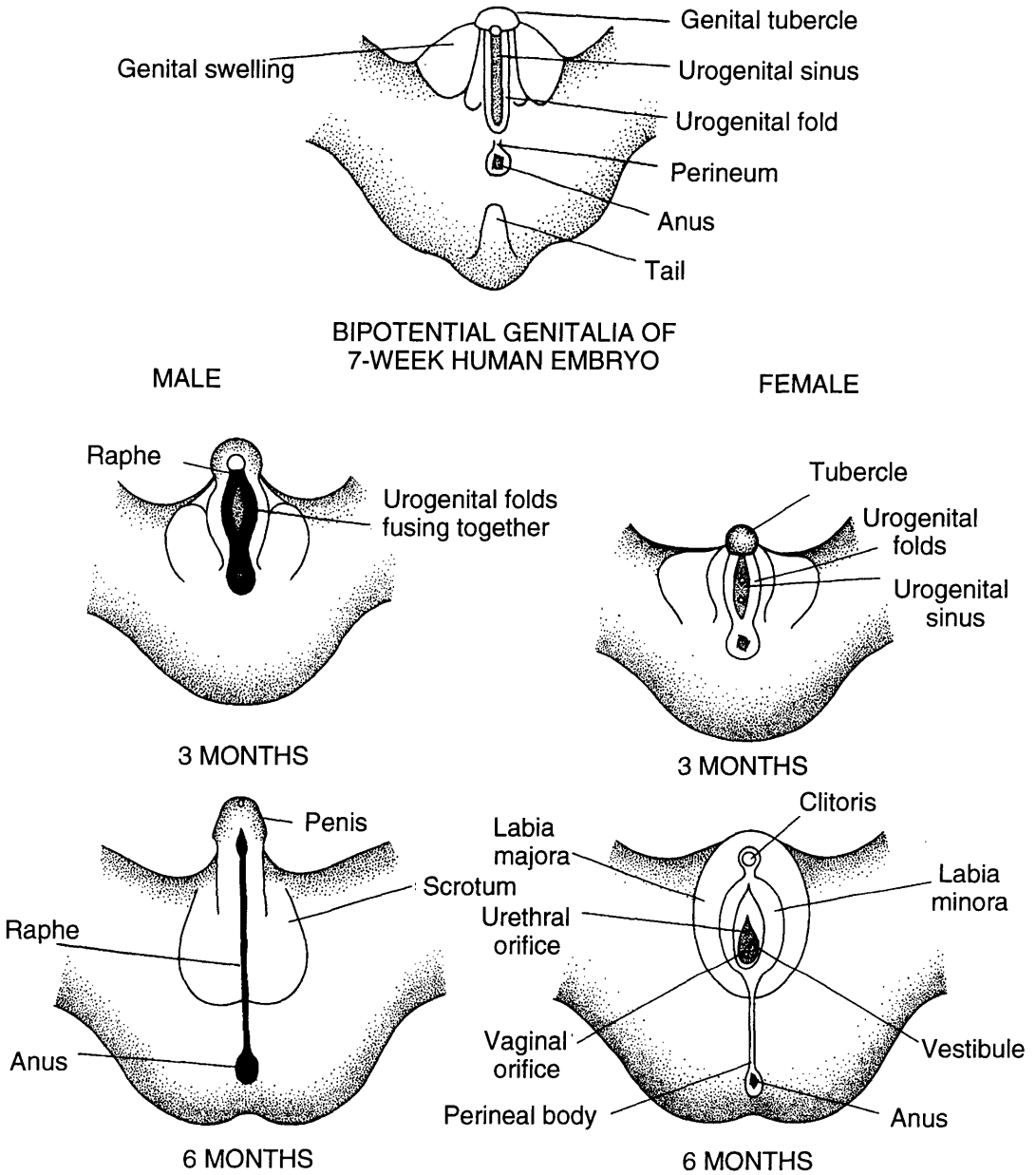


Figure 1–7. Development of the external genitalia from the bipotential primordia of the embryo in the male and female. Modified from *Human Reproduction and Development*, 1983, by C. T. Grabowski with permission from Holt, Rinehart, & Winston Publishers.

in the blood. This idea was subsequently challenged by experiments in which purified sex hormones were administered to embryos of other mammals. Specifically, hormonal treatments were unable to reverse sexual differentiation in mammals completely, as was possible for the freemartin and other vertebrates such as fish and amphibians (Moore 1944, 109-112). The ovary and the Müllerian duct, especially, were not 'reduced' by androgen administration (Jost 1953). Jost aimed to explore the validity of the hormonal theory with a direct surgical approach: castration of the fetus *in utero* to eliminate the gonadal hormones. Previously, castration experiments had been performed on mammalian subjects at birth; but as we have seen, sex is determined long before birth. Moore attempted to solve this problem by castration of opossum pouch young, which do not complete sexual development until some 100 days after birth. These animals are accessible, therefore, at stages of sexual development which occur *in utero* in placental mammals. Moore found that castration of the young just after gonadal determination has no effect on the differentiation of the sex ducts or glands, an apparent contradiction of the hormonal theory (Moore 1944, 123). More recently, investigators have found evidence for primary genetic control of somatic features of sexual development in another marsupial, the wallaby (Short, et al. 1988). Whether this explains Moore's results with opossums, which may have suffered from methodological problems, remains a mystery.

Jost's experimental design was simple, although his surgical technique was quite sophisticated. Gonadectomy was performed on rabbit embryos at various stages of sexual development, beginning just after the first appearance of morphological differences between male and female, and continuing until just before birth. The embryos ranged in size from about 26 to 62 mm. All embryos were sacrificed at birth and examined for the state of development of the external genital organs, vagina, oviducts, prostate, seminal vesicle, bulbo-urethral glands,

and vas deferens (Jost 1947, 278-9). Sexual development in females after ovariectomy was normal except for a slight reduction in the size of Müllerian duct derivatives (Jost 1947, Jost 1953). In males, however, a trend towards complete feminization with progressively earlier castration was clearly evident. The table reproduced below shows the essential results:

Table 1–2. Jost's results with fetal rabbits:
Condition of the Genital Tract of Castrated Male Fetuses, Studied on Day 28

Castration Date					
Stage	Days	Müllerian Duct	Wolffian Duct	Prostate	External Genitalia
I	19	Persistent	Absent	Absent	Female
II	20-21	Persistent	Occasional vestiges	Occasional buds	Female
III	22-23	Uterine & Vaginal Sections	Caudal Vestiges	+ or + +	Hypospadias
IV	23	Absent	Vas deferens absent, small seminal vesicle	+ + +	Male
V	24	Absent	Normal	+ + +	Male

Modified from Jost (1953), p. 390.

Castrating males early in development causes them to develop as females, whereas later castration has either an intermediate effect or no effect whatsoever, depending on the stage. From this data, Jost (1953, 386-387) concluded that

> the fetal testis exercises two kinds of effects: (*a*) a stimulative morphogenetic activity upon the common primordia (urogenital sinus wherefrom originates the prostate and external genitalia) and upon the Wolffian ducts which are "stabilized" for the remainder of life and allowed to differentiate; in the absence of the stabilizing action, these ducts undergo regression as does the mesonephros; (*b*) an inhibitory activity upon the Müllerian ducts which lead to their retrogression; these ducts persist if not suppressed by the testicular secretion.

Jost extended this conclusion in subsequent experiments by substituting a crystal of purified testosterone for the castrated gonad. The testosterone stimulated Wolffian duct development but failed to inhibit the Müllerian ducts, identifying testosterone

as the 'stimulative' but not the inhibitory secretion of the testis. Later, the peptide hormone MIS was found to be the inhibitory secretion.

Jost and his contemporaries realized immediately that his results have some broader implications for the study of sex determination. What he discovered is that the determination of gonadal type is the crucial developmental decision. The bipotential structures develop and follow the female developmental program unless the gonad becomes a testis. Once the embryo forms a testis, its sex is determined because the testis secretes hormones that inhibit the female program and initiate that of the male. If the testis does not form, the gonad becomes an ovary and the ducts follow the female program uninhibited. Hence, in the case of placental mammals, sex determination is equivalent to gonadal determination, i.e. the specification of gonadal type during development. Indeed, because of the pivotal role the testis plays in the binary decision between male and female, sex determination has been called testis determination, especially with regard to 'the testis determining factor' or *TDF*. Although the critical choice between male and female is already made at the point of gonadal determination, a great deal of sexual development is yet to come. For our purposes, sex determination applies only to gonadal specification. All subsequent sexual development is termed sex differentiation. Two examples will serve to illustrate the importance of distinguishing between these processes. Certain human subjects present with a female appearance and behavior pattern, failure of menarche but not of breast development at puberty, and absence of pubic and axillary hair. The external genitals are unambiguously female, but internally there are neither Wolffian nor Müllerian derivatives, and surprisingly, the gonad is an undescended testis. The karyotype is found to be 46, XY. This condition, testicular feminization syndrome (*Tfm*), is due to a defect in the intracellular androgen receptor. Although sex determination is unaffected, the androgens produced by the testis have no effect on the target tissues. Hence no further male

development occurs, except for the regression of the Müllerian ducts. There is no defect in sex determination, but the sex differentiation process is partially blocked, resulting in the appearance of a normal, albeit sterile and hairless, female. With respect to nomenclature, this individual is a male, though formerly, he would be classified as a male pseudohermaphrodite (Polani 1981). In contrast to the *Tfm* male, the XY female described previously illustrates the consequence of the failure of the sex determining mechanism. The karyotype is the same, but the gonadal development is ovarian and very limited (dysgenetic). Duct development is female in the absence of a testis. Clearly, there has been an error of sex determination in this individual, with a consequent failure of sex differentiation secondary to the gonadal dysgenesis. This important distinction will surface again in the next section when I discuss the identification of the testis determining factor.

All mammals exhibit chromosomal sex determining systems

This introduction would not be complete without a brief mention of the classification of mammals and their sex determining systems. Mammals first appear in the fossil record 200 million years ago (Graves and Schmidt 1992). To the taxonomist, mammals are a class of vertebrate animals that nourish their young from mammary glands and whose bodies are covered with hair. Two subclasses of mammals are recognized, the Prototheria and the Theria. The Prototheria are extinct except for one order, the monotremes, represented by the platypus and spiny ant eater of Australia. These odd creatures lay shelled eggs, like the reptilian ancestors of the earliest mammals. Theria is a larger subclass with two infraclasses, Metatheria and Eutheria. Commonly known as marsupials, the Metatheria have young that are blind, hairless, and essentially helpless, and must complete development in the mother's pouch after birth. Well-known examples of marsupials are the opossum, kangaroo, wallaby, and koala. Placental mammals, the Eutheria, are a diverse group with some 19 orders, amongst them the primates

(monkeys, marmosets, apes, humans), and the Rodentia (mice and rats, squirrels, porcupines, lemmings, and numerous others). All mammalian sex chromosomes are derived from a heteromorphic pair in the last common ancestor (some 150 million years ago), and thus share some degree of homology (Graves 1987) . Although all mammals have chromosomal sex-determining mechanisms, they are not all of the same pattern. In addition, we shall frequently refer to invertebrate species for examples and to make comparisons. The sex-determining systems we shall focus on fall into one of two categories: heterogamety and multiple factor systems. Heterogamety may be male (XX/XY) or female (ZZ/ZW). Humans and mouse species exhibit the dominant-Y form of male heterogamety, whereas the invertebrates *D. melanogaster* and *C. elegans* show recessive-X male heterogamety. Sex determination in the lemming *Myopus schisticolor* is via a multiple factor system (Bull 1983).

Conclusion

In conclusion, I have provided what I see as the key definitions for the purposes of this dissertation. After considering separately the general and/or specific meanings of sex, sex reversal, determination, differentiation, autosomes, and allosomes, what is sex determination? In its most concise definition, sex determination is the special case of an embryo committing to a particular sexual fate during development. I have attempted to show that this is a complex developmental process with some truly unique features. When sex is determined, the embryo makes a binary choice between the male and the female state, which is reflected in the bipotential nature of the gonad and the duct system. Rarely are developmental processes that are amenable to study so clearly delimited. Another striking feature of sex determination in the male is that testis determination represents a distinct transition from determinitive processes to hormone-mediated differentiation processes, thus placing distinct limits on the process we are attempting to

understand. Finally, individuals with defects in sex differentiation as well as determination are viable and relatively easy to assess at the phenotypic level. All of these characteristics made sex one of the first developmental systems in which molecular genetic approaches made major inroads, as we shall soon see.

Section II

What determines sex in mammals?

Insect sex chromosomes are described c. 1900

The best scientific thinking about sex determination prior to the revival of Gregor Mendel's ideas about inheritance was summarized by Geddes and Thomson in 1890. They conclude that the metabolic environment in the parents' bodies influences sex determination such that if catabolism or energy utilization is favored, male offspring are produced; whereas, if anabolism or energy storage is favored, female offspring are produced (Geddes and Thomson 1890). The first evidence to the contrary came from investigators studying the behavior of chromosomes during spermatogenesis in insects. McClung is credited with the re-discovery of sex chromosomes for establishing that an odd chromatin body previously observed in the nuclei of spermatogonia is indeed a chromosome (Henking 1890, McClung 1902). Henking first observed this element but labeled it 'x' for unknown. McClung studied spermatogenesis in several genera of locusts and confirmed that half the sperm in these insects have an extra chromosome, dubbed the accessory or 'X' chromosome, which is unpaired during meiosis. Accepting "the theory that chromatin is the bearer of hereditary qualities", McClung recognized that the accessory chromosome fulfills important theoretical expectations for a chromosomal sex determinant, primary among these being its distribution to half the male germ cells, which ensures that the sexes are represented in equal proportions amongst the offspring. McClung postulated that the accessory chromosome "is the bearer of those qualities which pertain to the male organism" (McClung 1902, 72), such that the sperm which carry it give rise to males. His error in not determining the chromosome constitution of the females became evident shortly thereafter, when reports of chromosome studies in Hemiptera (plant bugs) established that in some insect species, males have one *less* chromosome than the females (Wilson 1905).

In this XX female/XO male sex determining system, the X-bearing sperm is the one McClung identified as having the accessory chromosome, but it necessarily produces a female zygote after fertilization since all eggs already carry one X chromosome. The OO female/XO male sex determining system McClung postulated does exist (Bull 1983; 13, 220), but not in the Locustidae. Wilson (1905) also reported an alternative chromosomal sex determining system in Hemiptera which was independently observed in the beetle *Tenebrio molitor* the same year (Stevens 1905). Both sexes in these insects have the same number of chromosomes, but one in the male is much smaller than its homolog. The odd chromosome is, of course, the Y chromosome, making this an XX female/XY male system. As an historical aside, it is interesting to note that Wilson first applied the name Y to this chromosome (Wilson 1906).

It is interesting to note that McClung, writing as he did at the very beginning of the genetic era, listed the ability to respond to environmental cues as a requirement for a chromosomal sex determinant: "such disposition of the [sex-determining] element in the two forms of germ cells, paternal and maternal, should be made as to admit of the readiest response to the demands of the environment regarding the proportion of the sexes" (McClung 1902, 73). He clearly did not view his hypothesis as contradicting that of Geddes and Thomson. Indeed, he attempted to reconcile the behavior of the accessory chromosome in spermatogenesis with their ideas, suggesting that this element might determine sex by stimulating the catabolic male environment in the developing germ cells:

> If it be that the production of male elements is a sign of katabolic conditions,...then it would seem most natural that the [sex] determinant should be for the purpose of carrying the transformation beyond the production of ova to spermatozoa. It would therefore be a necessary content of the cells until they had passed through the stages of development beyond that at which they might pause and become laden with yolk ...It is conceivable that the production of four functional cells from one spermatogonium would call for the employment of more

> energy than would the formation of one functional egg from an oögonium...(McClung 1902, 73-74)

Despite McClung's uncertainty about the precise mechanism and the role of the environment, his hypothesis, confirmed and extended very shortly as it was by the work of Stevens and of Wilson, excited the nascent field of genetics with the prospect of a simple genetic rule to solve the age-old riddle of sex.

Chromosomes determine sex and eye color in Drosophila melanogaster

One investigator who was *not* excited about genetics was Stevens' mentor and Wilson's colleague, Thomas Hunt Morgan. He came to Columbia University early in his career convinced that Mendel's ideas were all wrong (Shine and Wrobel 1976). But by the experiments that he performed, he eventually convinced not only himself and his assistants, but indeed the entire world of science, that genes reside on chromosomes and control the form of every imaginable characteristic, the first and foremost of these being sex. Morgan studied *Drosophila* without making much progress for several years until a white-eyed "sport"(mutant) appeared in a pedigreed culture of red-eyed flies. This fly and its descendants allowed Morgan to describe in detail the 'sex limited' inheritance of eye color, which first appeared in the F2 generation of a cross between the white-eyed sport and normal red-eyed females. The F1 was predominantly composed of red-eyed flies as predicted for a recessive character; however, 3/1240 were white-eyed and male. Morgan ignored these "exceptions" as examples of further sporting (but see below). Intercrossing the red-eyed F1 offspring gave the predicted red:white ratio of approximately 3:1 (actually 3470 : 782), but all of the white-eyed offspring were male. The explanation that seems elementary today was a major break-through for Morgan– assuming that the F1 male is heterozygous for a sex factor (X–) and for red eyes (RW), the absence of white-eyed females in the cross is explained by assuming that the red-eyed trait and the sex factor *always go together* into half the spermatozoa.

After reviewing the results of numerous crosses designed to test the hypothesis, Morgan completes this landmark analysis with his inescapable conclusion: "It now becomes evident why we found it necessary to assume a coupling of R and X in one of the spermatozoa of the red-eyed F_1 hybrid...The fact is that this R and X are combined, and have never existed apart" (Morgan 1910, 122).

In that early publication, Morgan was very careful not to go beyond the data–by identifying a particular chromosome as a sex factor, for instance–but soon his assistant Calvin Bridges demonstrated directly that the X chromosomes are the determinants of both sex and eye color (Bridges 1913, Bridges 1914, Bridges 1916). He did this by studying the kind of exceptional flies that Morgan had attributed to 'further sporting' and cautiously suggested might result from chromosome non-disjunction (Morgan 1910, 122) . However, coming as they did three and more years after the original work, Bridges publications reflected a much greater level of understanding and methodological sophistication. There were already some fifty sex-linked mutations described in *Drosophila.* An ingenious system for ordering the characters on the chromosomes, devised by Alfred Sturtevant and based on Morgan's ideas about crossing-over, had generated a detailed map of the X chromosome (Bridges 1916, 8). Now Bridges could demonstrate the features of sex-linked inheritance and look for the all-important exceptions in one cross: a vermilion-eyed female by a wild-type male. The overwhelming majority of offspring showed "criss-cross" inheritance, meaning that the eye colors in the parents switch sexes in the F1. See **Table 1–3** (next page). Occasionally, however, Bridges observed a daughter like the mother (a "matroclinus" daughter), or a son like the father (a "patroclinus" son) and proposed the scheme shown in the table to explain these primary exceptions. It is important to note that this scheme assumes that XXY animals are female, and XO male. Once

he had bred the primary exceptions and documented the production of secondary exceptional offspring exactly as his model predicted, he was convinced that the eye

Table 1–3. Sex-linked inheritance of vermilion eye color in *Drosophila*, with and without sex chromosome non-disjunction:

	NORMAL CHROMOSOME DISJUNCTION	WITH CHROMOSOME NON-DISJUNCTION
P1 GENERATION	X^vX^v x X^RY vermilion female wild type (red) male	X^vX^v x X^RY vermilion female wild type male
GAMETES	X^v X^R Y	X^vX^v Ø X^R Y
F1 OFFSPRING	X^vX^R wild type female X^vY vermilion male	X^vX^vY vermilion female $X^RØ$ wild type male
LETHAL COMBINATIONS		$X^vX^vX^R$ Ø Y
INHERITANCE	"criss-cross"	"primary exceptions"

Adapted from Bridges (1916), pp. 5-10.

color genes and the sex chromosomes show the same distribution in the fruit flies. But there was no direct proof that the XXY chromosome constitution is female and XO male. Therefore, the chromosomes of the aneuploid flies were examined, and when they were found to conform exactly to his assumption, Bridges could conclude:

> The genetic and cytological evidence in the case of non-disjunction leaves no escape from the conclusion that the X chromosomes are the carriers of the genes for the sex-linked characters. The distribution of sex-linked genes has been demonstrated to be identical...with the distribution of the X chromosomes... Experimental proof is given that particular chromosomes, the X chromosomes, are the differentiators of sex; the X chromosome constitution of an individual is the cause of the development by that individual of a particular sex, and is not the result of sex already determined by some other agent (Bridges 1916, 161-2).

This discovery–hailed as proof of the chromosome theory of inheritance–ultimately changed all biological pursuit in that it heralded the molecular revolution in biology. With a deferential nod to this remarkable achievement, let us turn to a consideration of its implications for sex determination.

Drosophila exhibits recessive-X male heterogamety

At the time of his publications, Bridges concluded that the number of X chromosomes is the sex determining signal in *Drosophila* . Incontrovertible genetic and cytological evidence indicated that XY or XO individuals are male, while XX or XXY individuals are female. Therefore, two X chromosomes give a female, and one a male. The Y chromosome has a passive role, if any, in sex determination, whereas the X chromosome has an both an active and a passive role. It is active in that it exerts a feminizing influence in the XX and especially in the XXY individual. On the contrary, the XO or XY individual is male, as though the feminizing influence is recessive in some instances. For this reason, the *Drosophila* scheme for sex determination is sometimes called the recessive-X form of male heterogamety (Bull 1983, 20-22). Bridges' discovery of a role for the autosomes in sex determination resolved the paradox of the active/passive X chromosome. He obtained aneuploid flies with three sets of autosomes and XX or XXY sex chromosomes (denoted 2X:3A) and found they are not female but intersexual (Bridges 1921). His interpretation is the following:

> It is not the simple possession of two X-chromosomes that makes a female, and of one that makes a male. A preponderance of genes that are in the autosomes tend toward the production of male characters; and the net effect of the X is a tendency to the production of female characters. The ratio of *2X:2 sets autosomes*...produces a female, while *1X:2 sets autosomes* produces a male. An intermediate ratio, *2X:3 sets autosomes*, produces an intermediate condition–the intersex...
> (Bridges 1921, 253).

This statement installed the 'X:autosome ratio' as the unquestioned sex-determining signal in *Drosophila* for nearly seventy years. Recently, however, Cline (1993) has

suggested that Bridge's interpretation may not withstand the final test, the identification of the autosomal male-tendency genes (see below). Nevertheless, for the first time in the long history of sex determination, genes are proposed to be sex determinants, a remarkable revolution in thought to have been completed in just thirty years.

The human Y chromosome was first described in 1921

When the early cytologists turned their attention from the chromosomes of insects to those of mammals, formidable technical difficulties presented themselves. Austin *et al.* (1981; 5, 7) point out that mammalian chromosomes are small and numerous relative to those of insects, and that suitable material (*i.e.* fresh mitotic or meiotic tissue) was not readily available. Therefore the earliest reports of identification of sex chromosomes and of the true chromosome number in humans were based on studies of sectioned material (from testes or cornea), and they varied wildly. It is interesting to note in passing that the early investigators (Guyer 1910; Painter 1923) often included material from both black and white subjects, searching unsuccessfully for racial differences in chromosome constitution. Painter (1923, 312-3) summarized the confusion of his contemporaries in the form of a review of the literature which listed three proposals for the sex chromosome constitution of humans (female/male being XX/OO, XX/XO, or XX/XY), and no less than seven possible diploid chromosome numbers ranging from 16 to 47. His were the first reports to demonstrate the existence of a Y chromosome in humans (Painter 1921), and to propose the correct chromosome number (46, although his final conclusion was 48) (Painter 1924). The key questions were far from being settled, however, as the proponent of a rival hypothesis (von Winiwarter 1912) proposing a chromosome number of 2n = 47 in males (and hence an XX female/XO male sex determining system) had a substantial following as late as 1937. At that time, a well-received report on meiosis in spermatocytes confirmed Painter's observations

of both X and Y chromosomes in males, as well as his inflated chromosome count (Koller 1937). In the words of those who finally debunked this 'myth': "From then on, the value of 2n = 48 in both male and female remained unchallenged for nearly twenty years, and it seemed that the chromosome number of man had finally been established" (Ford and Hamerton 1956).

In any event, the notion that sex is determined chromosomally with XY males and XX females was widely accepted long before the definitive demonstration of the chromosome number in humans. And simply by making the obvious analogy, many geneticists assumed that humans would show the recessive-X sex determining system demonstrated for *Drosophila*. The barriers to progress in human cytology were ultimately crossed by innovations like pre-treatment of cells with hypotonic solutions alone (Hsu 1952, Hughes 1952), or followed by colchicine (Ford and Hamerton 1956, Tjio and Levan 1956), as well as the application of novel cell-culture techniques. Thus in 1956, two reports identified the chromosome number as 46, Tjio and Levan in cultured fetal lung cells, and Ford and Hamerton in spermatocytes of surgically removed testis tissue. Modern scientists often attribute the persistence of the 2n = 47 or 48 hypotheses to the power of suggestion, but not so these investigators:

> Undoubtedly the adoption of [modern methods]...is bringing about a great change in mammalian chromosomal cytology, and it is to this technical improvement that the rectification of the error–if such it be–must primarily be attributed...The weary hours of toil which the pioneers must have spent at the microscope is reflected in de Winiwarter's *cri de coeur*, "J'ai perdu un temps énorme à répéter de numérations fatigantes et j'avoue aussi, très fastidieuses"[1]. The wonder is that there is so little to alter.
> (Ford and Hamerton 1956, 1023).

[1]"I have wasted enormous amounts of time repeating tiresome counts, and I avow they were also very fastidiously [performed]" (K. McNichols, unpublished translation)

Very soon the improvements in cytology were widely applied, and the result was a veritable explosion in sex chromosome research. For the first time, it was possible to perform Bridges' analysis, that is, to ascertain aneuploid individuals and assess their sex, in mammals.

Mammals exhibit dominant-Y male heterogamety

Three reports published shortly thereafter ushered in the modern era in the study of sex determination. Almost simultaneously, XO mice (Russell, et al. 1959, Welshons and Russell 1959) and humans (Ford, et al. 1959) were identified and shown to be female. Exceptional inheritance of sex-linked markers *à la* Bridges was demonstrated for color-blindness in humans, and *scurfy*(*sf*) and *Tabby* (*Ta*) in mice. In humans, the XO chromosome constitution is associated with gonadal dysgenesis and often a host of other congenital malformations collectively referred to as Turner's syndrome (TS), while mice suffer few if any effects. But if XO is female in mammals, would XXY be male or intersexual? It seemed likely that the rules from *Drosophila* would not be valid. Indeed, the chromosome constitution 47,XXY was reported in a male exhibiting the features of Klinefelter's syndrome (KS) that same year (Jacobs and Strong 1959). The authors report that the patient is an intersex, but describe testicular biopsy results clearly identifying him as a male in our nomenclature. Very shortly, 41,XXY mice were obtained in suitable genetic crosses, and they are sterile males like their human counterparts (Cattanach 1961, Russell and Chu 1961). In the mammalian sex-determining system, the Y chromosome therefore has a strong masculinizing influence which can overcome the presence of two (or even more) X chromosomes to produce a relatively normal, albeit sterile, male. Thus, mammals exhibit dominant-Y male heterogamety, a sex-determining system described only once previously: in the red campion, a flowering plant (Westergaard 1953).

Sex reversed humans make deletion mapping TDF feasible

It was not long after the dominant-Y sex determining mechanism for humans had been deduced that the first reports of exceptions to this rule, in the form of sex-reversed humans, were published (Court-Brown, et al. 1964, de la Chapelle, et al. 1964, Therkelsen 1964). The males who were identified as having a 46,XX karyotype were normal in all respects except for sterility and small testes. 46,XY females were also fairly normal as children, but failed to go through puberty (See Section IV for a detailed discussion of the phenotype of sex reversed humans). In a synthesis of current progress regarding the behavior of normal and abnormal sex chromosomes, Ferguson-Smith (1966) proposed that sex reversed males carry the male-determining loci of the Y chromosome on their paternally-derived X chromosome. Such a chromosome could arise if an illegitimate cross-over, i.e. one outside the pseudoautosomal region, resulted in the transfer of all the non-homologous Y chromosome loci distal to the break-point (including testis determinants), as well as the pseudoautosomal region, to the X chromosome. The hypothesis can explain some cases of anomalous inheritance of a blood group marker called Xg (Sanger, et al. 1964), as well as the origin of 46,XY females. See **Figure 1–8**. These individuals must carry the reciprocal product of the illegitimate cross-over: a Y chromosome from which the testis determining factor(s) (or *TDF*) have been 'deleted' by transfer to the X chromosome.
In that same year, a study of patients with abnormal Y chromosomes, most notably two females with isochromosomes for the long arm, led to the suggestion that the *TDF* resides on the short arm (Yp) (Jacobs and Ross 1966). In combination, these ideas led various investigators to hypothesize that the precise location of *TDF* could be found by determining just how much of the Y chromosome short arm was present in XX males or absent in XY females. The 'deletions' in the sex reversed patients could be used to construct a map of the Y chromosome and to position *TDF*

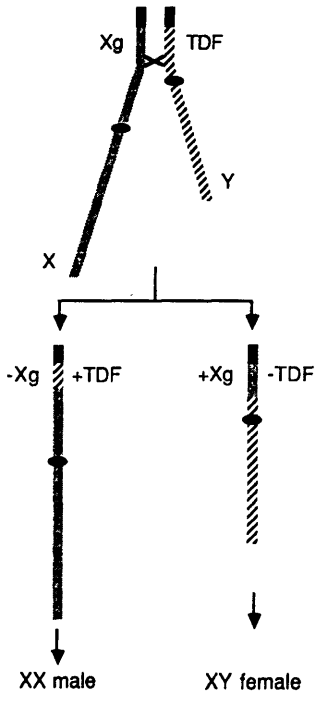


Figure 1–8. Hypothesis: XX males and XY females receive the reciprocal products of illegitimate cross-overs in paternal meiosis. Courtesy of David C. Page.

within it. Indeed, a deletion map of this sort is the only kind of map possible for the male-specific region of the Y chromosome because it does not normally undergo meiotic crossing-over with the X chromosome. Now the hunt for *TDF* was on, and in the twenty-five years that followed, the smallest region known to be sex-determining shrunk from the entire short arm down to just 35 kb of DNA, before the sex-determining gene, *SRY*, was cloned by chromosome walking.

Molecular & genetic approaches discredit two candidates for TDF

Initially, though, the search for *TDF* focused on two candidate sex determinants which were the subjects of numerous investigations. For nearly ten years, the prime candidate for *TDF* was the histocompatibility-Y (H-Y) antigen, a minor histocompatibility antigen specific to the heterogametic sex in both mammals and birds (Wachtel, et al. 1975, Wood 1988) Because this molecule is on the cell surface, hypotheses regarding its possible role in gonadal development emphasized

the potential importance of cell-cell interactions in testis determination. For instance, Ohno proposed that a gonad-specific receptor for H-Y antigen allows cells there to form the unique architecture of the seminiferous tubules, guaranteeing testis and not ovary organogenesis (Ohno 1976). Despite a great deal of data concerning the presence or absence of H-Y antigen expression in various circumstances of normal and abnormal sexual development, none could prove or disprove the hypothesis until a strain of mutant mice was discovered that develops testes, but lack H-Y antigen (McLaren, et al. 1984). The *Sex reversed* (*Sxr*) mutation in these mice involves a translocation of the sex determining region of the mouse Y chromosome to the X chromosome, with a subsequent deletion (ΔSxr^b) that removes H-Y antigen but not the testis determining locus (*Tdy*), demonstrating that the two are genetically separable. But a few years before McLaren refuted the H-Y antigen hypothesis, another candidate had appeared on the scene. This candidate was not a particular molecule, but a type of repetitive DNA conserved on the W or Y chromosome of snakes, birds, and mice. In a study of the evolution of sex chromosomes in snakes, repetitive or satellite DNA was tested for use as a molecular probe that would distinguish the DNA of male and female snakes. Two components of the snake DNA, Banded krait minor satellite (Bkm) DNA and Elaphe satellite IV, were found to be conserved amongst the suborder *Ophidia*, and then more widely to birds and mice. The most exciting result of the early studies was that the Bkm DNA shows a male-specific transcript in mouse (Epplen, et al. 1982), and co-localizes with the *Sxr* transposition, placing it near the primary sex-determining locus, *Tdy* (Jones and Singh 1981, Singh, et al. 1984). However, a detailed study of the distribution of Bkm DNA in humans demonstrated its absence from the Y chromosome by *in situ* hybridization, despite a strong signal from the X chromosome and two autosomes (Kiel-Metzger, et al. 1985). Without Y-specific localization, the Bkm sequence was no longer considered a candidate for *TDF*.

