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Sex From W to Z: Evolution of Vertebrate Sex Chromosomes and Sex Determining Genes

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ABSTRACT Sex determination in major vertebrate groups appears to be very variable, including systems of male heterogamety, female heterogamety and a variety of genetic and environmental sex determining systems. Yet comparative studies of sex chromosomes and sex determining genes now suggest that these differences are more apparent than real. The sex chromosomes of even widely divergent groups now appear to have changed very little over the last 300+ million years, and even independently derived sex chromosomes seem to have followed the same set of evolutionary rules. The sex determining pathway seems to be extremely conserved, although the control of the genes in this pathway is vested in different elements. We present a scenario for the independent evolution of XY male heterogamety in mammals and ZW female heterogamety in birds and some reptiles. We suggest that sex determining genes can be made redundant, and replaced by control at another step of a conserved sex determining pathway, and how choice of a gene as a sex switch has led to the evolution of new sex chromosome systems. *J. Exp. Zool. 290:449–462, 2001.*

Sex determining systems in the major groups of higher vertebrates—mammals, birds and reptiles—are apparently very variable. They include male heterogamety (the XX female:XY male system typified by mammals), female heterogamety (the ZW female:ZZ male system in birds and snakes), as well as a variety of genetic and environmental sex determining systems (for instance, temperature sensitive sex determination in alligators).

The difference in male and female heterogametic systems is more than skin deep. Gene mapping confirms our long-held view that there is no homology between the mammal XY and bird ZW pairs, and that they act in completely different ways, by virtue of different sex determining genes. In mammals, the small heterochromatic Y acts as a male-dominant factor to control testis development, by virtue of the testis determining gene *SRY*. In birds, a small heterochromatic W has at least some female-determining effect, although dosage of the Z seems to play the critical role. There is no sex-specific *SRY* in birds and reptiles, but the *DMRT1* gene, which is present on the chicken Z but absent from the W, is a good candidate sex determining gene. Many reptiles, such as alligators and turtles, have temperature-dependent sex determination and lack sex chromosomes, but chromosome painting reveals that the ZW pair remains intact throughout reptiles.

Although comparative gene mapping implies that the mammal XY and the bird ZW pairs are entirely nonhomologous, differentiation of the sex pair seems to have followed similar pathways in mammals, birds and snakes. From observations on snake sex chromosomes many decades ago, Ohno ('67) suggested that the W evolved from the Z by progressive degradation within a nonrecombining sex-specific region. This hypothesis also accounts for the different degrees of ZW homology in different bird groups. A similar pathway for mammal X-Y differentiation is implied by comparisons between the gene content of the X and Y chromosomes, but this is complicated in eutherian mammals by the enlargement of both sex chromosomes by the addition of autosomal segments. In mammals, differences in gene dosage between XX females and XY males are compensated by a large scale inactivation of one X in mammals, and this dosage compensation mechanism now seems to have a parallel in birds.

How did two completely different chromosomal sex determining mechanisms arise independently and drive sex chromosome evolution along simi-

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lar but independent pathways? Comparative studies of sex chromosomes and sex determining genes have provided many surprising answers.

Interpreting comparative genomic data requires a framework of relationships and approximate divergence dates provided by fossil and molecular evidence (Fig. 1). Extant mammals belong to three major groups. Two infraclasses, Eutheria ("placental" mammals) and Metatheria (marsupials) diverged 130–170 million years ago (MYA), and the subclass Theria, which contains them, diverged from Subclass Prototheria (the egg-laying monotremes) even earlier in the 200 million year history of mammals. Mammals diverged from a branch of reptiles (synapsids), which left no other descendants. The other two major branches of reptiles, anapsids (turtles) and diapsids (snakes and lizards, crocodiles and alligators, and the ancestors of birds) are traditionally thought to have diverged 300–350 MYA, although recent DNA sequence comparisons (Janke and Arnason, '97) suggest that turtles are a branch of the diapsids, despite the differences in their anatomy. The thousands of extant bird species belong to two major subclasses, the Ratitae (flightless birds) and the Carinitae, which diverged about 80 MYA. Reptiles, in turn, diverged from amphibians, which evolved from a branch of the bony fish 350–400 MYA.

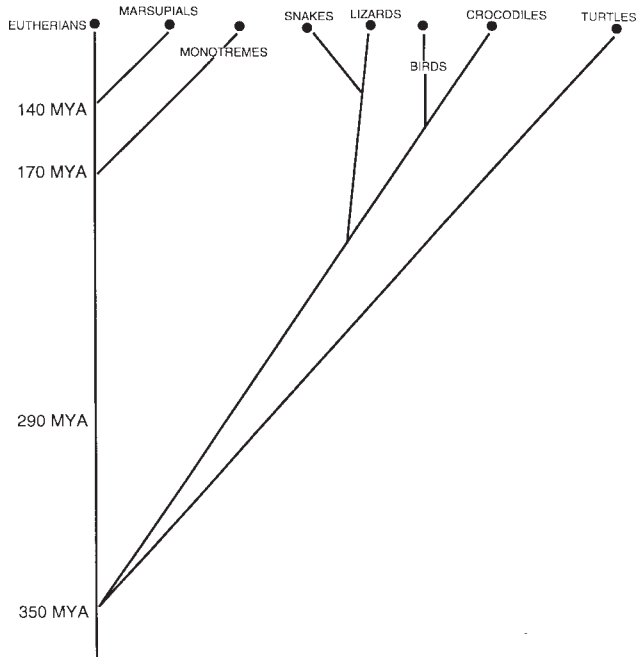

Fig. 1. Approximate divergence dates for the major classes of vertebrates based on fossil and molecular evidence.

VERTEBRATE SEX CHROMOSOMES

Outwardly, the XY pair of mammals and the ZW pair of birds and snakes appear rather similar, one member being large and gene-rich, and the other small and heterochromatic, and genetically depauperate.

What is the relationship between bird, snake and mammal sex chromosomes? The fact that the male is the heterogametic sex in mammals and the female in birds and snakes does not necessarily mean that the XY and ZW chromosomes are fundamentally different, since it is possible to devise a male-dominant system for male heterogamety in mammals, and a dosage-regulated system for female heterogamety from the same proto-sex chromosome pair. However, comparative mapping shows unequivocally that the mammalian XY pair and the bird and reptile ZW pair have entirely different gene contents and must have evolved independently from different autosome pairs containing different sex determining genes. In this section, we briefly review cytological and gene mapping evidence for the origin and evolution of sex chromosomes in mammals, birds and reptiles.

