14

Sex Determination

HOW AN INDIVIDUAL'S SEX IS DETERMINED has been one of the great questions of natural philosophy since antiquity. Aristotle claimed that sex was determined by the heat of the male partner during intercourse (Aristotle, ca. 335 BCE). The more heated the passion, said the sage, the greater the probability of male offspring. He also counseled elderly men to conceive in the summer if they wished to have male heirs.

Aristotle's hypothesis of sex determination was straightforward: women were men whose development was arrested too early. The female was "a mutilated male" whose development was forestalled when the coldness of the mother's womb overcame the heat of the father's semen. Women were therefore colder and more passive than men, and female sex organs did not mature to the point where they could provide active seeds. These Aristotelian views were accepted both by the Christian church and by the Greco-Roman physician Galen,* whose anatomy texts were the standard for more than a thousand years.

Indeed, until the twentieth century, the environment—temperature and nutrition, in particular—was believed to be important in determining sex. In 1890, Geddes and Thomson summarized all available data on sex determination and came to the conclusion that the "constitution, age, nutrition, and environment of the parents must be especially considered." They argued that factors favoring the storage of energy and nutrients predisposed one to have female offspring, whereas factors favoring the utilization of energy and nutrients influenced one to have male offspring.

This environmental view of sex determination remained the only major scientific theory until the identification (derived primarily from studies on insects) of the X and Y chromosomes and the correlation of the female sex with an XX karyotype (chromosome complement) and the male sex with either XY or XO karyotypes (Stevens 1905; Wilson 1905; see Gilbert 1978). This correlation suggested strongly that a specific nuclear component was responsible for directing the development of the sexual phenotype. Evidence that sex determination occurs by nuclear inheritance rather than by environmental happenstance continued to accumulate.

Today we know that both internal and environmental mechanisms of sex determination operate in different species. We will first discuss the chromoso-

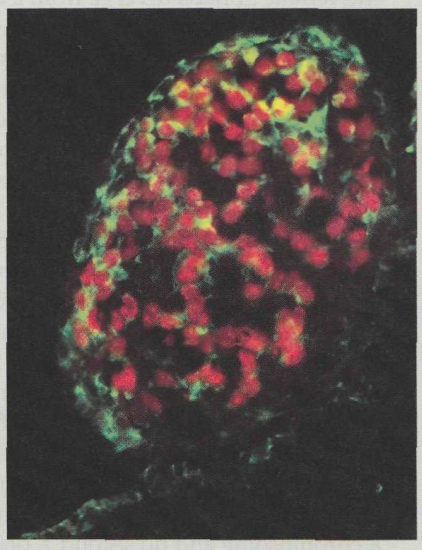
*The imperial physician to the Roman emperors Marcus Aurelius and Commodus, Galen first achieved fame as a physician to gladiators, from whose wounds and corpses he undoubtedly learned much anatomy.

Sexual reproduction is ... the masterpiece of nature.

ERASMUS DARWIN (1791)

It is quaint to notice that the number of speculations connected with the nature of sex have well-nigh doubled since Drelincourt, in the eighteenth century, brought together two hundred and sixty-two "groundless hypotheses," and since Blumenbach caustically remarked that nothing was more certain than that Drelincourt's own theory formed the two hundred and sixty-third.

J. A. THOMSON (1926)



mal mechanisms of sex determination and then consider the ways in which the environment can regulate sexual phenotype.

See WEBSITE 14.1 Social critique of sex determination research

CHROMOSOMAL SEX DETERMINATION

There are several ways chromosomes can determine the sex of an embryo. In mammals, the presence of either a second X chromosome or a Y chromosome determines whether the embryo is to be female (XX) or male (XY). In birds, the situation is reversed (Smith and Sinclair 2001): the male has the two similar sex chromosomes (ZZ), and the female has the unmatched pair (ZW). In flies, the Y chromosome plays no role in sex determination, but the number of X chromosomes appears to determine the sexual phenotype. In other insects (especially hymenopterans such as bees, wasps, and ants), fertilized, diploid eggs develop into females, while the unfertilized, haploid eggs become males (Beukeboom 1995; Gempe et al. 2009). This chapter will discuss only two of the many chromosomal modes of sex determination: sex determination in placental mammals and sex determination in *Drosophila*.

The Mammalian Pattern: Primary and Secondary Sex Determination

Primary sex determination is the determination of the gonads—the egg-forming ovaries or sperm-forming testes. In mammals, primary sex determination is chromosomal and is not usually influenced by the environment. The formation both of ovaries and of testes is an active, gene-directed process. Moreover, as we shall see, both the male and female gonads diverge from a common precursor, the **bipotential**, or **indifferent**, **gonad**.

In most cases, the female's karyotype is XX and the male's is XY (Figure 14.1A). Every individual must carry at least one X chromosome. Since the female is XX, each of her haploid eggs has a single X chromosome. The male, being XY, generates two populations of haploid sperm: half will bear an X chromosome, half a Y. If at fertilization the egg receives a second X chromosome from the sperm, the resulting individual is XX, forms ovaries, and is female; if the egg receives a Y chromosome from the sperm, the individual is XY, forms testes, and is male.

The Y chromosome is a crucial factor for determining male sex in mammals. The Y chromosome carries a gene that encodes a **testis-determining factor**, which organizes the gonad into a testis rather than an ovary. A person with five X chromosomes and one Y chromosome (XXXXXY) would be male. Furthermore, an individual with a single X chromosome and no second X or Y (i.e., XO) develops as a female and begins making ovaries (although the ovarian follicles cannot be maintained; for a complete ovary, a second X chromosome is needed).

Secondary sex determination involves the sexual phenotype outside the gonads. This includes the male or female duct systems and external genitalia. A male mammal has a penis, scrotum (testicle sac), seminal vesicles, and prostate gland. A female mammal has a vagina, clitoris, labia, cervix, uterus, oviducts, and mammary glands.* In many species, each sex has a sex-specific body size, vocal cartilage, and musculature. Secondary sex characteristics are usually determined by hormones and paracrine factors secreted from the gonads. In the absence of gonads, however, the female phenotype is generated. When Jost (1947, 1953) removed fetal rabbit gonads before they had differentiated, the resulting rabbits had a female phenotype, regardless of whether their genotype was XX or XY.

The general scheme of mammalian sex determination is shown in Figure 14.1B. If the embryonic cells have two X chromosomes (and no Y chromosome), the gonadal primordia develop into ovaries. The ovaries produce **estrogen**, a hormone that enables the development of the **Müllerian duct** into the uterus, oviducts, cervix, and upper end of the vagina (Fisher et al. 1998; Couse et al. 1999; Couse and Korach 2001). If embryonic cells contain both an X and

*Linnaeus named the mammals—our particular class of vertebrates—after this female secondary sexual trait. The politics of this decision is discussed in Schiebinger 1993.

FIGURE 14.1 Sex determination in mammals. (A) Mammalian chromosomal sex determination results in approximately equal numbers of male and female offspring. (B) Postulated cascades leading to male and female phenotypes in mammals. The conversion of the genital ridge into the bipotential gonad requires, among others, the *LHX9*, *SF1*, and *WT1* genes, since mice lacking any of these genes lack gonads. The bipotential gonad appears to be moved into the female pathway (ovary development) by the *WNT4*, *RSPO1*, and (perhaps) *DAX1* genes and into the male pathway (testis development) by the *SRY* gene (on the Y chromosome) in conjunction with autosomal genes such as *SOX9*. (Lower levels of Dax1 and Wnt4 are also present in the male gonad.) The ovary makes thecal cells and granulosa cells, which together are capable of synthesizing estrogen. Under the influence of estrogen (first from the mother, then from the fetal gonads), the Müllerian duct differentiates into the female reproductive tract, the internal and external genitalia develop, and the offspring develops the secondary sex characteristics of a female. The testis makes two major compounds involved in sex determination. The first, anti-Müllerian factor (AMF), causes the Müllerian duct to regress. The second, testosterone, causes differentiation of the Wolffian duct into the male internal genitalia. In the urogenital region, testosterone is converted into dihydrotestosterone (DHT), and this hormone causes the morphogenesis of the penis and prostate gland. (B after Marx 1995; Birk et al. 2000.)

a Y chromosome, testes form and secrete two major factors. The first is a TGF-β family paracrine factor called **anti-Müllerian factor** (**AMF**), traditionally called anti-Müllerian hormone, or AMH. It destroys the Müllerian duct, preventing formation of the uterus and oviducts. The second factor is the steroid hormone **testosterone**, which masculinizes the fetus, stimulating the formation of the penis, male duct system, scrotum, and other portions of the male anatomy, as well as inhibiting development of the breast primordia. We will now take a more detailed look at these events.

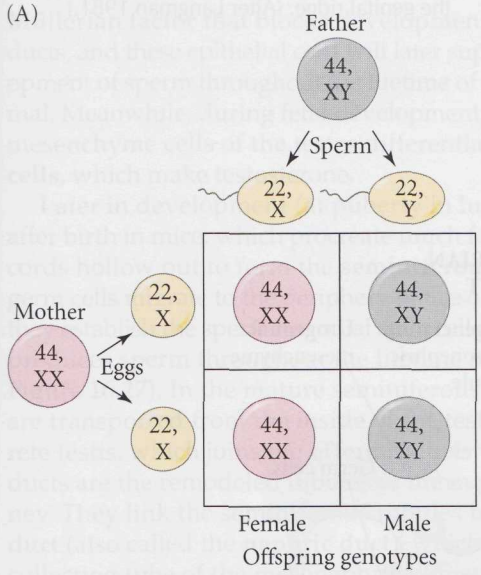
Primary Sex Determination in Mammals

The developing gonads

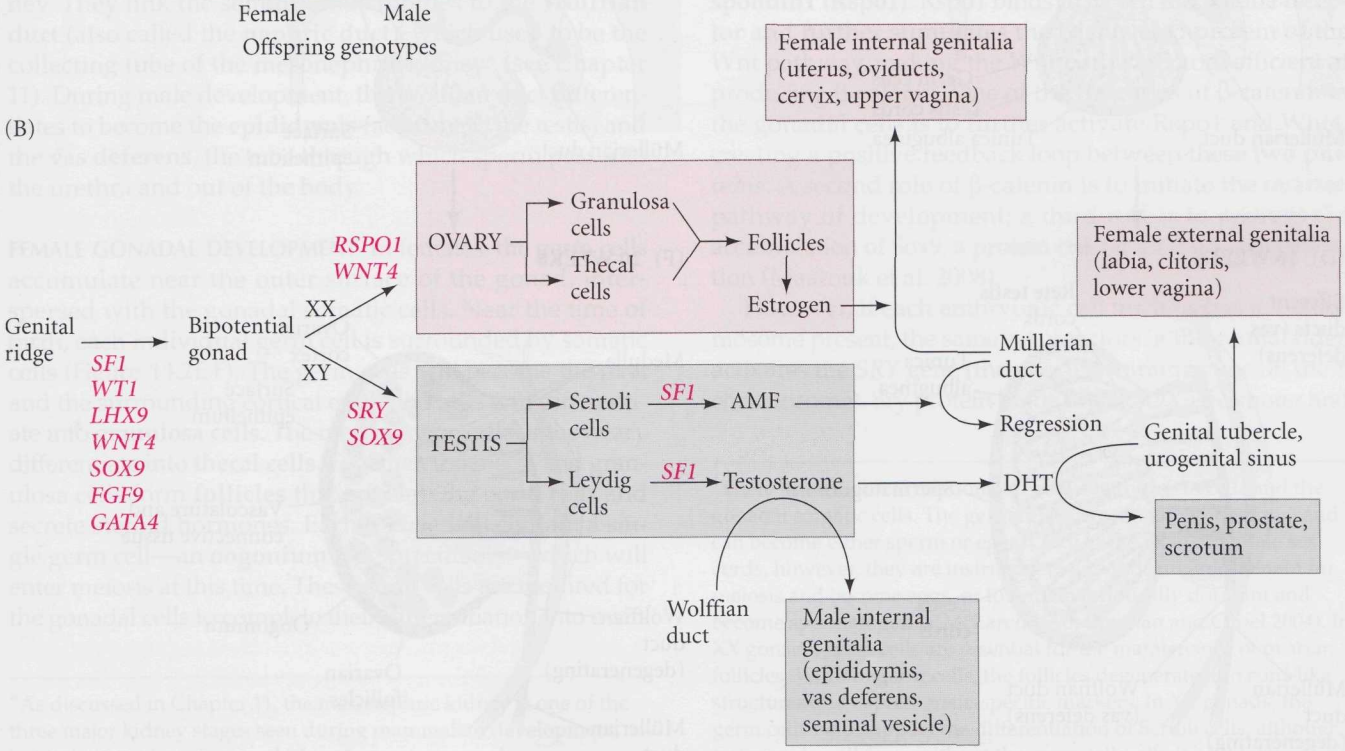
Mammalian gonads embody a unique embryological situation. All other organ rudiments normally can differentiate into only one type of organ. A lung rudiment can only become a lung, and a liver rudiment can develop only into a liver. The gonadal rudiment, however, has two options: it can develop into either an ovary or a testis. The path of differentiation taken by this rudiment is dictated by the genotype and determines the future sexual development of the organism (Lillie 1917). But before this decision is made, the mammalian gonad first develops through a **bipotential**, or **indifferent**, **stage**, during which time it has neither female nor male characteristics.

In humans, the bipotential gonadal rudiments appear during week 4 and remain sexually indifferent until week 7. The gonadal rudiments are paired regions of the intermediate mesoderm; they form adjacent to the developing kidneys. The ventral portions of these rudiments comprise the **genital ridge epithelium**. During the indifferent stage, the genital ridge epithelium proliferates (Figure 14.2A,B). These epithelial layers will form the somatic (i.e., non-germ cell) component of the gonads. The germ cells migrate into the gonad during week 6 and are surrounded by the somatic cells.

(A)



(B)



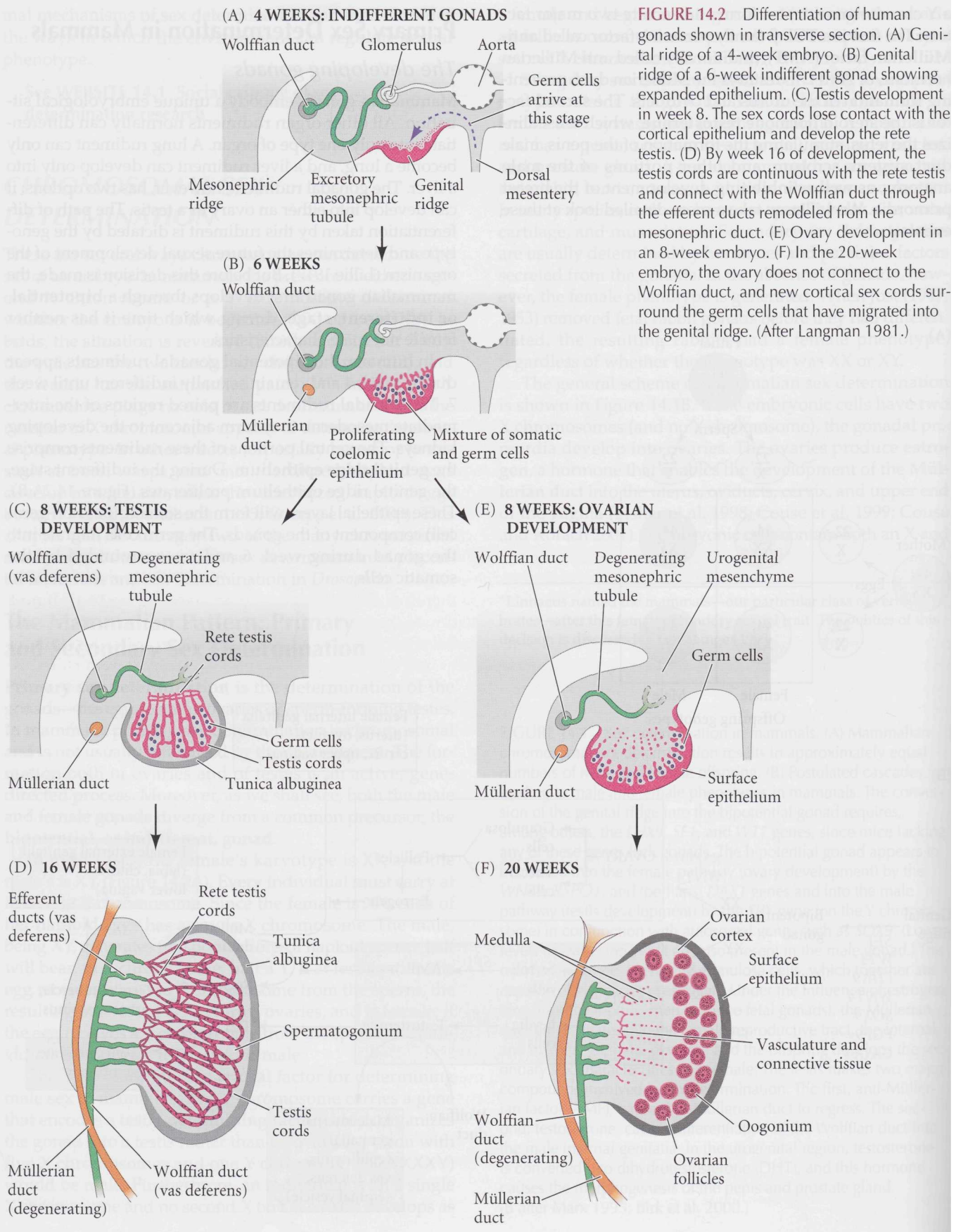


FIGURE 14.2 Differentiation of human gonads shown in transverse section. (A) Genital ridge of a 4-week embryo. (B) Genital ridge of a 6-week indifferent gonad showing expanded epithelium. (C) Testis development in week 8. The sex cords lose contact with the cortical epithelium and develop the rete testis. (D) By week 16 of development, the testis cords are continuous with the rete testis and connect with the Wolffian duct through the efferent ducts remodeled from the mesonephric duct. (E) Ovary development in an 8-week embryo. (F) In the 20-week embryo, the ovary does not connect to the Wolffian duct, and new cortical sex cords surround the germ cells that have migrated into the genital ridge. (After Langman 1981.)

MALE GONADAL DEVELOPMENT If the fetus is XY, the somatic cells continue to proliferate through the eighth week, and then initiate their differentiation into **Sertoli cells**. During week 8, the developing Sertoli cells surround the incoming germ cells and organize themselves into the **testis cords**. These cords form loops in the medullary (central) region of the developing testis and are connected to a network of thin canals, called the **rete testis**, located near the mesonephric duct (Figure 14.2C,D). Eventually, the region containing the testis cords and germ cells becomes enclosed by a thick extracellular matrix, the **tunica albuginea**. Thus, when the germ cells enter the male gonads, they will develop within the testis cords, inside the organ.

The Sertoli cells of the fetal testis cords secrete the anti-Müllerian factor that blocks development of the female ducts, and these epithelial cells will later support the development of sperm throughout the lifetime of the male mammal. Meanwhile, during fetal development, the interstitial mesenchyme cells of the testes differentiate into **Leydig cells**, which make testosterone.