Positional cloning localizes a new TDF candidate, ZFY

Then in the mid-1980s, aided by advances in molecular biology, accurate deletion maps of the Y chromosome led to another candidate for *TDF*. As hypothesized years earlier, deletion maps could be constructed based on the Y DNA that is present in an XX male, or absent in an XY female. It is reasoned that any segment of Y DNA that a given XX male carries may contain *TDF*, unless another XX male with an even smaller segment is discovered. If several of these patients consistently indicate an interval that must contain *TDF*, all or part of that interval should be deleted in the XY females who have Y chromosome deletions (Page 1986). The first reports of Y-specific DNA in human XX males confirmed the power of DNA hybridization techniques to detect abnormal sex chromosomes where cytogenetic techniques would fail (Guellaen, et al. 1984, Page, et al. 1985). Cytogenetic and DNA studies also confirmed the hypothesis that some XY females have small deletions of the critical male-determining region of the Y chromosome (Disteche, et al. 1986). In the few years that followed those early reports, many investigators collected patients and tested their chromosomes against a growing panel of Y-specific probes. The first deletion maps of the human Y chromosome localized *TDF* to distal Yp, and had about seven or eight intervals, each defined by a patient's deletion breakpoint (Page 1986, Vergnaud, et al. 1986). The number of articles related to deletion mapping the Y chromosome published around this time is evidence of the intense scrutiny the research community brought to bear on the question of *TDF* (Affara, et al. 1986, Muller, et al. 1986, Affara, et al. 1987, for example). Soon, a deletion mapping effort narrowed the critical segment containing *TDF* to region 1A2, an interval of just 140 kilobases (kb) or about 0.2 % of the Y chromosome, very near the pseudoautosomal boundary on Yp. A zinc-finger protein was cloned from this interval by chromosome walking using a

bacteriophage vector (Page, et al. 1987), and immediately became the prime candidate for the testis determining factor.

The hypothetical properties of the sex determinant act as a standard

Once the new candidate sex-determining gene was isolated, its properties were compared unfavorably with the hypothetical properties of the mammalian testis determinant, but none could disprove the zinc finger-Y (*ZFY*) hypothesis until new genetic evidence came to light. It is instructive in this regard to examine the validity of the hypothetical properties of the sex determinant, which we shall explore in a more general sense in Section IV. One of these properties is that the testis determinant of eutherian mammals should be conserved as such in marsupials. Soon after it was cloned, *ZFY* was shown to be autosomal in four species of marsupials from two separate orders (Sinclair, et al. 1988) . Although described as a surprising result, without extensive knowledge about the primary sex determinant in marsupials, the result is inconclusive. That is, it does not bear on the question of whether *ZFY* is testis determining in eutherian mammals–but if it is, this finding means that the sex determining mechanism of marsupials and eutherians differ. Although somewhat unexpected since the Y chromosome determines the fate of the marsupial gonad, theoretically it is not unlikely that the sex-determining mechanisms of these groups have diverged. The sex-determining mechanism may evolve extremely rapidly, leading to numerous mechanisms within a single taxonomic group, such as in the Diptera (Bull 1983) . In marsupials, in fact, there is good evidence that the Y chromosome does not control all sexual dimorphism (Sharman, et al. 1970) , possibly indicating some divergence in sex determination pathways.

Two other 'predictions' about the Y-linked testis determinant were not true in the case of ZFY: 1) that its X-linked homolog must be subject to X inactivation, and 2) that the gene's expression should be confined to the somatic portion of the

gonad, independent of the presence of germ cells. *ZFY* has an X-linked homolog, *ZFX*, a transcribed gene which is nearly identical. Since *ZFY* was postulated to be the primary sex determinant, the presence of an X chromosome homolog presents a theoretical difficulty in that males and females would not differ in terms of 'ZF' gene dosage unless *ZFX* is subject to X inactivation. If it is, then these genes could determine sex by the dosage of transcribed copies present in an embryo (Page, et al. 1987). The finding that *ZFX* escapes from X inactivation soon disproved this interesting model for the action of the mammalian sex determinant (Schneider-Gädicke, et al. 1989). The next test for the new candidate gene involved its expression pattern during development. Recall the development timeline (Figure 1–3) from Section I. The developing gonad begins to manifest a testicular or ovarian phenotype by 12.5 d*pc* in the mouse. There are very good arguments that the sex determinant must function in the somatic supporting cell lineage of the developing gonad around this time (McLaren 1991a, Bogan and Page 1994). Significantly, germ cells are not required for testis determination because it can proceed normally in their complete absence (Merchant 1975, Merchant-Larios and Centeno 1981, McLaren 1991a). Therefore, although *Zfy–1* (a mouse *ZFY* homolog) is expressed in the developing gonad between 10.5 and 14.5 d*pc,* when the expression was shown to be absent in gonads lacking germ cells due to a mutation at the *W* locus (Koopman, et al. 1989), the *ZFY* hypothesis was abandoned. This data suggested that *Zfy* is not the sex determinant because its expression appears to be germ-cell dependent. That is, it would not be transcribed in gonads that complete normal testis determination despite the complete lack of germ cells, and cannot be *TDF.* It is well worth noting, however, that the hypothetical properties of sex determinants, and the experimental assay systems to test them, are always based on imperfect understanding, and although *ZFY* is indeed not a sex determinant, the real disproof of the *ZF* hypothesis only came after the emergence of another candidate locus.

SRY is the mammalian Y-linked testis determinant

A publication reporting three Y+,*ZFY* − XX males and one Y+ XX hermaphrodite, marked the home stretch in the race to clone *TDF* (Palmer, et al. 1989). The individuals described redefined the minimal sex-determining interval of the Y chromosome, implicating a 60 kb region just proximal to the pseudoautosomal (ΨA) boundary as the most likely location. Although these patients exhibited some abnormalities of sexual differentiation, all showed some testicular tissue in gonad biopsy, and are therefore male or hermaphrodite in our nomenclature. This location (some 140 kb distal to the *ZFY* locus) was a slight paradox initially, as the previous study had ruled out the entire region (Page, et al. 1987). That location for *TDF* was based on a Y-chromosome deletion in a patient with a reciprocal translocation between the Y chromosome and chromosome 22. A more detailed study revealed that she had an additional deletion spanning some 600–1,900 kb, including the 60 kb region implicated by the *ZFY*− XX males and hermaphrodite (Page, et al. 1990). It wasn't long before a new candidate gene emerged from a search of the 35 kb of DNA between the ΨA boundary and the most distal breakpoints in the new patients (Sinclair, et al. 1990). Named *SRY* for *s*ex-determining *r*egion *Y*, this locus fulfills all expectations of the mammalian sex determinant. In mouse, it maps to the minimal male-determining region of the Y chromosome (*Sxr*^{b}), is deleted in a line of XY female mice known to have a mutation of *Tdy*(Gubbay, et al. 1990), and is expressed in the somatic compartment of the developing gonad between 10.5 and 12.5 d*pc*, regardless of the presence of germ cells (Koopman, et al. 1990). Even more telling, some human XY females were found to have *de novo* mutations in the *SRY* gene (Berta, et al. 1990, Jäger, et al. 1990, Jäger, et al. 1992). Ironically, the predicted protein product of the *SRY* locus shares a DNA binding motif with a protein called Mc, which is involved in sex determination (*i.e.* mating type switching) in the yeast *Schizzosaccharomyces*

pombe (Kelly, et al. 1988). Another relative of *SRY*, the *HMG1*, or high mobility group-1 protein, gives its name to the HMG–box DNA binding domain. Of course, because the action of the mammalian testis determinant is predicted to exert a dominant effect, the ultimate test of the *SRY* locus was to introduce it into mice, and assay its effects on those transgenic animals that are XX. The result of this experiment gave the final proof that a single gene determines whether the bipotential embryo become a male or a female: *Sry* induces male development of chromosomally female embryos (Koopman, et al. 1991) These experiments mark the end of a seventy-year search for the Y-linked sex determinant, and the beginning of a new phase in the study of mammalian sex determination.

Section III

Are the sex-determining genes all on sex chromosomes?

Although sex determination is chromosomal in all mammals, it is clear that not all sex-determining genes are located on the sex chromosomes. In Section II, I reviewed the evidence that the dominant male-determining effect of the Y chromosome is due to a single locus, *SRY*, located on the Y chromosome short arm in both humans and mice. Here I would like to present a summary of the theoretical, evolutionary, and genetic arguments which lead to the conclusion that autosomes carry sex determining genes as well. Related arguments could be made for the existence of sex-determining genes on the mammalian X chromosome, but I shall not treat this topic specifically. The genetic evidence for sex-determining loci on the X chromosome of humans and wood lemmings is summarized in Section IV. The first question to consider with regard to the autosomes is: must genes reside on sex chromosomes in order to control sex determination? Obviously not, because some species with separate sexes have no heteromorphic sex chromosomes at all. The apparent paradox of 'sex without sex chromosomes' is well-documented for two systems: 1) environmental sex determination, and 2) chromosomal sex determination in the absence of heteromorphic chromosomes. The latter may have been the ancestral pattern from which the diverse sex determining systems of modern vertebrates arose. In any event, mammals *do* have sex chromosomes; therefore, we must also ask whether or not autosomal sex determinants may continue to function, once sex chromosomes evolve. The short answer is that they clearly do, based on the existence of functional autosomal sex-determining genes in several modern-day vertebrate and invertebrate species.

Sex-determining genes need not reside on sex chromosomes

Environmental or chromosomal mechanisms may determine sex in the absence of overtly differentiated sex chromosomes. Environmental cues control the

sex ratio in a variety of species. Bull describes a marine worm in which adult females induce male development in larvae that settle upon them; several species of nematodes in which the proportion of males increases with population density; and various lizards, turtles, crocodiles, and a fish in which temperature determines sex (Bull 1983, 109-127 and references therein). In these species, no chromosome acts as the primary sex determining signal, yet there is no doubt that the environmental cue ultimately influences sex by establishing sexually dimorphic gene expression. These genes are autosomal, or 'proto-autosomal', sex-determining genes. The proto-autosomal designation is perhaps more appropriate since autosomes are only defined in relation to heteromorphic sex chromosomes (Rieger, et al. 1991, *s.v.* autosome). The second example of sex without sex chromosomes is more perplexing. Some species have heterogametic chromosomal sex determination in the absence of heteromorphic sex chromosomes. Allelic differences at one particular locus act as the primary sex-determining signal, but there is no cytogenetic difference between the chromosomes of the pair that carries that locus. Sometimes it is even possible to demonstrate, or 'diagnose', the heterogametic sex in such instances by visible sex-linked markers, or by breeding individuals whose sex is reversed by hormonal or other means (Bull 1983, 13-15). Most species of the Boidae (pythons and boas), one of the four modern snake families, show this pattern, whereas the remaining boids and the other families exhibit a varying degree of sex chromosome heteromorphism in the fourth chromosome pair. Female heterogamety is found in the three remaining groups, and is presumed to apply in the boids that have no chromosome heteromorphism. An interesting aspect of sex chromosome evolution in these snake families is that it roughly corresponds to their degree of divergence from a common ancestor, as judged by morphological development. That is, the morphologically primitive Boidae generally have undifferentiated sex chromosomes, whereas the highly derived Viperidae and

Elapidae exhibit extreme sex chromosome heteromorphism. An intermediate group, the Colubridae, shows moderate to extreme heteromorphism. The evidence from snakes is suggestive of an evolutionary progression from homomorphism to heteromorphism. This idea of a progression led to an evolutionary model in which the homomorphic chromosomes of an ancestral vertebrate gave rise to sex chromosomes independently several times, in a fairly arbitrary selection process (Ohno 1967). The great variety of sex chromosome mechanisms among mammals, birds, reptiles and fish supports this model for sex chromosome evolution (Graves and Schmidt 1992).

Theoretically, all sex determinants were originally autosomal

The model implies that sex-determining genes on the proto-autosomes were fairly numerous, and more or less interchangeable with respect to their potential to act as the primary sex determining signal on a sex chromosome. Perhaps the number of independently-derived sex chromosome pairs present in modern vertebrates represents a rough estimate of their number. Unfortunately, syntenic relationships among distantly related species cannot be assessed with certainty, making any such estimate unreliable. Even so, given that there are at least five different chromosomal sex-determining mechanisms known in vertebrate organisms (Bull 1983, Graves and Schmidt 1992), at least two of which probably derived from separate progenitor chromosomes (because they led to male or female heterogamety), it seems reasonable to predict that a fair number of sex determining genes will be present on the autosomes in modern species with sex chromosomes. Of course, we must assume that no mechanism acts to inactivate these autosomal genes once the sex chromosome pair differentiates, and that these genes have no tendency to translocate to the sex chromosomes. I shall evaluate these assumptions in Chapter Three when I consider the possibility of fundamental differences between autosomal and sex-chromosome-linked sex determining genes, but for

now, let us put all theory aside to consider the genetic evidence for autosomal sex-determining genes, and so conclude this discussion.

Genetic evidence for autosomal sex determinants

There is abundant evidence for the action of autosomal sex-determining genes in a variety of invertebrate and vertebrate species. The genetic and molecular studies in which the sex-determining systems in *Drosophila* and *C. elegans* were elucidated have been reviewed recently (Parkhurst and Meneely 1994). We shall examine these systems in more detail in Section IV. At present I shall just point out one aspect of these regulatory networks that is rarely remarked: in both systems, major regulators of the sexual phenotype map to autosomes. **Table 1–4** contains a list of key sex-determining genes in each organism, along with their location,

Table 1–4. Autosomal and X-Linked Sex-determining Genes in *D. melanogaster* and *C. elegans*

Organism & Gene	Location: X-linked	Autosomal	Reference
D. melanogaster			
Sxl	1–19.2		694†
sis-a	1–34.3		643
sis-b	1–0.0		50
sis-c	1–58.7		(Cline 1993)
run	1–65.8		603
dpn			
emc		3–0.0	197
da		2–41.5	140
tra		3–45	716
tra-2		2–70	717
dsx		3–48.1	174
ix		2–60.5	290
C. elegans			
xol-1	X - 0.6		
sdc-1	X 23.5		
sdc-2	X 4.0		
sdc-3		V 6.9	
her-1		V 2.1	520
tra-1		III 6.7	544
tra-2		II 0.2	544
tra-3		IV 11.6	544
fem-1		IV 2.0	518
fem-2		III - 26.8	518
fem-3		IV 4.1	518

† Numerical references are to the first page of the gene's entry in (Lindsley and Zimm 1992) for *Drosophila*, or in (Wood 1988) for *C. elegans*.

autosomal or X-linked. The Y chromosome has no sex-determining function in *Drosophila*, and is completely absent in *C. elegans*. Not all of the genes listed cause complete sex reversal in mutant animals, but of those that do (indicated in bold face type), some are autosomal and some X-linked in each species. Therefore even by this strict criteria, some sex-determining genes are autosomal in both these invertebrate species. In vertebrates, the field of sex determination has not advanced to the molecular level as in flies and worms, but there is ample genetic evidence for the existence of autosomal sex-determining genes (Eicher 1988). For instance, in a particular breed of goats, the dominant allele for hornlessness (*Polled*) causes epididymal defects in XY animals and pseudohermaphroditism or frank sex reversal in XX animals. Inheritance of *Polled* is autosomal, and it only causes sex reversal when homozygous (Soller, et al. 1969). More extensively studied is inherited XY sex reversal in the mouse. This phenomenon was discovered in two different types of experiments in which related but distinct strains or subspecies of mice were hybridized. The sex reversal becomes apparent when males bearing a 'foreign' Y chromosome are crossed and/or backcrossed to females of another strain. In the case of *T-associated* sex reversal, XY animals in the first generation develop as hermaphrodites or females only if: 1) the Y chromosome from the inbred strain AKR is present with a deletion in the *T* complex on chromosome 17, and 2) the normal chromosome 17 is derived from the C57BL/6J inbred strain (Washburn and Eicher 1983). In another apparently allele-specific form of sex reversal, the Y chromosome of the wild mouse *Mus domesticus poschiavinus* causes sex reversal in the first backcross generation with C57BL/6J (Eicher, et al. 1982) . To explain these phenomena, Eicher and others have hypothesized that *Tdy* ^{POS} or *Tdy* ^{AKR} cannot complete testis determination in a genetic background in which the alleles of key autosomal testis determining loci are 'mismatched' or 'incompatible'. The nature of these

autosomal sex determinants has been the subject of speculation for years, but it has only recently become clear that a finite number of loci can account for Y^{POS} sex reversal. A genetic mapping study implicating several discrete autosomal loci in the control of inherited sex reversal in the mouse is presented in Chapter 2. In humans, direct evidence for autosomal sex-determining genes is not as abundant as that for genes that control sexual differentiation (Austin and Edwards 1981). However, recent results indicate that a great number of sex-reversed human individuals lack mutations in the *SRY* locus. Presumably these individuals carry X-linked or autosomal sex-reversing mutations. General aspects of the genetics of sex reversal in the mouse and humans are discussed in more detail in Section IV of this Chapter. In conclusion, the notion that autosomal loci influence sex determination in mammals has strong theoretical and experimental support.

Section IV

How might genes determine sex in mammals?

In this section I would like to shift the emphasis from the historical to the theoretical, and finally to some practical issues in sex determination. Without the benefit of any experimental data, is it possible to predict the functions of sex determining genes? Predictions as to the nature and expression pattern of the testis determinant became important during the final stage of the search for TDF, but such criteria, based as they are on previous observations, by definition are not theoretical predictions. In purely theoretical terms, then, what must sex-determining genes do? The short answer is that they must activate or repress sex-specific processes. Consider the development of a female, for instance. Activation of female-determining genes is essential for proper sexual development, as is the repression of male-determining genes. In males the regulatory requirements are just the opposite: to repress female-determining genes and activate male-determining genes. Despite potential complications–the absence of a sex-determining gene from one sex might obviate the need for its repression, for example–this simple "four functions" paradigm is useful in analyzing sex-determining systems. Indeed, the ultimate goal in the molecular genetics of sex determination is a complete description of how the four functions are accomplished, for the particular organism under study. For example, **Figure 1–9** shows an outline for such a complete description for the genes to be discussed here and in Chapter Two. In this section I will examine well-understood sex-determining systems in light of this paradigm, and then explore its utility as a theoretical framework for investigations of mammalian sex determination. This evaluation will include a discussion of the practical issue of ascertaining mutations in the study of sex determination, and conclude with a consideration of whether or not sex determining genes are likely to be involved in certain specific developmental processes.

Figure 1–9. Early steps in sex determination showing four locations at which mammalian sex-determining genes may act, according to the four functions paradigm. Courtesy of David C. Page.

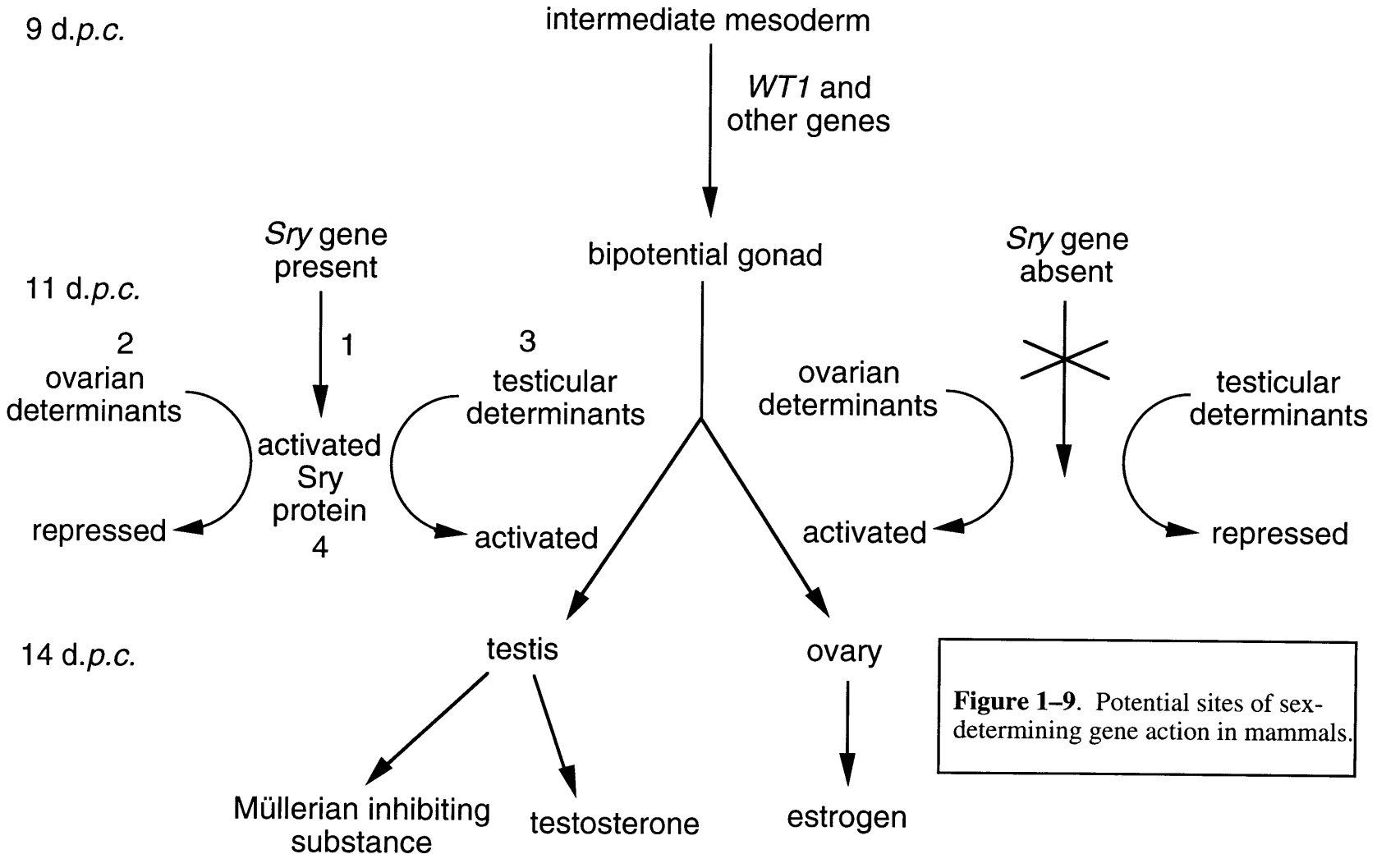


Figure 1–9. Potential sites of sex-determining gene action in mammals.

Sex determination pathways illustrate the four functions paradigm

The sex-determining system of the yeast *Saccharomyces cerevisiae* provides an example of how a regulatory system may consist of just a few activating and repressing functions. The 'sexes' in this unicellular organism, which are called mating types, may be haploid (**a** or α cells), or diploid (**a**/α cells). **a** cells mate with α cells and **a**/α cells with neither of the others. Pheromones control the specificity of the mating response. α cells secrete a unique peptide, α-factor, which is recognized by α-factor receptor, a G-protein-coupled receptor specific to **a** cells. Likewise, the **a** cells signal with **a**-factor, which the α cells recognize *via* the **a**-factor receptor. Clearly, the mating types exhibit dimorphic gene expression patterns. If we think of the mating types as analogous to the sexes in multicellular organisms, the four functions paradigm predicts that each cell type will have a specific activator as well as a repressor specific to the other type. This is indeed the case for α cells, which produce both a repressor and an activator, from the mating type locus MAT. The first of these, α1, is responsible for activating the α-specific genes (abbreviated αsg) such as *STE3*, the **a**-factor receptor, and *MFα1 & 2*, the structural genes that encode the pheromone. The second regulator, α2, has the expected properties: it represses **a**-specific genes (**a**sg) in α cells, including genes encoding the **a**-factor precursor (*MFA1 & 2*), α-factor receptor (*STE2*), and an enzyme that processes **a**-factor precursor (*STE6*). The α1 and α2 regulators are DNA binding proteins which recognize regulatory sites upstream of the genes they control. In **a** cells, our paradigm breaks down because no activating or repressing functions are found. The αsg set is not transcribed in the absence of α1 to activate it, and the basic transcription machinery of the cell transcribes the **a**sg set normally because the α2 repressor is absent (Herskowitz 1988, and references therein). The **a** state therefore appears to be the default state of the yeast cell, with additional regulators required to establish the α state, a regulatory scheme we shall see again

shortly. Although in the yeast *Saccharomyces cerevisiae* two of the four functions are not necessary because one sex has a 'default' gene expression pattern, repressors and activators of sex-specific functions are clearly the key determinants of mating type.

In *Drosophila*, Bridges originally recognized a male tendency of the autosomes in the studies on sex-chromosome aneuploids (reviewed in Section II), identifying the 'X:A ratio' as the primary sex-determining signal. The molecular genetics of this process are now understood in some detail. The general outline is shown schematically in **Figure 1–10**. The primary sex-determining signal is transmitted first to the master regulatory gene *Sex lethal (Sxl*) which, if fully activated, produces females *via* its effects on the splicing of the immediate downstream gene *transformer* (*tra*). Males are produced if the regulatory genes are spliced by the cell's default splicing apparatus. The male transcript includes an exon of *Sxl* containing a stop codon, so that no functional *Sxl* product is present. Hence, *Sxl* acts as a switch in the somatic sex determination pathway. The next gene in the pathway also depends on *Sxl* for a female-specific splice which produces the product which can give correct regulation of *doublesex* (*dsx*), which links the pathway to effector genes together with *intersex* (*ix*).

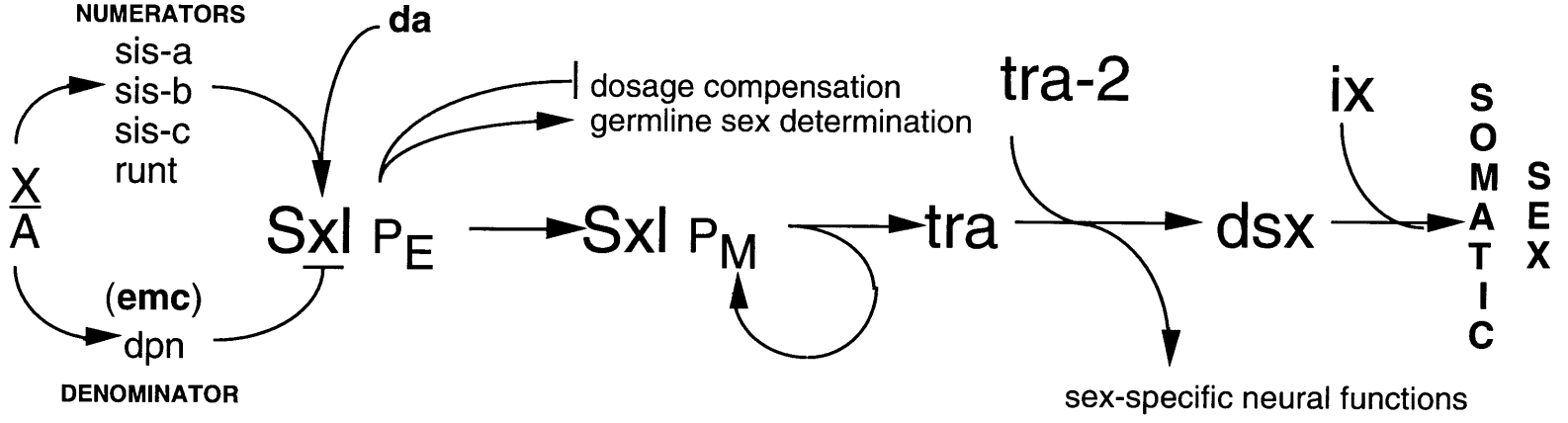
In terms of the four functions, it is obvious that activation of *Sxl*'s early promoter is a key, female-activating step in the pathway. As we saw in *Saccharomyces*, the default state of the cell defines one of the sexes, in this case the male. But subsequent investigations have shown that active repression of male differentiation occurs in females, and active repression of female differentiation occurs in males. These steps are quite far removed from the master regulator *Sxl*, and are in fact accomplished by alternative forms of the same protein. This bifunctional regulator is the gene *dsx*. In combination with *tra*, active *tra-2* ensures the female-specific splicing of *dsx* to yield DSX^F, a 427 amino acid female form of

the gene product. The male-specific transcripts yield DSX^M, which has 549 amino acids. In an elegant synthesis of genetic and biochemical approaches to the problem of how the alternative forms of *dsx* lead to sexually dimorphic gene expression, investigators demonstrated that DSX^M and DSX^F regulate *Yolk protein* (*Yp*) gene expression by transcription repression and activation, respectively (Burtis, et al. 1991, Coschigano and Wensink 1993). In fact, the DSX proteins were shown to bind to the same 127-bp regulatory sequence, the fat body enhancer (FBE), upstream of the *Yp* genes, with opposite effects. This example serves to illustrate another variation on the theme of the four functions, and is an amazing instance of the efficient use of regulators in a sex determining pathway. Other examples can be found in sex determination in the nematode worm *C. elegans*, but for utility I will simply refer the reader to a recent review of these topics (Parkhurst and Meneely 1994).

The four functions operate in mammals as well

In mammals the same four sex-specific functions clearly operate, though much less is known about their molecular identities than in the organisms discussed above. The model predicts that in males activation of male determinants and repression of female determinants is required. *Sry* acts as a dominant male determinant in that it stimulates testicular development of the bipotential gonad regardless of the sex chromosome constitution of the animal (Koopman et al. 1991), but the mechanism of *Sry*'s action remains a mystery. It could establish a 'male' pattern of gene expression directly, or it could repress some molecule that would otherwise lock in a 'female' pattern in the developing gonads. *Sry* could alternatively accomplish both of these functions. Combining the latter prediction that *Sry* might be involved in repressing a female determinant with knowledge of the role of hormones in sexual differentiation, investigators sought for evidence that *Sry* regulates sex-specific promoters. They found that a recombinant *Sry* peptide

Figure 1–10. Schematic representation of gene action in *Drosophila* somatic sex determination, with a summary of each gene's activity state in males and females. A gene which illustrates the four functions paradigm is *doublesex*, whose alternative polypeptide products repress inappropriate sexual development in both males and females. See text for further discussion.



Females 2X:2A	GENES THAT ACTIVATE SXL: ZYGOTIC: SIS A, B, C, AND RUNT MATERNAL: DA	EARLY PROMOTER ACTIVATED BY 2X LEVEL OF NUMERATORS SPLICED TRANSCRIPTS GIVE ACTIVE PROTEIN	MAINTENANCE PROMOTER TRANSCRIBED SXL-MEDIATED SPLICE GIVES ACTIVE PROTEIN + AUTOREGULATION ESTABLISHED	2 TRA TRANSCRIPTS: 1) FEMALE-SPECIFIC FUNCTIONAL, DEPENDS ON SXL FOR SPLICE 2) NON-SEX PECIFIC, GIVES NON-FUNCTIONAL PROTEIN	TRA-2 ACTIVE MULTIPLE TRANSCRIPTS AND PROTEINS DSX SEX-SPECIFIC RNAS GIVE 427 AA FEMALE PRODUCT THAT REPRESSES MALE DIFFERENTIATION IN CONJUNCTION WITH IX
Males 1X:2A	GENES THAT INHIBIT SXL: ZYGOTIC: DPN MATERNAL: EMC	EARLY PROMOTER NOT TRANSCRIBED NUMERATOR PROTEINS INACTIVATED BY DENOMINATORS DPN, EMC	MAINTENANCE PROMOTER TRANSCRIBED, DEFAULT SPLICE INCLUDES EXON WITH STOP CODON TRUNCATED SXL PROTEIN IS NOT FUNCTIONAL	ONE NON-SEX-SPECIFIC TRANSCRIPT GIVES NON-FUNCTIONAL PRODUCT	TRA-2 INACTIVE DSX SEX-SPECIFIC RNAS GIVE 549 AA MALE PRODUCT REPRESSES FEMALE DIFFERENTIATION

***Drosophila* Somatic Sex Determination**

(including only the HMG box domain) binds specifically *in vitro* to 'SRYe' sequences present in the promoter regions of the *MIS* gene and the P450 aromatase gene (Haqq, et al. 1993). The P450 aromatase gene encodes a female function which must be repressed in males: an enzyme that converts testosterone to estradiol. Confirmation that the interactions observed *in vitro* are relevant to regulation *in vivo* would demonstrate a direct link between the [master regulator] and markers specific to differentiated cells. Such a direct link has no precedent in the invertebrate systems discussed above, but there is no theoretical reason why it might not be true. However, two facts mitigate against the results and their interpretation: 1) the period of *MIS* transcription in male embryos (12.5–20 d*pc*) does not overlap significantly with *Sry* transcription (10.5 d*pc*–12.5 d*pc*), and 2) a subsequent report showed no binding of *Sry* to the SRYe binding site (Shen, et al. 1994). Clearly, more experiments are required to establish *Sry*'s true targets, but it is certain that some investigators are approaching this problem with a four functions model in mind.