Eutherian sex chromosomes

In all eutherian mammals, the large and gene-rich X chromosome and the small and heterochromatic Y are nonhomologous except over a small "pseudoautosomal region" (PAR) at one or both tips, where they pair and recombine at meiosis.

The eutherian X chromosome represents about 5% of the haploid genome, and bears a few thousand genes coding for a fairly typical mix of housekeeping and specialized functions. As first pointed out by Ohno ('67), the X is remarkably conserved in size and gene content between different eutherian species, perhaps because of strong selection against disruption of the chromosome-wide inactivation system. Conservation is demonstrated by comparative gene mapping and chromosome painting (O'Brien et al., '99). The few exceptions to "Ohno's Law" are three exceptional genes on the short arm of the human X, which map to autosomes in mice. However, these genes were probably exempt from Ohno's Law because they are (or were) pseudoautosomal (Graves et al., '98b). Since females have two copies of the X and males only one, there would be severe dosage imbalance between the sexes were it not for the inactivation of one X in all female somatic cells. There is ongoing debate as to why X inactivation evolved. Was

it simply a dosage compensation mechanism to ensure fair play between the sexes? Or did it represent a primitive dose-dependent sex determining mechanism?

Most eutherian mammals have a small, heterochromatic Y chromosome bearing few active genes amid a wasteland of repetitive sequence and pseudogenes. For instance, the human Y comprises only about 2% of the haploid complement, and the distal half is composed of repetitive, noncoding DNA. The phenotypes first ascribed to the Y were that of testis determination and a male-specific minor histocompatibility antigen (HYA), but more recently, functions in spermatogenesis, growth and cancer have been identified by deletion analysis. Exhaustive screening of cDNA libraries with human Y-specific YACs (yeast artificial chromosomes) has revealed only about twenty genes and pseudogenes on the differentiated region of the human Y, and 12 within the short PARs. The mouse Y contains a similar number of characterized genes all concentrated in the tiny short arm. The long arm of the mouse Y is largely composed of repetitive sequences but contains a terminal PAR.

Evidence that the eutherian Y chromosome is male determining is provided by the male phenotype of XXY and the female phenotype of XO in humans and other species. Testis determination, which is the first identifiable step in the sex determination pathway, is regulated by a "testis determining factor" (TDF) on the Y. Deletion analysis localized TDF to the distal region of the small short arm of the human Y, which was then intensively searched. Mapping studies excluded HYA, the first candidate for TDF, which was genetically separable from testis determination in humans and mice (Simpson et al., '87; McLaren et al., '88). Later *ZFY* (Page et al., '87), cloned from the human Y, was believed to be TDF, but was disqualified first by its autosomal location in marsupials and monotremes (Sinclair et al., '88), then by gene mapping (Palmer et al., '89). The *SRY* gene was cloned from a region on the human Y just proximal to the PAR (Sinclair et al., '90), and its identity as TDF was confirmed by mutation analysis in human sex reversed patients and transgenesis in mice (Koopman et al., '91; Hawkins et al., '92). *SRY* detected homologous genes on the Y in all eutherian mammals, including marsupials (Foster et al., '92).

Genes on the Y are particularly interesting for the insight they give us on the origin of the Y chromosome and its role in sex determination and differentiation, as well as on how genes may evolve a novel function. Several genes on the human Y are expressed specifically in testis, and may be involved in spermatogenesis. *RBMY* and *DAZ,* both repeated on the long arm of the human Y, have been put forward as candidates for the azoospermia factor AZF (Ma et al., '93; Reijo et al., '96), and other testis-specific repeated genes might have functions in spermatogenesis in humans and mice (Mitchell et al., '92; Burgoyne, '92; Sargent et al., '99). Genes on the differential region of the Y are inconsistent in their presence, copy number and activity (Graves and Foster, '94). For instance, *RPS4Y*, coding for a ribosomal protein, is present on the human but not the mouse Y, whereas *UBE1Y* is present on the mouse but not the human Y. Human *AMELY* is expressed, but its mouse homologue is an inactive pseudogene. Copy number, too, varies between species. *ZFY* is unique in humans, duplicated in mice, and multicopy in old world mice (Bianchi et al., '92). Several genes are expressed ubiquitously in human but are testis-specific in mice, suggesting that they may perform different functions in the two species. Even *SRY* is amplified in several mouse and rat species, and its expression pattern is much wider in man than in mouse (Clepet et al., '93; Nagamine, '94). There are rodent species with variant sex chromosome systems, the most extreme of which are two species of the mole vole *Ellobius*, which have entirely lost the Y chromosome and have no *SRY*. Thus, recent and radical change in the sex determining system is apparent even among extant eutherian mammals.

The PAR appears to be critical for XY pairing and fertility, at least in man and mouse. The human PAR1 contains nine functional genes, and a tenth, which straddles the PAR1 boundary and has been truncated and rendered inactive on the Y. Another four genes lie in the small PAR2. The gene content of the PAR is very inconsistent between species (Graves et al., '98b); for instance, *STS*, which is represented by a pseudogene near but not in the human PAR, is active and pseudoautosomal in bovine and sheep, and is the only gene in the 2 Mb mouse PAR.

Sex chromosomes of distantly related mammals

Spectacular sex chromosome variation is to be found among the most distantly related mammal groups, and comparisons of eutherians with marsupials and monotremes have provided unexpected insights. Like eutherians, marsupials have heteromorphic X and Y chromosomes, but the ba-

sic X is only about 3% of the haploid complement, and the Y is tiny. The marsupial X and Y do not undergo homologous pairing and recombination (Sharp, '82), and chromosome painting reveals no PAR (Toder et al., 2000). Monotremes are quite the opposite, with large X and Y chromosomes, which pair over the entire short arm of the X and long arm of the Y (Murtagh, '77), and are the first members of a unique translocation chain. X chromosome inactivation occurs in marsupials, but is different from the eutherian system in many respects, and it is not clear whether X inactivation occurs at all in monotremes (Cooper et al., '93).