Later in development (at puberty in humans; shortly after birth in mice, which procreate much faster), the testis cords hollow out to form the **seminiferous tubules**. The germ cells migrate to the periphery of these tubules, where they establish the spermatogonial stem cell population that produces sperm throughout the lifetime of the male (see Figure 16.27). In the mature seminiferous tubule, sperm are transported from the inside of the testis through the rete testis, which joins the **efferent ducts**. These efferent ducts are the remodeled tubules of the mesonephric kidney. They link the seminiferous tubules to the **Wolffian duct** (also called the **nephric duct**), which used to be the collecting tube of the mesonephric kidney* (see Chapter 11). During male development, the Wolffian duct differentiates to become the **epididymis** (adjacent to the testis) and the **vas deferens**, the tube through which sperm pass into the urethra and out of the body.

FEMALE GONADAL DEVELOPMENT In females, the germ cells accumulate near the outer surface of the gonad, interspersed with the gonadal somatic cells. Near the time of birth, each individual germ cell is surrounded by somatic cells (Figure 14.2E,F). The germ cells will become the ova, and the surrounding cortical epithelial cells will differentiate into **granulosa cells**. The mesenchyme cells of the ovary differentiate into **thecal cells**. Together, the thecal and granulosa cells form **follicles** that envelop the germ cells and secrete steroid hormones. Each follicle will contain a single germ cell—an **oogonium** (egg precursor)—which will enter meiosis at this time. These germ cells are required for the gonadal cells to complete their differentiation into ovarian tissue† (McLaren 1991). In females, the Müllerian duct remains intact and differentiates into the oviducts, uterus, cervix, and upper vagina. In the absence of adequate testosterone, the Wolffian duct degenerates. A summary of the development of mammalian reproductive systems is shown in Figure 14.3.

*As discussed in Chapter 11, the mesonephric kidney is one of the three major kidney stages seen during mammalian development, but it does not function as a kidney in most mammals.

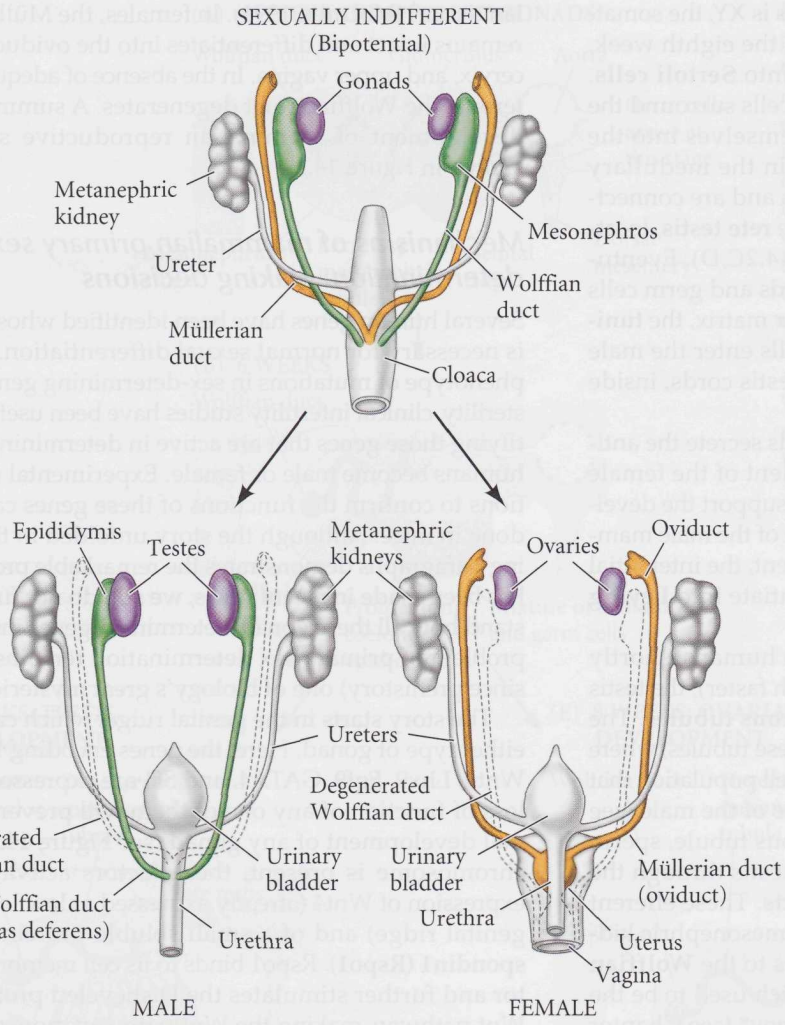
Mechanisms of mammalian primary sex determination: Making decisions

Several human genes have been identified whose function is necessary for normal sexual differentiation. Since the phenotype of mutations in sex-determining genes is often sterility, clinical infertility studies have been useful in identifying those genes that are active in determining whether humans become male or female. Experimental manipulations to confirm the functions of these genes can then be done in mice. Although the story unfolded in the following paragraphs demonstrates the remarkable progress that has been made in recent years, we still do not fully understand how all these gonad-determining genes interact. The problem of primary sex determination remains (as it has since prehistory) one of biology's great mysteries.

The story starts in the genital ridge, which can become either type of gonad. Here, the genes encoding Wt1, Sox9, Wnt4, Lhx9, Fgf9, GATA4, and Sf1 are expressed, and the loss of function of any one of them will prevent the normal development of any gonad (see Figure 14.1). If no Y chromosome is present, these factors activate further expression of Wnt4 (already expressed at low levels in the genital ridge) and of a small soluble protein called **R-spondin1 (Rspo1)**. Rspo1 binds to its cell membrane receptor and further stimulates the Disheveled protein of the Wnt pathway, making the Wnt pathway more efficient at producing β-catenin. One of the functions of β-catenin in the gonadal cells is to further activate Rspo1 and Wnt4, creating a positive feedback loop between these two proteins. A second role of β-catenin is to initiate the ovarian pathway of development; a third role is to prevent the accumulation of Sox9, a protein crucial for testis determination (Maatouk et al. 2008).

However, if each embryonic cell nucleus has a Y chromosome present, the same set of factors in the genital ridge activates the *SRY* gene (the sex-determining gene on the Y chromosome). Sry protein binds to the *SOX9* promoter and

†There is a reciprocal relationship between the germ cells and the gonadal somatic cells. The germ cells are originally bipotential and can become either sperm or eggs. Once in the male or female sex cords, however, they are instructed to either begin (and remain in) meiosis and become eggs, or to remain mitotically dormant and become spermatogonia (McLaren 1995; Brennan and Capel 2004). In XX gonads, germ cells are essential for the maintenance of ovarian follicles. Without germ cells, the follicles degenerate into cord-like structures and express male-specific markers. In XY gonads, the germ cells help support the differentiation of Sertoli cells, although testis cords will form without the germ cells, albeit a bit later.



GONADS		
Gonadal type	Testis	Ovary
Germ cell location	Inside testis cords (in medulla of testis)	Inside follicles of ovarian cortex
DUCTS		
Remaining duct	Wolffian	Müllerian
Duct differentiation	Vas deferens, epididymis, seminal vesicle	Oviduct, uterus, cervix, upper portion of vagina

FIGURE 14.3 Development of the gonads and their ducts in mammals. Note that both the Wolffian and Müllerian ducts are present at the indifferent gonad stage.

elevates expression of this key gene in the testis-determining pathway (Bradford et al. 2009b; Sekido and Lovell-Badge 2009). Sox9 and Fgf9 (and possibly Sry) act to block the ovary-forming pathway, probably by blocking the function of β-catenin (Bernard et al. 2008; Lau and Li 2008). One possible model of how sex determination can be initiated is shown in Figure 14.4.

Here we see an important rule of animal development: A pathway for organogenesis often has two components. One branch of the pathway says "Make A"; the other branch says "and don't make B." So the male pathway says "Make testes and don't make ovaries," while the female pathway says, "Make ovaries and don't make testes."

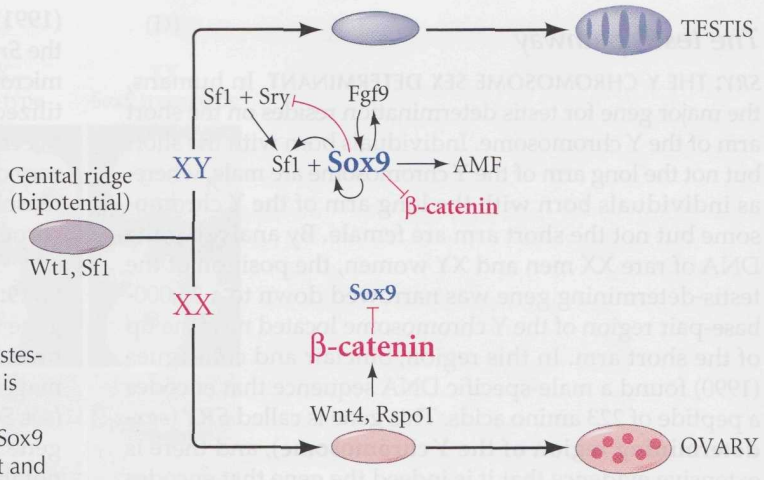


FIGURE 14.4 Possible mechanism for the initiation of primary sex determination in mammals. While we do not know the specific interactions involved, this model attempts to organize the data into a coherent sequence. In this model (others are possible), the interactions between paracrine and transcription factors in the developing genital ridge activate Wnt4 and Rspo1. Wnt4 activates the canonical Wnt pathway, which is made more efficient by Rspo1. The Wnt pathway causes the accumulation of β-catenin, and the large accumulation of β-catenin stimulates further Wnt4 activity. *This continual production of β-catenin both induces* the transcription of ovary-producing genes and blocks the testes-determining pathway by interfering with Sox9 activity. If Sry is present, it may block β-catenin signaling (thus halting ovary generation) and, along with Sf1, activate the gene for Sox9. Sox9 activates Fgf9 synthesis, which stimulates testis development and promotes further Sox9 synthesis. Sox9 also prevents β-catenin's activation of ovary-producing genes. In summary, then, a Wnt4/β-catenin loop specifies the ovaries, while a Sox9/Fgf9 loop specifies the testes. (After Sekido and Lovell-Badge 2009).

The ovary pathway: Wnt4 and R-spondin1

In mice, Wnt4 is expressed in the genital ridges of both sexes, but its expression later becomes undetectable in XY gonads (which become testes), whereas it is maintained in XX gonads as they begin to form ovaries. In transgenic XX mice that lack the *Wnt4* gene, the ovary fails to form properly, and the cells transiently express testis-specific markers, including Sox9, testosterone-producing enzymes, and AMF (Vainio et al. *1999*; Heikkila et al. 2005). Thus, Wnt4 appears to be an important factor in ovary formation, although it is not the only determining factor.

R-spondin1 is also critical in ovary formation, since in human case studies several XX individuals with *RSPO1* gene mutations became males (Parma et al. 2006). Rspo1 acts in synergy with Wnt4 to produce β-catenin, and this β-catenin appears to be critical both in activating further ovarian development and in blocking the synthesis of Sox9. Sox9 (as we will see below) is necessary in testis determination. In XY individuals with a duplication of the region on chromosome 1 that contains both the *WNT4* and *RSPO1* genes, the pathways that make β-catenin override the male pathway and cause a male-to-female sex reversal. Similarly, if XY mice are induced to overexpress β-catenin in their gonadal rudiments, they too will form ovaries rather than testes. Indeed, β-catenin appears to be a key "pro-ovarian" and "anti-testis" signaling molecule in all vertebrate groups, as it is seen in the female gonads (but not in the male gonads) of birds, mammals, and turtles. These groups have very different modes of sex determination, yet Rspo1 and β-catenin are made in the ovaries of each of them (Figure 14.5; Maatouk et al. 2008; Cool and Capel 2009; Smith et al. 2009).

Certain transcription factors that appear to be activated by β-catenin are found exclusively in the ovaries. One possible target for β-catenin is the gene encoding TAFII 105 (Freiman et al. 2002). This subunit of the TATA-binding protein for RNA polymerase binding is seen only in ovarian follicle cells. Female mice lacking this subunit have small ovaries with few, if any, mature follicles. *FoxL2* is another gene that is strongly upregulated in ovaries, and XX mice homozygous for mutant *FoxL2* alleles develop malelike gonad structure and upregulate Sox9 expression and testosterone production. Estrogen receptors (transcription factors that become active by binding estrogen) are expressed in the developing gonads of both sexes but become prevalent in the developing ovaries.

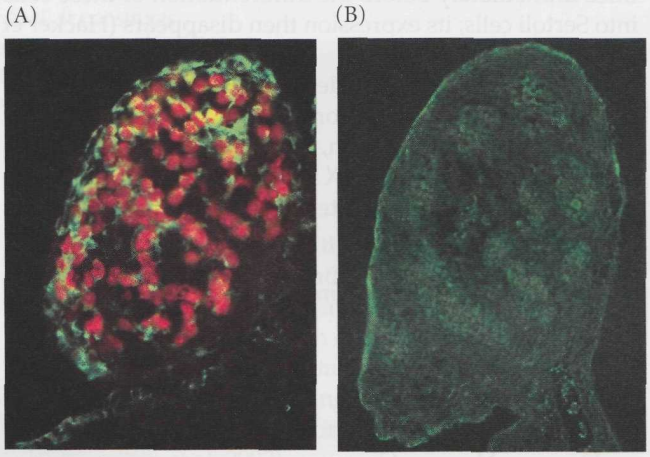


FIGURE 14.5 Localization of Rspo1 protein in embryonic day 14.5 mouse gonads. Immunofluorescent probes were used to identify Rspo1 (green) and the meiotic germ cell marker, Scp3 (red). (A) Rspo1 was found on somatic cells (arrowhead) and at the germ cell surface (arrow) of the ovaries. (B) These antibodies revealed neither Rspo1 nor Scp3 in the developing testes. (The germ cells in the male gonads have not entered meiosis at this *point in development, whereas the ovarian germ cells have.*) (From Smith et al. 2008; photograph courtesy of C. Smith.)

The testis pathway

SRY: THE Y CHROMOSOME SEX DETERMINANT In humans, the major gene for testis determination resides on the short arm of the Y chromosome. Individuals born with the short but not the long arm of the Y chromosome are male, whereas individuals born with the long arm of the Y chromosome but not the short arm are female. By analyzing the DNA of rare XX men and XY women, the position of the testis-determining gene was narrowed down to a 35,000-base-pair region of the Y chromosome located near the tip of the short arm. In this region, Sinclair and colleagues (1990) found a male-specific DNA sequence that encodes a peptide of 223 amino acids. This gene is called ***SRY*** (**sex-determining *r*egion of the *Y* chromosome**), and there is extensive evidence that it is indeed the gene that encodes the human testis-determining factor.

SRY is found in normal XY males and also in the rare XX males; it is absent from normal XX females and from many XY females. Approximately 15% of XY females have the *SRY* gene, but their copies of the gene contain point or frameshift mutations that prevent the Sry protein from binding to DNA (Pontiggia et al. 1994; Werner et al. 1995). If the *SRY* gene actually does encode the major testis-determining factor, one would expect that it would act in the genital ridge immediately before or during testis differentiation. This prediction has been found to be the case in studies of the homologous gene in mice. The mouse gene (*Sry*) also correlates with the presence of testes; it is present in XX males and absent in XY females (Gubbay et al. 1990; Koopman et al. 1990). The *Sry* gene is expressed in the somatic cells of the bipotential mouse gonads of XY mice immediately before the differentiation of these cells into Sertoli cells; its expression then disappears (Hacker et al. 1995; Sekido et al. 2004).

The most impressive evidence for *Sry* being the gene for testis-determining factor comes from transgenic mice. If *Sry* induces testis formation, then inserting *Sry* DNA into the genome of a normal XX mouse zygote should cause that XX mouse to form testes. Koopman and colleagues (1991) took the 14-kilobase region of DNA that includes the *Sry* gene (and presumably its regulatory elements) and microinjected this sequence into the pronuclei of newly fertilized mouse zygotes. In several instances, XX embryos injected with this sequence developed testes, male accessory organs, and a penis* (Figure 14.6). There are thus good reasons to think that *Sry/SRY* is the major gene on the Y chromosome for testis determination in mammals.

SOX9: AN AUTOSOMAL TESTIS-DETERMINING GENE The *SRY* gene is probably active for only a few hours. During this time, it synthesizes the Sry transcription factor whose primary (and perhaps only) role is to activate the *SOX9* gene (see Sekido and Lovell-Badge 2008). *SOX9* is an autosomal gene involved in several developmental processes, most notably bone formation. In the genital ridge, however, *SOX9* induces testis formation. XX humans who have an extra copy of *SOX9* develop as males, even if they have no *SRY* gene, and XX mice transgenic for *Sox9* develop testes (Figure 14.7; Huang et al. 1999; Qin and Bishop 2005). Knocking out the *Sox9* genes in the gonads of XY mice causes complete sex reversal (Barrionuevo et al. 2006). Thus, even if Sry is present, mouse gonads cannot form testes if Sox9 is absent—so it appears that Sox9 can replace Sry in testis formation. This is not altogether surprising: although the *Sry* gene is found specifically in mammals, *Sox9* is found throughout the vertebrate phyla.

Indeed, *Sox9* appears to be the central male-determining gene in vertebrates. In mammals, it is activated by Sry; in birds, frogs, and fish, it appears to be activated by the dosage of Dmrt1 (see Sidelights & Speculations, p. 521); and in those vertebrates with temperature-dependent sex determination, it is often activated (directly or indirectly) by the male-producing temperature. *Sox9* may thus be the older and more central sex determination gene (Pask and

See WEBSITE 14.2
Finding the male-determining genes

*These embryos did not form functional sperm—but they were not expected to. The presence of two X chromosomes prevents sperm formation in XXY mice and men, and the transgenic mice lacked the rest of the Y chromosome, which contains genes needed for spermatogenesis. There is biochemical evidence (not yet tested in living mice) that Sry may have a second function, namely, to block β-catenin-mediated transcription (Bernard et al. 2008; Lau et al. 2009).

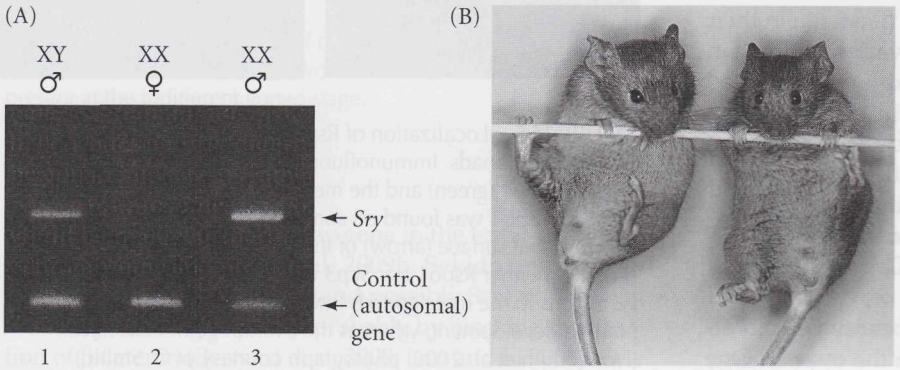


FIGURE 14.6 An XX mouse transgenic for *Sry* is male. (A) Polymerase chain reaction followed by electrophoresis shows the presence of the *Sry* gene in normal XY males and in a transgenic XX/*Sry* mouse. The gene is absent in a female XX littermate. (B) The external genitalia of the transgenic mouse are male (right) and are essentially the same as those in an XY male (left). (From Koopman et al. 1991; photographs courtesy of the authors.)

