Weird Animal Genomes and the Evolution of Vertebrate Sex and Sex Chromosomes

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Key Words

Abstract

Humans, mice, and even kangaroos have an XX female:XY male system of sex determination, in which the Y harbors a male-dominant sex-determining gene *SRY*. Birds have the opposite, ZZ males and ZW females, and may use a dosage-sensitive Z-borne gene. Other reptiles have genetic sex but no visible sex chromosomes, or determine sex by temperature of egg incubation. How can we make sense of so much variation? How do systems change in evolution? Studies of some unlikely animals—platypus and dragon lizards, frogs and fish—confirm that evolutionary transitions have occurred between TSD and GSD systems, between XY and ZW systems, and even between male and female heterogametic systems. Here I explore nonmodel systems that offer some new perspectives on some venerable questions of sex and sex chromosomes.

INTRODUCTION

Sex and sex chromosomes have engaged some of the greatest thinkers in biology, including Darwin, Haldane, and R.A. Fisher. Sex chromosomes are the most changeable part of any animal genome because they are subject to special selective forces. Understanding animal sex will bring understanding at many levels of genetic pathways, genome organization, function, and evolution.

Many species of animals have sex chromosomes that differ in males and females. For instance, mammals have an XX female:XY male system of male heterogamety. Birds and snakes have the opposite: a ZW female:ZZ system of female heterogamety. In both these systems, the sex-specific element (the Y or W) tends to be small and/or heterochromatic. However, many animal species have no obvious sex chromosomes. In many reptiles and most frogs and fish, sex is determined genetically, but no sex chromosomes can be distinguished. In many other reptiles and a few fish, the sex-determining trigger is an environmental stimulus, usually the temperature at which eggs are incubated.

The bewildering variety of sex-determining mechanisms just among higher vertebrates disguises a biochemical and histological commonality of the genetic pathway that induces gonad differentiation. The histology of gonad differentiation is almost indistinguishable among mice, kangaroos, and fish, and many genes are now known that are activated in testis differentiation in all vertebrates. This common pathway may be activated by a variety of triggers. The trigger gene is of intense genetic interest, because it appears to define a whole chromosome and determine its fate.

Much has been learned about the genetics of sex determination in our model animals, humans, and mice, *Drosophila melanogaster*, and *Caenorhabditis elegans*, which display similarities in the overall strategy of determining sex, but amazing differences in how a conserved sex-determining pathway is triggered. In this review, I focus on the contributions made by studying the genomes of nonmodel vertebrates, particularly the "alternative" mammals (marsupials and monotremes), and other higher vertebrates (birds, reptiles, and the few frogs and fish that have been studied). By comparing vertebrates that are more and more distantly related, we can retrace the evolutionary steps further and further back in time, gleaning information that is useful in undersanding sex determination in all mammals including humans.

I made this journey, rather reluctantly, in my own laboratory, insisting that "we are a mammal lab" until entrepeneurial students tackled sex in emus and alligators. It was a short step to turtles, snakes, and dragon lizards, while insisting on "no frogs"—until a visit from a colleague from Japan, and opportunities to study the cane toad that is devastating the north of Australia, and the beautiful and nearly extinct corroboree frog. Now a shaky line is drawn at fish. These anything-but-model animals (**Figure 1**) have enormously widened our understanding of how sex and sex chromosome systems have evolved in mammals, including humans, and how they can transition, sometimes in dramatic ways, between seemingly quite different states.

The advent of whole genome sequencing provides new opportunities to compare genomes across the evolutionary spectrum. Many mammals have now been sequenced to a depth of at least twofold, and comparisons between them have illuminated many questions of genome organization, function, and evolution. We should therefore gather information more broadly, particularly when studying a trait that is as variable and quixotic as sex.

VERTEBRATE RELATIONSHIPS

To trace the evolution of sex-determining systems, it is essential first to understand how vertebrate groups are related (**Figure 1**). Fish and tetrapods (limbed animals) diverged about 410 Mya, amphibians and amniotes (having a waterproof skin and egg) diverged ~360 Mya, and reptiles (including birds) and mammals diverged 310 Mya.

Placental mammals are only one of three major extant mammalian groups. This Infraclass Eutheria diverged ~180 Mya from the
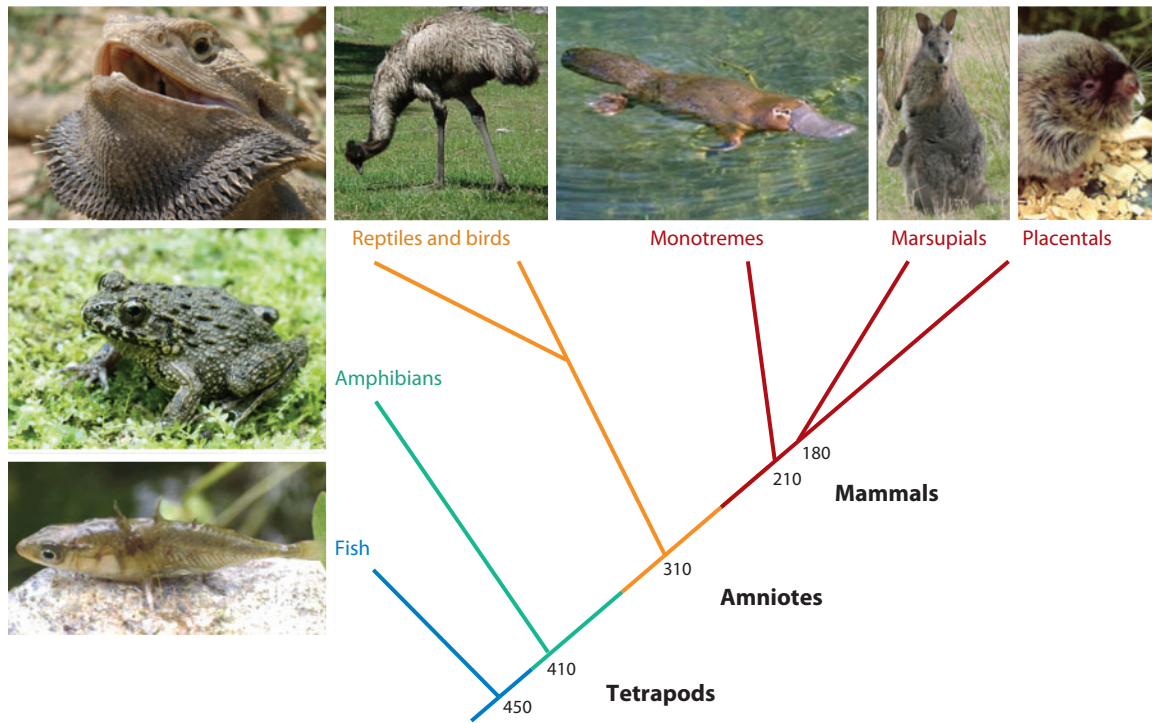
Figure 1

Relationships of vertebrates. Divergence times of vertebrate classes, and mammalian subclasses Prototheria (Monotremes) and Theria, which comprises infraclasses placentals (Eutheria) and marsupials (Metatheria). Dates are from Reference 92, which combines fossil and molecular data and proposes deep roots of the major clades. For other views see a recent meta-analysis (5). Pictured (*clockwise from bottom left*) are some of the non-model animals that are the subject of this review: the three-spined stickleback, the frog *Rana rugosa*, the central bearded dragon *Pogona vitticeps*, the emu *Dromaius novaehollandiae*, the platypus *Ornithorhynchus anatinus*, the tammar wallaby *Macropus eugenii*, and the Y chromosome-less mole vole, *Ellobius tancrei*.

marsupials (Infraclass Metatheria), with which they form mammalian subclass Theria. Australian and American marsupials diverged from each other ~70 Mya when the continents separated (54). Theria diverged from subclass Prototheria (monotremes) about 210 Mya. Marsupials and monotremes differ from placentals most notably in their mode of reproduction. Marsupial young are born at an early stage of development, and complete development attached to a teat often protected within a pouch. Monotremes, including only the fabled duck-billed platypus and several species of the spiny echidna, display an extraordinary amalgam of mammal-like and reptile-like character-

istics; they bear fur and feed their young with milk, but they lay eggs.