Comparative mapping of human X genes in marsupials and monotremes provided the first real exceptions to Ohno's Law of conservation of the X (Graves, '95). Genes from the long arm and the region around the centromere of the human X were mapped to the marsupial and monotreme X. This X conserved region (XCR) is likely to represent the original mammalian X, which has been retained for at least 170 million years. However, genes located more distally on the short arm of the human X were found to map to two autosomal clusters in both marsupials and monotremes. For instance, ten human Xp genes, including pseudoautosomal genes, co-localize on the short arm of the tammar wallaby chromosome 5. Since it is unlikely that the same region was removed independently from the marsupial and monotreme X, the best explanation is that an X added region (XAR) was recently translocated to the eutherian X, after the divergence of the marsupials, but before the major eutherian radiations. Thus, comparisons between the X chromosomes of the most distantly related mammals identifies the evolutionary origins of different regions of the human X chromosome.

The basic marsupial Y chromosome is the smallest of any mammal, weighing in at only 12 million base pairs (megabases, or Mb) in dasyurid marsupials. It is entirely differentiated from the X, and hybridization with DNA from a microdissected Y reveals no PAR (Toder et al., 2000). In kangaroos, the Y is larger, due to the recent addition of the ribosomal RNA genes and associated heterochromatin to the X and Y (Toder et al., '97). The marsupial Y chromosome is testis determining, but differentiation of the embryonic testis does not hormonally control all aspects of sexual differentiation (Sharman et al., '90) as it does in eutherians. XXY animals have testes, but a pouch with mammary glands replaces a scrotum. Conversely, XO animals lack testes, but have an empty scrotum in place of a pouch. Thus, a TDF gene on the marsupial Y determines testis, but dosage (or parental imprinting) of a gene on the X is important for scrotum/mammary gland choice (Cooper et al., '93).

Despite their different sizes, the marsupial and eutherian Y chromosomes have the same evolutionary origin, sharing five genes including homologues of the human testis determining gene *SRY* (Waters et al., 2001). In the tammar wallaby, all five genes map to the tiny short arm of the Y, the long arm being composed of heterochromatin shared with the short arm of the X. These conserved genes define a small Y conserved region (YCR). However, several genes on the human Y, including pseudoautosomal genes, map to the same cluster on kangaroo chromosome 5p as do genes within the XAR. This defines a Y added region (YAR), and implies that autosomal regions were added, not only to the eutherian X, but also to the Y.

The monotreme Y remains mysterious. It is a medium-sized chromosome, but its entire long arm is homologous to, and pairs with, the short arm of the X. The short arm of the Y also pairs with another unpaired element in the translocation chain, so it is unclear how much of the Y is male-specific. Only one gene has been identified on the platypus Y, and this maps within the large PAR and detects no male-specific sequence (Mitchell et al., '98). Indeed, no male-specific sequences have yet been detected in the platypus, and perhaps there are none. In the absence of XXY and XO monotremes, we have no idea whether the Y chromosome is sex determining. Our inability to identify a *SRY* homologue in the platypus may simply mean that it has diverged beyond recognition, but it is possible that monotreme sex determination depends on another unknown gene; a male dominant factor on the long arm of the Y, or a dosage-sensitive gene present on the long arm of the X and absent from the Y.

Sex chromosomes of birds and reptiles

Sex determining systems among birds and reptiles include male heterogamety (XX female:XY male), female heterogamety (ZW female:ZZ male), genetic sex determination (GSD) and environmental sex determination (ESD) in species with no heteromorphic sex chromosomes. Birds and snakes all have a ZZ male:ZW female system, in which Z chromosomes are similar in size (around 10% of the haploid set). Some lizards also have a ZZ male:ZW female system, but others exhibit XX:XY

male heterogamety like mammals. Most turtles and all Crocodylia have no recognizable sex chromosomes, and sex is determined by the temperature at which eggs are incubated (TSD).

In birds, the Z comprises a uniform 7% of the haploid genome in different families, but the W varies. Sex chromosomes of the ratites are homomorphic, having cytologically indistinguishable Z and W. However, cross-species chromosome painting (fluorescence in situ hybridization with DNA amplified from a whole isolated chromosome) with the chicken Z chromosome reveals a nonhybridizing segment on the emu W that presumably consists of repetitive elements not present on the Z (Shetty et al., '99). Carinate birds have a small heterochromatic W, which varies in size between different orders. For instance, the chicken W comprises only 1.5% of the genome (Clinton and Haines, '99), and pairs with the Z only over a small PAR at the tip of the short arm, which contains a single recombination nodule. It consists largely (65%) of families of repeated sequences, and has a GC-rich long arm.

Similarly, snake sex chromosomes show different levels of Z-W differentiation in different groups, from near homomorphy in primitive snakes to extreme differentiation in the higher snakes. The Z chromosomes of most snakes constitute 9–11% of the haploid set, but the W chromosome varies. In boid snakes, the Z and W are morphologically indistinguishable, and in colubrid snakes they are differentiated only by the position of the centromere. In higher snakes (vipers and elapids) the W is small and heterochromatic (Jones and Singh, '85). A female-specific repeated DNA sequence was first identified as a minor satellite from an Indian snake (the Banded Krait, hence *Bkm*), consisting of 12–26 tandem repeats of GATA and GACA sequences. It is distributed throughout the snake genome but concentrated on the W chromosome. This "Garden of Eden" sequence was originally proposed to determine sex, since there are similar sequences concentrated on the mouse Y. However, gene mapping separated *Bkm* from the TDF.

There has been almost no gene mapping in any bird or reptile species except chicken. Like the mammal X chromosome, the chicken Z is gene-rich, and many cloned genes have now been mapped to it. At least two of these also map to the Z chromosome in the emu. Most of the genes on the chicken Z map to human chromosome 9. Only four genes have been mapped to the small, heterochromatic chicken W chromosome, and all have homologues on the Z (reviewed, Ellegren 2000a). The large ratite W contains copies of both genes, which are on the Z but not the W chromosome in the chicken. There is no evidence of dosage compensation of the bird Z from early studies of enzyme dosage (Baverstock et al, '82), but recent quantification of mRNA transcribed by Z linked genes shows clear dosage compensation (McQueen et al., 2001). The two Z chromosomes in males replicate synchronously, implying absence of an inactivation mechanism similar to that of X inactivation.