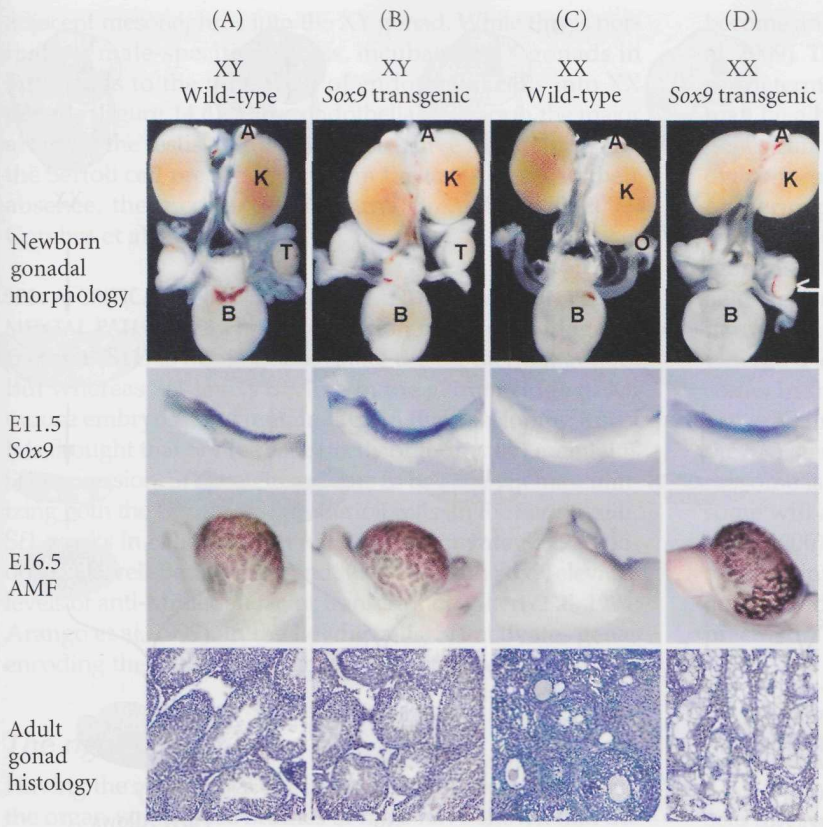


FIGURE 14.7 Ability of *Sox9* to generate testes. (A) A wild-type XY mouse embryo expresses *Sox9* in the genital ridge at 11.5 days, anti-Müllerian factor (AMF) in the embryonic gonad Sertoli cells at 16.5 days, and eventually forms descended testes (T) with seminiferous tubules. (B) An XY embryo with the *Sox9* transgene (a control for the effects of the transgene) also shows *Sox9* expression, AMF expression, and descended testes with seminiferous tubules. (C) The wild-type XX embryo shows neither *Sox9* expression nor AMF. It constructs ovaries with mature follicle cells. (D) An XX embryo with the *Sox9* transgene expresses the *Sox9* gene and has AMF in its 16.5-day Sertoli cells. It has descended testes, but the seminiferous tubules lack sperm (because of the presence of two X chromosomes in the Sertoli cells). K, kidneys; A, adrenal glands; B, bladder; T, testis; O, ovary. (From Vidal et al. 2001; photographs courtesy of A. Schedl.)

Graves 1999). Expression of the *Sox9* gene is specifically upregulated by the combined expression of Sry and Sf1 proteins in Sertoli cell precursors (Pask and Graves 1999; Sekido et al. 2004; Sekido and Lovell-Badge 2008). Thus, Sry may act merely as a "switch" operating during a very short time to activate *Sox9*, and the Sox9 protein may initiate the conserved evolutionary pathway to testis formation. So, borrowing Eric Idle's phrase, Sekido and Lovell-Badge (2009) propose that Sry initiates testis formation by "a wink and a nudge."

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The testis pathway

SRY: THE Y CHROMOSOME SEX DETERMINANT In humans, the major gene for testis determination resides on the short arm of the Y chromosome. Individuals born with the short but not the long arm of the Y chromosome are male, whereas individuals born with the long arm of the Y chromosome but not the short arm are female. By analyzing the DNA of rare XX men and XY women, the position of the testis-determining gene was narrowed down to a 35,000-base-pair region of the Y chromosome located near the tip of the short arm. In this region, Sinclair and colleagues (1990) found a male-specific DNA sequence that encodes a peptide of 223 amino acids. This gene is called ***SRY* (sex-determining *r*egion of the *Y* chromosome)**, and there is extensive evidence that it is indeed the gene that encodes the human testis-determining factor.

SRY is found in normal XY males and also in the rare XX males; it is absent from normal XX females and from many XY females. Approximately 15% of XY females have the *SRY* gene, but their copies of the gene contain point or frameshift mutations that prevent the Sry protein from binding to DNA (Pontiggia et al. 1994; Werner et al. 1995). If the *SRY* gene actually does encode the major testis-determining factor, one would expect that it would act in the genital ridge immediately before or during testis differentiation. This prediction has been found to be the case in studies of the homologous gene in mice. The mouse gene (*Sry*) also correlates with the presence of testes; it is present in XX males and absent in XY females (Gubbay et al. 1990; Koopman et al. 1990). The *Sry* gene is expressed in the somatic cells of the bipotential mouse gonads of XY mice immediately before the differentiation of these cells into Sertoli cells; its expression then disappears (Hacker et al. 1995; Sekido et al. 2004).

The most impressive evidence for *Sry* being the gene for testis-determining factor comes from transgenic mice. If *Sry* induces testis formation, then inserting *Sry* DNA into the genome of a normal XX mouse zygote should cause that XX mouse to form testes. Koopman and colleagues (1991) took the 14-kilobase region of DNA that includes the *Sry* gene (and presumably its regulatory elements) and microinjected this sequence into the pronuclei of newly fertilized mouse zygotes. In several instances, XX embryos injected with this sequence developed testes, male accessory organs, and a penis* (Figure 14.6). There are thus good reasons to think that *Sry/SRY* is the major gene on the Y chromosome for testis determination in mammals.

See WEBSITE 14.2
Finding the male-determining genes

SOX9: AN AUTOSOMAL TESTIS-DETERMINING GENE The *SRY* gene is probably active for only a few hours. During this time, it synthesizes the Sry transcription factor whose primary (and perhaps only) role is to activate the *SOX9* gene (see Sekido and Lovell-Badge 2008). *SOX9* is an autosomal gene involved in several developmental processes, most notably bone formation. In the genital ridge, however, *SOX9* induces testis formation. XX humans who have an extra copy of *SOX9* develop as males, even if they have no *SRY* gene, and XX mice transgenic for *Sox9* develop testes (Figure 14.7; Huang et al. 1999; Qin and Bishop 2005). Knocking out the *Sox9* genes in the gonads of XY mice causes complete sex reversal (Barrionuevo et al. 2006). Thus, even if Sry is present, mouse gonads cannot form testes if Sox9 is absent—so it appears that Sox9 can replace Sry in testis formation. This is not altogether surprising: although the *Sry* gene is found specifically in mammals, *Sox9* is found throughout the vertebrate phyla.

Indeed, *Sox9* appears to be the central male-determining gene in vertebrates. In mammals, it is activated by Sry; in birds, frogs, and fish, it appears to be activated by the dosage of Dmrt1 (see Sidelights & Speculations, p. 521); and in those vertebrates with temperature-dependent sex determination, it is often activated (directly or indirectly) by the male-producing temperature. *Sox9* may thus be the older and more central sex determination gene (Pask and

*These embryos did not form functional sperm—but they were not expected to. The presence of two X chromosomes prevents sperm formation in XXY mice and men, and the transgenic mice lacked the rest of the Y chromosome, which contains genes needed for spermatogenesis. There is biochemical evidence (not yet tested in living mice) that Sry may have a second function, namely, to block β-catenin-mediated transcription (Bernard et al. 2008; Lau et al. 2009).

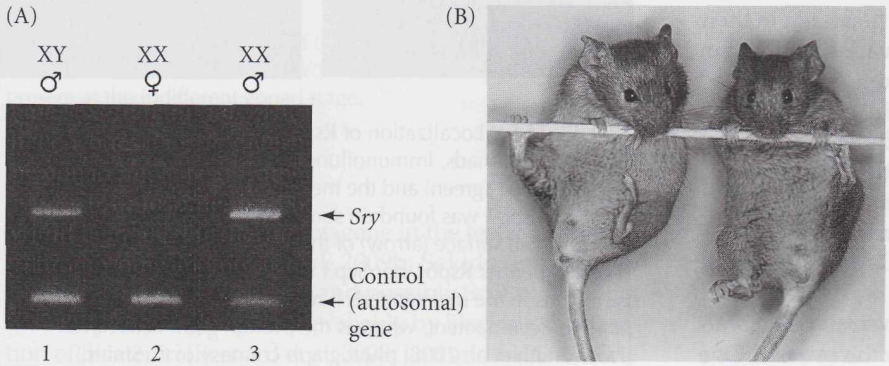
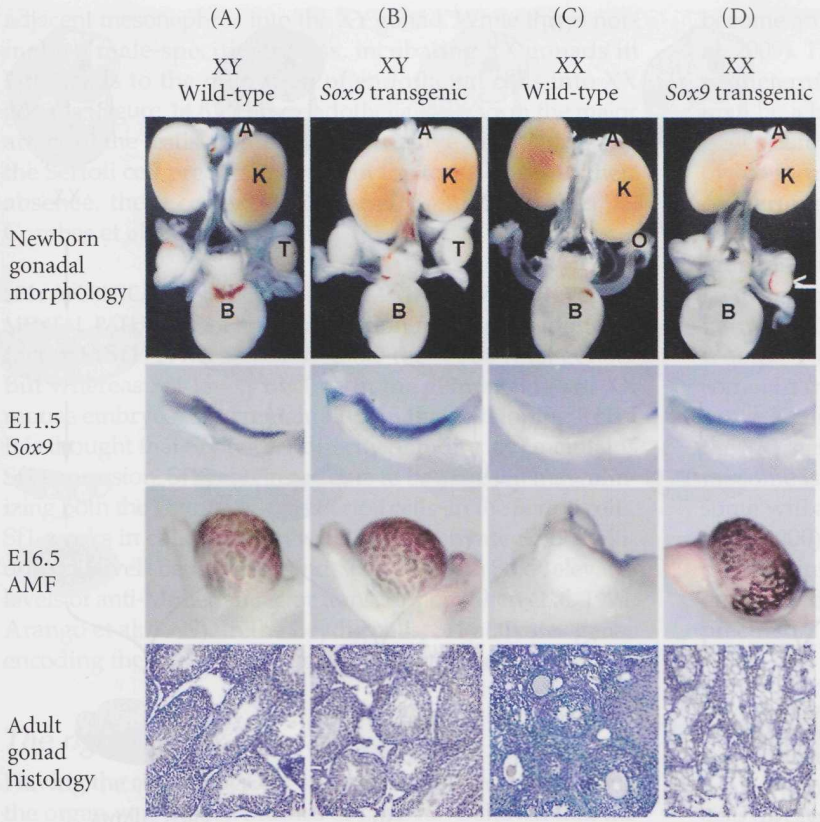


FIGURE 14.6 An XX mouse transgenic for *Sry* is male. (A) Polymerase chain reaction followed by electrophoresis shows the presence of the *Sry* gene in normal XY males and in a transgenic XX/*Sry* mouse. The gene is absent in a female XX littermate. (B) The external genitalia of the transgenic mouse are male (right) and are essentially the same as those in an XY male (left). (From Koopman et al. 1991; photographs courtesy of the authors.)



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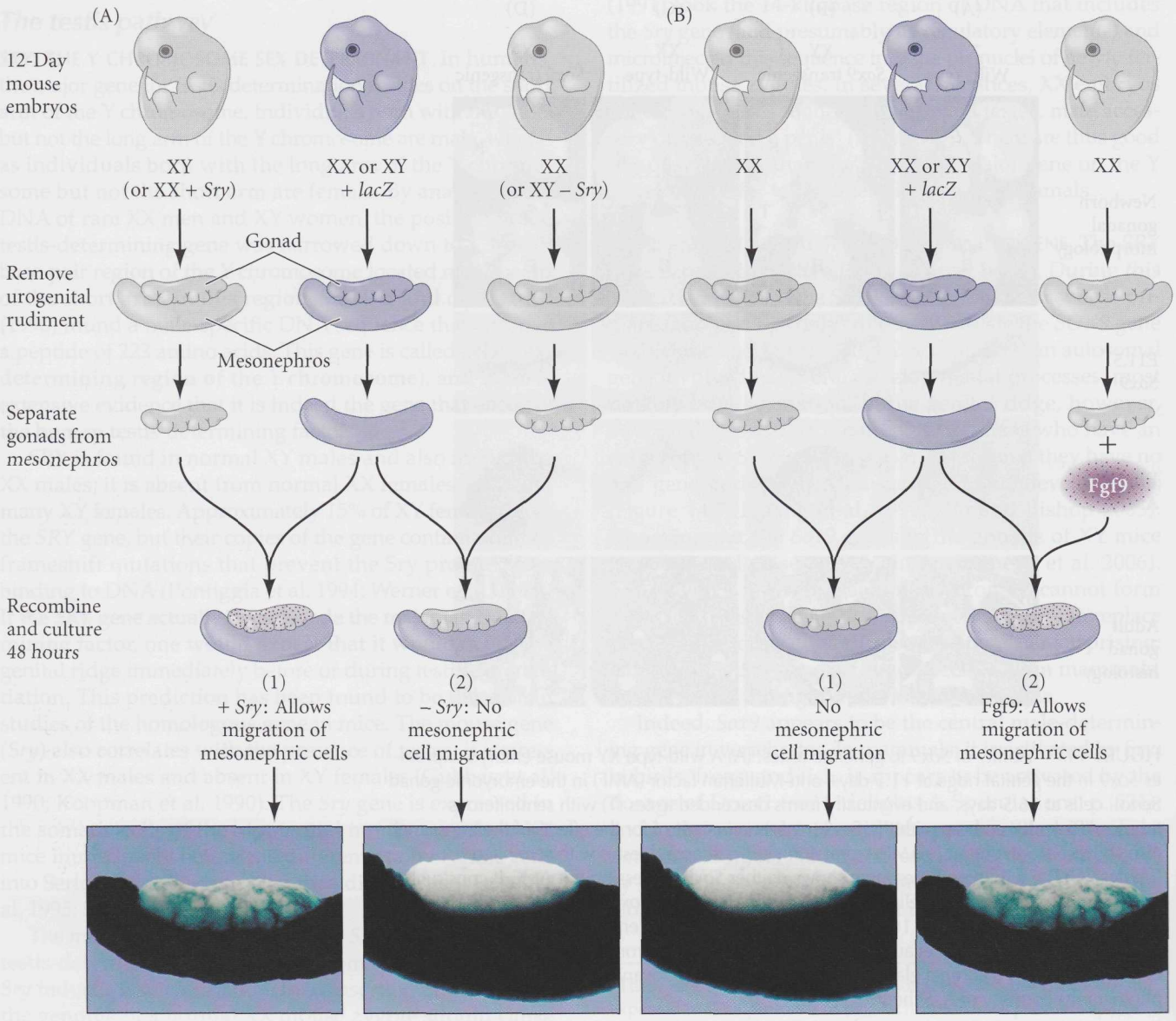

FIGURE 14.8 Migration of mesonephric endothelial cells into Sry^+ gonadal rudiments. In the experiment diagrammed, urogenital ridges (containing both the mesonephric kidneys and bipotential gonadal rudiments) were collected from 12-day embryonic mice. Some of the mice were marked with a β-galactosidase transgene (*lacZ*) that is active in every cell. Thus, every cell of these mice turned blue when stained for β-galactosidase. The gonad and mesonephros were separated and recombined, using gonadal tissue from unlabeled mice and mesonephros from labeled mice. (A) Migration of mesonephric cells into the gonad was seen (1) when the gonadal cells were XY or when they were XX with a *Sry* transgene. No migration of mesonephric tissue into the gonad was seen (2) when the gonad contained either XX cells or XY cells in which the Y chromosome had a deletion in the *Sry* gene. The sex chromosomes of the mesonephros did not affect the migration. (B) Gonadal rudiments for XX mice could induce mesonephric cell migration if these rudiments had been incubated with Fgf9. (C) Intimate relation between the mesonephric duct and the developing gonad in a 16-day male mouse embryo. The duct tissue will form the efferent ducts of the testes and has been stained for cytokeratin-8. (A,B after Capel et al. 1999, photographs courtesy of B. Capel; C from Sariola and Saarma 1999, courtesy of H. Sariola.)

adjacent mesonephros into the XY gonad. While this is normally a male-specific process, incubating XX gonads in Fgf9 leads to the migration of endothelial cells into XX gonads (Figure 14.8). These endothelial cells form the major artery of the testis and play an instructive role in inducing the Sertoli cell precursors to form the testis cords; in their absence, these cords do not form (Brennan et al. 2002; Combes et al. 2009).

SF1: A CRITICAL LINK BETWEEN SRY AND THE MALE DEVELOPMENTAL PATHWAYS The transcription factor **steroidogenic factor 1 (Sf1)** is necessary to make the bipotential gonad. But whereas Sf1 levels decline in the genital ridge of XX mouse embryos, they remain high in the developing testis. It is thought that Sry (either directly or indirectly) maintains Sf1 expression. Sf1 protein appears to be active in masculinizing both the Leydig and the Sertoli cells. In the Sertoli cells, Sf1 works in collaboration with Sry to activate Sox9 (Sekido and Lovell-Badge 2008) and, working with Sox9, elevates levels of anti-Müllerian factor transcription (Shen et al. 1994; Arango et al. 1999). In the Leydig cells, Sf1 activates genes encoding the enzymes that make testosterone.

The right time and the right place

Having the right genes doesn't necessarily mean you'll get the organ you expect. Studies of mice have shown that the *Sry* gene of some strains of mice failed to produce testes when bred onto a different genetic background (Eicher and Washburn 1983; Washburn and Eicher 1989; Eicher et al. 1996). This is attributed to a delay in Sry expression or to the failure of the protein to accumulate to the critical threshold level required to trigger Sox9 expression and launch the male pathway. By the time *Sox9* gets turned on, it is too late—the gonad is already following the path to become an ovary (Bullejos and Koopman 2005; Wilhelm et al. 2009). This provides an important clue to how primary sex determination may take place. Timing is critical. There may be a brief window during which the testis-forming genes can function. If these genes are not turned on, the ovary-forming pathway is activated.

Hermaphrodites are individuals in which both ovarian and testicular tissues exist; they have either ovotestes (gonads containing both ovarian and testicular tissue) or an ovary on one side and a testis on the other.* In humans, hermaphroditism is a very rare condition that can result when a Y chromosome is translocated to an X chromosome. In those tissues where the translocated Y is on the active X chromosome, the Y chromosome will be active and the *SRY* gene will be transcribed; in cells where the Y chromosome is on the inactive X chromosome, the Y chromosome will also be inactive (Berkovitz et al. 1992; Margarit et al. 2000). A gonadal mosaic for expression of *SRY* can develop into a testis, an ovary, or an ovotestis, depending on the percentage of cells expressing *SRY* in the Sertoli cell precursors (see Brennan and Capel 2004).