We used to think that humans and mouse gave a good representation of mammals, but we now know that they are rather closely related. More distantly related placentals must be considered if we want to discover the commonality, and the variations of sex chromosomes and sex determination. The familiar placental mammals are now divided into two supergroups that diverged about 105 Mya, one that includes primates and rodents, as well as bats, carnivores, and ungulates such as cows, and the other made up of groups of South American and African mammals (52).

WEIRD ANIMAL GENOMES

The type of information such comparisons can provide depends on how closely related are the species being compared. At one end of the spectrum, comparisons of the human and chimpanzee genomes inform us of changes that occurred recently in the hominid lineage and track regions of the genome that have changed very rapidly and may contain clues to our humanness. At the other end of the mammal scale, comparisons with the most distantly related mammals—elephants and armadillos, marsupials and monotremes—can tell us about an ancient common ancestor and identify events that occurred when mammals were first evolving from a reptile-like ancestor.

Sequencing the human genome, then the mouse, were vast undertakings with the technologies of the day, but rapid advances in technology and bioinformatic analysis now make resequencing of known or related genomes relatively inexpensive. This has allowed rapid sequencing and assembly of at least 2x genomes of mammals representing every major placental clade. Proposals to sequence representatives of the other two major mammal groups, marsupials and monotremes, began four years ago. The opossum was sequenced to a depth of sixfold (48), and a twofold sequence of a model kangaroo, the tammar wallaby, is now available and in assembly. The marsupial genome is much the same size as the eutherian genome, and contains a similar set of about 20,000 genes. However, noncoding functional elements showed much more turnover, and comparisons revealed that many conserved noncoding elements in the human genome arose from transposable elements inserted in the eutherian lineage. The principal marsupial-specific innovations appear to have been diversification of gene families involved in environmental interactions, and the principal eutherian innovation was modification of the X chromosome to facilitate epigenetic silencing.

A sixfold platypus genome sequence has also been assembled and published (86). As might be expected for such an extraordinary mammal, its genome contains a fascinating mixture of mammalian and reptilian characteristics. The genome is rather small, comparable to those of reptiles, and has fewer noncoding RNAs than therian genomes, suggesting that, like chicken, it has evaded extensive retrotransposition. The set of 18,527 platypus genes resembles that of therian mammals, although there are expansions (e.g., vomeronasal receptors, milk proteins, and natural killer cell receptors), some mammal-type proteins (e.g., sperm storage proteins) are lacking, and there are specialized functions (e.g., the 19 venom proteins, which evolved from normal components of pathways in parallel to snake venom). Egg yolk proteins, a characteristic that defines reptiles, have mostly been lost or mutated, and the caseins have appeared, consistent with a switch from nutrition of the young in the egg to nutrition by lactation.

Nonmammal vertebrates are still poorly represented among sequenced genomes. The chicken genome, sequenced some years ago (though there are still gaps), was joined recently by the zebra finch. Bird genomes are smaller than mammalian genomes, and contain macrochromosomes and gene-rich microchromosomes that contain little repetitive sequence. Few reptiles are represented; the genome of the green anole, an American lizard, was sequenced, but not assembled onto chromosomes, and a snake, turtle, and alligator are set for twofold sequencing. The frog *Xenopus tropicalis* (a diploid relative of the model *X. laevis*) has been sequenced but not assembled onto chromosomes. Three bony fish species (unfortunately all tetraploid) have been sequenced: the zebrafish (a common model for development), puffer fish (with its tiny genome and lack of repetitive sequence), and the medaka. Sequencing a shark (diploid) and the jawless hagfish will at last provide a fair (though still very mammal-heavy) representation of vertebrate lineages.

SEX-DETERMINING STRATEGIES IN VERTEBRATES

Vertebrates use two seemingly distinct strategies to trigger gonad differentiation: genetic

sex determination (GSD) or environmental sex determination (ESD; commonly temperature-dependent sex determination, TSD). Yet these systems sometimes interact.

In many reptiles, the temperature of egg incubation fixes the sex (69). In some species such as marine turtles, 100% males are produced at low temperatures and 100% females at high (MF pattern). It is the opposite in the alligator; 100% eggs hatch into females at low temperature and 100% into males at high temperature (FM). However, at even higher temperatures, females are produced once more; this FMF pattern suggests that males are produced over a narrow window of optimal temperature. The widespread FMF pattern may be general for all temperature sex determination, but obscured by the limited temperature range over which the animal is viable. In other species, including crocodiles and many lizards, the proportion of males never reaches 100%.

The sex ratio in ESD species may be far from 1:1, according to the environmental conditions. Classic theory of R.A. Fisher predicts that the sex ratio should tend toward 1:1, but there may be situations in which a female bias is selected. There is much ecological work on the advantages offered by these different sex-determination systems (and sex ratios), and the differential fitness of males and females at different temperatures (11). The molecular mechanism by which temperature triggers sex determination in these species is completely unknown, although work on the influence of hormones on sex ratios suggests that the sex-determining trigger acts through influencing the concentration of estrogen or its receptor (69).

In mammals, birds, amphibians, and many reptiles and fish, sex is determined genetically. GSD mechanisms range from those that depend on allelic variation at a single locus to those in which the sex-determining gene is borne on a pair of differentiated sex chromosomes that are obvious under the microscope. In between are many species in which sex-determining genes are borne on cryptic sex chromosomes, although special cytogenetic

methods (e.g., comparative genome hybridization) can sometimes distinguish them (18).

In all these systems, one sex makes gametes that are homozygous for the same sex allele or chromosome (homogametic) and the other makes equal proportions of two kinds of gametes that specify equal numbers of males and females in the offspring (heterogametic). There is a fundamental distinction between species with male heterogamety (XY male, XX female, such as human), and female heterogamety (ZZ male, ZW female such as birds). However, the two systems share many parallels; for instance, the X and Z chromosomes are usually larger and contain many more active genes than the sex-specific Y or W.

Environmental and genetic sex determination have traditionally been thought to constitute completely different triggers, but some reptile species have elements of both (63).

SEX CHROMOSOMES

Sex chromosomes are very atypical of the genome. Evidently the acquisition of a sex-determining allele confers on a chromosome special properties and a special fate.

Sex Chromosome Conservation

Sex chromosomes are considered to be the most variable region of the genome. However, this is true only for the sex-specific element (the Y or W). The mammal X and the bird Z are extremely conserved.

As noted decades ago by Susumo Ohno (57), the gene content of the X chromosome is almost identical between species of placental mammals, perhaps because rearrangements that disrupt the chromosome-wide X inactivation system are strongly selected against. Marsupials have a smaller X, and comparative gene mapping shows that it represents only the long arm and pericentric region of the human X: the rest of the human X is homologous to an autosomal region in marsupials (28). This defines an ancient X conserved region XCR that is on the X

GSD: genetic sex determination

ESD: environmental sex determination

TSD: temperature-dependent sex determination

in all therian mammals, and a region XAR that was recently added in the eutherian lineage.

The mammalian Y chromosome is much more variable, in size and gene content, between therian mammals. Only four genes on the human Y also lie on the tiny marsupial Y, identifying a (~4-Mb) conserved region (YCR) that was part of the original therian Y, and an added region (YAR) that constitutes the bulk of the Y (87).