It is not yet clear whether the bird/reptile ZZ male:ZW female chromosome system works via a female determining gene on the W, or dosage differences of a gene on the Z. The question could be easily answered by observation of the phenotypes of ZO and ZZW diploid birds, but only one has ever been described, despite lengthy search. Their absence suggests that, unlike mammals in which most X-borne genes are active in a single dose in both sexes, two copies of at least some W-Z shared genes are vital in birds. Somewhat contradictory information has come from chickens with three copies of the genome (triploid). ZZZ triploids were phenotypically normal males producing abnormal spermatozoa, and ZWW triploids died early during embryonic development. But ZZW triploids had a confused sexual identity, being female until sexual maturity, but then beginning to crow and adopting a male phenotype. The left gonad developed first as a normal ovary, which gradually turned into an ovotestis as sexual maturity was reached (Thorne and Sheldon, '93). Studies of chimeric birds with a variable proportion of ZZW triploid cells showed that the presence of a W chromosome had a feminizing effect, although Z dosage had a male effect. Thus, both dosages of a Z-borne gene(s) and a dominant W-borne switch gene could operate in birds to initiate sex determination. The recent identification of the *DMRT1* gene, which is involved in testis differentiation in all vertebrates and which maps to the chicken Z, has raised speculation that this is the bird sex determining gene.

ORIGIN AND EVOLUTION OF VERTEBRATE SEX CHROMOSOMES

Despite the differences in size and gene content between heteromorphic sex chromosomes, there is good evidence that they evolved from an ordinary autosomal pair. This hypothesis was originally put forward three decades ago on the basis of observations of intermediate stages in the dif-

ferentiation of the Z and W chromosomes in different snake families. Ohno suggested that the snake Z chromosome maintained its original size and gene content, but the W chromosome had progressively degenerated (Ohno, '67). Similarly, the mammalian X maintains the gene content of an original autosome, but the Y represents a degraded relic. Comparative mapping strongly supports this hypothesis and demonstrates that sex chromosome differentiation has occurred independently, at least in mammals and birds.

Evolution of mammal sex chromosomes

The considerable homology between the mammalian X and Y provides good evidence that mammalian sex chromosomes evolved from an autosomal pair as the Y was progressively degraded. The most obvious homology is in the PARs, but there are also many X-Y shared sequences even in the differentiated regions. Some of the homology is seen in tracts of repeated sequences shared between the X and Y (Affara and Ferguson-Smith, '93), and is probably the result of recent illegitimate exchange. However, many genes on the human Y chromosome have homologues on the X. Several genes in the differentiated region of the human Y, some within the conserved YCR, and some within the recently added YAR, all have homologues on the X. Other X-borne genes have mutated and inactive partners on the Y, as expected of a degraded relic of the ancient autosome from which the sex chromosomes evolved.

This "wimpy Y" view of the Y as a degraded relic of the X (Graves, 2000) has been challenged by Lahn and Page ('97). They asserted that, as well as genes with homologues on the X (referred to as Class I genes), there is a distinct class of genes, which are multicopy and testis-specific. These Class II genes have no homologues on the X, and therefore did not evolve from X-borne genes, but were recruited from autosomes by a "selfish Y" chromosome.

All the interesting genes with male-specific functions, such as the testis determining gene *SRY* and the spermatogenesis gene *RBMY*, were thought to be Y-specific, but both these prototype Class II genes have been recently shown to have an X-borne homologue just like all the Class I genes. *RBMY* detects an X-borne homologue *RBMX* (Delbridge et al., '99; Mazeyrat et al., '99), and even the sex determining gene *SRY* is closely related to *SOX3* on the X, from which it is thought to have evolved (Foster and Graves, '94). Both Y genes have undergone considerable change in structure and function during their evolution from genes with widespread functions in RNA metabolism (*RBMX*) and neural development (*SOX3*). Although there are several genes on the Y with obvious origins by transposition from autosomes (e.g., Lahn and Page, '99), many or most genes, including those with sex specific functions, evolved from progenitors originally on the autosome pair from which the sex chromosomes derived. A clear cutoff between Class I and Class II Y-borne genes is also hard to see because many Y-borne genes have different characteristics in different mammal species. For instance, many human "Class I" genes (such as *ZFY*) are multicopy and testis-specific in rodents.

We conclude that, with the exception of a few intronless genes that were copied from RNA transcripts, genes on the Y chromosome all have counterparts on the X, as predicted by the hypothesis that the X and Y were ultimately derived from a homologous pair of autosomes in a vertebrate ancestor. Thus, each Y-borne gene was evolved from its X-borne counterpart.

If the mammalian X and Y chromosomes were ultimately derived from an ordinary pair of autosomes in an ancestral mammal, we must ask what initiated the process, and what forces drove it. The first step in sex chromosome differentiation is generally supposed to be the acquisition of a sex determining allele by one or other proto-sex chromosome. In mammals, this might be assumed to be the advent of *SRY*. Degradation of the Y chromosome is thought to have occurred when alleles of genes near the sex determining locus acquired a male-specific function, for instance in spermatogenesis, making it advantageous to keep together a male-specific gene package by suppressing recombination between these genes. Mutations, deletions and insertions then rapidly accumulated in this nonrecombining region, as is observed in many regions of low recombination in many organisms. The reasons for this accumulation have been debated over decades, and include stochastic elimination of Y chromosomes with the fewest mutants in small populations ("Muller's ratchet") and selection for Y chromosomes which contain a favourable new variant as well as mutated genes (the "hitchhiker hypothesis") (Charlesworth, '91).

Degradation of a region of the proto-Y chromosome would immediately set up gene dosage inequality between the sexes. For at least some (though probably not many) genes, dosage differences might be deleterious, so this would rapidly select for spreading of X chromosome inactivation

into the unpaired region. This is why most genes on the X with no copy on the Y are subject to inactivation, while genes with an active, widely expressed copy on the Y are exempt. The few exceptional genes probably escape inactivation because degradation has been so recent that the X inactivation system has simply not had time to catch up (Graves et al., '98a).

Interspecies variation in the presence, number and activity of genes on the Y, and in the gene content of the PAR, can be easily explained by differences in the extent of degradation in different mammal lineages. Famously, the *UBE1Y* gene shows the full range, being pseudoautosomal in the platypus, present in the differential region in marsupials and most eutherian mammals, but absent in primates, except for a few pseudogene fragments (Mitchell et al., '98). The different sets of Y-borne genes in different mammals therefore seem to represent small, rather random subsets of genes from the X, caught in various stages of degradation and loss.