Regrettably even less is known about the repressor or activator functions of ovary determinants in females. Many investigators have described female sexual development as the default pathway. This description is justified in the sense that female development ensues any time that testis determination fails, but it should not be over-interpreted to mean that ovary determination is a passive process compared to testis determination, or that ovary determinants do not exist. The gene expression profile of differentiated ovarian cells is demonstrably different from that of testicular cells. This could result from active testicular determinants that alter a gene expression pattern that would otherwise lead to ovary formation. Alternatively, different regulators could be expressed in each lineage during their divergence from common precursor cell types. In either case, unique gene expression patterns are required for ovary determination. There is suggestive

genetic evidence that inappropriate early expression of an ovary-determining gene might cause inherited true hermaphroditism in mice (Eicher, et al. 1982, Eicher and Washburn 1983). In their model for sex-determining gene action, the fate of the bipotential gonad hinges critically on the activation of testis determining genes before the ovarian program is activated. In a mouse strain with an early-acting ovary determinant, a Y chromosome which carries a late-acting *Sry* allele could not suppress precocious ovarian development, resulting in hermaphrodite or female development of XY animals (Eicher and Washburn 1983, Palmer and Burgoyne 1991a). A proposal to explain this phenomenon without invoking active ovary determinants is that expression of an *Sry*-induced signalling molecule and its receptor is asynchronous such that testis determination is aborted if a Y chromosome bearing a late-acting *Sry* allele is crossed into an 'early receptor' strain (Lovell-Badge 1992). It is also possible that ovary determinants are activated in all embryos and subsequently repressed in males, if the appropriate *Sry* variant is present. That is, active repression of ovarian determinants could be required during gonadal determination, in a manner analogous to the repression of female duct development by *MIS* in males. The resolution of this on-going debate will no doubt prove interesting. As for the female requirement to repress male-determining genes, the absence of the Y chromosome in females obviates this need with respect to the primary testis determinant. But autosomal or X-linked male-determinants must still be considered. Recently, loss of function mutation in a gene that normally represses male development in females was invoked to explain the occurrence of four familial cases of XX sex reversal in humans (McElreavey, et al. 1993). The hypothetical Z gene would have to be activated in females, and inactivated in males, presumably by the direct or indirect action of *SRY*. A dominant mutation rendering Z unresponsive to *SRY*-mediated repression could result in female development in sex reversed XY individuals that have no detectable

mutations at the *SRY* locus (>90% of all XY females) (Hawkins, et al. 1992a, Hawkins, et al. 1992b). Once again this gene remains a theoretical possibility only. The four functions paradigm is not testable in mammals in the sense that it is generally not possible to design mutant screens for the hypothetical genes as it is in some laboratory organisms . However, any viable model for the action of sex determining genes must include some if not all of the four functions in the paradigm.

How are sex reversing mutations ascertained without genetic screens?

Because performing a genetic screen for sex determination mutations is practically and ethically impossible in humans, and costly in mice (but see below), the pool of mutant individuals is restricted to those that can be ascertained in a practical manner. In humans, this means that ascertainment of mutations in sex determination is limited to those which lead to congenital abnormalities of the external genitals, or those that individuals themselves are likely to bring to the attention of a physician later in life. Fortunately humans are acutely sensitive to their own phenotype, making 'self-report' a reliable means of ascertainment. In mice, effects on fertility may bring a mutation to the attention of geneticists. What follows is a catalogue of known mutations in humans and mice, all of which were ascertained in some straightforward way, in which I shall emphasize the observed phenotype, especially when it may reveal clues about the nature of sex determining gene action. The cardinal phenotype resulting from the mutation of any sex determining gene is sex reversal. To organize the following discussion, the mutations are grouped according to the location of the sex reversing mutation on the Y chromosome, the X chromosome, or an autosome.

Y chromosome-linked mutations cause sex reversal in humans and mice

Some human XX males and XY females have mutations of the Y chromosome

Y-linked mutations involving the *SRY/Sry* locus may cause sex reversal in humans and mice. Sex-reversed human individuals may be males or females and frequently have alterations of the Y chromosome. As discussed previously, 46,XX males frequently carry some portion of the sex-determining region of the Y chromosome on the paternally-derived X chromosome. The clinical features of sex reversal in these individuals are not that remarkable (Therkelsen 1964, de la Chapelle 1981, Polani 1981, Ferguson-Smith, et al. 1990, Ferguson-Smith 1992). These patients resemble those that have Klinefelter's syndrome (KS) with the following exceptions: XX males have below average height, normal intellectual function, and an increased frequency of breast development (Ferguson-Smith, et al. 1990, 520). The common features are a general masculine appearance and male psychosexual orientation, but with small testes, abnormal azoospermic testicular histology, and normal to low androgen levels (de la Chapelle 1981, 105-106). Polani (1981, 478) summarizes other relevant features:

> The internal genital tract is similar to that of normal males. Hypospadias has been found occasionally. The testes are generally small and of soft consistency in the older XX males. Hormonally, they resemble Klinefelter males: they have increased plasma levels of follicle stimulating hormone, with low levels of plasma testosterone... Histologically, in older children and adults, the testes resemble those of KS, with small and uneven testicular tubules that are more or less hyalinized , peritubular fibrosis, absent spermatogonia, and prominent uneven agglomerates of interstitial Leydig cells which may be present in greater quantities than normal ... [However] it would seem that in infants the testes can be normal histologically, and may contain spermatogonia ...

Ascertainment of these patients is usually a consequence of self-report for infertility, small testes, or abnormal secondary sexual characteristics such as gynecomastia (de la Chapelle 1981, 106). Human 46,XY females are traditionally

divided into two groups, those with a diagnosis of pure gonadal dysgenesis (PGD), and those that display the developmental somatic abnormalities of Turner's Syndrome as well as the gonadal phenotype. Both types of patients are sex-reversed according to our definition, and were useful in the search for *TDF* because they can have deletions of the Y chromosome which inactivate the *SRY* locus (Ferguson-Smith, et al. 1990, 520). 46,XY individuals with TS presumably have Y-linked mutations inactivating both testis-determinants and the hypothetical anti-Turner gene on the Y chromosome. We shall not consider TS any further, except to note that the gonadal dysgenesis observed is identical to that found in 46,XY PGD females. These individuals have a different and perhaps more severe syndrome of abnormal sexual development than do 46,XX males, but are fairly normal females early in life (Polani 1981, Ferguson-Smith 1992). Adults are sterile and secondary sex characteristics are absent after puberty. Their stature is slightly taller than that of XX females and of XX males, but shorter than that of XY males (Ogata and Matsuo 1992). Gonadal development in the XY female is markedly disturbed, the ovaries degenerating into thin streaks of ovarian stroma without follicles. The remnants may become malignant and form gonadoblastoma, a very rare cancer, that can progress into dysgerminoma. Possibly these cancers form from XY germ cells removed from their normal environment (Ferguson-Smith 1992, 521). The development of the Müllerian duct and external genitals is female since a testis is not formed. In the case of 46,XY pure gonadal dysgenesis, the patients present with primary amenorrhea or gonadoblastoma.

<u>In the mouse, both types of sex reversal have been observed</u>

A dominant mutation called *Sxr*^{a} causes sex reversal in XX mice (Cattanach, et al. 1971). *Sxr* ^{a} was originally interpreted to be either a Y-autosome translocation or a masculinizing mutation in an autosomal sex determining gene, because XY carrier males produce four types of offspring in equal numbers: XX

females, XY males, XY *Sxr^a* carrier males, and XX *Sxr^a* affected (sterile) males. The mutation is now known to be the result of duplication and transposition of a portion of the mouse Y chromosome short arm (Yp) to the end of the long arm, distal to the pseudoautosomal region (Singh and Jones 1982). The original genetic data agree with this pseudoautosomal location for *Sxr^a*, and more recently, a cytogenetic study utilizing a single-copy probe within the *Sxr^a* region demonstrated two copies of the region on the YSxr chromosome (Roberts, et al. 1988). The copy of *Sxr^a* on Yq is transferred to the X chromosome by an obligate chromatid exchange during meiosis in XY carrier males, such that 50% of the sperm they produce have an X^{Sxr} chromosome (Singh and Jones 1982, McLaren, et al. 1992). As noted earlier, a deleted derivative of the *Sxr^a* transposition, called *Sxr^b*, was important in refuting the *Hya* hypothesis of testis determination (McLaren, et al. 1984, and references in Section II). More recently, *Sxr^b* has been exploited to make a rough estimate of the order of the five genes known to reside in *Sxr^a*: *Sry*, *Zfy-1* and *Zfy-2*, *Hya*, and *Spy* (Simpson and Page 1991). Beyond these instances, the *Sxr* transposition never fulfilled its promise as a potential tool for cloning the testis determining locus. Mouse *Sry* was cloned by homology to its human homolog, which, as discussed above, was identified by deletion mapping and chromosome walking.

Initially even more mysterious than *Sxr^a*, a mutation leading to XY sex reversal in mice, *Tdy^{m1}*, is also the result of a Y-linked mutation. Anticipating the difficulty of a deletion mapping approach to cloning the mouse testis determinant, Lovell-Badge and Robertson (1990) used a retroviral mutagenesis approach to generate tagged mutations in the *Tdy* locus. The mutant screen they designed was ambitious, but certainly feasible (Lovell-Badge and Robertson 1990). Embryonic stem cells were multiply infected with a replication-defective retrovirus designed to mutate endogenous loci by insertion, mark particular cell lineages by multiple

integrations, and tag mutated loci for later identification. Several lines of mutagenized cells were injected in each host blastocyst, in hopes that at least one line would supply a normal testicular environment in which ES cells carrying a mutation of the primary sex determinant might still be able to contribute functional sperm. Founder male chimeras were screened for the ability to sire female offspring lacking paternal X chromosome markers. The marked females for the test mating were homozygous for the *phoshoglycerate kinase-1*^{a} allele (*Pgk-1*^{a}), or heterozygous for the *blotchy* allele of the *Mottled* locus. For *Pgk*, normal XX female daughters were expected to be *Pgk-1*$^{a/b}$ and anomalous daughters would lack the paternal *Pgk-1*^{b} allele. For *Mottled*, normal daughters would be +/+ or *blo*/+ (wild-type or *blotchy* phenotype, respectively), while anomalous daughters would be *blo*/Y (light-colored) or +/Y (wild-type). After testing only three chimeras, a male was found that gave rise to F1 female progeny with inappropriate phenotypes (*blo* or *Pgk-1*^{a}) at a frequency of about 3-4%. Eleven of thirteen of these females proved to have Y DNA by karyotypic or Southern blot analysis. The other two were anomalous due to an XO chromosome constitution or XO/XX mosaicism.

The phenotype of the mutant XY females proved surprising as well. Despite an abnormally low number of normal oocytes in the ovaries of these mice, two of the F1 progeny and numerous XY females in subsequent generations proved to be fertile. The reproductive lifespan is reduced, however, as is the size and frequency of their litters. Fertility in these females allowed the *Tdy*^{m1} mutation to be tested for complementation with three other altered mouse chromosomes: 'small y', X^{Sxra}, and X^{Sxrb}. The small y is a cytological marker Y chromosome, apparently functional but lacking much of the long arm, it is easily distinguished from the Y chromosome of typical inbred strains. All three chromosomes complement the *Tdy*^{m1} mutation, that is, both types of X^{Sxr}Y$^{Tdy.m1}$ and

XyY$^{Tdy.m1}$ animals are normal, fertile males. The latter mice are aneuploids that are produced quite frequently in the appropriate matings because the Y$^{Tdy.m1}$ chromosome segregates randomly in female meiosis, probably because it fails to pair with the X chromosome. This genetic data argues strongly that the sex-reversing mutation is located on the Y chromosome in the *Tdy* locus itself. Mysteriously, none of the retroviral insertions in the sex-reversed strain was found to segregate with the mutation. In fact, the investigators were able to deduce that the sex-reversing mutation arose subsequent to the retroviral mutagenesis because the mutant cell line carries the same 8 retroviral insertions found in other fertile XY mice derived from the same founder. Once again, a promising discovery failed to localize the mouse Tdy locus. Once the human SRY gene was used to clone the mouse locus, the likely cause of the Tdy.m1 mutation was revealed. The Sry locus lies within 2.8 kb of unique sequence which is situated within a large inverted repeat. Sequence from genomic DNA cloned from a XY$^{Tdy.m1}$ female indicates that a rearrangement involving the inverted repeats is the likely cause of the sex-reversing mutation. The rearrangement resulted in a small inversion and an 11 kb deletion encompassing the entire Sry coding sequence (Gubbay, et al. 1992). The paradoxical results of this mutant screen are finally understood.

X chromosome-linked mutations also cause sex reversal

X-linked mutations also cause sex reversal, but fewer have been reported. The best-studied example is the X* chromosome in the lemming species *Myopus schisticolor*, *Dicrostonyx torquatus* and *D. groenlandicus* (the wood lemming, varying lemming, and bog lemming, respectively). In these arctic rodents, an X-linked mutation has apparently converted the sex-determining system from dominant Y heterogamety to a multiple factor system (Bull 1983, 29-30; 79-80). The derivative X chromosome, called X* in wood lemmings, has the surprising property of a dominant female-determining factor, such that the sex chromosomes

of females may be XX, X*X, or X*Y, while those of males are XY (Fredga, et al. 1976). In some isolates of the *Dicrostonyx* species, the Y chromosome may be completely absent (XX, X*X, X*O females/XO males)(Gileva 1980). This dominant X* chromosome leads to an excess of females in captive lemming populations, with the observed proportion of females, especially X*Y females, being consistently higher than theoretical predictions (Bull 1983, 80). Fredga *et al.* originally proposed that XY sex reversal in lemmings is due to mutation of a major sex-determining gene on the X chromosome (Gropp, et al. 1976, Fredga, et al. 1977), based on the observation that the X* chromosome is cytologically distinguishable from the X chromosome in the wood lemming (Herbst, et al. 1978). The simplest interpretation of the cytogenetic data is that the X* chromosome carries a duplication of part of the X chromosome. A recent report shows that the X* and X chromosomes of the wood lemming are also distinguishable using molecular probes derived from the human *Zfy* locus, but *Zfx* itself does not appear to be a candidate for the X* chromosome mutation (Lau, et al. 1992).

Shortly after descriptions of the lemming chromosomes appeared, a duplication of the X chromosome short arm was found in two sex reversed human XY females. Each had a 46,dup(X)(p21->pter)Y karyotype and multiple somatic developmental abnormalities in addition to sex reversal (Bernstein, et al. 1980). The duplicated X chromosome was present in the mother, maternal grandmother, and sister, but its deleterious effects were nullified by non-random inactivation. The sex-reversal was noted in a 5-year-old child and a fetus at 20 weeks' gestation. Both appeared to be normal females except that in the 5-year-old, the gonad had degenerated to a streak of ovarian stroma with degenerating primordial follicles. The fetus had normal ovaries with abundant primordial follicles (Bernstein, et al. 1980, 294). Subsequently, two additional reports of X chromosome

rearrangements associated with XY sex reversal in three patients appeared in the literature, strengthening the argument for a sex-determining gene and narrowing the range of possible locations to Xp21.2–Xp22.2 (Scherer, et al. 1989, Ogata, et al. 1992). Gonadal histology was not performed for two of these three new cases, but in the third, streak gonads and gonadoblastoma were found together with normal female internal and external genitals at autopsy. It is interesting to note that the phenotype associated with the X-linked mutation causing sex reversal in humans is very different from that in the lemming. The XY female wood lemmings do not exhibit the multiple developmental somatic abnormalities nor the gonadal dysgenesis of their human counterparts. They are normal, fertile females, perhaps by virtue of a poorly understood non-disjunction mechanism by which the Y chromosome is lost and the X* chromosome duplicated in the development of the germ line (Fredga 1988). In any event, both of these examples show that there is clear genetic and cytogenetic evidence for a major sex-determining gene on the X chromosome.

Autosomal mutations may cause sex reversal:

T-associated sex reversal in the mouse

Mutations at autosomal loci are also known to cause sex reversal, and several have been studied extensively in the mouse. The existence of a locus capable of causing sex-reversal within the *T* (Brachyury) complex is well-documented (Washburn and Eicher 1983, Erikson, et al. 1987, Eicher 1988, Washburn, et al. 1990). Located on mouse chromosome 17, the *T* complex is a ~40 Mb (20 cM) region over which four non-overlapping inversions have occurred, effectively preventing recombination in heterozygous animals. The second inversion includes the *T* gene itself, and the genetic effects of the *T* complex were first described in conjunction with that gene's effects on tail phenotype. The general features of the *T* complex have been reviewed recently (Silver 1993). Among the

many genes which map to this region, *T* associated sex reversal, or *Tas*, was named for the following effect: when a spontaneous new *T* allele, T^{hp}, was crossed onto the C57BL/6J (B6) inbred background, no XY animal that inherited T^{hp} developed normal testes; ovaries or ovotestes were found instead (Washburn and Eicher 1983, 339). This phenomenon was also noted for T^{Orl}, and both T^{hp} and T^{Orl} were shown to have deletions within the *T* complex. Interestingly, the sex reversal is allele specific. That is, it was only evident when the source of the Y chromosome was the AKR/J inbred strain, and the *Tas* allele on the normal chromosome 17 came from C57BL/6J . At least two conclusions are indicated: 1) *Tas* must lie within the region of overlap of the T^{hp} with the T^{Orl} deletion, and 2) *Tdy* has to be genetically compatible with at least one autosomal locus to accomplish testis determination (Washburn and Eicher 1989).

Inherited true sex reversal in the mouse

The conclusion that the autosomes carry sex determining genes is substantiated by genetic mapping studies of inherited true sex reversal in the mouse. First reported by Eicher *et al.* (1982), sex reversal in this instance depends on the Y chromosome of the wild mouse *Mus domesticus poschiavinus* and the autosomal complement of the C57BL/6J inbred strain (of *Mus musculus musculus* origin). When the Y^{POS} is present in the C57BL/6J background, the phenotype of XY animals ranges from normal male to true hermaphrodite to sterile female, depending on the extent of the C57BL/6J contribution to the genome (reviewed by Eicher and Washburn, 1986). Again, the sex reversal was found to be allele-specific: the reciprocal situation of crossing the Y^{B6} onto the *M. d. poschiavinus* strain does not lead to sex reversal, and the Y^{POS} functions normally on several other inbred strain backgrounds. This phenomenon can be duplicated using some but not all mouse strains carrying a Y chromosome of *domesticus* origin (Eicher, et al. 1982, Biddle

and Nishioka 1988). For this reason, this phenomenon is sometimes called B6.Y^{DOM}, or simply Y^{DOM}, sex reversal.

In the first genetic investigation of Y^{DOM} sex reversal, Eicher reports that 103/185 XY progeny from a backcross (B6 females to B6.RI.Y^{POS} F1 males) show either partial or complete sex reversal (Eicher and Washburn 1983, 299). This suggests a 1:1 ratio, and consequently, that the sex reversal segregates as a single autosomal locus with recessive, 'incompatible' alleles in the B6 strain (*tda-1*^{b}) and dominant, 'compatible' alleles (*Tda-1*^{do}) in *M. d. poschiavinus* and other inbred strains (such as the RI strains reported or DBA/2J, BALB/cBy, or C58/J). Accordingly, the Y^{POS} was crossed into a panel of BXD recombinant inbred strains (Taylor 1978) to assess the chromosomal location of *tda-1* by the strain distribution pattern method. Unpublished reports of a possible location on chromosome 2 or chromosome 4 were never confirmed. One interpretation of these findings is that multiple loci are involved in Y^{POS} sex reversal. In another attempt at using RI strains to solve this problem, C57BL/6J.Y^{POS} hermaphrodites were bred to females of the NXSM recombinant inbred panel (Eicher 1988, Eicher and Lee 1990). The progenitor strains of this panel vary at *Tda-1* because sex reversal is observed when the Y^{POS} is placed on an NZB/BLNJ (N), but not on an SM/J (SM) background. Once again, unpublished reports of a *Tda-2* locus on chromosome 12 segregating between the N and SM strains of mice are as yet unconfirmed. In Chapter 2 of this dissertation, I will present evidence that at least two novel autosomal sex determining genes contribute to Y^{DOM} inherited true sex reversal.

The phenotype of gonadal hermaphrodites is instructive

The phenotype, or rather the distribution of the possible phenotypes, of sex reversed mice has been analyzed in detail, in the hope that it might shed light on the mode of action of the controlling genes. In the early reports of inherited true sex reversal, three general features of the phenotype of XY animals were noted

(abbreviations: O, ovary; OT, ovotestis; T, testis): 1) the O+O class (bilateral ovaries or 'complete' sex reversal) was rare, 2) the phenotype of hermaphroditism was varied (O+OT, OT+OT, and OT+T possible) and present in about half of the progeny of a backcross, 3) the O+T and T+O classes of hermaphrodites were never observed, and 4) in the asymmetrical classes (O+OT and T+OT), the testicular tissue is much more likely to be found on the left side of the animal's body (Eicher and Washburn 1983, Biddle and Nishioka 1988, Biddle, et al. 1994) Various investigators have addressed the first two features of the phenotype, but neither they nor the others have been satisfactorily explained. There are two hypotheses concerning the low frequency of O+O animals in early generations of Y^{POS} congenic line construction. The first is that the O+O phenotype depends on the action of several loci, which must all be homozygous tda^b/tda^b before complete sex reversal can be observed. The second is that the genetic background controls only the 'liability' to express testicular tissue, and stochastic events in the course of development result in a consistent phenotypic distribution amongst the hermaphrodites, including some animals with no testicular tissue at all. In defense of the first point of view, Eicher states:

> ...genes other than *Tda-1* also play a role in causing sex reversal of C57BL/6J-Y^{POS} mice. For example, all XY mice of the C57BL/6J-Y^{POS} strain develop either as females with two ovaries or as hermaphrodites, half of which have two ovotestes and half have an ovary and an ovotestis. No C57BL/6J-Y^{POS} XY mouse develops even a single testis. This result is in contrast to what was observed in first backcross XY offspring produced in matings involving C57BL/6J females mated to the F1 males...[carrying the Y^{POS}]. In these cases, although half of the backcross XY mice developed ovarian tissue, the ovarian tissue was usually present in an ovotestis and more often accompanied by a testis or another ovotestis than an ovary...To explain these contrasting results, we have suggested that there are other C57BL/6J-derived alleles at autosomal loci that, when present in the homozygous state in a $Tda\text{-}1^b/Tda\text{-}1^b$ XY^{POS} individual, increase the probability that ovarian tissue will develop (Eicher 1988, 111-112).

The proponent of the second hypothesis has recently rejected the earlier single-gene model, but not the idea that stochastic events in development can convert embryos with identical genotypes into phenotypically distinct animals in a predictable pattern (Biddle, et al. 1994). His recent, detailed study of the sex reversal phenotype focuses on the frequency of five possible phenotypic classes: O+O, O+OT, OT+OT, OT+T, and T+T, in a B6.Y^{POS} congenic line. When the phenotype is broken down into these 'graded' categories, patterns emerge that are obscured when all abnormal individuals are grouped together as hermaphrodites. For instance, in the recent study, various reciprocal backcross matings involving B6, D2, and the Y^{POS} gave a total of 303/560 XY animals with some degree of sex reversal. This would apparently support a single-gene model since the numbers approximate the 1:1 ratio expected in a backcross. However, the *distribution* of the phenotypes (observed % of O+O, O+OT, OT+OT, OT+T, T+T) is 2, 3, 28, 21, and 46%, quite different from the distribution of 25, 10, 13, 1, and 51 % expected if a single locus determines the phenotype (Biddle, et al. 1994, 301). In any event, this study provides a good theoretical foundation for the results in the next chapter.

To return to the question of what the phenotype can tell us about sex determining gene action, as opposed to simple predictions about numbers, the low frequency of the O+O phenotype in early generations of the Y^{POS} congenic lines is significant. Since the frequency increases with each generation up to about 50%, there must exist several loci at which Y^{POS}-compatible alleles are required for proper testis determination. This could be interpreted to mean that *Sry* functions in a 'region' of the mammalian sex determining pathway which is a regulatory web or network, instead of a linear region with one regulator and one target. An example of a web region from *Drosophila*'s sex determining pathway is the early steps in Figure 1-10 when the balance of numerator and denominator elements is determining the transcription status of *Sxl*'s early promoter. Many proteins interact

at that point, competing for binding sites in the other regulators, or within the promoter. The allele specificity of mouse Y chromosome-autosome incompatibility leads to the prediction that some form of direct interaction will be found between the *Sry* gene and the *tda* genes. Since these genes are apparently numerous, I suggest that *Sry* may function in a network of regulation instead of the branch-point which is the traditional view. The absence of the O+T and T+O classes amongst the hermaphrodite progeny raises interesting questions. The fate of the contralateral gonad can certainly yield some information about genetic *v.* stochastic events in development, since a contralateral gonad must start with the same genetic information. But it is quite possible that the developing gonads may influence each other. In this case, the observed phenotype is the sum of the genetic influences we are interested to discover, together with the effects of unpredictable events during embryogenesis. For instance, if a testis (but not an ovotestis) was able to induce testicular development of a contralateral gonad by a long-range induction effect, perhaps *via* hormones, then testes would only be found with contralateral testes or ovotestes, as is observed. Quite apart from the conspicuous absence of the O+T and T+O hermaphrodites, it is worthwhile to consider the implications of the asymmetrical classes that *are* found. If an ovary can develop in the same animal with an ovotestis, then the process of gonadal determination must be responsive to influences other than those that can be rigorously genetically programmed, such as inductive processes. Several lines of evidence point to the influence of an inductive process in testis determination (see below), but such an inductive force must be rather weak. Otherwise, testicular tissue of the ovotestes found in sex reversed mice would be capable of inducing the adjacent ovarian tissue to become testicular, so that no ovotestes would be observed. But perhaps the very basis of Y^{DOM} sex reversal is a defect in the reception of an inductive signal required for completion of testis development. In this case, tissue that is normally masculinized would remain

undifferentiated until the ovarian program is activated, resulting in ovotestis formation. Finally, although the left-side bias of testicular development in hermaphrodites is well-documented, its implications remain a mystery at present. Until the genes which control Y^{DOM} sex reversal are identified, we can only speculate about their functions, but the best foundation for such speculations is a thorough understanding of the mutant phenotype.

Unexplained sex reversal in humans

In humans the evidence for autosomal mutations that lead to sex reversal is mostly indirect at present, with the exception of a growing body of data implicating a locus on the long arm of chromosome 17. The proportion of cases of human sex reversal that is due to Y-chromosome-linked *versus* other mutations varies dramatically between 46,XX and 46,XY individuals. Most (probably >90%) of the 46, XX males in the medical literature have been shown to harbor an X chromosome that carries Y-specific DNA as a result of an aberrant exchange with the X chromosome during paternal meiosis (Ferguson-Smith 1966, Guellaen, et al. 1984). The remainder, who may be referred to as *SRY*$^{-}$ XX males, are alternatively supposed to carry constitutively activated alleles of a testis-determining gene on the X chromosome (Ferguson-Smith 1992), or two mutant copies of an autosomal gene which normally represses male development in females (McElreavey, et al. 1993). An interesting aspect of these cases is that the Y^{-} status of an XX male is positively correlated with abnormal external genitalia and an increased frequency of gynecomastia (Ferguson-Smith, et al. 1990). Any genetic explanation proposed for these individuals must certainly account for their incomplete masculinization. One possibility is that *SRY*$^{-}$ XX males have gain-of-function mutations in genes that operate downstream of a branch-point in a cascade of SRY-activated regulators. In this case, only those regulators after the branch-point would function properly, leading to incomplete masculinization. In marked

contrast, only about 10% of 46,XY females have demonstrable mutations on the Y chromosome (Hawkins, et al. 1992b), and most of these are point mutations or small deletions at the *SRY* locus, not the large deletions of Y DNA predicted to result from aberrant exchange (Ferguson-Smith 1992, 521). It has been suggested that a mutation in SRY in an XY female leads to a more severe form of gonadal dysgenesis than that seen in SRY^+ XY females (Hawkins, et al. 1992a). Whether or not this hypothetical genotype–phenotype correlation proves true, we know that the majority of XY females have no known defect of the Y chromosome or the testis determining locus, and could certainly have autosomal sex-reversing mutations.

Campomelic dysplasia in humans

An example of such a sex-reversing autosomal mutation in humans is the locus on the long arm of chromosome 17 (17q24–q25), which causes the 46,XY sex reversal sometimes associated with campomelic dysplasia (CMPD). This disorder is a semilethal, autosomal recessive trait which manifests as a characteristic array of serious skeletal defects involving the extremities, pelvic and shoulder girdles, and thoracic cage (Maraia, et al. 1991). Genetic studies indicate that CMPD is heterogeneous, and also associated with a high incidence of XY sex reversal (21 females and 2 intersexes among 33 campomelic individuals with a 46,XY karyotype in one early report). Although it is currently unclear whether CMPD and sex reversal are pleiotropic effects of the same mutation, or whether the phenotypes result from a contiguous deletion syndrome, the chromosomal rearrangements involving 17q reported recently make it certain that the nature of this sex determining locus will not remain a mystery much longer (Maraia, et al. 1991, Young, et al. 1992, Tommerup, et al. 1993).

What processes are sex determining genes likely to control?

After considering the substantial genetic evidence for sex determining genes throughout the genome, can we make any predictions about how these genes will function in sexual development? Genetics alone will not aid us in this area, but we can review the growing field of the molecular genetics of sex determination with our four functions paradigm in mind. It may be useful to refer back to Figures 1–9, 1–10, and Table 1–4 during the following discussion. For insight into the processes which sex determining genes are likely to control in mammals, we may consider invertebrate examples, what is known about the molecular action of the primary testis determinant SRY, experiments with chimeric animals aimed at dissecting the embryology of sex determination, and what we know about the existing mammalian mutants. From these lines of evidence, the following simple predictions emerge: genes most likely control mammalian sex determination through regulation of other genes, cell fate determination and intercellular communication during development, and perhaps even such basic processes as the timing and pace of embryonic growth.

Sex determinants are developmental regulators

In a comparison of sex determination in the invertebrates *D. melanogaster* and *C. elegans*, Hodgkin describes both systems in terms of a single paradigm of a developmental pathway originating at the primary sex determining signal, continuing to a set of master regulator genes, followed by intermediate regulators, and ending with terminal regulator genes (effectors) (Hodgkin 1990) . Many of the genes investigated have been found to be active during development and to regulate the action of another gene or genes in the pathway, or a gene responsible for some sexually dimorphic character. An examples from *C elegans* is the master regulator gene *sdc-1*, a zinc-finger motif DNA binding protein that, in conjunction with *sdc-2* and *3*, exert negative control over the transcription of the next gene in the pathway,

her-1 (Parkhurst and Meneely 1994, and references therein). In *Drosophila*, of the eleven genes in Table 1–4, one is a splicing factor that binds RNA, and at least seven are transcriptional regulators (Parkhurst and Meneely 1994, and references therein). Transcriptional control and RNA splicing are two emerging themes. Writing about mammals, McLaren echoes Hodgkin's paradigm with a pathway beginning at the sex determining 'switch', proceeding through sex determining intermediates, and terminating with sexual differentiation genes (McLaren 1988b). The genetic properties of the mammalian sex determinant *SRY* indicate it is the switch gene that initiates the pathway in males, and what is known about its molecular identity invites parallels with the invertebrate transcriptional regulators. *SRY* binds specifically to linear DNA and non-specifically to cruciform DNA (Harley, et al. 1992). Upon binding to linear DNA, *SRY* induces a dramatic bend (130^{o}) which would seem to have obligatory consequences within the cell (Giese, et al. 1992). However, it is not known whether this kind of DNA binding factor is expected to act as a repressor or an activator, so what is known thus far about the molecular details of *SRY*'s action do not let us place it precisely within the four functions paradigm. We may conclude with certainty, however, from the genetic as well as the biochemical evidence, that it is a regulator that we expect to act on other genes in a cell autonomous fashion.