Birds, too, have very conserved Z chromosomes. Even the most distantly related species share a Z chromosome, as demonstrated by chromosome painting (71), although gene mapping shows many internal rearrangements. Although the snake ZW system looks superficially the same, comprising the fourth largest chromosome pair in a very similar karyotype, surprisingly they share no homology (45). The W of both birds and snakes is more variable, differing in size and gene content between families. Other reptiles show a bewildering variety of sex chromosomes. Many turtles have XX:XY systems of different shapes and sizes, and marine turtles have strict TSD. Lizards may have XX:XY, or ZW:ZZ sex chromosomes, or TSD; in some groups TSD and GSD systems coexist in the same genus.

Amphibians have been much less studied but seem uniformly to have GSD. Sex linkage shows little genetic homology between species. In many frogs the sex chromosomes are cryptic and can be recognized only by comparative genome hybridization or replication banding.

The Autosomal Origins of Sex Chromosomes

Classic theory (51) proposes that sex chromosomes differentiated from an autosomal pair when an allele on one partner acquires a male-determining role. Supporting this hypothesis are observations that sex chromosomes in one group of animals may be autosomal in another (see below, **Figure 4**). For instance, the chicken Z is homologous to human chromosomes 9 and 5, whereas the mammal X is homologous to chicken chromosome 4 p and part of chromosome 1 (31, 53). The snake ZW is homologous to chicken chromosome 2, and the bird ZW is homologous to snake chromosome 2 (45). This would be expected if different autosomes were chosen to be sex chromosomes in different lineages.

Sex chromosomes, even highly differentiated XY or ZW pairs, usually retain some homology consistent with their derivation from autosomal homologues. For instance, the human X and Y, although they differ greatly in size and gene content, share a 2.6-Mb region at the terminus known as the pseudoautosomal region (PAR), over which pairing and recombination occur at meiosis in males.

In addition, most Y-borne genes have homologues on the X from which they clearly derived (30). Many widely expressed genes on the human Y have obvious homologues on the recently added region of the X. Initially, it was proposed that the male-specific genes on the human Y with functions in reproduction originated from other sites in the genome (43), and there are at least two genes on the Y (*DAZ* and *CDY*) that appear to have been transposed or retrotransposed from autosomes. However, several genes known to have male-specific functions have X homologues. For instance, the candidate spermatogenesis gene *RBMY* has a conserved X homologue *RBMX* (13) that is implicated in brain development (83), and the candidate gonadoblastoma gene *TSPY* has an X homologue with the hallmarks of a cell cycle gene (14). Even the sex-determining trigger *SRY* has a homologue in the X-borne *SOX*3 gene (22). The distinction between the two classes of Y genes that were originally proposed is therefore a function of their evolutionary stage, rather than of their ultimate origin.

Bird Z and W chromosomes also share homology. The chicken Z and W share a terminal PAR, over which they pair at female meiosis (79), and nearly all the genes in the female-specific part of the chicken W have partners on the Z. In ratites (large flightless birds that are distantly related to all other birds), almost complete homology between the Z and W was established by painting the chromosomes of a

female (ZW) emu with DNA from an isolated chicken Z (71), and genetic homology was confirmed by mapping genes to the Z and W (56).

There is great variability in the proportion of the sex chromosomes that are pseudoautosomal; for instance, although the bird Z is identical, the W ranges in size and pairing capacity (57). In some mammal species the sex chromosomes are completely nonhomologous; for instance, some rodents (80) in which the X and Y do not pair at all at male meiosis, and all marsupials, in which segregation of the X and Y is accomplished by means of attachment to a proteinaceous basal plate (59). Such variations in the size of the PAR in XY and ZW systems (27) support the hypothesis that sex-chromosome differentiation proceeded by progressive degeneration of the sex-specific heterogametic element, the Y or the W (**Figure 2**).

Degradation of the Sex-Specific Chromosome

The insight that vertebrate sex chromosomes differentiate as the sex-specific element degenerates first came from observations in snakes (57). Different families have an identical Z, but the W ranges from near identity in boids (pythons and their kin), through a large but rearranged (and therefore nonpairing) W in colubrids, to highly differentiated W in vipers. Ohno suggested that these families display different stages of differentiation of the W, and proposed that the Z and W chromosomes differentiated as the W chromosome was progressively degraded.

The same conclusion can be drawn from a comparison of the ZW systems of ratite and carinate birds. Carinate birds have highly differentiated W chromosomes that contain few active genes, but in ratites the W chromosome is nearly indistinguishable from the Z. Loss of active genes from the Z was directly confirmed by comparing gene content in a range of bird species (24).

Y degradation evidently also occurred independently in different mammal lineages. The size and gene content of the Y differs between
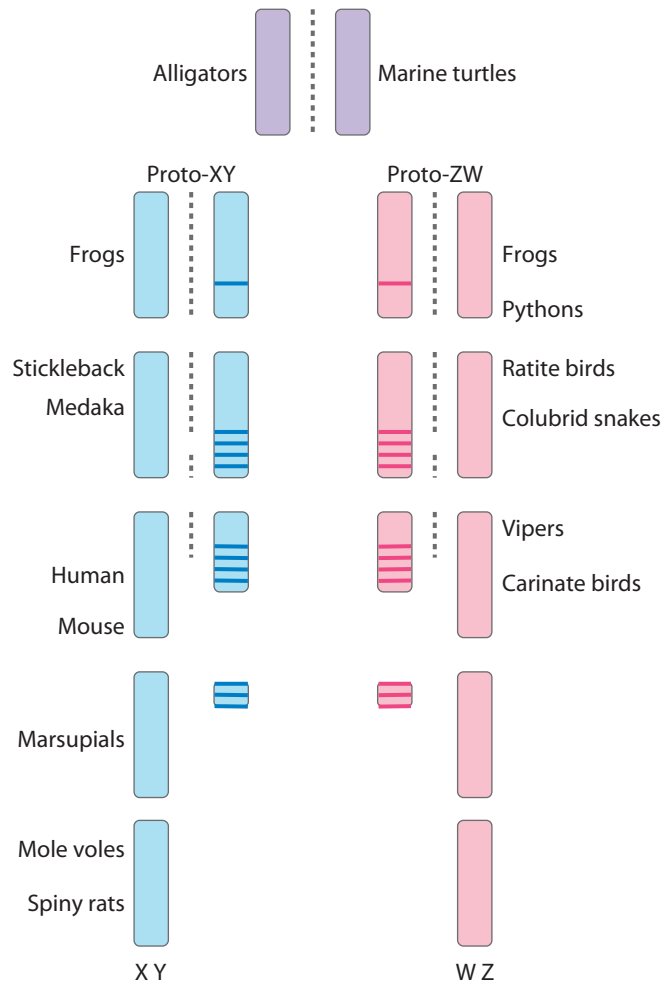

Figure 2

Degeneration of the sex-specific element (Y or W) from an original autosome (*purple*) to form more or less differentiated XY (*blue*) or ZW (*pink*) sex chromosome pairs. Degraded Y chromosomes harbor male-specific genes (*blue lines*) and W harbor female-specific genes (*pink lines*). Examples of vertebrate species that exhibit this level of differentiation of XY or ZW chromosomes are given on the left or right, respectively. Dotted lines represent pairing and recombination.

mammals (30), even between closely related species such as human and chimpanzee (36). In different species the Y evidently retained overlapping subsets of X-borne genes, some of which (like *SRY* and *RBMY*) evolved a critical male-specific function in a common ancestor. Others were lost randomly in some lineages and evolved a male-specific function in others, e.g., the X-borne *UBE1* gene has no copy on the human Y, but retains a copy on the mouse

Y that is required for spermatogenesis. The marsupial (but not the therian) Y retains a copy of *ATRX*. This *ATRY* has testis-specific expression and presumably a male-specific function (61). Some Y genes that seem to have retained housekeeping functions in human have evolved sex-specific functions in mice; for instance, the widely expressed *ZFY* appears to complement the transcription factor *ZFX* in humans, but its mouse homologue *Zfy* is testis-specific and has acquired an essential role in spermatogenesis.