In eutherian mammals, the picture is complicated by the recent addition of at least one large autosomal segment. As we described earlier, comparative mapping between eutherian and marsupial mammals implies that both the X and Y are composed of a conserved region present on the sex chromosomes in all mammals, and an added region, which is autosomal in marsupials and monotremes. The added region shows homology between the X and Y, so it must have been transferred to both sex chromosomes. It was probably added initially to an ancient PAR of one partially differentiated sex chromosome, then recombined onto the other (Graves, '95). The enlarged PAR subsequently became differentiated as the Y continued to degrade, until it was reduced to its present vestige.

Did the Y chromosome evolve by a creeping degradation, or by major rearrangements? Traces of ancient pseudoautosomal boundaries on the human and primate Y suggest a gradual attrition. However, there is evidence of several recent inversions in the human-chimpanzee Y lineage (Schempp and Toder, '93; Yen et al., '88), one of which cut a pseudoautosomal gene in half on the Y and produced a large region of the Y which no longer paired with a homologous region of the X. This nonrecombining region would have degraded rapidly. Lahn and Page ('99) have recently compared sequences from several X-Y shared genes to demonstrate five "geological strata", regions of the X which diverged in bursts at different times over the last 200 million years. These may represent different additions and/or major rearrangements (such as inversions), which eliminated homology in large regions and permitted rapid degradation of the newly unpaired Y region.

The upshot of comparative genome mapping between eutherian mammals, marsupials and monotremes is that most of the original mammal Y chromosome has been degraded and lost over the last 200 million years. The marsupial Y is all but gone— only about 10 Mb remain, containing a handful of genes. It is so disposable that it is eliminated in the somatic tissues of several marsupial species. The eutherian Y is larger and bears more genes, but it contains only two small regions (as little as 8% of the euchromatic region) of original Y (Waters et al., 2001), and most of it originated from a recent addition. Thus, the original Y chromosome is all but gone in eutherians, and the Y has been saved from extinction only by addition of an autosomal region.

The mole vole provides a chilling reminder of the mortality of the Y chromosome, for it completely lacks a Y chromosome and there is no *SRY* (Just et al., '95). Presumably, a new sex determining gene has arisen on another autosome, initiating a new cycle of sex chromosome differentiation.

Evolution of sex chromosomes in birds and reptiles

Despite the variety of sex determining systems, and differences between sex chromosomes in different reptile groups, the most strongly differentiated sex chromosome systems in snakes and birds show striking parallels of relative size and ranges of homology, and may even represent a conserved sex pair.

The bird Z chromosome is highly conserved in size and banding pattern, and limited gene mapping shows that genes located on the chicken Z are also on the Z in ratites. Homology is spectacularly confirmed by the complete painting of the emu Z chromosome with DNA from the flow-sorted chicken Z chromosome (Fig. 2a) (Shetty et al., '99). No gene mapping data are available in snakes, but the Z has a similar size (as the fourth or fifth largest element) in all snakes as well as birds, suggesting that the bird and the snake Z chromosomes are genetically homologous. Remarkably, chromosome painting establishes that the bird Z chromosome is also completely equivalent to chromosome 5 in the turtle (Fig. 2b), an ESD species with no sex chromosomes. The con-

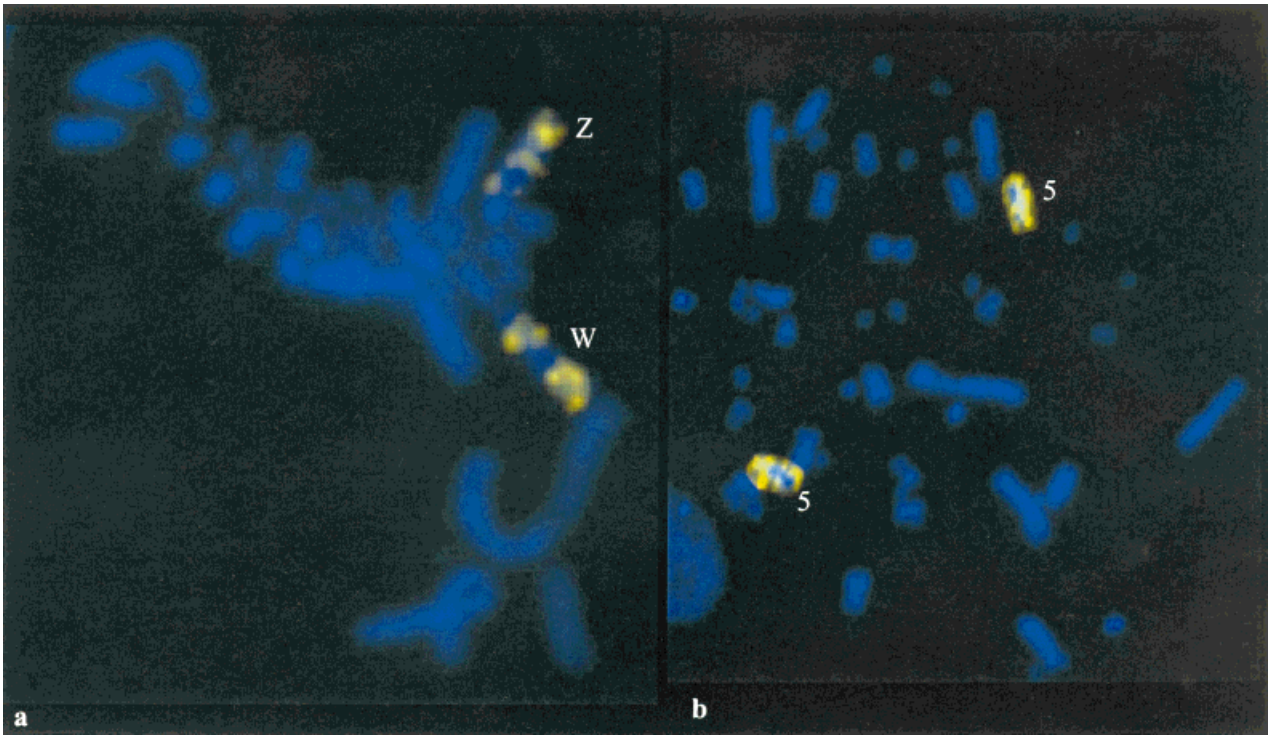

Fig. 2. Chicken (*Gallus domesticus*) chromosome Z hybridized to emu (*Dromaius novaehollandiae*) and turtle (*Chelodina longicollis*) metaphase spreads. (**a**) Chicken Z paint hybridized to Z and most of the W chromosome in a female emu. (**b**) Chicken Z hybridized to chromosome 5 in the turtle.

servation of the Z is typical of the extraordinary conservation of the whole karyotype in birds and even turtles, which diverged about 350 MYA. It will be of particular interest to test homology of the bird and snake Z by chromosome painting, and see whether *DMRT1* lies on the snake chromosome 5, as would be required of a conserved reptile-bird sex determining gene.