Cell fate decisions are important in the determination of sex

Mammalian embryologists have long interpreted the problem of gonadal determination as one of cell fate determination. As reviewed in Section I of this chapter, the embryonic mammalian gonad is composed of three bipotential cell types which follow different fates in each sex. The supporting cell, interstitial cell, and germ cell lineages give rise to Sertoli cells, Leydig cells, and prospermatogonia in the male embryo; whereas they become follicle cells, theca cells, and oocytes in the female (McLaren 1991b, Hodgkin 1992) Clearly, cell fate determination must

be at least indirectly involved in sex determination. Surprisingly, there is even some precedent for this amongst the invertebrate organisms, whose development was formerly thought to proceed by strictly cell autonomous mechanisms (Hodgkin 1992, and references therein). The data upon which this conclusion is based comes from a series of experiments performed using XO/XY or XO/XY/XYY mosaics or XX<–>XY chimeric mice made by aggregation or blastocyst injection (Burgoyne, et al. 1988, Patek, et al. 1991, Palmer and Burgoyne 1991b). For the chimeras, it was found that the XX/XY composition of all cell types in a particular animal were similar, but that in the Sertoli cells of the gonad, a marked skewing towards XY cells was observed, indicating that the Y chromosome is required for Sertoli cell determination. An initial study reported no XX Sertoli cells at all (Burgoyne, et al. 1988) , but improved methods and examination of fetal as well as prepuberal and adult mice demonstrated up to 20% XX Sertoli cells in some fetal chimeras; whereas this appears to drop to a constant low value of about 2% in adults (Palmer and Burgoyne 1991b). Burgoyne and others conclude from this data that the role of *Tdy* is to direct the cells of the supporting cell lineage to form Sertoli cells. The short burst of *Sry* expression from 10.5–12.5 d*pc* in the somatic compartment of the bipotential gonad offers indirect support for this notion. However, the Sertoli cells may then go on to influence the cells around them, recruiting XX cells into the Sertoli cell pool, for instance. This effect is presumably mediated via molecules induced by a hypothetical *Sry*-initiated cascade of gene expression, since a DNA binding protein is not expected to be secreted. This would mean that sex is not determined in mammals in the supporting cell lineage of the developing gonad in a fundamentally cell-autonomous fashion, but that some of intercellular signalling is involved. These experiments are reminiscent of those in which intercellular signalling was found to be important in the determination of sex in *C. elegans*. This process was likewise believed to proceed in a strictly cell-autonomous fashion

initially until mosaic analysis was applied to certain key regulators. In experiments I will not review here specifically, the master regulator gene *her-1* was found to be neither necessary nor sufficient for masculinizing marked cells in mosaic animals (Hunter and Wood 1992, Perry, et al. 1993) . The authors conclude that the *her-1* gene produces an intercellular signal, and suggest that the next gene in the pathway, *tra-2* , is a good candidate for the receptor. In conclusion, cell fate determination appears to be integral to sex determination, but we obviously have much to learn about the processes involved.

Sex determinants may be growth factors

The literature on sex determinants as growth factors or their regulators is continually growing. In the Future Directions section of this work, I specifically review recent manuscripts by Hurst in which he makes sound evolutionary arguments for the Y chromosome acting as an attractor for selfish growth factors. There is good evidence for a stature determinant on the human Y chromosome (Ogata and Matsuo 1992), but the arguments for sex determinants as growth-promoting genes rely more on an effect on timing and pace of growth during embryogenesis than on adult stature. Therefore it is unclear whether the stature determinant should be interpreted as support for such arguments. In this section, I would like to review the genetic evidence for a growth effect in male embryos influencing sex determination. Eicher and Washburn were the first to invoke the timing of developmental milestones as crucial determinants of testis or ovary development (Eicher and Washburn 1983, Eicher and Washburn 1986). They posit two genetic pathways, one leads to testis development and is shadowed in developmental time by the other, which leads to ovary development. A key feature of the model is that the first testis determining gene should pre-empt activation of the first ovary-determining gene, perhaps even inactivating it. The model is based on the observations reviewed in this section regarding Y^{DOM} sex reversal, namely,

that the Y^{DOM} is not capable of completing testis determination on some genetic backgrounds in the mouse. The developmental asynchrony model explains why the phenomenon is only observed for Y^{DOM} on a *M. m. musculus* background, and not vice versa. The Y^{DOM} is postulated to have a late-acting allele of *Tdy*, which allows the ovarian pathway to commence. The Y chromosome from a strain with early-acting ovary determinants ('incompatible') must have an early-acting *Tdy* allele, and hence the reciprocal cross presents no problems. McLaren offers an explanation of the gonadal development that ensues in such cases:

> In an XX embryo, the germ cells enter meiosis and induce the supporting cells to differentiate as follicle cells. But in a normal XY embryo, *Tdy* is expressed several days before the germ cells are due to enter meiosis: the supporting cell lineage differentiates as pre-Sertoli cells which come together to form testis cords...If, however, *Tdy* expression is delayed, ...the germ cells may enter meiosis before the XY supporting cells have been committed to a male pathway... As in an XX embryo, the supporting cells will start to develop as pre-follicle cells. Depending on the extent of the mismatch, few if any Sertoli cells will differentiate...and the embryo will develop as a female (McLaren 1991a, 153).

Although supported by a good deal of circumstantial evidence such as the preponderance of testicular tissue on the faster-growing left side of mice and rats hermaphrodites (Mittwoch 1989, Mittwoch 1992), the best experimental evidence for this developmental asynchrony comes from a careful analysis of testicular cord formation and limb development at 12.5d*pc* in hybrid embryos carrying the Y^{POS} or the Y^{B6} (Palmer and Burgoyne 1991a). These investigators measured mean gonad breadth for male and female embryos between 280 and 340 hours post coitum for two different outcrosses to B6.Y^{POS} and B6. For both crosses, they found no difference in the gonad growth for females fathered by B6 or B6.Y^{POS} sires, but there was a significant reduction in gonad size for the males bearing the Y^{POS} compared to those bearing the Y^{B6}. The reduction translated into an approximate 14 hour delay between the time when gonad growth in Y^{B6}-bearing males begins to accelerate compared to the female controls v. the growth spurt in

Y^{POS}-bearing males. If one role of Tdy is to cause the increased gonadal growth observed in males, and this growth is integral to the process of sex determination, then their conclusion, that Tdy^{DOM} acts later than Tdy^{B6}, is highly relevant to the sex-determinants-as-growth-factors hypothesis. It remains to be seen whether any of the autosomal components of either hypothesized pathways can be shown to act earlier in B6 than in *M. d. poschiavinus*.

What processes are sex determining genes* not *likely to control?

Organogenesis of the gonad during the bipotential period

A few words about processes which are unlikely to involve sex-determining genes will serve to close this discussion. We have classified genes as sex-determining based on what they do, as shown by mutant phenotypes. Considering what sex-determining genes *do not do* is also instructive. Recently, two groups have reported perturbations of mouse genital system development as a consequence of targeted mutagenesis of genes required during renal system development (Kriedberg, et al. 1993, Luo, et al. 1994), but the genital abnormalities are likely to be secondary to the renal defect. In the first instance, homozygous disruption of the WT-1 tumor suppressor gene causes the failure of kidney and gonad development with embryonic death between 13 and 15 d*pc*. The lack of a gonad makes it impossible to determine whether the WT-1 gene participates directly in normal gonad development. This is suggested by the urogenital malformations which accompany some germ-line mutations of WT-1 in humans (van Heyningen, et al. 1990, Pelletier, et al. 1991a, Pelletier, et al. 1991b, Bruening, et al. 1992), but because the bipotential gonad cannot develop properly in the mouse mutants, WT-1's role in the gonad during sex determination, if any, remains obscure. If its function influenced the decision between testicular and ovarian fate in a specific way, such as transcriptional regulation of *SRY*, it could be considered sex-determining; however, to date the available evidence favors an earlier requirement

for *WT-1* function in the formation of the bipotential gonad, with indirect effects on gonadal development (Bogan and Page 1994).

The second example of this kind is that of a nuclear receptor called steroidogenic factor 1 (*SF-1*). This gene encodes a protein with a zinc-finger DNA-binding domain, and is thought to regulate the steroid hydroxylases, enzymes important in androgen production (Luo, et al. 1994). Targeted mutagenesis of *SF-1* revealed that homozygous mutants lack adrenal glands and gonads and die of presumptive adrenocortical insufficiency before eight days of age. In this case, the kidney is normal, but again the absence of gonads makes the conclusion that *SF-1* is essential for sexual differentiation premature at best. Any agent that ablated the gonad would lead to the sex characteristics observed in these animals: female internal genitals regardless of the presence of *SRY*. Both of these examples illustrate the subtleties involved in studying genes 'upstream' of, *i.e.* with epistatic effects upon, sex determining processes. In both cases, proof of the gene's specific effects on sex determination, by genetic or biochemical means, is still lacking and will be required if they are ever recognized as sex determining genes. To summarize, although the function of a true sex determining gene may involve the formation of the urogenital ridge or the bipotential gonad, it obviously cannot be limited to such indeterminate processes.

Germ cell development and dosage compensation

Germ cell development and dosage compensation are two other processes related to sex determination which occur independently in mammals, with the exception of oocyte development inducing follicle cells in the ovary. In each of the mutant mice discussed above, development proceeds past the point at which developing germ cells normally colonize the gonad. Despite the disruption of the sex determining process, the germ cells migrate and proliferate normally (Kriedberg, et al. 1993, 681; Luo, et al. 1994,487). Of course, their final

maturation is dependent on the gonadal environment, and is not completed. The reverse situation of abnormal germ cell development with normal gonadal determination applies in the case of *W* or *Sl* mutant mice. In both mutants, the germ cells fail to migrate, due to a defect in either the migratory signal (encoded by *Sl*), or the cell-surface receptor (encoded by *W*). Consequently, the germ cells never leave the allantois, but testis determination is observed to proceed normally in the gonad (McLaren 1991a). Of course the animals are sterile, but in males gonadal histology is normal, apart from the absence of germ cells. In contrast, ovaries fail to develop when the germ cells are absent (Merchant-Larios and Centeno 1981). This is consistent with evidence suggesting the germ cells are required for the normal differentiation and maintenance of follicle cells (Merchant-Larios and Centeno 1981), and it may prove to be a fundamental difference between sex determining mechanisms in males and females. In general, though, we expect that genes which function in germ cell development are not those amongst which we will find sex-determining genes. Finally, dosage compensation is also independent of sex determination in mammals, in marked contrast to invertebrates such as *Drosophila* and *C. elegans*. The mechanism of dosage compensation in mammals is X inactivation, and although it normally occurs in XX females, it does not occur in XO females (Turner's Syndrome). Conversely, in males which have inherited an extra X chromosome (XXY), one X is inactivated in every cell, as for normal females. Obviously, then, this is another example of a process related to but completely independent of sex determination. Sex determining genes are unlikely to be involved in any such processes.

Conclusion

In this section we have looked in depth at a four functions paradigm for sex determination and assessed the sex determining schemes of some invertebrate and vertebrate organisms with this paradigm in mind. The four functions consist of the repression of male functions and activation of female functions in females, and the repression of female functions and activation of male functions in males. Although the paradigm is widely applicable, to *Saccharomyces* and *Drosophila*, for instance, we find that one sex or the other is often produced by a default pattern of gene expression that makes use of no specific regulators. Examples are the **a** cells of *Saccharomyces* and the males in *Drosophila*. In mammals, it is not yet clear which functions are accomplished by default and which by active regulation. The traditional view of the female as the default state of the developing mammalian embryo may be giving way in the face of new genetic evidence. Recalling Figure 1–9, it appears clear that the presence of the SRY gene product activates testicular determinants, but to what extent that requires the repression of ovarian determinants is unclear, given the possibility that testicular development may hinge on the activation of testicular determinants prior to the initiation of the ovarian program. In the absence of the SRY gene, ovarian determinants could be activated by default, but testicular determinants might require active repression, as suggested by the genetic arguments for the hypothetical Z gene. Consequently, the female state may not be the default sex as has been so widely hypothesized. The resolution of these key questions in mammalian sex determination must await future investigations.

References

Affara, N. A., Ferguson, S. M., Magenis, R. E., Tolmie, J. L., Boyd, E., Cooke, A., Jamieson, D., Kwok, K., Mitchell, M., and Snadden, L. (1987). Mapping the testis determinants by an analysis of Y-specific sequences in males with apparent XX and XO karyotypes and females with XY karyotypes. Nucleic Acids Res *15*, 7325-42.

Affara, N. A., Ferguson, S. M., Tolmie, J., Kwok, K., Mitchell, M., Jamieson, D., Cooke, A., and Florentin, L. (1986). Variable transfer of Y-specific sequences in XX males. Nucleic Acids Res *14*, 5375-87.

Austin, C. R., and Edwards, R. G. (1981). *Mechanisms of Sex Differentiation in Animals & Man.* London: Academic Press.

Bernstein, R., Jenkins, T., Dawson, B., Wagner, J., Dewald, G., Koo, G. C., and Wachtel, S. S. (1980). Female phenotype and multiple abnormalities in sibs with a Y chromosome and partial X chromosome duplication: H--Y antigen and Xg blood group findings. J Med Genet *17*, 291-300.

Berta, P., Hawkins, J. R., Sinclair, A. H., Taylor, A., Griffiths, B. L., Goodfellow, P. N., and Fellous, M. (1990). Genetic evidence equating SRY and the testis-determining factor. Nature *348*, 448-50.

Biddle, F. G., Eisner, J., and Eales, B. A. (1994). The testis-determining autosomal trait, *Tda-1*, of C57BL/6J is determined by more than a single autosomal gene when compared with DBA/2J mice. Genome *37*, 296-304.

Biddle, F. G., and Nishioka, Y. (1988). Assays of testis development in the mouse distinguish three classes of *domesticus*-type Y chromosome. Genome *30*, 870-878.

Bogan, J., and Page, D. C. (1994). Ovary? Testis? – A mammalian dilemma. Cell *76*, 603-607.

Bridges, C. B. (1913). Non-disjunction of the sex chromosomes of *Drosophila*. J Exp Zool *15*, 587-606.

Bridges, C. B. (1914). Direct proof through non-disjunction that the sex-linked genes of *Drosophila* are born by the X chromosome. Science *40*, 107-109.

Bridges, C. B. (1916). Non-disjunction as proof of the chromosome theory of heredity. Genetics *1*, 1-51, 107-163.

Bridges, C. B. (1921). Triploid intersexes in *Drosophila melanogaster*. Science *54*, 252-254.

Bruening, W., Bardeesy, N., Silverman, B. L., Cohn, R. A., Machin, G. A., Aronson, A. J., Housman, D., and Pelletier, J. (1992). Germline intronic and exonic mutations in the Wilms' tumour gene (*WT1*) affecting urogenital development. Nat Genet *1*, 144-8.

Bull, J. J. (1983). *Evolution of sex determining mechanisms.* Menlo Park, California: The Benjamin Cummings Publishing Co.,Inc.

Burgoyne, P. S., Buehr, M., Koopman, P., Rossant, J., and McLaren, A. (1988). Cell-autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XX----XY chimaeric mouse testes. Development *102*, 443-50.

Burtis, K. C., Coschigano, K. T., Baker, B. S., and Wensink, P. C. (1991). The *doublesex* proteins of *Drosophila melanogaster* bind directly to a sex-specific yolk protein gene enhancer. Embo J *10*, 2577-82.

Cattanach, B. M. (1961). XXY mice. Genet Res *2*, 156-158.

Cattanach, B. M., Pollard, C. E., and Hawker, S. G. (1971). Sex-reversed mice: XX and XO males. Cytogenetics *10*, 318-37.

Cline, T. (1993). The *Drosophila* sex determination signal: how do flies count to two? TIG *9*, 385-390.

Coschigano, K. T., and Wensink, P. C. (1993). Sex-specific transcriptional regulation by the male and female *doublesex* proteins of *Drosophila*. Genes Dev *7*, 42-54.

Court-Brown, W. M., Harnden, D. G., Jacobs, P. A., MacLean, N., and Mantle, D. J. (1964). Abnormalities of the sex chromosome complement in man. *Medical Research Council Special Report*, London: Her Majesty's Stationery Office, series 305.

de la Chapelle, A. (1981). The etiology of maleness in XX men. Hum Genet *58*, 105-116.

de la Chapelle, A., Hortling, H., Niemi, M., and Wennstrom, J. (1964). XX sex chromosomes in a human XX male: first case. Acta Med Scand (Suppl) *412*, 25-38.

Disteche, C. M., Casanova, M., Saal, H., Friedman, C., Sybert, V., Graham, J., Thuline, H., Page, D. C., and Fellous, M. (1986). Small deletions of the short arm of the Y chromosome in 46,XY females. Proc Natl Acad Sci U S A *83*, 7841-4.

Eicher, E. M. (1988). Autosomal genes involved in mammalian primary sex determination. Philos Trans R Soc Lond [Biol] *322*, 109-18.

Eicher, E. M., Beamer, W. G., Washburn, L. L., and Whitten, W. K. (1980). A cytogenetic investigation of inherited true hermaphroditism in BALB/cWt mice. Cytogenet Cell Genet *28*, 104-115.

Eicher, E. M., and Lee, B. K. (1990). The NXSM recombinant inbred strains of mice: genetic profile for 58 loci including the Mtv proviral loci. Genetics *125*, 431-46.

Eicher, E. M., and Washburn, L. L. (1983). Inherited sex reversal in mice: identification of a new primary sex-determining gene. J Exp Zool *228*, 297-304.

Eicher, E. M., and Washburn, L. L. (1986). Genetic control of primary sex determination in mice. Annu Rev Genet *20*, 327-60.

Eicher, E. M., Washburn, L. L., Whitney, J. 3., and Morrow, K. E. (1982). Mus poschiavinus Y chromosome in the C57BL/6J murine genome causes sex reversal. Science *217*, 535-7.

Epplen, J. T., McCarrey, J. R., Sutou, S., and Ohno, S. (1982). Base sequence of a cloned snake W-chromosome DNA fragment and identification of a male-specific putative mRNA in the mouse. Proc Natl Acad Sci U S A *79*, 3798-802.

Erikson, R. P., Durbin, E. J., and Tres, L. L. (1987). Sex determination in mice: Y and chromosome 17 interactions. Development (Supplement) *101*, 25-32.

Ferguson-Smith, M. A. (1966). X-Y chromosomal interchange in the aetiology of true hermaphroditism and of XX Klinefelter's syndrome. Lancet *2*, 475-6.

Ferguson-Smith, M. A. (1992). Abnormalities of Human Sex Determination. J Inher Metab Dis *15*, 518-525.

Ferguson-Smith, M. A., Cooke, A., Affara, N. A., Boyd, E., and Tolmie, J. L. (1990). Genotype-phenotype correlations in XX males and their bearing on current theories of sex determination. Hum Genet *84*, 198-202.

Ford, C. E., and Hamerton, J. L. (1956). The chromosomes of man. Nature *178*, 1020-3.

Ford, C. E., Jones, K. W., Polani, P. E., deAlmeida, J. C., and Briggs, J. H. (1959). A sex chromosome anomaly in a case of gonadal dysgenesis (Turner's Syndrome). Lancet *i*, 711-713.

Fredga, K. (1988). Aberrant chromosomal sex-determining mechanisms in mammals, with special reference to species with XY females. Philos Trans R Soc Lond [Biol] *322*, 83-95.

Fredga, K., Gropp, A., Winking, H., and Frank, F. (1976). Fertile XX- and XY-type females in the wood lemming *Myopus schisticolor*. Nature *261*, 225-7.

Fredga, K., Gropp, A., Winking, H., and Frank, F. (1977). A hypothesis explaining the exceptional sex ratio in the wood lemming (*Myopus schisticolor*). Hereditas *85*, 101-4.

Geddes, P., and Thomson, J. A. (1890). *The Evolution of Sex*. London: Walter Scott.

Giese, K., Cox, J., and Grosschedl, R. (1992). The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell *69*, 185-196.

Gilbert, S. F. (1991). *Developmental biology*. 3rd ed. Sunderland, Mass.: Sinauer Associates, Inc.

Gileva, E. A. (1980). Chromosomal diversity and an aberrant genetic system of sex determination in the Arctic lemming, *Dicrostonyx torquatus Pallas* (1779). Genetica *52/53*, 99-103.

Graves, J. A., and Schmidt, M. M. (1992). Mammalian sex chromosomes: design or accident? Curr Opin Genet Dev *2*, 890-901.

Graves, J. A. M. (1987). The evolution of mammalian sex chromosomes and dosage compensation: clues from marsupials and monotremes. Trends Genet *3*, 252-256.

Gropp, A., Winking, H., Frank, F., Noack, G., and Fredga, K. (1976). Sex-chromosome aberrations in wood lemmings(*Myopus schisticolor*). Cytogenet. Cell Genet. *17*, 343-58.

Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P., and Lovell, B. R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature *346*, 245-50.

Gubbay, J., Vivian, N., Economou, A., Jackson, D., Goodfellow, P., and Lovell-Badge, R. (1992). Inverted repeat structure of the Sry locus in mice. Proc Natl Acad Sci U S A *89*, 7953-7.

Guellaen, G., Casanova, M., Bishop, C., Geldwerth, D., Andre, G., Fellous, M., and Weissenbach, J. (1984). Human XX males with Y single-copy DNA fragments. Nature *307*, 172-3.

Haqq, C. M., King, C., Donahoe, P. K., and Weiss, M. A. (1993). SRY recognizes conserved DNA sites in sex-specific promoters. Proc. Natl. Acad. Sci. USA *90*, 1097-1101.

Harley, V. R., Jackson, D., Hextall, P., Hawkins, J. R., Berkowitz, G. D., Sokanathan, S., Lovell-Badge, R., and Goodfellow, P. N. (1992). DNA binding activity of recombinant SRY from normal males and XY females. Science *255*, 453-456.

Hawkins, J. R., Taylor, A., Berta, P., Levilliers, J., Auwera, B. V. d., and Goodfellow, P. N. (1992b). Mutational analysis of SRY: nonsense and missense mutations in XY sex reversal. Hum. Genet. *88*, 471-474.

Hawkins, J. R., Taylor, A., Goodfellow, P. N., Migeon, C. J., Smith, K. D., and Berkovitz, G. D. (1992a). Evidence for increased prevalence of SRY mutations in XY females with complete rather than partial gonadal dysgenesis. Am J Hum Genet *51*, 979-84.

Henking, H. (1890). Untersuchungen über die ersten Entwickelungsungsvorgänge in den Eiern der Insecten. II. Über Spermatogenese und deren Beziehung zur Eientwickelung bei *Pyrrhocoris apterus* M. Cited in McClung, 1902. Zeit wiss Zool *51*,

Herbst, E. W., Fredga, K., Frank, F., Winking, H., and Gropp, A. (1978). Cytological identification of two X-chromosome types in the wood lemming (*Myopus schisticolor*). Chromosoma *69*, 185-191.

Herskowitz, I. (1988). Life cycle of the budding yeast *Saccharomyces cerevisiae*. Microbiol. Rev. *52*, 536.

Hodgkin, J. (1990). Sex determination compared in *Drosophila* and *Caenorhabditis*. Nature *344*, 721-728.

Hodgkin, J. (1992). Genetic sex determination, mechanisms and evolution. Bioessays *14*, 253-261.

Hsu, T. C. (1952). Mammalian chromosomes in vitro. X. The karyotype of man. J. Hered. *43*, 167-172.

Hughes, A. (1952). Some effects of abnormal tonicity on dividing cells in chick tissue culture. Q. J. Microsc. Sci. *93*, 207-219.

Hunter, C. P., and Wood, W. B. (1992). Evidence from mosaic analysis of the masculinizing gene *her-1* for cell interactions in *C. elegans* sex determination. Nature *355*, 551-5.

Jacobs, P. A., and Ross, A. (1966). Structural abnormalities of the Y chromosome in man. Nature *210*, 352-4.

Jacobs, P. S., and Strong, J. A. (1959). A case of human intersexuality having a possible XXY sex-determining mechanism. Nature *183*, 302-303.

Jäger, R. J., Anvret, M., Hall, K., and Scherer, G. (1990). A human XY female with a frame shift mutation in the candidate testis-determining gene SRY. Nature *348*, 452-4.

Jäger, R. J., Harley, V. R., Pfeiffer, R. A., Goodfellow, P. N., and Scherer, G. (1992). A familial mutation in the testis-determining gene SRY shared by both sexes. Hum Genet *90*, 350-5.

Jones, K. W., and Singh, L. (1981). Conserved repeated DNA sequences in vertebrate sex chromosomes. Hum Genet *58*, 46-53.

Jost, A. (1947). Recherches sur la differenciation sexuelle de l'embryon de lapin. III. Role des gonades foetales dans la differentiation sexuelle somatique. Arch Anat Morphol Exp *36*, 271-315.

Jost, A. (1953). Problems of fetal endocrinology: the gonadal and hypophyseal hormones. Rec Prog Horm Res *8*, 379-418.

Kaufman, M. H. (1992). *The atlas of mouse development.* San Diego: Academic Press, Ltd.

Kelly, M., Burke, J., Smith, M., Klar, A., and Beach, D. (1988). Four mating-type genes control sexual differentiation in the fission yeast. EMBO J. *7*, 1537-1547.

Kiel-Metzger, K., Warren, G., Wilson, G. N., and Erickson, R. P. (1985). Evidence that the human Y chromosome does not contain clustered DNA sequences

(BKM) associated with heterogametic sex determination in other vertebrates. N Engl J Med *313*, 242-5.

Koller, P. (1937). Cited in Ford and Hamerton, 1956. Proc Roy Soc Edin, B *57*, 197.

Koopman, P., Gubbay, J., Collignon, J., and Lovell, B. R. (1989). Zfy gene expression patterns are not compatible with a primary role in mouse sex determination. Nature *342*, 940-2.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. Nature *351*, 117-21.

Koopman, P., Munsterberg, A., Capel, B., Vivian, N., and Lovell, B. R. (1990). Expression of a candidate sex-determining gene during mouse testis differentiation. Nature *348*, 450-2.

Kriedberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D., and Jaenisch, R. (1993). *WT-1* is required for early kidney development. Cell *74*, 679-691.

Langman, J. (1975). Urogenital System, Ch 11. *Medical Embryology*, Baltimore: The Williams & Wilkins Co., 160-200.

Lau, Y. F., Yang-Feng, T. L., Elder, B., Fredga, K., and Wiberg, U. H. (1992). Unusual distribution of Zfy and Zfx sequences on the sex chromosomes of the wood lemming, a species exhibiting XY sex reversal. Cytogenet Cell Genet *60*, 48-54.

Lillie, F. R. (1917). The free-martin; a study of the action of sex hormones in the foetal life of cattle. J Exp Zool *23*, 371-452.

Lindsley, D. L., and Zimm, G. G. (1992). *The genome of Drosophila melanogaster.* San Diego: Academic Press.

Lovell-Badge, R. (1992). Testis determination: soft talk and kinky sex. Curr Opin Genet Dev 2, 596-601.

Lovell-Badge, R., and Robertson, E. (1990). XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. Development *109*, 635-46.

Luo, X., Ikeda, Y., and Parker, K. (1994). A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell *77*, 481-490.

Maraia, R., Saal, H., and Wangsa, D. (1991). A chromosome 17q de novo paracentric inversion in a patient with campomelic dysplasia; case report and etiologic hypothesis. Clin Genet *39*, 401-408.

McClung, C. E. (1902). The accessory chromosome--sex determinant? Biol Bull *3*, 72-77.

McElreavey, K., Vilain, E., Abbas, N., Herskowitz, I., and Fellous, M. (1993). A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development. Pro. Natl. Acad. Sci. USA *90*, 3368-3372.

McLaren, A. (1988b). Sex determination in mammals. Trends Genet *4*, 153-7.

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

McLaren, A. (1991b). Sex determination in mammals, Ch 1. *Oxford Reviews of Reproductive Biology*, Ed. Mulligan, S. R. Oxford: Oford University Press, 13, 1-33.

McLaren, A., Simpson, E., Bishop, C. E., Mitchell, M. J., and Darling, S. M. (1992). Recombination between the X and Y chromosomes and the Sxr region of the mouse. Genet Res *60*, 175-84.

McLaren, A., Simpson, E., Tomonari, K., Chandler, P., and Hogg, H. (1984). Male sexual differentiation in mice lacking H-Y antigen. Nature *312*, 552-5.

Merchant, H. (1975). Rat gonandal and ovarian organogenesis with and without germ cells. An ultrastructural study. Devl. Biol. *44*, 1-21.

Merchant-Larios, H., and Centeno, B. (1981). Morphogenesis of the ovary from the sterile W/Wv mouse. *Advances in the Morphology of Cells and Tissues*, New York: Alan R. Liss, 383-392.

Mittwoch, U. (1989). Sex differentiation in mammals and tempo of growth: probabilities vs. switches. J Theor Biol *137*, 445-55.

Mittwoch, U. (1992). Sex determination and sex reversal: genotype, phenotype, dogma and semantics. Hum Genet *89*, 467-479.

Moore, C. R. (1944). Gonad hormones and sex differentiation. Am Naturalist *78*, 97-130.

Moore, K. L. (1988). The Urogenital System. Ch. 13. *The developing human*, Philadelphia: W B Saunders Co.,

Morgan, T. H. (1910). Sex-limited inheritance in *Drosophila*. Science *32*, 120-122.

Muller, U., Donlon, T., Schmid, M., Fitch, N., Richer, C. L., Lalande, M., and Latt, S. A. (1986). Deletion mapping of the testis determining locus with DNA probes in 46,XX males and in 46,XY and 46,X,dic(Y) females. Nucleic Acids Res *14*, 6489-505.

Ogata, T., Hawkins, J. R., Taylor, A., Matsuo, N., Hata, J., and Goodfellow, P. N. (1992). Sex reversal in a child with a 46,X,Yp+ karyotype: support for the existence of a gene(s), located in distal Xp, involved in testis formation. J Med Genet *29*, 226-30.

Ogata, T., and Matsuo, N. (1992). Comparison of adult height between patients with XX and XY gonadal dysgenesis: support ofr a Y specific growth gene(s). J. Med. Genet. *29*, 539-541.

Ohno, S. (1967). *Sex Chromosomes and Sex-Linked Genes.* Berlin: Springer-Verlag.

Ohno, S. (1976). Major regulatory genes for mammalian sexual development. Cell *7*, 315-21.

Otis, E. M., and Brent, R. (1954). Equivalent ages in mouse and human embryos. Anat Rec *120*, 33-56.

Oxford English Dictionary (1989) 2nd ed.

Page, D. C. (1986). Sex reversal: deletion mapping the male-determining function of the human Y chromosome. Cold Spring Harb Symp Quant Biol *1*, 229-35.

Page, D. C., de, l. C. A., and Weissenbach, J. (1985). Chromosome Y-specific DNA in related human XX males. Nature *315*, 224-6.

Page, D. C., Fisher, E. M., McGillivray, B., and Brown, L. G. (1990). Additional deletion in sex-determining region of human Y chromosome resolves paradox of X,t(Y; 22) female. Nature *346*, 279-81.

Page, D. C., Mosher, R., Simpson, E. M., Fisher, E. M., Mardon, G., Pollack, J., McGillivray, B., de, l. C. A., and Brown, L. G. (1987). The sex-determining region of the human Y chromosome encodes a finger protein. Cell *51*, 1091-104.

Painter, T. (1921). The Y chromosome in mammals. Science *53*, 503.

Painter, T. (1924). The sex chromosomes of man. Am. Nat. *58*, 506-524.

Palmer, M. S., Sinclair, A. H., Berta, P., Ellis, N. A., Goodfellow, P. N., Abbas, N. E., and Fellous, M. (1989). Genetic evidence that ZFY is not the testis-determining factor. Nature *342*, 937-9.

Palmer, S. J., and Burgoyne, P. S. (1991a). The Mus musculus domesticus Tdy allele acts later than the Mus musculus musculus Tdy allele: a basis for XY sex-reversal in C57BL/6-YPOS mice. Development *113*, 709-14.

Palmer, S. J., and Burgoyne, P. S. (1991b). In situ analysis of fetal, prepuberal and adult XX----XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. Development *112*, 265-8.

Parkhurst, S., and Meneely, P. (1994). Sex determination and dosage compensation:lessons from flies and worms. Science *264*, 924-932.

Patek, C. E., Kerr, J., Gosden, R., Jones, K., Hardy, K., Muggleton-Harris, A., Handyside, A., Whittingham, D., and Hooper, M. L. (1991). Sex chimerism, fertility, and sex determination in the mouse. Development *113*, 311-325.

Pelletier, J., Bruening, W., Kashtan, C. E., Mauer, S. M., Manivel, J. C., Striegel, J. E., Houghton, D. C., Junien, C., Habib, R., Fouser, L., and et, a.