*Hermaphroditos was the young man in Greek mythology whose beauty inflamed the ardor of the water nymph Salmacis. She wished to be united with him forever, and the gods, in their literal fashion, granted her wish. The language used to group these conditions is being debated. Hermaphroditism has often been called an "intersex" phenotype; but some activists, physicians, and parents wish to eliminate the term "intersex" to avoid confusion of these anatomical conditions with identity issues such as homosexuality. They prefer to call these conditions "disorders of sex development." In contrast, other activists do not want to medicalize this condition and find the "disorder" category offensive to individuals who do not feel there is anything wrong with their health. For a more detailed analysis of intersexuality, see Gilbert et al. 2005 or the websites listed at the end of this chapter.

SIDELIGHTS & SPECULATIONS

Mysteries of Gonad Differentiation

There are some genes that are definitely involved in primary sex determination, but we don't know how they act or what their normal functions are.

DAX1: A potential testis-suppressing gene on the X chromosome

One of the most puzzling loci in the catalog of sex-determining genes is *DAX1*, located on the X chromosome. In XY humans having a duplication of the *WNT4* region, Dax1 protein is overproduced and the gonads develop into ovaries despite the presence of the Y chromosome (Jordan et al. 2001). Moreover, in XY individuals having a duplication of the *DAX1* gene on their X chromosome, the *SRY* signal is inhibited (Figure 14.9; Bernstein et al. 1980; Bardoni et al. 1994). In XY embryos, *DAX1* normally would be suppressed; having two active copies of the gene, however, overrides this suppression.

The mouse homologue, *Dax1*, was cloned and shown to encode a member of the nuclear hormone receptor family (Muscatelli et al. 1994; Zanaria 1994). In mice, *Dax1* is initially expressed in the genital ridges of both male and female embryos prior to the

(Continued on next page)

SIDELIGHTS & SPECULATIONS (Continued)

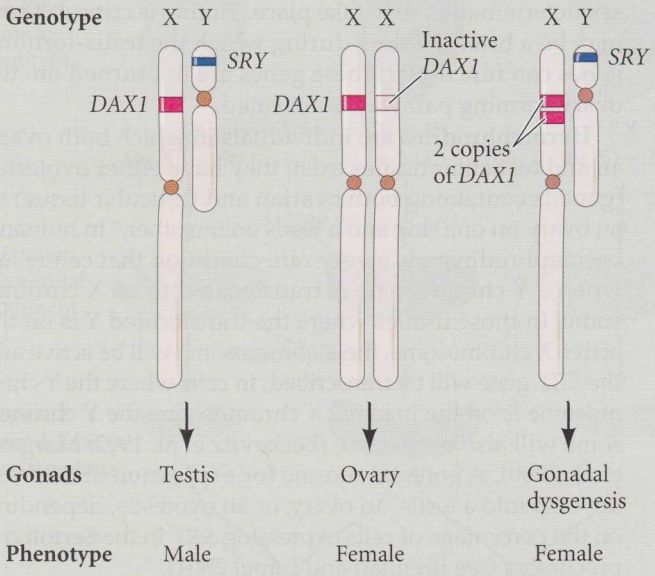

Figure 14.9 Phenotypic sex reversal in humans having two copies of the *DAX1* locus. *DAX1* (on the X chromosome) plus *SRY* (on the Y chromosome) produces testes. *DAX1* without *SRY* (since the other *DAX1* locus is on the inactive X chromosome) produces ovaries. Two active copies of *DAX1* (on the active X chromosome) plus *SRY* (on the Y chromosome) lead to a poorly formed gonad. Since the gonad makes neither anti-Müllerian factor nor testosterone, the phenotype is female. (From Genetics Review Group 1995.)

time that sex determination occurs, but gradually its expression becomes female-specific. As predicted from the data in humans, overexpression of *Dax1* in male mice appears to antagonize the function of *Sry* and *Sox9* and downregulates *Sf1* expression (Nachtigal et al. 1998; Swain et al. 1998; Iyer and McCabe 2004). However, unexpectedly, the *loss* of *Dax1* does not disrupt ovary development, but instead impairs testis formation (Meeks et al. 2003; Bauma et al. 2005). The apparent contradictory roles of this protein are still not understood.

Dmrt genes: Highly conserved testis-determining genes

The *Dmrt* (doublesex and mab-3-related transcription factor) genes are involved in male sex determination throughout the animal kingdom (Raymond et al. 1998). The male-specific Doublesex (Dsx^M) protein in *Drosophila* (which will be discussed later in the chapter) has a region of amino acids similar to the male-specific regulatory protein Mab-3 in *Caenorhabditis elegans*. Both proteins positively regulate male-specific development. Moreover, if the male-specific *doublesex* transcript from *Drosophila* is inserted into *C. elegans* males lacking *mab-3*, the newly introduced *Drosophila* message will restore male-specific neurogenesis to the mutant *C. elegans* strain at levels the same as the introduction of *mab-3*. The female-specific *doublesex* transcript will not work.

Dmrt genes play a critical role in the primary sex determination of Medaka fish, where sex is chromosomally specified by the Y chromosome (Matsuda et al. 2002). Here a *Dmrt* gene on the Y chromosomes encodes a protein that is made in the somatic cells of the developing testes (and not ovaries) and whose function is critical for testis formation. It probably binds to and activates the *Sox9* gene (Bagheri-Fam et al. 2009). In the chicken (and other birds), the male is the homogametic sex (ZZ), and the female is the heterogametic sex (ZW). Smith and colleagues (2009) have shown that the *Dmrt1* gene on the Z chromosome is the major male sex-determining gene, and that a double dose of this gene is needed for the gonad to become a testis. *Dmrt1*, too, appears to activate (either directly or indirectly) the *Sox9* gene.

In mammals (including humans), *Dmrt1*, which is autosomal, appears to function later in testis development. It is no longer involved in specification of the testis, rather it is important in testis differentiation. Kim and colleagues (2007; Hong et al. 2007) showed that *Dmrt1* in mice is not critical for sex determination but is crucial for Sertoli cell differentiation and germ cell survival.

Sex-specific microRNAs

There is evidence that sexual distinctions exist even at the blastocyst stage. Tavakoli and colleagues (2009) have found that XX embryonic stem cells appear to have greater differentiation potential (and slower growth rates) than XY stem cells. This difference may be the result of different populations of microRNAs found in the XY and XX embryonic stem cells. Ciaudo and colleagues (2009) have found that XX and XY human embryonic stem cells transcribe different populations of microRNAs, whose functions, though overlapping, may differentially regulate genes involved in pluripotency and cell cycling.

The apparent relationship between cell division rates and sex determination is provocative. Indeed, the rapid proliferation of Sertoli cell precursors has been hypothesized to be the unifying feature in testis determination (Mittwoch 1986; Schmahl and Capel 2003). In its most extreme form, this hypothesis predicts that anything that increases the division rate of somatic cell precursors in the gonad will direct gonadogenesis in the male direction. While we do not know if this is true under all conditions, we do know that in mice, one of the first distinguishing features of testis development is an increase in cell proliferation seen immediately after *Sry* expression. There appears to be a threshold number of Sertoli cells needed for mammalian testis development (Schmahl et al. 2000). In turtles and alligators, rapid division of Sertoli cell precursors is initiated during the critical stage for forming the males (Mittwoch 1986; Schmahl et al. 2003). From this perspective, anything that increases the division rate of Sertoli cell precursors might direct gonadogenesis in the male direction.

Secondary Sex Determination in Mammals: Hormonal Regulation of the Sexual Phenotype

Primary sex determination is the formation of either an ovary or a testis from the bipotential gonad. This process, however, does not give the complete sexual phenotype. In mammals, secondary sex determination is the development of the female and male phenotypes in response to hormones secreted by the ovaries and testes. Both female and male secondary sex determination have two major temporal phases. The first phase occurs within the embryo during organogenesis; the second occurs at puberty.

During embryonic development, hormones and paracrine coordinate the development of the gonads with the development of secondary sexual organs. In females, the Müllerian ducts persist and, through the actions of estrogen, will later differentiate to become the uterus, cervix, oviducts, and upper vagina (see Figure 14.3). The **genital tubercle** becomes differentiated into the clitoris, and the **labioscrotal folds** become the labia. The Wolffian ducts atrophy in females, since they need testosterone to persist, and become the male accessory organs. As mentioned earlier, if the bipotential gonads are removed from an embryonic mammal, the female phenotype is realized: the Müllerian duct develops and the Wolffian ducts degenerate. This pattern is also seen in certain humans who are born without functional gonads.

The coordination of the male phenotype involves the secretion of two testicular factors. The first of these is AMF, a BMP-like paracrine factor made by the Sertoli cells that causes the degeneration of the Müllerian duct. The second is the steroid hormone testosterone, which is secreted from the fetal Leydig cells. Testosterone causes the Wolffian ducts to differentiate into the epididymis, vas deferens, and seminal vesicles, and it causes the genital tubercle to develop into the penis and the labioscrotal folds to develop into the scrotum.

The mechanism by which testosterone (and, as we shall see, its more powerful derivative, dihydrotestosterone) masculinizes the external genitalia is thought to involve its interaction with the Wnt pathway (Figure 14.10). The Wnt pathway, which in the genital *ridge* activates the female trajectory, acts in the genital *tubercle* to activate male development. In one recent model, the mesenchyme in the XX urogenital swellings is seen to make inhibitors of the Wnt pathway (such as Dickkopf). This prevents the activity of Wnt in the mesenchyme and leads to the feminization of the genital tubercle by estrogens (Holderegger and Keefer 1986; Miyagawa et al. 2009). In this case, the genital tubercle becomes the clitoris and the labioscrotal folds become the labia majora. In males, however, testosterone and dihydrotestosterone bind to the androgen (testosterone) receptor in the mesenchyme and prevent expression of the Wnt inhibitors. Thus, when Wnt expression is permitted in the mesenchyme, male urogenital swellings are converted into the penis and the scrotum.

The genetic analysis of secondary sex determination

The existence of separate and independent AMF and testosterone pathways of masculinization is demonstrated by people with **androgen insensitivity syndrome**.

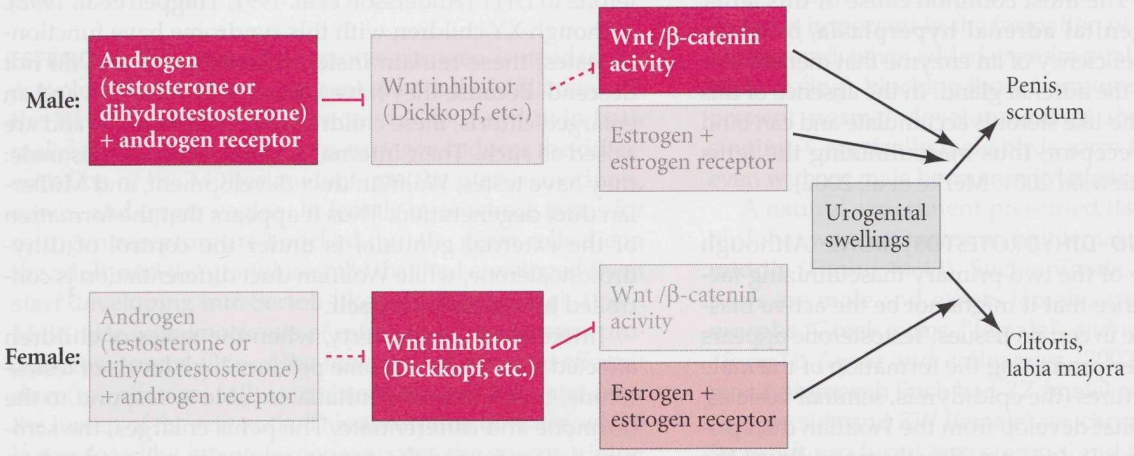

FIGURE 14.10 Model for the formation of external genitalia. In this schema, the mesenchyme in the urogenital swellings secretes inhibitors of Wnt signaling. In the absence of Wnt signaling, estrogen modifies the genital tubercle into the clitoris and the labioscrotal folds into the labia majora surrounding the vagina. In males, however, androgens (such as testosterone and dihydrotestosterone) bind to the androgen receptor in the mesenchymal cells and prevent the synthesis of the Wnt inhibitors. Wnt signaling is permitted, and it causes the genital tubercle to become the penis and the labioscrotal folds to become the scrotum. (After Miyagawa et al. 2009.)

These XY individuals have the *SRY* gene and thus have testes that make testosterone and AMF. However, they have a mutation in the gene encoding the androgen receptor protein that binds testosterone and brings it into the nucleus. Therefore these individuals cannot respond to the testosterone made by their testes (Meyer et al. 1975). They can, however, respond to the estrogen made by their adrenal glands (which is normal for both XX and XY individuals), so they develop female external sex characteristics (Figure 14.11). Despite their distinctly female appearance, these individuals have testes, and even though they cannot respond to testosterone, they produce and respond to AMF. Thus, their Müllerian ducts degenerate. Persons with androgen insensitivity syndrome develop as normal-appearing but sterile women, lacking a uterus and oviducts and having internal testes in the abdomen.

Such phenotypes, in which male and female traits are seen in the same individual, are called **intersex** conditions. Indeed, although most people have a reasonably good correlation of their genetic and anatomical sexual phenotypes, about 0.4–1.7% of the population differs from the strictly dimorphic condition (Blackless et al. 2000; Hull 2003). Androgen insensitivity syndrome is one of several intersex conditions that have traditionally been labeled **pseudohermaphroditism**. In pseudohermaphrodites, there is only one type of gonad, but the secondary sex characteristics differ from what would be expected from the gonadal sex. In humans, male pseudohermaphroditism (wherein the gonadal sex is male and the secondary sex characteristics are female) can be caused by mutations in the androgen (testosterone) receptor or by mutations affecting testosterone synthesis (Geissler et al. 1994).

Female pseudohermaphroditism (in which the gonadal sex is female but the person is outwardly male) can be caused by the overproduction of androgens in the ovary or adrenal gland. The most common cause of this latter condition is **congenital adrenal hyperplasia**, in which there is a genetic deficiency of an enzyme that metabolizes cortisol steroids in the adrenal gland. In the absence of this enzyme, testosterone-like steroids accumulate and can bind to the androgen receptor, thus masculinizing the fetus (Migeon and Wisniewski 2001; Merke et al. 2002).

TESTOSTERONE AND DIHYDROTESTOSTERONE Although testosterone is one of the two primary masculinizing factors, there is evidence that it might not be the active masculinizing hormone in certain tissues. Testosterone appears to be responsible for promoting the formation of the male reproductive structures (the epididymis, seminal vesicles, and vas deferens) that develop from the Wolffian duct primordium. However, it does not directly masculinize the male urethra, prostate, penis, or scrotum. These latter functions are controlled by **5α-dihydrotestosterone**, or **DHT** (Figure 14.12). Siiteri and Wilson (1974) showed that testosterone is converted to DHT in the urogenital sinus and swellings, but not in the Wolffian duct. DHT appears to be a more potent hormone than testosterone.

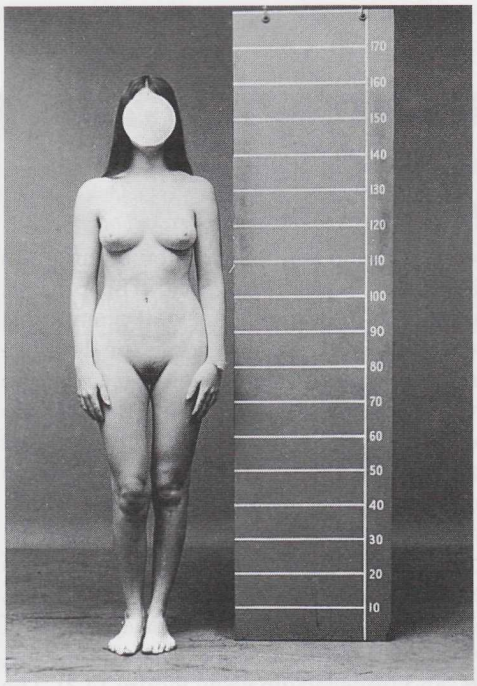

FIGURE 14.11 Androgen insensitivity syndrome. Despite having the XY karyotype, individuals with this syndrome appear female. They cannot respond to testosterone but can respond to estrogen, so they develop female secondary sex characteristics (i.e., labia and a clitoris rather than a scrotum and a penis). Internally, they lack the Müllerian duct derivatives and have undescended testes. (Courtesy of C. B. Hammond.)

The importance of DHT was demonstrated by Imperato-McGinley and her colleagues (1974). They found a small community in the Dominican Republic in which several inhabitants lacked a functional gene for the enzyme 5α-ketosteroid reductase 2—the enzyme that converts testosterone to DHT (Andersson et al. 1991; Thigpen et al. 1992). Although XY children with this syndrome have functional testes, these remain inside the abdomen and do not descend. Because they have a blind vaginal pouch and an enlarged clitoris, these children appear to be girls, and are raised as such. Their internal anatomy, however, is male: they have testes, Wolffian duct development, and Müllerian duct degeneration. Thus it appears that the formation of the external genitalia is under the control of dihydrotestosterone, while Wolffian duct differentiation is controlled by testosterone itself.

Interestingly, at puberty, when the testes of children affected with this syndrome produce high levels of testosterone, their external genitalia are able to respond to the hormone and differentiate. The penis enlarges, the scrotum descends, and the person originally believed to be a girl is revealed to be a young man.

See WEBSITE 14.3
Dihydrotestosterone in adult men

See WEBSITE 14.4
Insulin-like hormone 3

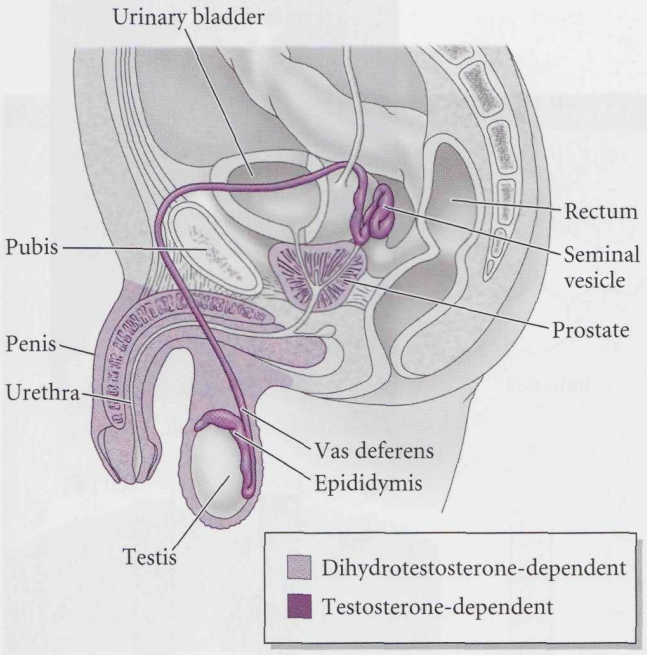

FIGURE 14.12 Testosterone- and dihydrotestosterone-dependent regions of the human male genital system. (After Imperato-McGinley et al. 1974.)