The Z and W chromosomes show obvious homology. Pairing extends over the undifferentiated Z and W in ratite birds, but is confined to one end (a putative PAR) in carinate birds with a small heterochromatic W. Gene mapping is entirely consistent with these observations of homology, for the three genes mapped to the chicken W all have homologues on the Z. Two other genes that are X-specific in the chicken both have homologues on the homomorphic W as well as the Z in the emu. Chromosome painting also demonstrates the near complete homology of the ratite Z and W, for the chicken Z paint, as well as completely hybridizing the emu Z chromosome, paints all of the W except a region near the centromere (Fig. 2a). In the absence of gene mapping, Z-W homology in snakes is demonstrated by homologous pairing at female meiosis, which extends over the undifferentiated Z and W in boid snakes, but is confined to one end (presumably a PAR) in snakes with a small heterochromatic W.

Thus, both snakes and birds illustrate different degrees of Z-W homology, which represent stages in W chromosome degradation. Boid snakes and ratite birds represent an early stage in Z-W differentiation, whereas higher snakes and carinate birds represent almost complete differentiation. The process in which the W chromosome in different bird and reptile lineages has become smaller and more heterochromatic, and has lost genetic markers found on the Z, is likely to be similar to the differentiation of the human X and Y from ancient near-homology (represented by monotremes), through partial differentiation (eutherians) to complete differentiation (marsupials). Thus, the progression first noted in snakes (Ohno, '67) can be used as a model for the evolution of all sex chromosome systems from ordinary pairs of autosomes.

The XY male:XX female system of mammals and the ZZ male:ZW female system of birds and snakes could represent totally different means of regulating sex determination. Yet it is possible to devise a system in which the same sex determin-

ing gene on the same pair of sex chromosomes might regulate sex in birds/ reptiles by dosage differences, and in mammals by a male-dominant factor on the Y. It is important, therefore, to test the hypothesis that the bird/reptile ZW pair is equivalent to the mammal XY pair.

The answer from comparative gene mapping is unequivocally no. Of the 17 genes now mapped to the chicken Z, most lie on human chromosome 9 and none lie on the mammalian X. Conversely, of the six genes on the human X that have been mapped in chicken, three lie on chicken 1 and three on chicken 4; none lie on the chicken Z (Fig. 3). Thus, the bird Z and the basic mammalian X do not share any genes at all. Since the bird W is a relic of the Z, and the mammal Y a relic of the X, it follows that the small heterochromatic W and Y chromosomes in the two lineages bear no relationship to each other. The complete absence of homology between the mammalian X and the bird Z therefore implies that the XX:XY and ZW:ZZ systems evolved independently from different autosomal pairs in a primitive reptile.

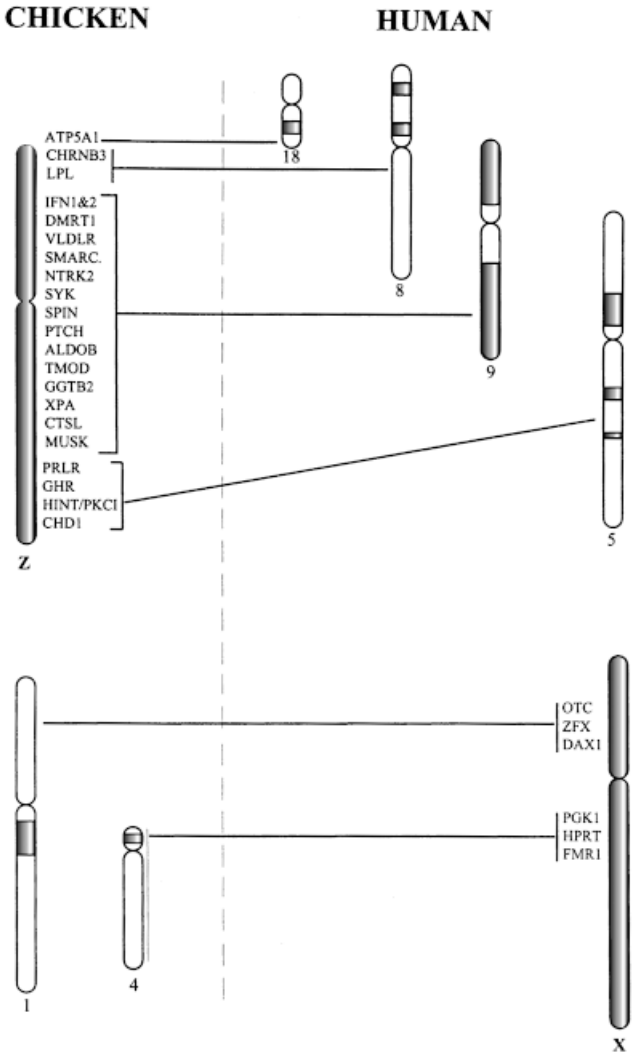

Fig. 3. Comparative positions of genes that have been mapped to the Z chromosome in birds and the X chromosome in humans. (Exact positions of human X genes on chicken chromosomes 1 and 4 are not yet defined.)

Since mammals and bird sex chromosomes are not related, they are unlikely to share a sex determining gene. It is not surprising, therefore, that the Y-borne human *SRY* detects no sex-specific bands in birds or reptiles (Griffiths, '91), and the *SOX3* gene from which mammalian *SRY* evolved is autosomal in the emu (Swathi Shetty, personal communication). Conversely, the Z-borne bird candidate sex determining gene *DMRT1* has a homologue in mammals that lies on human chromosome 9.

Thus, the independent evolution of XY and ZW sex chromosomes from different autosome pairs must have been initiated by different sex determining genes. Although the starting autosome pair was different, and the sex determining allele was different, the process of sex chromosome evolution in mammals and birds/reptiles has been entirely parallel, involving acquisition of an allele which controls a step in gonad differentiation, suppression of recombination around a cluster of sex-specific genes, and degradation of this nonrecombining region. Suggestions that the mammalian Y and the bird/reptile W were intrinsically unstable because of their repetitive structure, or because of their low representation in the population, were recently laid to rest by the finding (Ellegren, 2000b) of greater interspecies variation in mammal Y genes than in bird W genes. This implies that variation is male-driven, presumably because of the many mitotic divisions that the mammal Y (but not the chicken W) is subjected to in the testis.