The degradation of the Y seems to have proceeded in steps as large regions of the Y were denied recombination with the X. Evolutionary tidemarks are revealed by comparing sequence divergence of different XY pairs. These cluster into groups that represent the ancient and added regions of the X and Y, and subdivide the added region into parts that lost recombination at different times, perhaps by Y inversion (44). Differentiation of the bird ZW also seems to have occurred in stages (34) and independently in different lineages (16).

The endpoint of Y degradation is exhibited by some exceptional rodents that have lost the entire Y chromosome. Two species of mole vole (*Ellobius*) have no Y, no *SRY*, nor *ZFY* (38); their functions must have been taken over by other genes, as yet unknown. The Ryukyu spiny rat, too, has no Y chromosome and no *SRY* gene (81). How was the entire Y lost, when we know that, as well as the sex-determining trigger, the mammal Y contains several genes that are essential for spermatogenesis? Perhaps the last few genes were replaced one by one by genes that assume their functions. Alternatively, the remaining genes on the Y could be transferred holus bolus onto the X or an autosome. This seems to have occurred in the Ryukyu spiny rat, for several Y genes other than *SRY* have been relocated on the X chromosome (2). Could the Y disappear entirely from other mammals? Calculating the average rate at which genes have been lost from the human Y over the past 310 MY predicted that, at this rate of degradation, it will last only a few million more years (1, 30).

The loss of active genes by the mammal Y (or the bird or snake W) flies in the face of positive selection arguments. Why are mutated and deleted Y chromosomes not selected against, particularly if many genes lost have essential functions in fertility? The degradation process has been explored in detail elsewhere (3, 10, 30). It is proposed that once an allele on one partner of an autosomal pair acquires a male-determining role, other genes that confer a male advantage accumulate around the sex-determining locus. Selection to maintain this male-specific package leads to suppression of recombination with the X. Loss of recombination permits accumulation of mutations and deletions, leading to rapid degeneration. The reverse situation applies if a female-determining gene arises on the original autosome pair: Accumulation of female-advantage alleles and loss of recombination between the W and the Z leads to degradation of the W.

Evidence for molecular changes in the Y (W) comes from observations on all manner of sex chromosome systems. Rearrangements and insertions of repetitive sequence that disrupt recombination are frequent even in newly initiated sex chromosomes such as those in the medaka fish (39) and stickleback (62). Major rearrangements (particularly inversions) are also apparent in the comparison of the Y of humans and great apes (25, 36).

Are there special rules that govern the evolution of sex chromosomes? Basically, without recombination, selection acts on the Y as a unit, for good or ill, so that mutated genes can hitchhike to fixation on a good Y, and good genes are lost if they are on a bad Y. Loss is exacerbated by drift, especially in small populations, and by the low representation of the Y compared to X and autosomes. Accelerating their degeneration is the high level of Y chromosome variation, attested by the high interspecies variation. The Y is at special risk because each generation it must cycle through the testis, a site with active mitosis and poor DNA repair (1). There is no recombination to remove retrotransposed and amplified sequences, which accumulate on the Y and facilitate deletions. Some of the highly amplified regions on the human Y are present

as inverted repeats, palindromic sequences that can form physical loops, within which amplification and gene conversion may occur, homogenizing sequence (68).

The highly repetitive nature of the gene-poor bird W suggests that similar processes degraded the W chromosome in female heterogametic systems, except that the W, being female-specific, is not disadvantaged by a male-effect (17). Thus the heterogametic sex chromosome in XY and ZW systems seems doomed to degenerate.

GENES INVOLVED IN SEX DETERMINATION

In all vertebrate TSD and GSD systems, a similar gonad differentiation pathway is initiated by a variety of genetic or environmental triggers. Sex in vertebrates is a dichotomous trait; animals are either male or female (there is little selective value in being something in between). Yet the pathways that bring about this phenotype are governed by thresholds of continually varying states (69); in TSD it is temperature, but in GSD, too, many genes are dose-dependent, meaning that a threshold concentration is required.

Sex-Determining Pathways

In all vertebrates, the undifferentiated (bipotential) gonad arises from a ridge of cells on the embryonic kidney. This appears identical in males and females until a late stage (12 weeks after conception in humans, 10.5 days in mouse, and after birth in marsupials), then forms testis in males or (later) an ovary in females (**Figure 3**). The testis is composed of germcells (which migrate from outside the gonad) and somatic cells, the most important components of which are Sertoli cells (in which the sex-determining signals act) and steroidogenic Leydig cells.

We still lack a complete picture of the genetic pathway by which a testis forms in mammals, and have even less information about the genes that are involved in ovary differentiation. Many steps have been detected by study-
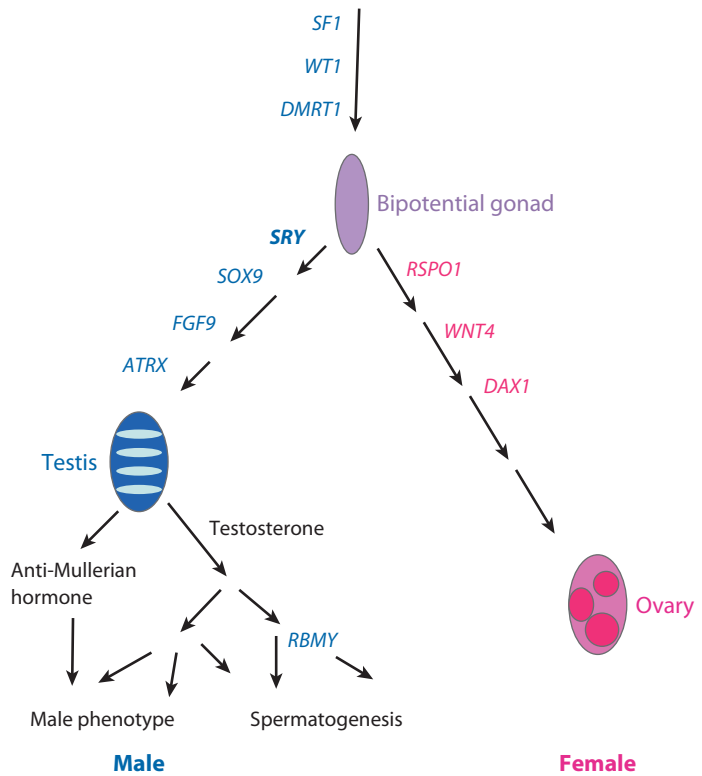

Figure 3

Some of the genes in the mammalian gonad differentiation pathway. A bipotential gonad differentiates into testis (*left*) under the influence of *SRY*, and testis hormones do the rest. If no testis forms, genes such as *RSPO1* promote the differentiation of the bipotential gonad into ovary.

ing animal mutants, or patients with sex reversal syndromes (e.g., haploinsufficiency of *SOX9* and *DMRT1* cause male-to-female reversal). An outline of genes involved in the human and mouse sex-determining pathway is presented in **Figure 3**, and the subject has been reviewed in detail (15, 90). A pivotal gene *SOX*9 is upregulated in the testis in all vertebrates studied; too little SOX9 product causes male-to-female reversal and too much produces XX male development. Several genes regulate or stabilize *SOX*9 to promote the male pathway, and upregulation of *RSPO1* favors the female pathway. Several genes act upstream of *SOX*9 in all mammals; mutations in these cause gonadal abnormalities in both sexes. Some genes in the pathway, including *SOX*9 and *DMRT1*, appear to be dosage sensitive.