ANTI-MÜLLERIAN FACTOR Anti-Müllerian factor, or AMF, is a member of the TGF-β family of growth and differentiation factors. It is secreted from the fetal Sertoli cells and causes the degeneration of the Müllerian duct (Tran et al. 1977; Cate et al. 1986). AMF is thought to bind to the mesenchyme cells surrounding the Müllerian duct, causing these cells to secrete a paracrine factor that induces apoptosis in the duct's epithelium and breaks down the basal lamina surrounding the duct (Trelstad et al. 1982; Roberts et al. 1999).

ESTROGEN The steroid hormone estrogen is needed for complete postnatal development of both the Müllerian and the Wolffian ducts, and is necessary for fertility in both males and females. In females, estrogen induces the differentiation of the Müllerian duct into the uterus, oviducts, cervix, and upper vagina. In female mice whose genes for estrogen receptors are knocked out, the germ cells die in the adult, and the granulosa cells that had enveloped them start developing into Sertoli-like cells (Couse et al. 1999). Male mice with knockouts of estrogen receptor genes produce few sperm. One of the functions of the male efferent duct (vas efferens) cells is to absorb most of the water from the lumen of the rete testis. This absorption of water, which is regulated by estrogen, concentrates the sperm, giving them a longer life span and providing more sperm per ejaculate. If estrogen or its receptor is absent in male mice, water is not absorbed and the mouse is sterile (Hess et al. 1997). Although, in general, blood concentrations of estrogen are higher in females than in males, the concentration of estrogen in the rete testis is even higher than in female blood.

Brain sex: Secondary sex determination through another pathway?

We have known for a long time that the brain, like other tissues, is responsive to the steroid hormones produced by the gonads. Now, recent evidence suggests that sex differences in the brain can be observed even before the gonads mature, and that the brain may experience direct regulation by the X and Y chromosomes (Arnold and Burgoyne 2004).

The first indication that something besides the gonadal hormones testosterone and estrogen was important in forming sexually different structures in the brain came from studies on Parkinson disease, during which embryonic rat brains were dissected before the gonads matured. These studies indicated that brains from XX embryos had more epinephrine-secreting neurons than XY embryonic brains (Beyer et al. 1991). Later studies, using microarrays and PCR, demonstrated that more than 50 genes in the mouse brain are expressed in sexually dimorphic patterns *before gonad differentiation has occurred* (Dewing et al. 2003). Moreover, the mouse *Sry* gene, in addition to being expressed in the embryonic testes, is also expressed in the fetal and adult brain. The human *SRY* gene appears to be expressed in the adult brain as well (Lahr et al. 1995; Mayer et al. 1998, 2000). *SRY* is specifically active in the substantia nigra of the male hypothalamus, where it helps regulate the gene for tyrosine hydoxylase, an enzyme that is critical for the production of the neurotransmitter dopamine (Dewing et al. 2006).

Stunning demonstrations that sexual dimorphism in the brain can be caused before gonadal hormone synthesis come from natural and experimental conditions in birds. One big difference between male and female finches is that large regions of the male brain are devoted to producing songs. Male finches sing; the females do not. While testosterone is important in the formation of the song centers in finches (and, when added experimentally, can cause female birds to sing), blocking those hormones in males does not prevent normal development of the song centers or singing. Genetically male birds form these brain regions even without male hormones (Mathews and Arnold 1990).

A natural experiment presented itself in the form of a bird that was half male and half female, divided down the middle (Figure 14.13). Such animals, where some body parts are male and others female, are called **gynandromorphs** (Greek *gynos*, "female"; *andros*, "male"; *morphos*, "form"). Agate and colleagues (2002) showed that the gynandromorph finch had ZZ (male) sex chromosomes on its right side and ZW (female) sex chromosomes on its left. Its testes produced testosterone, and the bird sang like a male and copulated with females. However, although many brain structures were similar on both sides, some brain regions differed between the male and female halves. The song circuits on the right side had a more masculine phenotype than similar structures on the left, showing that both intrinsic and hormonal influences were important.

Gahr (2003) generated his own avian sexual chimeras by surgically switching the forebrain regions (which control adult sexual behaviors) between ZZ and ZW quail embryos before their gonads had matured. If hormones were all that mattered, the brains of the resulting birds would be appropriate to the gonad that developed. For the females that received male forebrains, this was indeed the case: they looked and behaved like normal female quail. However, male birds given female forebrains did not act normally; they did not crow to attract mates, nor did they attempt copulation. Moreover, their testes failed to develop normally, suggesting that (in quail, at least) a chromosomally male brain is needed to complete development of the testes.

Thus, although brain sex is usually correlated with gonadal sex, it seems probable that this harmony is created both by intrinsic, cell-autonomous differences as well as hormonal regulation from outside the cell.*

*The sexual characteristics of the tammar wallaby (*Macropus eugenii*) appear to be another case where secondary sex traits are controlled intrinsically by chromosomal genes rather than extrinsically by circulating hormones. In this marsupial, the pouch and its mammary glands are found only in the female, while the scrotum is made only in males. However, the marsupial pouch, mammary glands, and scrotum are each made before the gonads have matured and are producing hormones (O et al. 1988; Glickman et al. 2005). Renfree and Short (1988) showed that the number of X chromosomes determines whether the wallaby develops a pouch and mammary glands or develops a scrotum for its still undeveloped testes.

FIGURE 14.13 Gynandromorph finch with ZZ (male) cells on its right side and ZW (female) cells on its left side. Since plumage is controlled by genes on the sex chromosomes, the adult finch has male plumage on its right and female plumage on its left. Micrographs show the difference in brain regions between the right and left sides, indicated by staining of the neurons of the HVC nucleus (a neuron cluster involved in bird song production). (From Agate et al. 2002.)

SIDELIGHTS & SPECULATIONS

Brain Sex: Sex Determinants and Behaviors in Mice and Rats

Does prenatal (or neonatal) exposure to particular steroid hormones impose permanent sex-specific changes on the central nervous system? Such sex-specific neural changes have been shown in regions of the brain that regulate involuntary sexual physiology. The cyclic secretion of luteinizing hormone by the pituitary in adult female rats, for example, is dependent on the lack of testosterone during the first week of the animal's life. The luteinizing hormone secretion of female rats can be made noncyclical by giving them testosterone 4 days after birth. Conversely, the luteinizing hormone secretion of males can be made cyclical by removing their testes within a day of birth (Barraclough and Gorski 1962).

α-Fetoprotein and the organization/activation hypothesis

It is believed that sex hormones act during the fetal or neonatal stage of a mammal's life to organize the nervous system in a sex-specific manner; and that during adult life, the same hormones may have transitory motivational (or "activational") effects. This model of the hormonal basis of sex-specific brain development and behavior is called the **organization/activation hypothesis**. Ironically, the hormone chiefly responsible for determining the male neural pattern is **estradiol**, a type of estrogen. Testosterone from fetal or neonatal blood can be converted into estradiol by the enzyme **aromatase** (Figure 14.14A).* This conversion

*The terms *estrogen* and *estradiol* are often used interchangeably. However, estrogen refers to a class of steroid hormones responsible (among other functions) for establishing and maintaining specific female characteristics. Estradiol is one of these hormones, and in most mammals (including humans) it is the most potent of the estrogens. The enzyme's name, *aromatase*, has nothing to do with aroma (although aromas are certainly crucial to rodent sex), but refers to the destabilization of hydrogen bonds in the steroid ring structure.

SIDELIGHTS & SPECULATIONS (Continued)

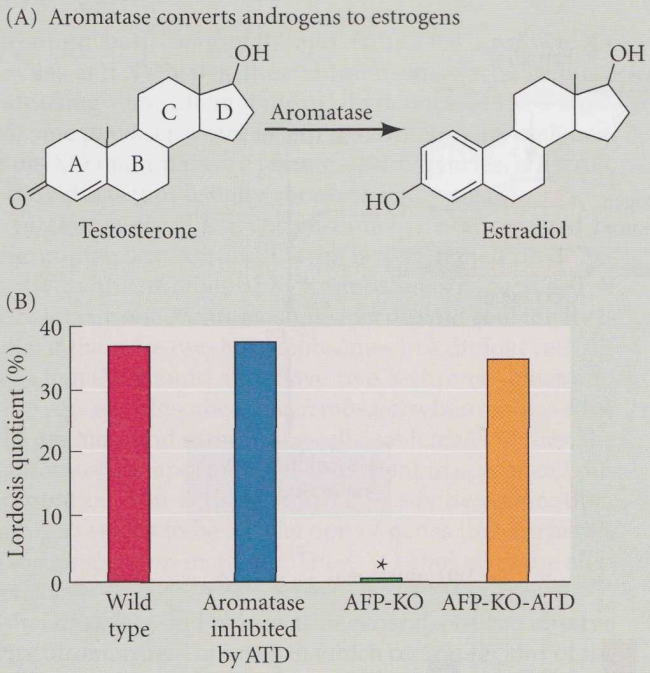

Figure 14.14 Organization of brain development by hormones. (A) The enzyme aromatase converts androgens (such as testosterone) into estrogens (such as estradiol). The name of the enzyme comes from its ability to aromatize the six-carbon ring by reducing the ring-stabilizing keto group (=O) to a hydroxyl group (—OH). This biochemical change allows the hormones to bind to different receptors (dormant transcription factors) and activate different genes. (B) Female lordosis behavior (in which the female mouse bends her spine so the male can readily mate with her) remains present in mice administered an inhibitor of aromatase; the behavior is abolished when the genes for α-fetoprotein are knocked out (AF–KO). However, when aromatase is blocked in female mice without α-fetoprotein (AFP–KO–ATD), lordosis behavior is restored. (After Bakker and Baum 2008.)

occurs in the hypothalamus and limbic system—two areas of the brain known to regulate hormone secretion and reproductive behavior (Reddy et al. 1974; McEwen et al. 1977). Thus, *testosterone exerts its effects on the* nervous system by being converted into estradiol in the brain.

But the fetal environment is rich in estrogens from the gonads and placenta. What stops these estrogens from masculinizing the nervous system of a female fetus? In both male and female rats, fetal estrogen is bound by **α-fetoprotein**, which binds and inactivates estrogen, but not testosterone. Human fetuses, by contrast, do not make a strong estrogen-binding protein and have a much higher level of free estrogen than do rodent embryos (see Nagel and vom Saal 2003). So although the organization/activation hypothesis explains many of the hormonal effects on rodent development, one of its fundamental assumptions—that α-fetoprotein strongly binds estrogens during prenatal development—does not in fact hold true for humans.

Relationships among estradiol, aromatase, and α-fetoprotein have been analyzed by observing sexual behaviors in mice that have loss-of-function mutations for aromatase and α-fetoprotein. The brain and the behaviors of mice lacking α-fetoprotein have been defeminized, showing that α-fetoprotein prevents the female brain from receiving circulating estrogens.

Indeed, female mice whose α-fetoprotein genes have been knocked out are sterile because the brain genes controlling ovulation (such as those for gonadotropin-releasing hormone) are downregulated. However, this lack of ovulation can be cured (and the normal female pattern of gene expression established) if such mice are also given drugs that block aromatase. Similarly, the amount of lordosis (a swayback posture taken by female rodents that permits males to mate with them) is almost completely abrogated in female mice lacking functional α-fetoprotein genes. This behavior, too, can be restored by treating the mice prenatally with aromatase inhibitors (Figure 14.14B; Bakker et al. 2006; De Mees et al. 2006; Bakker and Baum 2008).

While the *prenatal* lack of estrogen and testosterone may be critical for the formation of female brains, the feminization of the rodent brain may require estrogens *after* birth. This is suggested by the behavioral phenotypes of mice whose aromatase genes have been knocked out. Their female-specific behaviors (lordosis; the ability to discriminate male pheromones) are also impaired (Bakker and Baum 2008).

Pheromones

Pheromones appear to play a major role in sexual behaviors in rodents. If the vomeronasal organ (responsible for sensing pheromones) or the genes involved in pheromone recognition are removed from male mice, they fail to discriminate between males and females and attempt to mate with both. If this pheromone recognition system is removed from female mice, they lack certain female behaviors and acquire the full set of male courtship behaviors (including mounting, pelvic thrusting, and solicitation of females). *In females, pheromones act to repress* male behavior patterns and promote female-specific actions. Thus, it appears that the neural circuitry for both male and female behaviors exists in each mouse brain, but the interpretation of pheromone signals is what distinguishes male from female brains. In females, the "feminine" pattern of behavior is activated (sexual receptivity to males, lactating behavior toward pups), while the "masculine" pattern of behavior (fight if male, mount if female) is repressed. In males, the pheromones activate this "masculine" pattern, while the "feminine" pathway is suppressed (Kimchi et al. 2007). The interpretation of pheromone signals is thought to take place in the medial preoptic area/anterior hypothalamus region of the brain, and we know this region to be sexually dimorphic as a result of prenatal estrogen exposure. Thus, the organizational abilities of

SIDELIGHTS & SPECULATIONS (Continued)

testosterone may act largely to effect changes in this small area of the brain, and once this region is organized, it will interpret the pheromone signals to activate either the male or the female sets of neurons (Baum 2009).

The role of testosterone

If testosterone's conversion to estrogen is involved in brain masculinization, then what exactly is going on? It appears that many of testosterone's orders are carried out by **prostaglandin E2** (**PGE2**). PGE2 is made from arachidonic acid by the enzyme **cyclooxygenase-2** (**COX2**); COX2 is induced by estrogen in the brain. So estrogen acts to produce more PGE2 (Figure 14.15A).

Studies by Amateau and McCarthy (2004) demonstrated that PGE2 is stimulated by estradiol in the newborn rat brain, and that PGE2 induces the growth and differentiation of those regions of the brain involved in male sexual behaviors. They found that PGE2 was as effective as estrogen in masculinizing these brain regions, and that PGE2 induces male-specific morphology and behaviors when injected into female rat brains. Moreover, when male rats were given COX2 inhibitors, their brains became similar to those of females. Not only did their brain anatomy change, but so did their behaviors. The PGE2-treated females acted like males, attempting to mount and copulate with other females, while male mice treated with COX2 inhibitors lost their sexual drive and did not exhibit male behaviors (Figure 14.15B). Studies comparing the structure of brain regions responding to pheromones in mice treated with PGE2 or estrogens indicate that the processing of pheromone signals is a sexually dimorphic phenomenon regulated by early exposure to PGE2 and estradiol.

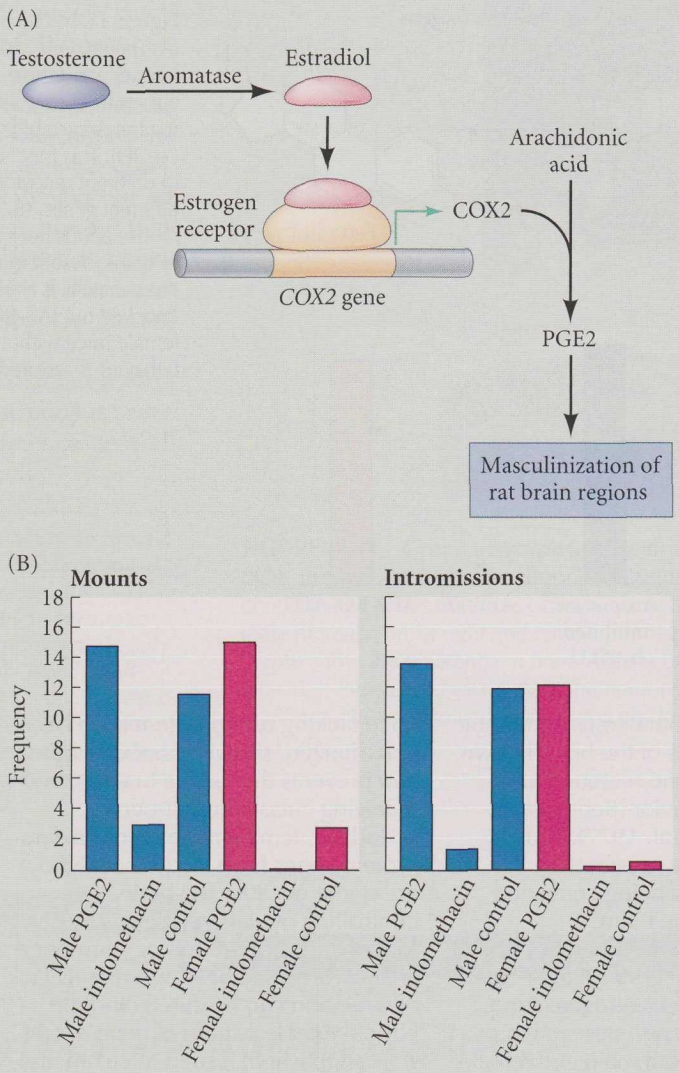

Figure 14.15 Masculinization of the brain by hormones. (A) Testosterone mediates its effect on the rodent brain by being converted to estradiol by aromatase. Estradiol activates the gene for cyclooxygenase, which converts arachidonic acid to PGE2. (B) PGE2 promotes male behavior, and its inhibition by COX2 inhibitors prevents that behavior. Males treated with indomethacin (COX2 inhibitor) did not display the mounting behavior expected of males, nor did they attempt intercourse ($P < 0.001$ between control and treated males). Conversely, females given PGE2 acted like control males in both cases and had significantly more mounting and mating behavior than control females ($P < 0.001$). (After Amateau and McCarthy 2004.)

The human element

Extrapolating from rodents to humans is a very risky business. No sex-specific behavior has yet been identified in humans, humans do not use α-fetoprotein to bind circulating estradiol, and humans do not use pheromones as a primary sexual attractant (sight and touch being far more critical). No "gay gene" has been discovered, and the concordance of gender identity between identical twins is only 30%—far from the 100% expected if sexual orientation were strictly genetic (Bailey et al. 2000; CRC 2006). Moreover, behaviors that are seen as "masculine" in one culture may be considered "feminine" in another, and vice versa (see Jacklin 1981; Bleier 1984; Fausto-Sterling 1992; Kandel et al. 1995). How humans acquire their gendered behavior appears to involve a remarkably complex set of interactions between genes and environment. Like many other behavioral phenotypes, how individuals acquire sexual behaviors has yet to be determined.

Chromosomal Sex Determination in *Drosophila*

Although both mammals and fruit flies produce XX females and XY males, their chromosomes achieve these ends using very different means. In mammals, the Y chromosome plays a pivotal role in determining the male sex. Thus, XO mammals are females, with ovaries, a uterus, and oviducts (but usually very few, if any, ova).

In *Drosophila*, the Y chromosome is not involved in determining sex. A fruit fly's sex is determined predominantly by the number of X chromosomes in each cell. If there is only one X chromosome in a diploid cell, the fly is male. If there are two X chromosomes in a diploid cell, the fly is female. Should a fly have two X chromosomes and three sets of autosomes, it is a mosaic, where some of the cells are male and some of the cells are female. In flies, the Y chromosome appears to be important in sperm cell differentiation, but it plays no role in sex determination. Rather, it seems to be a collection of genes that are active in forming sperm in adults. Thus, XO *Drosophila* are sterile males.