If XY and ZW differentiation from two different autosomal pairs in primitive mammals and reptiles were independent events, we must ask what common system did they take over from? The occurrence of ESD in Crocodylia and turtles, as well as in many fish species, suggests that ESD is the ancestral vertebrate condition. The evolution of the ZW system in birds and the XY system in mammals could therefore have occurred independently in the two lineages from a ground state in which there was no genetic sex determination. It is tempting to think of a ground state, in which sex determination is environmentally controlled and there are no sex chromosomes. This is conceivable, since two distantly related reptile

groups (turtles and alligators) both have ESD, as do many fish. It would be easy to account for the independent evolution of genetic sex determination in two groups (birds and mammals), which are homeothermic, and could hardly make use of TSD. There are fish species with highly differentiated sex chromosomes whose close relatives rely on ESD; indeed, there is one instance of a GSD/ESD polymorphism within the same species (Lagomarsino and Conover, '93), which seems to be controlled by only a few genes, implying that ESD and GSD are readily interchangeable.

EVOLUTION OF VERTEBRATE SEX DETERMINING GENES

Ohno's hypothesis predicts that the Y chromosome, and all the genes on it, was derived from the original X, and that the W, and all the genes on it, was derived from the original Z over the last 200–300 million years. This implies that genes involved in sex determination acquired their sex-specific roles quite recently in evolution. Comparisons between X- and Y-borne homologues, or Z- and W-borne homologues may reveal changes in gene structure and sequence that accompanied the acquisition of testis-specific expression and sex-specific function of *SRY* in mammals, and *DMRT1* in birds, and help deduce how these sex determining systems are related to each other.

Evolution of sex determining genes in eutherian mammals

SRY belongs to Class I of Y-borne genes, which have homologues on the X chromosome. For other Class I genes, wide expression of the X linked gene implies an important general role in both sexes. The Y homologue may have a similar expression profile (for instance, the ribosomal protein *RPS4*) implying that it has retained its original function, presumably because of selection for dosage balance, or simply because it has not yet had time to diverge. Alternatively, genes on the Y may have become testis-specific (such as mouse *Zfy*), and may have acquired a male-specific function, as has evidently the candidate spermatogenesis gene *RBMY*. In the same way, the male-specific *SRY* appears to have evolved from *SOX3* with a developmental function in both sexes.

Both *SRY* and *SOX3* are intronless members of the *SOX* gene family, members of which code for proteins containing an 80 amino acid HMG box shared with High Mobility Group proteins (Gubbay et al., '90; Sinclair et al., '90). The products of both genes bind DNA at an AACAAT target site and bend it through a specific angle, presumably bringing together sequences on either side of the target, or proteins bound to them, and causing changes in chromatin configuration and gene activity (Harley et al., '92). *SOX3* is expressed in the developing central nervous system in both sexes in mouse, human and marsupial, and specifies almost identical products, which presumably have a role in brain development. *SOX3* also has some expression in the undifferentiated gonad in the mouse (Collignon et al, '96). In contrast, mouse *SRY* is expressed only in the undifferentiated gonad, although human and marsupial *SRY* are more widely expressed.

There are striking differences in the structure and sequence of the *SRY* and *SOX3* genes. *SOX3* codes for a 141 amino acid protein containing, as well as the conserved HMG box, conserved N- and C-terminal regions and a polyalanine repeat, domains that are presumably important to function. In contrast, *SRY* is only moderately conserved between species within the 80 amino acid box, and cannot even be aligned outside it (Foster et al., '92). All the activity of human *SRY* must lie within the box, since sex reversing mutations cluster in this region. Thus, *SRY* appears to be a truncated version of *SOX3*.

Can the acquisition of a sex determining function by *SRY* be attributed to these changes? Because of the major structural and sequence differences between the two genes, it seems unlikely that *SRY* and *SOX3* have the same action and differ only in the time and tissue of transcription. Indeed, it is possible that *SRY* has a completely opposite action as a repressor, as has been demonstrated for a truncated *SOX9* gene (Südbeck et al., '96). *SRY* could have its male-dominant effect via a double inhibition (McElreavey et al., '93), perhaps by inhibiting the action of another *SOX* gene (*SOX3*), which in turn represses another *SOX* gene (*SOX9*) known to be critical in sex determination in all vertebrates (Graves et al, '98b).

How did a brain-determining gene like *SOX3* became a testis-determining gene? Perhaps *SOX3* had some ancestral role in gonadogenesis revealed by its minor expression in the mouse genital ridge, and by the ovary-specific expression of an amphibian homologue (Koyano et al., '97; Penzel et al., '97). If *SOX3* already interacted with *SOX9*, it is not hard to imagine how a Y-borne allele could have been altered to perform an inhibitory function. The first step may have been mutation to an inactive (null) form of *SOX3*, which took over sex determination first by a dose dependent inhi-

bition of *SOX9*. Homozygotes with two copies of normal *SOX3* inhibited *SOX9* to produce a female. Heterozygotes for the null allele had only a single active copy of *SOX3*, insufficient to inhibit *SOX9*, permitting testis development. This system later evolved into the more robust male dominant system by the truncation of *SOX3* to form *SRY*, which acted as an effective repressor of *SOX3*.Thus, the transcriptional activator *SOX3* was deleted and mutated to shape a truncated *SRY* gene with a repressor function.

Sequence difference between *SRY* in different species initially made it seem likely that regions outside the HMG box were tacked onto the *SRY* gene as a result of random genetic accidents to the Y and perform no function at all. However, mice transgenic for *SRY* with different amounts of 3′ sequence developed as males only when they had received the glutamine-rich 3′ domain that is unique to mouse (Bowles et al., '99). This domain must therefore have endowed the *SRY* gene with additional functions. The wider expression of *SRY* in some humans and marsupials may also mean that *SRY* retains other functions (e.g., in the brain) in species other than rodents.