DMRT1: *doublesex* and *mab-3* related transcription factor 1

These genes are also gonad-specific in birds (76, 77), and even reptiles with TSD (73), but the timing of their expression relative to other genes in the pathway may be different (50), as well as their expression levels in males and females and their sensitivity to dosage, which is more marked in human than in mouse. Thus the pathway has undergone some reordering during vertebrate evolution, and genes may have acquired new roles in sex determination. Particularly extraordinary are some genes that appear to have completely opposite effects on sex in different animals, appearing as either male or female determiners.

Sex-Determining Strategies

Gonad differentiation may be initiated by apparently quite different triggers, including environmental factors and different genes. We know nothing about the genes involved in environmental sex determination; presumably, some step in the pathway of a TSD animal (perhaps in a step of a hormone biosynthesis or receptor pathway) must be governed by a thermosensitive product of one or more genes, whose concentration of active product must exceed a threshold in order to trigger testis differentiation. We know little about the identity or conservation of this product. The gene(s) that direct sexual differentiation into the male or female pathway have been identified in only a few vertebrates.

GSD could be accomplished by different molecular strategies (**Figure 4**). The most straightforward sex-determining mechanism is via a dominant male-determining gene on the Y in a species with male heterogamety, or a dominant female-determining gene on the W in a species with female heterogamety. However, control can also be wielded by a dosage-dependent gene on the homogametic sex chromosome (X or Z) that triggers the pathway when present in two copies, but not one (e.g., a Z-specific gene could specify male determination in a ZZ male:ZW female system).
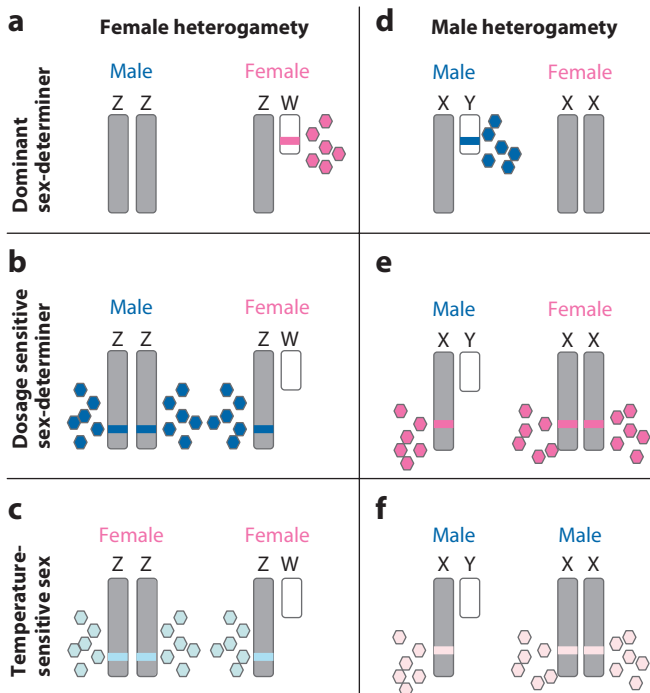

Figure 4

Modes of sex determination. Genes (lines on chromosomes) and their protein products (hexagons) are coded blue for male-determining and pink for female-determining. (*a-c*) ZZ male: ZW female systems of female heterogamety. (*d-f*) XY male: XX female systems of male heterogamety. (*a*) Dominant female-determining gene on the W. Male-determination is the default pathway. (*b*) Dosage-sensitive male-determining gene on the Z. Two copies of this gene are required to make sufficient male-determining protein. (*c*) Temperature-sensitive male-determining product is inactivated at suboptimal temperatures, producing ZZ as well as ZW females. (*d*) Dominant male-determining gene on the Y. Female-determination is the default pathway. (*e*) Dosage-sensitive female-determining gene on the X. Two copies of this gene are required to make sufficient female-determining protein. Temperature-sensitive female-determining product is inactivated at suboptimal temperatures, producing XX as well as XY males.

The Mammalian Sex-Determining Gene *SRY*

The first vertebrate sex-determining gene was discovered by positional cloning on the human Y chromosome. DNA from patients with Y chromosome deletions and additions was scanned to discover which parts were associated with male determination. A critical region was established on the short arm of the Y near the terminal PAR, and *ZFY*, the first gene to be isolated from the human Y, was proposed to be the long-sought testis-determining gene (58).

However, the autosomal location of this gene in marsupials (75), then the demonstration that it was lacking from some XY female patients, led to the isolation of the *SRY* gene from a region even nearer to the PAR (74) and the finding that it is on the Y in all placental mammals. *SRY* was mutated in sex-reversed XY females with *SRY*, and its mouse orthologue could impose maleness when injected into XX embryos (41). *SRY* from other placental mammals will substitute for mouse *SRY* in transgenic experiments, attesting to a conserved function (60).

The tiny marsupial Y, too, bears an orthologue of *SRY*, consistent with a conserved sex-determining role in therian mammals (21). In the absence of mutation analysis, transgenesis, and knockouts in marsupials, we cannot verify directly that *SRY* is the sex-determining gene, and there is a rival candidate *ATRY*, the testis-specific Y copy of the sex-reversing *ATRX* gene.

SRY is a small intronless gene. It is the founding member of a large family (*SOX*) (7) of important chromatin architecture genes whose products bind to DNA at an HMG box domain and bend it through specific angles. Many *SOX* genes have fundamental roles in development in all mammals and other vertebrates. In mammals, the *SRY*-related *SOX*1 is a neural factor and *SOX*2 is required for pluripotency.

Surprisingly for a gene with such a critical function, *SRY* is poorly conserved. There are many nonsynonomous substitutions between species, even within the HMG box, and outside the HMG box sequence is hard to align (21). Mouse *SRY* produces a protein with a polyglutamine tail, essential for activity of transgenes, but this is missing from the gene outside rodents (6).

SRY must be at least 180 MY old, because it is present in marsupials as well as eutherians. However, it cannot be demonstrated outside of mammals. Where did *SRY* come from and when did it evolve? Attempts to clone marsupial *SRY* led to the discovery of a *SOX* gene on the X chromosome (22). *SOX*3 is the closest relative of *SRY*, suggesting that it is the gene from which *SRY* evolved. Thus *SRY* is typical of genes on

the Y, which are almost all relics of genes on the X.

Is *DMRT1* Sex Determining in Vertebrates?

DMRT1 is part of the sex-determining pathway in all vertebrates studied. There are suspicions that it might be something more—the sex-determining trigger—in birds, a frog, and medaka fish.

Birds have ZW females and ZZ males, so sex could be determined by a female-dominant gene on the W, or a dosage-sensitive male determiner on the Z, or both. In the absence of confirmed ZZW and ZO diploid birds, which are proposed to be lethal because of a W-controlled Z dosage mechanism (26), it is difficult to distinguish between these possibilities.

The chicken W chromosome has been scanned in detail in the hope of identifying a female-dominant gene comparable to *SRY* (76). Several W-borne genes have been identified, but most are too similar to alleles on the Z. *ASW* (also called HINTW and *Wpkci*) was the most promising because its Z and W alleles show major differences in amino acid coding potential, and *ASW* is expressed in the genital ridge in females (76). However, ratites have no W-specific allele, so it could not be a universal bird sex determiner. A W-specific gene, *FET1*, is also expressed in the female genital ridge, but without good systems to knock out or transfer genes in chicken eggs, it is difficult to test these candidates directly.