In *Drosophila*, and in insects in general, one can observe gynandromorphs—animals in which certain regions of the body are male and other regions are female (Figure 14.16; see also Figure 14.13). Gynandromorph fruit flies result when an X chromosome is lost from one embryonic nucleus. The cells descended from that cell, instead of being XX (female), are XO (male). The XO cells display male characteristics, whereas the XX cells display female traits, suggesting that, in *Drosophila*, each cell makes its own sexual "decision." Indeed, in their classic discussion of gynandromorphs, Morgan and Bridges (1919) concluded, "Male and female parts and their sex-linked characters are strictly self-determining, each developing according to its own aspiration," and each sexual decision is "not interfered with by the aspirations of its neighbors, nor is it overruled by the action of the gonads."

Although there are organs that are exceptions to this rule (notably the external genitalia), it remains a good general principle of *Drosophila* sexual development. Moreover, molecular data show that differential RNA splicing to create male- and female-specific transcription factors is critical in determining fly sex and can explain how XX individuals become females and XY individuals become males.

The Sex-lethal *gene*

Although it had long been thought that a fruit fly's sex was determined by the X-to-autosome (X:A) ratio (Bridges 1925), this assessment was based largely on the fact that flies have aberrant numbers of chromosomes. Recent molecular analyses suggest that X chromosome number alone is the primary sex determinant in normal diploid insects (Erickson and Quintero 2007). The main basis for this assertion is the fact that the X chromosome contains genes encoding transcription factors that activate the critical gene in *Drosophila* sex determination, the autosomal locus ***Sex-lethal*** (***Sxl***).

ACTIVATING *SEX-LETHAL* The *Sxl* gene has two promoters. The early promoter is active only in XX cells; the later one is active in both XX and XY cells. The X chromosome appears to encode four protein factors that activate the early promoter of *Sxl*. Three of these proteins are transcription factors—SisA, Scute, and Runt—which bind to the early promoter to activate transcription (Figure 14.17). The fourth protein, Unpaired, is a secreted factor that reinforces the

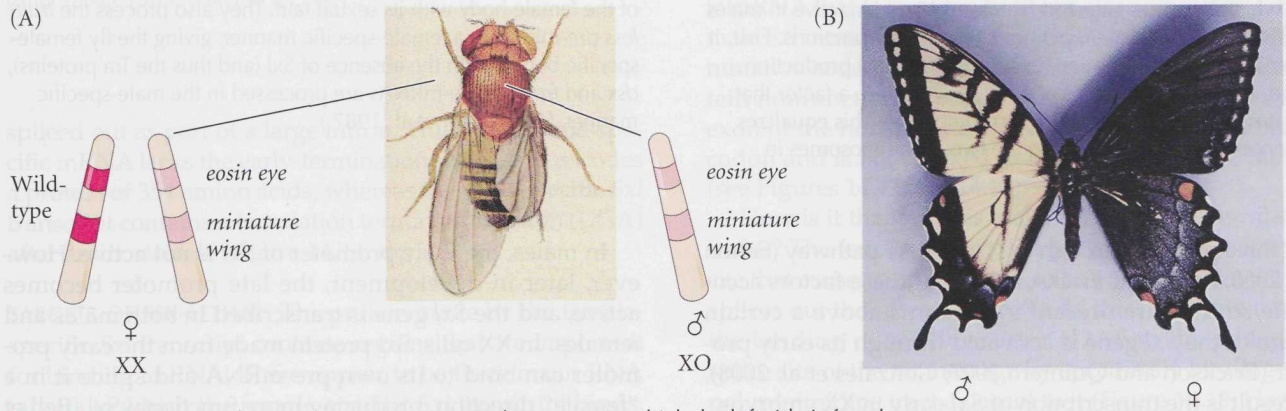

FIGURE 14.16 Gynandromorph insects. (A) *D. melanogaster* in which the left side is female (XX) and the right side is male (XO). The male side has lost an X chromosome bearing the wild-type alleles of eye color and wing shape, thereby allowing expression of the recessive alleles *eosin eye* and *miniature wing* on the remaining X chromosome. (B) Tiger swallowtail butterfly *Paplio glaucus*. The left half (yellow) is male, while the right half (blue-black) is female. (A, drawing by Edith Wallace from Morgan and Bridges 1919; B, photograph courtesy of J. Adams © 2005.)

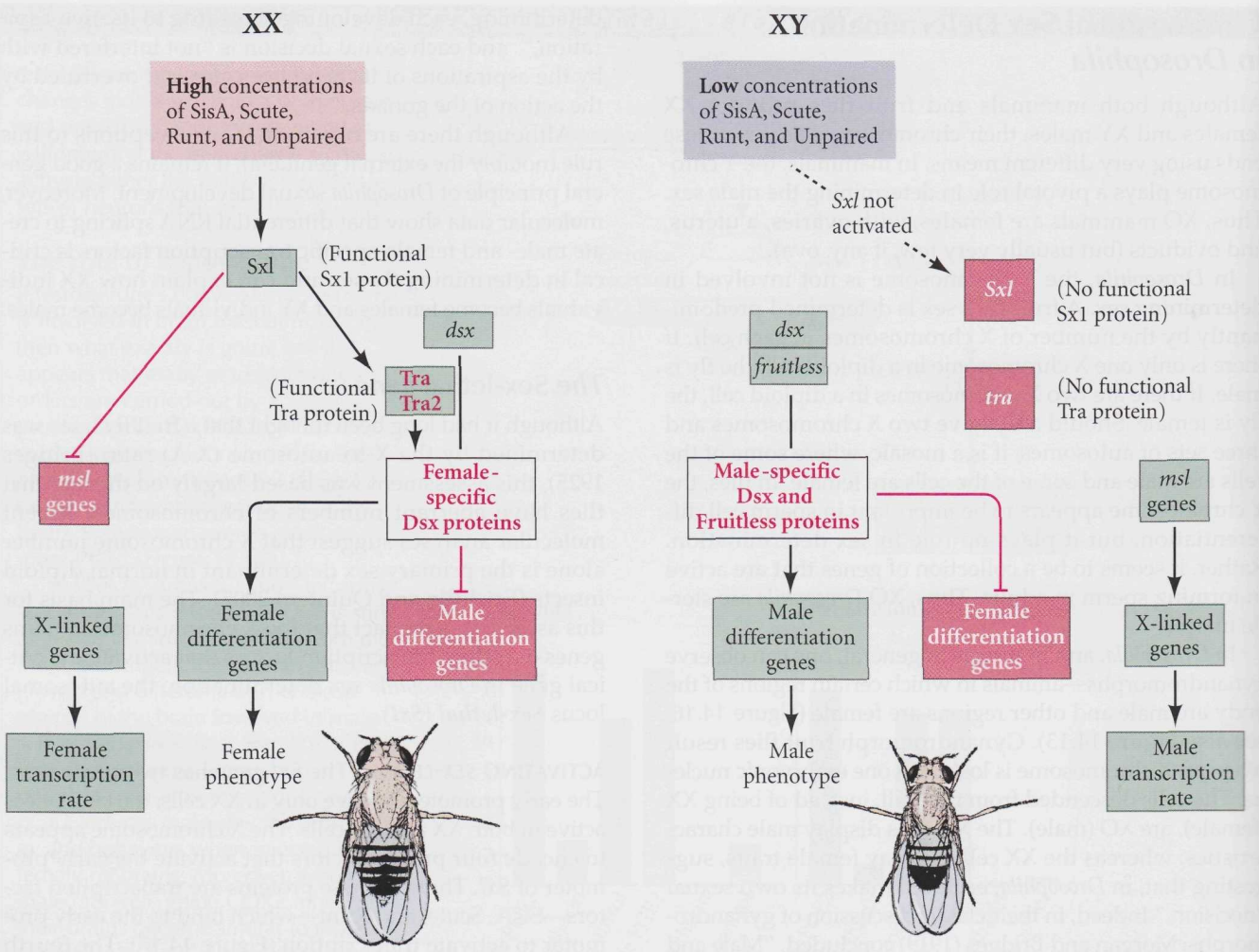

FIGURE 14.17 Proposed regulatory cascade for *Drosophila* somatic sex determination. Transcription factors from the X chromosomes and autosomes compete to activate or repress the *Sxl* gene, which becomes active in females (XX) and inactive in males (XY). The Sex-lethal protein performs three main functions. First, it activates its own transcription, ensuring further Sxl production. Second, it represses the translation of *msl2* mRNA, a factor that facilitates transcription from the X chromosome. This equalizes the amount of transcription from the two X chromosomes in females with that of the single X chromosome in males. Third, Sxl activates the *transformer* (*tra*) genes. The Tra proteins process *doublesex* pre-mRNA in a female-specific manner that provides most of the female body with its sexual fate. They also process the *fruitless* pre-mRNA in a female-specific manner, giving the fly female-specific behavior. In the absence of Sxl (and thus the Tra proteins), *dsx* and *fruitless* pre-mRNAs are processed in the male-specific manner. (After Baker et al. 1987.)

other three proteins through the JAK-STAT pathway (Sefton et al. 2000; Avila and Erickson 2007). If these factors accumulate so they are present in amounts above a certain threshold, the *Sxl* gene is activated through its early promoter (Erickson and Quintero 2007; Gonzales et al. 2008). The result is the transcription of *Sxl* early in XX embryos.

The *Sxl* pre-RNA transcribed from the early promoter lacks exon 3, which contains a stop codon. Thus, Sxl protein that is made early is spliced in a manner such that exon 3 is absent and a complete protein can be made. This functional Sxl binds to its own late promoter to keep the *Sxl* gene active.

In males, the early promoter of *Sxl* is not active. However, later in development, the late promoter becomes active, and the *Sxl* gene is transcribed in both males and females. In XX cells, Sxl protein made from the early promoter can bind to its own pre-mRNA and splice it in a "female" direction, producing more functional Sxl (Bell et al. 1988; Keyes et al. 1992). In XY cells, however, there is no early Sxl protein, and the "male" pre-mRNA is spliced in a manner that yields eight exons, and the termination codon is in exon 3. Protein synthesis thus ends at the third exon, and the protein is nonfunctional. In females, RNA processing yields seven exons, with the male-specific exon 3 being

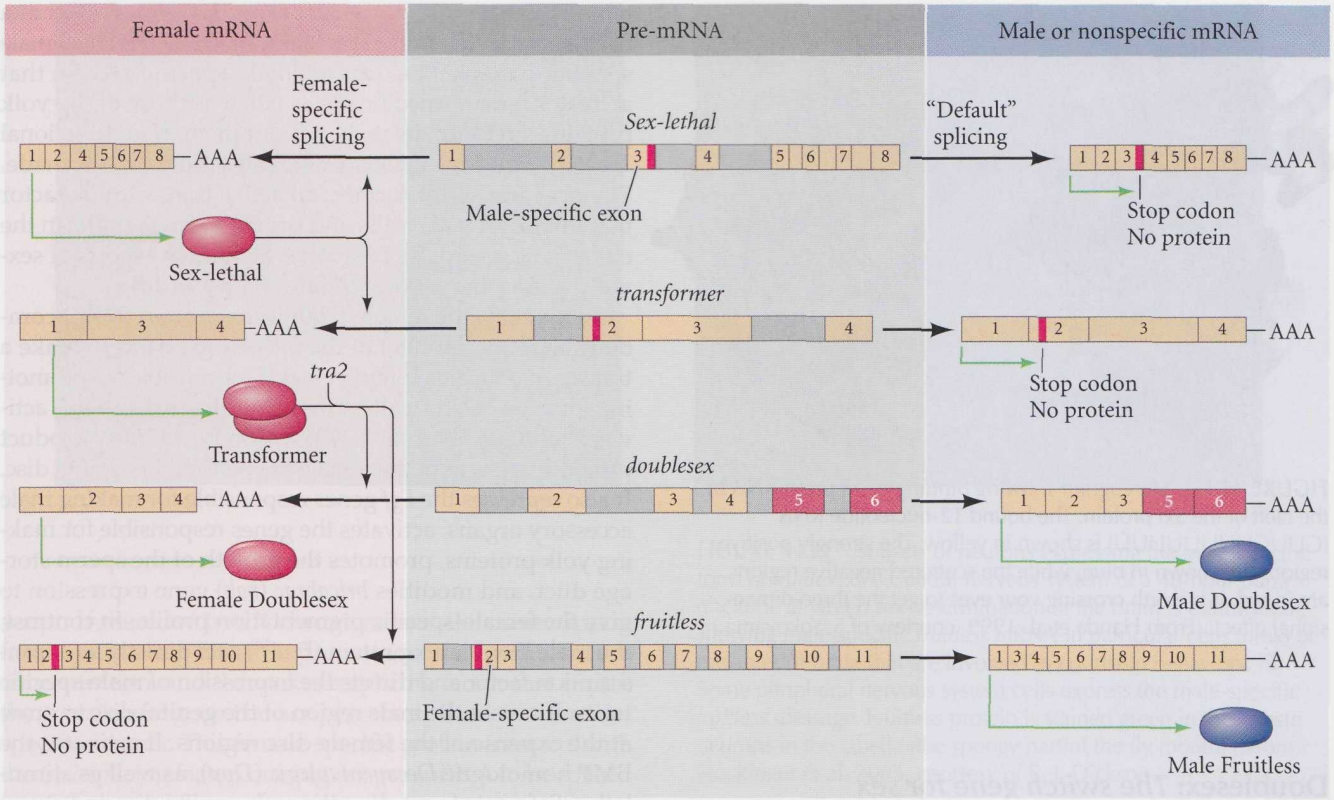

FIGURE 14.18 Sex-specific RNA splicing in four major *Drosophila* sex-determining genes. The pre-mRNAs (shown in the center of diagram) are identical in both male and female nuclei. In each case, the female-specific transcript is shown at the left, while the default transcript (whether male or nonspecific) is shown to the right. Exons are numbered, and the positions of termination codons are marked. *Sex-lethal*, *transformer*, and *doublesex* are all part of the genetic cascade of primary sex determination. The transcription pattern of *fruitless* determines the secondary characteristic of courtship behavior (see pp. 533–534). (After Baker 1989; Baker et al. 2001.)

spliced out as part of a large intron. Thus, the female-specific mRNA lacks the early-termination codon and encodes a protein of 354 amino acids, whereas the male-specific *Sxl* transcript contains a translation termination codon (UGA) after amino acid 48 (Figure 14.18, top).

TARGETS OF SEX-LETHAL The protein made by the female-specific *Sxl* transcript contains regions that are important for binding to RNA. There appear to be three major RNA targets to which the female-specific *Sxl* transcript binds. One of these, as mentioned above, is the pre-mRNA of *Sxl* itself. Another target is the *msl2* gene that controls dosage compensation (see below). Indeed, If the *Sxl* gene is nonfunctional in a cell with two X chromosomes, the dosage compensation system will not work, and the result is cell death (hence the gene's macabre name). The third target is the pre-mRNA of *transformer* (*tra*)—the next gene in the cascade (Bell et al. 1988; Nagoshi et al. 1988).

The pre-mRNA of *transformer* (so named because loss-of-function mutations turn females into males) is spliced into a functional mRNA by Sxl protein. The *tra* pre-mRNA is made in both male and female cells; however, in the presence of Sxl protein, the *tra* transcript is alternatively spliced to create a female-specific mRNA, as well as a nonspecific mRNA that is found in both females and males. Like the male *Sxl* message, the nonspecific *tra* mRNA contains a termination codon early in the message that renders the protein nonfunctional (Boggs et al. 1987). In *tra*, the second exon of the nonspecific mRNA contains the termination codon and is not utilized in the female-specific message (see Figures 14.17 and 14.18).

How is it that females make a different transcript than males? The female-specific Sxl protein activates a 3′ splice site that causes *tra* pre-mRNA to be processed in a way that splices out the second exon. To do this, Sxl protein blocks the binding of splicing factor U2AF to the nonspecific splice site of the *tra* message by specifically binding to the polypyrimidine tract adjacent to it (Figure 14.19; Handa et al. 1999). This causes U2AF to bind to the lower-affinity (female-specific) 3′ splice site and generate a female-specific mRNA (Valcárcel et al. 1993). The female-specific Tra protein works in concert with the product of the *transformer-2* (*tra2*) gene to help generate the female phenotype by splicing the *doublesex* gene in a female-specific manner.

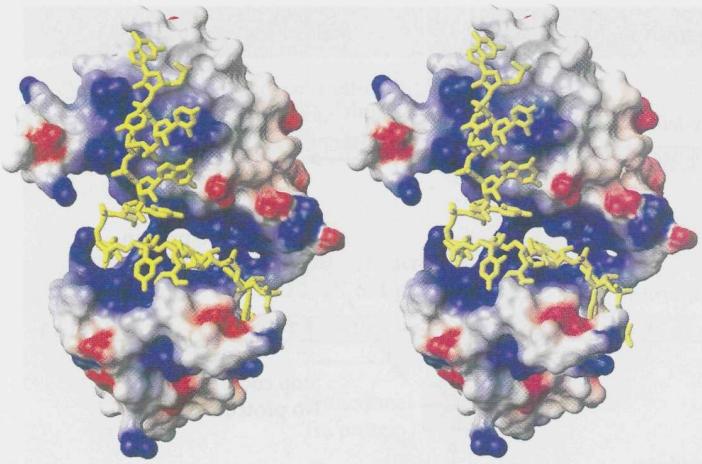

FIGURE 14.19 Stereogram showing binding of *tra* pre-mRNA by the cleft of the Sxl protein. The bound 12-nucleotide RNA (GUUGUUUUUUUU) is shown in yellow. The strongly positive regions are shown in blue, while the scattered negative regions are in red. It is worth crossing your eyes to get the three-dimensional effect. (From Handa et al. 1999, courtesy of S. Yokoyama.)

Doublesex: *The switch gene for sex determination*

The ***doublesex*** (***dsx***) gene is active in both males and females, but its primary transcript is processed in a sex-specific manner (Baker et al. 1987). This alternative RNA processing is the result of the action of the *tra* and *tra2* gene products on the *dsx* gene (see Figures 14.17 and 14.18; see also Figure 2.28). If the Tra2 and female-specific Tra proteins are both present, the *dsx* transcript is processed in a female-specific manner (Ryner and Baker 1991). The female splicing pattern produces a female-specific protein that activates female-specific genes (such as those of the yolk proteins) and inhibits male development. If no functional Tra is produced, a male-specific transcript of *dsx* is made. The male transcript encodes an active transcription factor that inhibits female traits and promotes male traits. In the embryonic gonad, Dsx regulates all known aspects of sexually dimorphic gonad cell fate (Figure 14.20).

In XX flies, the female Doublesex protein (Dsx^F) combines with the product of the *intersex* gene (Ix) to make a transcription factor complex that is responsible for promoting female-specific traits. This "Doublesex complex" activates the *Wingless* gene (*Wg*), whose Wnt-family product promotes growth of the female portions of the genital disc. It also represses the *Fgf* genes responsible for making male accessory organs, activates the genes responsible for making yolk proteins, promotes the growth of the sperm storage duct, and modifies *bricabrac* (*bab*) gene expression to give the female-specific pigmentation profile. In contrast, the male Doublesex protein (Dsx^M) acts directly as a transcription factor and directs the expression of male-specific traits. It causes the male region of the genital disc to grow at the expense of the female disc regions. It activates the BMP homologue *Decapentaplegic* (*Dpp*), as well as stimulating *Fgf* genes to produce the male genital disc and accessory structures. Dsx^M also converts certain cuticular structures into claspers and modifies the *bricabrac* gene to produce the male pigmentation pattern (Ahmad and Baker 2002; Christiansen et al. 2002).