Evolution of sex determining genes in vertebrates

The evolution of the *SRY* gene may be only a very recent chapter of the evolutionary story of mammalian sex determination. There appears to be no male-specific *SRY* gene in monotreme mammals, and although there is an *SRY* homologue on the marsupial Y chromosome, there is no direct evidence that it is testis determining. Indeed, the poor homology with human and mouse *SRY*, and its ubiquitous expression in marsupials, casts doubt on the function of this gene in marsupial sex determination.

The surprising finding (Pask and Graves, '99) that a human X-specific sex reversing gene *ATRX* recognizes homologues on the Y as well as the X chromosome in marsupials provides an alternative candidate sex determining gene in this mammal group. *ATRX* (for alpha thalassemia, mental retardation syndrome on the X) is accompanied by male-to-female sex reversal in XY individuals with a mutation in the X-borne *ATRX* gene. *ATRX* is expressed ubiquitously in humans and mice, and codes for a helicase, which is similar to other proteins with effects on chromatin. In marsupials, there is division of labour; the X homologue is expressed everywhere except the gonads, and a Y-borne *ATRY* is expressed specifically in the testis. *ATRY* may therefore be a better candidate sex determining gene than *SRY*. If there proves to be a Y-borne testis-specific *ATRY* gene in monotremes, it may imply that *ATRY*, not *SRY* or even *SOX3*, was the original mammalian sex determining gene, and the real initiator of X-Y chromosome differentiation.

SRY is certainly unlikely to have been the original sex determining gene in the common ancestor of all higher vertebrates. There is no sex-specific *SRY* homologue in birds or any reptile. Recently, a sex reversing syndrome associated with deletions of human chromosome 9p has been pinpointed to a critical region at the end of the short arm, and a gene *DMRT1* was cloned from this interval. (Raymond et al., '98). Since human chromosome 9 is equivalent to the bird Z chromosome, it is not surprising to find that *DMRT1* maps to the chicken Z (Nanda et al., '99). There is no copy on the W, so there is a dosage difference of two copies (male) to one (female). The gene is expressed specifically in the testis at about the same time as *SOX9* in both mice and chickens, so is obviously an ancient component of the vertebrate sex determining pathway (Smith et al., '99). In fact, the name of this gene is derived from its relatedness to two sex determining genes in invertebrates, Drosophila *doublesex* and the nematode *Mab g*ene. *DMRT1* makes *SRY* look like a real newcomer, having been associated with sex determination for perhaps a billion years.

DMRT1 therefore becomes our best guess for the bird/reptile sex determining gene. If the Z is equivalent in birds and snakes, we suggest the defining event must have occurred early in reptile evolution. The extraordinary conservation of the Z chromosome implies that the proto-ZW chromosome pair bore *DMRT*. How did this gene become sex determining? *DMRT1* may have been originally involved in the sex determining pathway but was not the control switch. In an event paralleling X-Y evolution, we surmise that *DMRT1* dosage came to control a step in the sex determining pathway with the evolution of a null mutation. This produced a dosage difference in *DMRT1*; homozygous individuals had the two doses required for male development, whereas heterozygotes for the null allele had a single dose, which was insufficient to induce maleness. This led to the differentiation of the Z (containing normal *DMRT1*) and the female-specific W (which lacked normal *DMRT1*). In humans, too, deletion of one copy of *DMRT1* on chromosome 9p interrupts the testis-determining pathway,

and switches gonad development to the female pathway.

This evolution of control by *DMRT1* dosage is similar to the proposal that dosage of *ATRY* or *SOX3* may have originally evolved as a sex determining switch in primitive mammals. However, the genes selected for this role were entirely independent, and lay on different autosomes. We can only speculate about the original sex determining system in the common ancestor of reptiles and mammals. Was it *DMRT1* or *ATRY* or *SOX3*, or another ancestral sex determining gene?

Changes of sex determining genes in vertebrate evolution

We suggest that the genes, which took over control of sex determination in different species, were components of an ancient and conserved sex determining pathway. Testis determination in all vertebrates is histologically almost identical, and it is expected that the steps, and the genes that control them, are all highly conserved. These steps are slowly being worked out by studies of sex reversing mutations in humans and mice, as well as by interspecific variation between animals as distantly related as humans, mice, marsupials, chickens and alligators. A number of genes have been identified, including *SOX9*, *DMRT1* and *ATRX*, although their precise roles and the order in which they act is still hotly debated. Such a conserved testis determining pathway could obviously be controlled at any step, so that different steps provide the control switch in different groups.

The same pathway could be controlled by a build-up of an unknown temperature-sensitive product in reptiles with ESD, by *DMRT1* in birds and reptiles, *ATRY* in monotremes and marsupials and *SRY* in eutherians. Mouse *SRY* could have recently evolved new functions with the acquisition of a unique 3′ domain. Control has evidently switched from one point in the pathway to another during evolution (Fig. 4).

It is not hard to see how a new gene could superimpose its function on sex determination, either by interacting with the old gene or its target, extending the chain of command, or by short-circuiting it. This has evidently occurred quite recently in the mole voles, which have completely lost their Y chromosome. It is likely that an emerging sex determining gene has short-circuited the unnecessarily complex *SRY*-controlled double inhibition, making *SRY*, and hence the whole Y chromosome, redundant. Evidently, these animals are

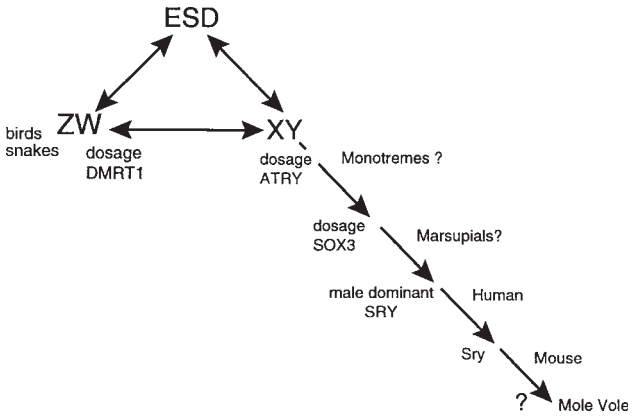

Fig. 4. Evolutionary changes in control of the sex determination pathway in higher vertebrates.

in the initial stages of evolution of a completely new sex chromosome system, initiated by another sex determining allele. Another autosome has begun to differentiate— perhaps into a large gene-rich U chromosome and a small heterochromatic V chromosome. A few million years in the future, this will make it necessary to rename an update of this review "Sex from U to Z."

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