The most promising candidate remains a sex-reversal gene on human chromosome 9 (19, 66), whose orthologue lies on the chicken Z, but not the W even in ratites with homomorphic sex chromosomes (72). *DMRT1* is not subject to dosage compensation (47) and is upregulated in the testis in birds as well as mammals. A locus near *DMRT1* (MHM) lies in a region of the Z that is hyperacetylated and transcribed only in ZW females, but is extensively methylated in ZZ males, and its transcript accumulates at the site on the Z (82). MHM transcription and

RNA accumulation seems to be influenced by the presence of a W chromosome and could be involved in sex determination by further down-regulating *DMRT1* in females.

Despite the lack of direct evidence that it is the bird sex-determining switch, *DMRT1* remains of great interest because it appears to have an ancient connection with sex. In TSD reptiles, only *DMRT1* is expressed more highly at the male-determining temperature (70). Its invertebrate orthologues are involved in sexual differentiation (indeed its name derives from mutations in *Drosophila* and *C. elegans*), arguing for a deep homology underlying sexual differentiation in animals.

WEIRD ANIMAL GENOMES PROVIDE NEW INSIGHTS ON SEX-DETERMINING SYSTEM

Some of the most informative new observations on vertebrate sex and sex chromosomes have come from animals that are far from being models—platypus, dragons, frogs, and fish.

Novel Sex-Determining Systems in Fish and Frogs

To understand how a new sex chromosome system is initiated, it would be valuable to study nascent sex chromosomes. Recently evolved sex-determining systems have been described in *Drosophila* (4, 9), but in most vertebrates, the trail has gone rather cold. Two systems have been described recently that are rare examples of sex chromosomes at a very early stage of their differentiation.

The three-spined stickleback has GSD with male heterogamety but no obvious sex chromosomes, although close relatives have XY systems. A sex-specific marker was discovered that led to the identification of a small differential region that is male specific (62). This region has already lost active genes and accumulated mutations, deletions, and many insertions. The sex-determining gene is unknown, but it should be possible to find a male-dominant gene within

the small male-specific region on the Y, or a dosage-dependent female-determining gene within the corresponding interval of its partner.

In a surprise twist, sex in the medaka was recently found to be controlled by an intronless *DMRT1*-like gene (*DMY*) on an almost undifferentiated Y chromosome, bearing a 43 kb duplication (39, 40). Since closely related species lack this gene, it must have been retrotransposed recently to a new site on an autosome, defining a new male-specific Y chromosome, unrelated to XY and ZW systems in close relatives. This discovery suggests that *DMRT1* and its relatives have a conserved testis-determining role.

However, a copy of *DMRT1* on the W chromosome (*DM-W*) was recently discovered in the toad *Xenopus laevis*, which has female heterogamety, but cryptic ZW chromosomes. *DM-W* was expressed transiently in the genital ridge in females during sex determination, and shown to control ovary differentiation in ZZ tadpoles injected with a *DM-W* transgene (94). Its absence from the closely related *X. tropicalis* implies that DM-W must be a recent transposition.

The presence of *DMRT1*-like genes at the head of female- and male-determining pathways in fish and frogs suggests not only that members of a gene family can acquire roles up or downstream in the sex-determining pathway, but that they can also take on apparently entirely opposite roles in sex determination.

Platypus Sex Chromosomes are Chicken-like, Not Mammal-like

Platypuses have particularly weird sex chromosomes. Early work established that male karyotypes include several chromosomes that do not pair, and these elements form a chain at male meiosis typical of chromosomes that have undergone repeated translocations (32). Chromosome-specific "paints" made by flow sorting or microdissecting individual platypus chromosomes were hybridized to male and female platypus chromosomes. Some paints

hybridized only to a single chromosome in males (defined as Y chromosomes), and others painted a single chromosome in males and a pair in females (defined as X chromosomes) (67). This showed that males have five X chromosomes and five Y chromosomes; females have five pairs of X chromosomes.

Early gene mapping using radioactive in situ hybridization suggested that the largest X chromosome (X_1 at one end of the chain) had homology to the conserved region of the human X (XCR) (88). This implied that the common ancestor of all mammals 310 Mya had an XY chromosome pair like present-day marsupials and that the chain was derived by progressive fusions of this ancestral XY pair with autosomes. This conservation predicted that the monotreme sex-determining gene, as for therian mammals, would prove to be *SRY*. However, a long and tedious search for a platypus *SRY* homologue detected no trace of any male-specific *SOX*-like gene. It was difficult to prove that the gene is really absent, rather than diverged beyond recognition, and the question remained unresolved for a decade.

Answers have now emerged from mapping and sequencing the platypus genome. First, several platypus BACs bearing orthologues of human X genes were mapped, not on platypus X_1, but on chromosome 6 (84, 86). Physical mapping of sequenced contigs verified that no human X genes lie on any platypus X. Instead, XCR genes map together on platypus chromosome 6, which is therefore genetically identical to the marsupial X (**Figure 5**). Thus the therian XY chromosome pair, which is autosomal in platypus, as well as birds and reptiles, was still an autosome 210 Mya, not as long ago as previously supposed.

So what genes do lie on the platypus X and Y chromosomes, and which is the sex-determining switch? The platypus orthologue of *DMRT1* was, unexpectedly, discovered to lie on the large X_5 at the far end of the platypus chain (33). Sequencing now shows that X_5 has almost complete homology to the chicken Z chromosome. There are also small regions of

homology to the chicken Z scattered on other X chromosomes throughout the chain, consistent with repeated translocations (84) from the original XY pair. X_5 Y_5 is the most differentiated pair and so is thought to represent the original sex chromosome pair that first entered into a translocation. Homology between platypus and chicken sex chromosomes suggests these may have been present in a common ancestor 310 Mya (**Figure 5**).

So which gene(s) determines sex in platypus? *SRY* is absent, and *SOX3* (the ancestor of the mammal *SRY*) lies on chromosome 6 among other human X-borne genes (85). The only obvious candidate sex-determining gene on platypus sex chromosomes is *DMRT* on X_5, so the possibility that *DMRT1* determines sex in platypus must be considered. *DMRT1* is required in two doses for male determination in both birds (where it might be the sex-determining trigger) and mammals. How then could this gene trigger sex determination in platypus, in which it has the opposite dosage relationship; two copies in females and one in males?

One possibility is that a copy of the *DMRT1* gene was retained on the tiny Y_5 and mutated or amplified into a dominant male determiner like the medaka *DM-Y*. Although no male-specific relative of *DMRT1* was detected in platypus, PCR or Southern blotting may have missed a diverged copy. Alternatively, *DMRT1* might have a dosage-sensitive female-determining action in the platypus, as it evidently does in *X. laevis*.