(1991b). Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. Cell *67*, 437-47.

Pelletier, J., Bruening, W., Li, F. P., Haber, D. A., Glaser, T., and Housman, D. E. (1991a). WT1 mutations contribute to abnormal genital system development and hereditary Wilms' tumour. Nature *353*, 431-4.

Perry, M. D., Li, W., Trent, C., Robertson, B., Fire, A., Hageman, J. M., and Wood, W. B. (1993). Molecular characterization of the her-1 gene suggests a direct role in cell signaling during Caenorhabditis elegans sex determination. Genes Dev *7*, 216-28.

Polani, P. E. (1981). Abnormal Sex Development in Man I. Anomalies of Sex-determining Mechanisms, Ch. 12. *Mechanisms of Sex Differentiation in Animals & Man*, Ed. Austin, C. R., and Edwards, R. G. London: Academic Press, 465-547.

Rieger, R., Michaelis, A., and Green, M. M. (1991). *Glossary of Genetics*. 5th. Berlin: Springer-Verlag.

Roberts, C., Weith, A., Passage, E., Michot, J. L., Mattei, M. G., and Bishop, C. E. (1988). Molecular and cytogenetic evidence for the location of Tdy and Hya on the mouse Y chromosome short arm. Proc Natl Acad Sci U S A *85*, 6446-9.

Ross, G. T., and Schreiber, J. R. (1986). The ovary. Ch. 4. *Reproductive endocrinology: physiology, pathophysiology, and clinical management*, Ed. Yen, S., and Jaffe, R. Philadelphia: W B Saunders Company,

Russell, L. B., and Chu, E. H. Y. (1961). An XXY male in the mouse. Proc Natl Acad Sci *47*, 571-575.

Russell, W. L., Russell, L. B., and Gower, J. S. (1959). Exceptional inheritance of a sex-linked gene in the mouse explained on the basis that the X/O cromosome constitution is female. Proc Natl Acad Sci USA *45*, 554-560.

Sanger, R., Race, R. R., Tippett, P., Gavin, J., Hardisty, R. M., and Dubowitz, V. (1964). Unexplained inheritance of the Xg groups in two families. Lancet *I*, 955-956.

Scherer, G., Schempp, W., Baccichetti, C., Lenzini, E., Bricarelli, F. D., Carbone, L. D., and Wolf, U. (1989). Duplication of an Xp segment that includes the ZFX locus causes sex inversion in man. Hum Genet *81*, 291-4.

Schneider-Gädicke, A., Beer-Romero, P., Brown, L., Nussbaum, R., and Page, D. C. (1989). ZFX has a gene structure similar to ZFY, the putative human sex determinant, and escapes X inactivation. Cell *57*, 1247-1258.

Sharman, G. B., Robinson, E. S., Walton, S. M., and Berger, R. J. (1970). Sex chromosomes and reproductive anatomy of some intersexual marsupials. J Reprod Fertil *21*, 57-68.

Shen, W., Moore, C. C. D., Ikeda, Y., Parker, K. L., and Ingraham, H. A. (1994). Nuclear receptor steroidogenic factor 1 regulates the Müllerian inhibiting substance gene: a link to the sex determination cascade. Cell *77*, 651-661.

Shine, I., and Wrobel, S. (1976). *Thomas Hunt Morgan: pioneer of genetics.* Lexington: University of Kentucky Press.

Short, O., Renfree, R., and Shaw, G. (1988). Primary genetic control of somatic sexual differentiation in a mammal. Nature *331*, 716-717.

Silver, L. (1993). The peculiar journey of a selfish chromosome: mouse *t* haplotypes and meiotic drive. TIG *9*, 250-254.

Simpson, E. M., and Page, D. C. (1991). An interstitial deletion in mouse Y chromosomal DNA created a transcribed Zfy fusion gene. Genomics *11*, 601-8.

Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A. M., Lovell, B. R., and Goodfellow, P. N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature *346*, 240-4.

Sinclair, A. H., Foster, J. W., Spencer, J. A., Page, D. C., Palmer, M., Goodfellow, P. N., and Graves, J. A. (1988). Sequences homologous to ZFY, a candidate human sex-determining gene, are autosomal in marsupials. Nature *336*, 780-3.

Singh, L., and Jones, K. W. (1982). Sex reversal in the mouse (Mus musculus) is caused by a recurrent nonreciprocal crossover involving the x and an aberrant y chromosome. Cell *28*, 205-16.

Singh, L., Phillips, C., and Jones, K. W. (1984). The conserved nucleotide sequences of Bkm, which define Sxr in the mouse, are transcribed. Cell *36*, 111-20.

Soller, M., Padeh, B., Wysoki, M., and Ayalon, N. (1969). Cytogenetics of Saanen goats showing abnormal development of the reproductive tract associated with the dominant gene for polledness. Cytogenetics *8*, 51-67.

Stevens, N. M. (1905). Studies in spermatogenesis with especial reference to the "accessory chromosome". Part I. Carnegie Inst. of Washington Publication *36*, 1-32.

Taylor, B. A. (1978). Recombinant inbred strains: use in genetic mapping. *Origins of Inbred Mice*, Ed. Morse, H. New York: Academic Press,

Therkelsen, A. J. (1964). Sterile male with the chromosome constitution 46 XX. Cytogenetics *3*, 207-218.

Tjio, J. H., and Levan, A. (1956). The chromosome number of man. Hereditas *42*, 1-6.

Tommerup, N., Schempp, W., Meinecke, P., Pedersen, S., Bolund, L., Brandt, C., Goodpasture, C., Guldberg, P., Held, K. R., Reinwein, H., Saugstad, O. D., Scherer, G., Skjeldal, O., Toder, R., Westvik, J., Hagen, C. B. v. d., and Wolf, U. (1993). Assignment of an autosomal sex reversal locus and campomelic dysplasia to 17q24.3–q25.1. Nat Genet *4*, 170-174.

van Heyningen, V., Bickmore, W. A., Seawright, A., Fletcher, J. M., Maule, J., Fekete, G., Gessler, M., Bruns, G. A., Huerre-Jeanpierre, C., and Junien, C. (1990). Role for the Wilms tumor gene in genital development? Proc Natl Acad Sci U S A *87*, 5383-6.

van Wagenen, G., and Simpson, M. E. (1965). *Embryology of the ovary and testis: Homo sapiens and Macaca mulatta.* New Haven: Yale University Press.

Vergnaud, G., Page, D. C., Simmler, M. C., Brown, L., Rouyer, F., Noel, B., Botstein, D., de, l. C. A., and Weissenbach, J. (1986). A deletion map of the human Y chromosome based on DNA hybridization. Am J Hum Genet *38*, 109-24.

von Winiwarter, H. (1912). Etudes sur la spermatogeneses humaine. Arch Biol *27*, 91-188.

Wachtel, S. S., Ohno, S., Koo, G. C., and Boyse, E. A. (1975). Possible role for H--Y antigen in the primary determination of sex. Nature *257*, 235-6.

Washburn, L. L., and Eicher, E. M. (1983). Sex reversal in XY mice caused by dominant mutation on chromosome 17. Nature *303*, 338-40.

Washburn, L. L., and Eicher, E. M. (1989). Normal Testis Determination in the Mouse Depends on Genetic Interaction of a Locus on Chromosome 17 and the Y Chromosome. Genetics *123*, 173-179.

Washburn, L. L., Lee, B. K., and Eicher, E. M. (1990). Inheritance of *T*-associated sex reversal in mice. Gent Res Camb *56*, 185-191.

Webster's Third New International Dictionary (1981) 3rd ed.

Welshons, W. J., and Russell, L. B. (1959). The Y chromosome as the bearer of male determining factors in the mouse. Proc Natl Acad Sci USA *45*, 560-566.

Westergaard, M. (1953). Über den Mechanismus der Geschlechtsbestimmung bei *Melandrium album* (Cited in Therkelson 1964). Cytogenetics *40*, 253-60.

Wilson, E. B. (1905). The chromosomes in relation to the determination of sex in insects. Science *22*, 500-502.

Wilson, E. B. (1906). Studies on chromosomes. III. The sexual differences of the chromosome-groups, with some considerations on the determination and inheritance of sex. J. Exp. Zool. *3*, 1-40.

Wilson, J. D., and Goldstein, J. L. (1975). Classification of hereditary disorders of sexual development. Birth Defects Orig. Article Series *11*, 1.

Wood, W. B. (1988). *The Nematode Caenorhabditis elegans.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Young, I. D., Zuccollo, J. M., Maltby, E. L., and Broderick, N. J. (1992). Campomelic dysplasia associated with a de novo 2q;17q reciprocal translocation. J Med Genet *29*, 251-252.

Chapter Two

Multiple Sex Determining Genes Located on Mouse Autosomes

Affect Y^{POS} Sex Reversal

by M. Jodeane Pringle[1]

Abstract

Testis determination is compromised in mice carrying the Y chromosome of the wild mouse *Mus domesticus poschiavinus* on the autosomal background of certain *Mus musculus musculus* inbred strains. For instance, the *M. d. poschiavinus* Y chromosome produces hermaphrodites or females in C57BL/6J congenic strains, but functions normally on a DBA/2J inbred background. We have exploited the natural variation between these two strains to map autosomal sex determinants in this system. We report segregation analysis of a large (n=529) backcross of F1 females (C57BL/6J X DBA/2J) by C57BL/6J.Y^{POS} congenic fertile hermaphrodites. All XX backcross progeny developed ovarian tissue exclusively. XY progeny were either normal males with testicular tissue only, males that exhibited an abnormal delay in testicular development, or hermaphrodites with both testicular and ovarian tissue. We used some 225 microsatellite repeat markers for genotypic analysis of a subset of the progeny. We report strong evidence for the localization of three sex-determining loci on mouse autosomes 2, 4 and 5, as well as weaker evidence for the possible existence of two additional loci on other autosomes.

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[1]The following report represents the results of a collaborative effort initiated by Jodeane Pringle, DavidC. Page, Linda L. Washburn, and Eva M. Eicher. In the Page lab at the Whitehead Institue, Jodeane Pringle and Xiaoling Xu collected and analyzed the genotype data, and Robert Dredge provided computer support and statistical analysis. Linda L.Washburn performed the phenotypic analysis and related mouse work in the Eicher lab at the Jackson Laboratories.

Introduction

Although sex determination is chromosomal in all mammals, it is clear that not all sex-determining genes are located on the sex chromosomes. The Y chromosome-linked testis determinant in mice and humans is *Sry/SRY,* a DNA-binding protein that regulates the choice between testicular and ovarian fate in the developing gonad (Berta, et al. 1990, Koopman, et al. 1991, Harley, et al. 1992). In humans and the wood lemming, an arctic rodent, there is also genetic and cytogenetic evidence for a sex-determining locus on the X chromosome which causes sex reversal if duplicated in XY individuals (Fredga, et al. 1977, Herbst, et al. 1978, Bardoni, et al. 1994). Although an intact *Sry* locus is the basis for the dominant masculinizing property of the mammalian Y chromosome, the molecular details of this genetic effect are still unknown. It is nonetheless very likely that several other genes besides for the hypothesized X-linked locus participate directly in the decision between male and female development. By analogy, a major regulator of sex in the fruit fly *Drosophila melanogaster*, *Sex lethal,* has seven regulators and two targets in the somatic sex determination pathway, as well as other targets that affect sex determination in the germ line, and dosage compensation (see Parkhurst and Meneely 1994 for a review). To find the remaining sex-determining genes in mammals, it is therefore essential to consider the role of the autosomes.

There is abundant evidence for the action of autosomal sex-determining genes in a variety of mammalian species. For instance, in a particular breed of goats, the dominant allele for hornlessness (*Polled*) causes epididymal defects in XY animals, and hermaphroditism or frank sex reversal in XX animals. Inheritance of *Polled* is autosomal, and it only causes sex reversal when homozygous (Soller, et al. 1969). More extensively studied is XY sex reversal in

the mouse. This phenomenon was discovered in two instances in which related but distinct strains or subspecies of mice were hybridized. The sex reversal first becomes apparent when males bearing a *domesticus*-type Y chromosome are crossed or backcrossed to females of another strain. In the case of *T-associated* sex reversal, XY animals that carry a deletion of the *T* complex together with the Y chromosome from the inbred strain AKR (Y^{AKR}) develop as females or hermaphrodites when crossed to C57BL6/J (B6) (Washburn and Eicher 1983). In yet another type of mouse of sex reversal, the Y chromosome of the wild mouse *Mus domesticus poschiavinus* (Y^{POS}), as well as that of some other strains that bear a *domesticus*-type Y chromosome (Y^{DOM}), causes sex reversal in the first backcross generation with B6 (Eicher, et al. 1982, Biddle and Nishioka 1988) . In other strains, the Y^{POS} may function normally (Nagamine, et al. 1987b). In humans, direct evidence for autosomal sex-determining genes is not as abundant as that for genes that control sexual differentiation (Austin and Edwards 1981), with the exception of a growing body of data implicating a locus associated with sex reversal and campomelic dysplasia on the long arm of chromosome 17 (Maraia, et al. 1991, Young, et al. 1992, Tommerup, et al. 1993). In addition, recent results indicate that many sex-reversed human individuals lack mutations in the *SRY* locus. Presumably, they carry X-linked or autosomal sex-reversing mutations (Ferguson-Smith 1992, McElreavey, et al. 1993).

The mouse is a promising system for the study of autosomal sex determinants. The genetic analysis of *T-as* sex reversal has located at least one testis-determining autosomal (*tda*) locus in the region deleted in common in T^{hp} and T^{Orl}. *T-as* sex reversal is allele-specific; that is, it is only evident in an animal with one of the *T* deletions together with the Y^{AKR}, and a normal chromosome 17 derived from B6. Y^{DOM} sex reversal likewise appears to be allele-specific because, when tested against a particular *domesticus*-type Y chromosome, some *musculus*

strains support normal testis development, and some exhibit sex reversal (Nagamine, et al. 1987a). In addition, some *domesticus*-type Y chromosomes vary with respect to their ability to masculinize the developing embryo. Some are capable of fully masculinizing the embryo in some *musculus*-derived backgrounds, while others are not (Eicher, et al. 1982, Biddle and Nishioka 1988). To explain these phenomena, Eicher and others have hypothesized that certain *Tdy* alleles cannot complete testis determination in a genetic background in which the alleles of key autosomal testis determining loci are incompatible, due perhaps to epistatic effects or possibly to a timing mismatch during gonadal development (Eicher and Washburn 1983, Eicher and Washburn 1986, Mittwoch 1989, Palmer and Burgoyne 1991a). These allelic variants of the sex-determining genes, which occur naturally in the mouse, could be involved in initiating speciation in the wild (Eicher, et al. 1982) . Regardless of their origins, however, they represent a unique genetic system in which to localize autosomal testis determinants.

The best characterized *domesticus*-type Y chromosome in terms of sex reversal is the Y^{POS}. The earliest studies of Y^{POS} showed that it gives XY embryos with ovaries or ovotestes on a B6 background (Eicher, et al. 1982, Eicher and Washburn 1983); hence, B6 is a feminizing (or incompatible) strain. As discussed above, subsequent studies identified masculinizing inbred mouse strains in which XY animals congenic for the Y^{POS} developed testes, such as SJL, SM/J, and DBA2/J (D2) (Nagamine, et al. 1987b, Eicher 1988). The first attempt to localize the loci responsible focused on genetic differences between the B6 and D2 inbred strains. The results of this study, which utilized a panel of BXD recombinant inbred (RI) strains (Taylor 1978), were inconclusive (reviewed in Eicher and Washburn 1986). One reason for the failure of the RI strain distribution pattern method to localize *tda* would be that multiple loci are responsible for sex reversal. In our study of Y^{POS} sex reversal, therefore, we performed genetic

analysis more analogous to quantitative trait dissection, by constructing an F1 between the feminizing and the masculinizing parental strains (B X D in our case). Next we performed a backcross to the feminizing parent (B), since the F1 does not exhibit sex reversal. See **Figure 2–1**. For this strain combination, XY animals from the backcross develop testes or ovotestes. Based on previous genetic data and the observation that some eleven percent of the XY animals in the backcross are hermaphrodites (28/240), we reasoned that a small number of autosomal genes with recessive, feminizing alleles contributed by the B6 strain, and all more or less strictly required for hermaphrodite development, might be the basis for sex reversal in this back cross. In order to detect several loci segregating simultaneously, we constructed a genome-wide genetic profile for each animal using polymerase chain-reaction (PCR)-based simple sequence repeat markers to streamline the genotype collection (Hearne, et al. 1992). Our results indicate that at least five loci with variable effects on testis development segregate in this cross. These candidate *tda* genes are located on mouse autosomes 2, 4, 5, 8, and 16 .

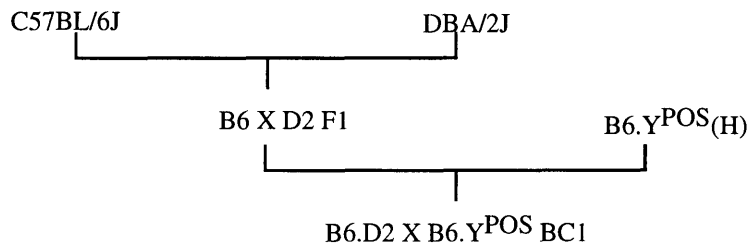

Figure 2–1. Diagram of the backcross analyzed in this study. Abbreviations: H, hermaphrodite; BC1, backcross generation 1; POS, *poschiavinus*.

Results

Sex reversal is evident in an intraspecific backcross

We observed sex reversal in a small percentage of embryos from a large backcross (n = 529) of (BXD) F1 females by C57BL6/J.Y^{POS} congenic hermaphrodites that could breed as males. See Figure 2–1. **Figure 2–2** shows the microscopic

appearance of some normal and abnormal embryonic gonads for the B6.Y^{POS} paternal line. Sex reversal was not complete, as the abnormal gonads observed in the embryos from the backcross resembled the ovotestes shown in Figure 2–1. Each gonad examined at E14.5 to E16 was categorized as a normal ovary, normal testes, an ovotestis, or a testis which appeared significantly delayed in its development but had no ovarian regions. Although the non-testicular regions of the ovotestes rarely showed any definitive characteristics of ovarian development, we refer to these regions as 'presumptive ovarian' regions because they differ in gross morphology from similar regions of the occasional testis which exhibits delayed development. The chromosome constitution was inferred by PCR analysis for a Y-specific marker. We found that all embryos from this cross that have an XX chromosome constitution develop bilateral ovaries and hence are normal females. On some backgrounds, the Y^{POS} may lead to complete sex reversal, i.e. bilateral ovaries in XY individuals, but this is clearly not the case for this strain combination. Amongst the XY animals, about 11% were hermaphrodites (28/240); that is, they had at least one ovotestis. See Table 2–1 below. Another 11% of the embryos were 'abnormal' males with one or both testes that appeared delayed, but was not an ovotestis. The remaining XY embryos were males with normal testicular development in the embryonic assay.

Constructing a dense genetic map

For genotypic analysis, we selected various groups of embryos that finally included 75 individual animals. The composite mapping panel includes the 28 hermaphrodites, the 29 abnormal males, and 18 of the normal males. All of these animals were tested with a total of 228 markers. The genetic map distances compiled with the MAPMAKER software package (Lander, et al. 1987) reflect female meiotic recombination frequencies as expected, but since our map was

Figure 2–2. Appearance of gonads from E14.5 to E16 C57BL/6J-Y^{POS} fetuses. Each freshly dissected gonad with attached mesonephros was photographed with a Zeiss inverted microscope . (a) Ovary from XX fetus. (b) Testis from normal C57BL/6J male fetus. (c) Ovary from XY female. (d to f) Ovotestes from XY hermaphrodites, each containing areas of ovarian and testicular tissue. Reproduced from Eicher, et al. 1982.

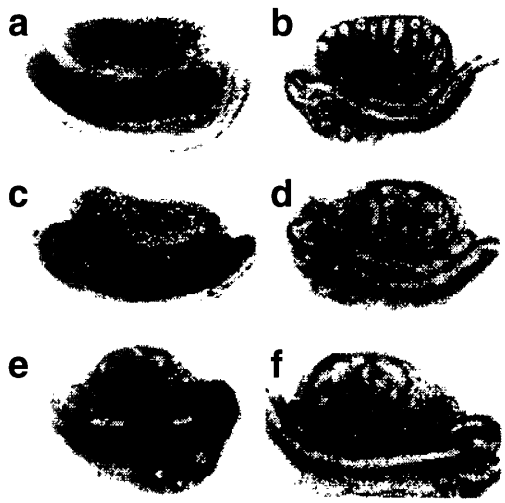

Figure 2–2. Appearance of gonads from E14.5 to E16 C57BL/6J-Y^{POS} fetuses.

Table 2–1. (BXD)F1 X C57BL6/J.Y^{POS} progeny: chromosome constitution and three phenotypic categories observed for XY animals.

	Chromosome constitution			Phenotype of XY mice†		
	XY	XX	TOTAL	Male	A–Male	Herm
Group 1	86	131	217	59	13	13
Group 2	151	161	312	124	12	12
W group	7	0	7	0	4	3
Pooled	244	292	529	183	29	28

† Abbreviations: A-Male, abnormal male (see text); Herm, hermaphrodite. Note: one XY embryo from group 1 and three from group 2 are not included in the breakdown according to phenotype.

otherwise consistent with the 4,000 marker reference mouse map completed recently, the distances reported here correspond to that map (Dietrich, et al. 1994). The resolution of our map is lower, based as it is on only 75 meioses, but it covers all twenty mouse chromosomes, in intervals no larger than 15 cM. Since it is sufficient to detect linkage to the entirety of the mouse genome, and the map is sufficiently dense to detect >99% of potentially misleading double crossover events, we are confident that this map has allowed us to detect all the loci of interest that are segregating in the backcross, within the inherent limits of our sample size (see Materials & Methods).

Linkage analysis implicates multiple autosomal loci

We detected a significant skewing in favor of the B/B genotype in the 28 hermaphrodites at five distinct autosomal locations: on proximal chromosome 2 near D2MIT88, distal chromosome 4 near *Dvl*, chromosome 5 near D5MIT157, chromosome 8 near D8MIT84, and chromosome 16 near D16MIT48. For all loci tested, **Table 2–2** shows a summary of the number of XY animals in the three phenotypic categories with each of the two possible genotypes. A χ^2 analysis with the Mendelian expectation of equal numbers in each genotypic categories the null

Table 2–2. Summary of the distribution of genotypes (B/B or D/B) at each locus tested for the XY animals in the mapping panel, which consisted of 28 hermaphrodites, 29 abnormal males, and 18 normal males (or 59 for selected loci), as well as XX animals (129) tested as controls. χ^2 analysis was performed based on the expectation that in these backcross progeny, half the animals would be homozygous and half heterozygous in all phenotypic categories. The females, normal and abnormal males deviated from this expectation in only a single instance, at D6MIT23 for abnormal males, but for five clusters of markers, significant deviations were detected amongst the hermaphrodites: near D2MIT88, *Dvl*, D5MIT157, D8MIT84, and D16MIT48. The strongest skewing occurs at *Dvl* and nearby markers on chromosome 4, followed by the chromosome 2 and 5 loci.

Table 2–2

	Hermaphrodites			Abnormal males			Normal males			Females		
Locus	B/B	D/B	Chi-sq >5.0	B/B	D/B	Chi-sq >5.0	B/B	D/B	Chi-sq >5.0	D/B	B/B	Chi-sq >5.0
D1Mit1	18	10	.	15	12	.	11	5	.	71	57	.
D1Mit70	19	8	.	12	17	.	9	9	.			
D1Mit5	17	10	.	14	15	.	8	9	.			
D1Mit7	17	10	.	15	14	.	8	9	.			
D1Mit11	16	9	.	14	14	.	9	9	.			
D1Mit54	17	10	.	15	13	.	8	8	.			
D1Mit30	16	12	.	16	12	.	10	5	.	66	62	.
D1Mit16	14	13	.	15	12	.	10	8	.			
D1Mit150	13	12	.	14	12	.	10	7	.			
D1Mit17	13	14	.	16	12	.	9	8	.			
D2Mit1	18	10	.	19	9	.	32	27	.	57	70	.
D2Mit6	20	8	5.1	18	11	.	34	25	.	64	64	.
D2Mit151	21	7	7.0	16	11	.	30	28	.			
D2Mit7	24	4	14.3	16	12	.	30	29	.	62	65	.
D2Mit203	24	4	14.3	13	11	.	30	29	.			
D2Mit154	25	3	17.3	15	12	.	31	27	.			
D2Mit88	25	3	17.3	15	12	.	31	28	.			
D2Mit241	25	3	17.3	15	11	.	9	8	.			
D2Mit269	24	3	16.3	15	12	.	31	27	.			
D2Mit157	24	4	14.3	15	12	.	29	30	.			
D2Mit156	24	4	14.3	15	12	.	29	30	.			
D2Mit61	24	4	14.3	15	12	.	29	30	.			
D2Mit9	23	5	11.6	15	14	.	27	32	.			
D2Mit91	22	6	9.1	14	12	.	27	32	.			
D2Mit125	22	6	9.1	14	13	.	26	33	.			
D2Mit10	20	7	6.3	14	13	.	10	8	.			
D2Mit92	20	7	6.3	15	12	.	10	7	.			
D2Mit66	20	8	5.1	15	14	.	29	30	.	69	59	.
D2Mit13	16	11	.	15	14	.	9	9	.			
D2Mit12	16	11	.	14	12	.	9	9	.			
D2Nds1	16	10	.	15	12	.	9	9	.			
D2Mit17	17	10	.	15	13	.	10	8	.			
D2Mit21	14	12	.	15	14	.	10	7	.			
D2Mit53	13	14	.	12	15	.	9	7	.			
D3Mit54	18	10	.	16	12	.	6	10	.	64	62	.
D3Mit21	19	8	.	13	13	.	8	9	.			
D3Mit6	18	8	.	15	13	.	7	11	.			
D3Mit22	16	9	.	18	10	.	6	12	.			
D3Mit10	15	12	.	20	8	5.1	6	12	.			
D3Mit43	15	11	.	16	9	.	5	12	.			
D3Mit17	16	10	.	17	10	.	6	11	.			
D3Mit19	16	11	.	17	11	.	7	11	.			
D4Mit18	17	10	.	15	14	.	30	29	.	63	65	.
D4Mit39	16	12	.	16	13	.	10	8	.			
D4Mit17	16	11	.	18	11	.	10	8	.			
D4Mit9	17	8	.	17	11	.	9	9	.			
D4Mit31	21	6	8.3	18	9	.	8	9	.			
D4Mit12	24	3	16.3	18	10	.	8	10	.			
D4Mit16	24	3	16.3	17	11	.	8	10	.			
D4Mit13	25	3	17.3	21	8	5.8	7	11	.			
D4Mit14	26	2	20.6	19	10	.	7	11	.			
D4Mit42	26	2	20.6	19	10	.	25	34	.	63	64	.
D4Mit180	26	2	20.6	19	10	.	25	34	.	61	66	.
D4Smh6b	27	1	24.1	19	10	.	25	34	.			
Dvl	27	1	24.1	19	10	.	25	34	.			
MovE-5,7,8	24	2	18.6	20	9	.	8	10	.			
Telq4	23	2	17.6	19	8	.	8	10	.			
D5Mit1	16	12	.	17	12	.	8	10	.			
D5Mit11	20	8	5.1	19	10	.	6	12	.			
D5Mit55	19	9	.	19	10	.	6	12	.			
D5Mit15	21	7	7.0	20	8	5.1	7	10	.			
D5Mit58	21	7	7.0	20	9	.	8	10	.			
D5Mit12	21	7	7.0	20	9	.	7	11	.			
D5Mit7	22	6	9.1	19	10	.	27	32	.	63	62	.
D5Mit10	21	6	8.3	18	11	.	7	11	.			
D5Mit41	22	6	9.1	18	11	.	27	32	.			
D5Mit157	22	5	10.7	16	11	.	27	31	.			
D5Mit25	21	7	7.0	19	10	.	6	11	.			

Table 2–2

	Hermaphrodites			Abnormal males			Normal males			Females		
Locus	B/B	D/B	Chi-sq >5.0	B/B	D/B	Chi-sq >5.0	B/B	D/B	Chi-sq >5.0	D/B	B/B	Chi-sq >5.0
D5Mit24	19	7	5.5	12	5	.						
D5Mit65	21	7	7.0	19	10	.	7	11	.			
D5Mit32	17	7	.	12	7	.						
D5Mit99	20	7	6.3	19	10	.	9	9	.			
D6Mit50	18	9	.	19	10	.	10	8	.			
D6Mit33	19	8	.	21	8	5.8	7	11	.			
D6Mit16	14	9	.	22	7	7.8	8	10	.			
D6Mit9	17	10	.	20	9	.	8	10	.			
D6Mit31	17	9	.	21	7	7.0	9	9	.			
D6Mit23	17	11	.	22	6	9.1	11	7	.			
D6Mit13	19	9	.	20	7	6.3	12	6	.	66	61	.
D6Mit14	15	13	.	19	9	.	13	5	.	61	67	.
D7Mit21	14	13	.	19	9	.	9	9	.			
D7Mit57	11	16	.	20	9	.	9	9	.			
D7Nds5	9	17	.	15	14	.	8	9	.			
D7Mit85	9	18	.	13	16	.	9	9	.			
D7Mit89	9	17	.	11	17	.	9	9	.			
D7Mit30	9	17	.	9	20	.	10	8	.			
D7Mit40	12	15	.	10	19	.	10	8	.			
D7Mit12	11	16	.	12	17	.	9	9	.			
D8Mit3	14	14	.	18	10	.	5	13	.	65	63	.
D8Mit24	18	10	.	17	11	.	7	10	.			
D8Mit8	18	10	.	20	9	.	6	12	.			
D8Mit41	19	9	.	18	11	.	7	11	.	61	66	.
D8Mit109	18	8	.	17	9	.	22	31	.			
D8Mit84	20	8	5.1	16	10	.	26	32	.			
D8Mit47	20	8	5.1	16	13	.	28	31	.	68	60	.
D8Mit86	15	5	5.0	13	9	.						
D8Mit88	18	10	.	18	11	.	8	10	.			
D8Mit42	16	9	.	14	14	.	11	7	.			
D9Mit65	15	13	.	16	13	.	13	5	.	61	66	.
D9Mit67	16	12	.	19	10	.	11	7	.	59	68	.
D9Mit4	14	12	.	18	8	.	10	6	.	57	70	.
D9Mit21	13	13	.	18	9	.	12	6	.			
D9Mit8	12	13	.	18	9	.	10	8	.			
D9Mit50	12	14	.	20	8	5.1	9	9	.			
D9Mit35	13	14	.	19	9	.	9	9	.			
D9Mit12	13	14	.	19	9	.	10	8	.			
D9Mit20	15	10	.	18	9	.	9	8	.			
D9Mit19	15	12	.	17	12	.	8	10	.			
D10Mit3	8	19	.	12	17	.	12	6	.			
D10Mit40	9	18	.	12	17	.	12	6	.			
D10Mit61	11	15	.	12	17	.	11	7	.			
D10Mit42	13	14	.	14	15	.	10	7	.			
D10Mit10	14	13	.	14	15	.	13	5	.			
D10Nds2	15	12	.	16	13	.	12	6	.			
D10Mit14	14	13	.	16	13	.	11	7	.			
D11Mit63	12	15	.	17	12	.	7	11	.			
D11Mit53	12	15	.	17	12	.	8	10	.			
D11Mit20	14	13	.	16	13	.	4	12	.	49	33	.
D11Mit112	14	13	.	15	13	.	6	12	.			
D11Mit4	15	13	.	15	13	.	6	12	.	63	57	.
D11Mit41	13	15	.	16	13	.	6	12	.	64	63	.
D11Mit14	14	13	.	14	13	.	7	10	.			
D11Mit48	15	12	.	18	11	.	10	8	.			
D11Mit104	15	11	.	16	12	.	10	8	.			
D12Mit12	11	16	.	11	18	.	10	8	.			
D12Mit46	12	15	.	12	17	.	10	8	.			
D12Mit2	13	14	.	12	17	.	9	9	.			
D12Mit36	14	13	.	13	16	.	9	9	.			
D12Mit34	12	15	.	12	17	.	11	7	.			
D12Mit114	13	15	.	10	17	.	31	28	.			
D12Mit128	13	15	.	12	15	.	33	26	.			
D12Mit4	12	15	.	12	17	.	12	6	.			
D12Mit5	12	15	.	12	17	.	11	7	.			