According to this model, the result of the sex determination cascade summarized in Figure 14.17 comes down to the type of mRNA processed from the *doublesex* tran-

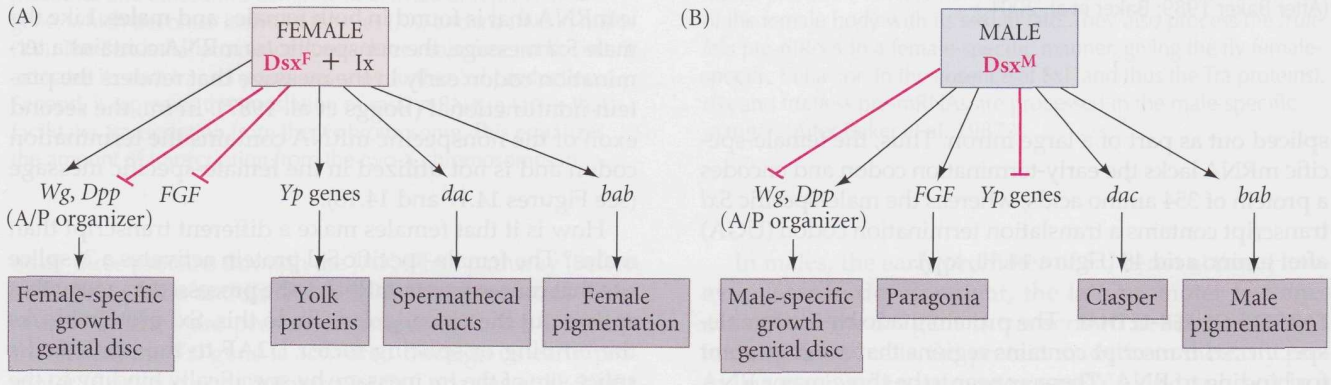

FIGURE 14.20 Roles of Dsx^M and Dsx^F proteins in *Drosophila* sexual development. (A) Dsx^F functions with Intersex (Ix) to promote female-specific expression of those genes that control the growth of the genital disc, the synthesis of yolk proteins, the formation of spermathecal ducts (which keep sperm stored after mating), and pigment patterning. (B) Conversely, Dsx^M acts as a transcription factor to promote the male-specific growth of the genital disc, the formation of male genitalia, the conversion of cuticle into claspers, and the male-specific pigmentation pattern. In addition, Dsx^F represses certain genes involved in specifying male-specific traits (such as the paragonia), and Dsx^M represses certain genes involved in synthesizing female-specific proteins such as yolk protein. (After Christiansen et al. 2002.)

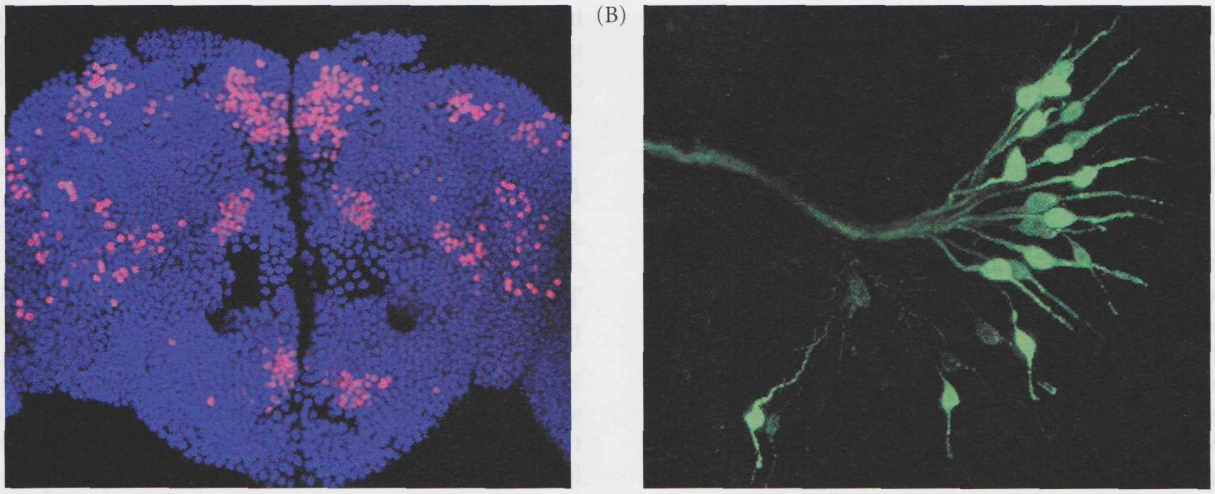

FIGURE 14.21 Subsets of neurons expressing the male-specific form of *fruitless*. (A) Central nervous system of a 48-hour pupa (the time at which sex determination of the brain is established), showing male-specific *fruitless* mRNA in particular cells (pink) of the anterior brain that are involved in courtship behaviors. (B) Some peripheral nervous system cells express the male-specific *fruitless* message. Fruitless protein is stained green in these taste neurons in the labella (the spongy part of the fly mouth). (From Stockinger et al. 2005, courtesy of B. J. Dickson.)

script. If there are two X chromosomes, the transcription factors activating the early promoter of *Sxl* reach a critical concentration, and *Sxl* makes a splicing factor that causes the *transformer* gene transcript to be spliced in a female-specific manner. This female-specific protein interacts with the *tra2* splicing factor, causing *dsx* pre-mRNA to be spliced in a female-specific manner. If the *dsx* transcript is not acted on in this way, it is processed in a "default" manner to make the male-specific message.

Brain Sex in Drosophila

Our discussion of sexual dimorphism in *Drosophila* has so far been limited to nonbehavioral aspects of development. However, as in mammals, there appears to be a separate "brain-sex" pathway in *Drosophila* that provides individuals of each sex with the appropriate set of courtship and aggression behaviors. Among *Drosophila*, there are no parents or other conspecifics to teach "proper" mating behavior, and mating takes place soon after the flies emerge from their pupal cases. So the behaviors must be "hard-wired" into the insect genome.*

Among male *Drosophila*, there is one very simple rule of behavior: If the other fly is male, fight it; if the other fly is female, court it (Certel et al. 2007; Billeter et al. 2008; Wang and Anderson 2010). Although the outcomes of this algorithm are simple, the behaviors of *Drosophila* courtship and mating are quite complicated. A courting male must first confirm that the individual he is approaching is a female. Once this is established, he must orient his body toward the female and follow a specific series of movements that include following the female, tapping the female, playing a species-specific courtship song by vibrating his wings, licking the female, and finally, curling his abdomen so that he is in a position to mate. Each of these sex-specific courtship behaviors appear to be regulated by the products of *fruitless*, a gene expressed in certain sets of neurons involved with male sexual behaviors (Figure 14.21). These include subsets of neurons involved in taste, hearing, smell, and touch, and in total they represent about 2% of all the neurons in the adult male (Lee et al. 2000; Billeter and Goodwin 2004; Stockinger et al. 2005). Fruitless also retains certain male-specific neural circuits; the neurons in these circuits die during female development (and in *fruitless* mutants; see Kimura et al. 2005).

As with *doublesex* pre-mRNA, the Tra and Tra2 proteins splice *fruitless* pre-mRNA into a female-specific message; the default splicing pattern is male (see Figure 14.18). So the female makes Tra protein and processes the *fruitless* pre-mRNA in one way, whereas the male, lacking the Tra protein, processes the *fruitless* message in another way. However, female *fruitless* mRNA includes a termination sequence in an early exon; therefore the female does not make functional Fruitless protein. The male, however, makes an mRNA that does not contain the stop codon (Heinrichs et al. 1998), and the protein it transcribes is a zinc-finger transcription factor. Using homologous recombination to force the transcription of particular splicing forms, Demir and Dickson (2005) showed that it is Fruit-

*This is not to say that flies don't learn; indeed, one thing they do learn is to avoid bad sexual encounters. A male who has been brushed off (quite literally) by a female because she has recently mated hesitates before starting to court another female (Siegel and Hall 1979; MacBride et al. 1999).

less, and not the flies' anatomy, that controls their sexual behavior. When female flies were induced to make the male-specific Fruitless protein, they performed the entire male courtship ritual and tried to mate with other females.

In normal females, the courtship ritual is not as involved as in males. However, females have the ability to be receptive to a male's entreaties or to rebuff them. The product of the *retained* gene (*rtn*) is critical in this female mating behavior. Both sexes express this gene, since it is also involved in axon pathfinding. However, female flies with a loss-of-function allele of *rtn* resist male courtship and are thus rendered sterile by their own behavior (Ditch et al. 2005).

The splicing of the *fruitless* transcripts not only regulates sex-specific courtship patterns, it also regulates sex-specific aggression patterns as well. Female flies having a male Fruitless protein not only tend to court females, they also will fight males and try to establish themselves at the top of a dominance hierarchy. Male flies having a mutant *fruitless* allele will show female-specific aggression against other females (Vrontou et al. 2006).

Dosage Compensation

In animals whose sex is determined by sex chromosomes, there has to be some mechanism by which the amount of X chromosome gene expression is equalized for males and females. This mechanism is known as **dosage compensation**. In Chapter 2 we discussed mammalian X-chromosome inactivation, whereby one of the X chromosomes is inactivated so that the transcription product level is the same in both XX cells and XY cells. In the worm *Caenorhabditis elegans*, dosage compensation occurs by lowering the transcription rates of *both* X chromosomes so that product levels are the same as those of XO individuals.

In *Drosophila*, the female X chromosomes are not suppressed; rather, the male's single X chromosome is hyperactivated. This "hypertranscription" is accomplished at the level of translation, and it is mediated by the Sxl protein. Sxl protein (which you will recall is made by the female cells) binds to the 5′ leader sequence and the 3′ untranslated regions (UTRs) of the *msl2* message. The bound Sxl inhibits the attachment of *msl2* mRNA to the ribosome and prevents the ribosome from getting to the mRNA's coding region (Beckman et al. 2005). The result is that female cells do not produce Msl2 protein (see Figure 14.17). However, Msl2 is made in male cells, in which Sxl is not present. Mls2 is part of a protein-mRNA complex that targets the X chromosome and loosens its chromatin structure by acetylating histone 4 (see Figure 2.4). In this way, transcription factors gain access to the X chromosome at a much higher frequency in males than in females—hence, "hypertranscription."

ENVIRONMENTAL SEX DETERMINATION

Temperature-Dependent Sex Determination in Reptiles

While the sex of most snakes and lizards is determined by sex chromosomes at the time of fertilization, the sex of most turtles and all species of crocodilians is determined *after* fertilization, by the embryonic environment. In these reptiles, the temperature of the eggs during a certain period of development is the deciding factor in determining sex, and small changes in temperature can cause dramatic changes in the sex ratio (Bull 1980; Crews 2003). Often, eggs incubated at low temperatures produce one sex, whereas eggs incubated at higher temperatures produce the other. There is only a small range of temperatures that permits both males and females to hatch from the same brood of eggs.*

Figure 14.22 shows the abrupt temperature-induced change in sex ratios for the red-eared slider turtle. If a brood of eggs is incubated at a temperature below 28°C, all the turtles hatching from the eggs will be male. Above 31°C, every egg gives rise to a female. At temperatures in between, the brood will give rise to individuals of both sexes. Variations on this theme also exist. The eggs of the

*The evolutionary advantages and disadvantages of temperature-dependent sex determination are discussed in Chapter 19.

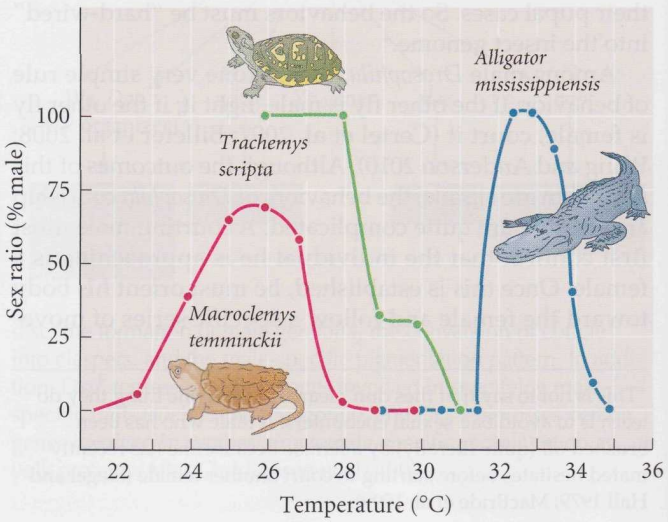

FIGURE 14.22 Temperature-dependent sex determination in three species of reptiles: the American alligator (*Alligator mississippiensis*), red-eared slider turtle (*Trachemys scripta elegans*), and alligator snapping turtle (*Macroclemys temminckii*). (After Crain and Guillette 1998.)

snapping turtle *Macroclemys*, for instance, become female at either cool (22°C or lower) or hot (28°C or above) temperatures. Between these extremes, males predominate.

As we will see in Chapter 18, there can be multiple pathways of sex determination in the same individual. Under normal temperature conditions, the sex of the lizard *Bassiana duperreyi* is determined by sex chromosomes (XY males; XX females). However, at low temperatures, the environmental component overrides the genetic sex-determining mechanism, and all the offspring in cool nests are male (even if their chromosomes are XX; Radder et al. 2008).

One of the best-studied reptiles is the European pond turtle, *Emys obicularis*. In laboratory studies, incubating *Emys* eggs at temperatures above 30°C produces all females, while temperatures below 25°C produce all-male broods. The threshold temperature (at which the sex ratio is even) is 28.5°C (Pieau et al. 1994). The developmental "window" during which sex determination occurs can be discovered by incubating eggs at the male-producing temperature for a certain amount of time and then shifting them to an incubator at the female-producing temperature (and vice versa). In *Emys*, the middle third of development appears to be the most critical for sex determination, and it is believed that the turtles cannot reverse their sex after this period.

The aromatase hypothesis for environmental sex determination

The enzyme aromatase, which converts testosterone into estrogen (see Sidelights & Speculations, p. 528), appears to be a particularly important target for environmental triggers. Unlike the situation in mammals, whose primary sex determination is a function of the X and Y chromosomes, primary sex determination in reptiles and birds is influenced by hormones, and estrogen is essential if ovaries are to develop. In reptiles, estrogen can override temperature, inducing ovarian differentiation even at masculinizing temperatures. Similarly, experimentally exposing eggs to inhibitors of estrogen synthesis produces male offspring, even if the eggs are incubated at temperatures that usually produce females (Dorizzi et al. 1994; Rhen and Lang 1994). The sensitive time for the effects of estrogens and their inhibitors coincides with the time when sex determination usually occurs (Bull et al. 1988; Gutzke and Chymiy 1988).

The estrogen-synthesis inhibitors used in the experiments mentioned above worked by blocking aromatase action, showing that experimentally low aromatase levels yield male offspring.* This correlation appears to hold under natural conditions as well. The aromatase activity of *Emys* is very low at the male-promoting temperature of 25°C. At the female-promoting temperature of 30°C, aromatase activity increases dramatically during the critical period for sex determination (Desvages et al. 1993; Pieau et al. 1994). Temperature-dependent aromatase activity is also seen in diamondback terrapins, and its inhibition masculinizes their gonads (Jeyasuria et al. 1994). Aromatase appears to be involved in the temperature-dependent differentiation of lizards and salamanders as well (Sakata et al. 2005). It is possible that aromatase expression is activated differently in different species. In some species, the aromatase *protein* itself may be temperature sensitive. In other species, the expression of the aromatase *gene* may be differentially activated at high temperatures (see Murdock and Wibbels 2006; Shoemaker et al. 2007).

When turtle gonads are taken out of the embryos and placed in culture, they become testes or ovaries depending on the temperature at which they are incubated (Moreno-Mendoza et al. 2001; Porter et al. 2005). This shows that sex determination is a local activity of the gonadal primordia and not a global activity directed by the pituitary or the brain. By analyzing the timing and regulation of sex-determining genes in such cultured gonads, Shoemaker and colleagues (2005, 2007) proposed that female temperatures not only activate aromatase (which would cause ovary formation), but also activate the *Wnt4* gene (which suppresses the formation of testes in mammals). Conversely, male temperatures appear to activate *Sox9* to a higher level in the male than in the female, promoting testis development (Moreno-Mendoza et al. 2001).

Estrogens, aromatase, sex reversal, and conservation biology

Over the last two decades, data has emerged showing that several of the polychlorinated biphenyl compounds (PCBs), a class of widespread pollutants introduced into the environment by humans, can act as estrogens (e.g., see Bergeron et al. 1994, 1999). PCBs can reverse the sex of turtles raised at "male" temperatures. This knowledge has important consequences in environmental conservation efforts to protect endangered species (such as turtles, amphibians, and crocodiles) in which hormones can effect changes in primary sex determination. Indeed, some reptile conservation biologists advocate using hormonal treatments to elevate the percentage of females in endangered species (www.reptileconservation.org).

The survival of some amphibian species may be at risk from herbicides that promote or destroy estrogens. One such case involves the development of hermaphroditic and demasculinized frogs after exposure to extremely low doses of the weed killer atrazine, the most widely used herbicide in the world (Figure 14.23). Hayes and colleagues (2002a) found that exposing tadpoles to atrazine concentrations as low as 0.1 part per billion (ppb) produced gonadal and other sexual anomalies in male frogs. At 0.1 ppb and higher, many male tadpoles developed ovaries in

*One remarkable finding is that the injection of an aromatase inhibitor into the eggs of an all-female, parthenogenetic species of lizards causes the formation of males (Wibbels and Crews 1994).

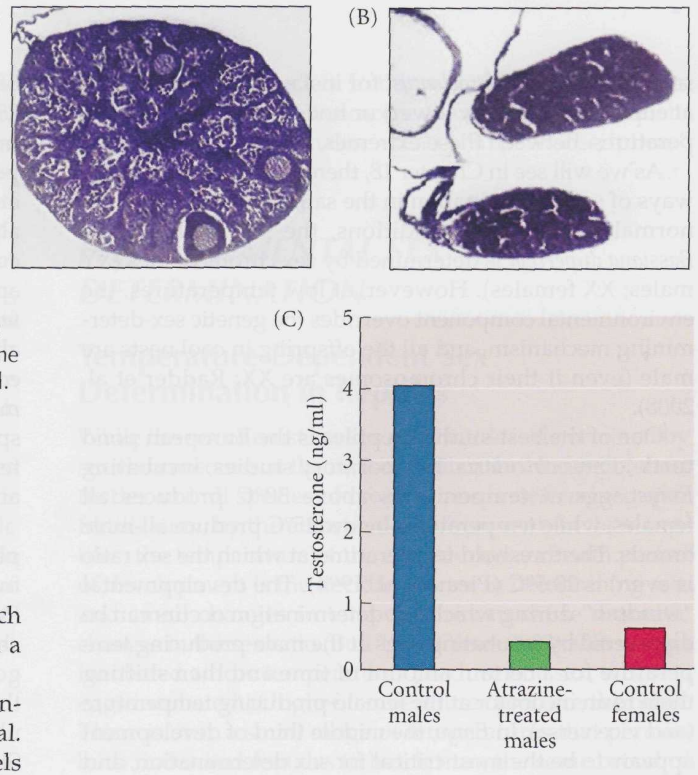

FIGURE 14.23 Demasculinization of frogs by low amounts of atrazine. (A) Testis of a frog from a natural site having 0.5 parts per billion (ppb) atrazine. The testis contains three lobules that are developing both sperm and an oocyte. (B) Two testes of a frog from a natural site containing 0.8 ppb atrazine. These organs show severe testicular dysgenesis, which characterized 28% of the frogs found at that site. (C) Effect of a 46-day exposure to 25 ppb atrazine on plasma testosterone levels in sexually mature male *Xenopus*. Levels in control males were some tenfold higher than in control females; atrazine-treated males had plasma testosterone levels at or below those of control females. (A,B after Hayes et al. 2003, photographs courtesy of T. Hayes; C after Hayes et al. 2002a.)

addition to testes. At 1 ppb atrazine, the vocal sacs (which a male frog must have in order to signal and obtain a potential mate) failed to develop properly.