This raises the extraordinary prospect that the same sex chromosome pair, and even the same sex-determining gene, could function in opposite ways to produce a dosage-dependent male factor on the Z in birds (with female heterogamety) and a male-dominant *DMY*-like gene on the primitive Y, or a female-determining dosage-dependent *DMRT1* on the X in mammals (with male heterogamety). This was previously thought to be impossible; however, we now have an example of just such a sex-determining flip-flop in a frog.
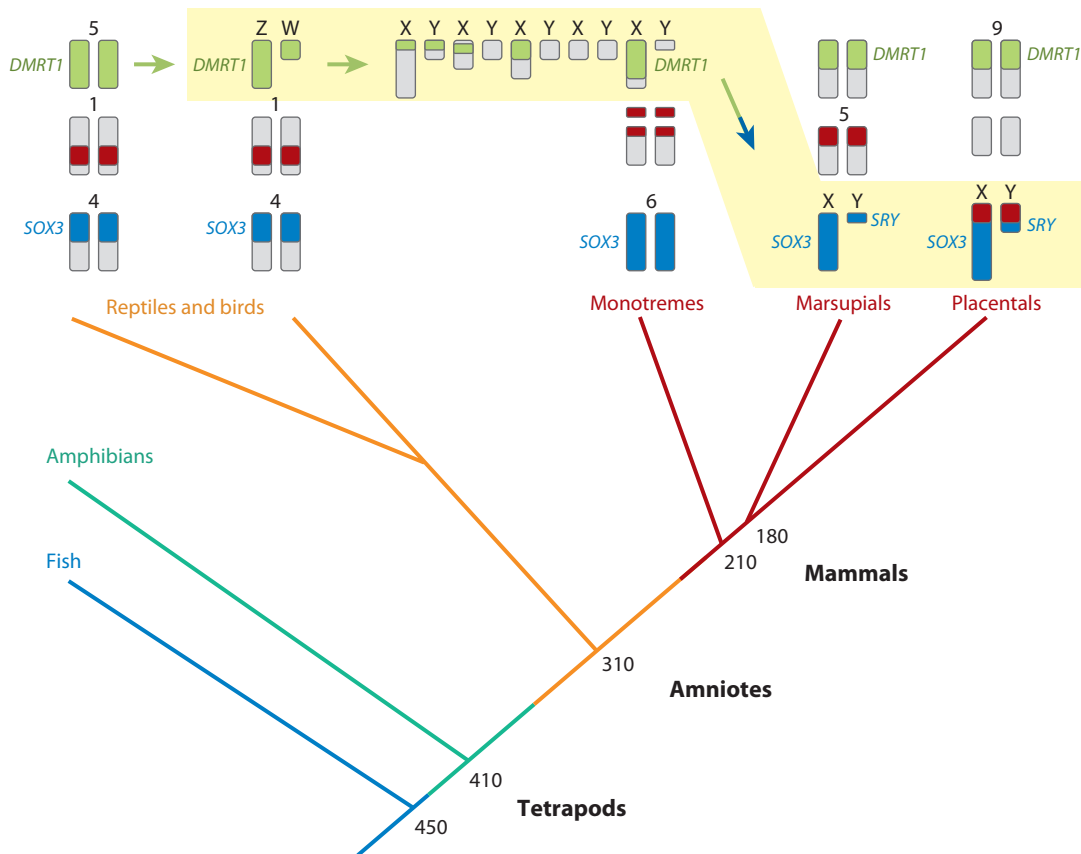
Figure 5

Some major transitions (indicated by *yellow band*) in the sex-determining system of higher vertebrates. A chromosome (turtle 5) that was originally an autosome in TSD reptiles acquired a role as ZW in sex determination in birds (*green*), possibly by means of candidate gene *DMRT1*. The same (*green*) chromosome is involved in sex determination by multiple XY chromosome in the platypus. And a completely different chromosome pair (*blue*, represented by turtle and chicken 4, platypus 6) assumed a sex-determining role as a new XY in therian mammals as *SRY* evolved from ancient gene *SOX3*. This pair was recently augmented by yet another autosomal region (*red*) in placental mammals.

The Frog that Flip-Flops Between XY and ZW Systems

Careful work on the Japanese frog *Rana rugosa* reveals that different populations have sex chromosome systems that are exact opposites. Two populations have male heterogamety but homomorphic sex chromosomes, and another has an XX female:XY male system. However, a fourth population has ZW females and ZZ males (49). Curiously, part of the ZZ:ZW population is more closely related to an XX:XY population, suggesting that an XY to ZW transition occurred twice (55). Hybrids and backcrosses can be made in which all manner of sex chromosome conditions coexist, including XW and WY. Sexual phenotypes of these hybrids and triploids suggest that the Y is dominant over the X or Z, but since WY hybrids are females, the W appears to be dominant over the Y.

The most extraordinary observation is that the XY and ZW pairs are basically the same chromosome, as demonstrated by their identical size (the seventh largest element), banding patterns and gene locations (83a) and ability to pair in hybrids. Without knowing the sex-determining gene in either population, we

cannot test the hypothesis that the same gene has opposite effects in these populations. The most obvious candidate, *DMRT1*, has been isolated and found to be autosomal. It is upregulated in testis, but only after differentiation, so must act downstream of an unknown trigger gene.

If ZW → XY transitions occurred in *Rana rugosa*, why not in the ancestral mammal?

Sex in Dragons: Transitions Between TSD and GSD

The Australian dragons demonstrate even more fundamental flip-flops between TSD and GSD sex-determining systems, and modeling predicts that transitions between ZW and XY sex chromosome systems can also occur. The Australian agamid lizards include many species with GSD, and many with TSD; surprisingly, they are all jumbled in the phylogeny so it is not clear which was the original group, or what changed to what. Either way, evolutionary change seems to have occurred frequently, so that even sister taxa differ in sex-determining mode.

The central bearded dragon *Pogona vitticeps* has ZW chromosomes that can be distinguished by several staining methods (18), and female-specific molecular markers have been identified. It has a 1:1 sex ratio over its normal temperature range. However, at higher temperatures, all hatchlings are female, and many of these animals can be shown to be ZZ sex-reversed females because they lack the W-specific marker (63). This animal therefore has both GSD with differentiated chromosomes and TSD at high temperature. The suggestion was made that sex in this species is controlled by a dosage-sensitive product of a Z-borne gene that is inactivated at high temperature. Two copies are necessary to make sufficient product to induce testis formation, so ZW can never be male. At extremes of temperature, this protein is inactivated, so that some or all ZZ have insufficient product, and develop as sex-reversed females (**Figure 4c**).

The skink *Bassiana* also shows a mix of GSD and TSD. It has an XX female XY male

mechanism over a wide temperature range, but at very low temperature, all animals are males, including XX males that lack the Y-specific molecular marker (64). This suggests the opposite situation: a dosage-sensitive thermolabile female-determining gene on the X (**Figure 4f**). Interactions between genes and temperature may therefore be common in reptiles. However, where the sex-reversing temperature lies outside of the viability range, it will not be obvious.

TRANSITIONS BETWEEN VERTEBRATE SEX-DETERMINING SYSTEMS

The great variety of sex-determining systems in mammals, birds, and reptiles makes it hard to deduce the sex-determining system of a common amniote ancestor 310 Mya. However, some startling homologies may reveal more commonality than has been recognized, and the occurrence of major transitions between different sex-determining systems.

The Sex-Determination System of an Ancient Amniote

Mammals, birds, and snakes all have very stable sex chromosome systems, and it has been difficult to imagine how one could possibly transform into another. Birds and snakes, which diverged ~285 Mya, have very similar karyotypes, but their ZW chromosome pairs are nonhomologous. A change in sex-determining system could have come about by the acquisition of a novel female-determining gene on another chromosome, which overrode the old system (as the W chromosome does in hybrid *R. rugosa*). Alternatively, a rearrangement might have created a neo-Z and neo-W, which is suggested by the occurrence of sex-specific bird-like repetitive elements on the snake ZW (D. O'Meally, unpublished).

Particularly difficult has been to imagine how male and female heterogamety can interconvert. It has therefore been proposed that a common ancestor had a TSD system with no

sex chromosomes, and the mammal and bird systems developed by independent acquisition of different sex-determining genes on different autosomes.

Such a ZW to XY transition seems to have occurred in a reptile-like mammal ancestor, because the newly recognized homology between the sex chromosomes of birds and monotreme mammals suggests that the common ancestor of reptiles and mammals had bird-like ZW chromosomes and sex determination.