Table 2–2

	Hermaphrodites			Abnormal males			Normal males			Females		
Locus	B/B	D/B	Chi-sq >5.0	B/B	D/B	Chi-sq >5.0	B/B	D/B	Chi-sq >5.0	D/B	B/B	Chi-sq >5.0
D12Mit7	12	15	.	15	14	.	10	8	.			
D12Mit8	12	15	.	13	16	.	9	9	.			
D13Mit3	11	16	.	19	10	.	10	8	.			
D13Mit18	11	16	.	17	11	.	11	7	.			
D13Mit23	11	15	.	17	12	.	11	7	.			
D13Mit27	11	15	.	17	12	.	8	10	.			
D13Mit45	12	13	.	17	11	.	9	8	.			
D13Mit35	13	14	.	14	15	.	8	10	.			
D14Mit1	11	16	.	20	9	.	7	11	.			
D14Mit2	10	16	.	20	9	.	7	11	.			
D14Mit45	10	17	.	18	11	.	6	12	.			
D14Mit4	11	16	.	14	12	.	8	10	.			
D14Mit28	10	17	.	15	14	.	7	10	.			
D14Mit7	10	17	.	14	14	.	9	9	.			
D14Mit94	13	14	.	14	14	.	11	7	.			
D14Mit75	15	12	.	12	16	.	11	7	.			
D15Mit12	14	13	.	12	17	.	9	9	.			
D15Mit8	16	11	.	15	14	.	7	11	.			
D15Mit26	15	12	.	14	15	.	7	11	.			
D15Mit3	13	14	.	16	13	.	7	11	.			
D15Mit37	11	16	.	16	13	.	10	8	.			
D15Mit39	9	18	.	17	11	.	9	9	.			
D15Mit42	9	18	.	18	11	.	9	9	.			
D15Mit16	9	18	.	17	12	.	9	9	.			
D16Mit9	14	14	.	16	13	.	8	10	.			
D16Mit101	16	11	.	15	11	.	8	9	.			
D16Mit4	20	8	5.1	17	12	.	9	9	.			
D16Mit5	20	8	5.1	15	14	.	9	9	.			
D16Mit48	21	7	7.0	13	12	.	28	30	.			
D16Mit19	20	8	5.1	15	12	.	30	27	.			
D16Mit50	20	8	5.1	17	12	.	29	30	.			
D16Mit6	20	8	5.1	17	12	.	8	10	.			
D16Mit52	14	6	.	14	9	.						
D17Mit46	12	14	.	19	7	5.5	6	12	.			
D17Mit24	11	15	.	18	9	.	6	12	.			
D17Mit10	11	15	.	16	10	.	6	12	.			
D17Mit6	12	14	.	18	10	.	6	12	.			
D17Mit3	11	15	.	15	11	.	5	11	.			
D17Mit38	8	17	.	16	11	.	5	13	.			
D17Mit41	9	17	.	17	10	.	4	14	5.6			
D18Mit19	16	11	.	15	14	.	12	6	.			
D18Mit68	16	11	.	12	16	.	11	7	.			
D18Mit17	16	10	.	14	14	.	10	8	.			
D18Mit35	16	9	.	14	13	.	11	7	.			
D18Mit9	15	11	.	15	14	.	9	9	.			
D18Mit33	14	12	.	14	15	.	8	9	.			
D18Mit4	16	9	.	15	11	.	8	10	.			
D19Mit16	15	11	.	18	10	.	10	8	.			
D19Mit40	16	11	.	16	12	.	9	9	.			
D19Mit11	15	10	.	14	15	.	8	9	.			
D19Mit1	14	12	.	14	14	.	8	10	.			
D19Mit6	14	12	.	15	14	.	8	10	.			
DXMit48	10	16	.	12	15	.	11	7	.			
DXMit25	12	16	.	12	15	.	32	26	.	48	42	.
DXMit1	12	16	.	13	16	.	11	7	.			
DXMit64	12	14	.	13	13	.	10	8	.			
DXMit19	14	14	.	14	15	.	30	29	.	62	66	.
DXMit10	15	12	.	16	12	.	10	8	.			

Figure 2–2. Graphical representation of the percentage of hermaphrodite progeny that have the B/B genotype for the markers tested on each chromosome. Proportions above or below the horizontal lines marking 75% and 25% are significantly distorted given the sample size of 28 ($p<0.01$). No distortions below 25% were found, but five chromosome regions have proportions at or above 75%

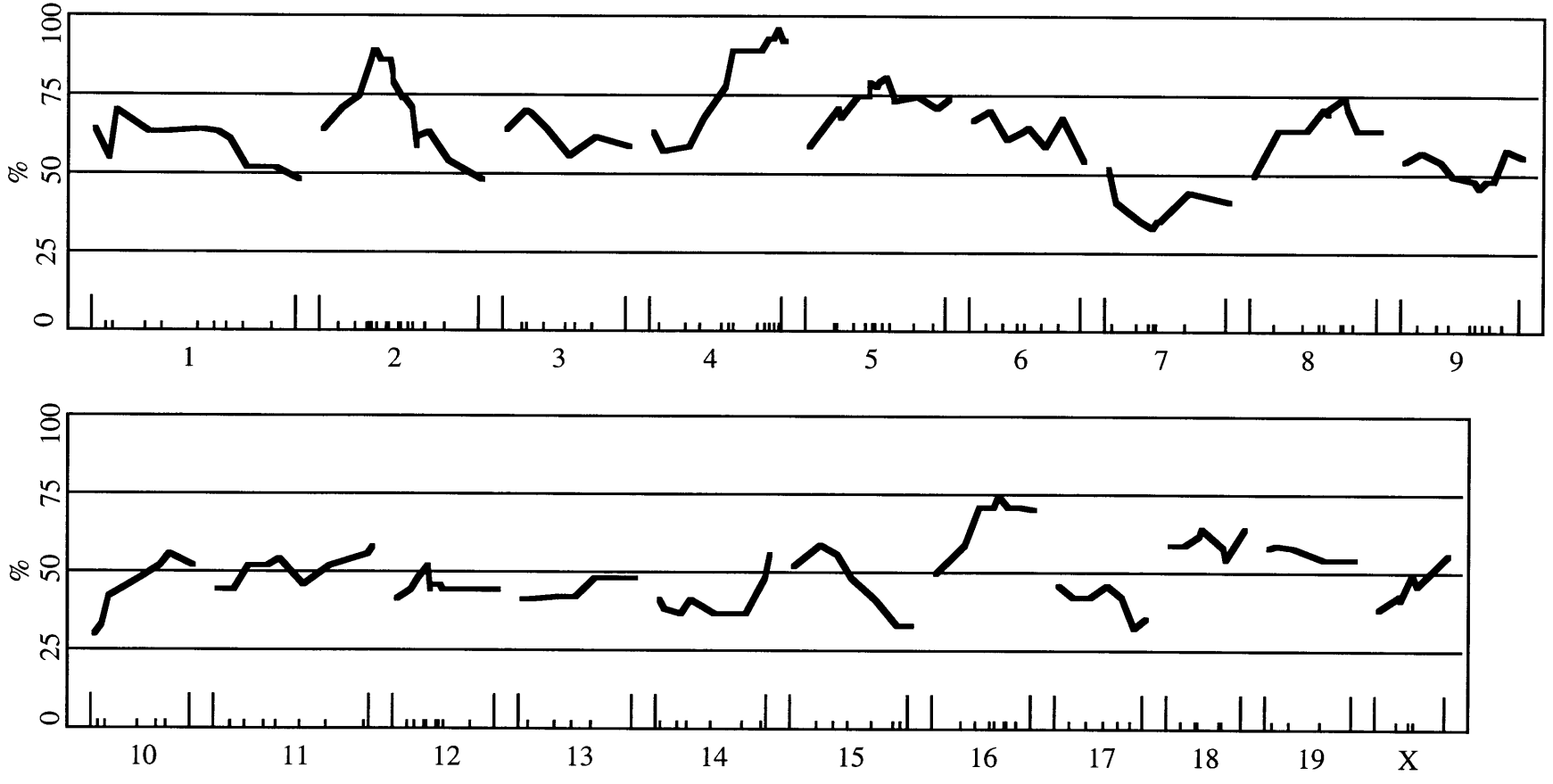

Figure 2–2. Percentage of all hermaphrodite progeny homozygous for B6-derived alleles at each locus tested. Numbers below the graph represent the mouse chromosome.

hypothesis is also shown. This skewing in favor of the B/B genotype amongst hermaphrodites is precisely the behavior predicted for a *tda* locus, since previous genetic analyses indicated a recessive effect of B6-derived alleles. Of interest is that no loci were significantly skewed in favor of D2 alleles in any group. For the most part, the skewing to B6 alleles was restricted to the hermaphrodites, but a notable exception to that was the observation of 22/28 abnormal males with the B/B genotype at D6MIT23. This effect is stronger than the two weakest associations in the hermaphrodites (at D8MIT84 and D16MIT48), but D6MIT23 shows no association amongst the hermaphrodites. Conversely, those loci which exhibit strong genotypic associations with the hermaphrodite phenotype show little or, more often, no association amongst the abnormal males. The abnormal males therefore did not figure prominently in the analysis.

Genetic properties of the candidate tda loci

The above analysis made it clear immediately that no single locus is strictly correlated with the phenotype. Even for the strongest locus (near *Dvl*), one hermaphrodite is clearly heterozygous for the critical region of distal chromosome 4. See Table 2–3. Conversely, for each *tda* locus, about half of the normal males have the 'hermaphrodite' (B/B) genotype. In ranking the loci, it is therefore useful to consider a relative risk (RR) score, which is a measure of the increased risk for sex reversal coincident with the B6 homozygous genotype. If there is no increased risk at all, the RR = 1. For D4SmhB6, D2MIT88, D5MIT41, D16MIT48, and D8MIT84, the RR scores are 17, 5.9, 2.7, 2.4, and 2.0. That is, the B6 homozygous genotype at a particular locus may increase an embryo's risk of hermaphrodite development from 2- to 17-fold, depending on the locus.

Utilizing our whole-genome genetic profiles, we tested models involving compound genotypes, to determine whether these loci might determine the hermaphrodite phenotype in some combinatorial fashion. For instance, considering

the strongest three loci, a reasonable model is that the combination of the B6 homozygous genotypes at three critical genes is required for hermaphrodite development. This model does not fit the data for two reasons: 1) some hermaphrodites are homozygous at only two of the loci (as noted above), and 2) some normal males are homozygous at all three loci (data not shown). Therefore, instead of a strict requirement for the B6 homozygous genotype at all three loci, we considered the possibility that homozygosity at two of the three loci is all that is required. All 28 hermaphrodites are homozygous at two of the three strongest loci; however, one must consider that homozygosity for two of three and even three of three of the loci is also compatible with male development (data not shown). Considering the two weakest loci likewise led to no simple genotype-to-phenotype correlation. Therefore, no locus or combination of loci is strictly necessary for hermaphrodite development, but rather, the B6 homozygous genotype at each one may be seen as a predisposition to sex reversal.

Sex reversal is independent of sex chromosome distortion

We considered the possibility that the skewing of genotypes amongst the hermaphrodites might be related to the sex chromosome distortion evident in Group 1. See Table 2–1. This group showed a significant deficit in the number of XY animals (n = 86) vs. XX animals (n = 131) ($p< 0.005$, $\chi^2 = 9.33$), but this deficit was not evident in the second group. The proportion of XY animals in the two samples is significantly different ($p < 0.05$ in a two-sided, two-sample normal theory test for independent proportions). The deficit in XY animals could be associated with a particular group of XY animals, e.g. XY females with a particular genotype. This would explain both the deficit of XY animals and the lack of XY females in the cross. Of course, other indeterminate variables affecting both sex-chromosome constitution and gonadal phenotype, or which increase the proportion

of B6 alleles transmitted at a particular locus or in general, could confound the observed associations.

We reasoned that a fairly simple test would show whether genotype-specific lethality amongst XY embryos, or a generalized skewing in favor of B6 alleles, might operate in the cross, in which case some associations are questionable. The test is to determine the genotype of a great enough number of normal XY males and XX females to detect a generalized skewing if it exists. Accordingly, we determined the genotype of an additional 41 normal males (for a total of 115 XY mice) and a panel of 129 normal XX females with selected markers from the map. The data for the males included 32 loci from chromosomes 2, 4, 5, 8, 9, 12, 16, and X. For the females, 22 loci were tested from chromosomes 1–6, 8, 9, 11, 12, and X. In no case were any significant deviations from the Mendelian expectation detected (data not shown). Although we have not determined the cause of the deficit in XY animals in Group 1, we can conclude with confidence the skewed genotypes of the hermaphrodites are not a consequence of a general skewing in the cross, or in the XY^{POS} progeny. Consequently, the data from all groups of embryos was pooled and analyzed as one data set.

Discussion

In this study we have exploited the natural variation amongst inbred mouse strains with respect to their compatibility with the Y^{POS} chromosome to map sex determinants by segregation analysis. We present evidence that at least five autosomal genes affect Y^{POS} sex reversal in an intraspecific backcross between C57BL/6J.Y^{POS} and (DBA/2J X C57BL/6J) F1 hybrids. The major determinants of sexual phenotype in this cross are two loci which map to mouse autosomes 2 and 4. Our data effectively refute two of the earliest models put forward to explain sex reversal; namely, that a single autosomal locus controls the trait, or alternatively, that any non-specific interference with male development capable of causing a

developmental growth delay will lead to sex reversal. Eicher and Washburn initially advocated the first hypothesis, but later abandoned it (Eicher, et al. 1982, Eicher and Washburn 1983, Eicher and Washburn 1986) on further genetic investigation. Biddle revived it recently, only to reject it based on his observations of the hermaphrodite phenotype (Biddle, et al. 1994). Cattanach advocated the latter hypothesis (unpublished data, but see Eicher 1988), citing experiments in which the Y^{AKR} caused sex reversal on a B6 background when present with the W^{19} deletion of chromosome 5. It will be very interesting to determine whether the *tda* locus we mapped to chromosome 5 is contained within that deletion, possibly explaining what seemed an impossible coincidence to Cattanach. Our demonstration the a finite number of loci affect Y^{POS} sex reversal in at least this one cross casts doubt on the non-specific growth effect hypothesis, although it is still plausible that one or more of the loci we have identified encode growth factors.

The central questions which our study leaves unanswered revolve around the number of loci that are significant in determining the male or hermaphrodite phenotype of XY animals: how much of the observed variation is due to developmental noise and how much to allelic variation at *tda* loci? The loci we found predispose animals to sex reversal, with an increased risk of from 2- to 17-fold, depending on the locus. It is appropriate to classify them as predisposing loci since no one locus is necessary nor sufficient to cause hermaphrodite development. Here our data invite comparison with genetic studies of other traits which are affected by inherited predispositions, such as nonobese diabetes (NOD) and the modifier of *Min* (*Mom-1*) in the mouse, and hypertension in the rat (Jacob, et al. 1991, Todd, et al. 1991, Dietrich, et al. 1993, Ghosh, et al. 1993). The example of the NOD mouse comes from ambitious studies conducted by John Todd and his colleagues (Todd, et al. 1991, Ghosh, et al. 1993). In a cross designed as ours was to locate loci whose alleles have recessive effects, ten loci named *Idd*–1-10 (for

*I*nsulin-*d*ependent *d*iabetes mellitus) were mapped to nine mouse chromosomes. About 100 BC1 progeny from a cross of the 'sensitive' parent to the 'resistant' F1 hybrid were analyzed to allow Todd to conclude that they have found all major susceptibility genes with recessive or partially dominant alleles (dependent on the MHC background) segregating in the cross.

With respect to the criteria applied for significant linkage, a χ^2 value greater than 10.8 (or $p < 0.001$) was demanded, and a multiple polychotomous regression method was applied to test the sufficiency of the loci mapped to explain the phenotypes. Only two of our *tda* loci fit this strict criterion, which must be relaxed to $\chi^2 = 5.14$ (or $p < 0.025$) to include all five, and we have as yet no model for the interactions of these loci to identify a compound genotype sufficient to cause hermaphroditism. However, as we found for the *tda* loci, the *Idd* genes show effects that are graded from major to minor. Todd's regression analysis allows him to conclude "that several different combinations of genes are capable of causing diabetes in mice and that even in this experimental model the trait is genetically heterogeneous." (Ghosh, et al. 1993), 407). This conundrum could result from developmental noise, perhaps in the initial number of beta cells a diabetic mouse begins life with, or it could be an indication of very complicated gene interactions in development. For instance, one way an organism could more efficiently deal with redundant genetic pathways would be to switch off all but one. Of course, this process would probably proceed in the absence of feedback as to whether one or the other pathway is partially defective, leading to many potential routes to diabetes or sex reversal, and at least as many genes as redundant pathways. Previous genetic data suggests that Y^{POS} sex reversal is heterogeneous with respect to different strain combinations (Eicher and Washburn 1986, Eicher 1988), and although it could well be heterogeneous within the cross we have analyzed, investigation of this fascinating issue must await future analysis.

We mapped the autosomal sex determinants reported here on the basis of their allelic variation in the inbred 'host' strains, but variation must also exist at *Tdy* since the Y^{D2} does not cause sex reversal on a B6 background. In fact, Washburn has shown that a 14 kb cosmid carrying the cloned *Sry* gene from the mouse can rescue *T-as* sex reversal (Linda L. Washburn, Ph.D. Thesis, University of Maine; Koopman, et al. 1991) , and this would presumably hold true for Y^{POS} sex reversal as well. Although this kind of allele-specificity is frequently taken to indicate an interaction between variant forms of two proteins, these experiments leave open the question of precisely how *Sry* interacts with the autosomal determinants. Besides for protein-protein interactions, two other possibilities include joint participation in a single pathway or process, or indirect signalling in a receptor-ligand relationship. These ambiguities lead directly to the question: what functions are the *tda* genes likely to control? In the absence of direct evidence, we can only speculate that they could be developmental regulators such as specific transcription or splicing factors, determinants of cell fate possibly involved in intercellular communication, ovary determinants, or even growth factors controlling the pace of testis development. There is a great deal of precedent for sex determining genes functioning to control other genes in the pathway from the invertebrate organisms *C. elegans* and *D. melanogaster*. For instance, a master regulator gene in C. elegans, *sdc-1*, encodes a zinc-finger motif DNA binding protein that, in conjunction with *sdc-2* and *3*, exerts negative control over the transcription of *her-1*, the next gene in the pathway (Parkhurst and Meneely 1994, and references therein). In *Drosophila*, of approximately eleven genes involved in somatic sex determination, one is a splicing factor that binds RNA, and at least seven are transcriptional regulators (Parkhurst and Meneely 1994, and references therein). Transcriptional control and RNA splicing are two themes that emerge

quite readily, and the *tda* loci could function as such in mammals, perhaps interacting directly with *Sry*.

Looking at the issue from another angle, mammalian embryologists have long interpreted the problem of gonadal determination as one of cell fate determination. The embryonic mammalian gonad is composed of three bipotential cell types which follow different fates in each sex (McLaren 1991a). A series of experiments performed using XO/XY or XO/XY/XYY mosaics or XX<–>XY chimeric mice made by aggregation or blastocyst injection (Burgoyne, et al. 1988, Patek, et al. 1991, Palmer and Burgoyne 1991b) suggests that sex determinants may function within and between cells of the developing gonad to determine their fate. For the chimeras, the experiments showed that the XX/XY composition of almost all cell types in a particular animal were similar, but that in the Sertoli cells of the gonad, a marked skewing towards XY cells was observed, indicating that the Y chromosome is required for Sertoli cell determination. An initial study reported no XX Sertoli cells at all (Burgoyne, et al. 1988) , but improved methods and examination of fetal as well as prepuberal and adult mice demonstrated up to 20% XX Sertoli cells in some fetal chimeras; whereas this appears to drop to a constant low value of about 2% in adults (Palmer and Burgoyne 1991b). Burgoyne and others conclude from this data that the role of *Tdy* is to direct the cells of the supporting cell lineage to form Sertoli cells. The short burst of *Sry* expression from 10.5–12.5 d*pc* in the somatic compartment of the bipotential gonad offers indirect support for this notion. However, since *Sry* is a DNA binding protein that is unlikely to be secreted, it seems likely that other gene products must assist *Sry* in producing the extracellular signal. These gene products might be the autosomal sex determinants.

Alternatively, the autosomal genes could be ovary determinants. The arguments for ovary determinants rely on an effect on timing and pace of growth

during embryogenesis. Eicher and Washburn were the first to invoke the timing of developmental milestones as crucial determinants of testis or ovary development (Eicher and Washburn 1983, Eicher and Washburn 1986). They posit two genetic pathways, one which leads to testis development and is shadowed in developmental time by the other, which leads to ovary development. A key feature of the model is that the first testis determining gene should pre-empt activation of the first ovary-determining gene, perhaps even inactivating it. This developmental asynchrony model explains why the phenomenon is only observed for Y^{DOM} on a *M. m. musculus* background, and not vice versa. The Y^{DOM} is postulated to have a late-acting allele of *Tdy*, which allows the ovarian pathway to commence. The Y chromosome from a strain with early-acting ovary determinants ('incompatible') must have an early-acting *Tdy* allele, and hence the reciprocal cross presents no problems. There is even good experimental evidence that the Y^{POS} *Sry* allele acts later than that of the Y^{B6} (Palmer and Burgoyne 1991b). However, Lovell-Badge has outlined an auto-induction hypothesis for Sertoli cell differentiation that requires neither protein-protein interactions between autosomal genes and *Sry*, nor ovary determinants. According to his hypothesis, *Sry* initiates Sertoli cell differentiation and controls the expression of a ligand which will allow neighboring pre-Sertoli cells to induce themselves, if enough are present (Lovell-Badge 1992). He goes on to observe that if the timing of the receptor for the hypothetical ligand were critical, then this system could also result in a 'timing mismatch' form of sex reversal without invoking ovary determinants.

The questions posed above as well as many others yet to be raised can only be answered by the determination of the nature of the autosomal sex determinants by molecular cloning. The future experimental directions for the *tda* system are simply summarized as follows: (1) to determine the identity of all loci which can be mapped, by positional cloning and/or syntenic relationships to cloned human genes,

and (2) to extend the identical analysis presented here to other strain combinations, to further elucidate the allele-specificity of the system. The techniques for such positional cloning efforts are both widely applied and thoroughly understood. Although the *tda* loci will almost certainly present some unique challenges, here we must simply refer the reader to some recent publications for information about the methods and strategies, such as those employed in the cloning of the Agouti locus in the mouse (Bultman, et al. 1992, Miller, et al. 1993). In conclusion, this study has successfully extended the hunt for sex determinants onto the autosomes, and therefore represents a foundation for a great deal of future progress in mammalian sex determination research.

Materials & Methods

Embryo collection As described previously (Eicher, et al. 1980), (embryos were collected from timed matings between E14.5 and E16. The embryos reported here were collected in three groups from matings of (BXD) F1 females to N24 (or greater) generation B6.Y^{POS} hermaphrodites that could breed as males. Gonadal type was determined by examination with a dissecting microscope. Any abnormal gonads were also analyzed by histological sectioning. The chromosome constitution of each embryo was inferred by testing with a PCR assay for the presence or absence of the *Zfy-1* locus. Genomic DNA was isolated from a subset of the embryos as previously described (Page, et al. 1987).

Genotype determination Initially we chose ~130 genetic markers for genotypic analysis of selected embryos. Virtually all of the markers were designed for "rapid typing"; that is, they are defined by PCR primers chosen such that they amplify simple sequence repeats (SSRs) previously found to differ in length between B6 and D2 . Whenever possible, the data collection was blind, in that the identity of the DNA samples was concealed using code numbers. After the preliminary phase of our study, certain chromosome regions emerged that appeared likely to contain a

locus of interest, and more genetic markers were added to the map, for a total of 228.

Genotype data analysis We applied two types of analysis to the genotype data: genetic map construction and tests for association between genotype and the hermaphrodite phenotype. A genetic map was constructed using the MAPMAKER program as described previously (Lander, et al. 1987). In addition, two tests of association were applied. Log of the Odds Ratio (LOD) scores were calculated for the hermaphrodite animals using MAPMAKER's two-point analysis, assuming sex-determining loci at which the B6 homozygous genotype (B/B) is strictly necessary for hermaphrodite development. For each marker, the LOD score is the $\log_{10}$ (odds of observing the data given linkage at $\theta = x$ cM to the hypothetical locus/ odds given no linkage). MAPMAKER performs several iterations of the LOD score calculation, varying x, until a genetic distance is found which maximizes the LOD score. Although one locus initially gave a significant LOD score (>3.0 is considered significant by conventional mathematical arguments) at the most likely genetic distance, it became clear that our assumption that the B/B genotype is required in hermaphrodites is not valid. That is, a significant number of hermaphrodites are heterozygous (D/B) at the loci which show the strongest association with the phenotype (see Discussion). We therefore applied a relative risk (RR) calculation to our data, to reduce the number of assumptions we made about the loci involved. The RR is the overall probability of being a hermaphrodite given a particular genotype (B/B) divided by the probability of being a hermaphrodite given any other genotype (only D/B in this cross). A RR = 1.0 indicates no association exists between phenotype and genotype.

Statistical tests & power considerations The sex chromosome constitution and proportion of male, abnormal, and hermaphrodite animals for the two large groups collected were subjected to two-sided and/or two-sample normal theory tests of

independent proportions before being combined and analyzed as a whole. (Pagano and Gauvreau 1993) The genotypes of a panel of normal males and females were determined for certain loci to address the possibility of a general skewing in favor of the D/B or B/B genotype in this cross (see Results). The small percentage of hermaphrodites produced in this cross limited our power to detect significant deviations from Mendelian expectations. For instance, if we adopt the statistical definition of power as the probability of rejecting the null hypothesis, given that it is false, our power to detect a deviation of the magnitude observed at the strongest locus is 86.9% (with $\alpha = 0.01$), but for the weakest association, the power falls off significantly. Since statistical power increases with increasing sample size (Pagano and Gauvreau 1993), it is not unlikely that expanding the number of animals sampled would lead to the identification of more loci affecting hermaphroditism in this cross.

References

Austin, C. R., and Edwards, R. G. (1981). *Mechanisms of Sex Differentiation in Animals & Man.* London: Academic Press.

Bardoni, B., Zanaria, E., Guioli, S., Floridia, G., Worley, K. C., Tonini, G., Ferrante, E., Chiumello, G., McCabe, E. R. B., Fraccaro, M., Zuffardi, O., and Camerino, G. (1994). A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. Nat Genet *7*, 497-501.

Berta, P., Hawkins, J. R., Sinclair, A. H., Taylor, A., Griffiths, B. L., Goodfellow, P. N., and Fellous, M. (1990). Genetic evidence equating SRY and the testis-determining factor. Nature *348*, 448-50.

Biddle, F. G., Eisner, J., and Eales, B. A. (1994). The testis-determining autosomal trait, *Tda-1*, of C57BL/6J is determined by more than a single autosomal gene when compared with DBA/2J mice. Genome *37*, 296-304.

Biddle, F. G., and Nishioka, Y. (1988). Assays of testis development in the mouse distinguish three classes of *domesticus*-type Y chromosome. Genome *30*, 870-878.

Bultman, S. J., Michaud, E. J., and Woychik, R. P. (1992). Molecular characterization of the mouse *agouti* locus. Cell *71*, 1195-204.

Burgoyne, P. S., Buehr, M., Koopman, P., Rossant, J., and McLaren, A. (1988). Cell-autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XX----XY chimaeric mouse testes. Development *102*, 443-50.

Dietrich, W. F., Lander, E. S., Smith, J. S., Moser, A. R., Gould, K. A., Luongo, C., Borenstein, N., and Dove, W. (1993). Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse. Cell *75*, 631-9.

Dietrich, W. F., Miller, J., Steen, R., Merchant, M., Damron, D., Nahf, R., Joyce, D., Wessel, M., Dredge, R., Marquis, A., Stein, L., Goodman, N., Page, D. C., and Lander, E. S. (1994). A genetic map of the mouse with 4,006 simple sequence length polymorphisms. Nat Genet *7*, 220-245.

Eicher, E. M. (1988). Autosomal genes involved in mammalian primary sex determination. Philos Trans R Soc Lond [Biol] *322*, 109-18.

Eicher, E. M., Beamer, W. G., Washburn, L. L., and Whitten, W. K. (1980). A cytogenetic investigation of inherited true hermaphroditism in BALB/cWt mice. Cytogenet Cell Genet *28*, 104-115.

Eicher, E. M., and Washburn, L. L. (1983). Inherited sex reversal in mice: identification of a new primary sex-determining gene. J Exp Zool *228*, 297-304.

Eicher, E. M., and Washburn, L. L. (1986). Genetic control of primary sex determination in mice. Annu Rev Genet *20*, 327-60.

Eicher, E. M., Washburn, L. L., Whitney, J. 3., and Morrow, K. E. (1982). *Mus poschiavinus* Y chromosome in the C57BL/6J murine genome causes sex reversal. Science *217*, 535-7.

Ferguson-Smith, M. A. (1992). Abnormalities of Human Sex Determination. J Inher Metab Dis *15*, 518-525.

Fredga, K., Gropp, A., Winking, H., and Frank, F. (1977). A hypothesis explaining the exceptional sex ratio in the wood lemming (*Myopus schisticolor*). Hereditas *85*, 101-4.

Ghosh, S., Palmer, S., Rodrigues, N., Cordell, H., Hearne, C., Cornall, R., Prins, J., McShane, P., Lathrop, G., Peterson, L., Wicker, L., and Todd, J. A. (1993). Polygenic control of autoimmune diabetes in nonobese diabetic mice. Nat Genet *4*, 404-409.

Harley, V. R., Jackson, D., Hextall, P., Hawkins, J. R., Berkowitz, G. D., Sokanathan, S., Lovell-Badge, R., and Goodfellow, P. N. (1992). DNA binding activity of recombinant SRY from normal males and XY females. Science *255*, 453-456.

Hearne, C. M., Ghosh, S., and Todd, J. A. (1992). Microsatellites for linkage analysis of genetic traits. TIG *8*, 288-294.

Herbst, E. W., Fredga, K., Frank, F., Winking, H., and Gropp, A. (1978). Cytological identification of two X-chromosome types in the wood lemming (*Myopus schisticolor*). Chromosoma *69*, 185-191.

Jacob, H. J., Lindpaintner, K., Lincoln, S. E., Kusumi, K., Bunker, R. K., Mao, Y. P., Ganten, D., Dzau, V. J., and Lander, E. S. (1991). Genetic mapping of a

gene causing hypertension in the stroke-prone spontaneously hypertensive rat. Cell *67*, 213-24.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. Nature *351*, 117-21.

Lander, E. S., Green, P., Abrahamson, J., Barlow, A., Daly, M., Lincoln, S., and Newburg, L. (1987). MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics *1*, 174-181.

Lovell-Badge, R. (1992). Testis determination: soft talk and kinky sex. Curr Opin Genet Dev *2*, 596-601.

Maraia, R., Saal, H., and Wangsa, D. (1991). A chromosome 17q de novo paracentric inversion in a patient with campomelic dysplasia; case report and etiologic hypothesis. Clin Genet *39*, 401-408.

McElreavey, K., Vilain, E., Abbas, N., Herskowitz, I., and Fellous, M. (1993). A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development. Proc Natl Acad Sci USA *90*, 3368-3372.

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

Miller, M. W., Duhl, D. M., Vrieling, H., Cordes, S. P., Ollmann, M. M., Winkes, B. M., and Barsh, G. S. (1993). Cloning of the mouse *agouti* gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. Genes Dev *7*, 454-67.

Mittwoch, U. (1989). Sex differentiation in mammals and tempo of growth: probabilities vs. switches. J Theor Biol *137*, 445-55.

Nagamine, C. M., Taketo, T., and Koo, G. C. (1987a). Morphological development of the mouse gonad in tda-1 XY sex reversal. Differentiation *33*, 214-22.

Nagamine, C. M., Taketo, T., and Koo, G. C. (1987b). Studies on the genetics of tda-1 XY sex reversal in the mouse. Differentiation *33*, 223-31.

Pagano, M., and Gauvreau, K. (1993). *Principles of Biostatistics*. Belmont, California: Duxbury Press.

Page, D. C., Mosher, R., Simpson, E. M., Fisher, E. M., Mardon, G., Pollack, J., McGillivray, B., de, l. C. A., and Brown, L. G. (1987). The sex-determining region of the human Y chromosome encodes a finger protein. Cell *51*, 1091-104.

Palmer, S. J., and Burgoyne, P. S. (1991a). 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. Development *113*, 709-14.

Palmer, S. J., and Burgoyne, P. S. (1991b). In situ analysis of fetal, prepuberal and adult XX----XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. Development *112*, 265-8.