Atrazine induces aromatase, and aromatase can convert testosterone into estrogen (Crain et al.1997; Fan et al. 2007). In laboratory experiments, the testosterone levels of adult male frogs were reduced by 90% (to levels of control females) by 46 days of exposure to 25 ppb atrazine—an ecologically relevant dose, since the allowable amount of atrazine in U.S. drinking water is 3 ppb, and atrazine levels can reach 224 ppb in streams of the midwestern United States (Battaglin et al. 2000; Barbash et al. 2001).

Given the amount of atrazine in the water supply and the sensitivity of frogs to this compound, the situation could be devastating to wild populations. In a field study, Hayes and his colleagues collected leopard frogs and water at eight sites across the central United States (Hayes et al. 2002b, 2003). They sent the water samples to two separate laboratories for the determination of atrazine, coding the frog specimens so that the technicians dissecting the gonads did not know which site the animals came from. The results showed that all but one site contained atrazine—and this was the only site from which the frogs had no gonadal abnormalities. At concentrations as low as 0.1 ppb, leopard frogs displayed testicular dysgenesis (stunted growth of the testes) or conversion to ovaries. In many examples, oocytes were found in the testes (see Figure 14.23A).

Concern over atrazine's apparent ability to disrupt sex hormones in both wildlife and humans has resulted in bans on the use of this herbicide by France, Germany, Italy, Norway, Sweden, and Switzerland (Dalton 2002). Many geographical and social concerns mediate the amount of atrazine use (Figure 14.24), and the company making atrazine has lobbied against the work of independent researchers whose research indicates that it might cause reproductive malfunctions or cancers in wildlife and humans (see Blumenstyk 2003). Endocrine disruptors will be discussed in more detail in Chapter 17.

Location-Dependent Sex Determination

Environmental factors other than temperature can also be sex determinants in some species. For example, it has been known since the nineteenth century that the sex of the echiuroid worm *Bonellia viridis* depends on where a larva settles (Baltzer 1914). If a *Bonellia* larva lands on the ocean floor, it develops into a 10-cm-long female. If the larva is attracted to a female's proboscis, it travels along the tube until it enters the female's body. There it differentiates into a minute (1–3 mm long) male that is essentially a sperm-producing symbiont of the female (Figure 14.25).

Another species in which sex determination is affected by the location of the organism is the slipper snail *Crepidula fornicata*. In this species, individuals pile up on top of one another in a great mound. Young individuals are always male, but this phase is followed by the degeneration of the male reproductive system and a period of lability. The next phase can be either male or female, depending on the animal's position in the mound. If the snail is attached to a female, it will become male. If such a snail is removed from its attachment, it will become female. Similarly, the presence of large numbers of males causes some of the males to become females. However, once an individual becomes female, it will not revert to being male (Coe 1936; Collin 1995; Warner et al. 1996).

Many fish change sex based on social interactions; such changes are mediated by the neuroendocrine system (Godwin et al. 2003, 2009). Interestingly, although the trigger of the sex change may be stress hormones (e.g., cortisol) that induce sex-specific neuropeptides, the effector of the change may be (once again) aromatase. There are two vari-

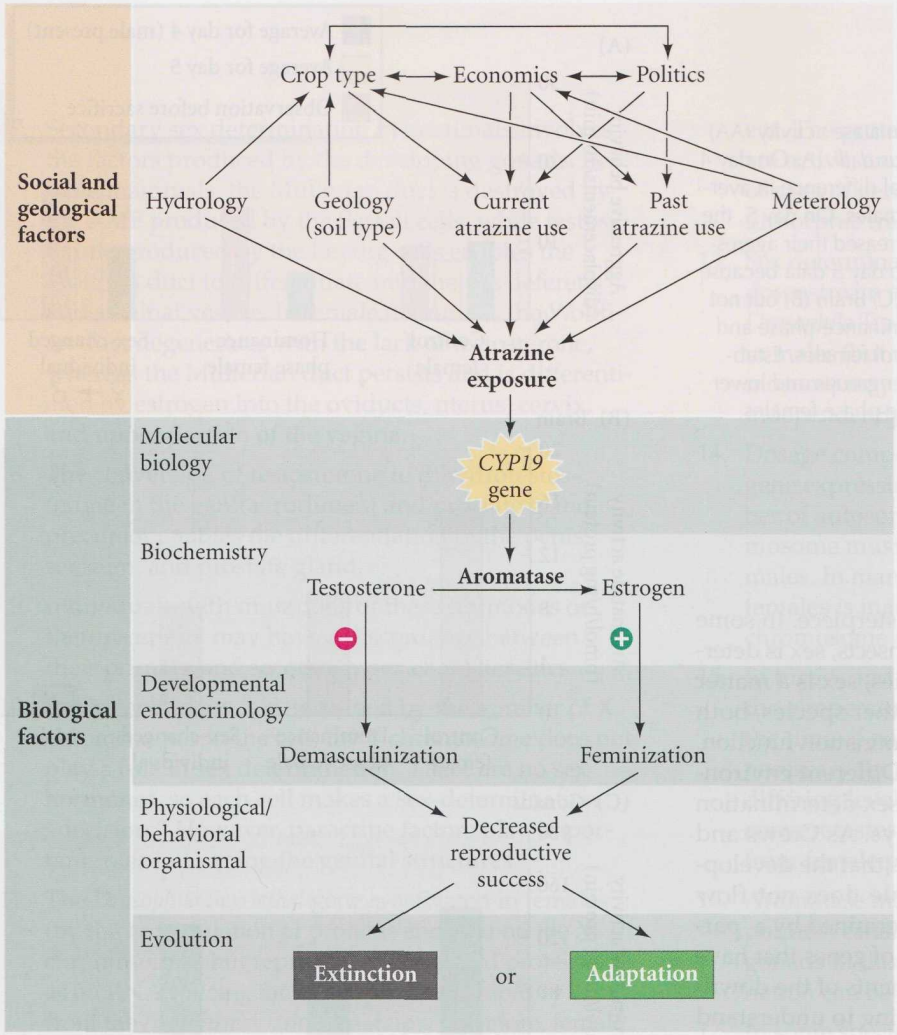

FIGURE 14.24 Possible chain of causation leading to the feminization of male frogs and the decline of frog populations in regions where atrazine has been used to control weed populations. Social, geological, and biological agents are shown. *CYP19* is the gene encoding aromatase, and it has been shown that transcription of the human *CYP19* gene is induced by atrazine (Sanderson et al. 2000). (From Hayes 2005.)

ants of aromatase in many animals, one expressed in the brain and one in the gonads. Black and colleagues (2005) showed a striking correlation between changes in brain aromatase levels and changes in sexual behavior during the sexual transitions of gobies, in which the school typically contains only one male and multiple females. The removal of the male from a stable group caused a rapid increase (>200%) in the aggressive behavior of the largest female, which then became a male in about a week's time. This transformation may have resulted from an increase in brain testosterone levels, since within hours upon removal of the male, these dominant females developed a lower brain aromatase than the other females (Figure 14.26). The *gonadal* aromatase levels, however, stayed the same, and gonadal sex change came later. In porgy fish, aromatase inhibitors can block the natural sex change and induce male development (Lee et al. 2002). Thus, changes in the social group, perceived by the nervous system, became expressed by the hormonal system within hours, thereby changing the behavioral phenotype of the female fish. Interestingly, when it comes to behaviors in fish, sex is in the brain before it is in the gonads.

See WEBSITE 14.5 Forms of hermaphroditism

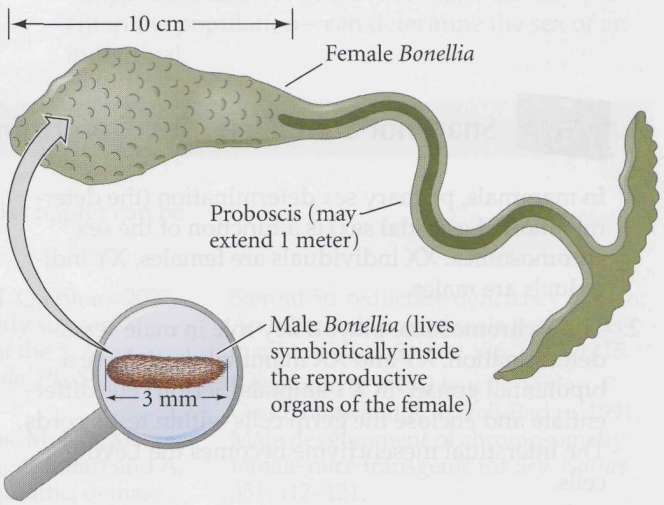

FIGURE 14.25 Sex determination in *Bonellia viridis*. Larvae that settle on the ocean floor become female. The mature female's body is about 10 cm long, with a 1-meter-long proboscis that emits chemicals which attract other *B. viridis* larvae. Larvae that land on the proboscis are taken into the female's body, where they develop and live symbiotically as tiny (1–3 mm long) males.

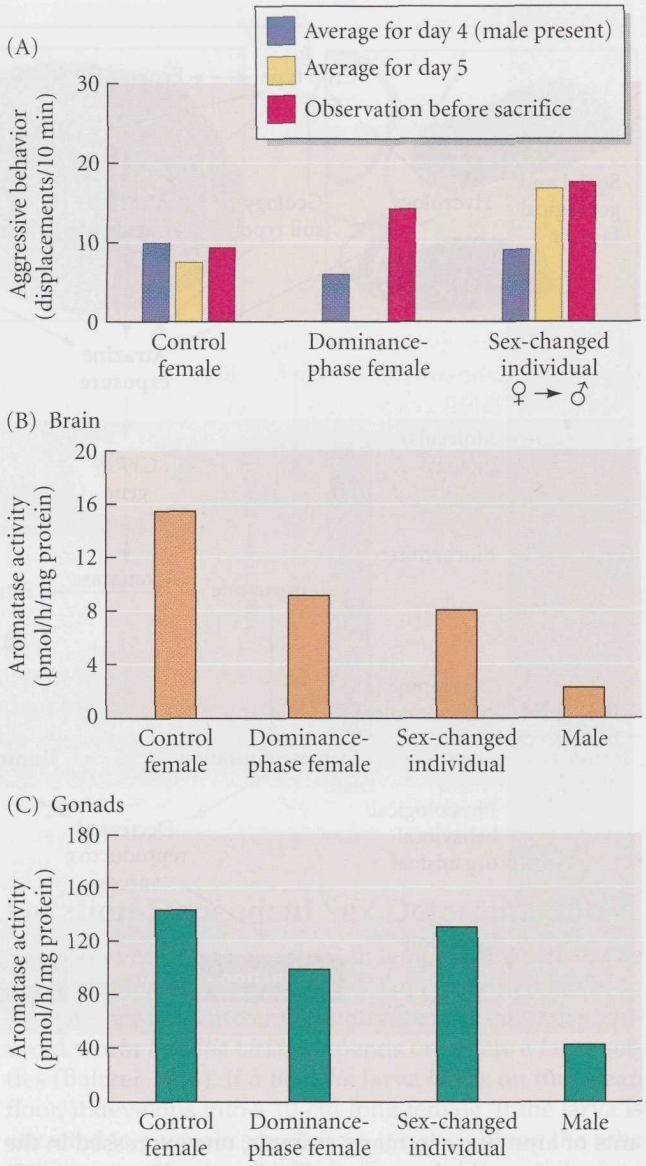

FIGURE 14.26 Aggressive behavior and aromatase activity (AA) in the brain and gonads of the goby *Lythrypnus dalli*. (A) On day 4 (prior to male removal), there was no statistical difference in average daily displacements among the largest females. On day 5, the male was removed and dominant females increased their aggressive behavior. (Dominance-phase fish have no day 5 data because they were sacrificed on day 5 or just after.) (B,C) Brain (B) but not gonadal (C) AA was significantly lower in dominance-phase and sex-changed individuals compared with control females. Established males had lower brain AA than all other groups and lower gonadal AA than all groups except dominance-phase females. (After Black et al. 2005.)

Coda

Nature has many variations on her masterpiece. In some species, including most mammals and insects, sex is determined by chromosomes; in other species, sex is a matter of environmental conditions. In yet other species, both environmental and genotypic sex determination function, often in different geographical areas. Different environmental or genetic stimuli may trigger sex determination through a series of conserved pathways. As Crews and Bull (2009) have reflected, "it is possible that the developmental decision of male versus female does not flow through a single gene but is instead determined by a 'parliamentary' system involving networks of genes that have simultaneous inputs to several components of the downstream cascade." We are finally beginning to understand the mechanisms by which this "masterpiece of nature" is created.

Snapshot Summary: *Sex Determination*

1. In mammals, primary sex determination (the determination of gonadal sex) is a function of the sex chromosomes. XX individuals are females, XY individuals are males.
2. The Y chromosome plays a key role in male sex determination. XY and XX mammals both have a bipotential gonad. In XY animals, Sertoli cells differentiate and enclose the germ cells within testis cords. The interstitial mesenchyme becomes the Leydig cells.
3. In XX individuals, the germ cells become surrounded by follicle cells in the cortex of the gonadal rudiment. The epithelium of the follicles becomes the granulosa cells; the mesenchyme becomes the thecal cells.
4. In humans, the *SRY* gene is the testis-determining factor on the Y chromosome. It synthesizes a nucleic acid-binding protein that may function as either a transcription factor or as an RNA splicing factor. It activates the evolutionarily conserved *SOX9* gene.
5. The *SOX9* gene product can also initiate testes formation. Functioning as a transcription factor, it binds to the gene encoding anti-Müllerian factor is responsible for activating *FGF9*. Fgf9 and Sox9 proteins have a positive feedback loop that activates testicular development and suppresses ovarian development.
6. Wnt4 and Rspo-1 are involved in ovary formation. These proteins upregulate production of β-catenin; the functions of β-catenin include promoting the ovarian pathway of development while blocking the testicular pathway of development

7. Secondary sex determination in mammals involves the factors produced by the developing gonads. In male mammals, the Müllerian duct is destroyed by the AMF produced by the Sertoli cells, while testosterone produced by the Leydig cells enables the Wolffian duct to differentiate into the vas deferens and seminal vesicle. In female mammals, the Wolffian duct degenerates with the lack of testosterone, whereas the Müllerian duct persists and is differentiated by estrogen into the oviducts, uterus, cervix, and upper portion of the vagina.
8. The conversion of testosterone to dihydrotestosterone in the genital rudiment and prostate gland precursor enables the differentiation of the penis, scrotum, and prostate gland.
9. Individuals with mutations of these hormones or their receptors may have a discordance between their primary and secondary sex characteristics.
10. In *Drosophila*, sex is determined by the number of X chromosomes in the cell; the Y chromosome does not play a role in sex determination. There are no sex hormones, so each cell makes a sex-determination "decision." However, paracrine factors play important roles in forming the genital structures.
11. The *Drosophila Sex-lethal* gene is activated in females (by the accumulation of proteins encoded on the X chromosomes) but repressed in males. Sxl protein acts as an RNA splicing factor to splice an inhibitory exon from the *transformer* (*tra*) transcript. Therefore, female flies have an active Tra protein, while males do not.
12. The Tra protein also acts as an RNA splicing factor to splice exons from the *doublesex* (*dsx*) transcript. The *dsx* gene is transcribed in both XX and XY cells, but its pre-mRNA is processed to form different mRNAs, depending on whether Tra protein is present. The proteins translated from both *dsx* messages are active, and they activate or inhibit transcription of a set of genes involved in producing the sexually dimorphic traits of the fly.
13. Sex determination of the brain may have different downstream agents than in other regions of the body. *Drosophila* Tra proteins also activate the *fruitless* gene in males (but not in females); in mammals, the Y chromosome may activate brain sexual differentiation independently from the hormonal pathways.
14. Dosage compensation is critical for the regulation of gene expression in the embryo. With the same number of autosomes, the transcription from the X chromosome must be equalized for XX females and XY males. In mammals, one X chromosome of XX females is inactivated. In *Drosophila*, the single X chromosome of XY males is hyperactivated.
15. In turtles and alligators, sex is often determined by the temperature experienced by the embryo during the time of gonad determination. Because estrogen is necessary for ovary development, it is possible that differing levels of aromatase (an enzyme that can convert testosterone into estrogen) distinguish male from female patterns of gonadal differentiation.
16. Aromatase may be activated by environmental compounds, causing demasculinization of the male gonads in those animals where primary sex determination can be effected by hormones.
17. In some species, such as *Bonellia* and *Crepidula*, sex is determined by the position of the individual with regard to other individuals of the same species. In fish, numerous environmental factors—especially temperature and the number of males already present in the population—can determine the sex of an individual.

For Further Reading

Complete bibliographical citations for all literature cited in this chapter can be found at the free-access website **www.devbio.com**

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Go Online

WEBSITE 14.1 Social critique of sex determination research. In numerous cultures, women are seen as the "default state" and men are seen as having "something extra." Historians and biologists show that, until recently, such biases characterized the scientific study of human sex determination.

WEBSITE 14.2 Finding the male-determining genes. The mapping of the testis-determining factor to the SRY region took scientists more than 50 years to accomplish. Moreover, other testis-forming genes that act downstream of SRY have been found on autosomes.

WEBSITE 14.3 Dihydrotestosterone in adult men. The drug finasteride, which inhibits the conversion of testosterone to dihydrotestosterone, is being used to treat prostate growth and male pattern baldness.

WEBSITE 14.4 Insulin-like hormone 3. In addition to testosterone, the Leydig cells secrete insulin-like hormone 3 (INSL3). This hormone is required for the descent of the gonads into the scrotum. Males lacking INSL3 are infertile because the testes do not descend. In females, lack of this hormone deregulates the menstrual cycle.

WEBSITE 14.5 Forms of hermaphroditism. In *C. elegans* and many other invertebrates, hermaphroditism is the general rule. These animals may be born with both ovaries and testes, or they may develop one set of gonads first and the other later (sequential hermaphroditism). In some fish, sequential hermaphroditism is seen, with an individual fish being female in some seasons and male in others.

Outside Sites

For discussions of disorders of sexual development, including intersex conditions, see the American Academy of Pediatrics site at **http://www.pediatriccareonline.org/pco/ub/view/Point-of-Care-Quick-Reference/397188/all/disorders_of_sexual_development**.

The Intersex Society of North America website, **http://isna.org**, is an excellent resource for information on human intersex conditions. The Wikipedia entry on intersexuality, **http://en.wikipedia.org/wiki/Intersexuality**, has a good discussion about the language used to describe these conditions.