How Do Transitions Between Sex-Determining Systems Occur?

Previous models proposed that different sex-determining systems can arise either by cycling through a TSD phase with no sex chromosomes and then differentiation of a novel sex pair, or by "capture" of the trigger function by a novel gene (8, 69).

Reptiles with a genetic sex chromosome mechanism could evolve a temperature-dependent mechanism by acquiring thermosensitivity of one gene product somewhere in the sex-determination pathway. For instance, if mutation affects the thermal stability of a gene product needed at a threshold level for male development, ZZ females will be produced outside the temperature optimum and be selected for because they have more sons. The opposite transition, from TSD to GSD, can be the reverse unless the W has completely disappeared. A strict TSD system with no sex chromosomes is immune from such changes; however, many species thought to have strict TSD never hatch 100% males at any temperature (e.g., saltwater crocodiles), so may harbor a W chromosome at low frequency.

If the W is really gone, transition to a GSD system will require the acquisition of a novel sex-determining gene on the same or a different chromosome that overrides the temperature-dependent step and defines a new proto W or Y. Acquisition of a novel master switch can also over ride GSD systems by capturing the control of the gonadal differentiation pathway. Wilkins

(91) has proposed that such capture is usually at the expense of downstream genes, so pathways get longer from the top. In line with this idea is the observation that sex-determining pathways often seem to involve genes with the same sort of strategies (e.g., RNA splicing in *Drosophila*, and chromatin architecture in mammals).

It is not difficult to see how replacement of one male-determining gene by another might occur, because we know that mutations in several genes in the mammal sexual differentiation pathway are sex reversing. However, for such a mutation to become fixed, it would need to have no detrimental phenotype, and to be fertile, and this is not the case for any known mutant in the mammal pathway. Nevertheless, there are many rodents with fertile XY females, for instance, the wood lemming with a modified male-suppressing X (23), and akodont rodents with a mutant Y (35). A novel male- or female-dominant gene, often a copy of a gene in the pathway retrotransposed to an autosome, can lead to the evolution of a novel Y (e.g., sticklebacks and medaka) or W (e.g., *X. laevis*).

The modeling of changes in species in which both genetic and environmental triggers interact (A.E. Quinn, submitted) provides a third possibility; direct transition between ZW systems of female heterogamety to XY systems of male heterogamety, involving the same chromosomes and even the same genes. Lowering the threshold in a system in which GSD and temperature (or other continuous factor) interact can produce ZW males as well as ZW females, with the prospect of viable WW (female) offspring if the W is not too degraded. This ZW male:WW female system is identical to an XY male:XX female system. This could be the basis of the flip-flop between male and female heterogamety in *R. rugosa* that involves the same chromosome pair. And maybe it could explain how an ancient bird-like ZW system in reptiles was transformed into an XY system in monotreme mammals and our mammal ancestors, starting with basically the same ZW pair and sex-determining (*DMRT1*?) gene.

Evolution of the Mammal XY System

The recent evolution of the therian XY system beautifully demonstrates the takeover of an ancient sex-determining pathway by a novel male-dominant gene.

We can now deduce rather precisely when the XY pair of therian mammals got its start because we know that it is represented by an autosome pair in monotreme mammals, as well as in birds and reptiles. This dates the origin of the therian XY pair to 210–180 Mya, far more recently than originally thought. It also predicts a more imminent extinction of the human Y.

The therian male-dominant gene *SRY* was clearly derived from the *SOX*3 gene, which lies on platypus chromosome 6. *SOX*3 is associated with sexual differentiation in amphibians and reptiles, being expressed in the developing ovaries of *Xenopus* and throughout gonadagenesis in both male and female chickens (42) and also in fish during primordial germcell differentiation (93). However, until recently, *SOX*3 was thought to be involved only in brain development or function in mammals, since it is expressed mainly in the central nervous system (12). Deficiency leads to mental retardation, growth hormone deficiency, and failure of spermatogenesis (65), but is not sex-reversing (89). More recent studies show that it is expressed also in the indifferent gonad, at least in humans and mice, consistent with a role in sex determination. Thus it remains possible that *SOX*3, as well as *SOX*9, plays some role in the mammal sex-determining pathway.

How did *SOX*3 evolve into a male-dominant *SRY*? Comparison of the sequence of these two genes reveals homology only in the HMG box that binds and bends DNA, and even this is poorly conserved. It may be that the *SOX*3 gene on one member of the autosomal pair was truncated, or fused with another gene(s). Its poor conservation suggested that *SRY* is a repressor of testis-inhibiting genes (29), perhaps as the hypothetical factor Z that represses the repressors of testis-differentiating genes (46). However, it now seems more likely that *SOX*3, as well as *SRY*, has a positive role in testis determination, perhaps by triggering, directly or indirectly, *SOX*9 upregulation. The original mutation in *SOX*3 may have enhanced the male-determining function of the *SOX*3 gene, perhaps by removing regulators that prevented its function in females.

CONCLUSIONS

Comparative genomics has great power to unravel the tortuous pathways by which sex evolved in mammals, and several nonmodel vertebrate species have recently yielded new insights into sex determination. Sex chromosomes of therian mammals (humans, mice, kangaroos), far from being archetypal, evolved relatively recently from a chromosome that had no role in sex determination, and is represented by autosomes in other vertebrates, and even the platypus, a monotreme mammal. Other vertebrates, although they share many elements of the sex-determining pathway, have a bewildering variety of sex-determining triggers, including unrelated XY systems of male heterogamety, ZW systems of female heterogamety, and environmental (usually temperature) cues. Yet there may be more commonality and more frequent transitions between these systems than previously recognized.

SUMMARY POINTS

1. Comparisons between genomes of distantly related mammals and other vertebrates can inform us about mammalian (including human) genomes, including the organization, function, and evolution of sex chromosomes and sex-determining genes.

2. Similar sex-determining pathways in vertebrates may be triggered by environmental factors (e.g., temperature) or different genes in different lineages.

3. Differentiated sex chromosomes (e.g., the XY of mammals and the ZW of birds) evolved from autosomal pairs after acquiring a novel sex-determining allele.

4. Sex chromosomes of placental and marsupial mammals (therians) are represented by an autosome in monotreme mammals, implying a recent origin of *SRY* and the human sex chromosomes.

5. Homology between platypus and bird sex chromosomes suggests that a common amniote ancestor had an ancient ZW sex-determining system, perhaps sharing the same conserved master switch gene.

6. Studies in frogs and lizards show that temperature and genetic sex determination commonly interact and may provide transitions between these systems, and even between male and female heterogamety.

FUTURE ISSUES

1. Where and what is the new sex-determining gene(s) in mammals that lost their Y? What happened to the Y-borne spermatogenesis genes in these mammals? Is the Y of related vole species *Ellobius fuscocapillus* already depauperate?

2. How did *SOX3* acquire a dominant male-determining role as *SRY* in therian mammals?

3. What is the sex-determining gene(s) on the bird sex chromosomes? Is it a dominant female-determining gene on the W or a dosage-dependent male gene (e.g., *DMRT1*) on the Z? Or both? Is it the same/related gene on a platypus X or Y? On a snake Z or W?

4. What is the sex-determining gene on the XY or ZW populations of the frog *Rana rugosa*? Do they determine sex by the same or different trigger genes? How do they interact in hybrids?

5. If heterogametic sex chromosomes (Y and W) are doomed to degenerate, why do many GSD systems (e.g., frogs) have cryptic sex chromosomes? We need more data on amphibians!

6. What is the gene product that is temperature sensitive in TSD reptiles? How conserved is it? What are the genetic triggers of sex determination in GSD reptiles and how do they interact with temperature?

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

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Contents

Errata

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