Parkhurst, S., and Meneely, P. (1994). Sex determination and dosage compensation: lessons from flies and worms. Science *264*, 924-932.

Patek, C. E., Kerr, J., Gosden, R., Jones, K., Hardy, K., Muggleton-Harris, A., Handyside, A., Whittingham, D., and Hooper, M. L. (1991). Sex chimerism, fertility, and sex determination in the mouse. Development *113*, 311-325.

Soller, M., Padeh, B., Wysoki, M., and Ayalon, N. (1969). Cytogenetics of Saanen goats showing abnormal development of the reproductive tract associated with the dominant gene for polledness. Cytogenetics *8*, 51-67.

Taylor, B. A. (1978). Recombinant inbred strains: use in genetic mapping. *Origins of Inbred Mice*, Ed. Morse, H. New York: Academic Press,

Todd, J. A., Aitman, T. J., Cornall, R. J., Ghosh, S., Hall, J. R., Hearne, C. M., Knight, A. M., Love, J. M., McAleer, M. A., Prins, J. B., and et, a. (1991). Genetic analysis of autoimmune type 1 diabetes mellitus in mice. Nature *351*, 542-7.

Tommerup, N., Schempp, W., Meinecke, P., Pedersen, S., Bolund, L., Brandt, C., Goodpasture, C., Guldberg, P., Held, K. R., Reinwein, H., Saugstad, O. D., Scherer, G., Skjeldal, O., Toder, R., Westvik, J., Hagen, C. B. v. d., and Wolf, U. (1993). Assignment of an autosomal sex reversal locus and campomelic dysplasia to 17q24.3–q25.1. Nat Genet *4*, 170-174.

Washburn, L. L., and Eicher, E. M. (1983). Sex reversal in XY mice caused by dominant mutation on chromosome 17. Nature *303*, 338-40.

Young, I. D., Zuccollo, J. M., Maltby, E. L., and Broderick, N. J. (1992). Campomelic dysplasia associated with a de novo 2q;17q reciprocal translocation. J Med Genet *29*, 251-252.

Chapter Three
Future Directions

Will autosomal and allosomal sex-determining genes differ ?

by Jodeane Pringle

Introduction

The future experimental directions for the *tda* system I have analyzed extensively throughout this dissertation are simply summarized as follows: (1) to determine the identity of all loci which can be mapped, by positional cloning and/or syntenic relationships to cloned human genes, and (2) to extend the identical analysis presented in Chapter Two to other strain combinations, to further elucidate the allele-specificity of the system. As the techniques for such positional cloning efforts are both widely applied and thoroughly understood, instead of reviewing them here, it is of interest to speculate in an informed way as to the identity of the genes, once cloned, and to compare the hypothetical *tda* molecules to the primary sex determinant. Of course, the *tda* loci will present some unique challenges to those who attempt their positional cloning, but here I must simply refer the reader to some recent publications for information about the methods and strategies (Bultman, et al. 1992, Miller, et al. 1993). To predict the nature of a sex determinant, it is logical to start with a consideration of known sex determinants, and extrapolate from them. In mammals, the only sex determinant known at the molecular level is *Sry*, the Y-linked sex determinant. A prevailing notion is that the autosomes and the sex chromosomes cooperate in the determination of sex under the common constraint of producing fertile individuals to reproduce the next generation. However, evidence from invertebrate and mammalian systems suggests there may be a functional dichotomy between autosomal genes involved in sex determination and the allosomal sex determining genes. Given such a

dichotomy, the central question we must pose becomes this: will autosomal and allosomal sex-determining genes differ simply by virtue of their chromosomal position, and if so, how will they differ?

Evidence for a functional dichotomy

Drosophila has a paucity of autosomal sex determining genes

Some fascinating recent discoveries in the fruit fly *Drosophila melanogaster* demonstrate that in this recessive-X sex determining system, there is a relative paucity of autosomal sex determining genes, and that their nature is fundamentally different from the X-linked sex determinants (recall the Y chromosome does not participate in sex determination). One possible conclusion from the recent data is that allosomal gene function is restricted to sex determination only, whereas autosomal genes participate in other processes as well. The observations that support this notion are the following. (It may be useful to recall Figure 1–9 and Table 1–4). Some genes that participate in the primary sex determining signal were found to have functions restricted to sex determination, or sex determination and dosage compensation: *Sxl, sis-a* and *sis-c* are three such genes. Others were found that function in segmentation (*run*) or neurogenesis (*sis-b, dpn*, *da* and *emc*) as well as sex determination. Three out of five of the 'bifunctional' genes listed above are autosomal (*dpn*, *da,* and *emc*), and therefore their dosage remains constant between the sexes (Cline 1993). Although they are part of the primary sex determining signal, they must be considered separately from those numerator genes whose varying dosage sends the signal in the embryo: *sis-a,b,c,* and *run* . Cline states that the "operation of the numerator elements is necessarily related to their evolution as part of the sex signal" (Cline 1993, 389). The other components evolved in a context that "may have had little or nothing to do with sex determination *per se*" (Cline 1993, 389). Thus, the rule seems to be that the genes generating the primary sex determining signal (which are allosomal for the most

part in *Drosophila*) have restricted functions, whereas the other (autosomal) components of the system participate in multiple developmental processes. Of course, we must consider that the X-linked pro-neural gene *sis-b* and the segmentation gene *run,* represent exceptions to the rule that mulifunctional sex determining genes will be on the autosomes. Their existence on the X chromosome does not wholly invalidate the hypothesis, however, because they could represent genes recently added to the X chromosome which are in the process of losing their superfluous functions. The large number of sex determining genes on the *Drosophila* X chromosome has led at least one investigator to posit a process by which the X chromosome accumulates such genes (Hodgkin 1992). As an evaluation of this hypothesis is beyond the scope of the present work, we must simply note that the possibility of a division of labor between the sex chromosomes and autosomes in *Drosophila*, imperfect as it is, represents an intriguing hypothesis.

Mammalian autosomes may evolve in conflict with the allosomes

Another suggestion regarding possible differences between the autosomal and allosomal sex determining genes is that they may be involved in inter-genomic conflict, with the Y chromosome harboring selfish growth factors and the autosomal sex determinants being suppressors subject to parental imprinting. (Hurst 1994a, Hurst 1994b, in press). Hurst makes a cogent argument for the location of selfish fetal growth factors on the Y chromosome based on the following: (1) the conditions for the initial evolution and spread of growth promoting factors on the Y chromosome are relaxed in theory, and (2) the mammalian Y chromosome is known to carry both growth factors and multiple fetally expressed genes (Hurst 1994a, and references therein). The presence of selfish Y-linked growth factors creates the conditions for the evolution of an opposing suppressor which may be X-linked or autosomal. If autosomal, the

growth-suppressing gene must be expressed in males and not in females in order to provide the correct balance, unless the suppressor acts merely to negate the growth effects of the Y factor. In the former case parental imprinting would be required for an autosomal gene to ensure expression in males (Hurst 1994b). Suppression could also be achieved by an X-linked gene, but as Hurst points out, if the selfish growth-promoting gene is *Sry*, an X-linked suppressor could cause problems with sex determination. This is documented for the dosage-sensitive sex reversal (DSS) locus on Xp21 that was discussed in Chapter One, Section IV (Bardoni, et al. 1994), and note that the hypothesis is in general agreement with the expectations for sex-determining gene function discussed in that same Section.

The evidence for inter-genomic warfare involving *SRY* is based largely on two studies (Tucker and Lundrigan 1993, Whitfield, et al. 1993) These two groups found that both for rodents and for primates, the evolution of *SRY* sequences outside the HMG box (DNA binding) domain is evolving extremely rapidly, and concluded that either these 'flanking' domains have no functional significance or that rapid, directional selection has occurred. Hurst favors the directional selection models, noting that the ratio of non-synonymous to synonymous substitutions in the DNA sequence (K_A/K_S) observed for *SRY* in the most extreme primate (1.88) is 10 to 37 times greater than a usual figure for this parameter (Hurst 1994b, in press). Another observation in favor of directional selection is that *SRY* is virtually monomorphic within the species tested, suggesting that a single variant of *SRY* can sweep through a population, as the inter-genomic conflict hypothesis would require when a new variant of the driving suppressor arises (Whitfield, et al. 1993, Hurst 1994b). Regardless of the validity of this hypothesis, some evidence has accrued suggesting that the allosomal component of the mammalian sex determining system is evolving under constraints which are very different than those encountered by all autosomal components not involved in inter-genomic conflict. Of course, the

autosomal genes which are hypothetically involved must be co-evolving with the allosomes. Hence it seems reasonable that the autosomal genes will have very different molecular identities contingent upon their divergent evolutionary histories.

Autosomal sex determining genes encounter a unique selective environment

Given the kind of functional dichotomy proposed above, we must ask how the system evolved in order to predict the consequences for modern-day genes. To understand these evolutionary hypotheses, it is essential to consider the unique selective environment that autosomal and allosomal sex-determining genes encounter. Probably the most consequential difference is that the proportion of all alleles that reside in a functionally selective environment differs between the two types of genes; for instance, autosomal female-determining genes are not subject to selection in a male, except that they must not express their sex-determining function, and *vice versa*. Because half of all alleles of an autosomal sex-determining gene reside in females and half in males in each generation, not all are being selected for their sex determining function at all times. On the contrary, all functional alleles of the primary testis determinant reside in males under dominant-Y heterogamety. Of course, X-linked loci differ according to whether a functional Y homolog is present. If so, half are in males and half in females as for an autosomal locus. If not, two-thirds are in females and one-third in males. It was this type of reasoning that led Hurst to suggest that the X chromosome might be a likely location for a growth-suppressor locus, but he also noted the great excess of autosomes over X chromosomes, indicating the latter as the most likely location. It is not difficult to imagine that under this type of selection, an autosomal sex determining gene in a female which acquired a mutation that led to an improved non-sex related function with a concomitant decrease in fitness due to decreased male fertility, might segregate amongst females long enough for a compensatory

mutation to occur and correct the decreased male fertility. This is just one scenario by which the autosomal sex determining genes may diversify in comparison with the allosomal sex determining genes due to their unique selective environment.

Two possibilities for the evolution of the dichotomy

It seems plausible that this kind of selection might result in sex chromosome-linked sex determining genes having functions limited to sex determination only, compared to autosomal genes which are able to retain other functions or evolve novel, or related functions more rapidly, as hypothesized above. This notion also fits with the general expectation that allosomal sex determining genes must be specific regulators of the primary sex determining signal, whereas autosomal genes are more likely to be downstream regulators or effectors (and hence more likely to be multifunctional). But what of the evolutionary history of such systems? Could they evolve by known mechanisms from the hypothetical ancestors of modern vertebrates? One plausible model assumes that one of a collection of multiple, multifunctional genes which happen to be involved in sex determination is randomly selected to become the primary sex determinant of the proto-allosomes, when the sex chromosomes begin to differentiate. This model is supported by the great diversity of sex determining mechanisms of modern vertebrates, since the initial selection process is expected to be random in such a system (Graves and Schmidt 1992). Another model posits two primitive classes of sex determining genes, specific regulators and multifunctional effectors, with the former selected as the primary allosomal sex determinant. This system would be expected to lead to a more restricted set of modern mechanisms, barring subsequent diversification. One last possibility must be mentioned before we leave the topic of selective environments behind. That is the hypothesis that autosomal sex determining genes diversify not simply because they *can*, but because they *must*. Of course, one rationale here is that they are

involved in inter-genomic conflict. Another rationale is that they must have another function to preserve them from random forces which might eliminate them if they were too long in a non-selective environment. All types of selective pressure seem equally feasible, and indeed, all may function simultaneously in a given species.

In any event, both schemes result in multifunctional autosomal sex determining genes with specific regulators as the primary sex determinants. Under the first model, the allosomal genes progressively lose functions unrelated to sex determination, while autosomal genes retain previous functions and perhaps evolve new functions more readily due to the unique selective forces that act upon them (discussed above). There is good evidence for the loss of gene function from one member of a heteromorphic pair of sex chromosomes . Under the second model, the allosomal genes never had multiple functions, but the autosomal genes may evolve new functions and retain old functions as above. Whatever the mechanism, the end-result of such processes fits the *Drosophila* data, with one small modification, and may well apply to vertebrates such as mouse. The modification, that multifunctional autosomal sex determining genes may accumulate on the X chromosome of *Drosophila* after its differentiation, appears to be an example of the process alluded to in Section III of Chapter One, in which translocations to the allosomes may leave an organism rather depleted of autosomal sex determining genes. This process may be unique to organisms with *Drosophila*'s recessive X system of male heterogametic sex determination . In conclusion, I refer the reader to **Table 3–1** which summarizes the foregoing arguments; namely, that reasonable predictions for the difference between allosomal and autosomal sex determining genes are that the latter will be involved in other, or more, developmental processes than the former, and may be subject to parental imprinting.

Conclusion

Table 3–1. Summarized properties of SRY in comparison to those of hypothetical *tda* loci

PROPERTIES	*SRY*	*HYPOTHETICAL TDA*
Chromosomal Location	Y Chromosome	Autosomes
Mutant (Variant) Phenotype	XY Sex reversal	XY Sex reversal
Genetic Action	Testis Activating	?Testis Activating ?Suppressing
Molecular identity	DNA-binding protein	?
Molecular action(s)	?Promote fetal growth ?Activate male determinants ?Repress female determinants	?Suppress fetal growth ?Imprinted
Function(s)	Sex determination (?Through growth effect)	Sex Determination ?Multiple others

References

Bardoni, B., Zanaria, E., Guioli, S., Floridia, G., Worley, K. C., Tonini, G., Ferrante, E., Chiumello, G., McCabe, E. R. B., Fraccaro, M., Zuffardi, O., and Camerino, G. (1994). A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. Nat Genet *7*, 497-501.

Bultman, S. J., Michaud, E. J., and Woychik, R. P. (1992). Molecular characterization of the mouse *agouti* locus. Cell *71*, 1195-204.

Cline, T. (1993). The *Drosophila* sex determination signal: how do flies count to two? TIG *9*, 385-390.

Graves, J. A., and Schmidt, M. M. (1992). Mammalian sex chromosomes: design or accident? Curr Opin Genet Dev *2*, 890-901.

Hodgkin, J. (1992). Genetic sex determination, mechanisms and evolution. Bioessays *14*, 253-261.

Hurst, L. D. (1994a). Embryonic growth and the evolution of the mammalian Y chromosome. I. The Y as an attractor for selfish growth factors. Heredity (in press).

Hurst, L. D. (1994b). Embryonic growth and the evolution of the mammalian Y chromosome. II. Suppression of selfish Y-linked growth factors may explain escape from X-inactivation and rapid evolution of *Sry*. Heredity (in press).

Miller, M. W., Duhl, D. M., Vrieling, H., Cordes, S. P., Ollmann, M. M., Winkes, B. M., and Barsh, G. S. (1993). Cloning of the mouse *agouti* gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. Genes Dev *7*, 454-67.

Tucker, P. K., and Lundrigan, B. L. (1993). Rapid evolution of the sex determining locus in Old World mice and rats. Nature *364*, 715-7.

Whitfield, L. S., Lovell-Badge, R., and Goodfellow, P. N. (1993). Rapid sequence evolution of the mammalian sex-determining gene SRY. Nature *364*, 713-5.

Appendix

Genotype data from a preliminary study of Y^{POS} sex reversal in the mouse

by Jodeane Pringle and Xiaoling Xu

The following appendix contains genotype data collected for the intraspecific backcross described in Chapter Two between January and December of 1992, which represent a subset of the data upon which the conclusions in that chapter are based. C57BL6/J-derived alleles are abbreviated B and DBA/2J alleles are abbreviated D. Only the maternally-derived chromosome is shown since the paternal chromosome is uniformly B6-derived. Phenotypes are abbreviated as follows: H, hermaphrodite; M, male; MA, abnormal male (see Chapter Two for a complete description of the phenotypic categories.)

Appendix

PHENOTYPE	MA	M	MA	M	MA	H	M	MA	M	MA	M
EMBRYO	3	1	6	11	8	14	15	32	20	47	21
PANEL I NUMBER	1	2	3	4	5	6	7	8	9	10	11
PANEL II NUMBER											
PANEL III NUMBER						1					
D1MIT1 L33	B	B	D	B	B	D	B	D	.	.	D
D1MIT5 L20	D	D	B	B	B	D	.	D	.	B	.
D1MIT7 A80	D	D	B	B	B	D	B	D	D	B	D
D1MIT11 M17	D	D	B	B	B	D	B	B	D	B	D
D1MIT54 B533	D	D	B	.	B	D	.	B	D	B	D
D1MIT30 P100	D	.	B	B	B	D	.	B	D	B	D
D1MIT16 L46	D	D	B	B	B	D	B	B	D	D	D
D1MIT17 M41	B	D	D	D	B	D	B	B	D	.	D
D2MIT1 M128	D	B	B	B	D	B	B	B	D	D	D
D2MIT6 L18	D	B	.	B	.	B	B	B	D	D	B
D2MIT7 L44	D	D	B	B	D	B	B	D	D	D	B
D2MIT9 M85	D	D	B	B	D	.	.	D	D	D	B
D2MIT10 M39	D	D	B	B	D	D	B	D	B	D	B
D2NDS1 T19	D	D	B	B	D	D	B	D	B	D	B
D2MIT13 M179	D	D	B	B	D	.	B	D	B	D	B
D2MIT12 M130	D	D	B	B	D	D	B	D	B	D	B
D2MIT17 M246	D	D	B	B	B	D	B	D	B	B	B
D2MIT21 M184	D	D	B	B	B	D	B	D	B	B	B
D2MIT53 B342	D	D	B	.	B	D	B	D	B	B	D
D3MIT54 B572	D	.	B	.	D	B	B	D	D	D	B
D3MIT21 D31	.	B	.	B	D	D	B	D	D	D	D
D3MIT6 M149	B	B	B	B	D	D	B	D	D	D	D
D3MIT22 D122	B	B	B	B	D	.	B	D	D	D	D
D3MIT10 M34	B	D	B	B	D	D	B	B	D	B	D
D3MIT43 B391	B	D	B	.	.	D	B	B	B	B	D
D3MIT17 M235	B	D	B	B	D	D	B	B	B	B	D
D3MIT19 M141	B	D	B	B	D	D	D	B	B	B	B
D9MIT4 M151	.	.	B	B	D	.	.	B	B	B	B
D9MIT21 D15	D	B	B	B	D	.	B	B	B	B	B
D9MIT8 M211	D	B	B	B	D	.	B	B	B	B	D
D9MIT35 B257	D	B	B	B	D	B	B	B	B	B	D
D9MIT12 M73	D	B	B	B	D	B	B	B	B	B	D
D9MIT20 L64	.	B	B	B	D	B	B	B	B	B	D
D9MIT19 M157	B	B	B	B	D	B	B	B	D	B	D

PHENOTYPE	M	MA	M	M	M	M	H	M	M	H	M
EMBRYO	22	51	23	25	33	34	35	37	38	39	40
PANEL I NUMBER	12	13	14	15	16	18	20	21	23	24	25
PANEL II NUMBER											
PANEL III NUMBER		4					2			3	
D1MIT1 L33	B	B	D	B	B	.	B	D	B	B	B
D1MIT5 L20	B	B	D	D	B	D	B	B	D	B	B
D1MIT7 A80	B	B	D	D	B	B	D	D	B	B	B
D1MIT11 M17	B	B	D	D	B	B	D	D	B	B	B
D1MIT54 B533	B	B	D	B	B	B	D	D	B	B	B
D1MIT30 P100	B	B	B	B	B	B	D	D	B	B	B
D1MIT16 L46	B	B	B	B	B	D	D	D	B	B	B
D1MIT17 M41	B	B	B	B	B	D	D	D	.	.	B
D2MIT1 M128	B	D	B	D	D	B	D	B	B	D	B
D2MIT6 L18	B	D	B	D	D	B	D	D	B	B	B
D2MIT7 L44	D	D	B	D	D	B	D	D	.	B	B
D2MIT9 M85	D	D	B	D	D	D	B	D	B	B	B
D2MIT10 M39	D	B	B	D	D	D	D	D	B	B	B
D2NDS1 T19	D	B	B	D	D	D	D	D	D	B	B
D2MIT13 M179	D	B	B	D	D	D	D	D	D	D	B
D2MIT12 M130	D	B	B	D	D	D	D	D	D	D	B
D2MIT17 M246	D	B	B	D	D	D	D	D	D	D	B
D2MIT21 M184	D	B	B	D	D	.	D	D	D	D	B
D2MIT53 B342	D	B	B	D	D	D	D	B	D	D	B
D3MIT54 B572	D	D	D	D	D	B	B	D	D	D	B
D3MIT21 D31	D	D	B	D	D	B	B	D	.	B	B
D3MIT6 M149	D	D	B	D	D	B	B	D	D	B	B
D3MIT22 D122	D	.	B	D	D	D	D	D	D	B	B
D3MIT10 M34	D	D	B	D	D	D	D	D	D	B	B
D3MIT43 B391	D	D	D	D	D	D	D	D	D	B	B
D3MIT17 M235	D	.	D	D	D	.	B	D	D	B	B
D3MIT19 M141	D	D	D	D	D	B	B	D	D	B	B
D9MIT4 M151	D	.	D	B	D	B	B	B	B	D	D
D9MIT21 D15	D	.	D	B	D	B	B	B	B	D	D
D9MIT8 M211	D	.	D	B	D	B	B	B	B	.	D
D9MIT35 B257	B	B	D	B	D	B	D	D	B	D	D
D9MIT12 M73	B	B	D	B	D	B	D	D	B	D	D
D9MIT20 L64	B	B	D	.	D	B	.	D	B	D	D
D9MIT19 M157	B	B	D	B	D	B	D	D	D	D	D

Appendix

PHENOTYPE	M	H	H	H	M	M	M	M	MA	MA	H
EMBRYO	54	59	62	64	65	66	67	75	92	103	106
PANEL I NUMBER	27	28	29	30	32	34	35	37	17	19	38
PANEL II NUMBER									1	2	3
PANEL III NUMBER		5	6	7					8	9	10
D1MIT1 L33	B	B	D	B	D	B	B	D	B	B	B
D1MIT5 L20	D	B	D	B	D	B	B	B	D	B	B
D1MIT7 A80	D	B	B	B	D	B	.	D	D	B	B
D1MIT11 M17	D	B	B	.	D	B	B	D	D	B	B
D1MIT54 B533	D	B	B	D	D	B	B	D	D	B	B
D1MIT30 P100	D	B	B	D	.	B	B	D	D	B	D
D1MIT16 L46	D	B	B	D	D	B	B	D	B	B	D
D1MIT17 M41	B	B	D	B	D	B	B	D	B	B	D
D2MIT1 M128	B	D	B	D	D	D	D	B	B	D	D
D2MIT6 L18	B	D	D	D	D	D	D	B	B	D	D
D2MIT7 L44	B	.	D	B	D	D	D	B	B	D	D
D2MIT9 M85	.	D	D	B	D	D	B	B	B	D	D
D2MIT10 M39	B	D	D	B	D	D	B	B	B	D	D
D2NDS1 T19	B	D	D	.	D	D	B	B	B	.	D
D2MIT13 M179	B	D	D	B	D	D	B	B	B	D	D
D2MIT12 M130	B	D	B	B	D	D	B	B	B	D	D
D2MIT17 M246	B	D	B	B	D	B	B	B	B	D	D
D2MIT21 M184	B	B	B	B	D	B	B	B	D	B	D
D2MIT53 B342	B	B	D	B	.	B	B	B	D	B	D
D3MIT54 B572	B	B	D	B	D	B	D	D	.	D	D
D3MIT21 D31	B	B	D	B	D	B	D	D	B	D	B
D3MIT6 M149	D	B	D	B	D	B	D	D	B	B	B
D3MIT22 D122	D	B	D	B	D	B	D	D	B	B	B
D3MIT10 M34	D	B	D	B	D	B	B	D	B	B	B
D3MIT43 B391	D	B	D	B	B	D	B	D	B	B	B
D3MIT17 M235	D	D	D	B	B	D	B	D	B	B	B
D3MIT19 M141	D	D	D	B	B	D	B	D	B	B	B
D9MIT4 M151	B	B	D	B	D	B	B	D	B	D	D
D9MIT21 D15	B	B	D	B	D	B	B	D	B	D	D
D9MIT8 M211	D	B	D	B	D	B	B	D	B	D	D
D9MIT35 B257	D	B	D	B	D	B	D	D	B	B	B
D9MIT12 M73	D	B	D	B	B	B	D	D	B	B	B
D9MIT20 L64	.	B	.	B	B	B	D	D	B	B	B
D9MIT19 M157	B	B	D	B	D	D	D	D	B	B	B

Appendix

PHENOTYPE	H	MA	H	H	H	H	MA	MA	MA	MA	MA
EMBRYO	111	118	137	169	170	179	182	191	199	212	W1
PANEL I NUMBER	39	22	41	42	43	44	26	31	33	36	40
PANEL II NUMBER	4	5	6	7	8	9	10	11	12	13	15
PANEL III NUMBER		12	13	14	15	16		17		18	20
D1MIT1 L33	B	D	D	D	B	B	D	D	D	D	B
D1MIT5 L20	B	D	D	D	B	B	D	D	D	D	B
D1MIT7 A80	B	B	D	D	B	B	D	D	D	D	B
D1MIT11 M17	B	B	.	D	B	B	D	D	D	D	B
D1MIT54 B533	B	B	D	D	D	D	.	D	D	D	B
D1MIT30 P100	B	D	D	B	D	D	.	D	D	D	B
D1MIT16 L46	B	D	D	B	D	D	D	D	D	.	B
D1MIT17 M41	D	D	B	B	D	B	D	D	B	D	B
D2MIT1 M128	B	B	D	B	D	D	.	D	B	B	B
D2MIT6 L18	B	.	B	B	D	D	B	D	B	B	B
D2MIT7 L44	B	B	B	B	B	B	B	D	B	B	D
D2MIT9 M85	B	B	B	B	B	B	B	D	B	B	D
D2MIT10 M39	D	B	B	B	B	B	.	D	B	B	D
D2NDS1 T19	D	B	B	D	B	B	B	B	D	B	D
D2MIT13 M179	D	B	B	D	B	B	B	B	D	B	D
D2MIT12 M130	D	B	B	D	B	B	B	B	D	B	D
D2MIT17 M246	D	D	B	D	B	B	B	B	D	B	D
D2MIT21 M184	D	D	B	D	B	.	B	B	D	B	D
D2MIT53 B342	D	D	B	B	B	D	.	B	D	B	D
D3MIT54 B572	B	B	D	D	B	B	B	B	D	D	B
D3MIT21 D31	B	B	D	D	B	B	B	B	D	D	B
D3MIT6 M149	D	B	D	B	B	.	B	B	D	D	B
D3MIT22 D122	D	B	D	B	B	D	B	B	B	D	B
D3MIT10 M34	D	B	B	B	B	D	B	B	B	D	B
D3MIT43 B391	D	B	B	B	B	D	.	B	D	D	B
D3MIT17 M235	D	B	B	B	B	D	B	B	D	D	B
D3MIT19 M141	B	D	B	D	D	D	B	B	D	D	B
D9MIT4 M151	.	B	B	B	B	B	D	B	B	B	B
D9MIT21 D15	D	B	B	B	B	B	D	B	B	B	B
D9MIT8 M211	D	B	B	B	B	D	D	B	B	B	B
D9MIT35 B257	B	B	B	B	B	D	D	B	D	B	B
D9MIT12 M73	B	B	B	B	B	D	D	B	D	B	B
D9MIT20 L64	B	D	B	B	B	D	D	B	D	B	B
D9MIT19 M157	B	D	B	B	B	D	D	B	D	D	B

PHENOTYPE	MA	M	MA	M	MA	H	M	MA	M	MA	M
EMBRYO	3	1	6	11	8	14	15	32	20	47	21
PANEL I NUMBER	1	2	3	4	5	6	7	8	9	10	11
PANEL II NUMBER											
PANEL III NUMBER						1					
D4MIT17 D1	B	B	D	B	D	D	D	B	D	D	B
D4MIT9 M241	B	B	D	B	B	D	D	B	D	D	B
D4MIT12 M15	B	D	D	D	B	D	D	B	B	D	D
D4MIT16 A65	B	D	D	D	B	D	D	D	B	D	D
D4MIT14 A69	D	D	D	D	B	B	D	D	B	B	D
D4MIT13 M169	B	D	D	D	D	B	D	D	B	B	D
D4MIT42 J5	D	D	D	D	B	B	D	D	B	B	D
D4SMH6B	D	D	D	D	B	B	D	D	B	B	D
DVL	D	D	D	D	B	B	D	D	B	B	D
TELQ4	D	D	D	D	B	B	D	D	B	B	D
D5MIT1 A82	B	D	B	D	D	D	D	D	B	D	D
D5MIT11 M97	B	D	B	D	D	D	D	D	B	B	D
D5MIT55 A1106											
D5MIT15 B223											
D5MIT12 D128	B	D	B	D	D	B	D	D	.	B	D
D5MIT7 M154	.	D	B	D	D	B	D	D	B	B	D
D5MIT10 M207	B	D	B	D	D	B	D	D	B	B	D
D5MIT41 B247											
D5MIT25 B147											
D5MIT65 B560											
D6MIT50 B497											
D6MIT33 A1094											
D6MIT16 D11											
D6MIT9 L23											
D6MIT31 A718											
D6MIT23 B385											
D6MIT13 D34											
D6MIT14 M190											
D7MIT21 A771											
D7MIT57 D515											
D7NDS5 T62											
D7MIT30 B175											
D7MIT40 B326											
D7MIT12 M23											

PHENOTYPE	M	MA	M	M	M	M	H	M	M	H	M
EMBRYO	22	51	23	25	33	34	35	37	38	39	40
PANEL I NUMBER	12	13	14	15	16	18	20	21	23	24	25
PANEL II NUMBER											
PANEL III NUMBER		4					2			3	
D4MIT17 D1	B	D	D	B	D	B	B	B	B	B	D
D4MIT9 M241	B	D	D	B	D	B	B	B	B	B	D
D4MIT12 M15	B	D	D	B	B	B	B	B	B	B	D
D4MIT16 A65	B	D	D	B	B	B	B	B	B	B	D
D4MIT14 A69	B	D	B	B	B	B	B	D	B	B	D
D4MIT13 M169	B	D	B	B	B	B	.	D	D	B	D
D4MIT42 J5	B	D	B	B	B	B	B	D	B	B	D
D4SMH6B	B	D	B	B	B	B	B	D	B	B	D
DVL	B	D	B	B	B	B	B	D	B	B	D
TELQ4	B	D	B	B	B	B	B	D	B	B	D
D5MIT1 A82	B	B	D	B	B	D	.	B	B	B	D
D5MIT11 M97	B	B	D	B	B	D	B	D	.	B	D
D5MIT55 A1106											
D5MIT15 B223											
D5MIT12 D128	B	D	D	B	B	D	B	D	B	B	D
D5MIT7 M154	B	D	D	B	B	D	B	D	.	B	D
D5MIT10 M207	B	D	D	B	B	D	B	D	B	B	D
D5MIT41 B247											
D5MIT25 B147											
D5MIT65 B560											
D6MIT50 B497											
D6MIT33 A1094											
D6MIT16 D11											
D6MIT9 L23											
D6MIT31 A718											
D6MIT23 B385											
D6MIT13 D34											
D6MIT14 M190											
D7MIT21 A771											
D7MIT57 D515											
D7NDS5 T62											
D7MIT30 B175											
D7MIT40 B326											
D7MIT12 M23											

PHENOTYPE	M	H	H	H	M	M	M	M	MA	MA	H
EMBRYO	54	59	62	64	65	66	67	75	92	103	106
PANEL I NUMBER	27	28	29	30	32	34	35	37	17	19	38
PANEL II NUMBER									1	2	3
PANEL III NUMBER		5	6	7					8	9	10
D4MIT17 D1	B	B	B	D	B	D	D	D	B	D	D
D4MIT9 M241	D	.	.	B	B	D	D	B	B	D	B
D4MIT12 M15	D	B	B	B	B	D	D	D	B	D	B
D4MIT16 A65	D	B	B	B	B	D	D	D	B	D	B
D4MIT14 A69	D	B	B	B	D	D	D	D	D	D	B
D4MIT13 M169	D	B	B	B	D	D	D	D	D	B	B
D4MIT42 J5	D	B	B	B	D	D	D	D	D	D	B
D4SMH6B	D	B	B	B	D	D	B	D	D	D	B
DVL	D	B	B	B	D	D	B	D	D	D	B
TELQ4	D	B	B	.	D	D	B	D	D	D	B
D5MIT1 A82	D	B	B	B	B	B	D	D	B	B	B
D5MIT11 M97	D	B	B	B	D	B	D	D	B	B	B
D5MIT55 A1106									B	B	B
D5MIT15 B223									B	B	B
D5MIT12 D128	D	B	B	B	D	D	B	B	.	B	B
D5MIT7 M154	D	B	B	B	D	D	B	B	D	.	B
D5MIT10 M207	D	B	B	B	D	D	B	B	D	D	B
D5MIT41 B247									D	D	B
D5MIT25 B147									D	D	B
D5MIT65 B560									D	D	B
D6MIT50 B497									B	D	D
D6MIT33 A1094									B	D	D
D6MIT16 D11									B	D	.
D6MIT9 L23									B	B	D
D6MIT31 A718									B	.	.
D6MIT23 B385									B	B	B
D6MIT13 D34									B	.	B
D6MIT14 M190									B	B	B
D7MIT21 A771									B	B	B
D7MIT57 D515									B	B	D
D7NDS5 T62									B	D	D
D7MIT30 B175									D	D	D
D7MIT40 B326									D	D	B
D7MIT12 M23									D	D	B

Appendix

PHENOTYPE	H	MA	H	H	H	H	MA	MA	MA	MA	H
EMBRYO	111	118	137	169	170	179	182	191	199	212	217
PANEL I NUMBER	39	22	41	42	43	44	26	31	33	36	
PANEL II NUMBER	4	5	6	7	8	9	10	11	12	13	14
PANEL III NUMBER		12	13	14	15	16		17		18	19
D4MIT17 D1	B	B	D	D	D	D	B	B	B	D	
D4MIT9 M241	B	D	D	D	D	D	B	B	B	D	
D4MIT12 M15	B	D	D	B	B	B	B	B	B	D	
D4MIT16 A65	B	D	D	B	B	B	B	B	B	D	
D4MIT14 A69	B	B	D	B	B	B	B	B	B	.	.
D4MIT13 M169	B	B	D	B	B	B	B	B	B	D	B
D4MIT42 J5	B	B	D	B	B	B	B	B	B	D	.
D4SMH6B	B	B	B	B	B	B	B	B	B	D	B
DVL	B	B	B	B	B	B	B	B	B	D	B
TELQ4	B	B	B	B	B	B	B	.	B	B	.
D5MIT1 A82	B	B	D	B	B	D	D	D	D	B	B
D5MIT11 M97	B	B	B	B	B	B	B	D	D	B	
D5MIT55 A1106	B	B	B	B	B	B	B	D	D	B	B
D5MIT15 B223	B	.	B	B	B	B	B	D	D	B	B
D5MIT12 D128	B	B	B	B	B	B	.	B	D	B	B
D5MIT7 M154	B	.	B	B	B	B	B	B	D	B	
D5MIT10 M207	B	B	B	B	B	B	B	B	D	B	
D5MIT41 B247	B	B	B	B	B	B	B	B	D	B	B
D5MIT25 B147	B	B	B	B	D	B	B	B	D	B	B
D5MIT65 B560	B	D	B	B	D	B	B	B	D	B	B
D6MIT50 B497	D	B	D	B	B	D	B	B	B	B	B
D6MIT33 A1094	B	B	D	D	B	D	B	B	B	B	B
D6MIT16 D11	.	B	D	.	D	D	B	B	B	B	D
D6MIT9 L23	B	B	D	D	D	D	B	B	B	B	D
D6MIT31 A718	B	B	D	D	D	D	B	B	B	B	D
D6MIT23 B385	B	B	D	D	D	D	B	B	B	B	D
D6MIT13 D34	B	D	B	B	D	D	B	B	B	D	D
D6MIT14 M190	D	D	B	B	D	D	B	B	B	D	D
D7MIT21 A771	B	B	D	B	D	B	B	B	B	D	D
D7MIT57 D515	B	B	D	B	D	B	B	B	B	D	D
D7NDS5 T62	D	B	D	B	D	B	B	B	B	D	D
D7MIT30 B175	D	B	D	D	D	B	B	D	B	D	D
D7MIT40 B326	B	D	D	D	D	B	B	D	B	D	B
D7MIT12 M23	B	D	B	D	D	D	B	D	B	D	B

Appendix

PHENOTYPE	MA	H	MA	MA	H	MA	MA	H	H	H	MA
EMBRYO	W1	W2	W3	W4	W5	304	319	358	362	377	381
PANEL I NUMBER	40										
PANEL II NUMBER	15	16	17	18	19	20	21	22	23	24	25
PANEL III NUMBER	20	21	22	23	24	25	26	27	28	29	30
D4MIT17 D1	D										
D4MIT9 M241	D										
D4MIT12 M15	D										
D4MIT16 A65	D										
D4MIT14 A69	B	.	.	B	.	B	B	B	B	B	B
D4MIT13 M169	B	B	D	B	B	B	B	B	B	B	B
D4MIT42 J5	B	B	D	B	B	B	B	B	B	B	B
D4SMH6B	B	B	D	B	B	B	B	B	B	B	B
DVL	B	B	D	B	B	B	B	B	B	B	B
TELQ4	B	B	D	B	B	B	B	B	D	D	B
D5MIT1 A82	D	B	D	B	D	D	B	D	B	B	B
D5MIT11 M97	D										
D5MIT55 A1106	D	B	D	B	B	B	B	D	D	B	B
D5MIT15 B223	B	B	D	B	B	B	B	D	D	B	B
D5MIT12 D128	B	B	D	B	B	B	B	D	D	B	B
D5MIT7 M154	B										
D5MIT10 M207	B										
D5MIT41 B247	B	B	D	B	B	B	B	D	D	B	B
D5MIT25 B147	B	B	D	B	B	B	B	D	D	B	B
D5MIT65 B560	B	B	D	B	B	B	B	D	D	B	B
D6MIT50 B497	B	D	D	B	B	D	D	B	B	B	D
D6MIT33 A1094	B	B	D	B	B	B	D	B	B	B	B
D6MIT16 D11	B	B	D	B	.	B	D	B	D	B	B
D6MIT9 L23	B	B	D	B	B	B	D	B	D	B	B
D6MIT31 A718	B	B	B	B	B	B	B	B	D	B	B
D6MIT23 B385	B	B	B	B	B	B	B	B	D	D	B
D6MIT13 D34	B	B	B	B	B	B	B	B	D	D	B
D6MIT14 M190	B	B	B	B	B	B	B	D	D	D	B
D7MIT21 A771	B	D	D	B	D	B	B	B	D	D	D
D7MIT57 D515	B	D	D	B	D	B	D	B	D	D	D
D7NDS5 T62	D	.	D	B	D	B	D	D	D	D	D
D7MIT30 B175	D	D	D	B	D	D	D	D	D	D	D
D7MIT40 B326	D	D	D	B	D	D	D	D	D	D	D
D7MIT12 M23	D	D	B	B	D	D	D	D	D	D	D

PHENOTYPE	MA	H	MA	H	H	H	MA	H	MA	MA	H
EMBRYO	382	390	399	423	431	436	438	440	446	449	451
PANEL I NUMBER											
PANEL II NUMBER	26	27	28	29	30	31	32	33	34	35	36
PANEL III NUMBER	31	32	33								
D4MIT17 D1											
D4MIT9 M241											
D4MIT12 M15											
D4MIT16 A65											
D4MIT14 A69	B	B	B	B	B	B	B	B	B	B	B
D4MIT13 M169	B	B	B	B	B	B	B	B	B	B	B
D4MIT42 J5	B	B	B	B	B	B	B	B	B	B	B
D4SMH6B	B	B	B	B	B	B	B	B	B	B	B
DVL	B	B	B	B	B	B	B	B	B	B	B
TELQ4	B	B	B	.	.	.	.	.	.	.	.
D5MIT1 A82	D	D	B	.	D	D	B	D	B	D	B
D5MIT11 M97											
D5MIT55 A1106	D	D	B	B	D	D	B	D	B	D	B
D5MIT15 B223	D	D	B	B	D	D	B	D	B	D	B
D5MIT12 D128	D	D	B	B	D	D	B	D	B	D	B
D5MIT7 M154											
D5MIT10 M207											
D5MIT41 B247	D	D	B	B	D	D	D	D	B	D	B
D5MIT25 B147	D	D	B	B	D	D	D	D	B	D	B
D5MIT65 B560	D	D	B	B	D	D	D	D	B	D	B
D6MIT50 B497	B	B	D	D	B	D	B	B	B	B	B
D6MIT33 A1094	B	B	D	D	B	D	B	B	B	B	B
D6MIT16 D11	B	B	D	D	B	B	B	B	B	B	B
D6MIT9 L23	B	B	D	D	B	B	B	B	B	B	B
D6MIT31 A718	B	B	D	D	B	B	B	B	B	B	B
D6MIT23 B385	B	B	B	B	B	D	B	B	B	B	B
D6MIT13 D34	B	B	D	B	B	D	B	B	B	B	B
D6MIT14 M190	.	B	B	B	D	D	D	B	B	D	B
D7MIT21 A771	B	B	D	B	B	D	B	D	B	B	D
D7MIT57 D515	B	D	D	B	B	D	B	D	B	B	D
D7NDS5 T62	B	D	D	B	D	D	B	B	B	B	D
D7MIT30 B175	D	D	B	B	D	D	B	B	B	D	B
D7MIT40 B326	D	D	B	B	D	B	B	B	B	B	B
D7MIT12 M23	D	D	B	B	D	B	D	B	B	B	B

PHENOTYPE	MA	MA	MA	H	MA	MA	H	F
EMBRYO	452	469	470	480	W6	491	499	110
PANEL I NUMBER								
PANEL II NUMBER	37	38	39	40	41	42	43	
PANEL III NUMBER								11
D4MIT17 D1								
D4MIT9 M241								
D4MIT12 M15								
D4MIT16 A65								
D4MIT14 A69	B	B	B	B	.	.	.	
D4MIT13 M169	D	B	B	B	.	.	.	
D4MIT42 J5	D	B	B	B	.	.	.	
D4SMH6B	D	B	B	.	.	.	.	
DVL	D	B	B	B	.	.	.	
TELQ4	.	.	.	.	.	.	.	
D5MIT1 A82	B	B	B	B	D	B	D	n
D5MIT11 M97								
D5MIT55 A1106	B	B	B	B	D	D	D	
D5MIT15 B223	B	B	B	B	B	D	B	n
D5MIT12 D128	B	B	B	B	B	D	B	n
D5MIT7 M154								
D5MIT10 M207								
D5MIT41 B247	B	B	B	B	B	D	B	
D5MIT25 B147	B	B	B	B	B	D	B	
D5MIT65 B560	B	B	B	B	B	B	B	
D6MIT50 B497	D	B	D	B	B	B	D	
D6MIT33 A1094	D	B	D	B	B	B	D	
D6MIT16 D11	D	B	D	B	B	B	D	
D6MIT9 L23	D	D	D	B	B	D	B	
D6MIT31 A718	D	D	D	B	B	D	B	
D6MIT23 B385	B	D	D	B	B	D	B	
D6MIT13 D34	B	.	D	B	B	B	B	
D6MIT14 M190	B	D	D	B	B	B	B	
D7MIT21 A771	D	.	B	B	B	B	B	
D7MIT57 D515	D	B	B	B	B	B	B	
D7NDS5 T62	D	D	B	B	D	D	B	
D7MIT30 B175	D	D	D	B	D	D	B	
D7MIT40 B326	D	D	D	D	B	D	B	
D7MIT12 M23	D	D	B	D	B	D	D	

PHENOTYPE	MA	MA	H	H	MA	H	H	H	H	MA	MA
EMBRYO	92	103	106	111	118	137	169	170	179	182	191
PANEL I NUMBER	17	19	38	39	22	41	42	43	44	26	31
PANEL II NUMBER	1	2	3	4	5	6	7	8	9	10	11
PANEL III NUMBER	8	9	10		12	13	14	15	16		17
D8MIT24 A737	B	D	D	B	B	B	D	B	B	B	D
D8MIT8 M257	B	D	D	D	B	B	D	B	B	B	B
D8MIT41 B171	B	D	D	D	B	B	B	B	B	D	B
D8MIT47 B591	B	D	D	B	B	B	B	B	B	D	B
D8MIT42 A754	B	D	B	B	D	.	B	B	B	D	B
D10MIT3 A114	B	B	B	D	B	B	D	D	D	D	D
D10MIT40 B184	D	B	B	D	B	B	D	D	D	D	D
D10MIT42 B484	D	B	B	D	B	D	B	D	D	D	B
D10MIT10 M7	D	B	B	D	B	D	B	D	D	D	B
D10NDS2 T32	D	B	B	D	B	D	B	D	D	D	B
D10MIT14 M175	D	B	B	D	D	D	B	D	D	D	B
D11MIT63 B675	D	D	D	D	B	B	D	D	D	B	B
D11 MIT53 D548	D	D	D	D	B	B	D	D	D	B	B
D11MIT20 A755	D	D	D	D	B	B	D	B	D	D	B
D11MIT4 A124	D	D	D	D	B	B	D	B	D	D	B
D11MIT41 B279	D	D	D	D	D	B	D	B	D	D	B
D11MIT14 D2	D	D	D	B	D	B	D	B	B	D	B
D11MIT48 B121	D	D	D	D	D	B	B	B	B	B	D
D12MIT38 D135	D	D	B	D	B	D	D	D	B	B	D
D12MIT46 P82	B	D	B	B	D	D	B	B	D	B	B
D12MIT3 L41	D	D	B	D	B	D	B	B	D	D	D
D12MIT12 B269	B	D	D	B	D	D	B	B	D	B	B
D12MIT2 M27	B	D	B	B	D	D	B	B	D	B	B
D12MIT36 B297	B	D	B	B	B	D	B	B	D	B	B
D12MIT34 B176	B	D	B	D	B	D	B	B	D	B	D
D12MIT4 A64	D	D	B	D	B	D	B	B	D	D	D
D12MIT5 L58	D	D	B	D	B	D	B	B	D	D	D
D12MIT7 M62	D	D	B	D	B	D	B	B	D	D	D
D12MIT D7	D	D	B	D	B	D	B	B	D	D	D

PHENOTYPE	MA	MA	H	MA	H	MA	MA	H	MA	MA	H
EMBRYO	199	212	217	W1	W2	W3	W4	W5	304	319	358
PANEL I NUMBER	33	36		40							
PANEL II NUMBER	12	13	14	15	16	17	18	19	20	21	22
PANEL III NUMBER		18	19	20	21	22	23	24	25	26	27
D8MIT24 A737	D	B	B	B	B	B	D	B	B	B	B
D8MIT8 M257	D	B	B	B	B	B	B	B	B	B	B
D8MIT41 B171	D	B	B	B	B	B	B	B	B	B	B
D8MIT47 B591	D	B	B	B	B	B	B	B	B	B	B
D8MIT42 A754	.	B	B	B	B	B	B	.	B	B	B
D10MIT3 A114	B	D	B	B	B	D	B	D	B	D	D
D10MIT40 B184	B	D	B	B	B	D	B	D	B	D	D
D10MIT42 B484	B	D	B	B	B	D	B	D	B	D	D
D10MIT10 M7	B	D	B	B	B	D	B	D	B	D	D
D10NDS2 T32	B	B	B	B	B	D	B	D	B	D	B
D10MIT14 M175	B	B	D	B	B	D	B	D	B	D	B
D11MIT63 B675	D	B	B	B	B	B	B	B	D	B	D
D11 MIT53 D548	D	B	B	B	B	B	B	B	D	B	D
D11MIT20 A755	D	B	B	B	B	B	B	B	D	B	D
D11MIT4 A124	D	D	B	B	B	B	.	B	B	B	D
D11MIT41 B279	D	D	B	B	D	B	B	B	B	B	D
D11MIT14 D2	D	D	B	B	D	B	B	B	B	B	D
D11MIT48 B121	D	B	B	B	D	B	B	B	B	B	B
D12MIT38 D135	B	D	B	D	D	D	B	D	D	D	D
D12MIT46 P82	D	D	B	D	D	B	B	B	D	D	D
D12MIT3 L41	B	D	B	D	B	B	B	B	B	B	B
D12MIT12 B269	D	D	B	D	D	B	B	D	D	D	D
D12MIT2 M27	D	D	B	D	D	B	B	B	D	D	D
D12MIT36 B297	B	D	B	D	B	B	B	B	D	D	D
D12MIT34 B176	B	D	B	D	B	B	B	B	D	D	D
D12MIT4 A64	B	D	B	D	B	B	B	B	B	D	D
D12MIT5 L58	B	D	B	D	B	B	B	B	B	D	D
D12MIT7 M62	B	D	B	B	B	B	B	D	B	D	D
D12MIT D7	B	D	B	B	D	B	B	D	B	D	D

PHENOTYPE	H	H	MA	MA	H	MA	H	H	H	MA	H
EMBRYO	362	377	381	382	390	399	423	431	436	438	440
PANEL I NUMBER											
PANEL II NUMBER	23	24	25	26	27	28	29	30	31	32	33
PANEL III NUMBER	28	29	30	31	32	33					
D8MIT24 A737	D	D	B	B	B	D	B	B	B	B	B
D8MIT8 M257	D	B	B	B	B	D	B	B	B	B	B
D8MIT41 B171	D	B	B	D	B	D	B	B	B	B	B
D8MIT47 B591	D	B	B	D	B	D	B	B	B	D	B
D8MIT42 A754	D	B	B	D	B	D	D	B	B	D	D
D10MIT3 A114	D	D	B	D	D	B	D	D	D	B	D
D10MIT40 B184	D	D	B	D	D	B	D	D	D	B	B
D10MIT42 B484	D	D	B	D	D	B	B	B	D	D	B
D10MIT10 M7	D	D	B	D	D	B	B	B	D	D	B
D10NDS2 T32	D	D	B	D	D	B	B	B	D	D	B
D10MIT14 M175	B	D	B	D	D	B	B	B	D	D	D
D11MIT63 B675	D	D	D	D	D	B	D	D	B	B	D
D11 MIT53 D548	D	D	D	D	D	B	D	D	B	B	D
D11MIT20 A755	D	D	D	D	D	B	D	B	B	B	D
D11MIT4 A124	D	D	D	D	B	D	D	B	D	B	D
D11MIT41 B279	D	D	B	D	B	D	D	B	D	B	D
D11MIT14 D2	D	D	B	D	B	.	D	B	D	B	D
D11MIT48 B121	D	D	B	B	B	D	D	B	D	B	D
D12MIT38 D135	D	D	D	D	D	B	B	D	B	B	B
D12MIT46 P82	D	B	D	B	B	D	D	D	D	B	B
D12MIT3 L41	B	B	B	B	B	D	B	B	B	B	B
D12MIT12 B269	B	B	D	D	B	D	D	D	D	B	B
D12MIT2 M27	D	B	D	B	B	D	D	B	D	B	B
D12MIT36 B297	D	D	D	B	B	D	D	B	D	B	B
D12MIT34 B176	D	D	D	B	B	D	D	B	D	B	D
D12MIT4 A64	D	D	D	B	B	D	D	B	D	B	D
D12MIT5 L58	D	D	D	B	B	D	D	B	D	B	D
D12MIT7 M62	D	D	B	B	D	D	B	B	D	B	D
D12MIT D7	D	D	B	D	B	D	B	B	D	D	D

Appendix

PHENOTYPE	MA	MA	H	MA	MA	MA	H	MA	MA	H	F
EMBRYO	446	449	451	452	469	470	480	W6	491	499	110
PANEL I NUMBER											
PANEL II NUMBER	34	35	36	37	38	39	40	41	42	43	
PANEL III NUMBER											11
D8MIT24 A737	D	B	D	B	B	D	B	D	D	B	
D8MIT8 M257	D	B	D	B	B	D	B	D	D	B	
D8MIT41 B171	D	B	D	B	B	D	B	D	D	B	
D8MIT47 B591	D	B	B	B	B	D	B	D	D	D	
D8MIT42 A754	D	D	B	D	B	B	D	D	D	D	
D10MIT3 A114	D	D	D	D	B	D	B	D	B	D	
D10MIT40 B184	D	D	D	D	B	B	B	D	B	D	
D10MIT42 B484	D	B	D	B	B	B	B	D	B	B	
D10MIT10 M7	D	B	D	B	B	B	B	D	B	B	
D10NDS2 T32	B	B	D	B	B	B	D	D	B	B	
D10MIT14 M175	B	B	D	B	B	B	D	D	B	B	
D11MIT63 B675	B	B	B	B	D	D	B	D	B	D	
D11 MIT53 D548	B	D	B	B	D	D	B	B	B	D	
D11MIT20 A755	B	D	B	B	D	D	B	B	B	D	
D11MIT4 A124	B	D	B	B	D	D	B	B	B	B	
D11MIT41 B279	B	D	B	B	D	D	B	B	B	B	
D11MIT14 D2	B	D	B	.	D	D	D	D	B	B	
D11MIT48 B121	D	B	B	D	D	B	D	D	B	B	
D12MIT38 D135	B	D	B	D	D	D	B	D	D	B	
D12MIT46 P82	B	D	D	B	D	D	D	D	B	B	
D12MIT3 L41	B	B	B	B	B	B	B	B	B	B	
D12MIT12 B269	B	D	D	B	D	D	D	D	B	B	
D12MIT2 M27	B	D	D	B	D	D	D	D	B	B	
D12MIT36 B297	D	D	D	B	D	D	D	D	B	B	
D12MIT34 B176	D	D	D	B	D	D	D	D	B	B	
D12MIT4 A64	D	D	D	B	D	D	D	D	B	B	
D12MIT5 L58	D	D	D	B	D	D	D	D	B	B	
D12MIT7 M62	D	D	D	B	D	D	D	D	B	D	
D12MIT D7	D	D	D	B	D	D	D	D	B	D	

Appendix

PHENOTYPE	MA	MA	H	H	MA	H	H	H	H	MA	MA
EMBRYO	92	103	106	111	118	137	169	170	179	182	191
PANEL I NUMBER	17	19	38	39	22	41	42	43	44	26	31
PANEL II NUMBER	1	2	3	4	5	6	7	8	9	10	11
PANEL III NUMBER	8	9	10		12	13	14	15	16		17
D13MIT3 M79	D	B	D	D	B	D	D	B	B	D	B
D13MIT18 A890	D	B	D	D	B	D	D	B	B	D	D
D13MIT23 J9	B	B	D	D	B	D	D	D	D	D	D
D13MIT27 A1130	B	B	B	D	B	D	D	D	D	D	D
D13MIT45 P51	B	B	B	D	B	B	D	D	B	D	D
D13MIT35 A1107	B	B	B	D	B	B	D	D	B	D	B
D14MIT1 A103	D	D	D	D	B	B	D	B	B	B	B
D14MIT2 A24	D	D	D	D	B	B	D	B	B	B	B
D14MIT45 B441	D	D	D	D	B	B	D	B	B	B	B
D14MIT4 M228	B	D	D	D	B	B	D	B	B	B	B
D14MIT28 D539	B	D	D	D	B	B	D	B	B	B	B
D14MIT7 L27	B	D	D	B	B	B	D	D	B	B	B
D15MIT12 A34	B	B	D	B	B	B	D	D	D	B	D
D15MIT38 A79	B	B	B	B	B	B	D	D	B	B	D
D15MIT26 A787	D	B	B	D	B	B	D	D	B	B	D
D15MIT3 L78	D	D	B	D	B	D	D	D	B	B	B
D15MIT37 B162	D	D	B	D	B	D	D	D	B	B	B
D15MIT42 D654	D	D	B	D	B	D	D	D	B	B	B
D15MIT16 D131	D	D	B	D	B	D	D	D	B	B	B
D16MIT9 B159	B	D	D	B	D	B	B	B	D	B	D
D16MIT4 M203	B	D	D	B	D	B	B	B	B	B	B
D16MIT5 A38	B	D	D	B	D	B	B	B	B	B	B
D16MIT6 L7	B	D	D	B	B	B	B	B	B	B	B
D17MIT46 D578	D	B	D	B	B	B	B	B	D	B	B
D17MIT24 D12	B	B	D	B	B	B	B	D	D	B	B
D17MIT10 L36	B	B	D	B	B	D	B	D	D	B	D
D17MIT3 L28	B	B	D	B	B	B	B	D	B	B	B
D17MIT38	B	B	D	B	B	B	.	D	B	B	B
D17MIT41 B306	B	B	B	B	B	B	B	D	B	B	B
DXMIT1 L43	D	D	B	B	B	D	D	D	B	D	B
DXMIT10 A1124	D	D	B	B	D	B	B	D	B	D	B

PHENOTYPE	MA	MA	H	MA	H	MA	MA	H	MA	MA	H
EMBRYO	199	212	217	W1	W2	W3	W4	W5	304	319	358
PANEL I NUMBER	33	36		40							
PANEL II NUMBER	12	13	14	15	16	17	18	19	20	21	22
PANEL III NUMBER		18	19	20	21	22	23	24	25	26	27
D13MIT3 M79	D	B	B	B	B	B	B	D	D	D	D
D13MIT18 A890	D	B	B	B	B	B	B	D	B	D	D
D13MIT23 J9	D	B	B	B	B	B	B	B	B	D	D
D13MIT27 A1130	D	B	B	B	B	B	B	B	B	D	D
D13MIT45 P51	D	D	D	B	B	B	B	B	B	.	B
D13MIT35 A1107	D	D	D	B	B	B	B	B	B	D	B
D14MIT1 A103	B	D	B	B	D	B	D	B	B	B	D
D14MIT2 A24	B	D	B	B	D	B	D	B	B	B	D
D14MIT45 B441	B	D	B	B	D	D	D	B	D	B	D
D14MIT4 M228	B	D	B	B	D	D	D	B	D	B	B
D14MIT28 D539	B	D	B	B	D	D	D	D	D	B	B
D14MIT7 L27	.	D	B	B	B	D	D	D	D	B	B
D15MIT12 A34	D	D	B	B	D	D	D	D	D	B	B
D15MIT38 A79	B	D	B	B	D	D	D	D	D	B	B
D15MIT26 A787	B	D	D	B	D	D	D	D	D	B	B
D15MIT3 L78	B	D	D	B	D	D	D	D	D	B	B
D15MIT37 B162	B	D	D	B	D	D	B	D	D	D	B
D15MIT42 D654	B	B	B	B	B	B	B	D	D	D	D
D15MIT16 D131	D	B	B	B	B	B	B	D	D	D	D
D16MIT9 B159	D	B	B	D	B	B	B	D	B	D	B
D16MIT4 M203	D	B	B	D	D	B	B	D	B	D	B
D16MIT5 A38	D	B	B	D	D	B	B	D	B	D	B
D16MIT6 L7	D	B	D	D	D	B	B	D	B	D	B
D17MIT46 D578	B	B	B	D	D	B	B	D	D	B	B
D17MIT24 D12	B	B	B	D	D	B	B	D	D	B	B
D17MIT10 L36	B	D	B	D	D	D	B	D	D	B	B
D17MIT3 L28	.	D	B	B	D	D	B	D	B	D	B
D17MIT38	B	D	B	B	D	D	B	D	B	D	D
D17MIT41 B306	B	D	B	B	D	D	B	D	B	D	D
DXMIT1 L43	D	B	D	B	D	D	B	D	B	D	B
DXMIT10 A1124	D	B	D	B	B	B	B	D	B	B	B

PHENOTYPE	H	H	MA	MA	H	MA	H	H	H	MA	H
EMBRYO	362	377	381	382	390	399	423	431	436	438	440
PANEL I NUMBER											
PANEL II NUMBER	23	24	25	26	27	28	29	30	31	32	33
PANEL III NUMBER	28	29	30	31	32	33					
D13MIT3 M79	B	D	D	B	D	B	B	D	B	B	D
D13MIT18 A890	B	D	D	B	D	B	B	D	B	B	D
D13MIT23 J9	B	D	D	B	D	D	B	B	B	B	D
D13MIT27 A1130	B	D	D	D	D	D	B	B	B	B	D
D13MIT45 P51	B	.	D	D	D	D	D	B	.	B	D
D13MIT35 A1107	D	B	D	D	D	D	D	B	B	B	D
D14MIT1 A103	D	D	D	D	D	B	D	D	B	B	D
D14MIT2 A24	D	D	D	D	D	B	.	D	D	B	D
D14MIT45 B441	D	D	D	B	D	B	D	D	D	B	D
D14MIT4 M228	D	D	D	B	D	B	D	D	D	D	D
D14MIT28 D539	D	D	D	B	D	B	D	D	D	D	D
D14MIT7 L27	D	D	D	B	D	B	D	D	D	D	D
D15MIT12 A34	B	B	D	D	D	D	B	B	D	D	D
D15MIT38 A79	B	B	D	D	D	D	B	B	D	B	D
D15MIT26 A787	B	B	D	D	D	D	B	B	D	B	D
D15MIT3 L78	B	B	D	D	D	B	B	B	D	B	D
D15MIT37 B162	B	B	D	D	D	B	B	B	D	B	B
D15MIT42 D654	B	D	D	D	D	B	B	D	D	D	B
D15MIT16 D131	B	D	D	D	D	B	B	D	D	D	B
D16MIT9 B159	D	B	D	D	D	D	B	D	D	D	D
D16MIT4 M203	B	B	D	D	B	D	B	B	D	D	D
D16MIT5 A38	B	B	D	D	B	D	B	B	D	D	D
D16MIT6 L7	B	B	D	D	B	B	B	B	B	D	D
D17MIT46 D578	D	D	D	D	B	B	B	D	D	B	D
D17MIT24 D12	D	D	D	D	B	B	B	B	D	D	D
D17MIT10 L36	D	D	D	D	B	B	B	B	D	D	D
D17MIT3 L28	D	D	D	D	B	B	D	B	D	D	D
D17MIT38	D	D	D	D	B	B	D	B	D	D	D
D17MIT41 B306	D	D	B	D	B	B	D	B	D	D	D
DXMIT1 L43	B	D	D	B	B	D	D	D	D	D	B
DXMIT10 A1124	B	D	B	B	D	D	D	B	D	D	B

PHENOTYPE	MA	MA	H	MA	MA	MA	H	MA	MA	H	F
EMBRYO	446	449	451	452	469	470	480	W6	491	499	110
PANEL I NUMBER											
PANEL II NUMBER	34	35	36	37	38	39	40	41	42	43	
PANEL III NUMBER											11
D13MIT3 M79	B	B	B	D	B	B	D	B	D	D	
D13MIT18 A890	B	D	B	D	B	B	D	B	D	D	
D13MIT23 J9	B	D	B	D	B	B	D	B	D	D	
D13MIT27 A1130	B	D	D	D	B	B	D	B	B	D	
D13MIT45 P51	B	B	D	D	B	B	D	B	B	D	
D13MIT35 A1107	B	B	D	D	B	D	D	B	B	D	
D14MIT1 A103	B	B	D	B	B	D	B	B	B	D	
D14MIT2 A24	B	B	D	B	B	D	B	B	B	D	
D14MIT45 B441	D	B	D	B	B	D	B	B	B	D	
D14MIT4 M228	D	B	D	B	B	.	B	.	D	D	
D14MIT28 D539	D	B	D	B	B	D	B	B	D	D	
D14MIT7 L27	D	B	D	B	B	B	D	D	D	D	
D15MIT12 A34	B	D	B	B	D	D	B	B	B	D	
D15MIT38 A79	B	D	B	B	D	D	B	B	B	B	
D15MIT26 A787	B	D	B	B	D	D	B	B	B	B	
D15MIT3 L78	B	D	B	B	D	D	B	B	B	D	
D15MIT37 B162	B	D	D	B	D	D	B	B	B	D	
D15MIT42 D654	B	D	D	B	D	D	D	B	B	D	
D15MIT16 D131	B	D	D	B	D	D	D	B	B	D	
D16MIT9 B159	B	B	B	B	D	D	B	B	D	B	
D16MIT4 M203	D	B	B	B	D	B	B	B	B	B	
D16MIT5 A38	D	B	B	B	D	B	B	B	B	B	
D16MIT6 L7	D	B	B	B	D	B	B	B	B	B	
D17MIT46 D578	.	D	D	D	B	B	D	.	.	.	
D17MIT24 D12	D	D	D	D	B	B	D	.	.	.	
D17MIT10 L36	D	D	D	D	B	B	D	.	.	.	
D17MIT3 L28	D	D	D	D	B	B	B	.	.	.	
D17MIT38	D	D	D	D	B	B	D	.	.	.	
D17MIT41 B306	D	D	D	D	B	B	D	.	.	.	
DXMIT1 L43	B	B	D	D	D	D	B	D	B	D	
DXMIT10 A1124	B	B	D	D	D	D	D	B	B	D	