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Sex chromosomes and sex-determining genes: insights from marsupials and monotremes

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Abstract. Comparative studies of the genes involved in sex determination in the three extant classes of mammals, and other vertebrates, has allowed us to identify genes that are highly conserved in vertebrate sex determination and those that have recently evolved roles in one lineage. Analysis of the conservation and function of candidate genes in different vertebrate groups has been crucial to our understanding of their function and positioning in a conserved vertebrate sex-determining pathway. Here we review comparisons between genes in the sex-determining pathway in different vertebrates, and ask how these comparisons affect our views on the role of each gene in vertebrate sex determination.

Key words. Vertebrate sex determination; comparative analysis; DAX1; SOX9; SOX3; SF1; SRY.

Introduction

Sex determination is critical for reproduction. One would think, therefore, that the mechanisms determining sex and sexual dimorphism would be extremely conserved in evolution. Certainly the process of gonadogenesis appears to be similar, at least at the histological level. But surprisingly, the control of this critical phenotype seems to be subject to great variation within mammals, and between mammals and other vertebrates. We can use this variation to ask how the sex-determining pathway evolved and how it functions.

Particularly enlightening have been comparisons between the three major extant groups of mammals. Marsupials and monotremes represent the mammals most distantly related to humans and mice, having diverged from eutherians about 130 and 170 million years before present (MYrBP), respectively [1], early in the 200 million year history of Class Mammalia. In turn, mammals diverged from reptiles and birds about 350 MYrBP (fig. 1).

Sex determination in mammals is accomplished by a chromosomal mechanism. Females (the homogametic sex) have two X chromosomes, and males (the heterogametic sex) a single X and a Y. In eutherian ('placental') mammals, observations of the phenotypes of XO females and XXY males show that the presence of a Y chromosome determines a male phenotype, no matter how many copies of the X are present. This has been ascribed to the presence on the Y of a dominant 'testis-determining factor' (TDF), which activates a testis-determining pathway. Once a testis is differentiated, the hormones it produces control all other aspects of male phenotype.

In marsupials, the control of testis determination is also vested in the Y, for XXY animals have testes and XO do not. However, not all aspects of male phenotype are fixed by testicular androgens [2]. XXY animals have no scrotum, but possess a pouch with mammary glands. XO, though more variable, are the reverse, lacking pouch and mammary glands, but having a scrotum. This suggests that there is a gene on the marsupial X which controls a scrotum/mammary switch. This gene must be either dosage-sensitive such that one copy determines scrotum development and two mammary development, or imprinted such that a gene(s) on the paternal X switches the potentiality from scrotum to mammary gland development [3].

There are several different modes of sex determination in other vertebrates. Birds as well as snakes subscribe to a ZZ male:ZW female sex chromosome system in which

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the female, not the male, is the heterogametic sex, and the heterochromatic W chromosome is not strongly female determining. Reptiles have a great variety of chromosomal and genetic sex determination mechanisms, as well as environmental sex determination, in which factors such as temperature of incubation determine the sex of the eggs. Do all these control mechanisms operate through the same genetic pathway? Are the same sex determining genes active in testis determination—and ovary determination—in all vertebrates?

Here we will review the evidence for the identity of the testis-determining factor and other genes in the sex-determining pathway in mammals, and ask whether the same genes operate in the same pathway in all mammal groups and other vertebrates. Comparisons of homologous genes between closely and distantly related species have provided some surprising information on the complexity and variation of the controls on sex determination, and have demonstrated that a knowledge of evolution can sometimes be required for an understanding of function.

Sex chromosome organization, function and evolution

In mammals, the X and Y chromosomes are strikingly nonhomologous, having different sizes and quite different gene contents. The human X chromosome comprises about 5% of the haploid genome, and accounts for 3000 or 4000 genes. These code for a mixture of classic housekeeping enzymes and products with specialised functions, although it has been argued that there are a disproportionate number of X-linked conditions with an effect on gonads or reproduction. The X is highly conserved between different eutherian species, perhaps because of a chromosome-wide inactivation mechanism which ensures dosage compensation between males and females.

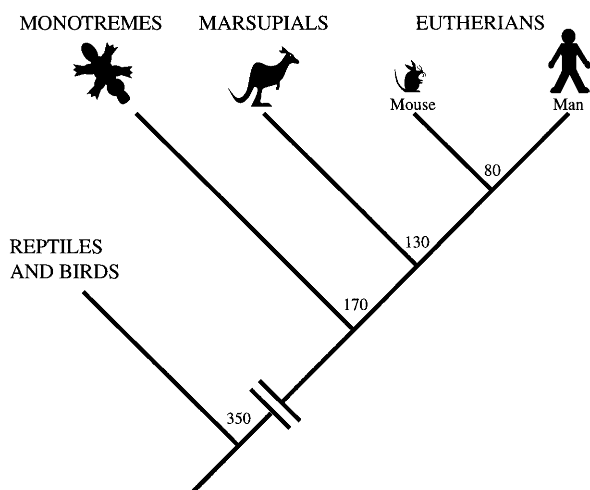

Figure 1. Phylogeny of class Mammalia, indicating the divergence of marsupials, monotremes, reptiles and birds. Numbers indicate estimated times of divergence in millions of years before present.

The human Y chromosome is much smaller and largely heterochromatic. It recombines with the X only over a tiny 'pseudoautosomal region' (PAR) at the tips of the short arms, and a second smaller homologous region at the tips of the long arms. Only three phenotypes were initially ascribed to the Y: the testis-determining factor, TDF; a minor male-specific antigen, HYA; and a region AZF whose deletion confers sterility in azoospermic men. However, several genes and pseudogenes have been isolated from cloned regions of the Y, or by homologous cloning using probes which map elsewhere on the genome [4]. Many sequences on the differential part of the Y detect a homologue on the X chromosome. Unlike the X, the Y is poorly conserved between species, and there are several genes which are active in one species and inactive in another. The gene content of the PAR is not conserved, but represents different subsets of markers present on human Xp [5]. The content and activity of genes on the Y chromosome has therefore changed rapidly during recent eutherian evolution.

A useful way to unravel ancient events in the evolution of the mammalian sex chromosomes has been to look for variation of sex chromosomes among the three major extant groups of mammals. The basic marsupial X is smaller than the eutherian X, and the basic Y is tiny (estimated at 12 Mb). The marsupial X and Y seem not to undergo homologous pairing at meiosis [6], and presumably have no pseudoautosomal region. In contrast, the monotreme sex chromosomes are large, and the X and Y pair at meiosis over the entire short arm of the X and long arm of the Y [7].

In order to detect genetic homology between the sex chromosomes of these three groups, comparative mapping has been undertaken (reviewed in [8]). Somatic cell genetics and in situ hybridization have shown that the X chromosome of marsupials includes all the genes on the long arm and the pericentric region of the human X. The same suite of genes lies on the monotreme X, and so must represent a highly conserved original mammalian X. However, the marsupial and monotreme X lack the genes on the rest of the short arm of the human X. Since marsupials and monotremes diverged independently from eutherians, the most parsimonious explanation is that this region was recently added to the X in the eutherian lineage. The observation that most genes within this region map to two similar clusters in marsupials and monotremes suggests that there have been at least two additions to the X (fig. 2).

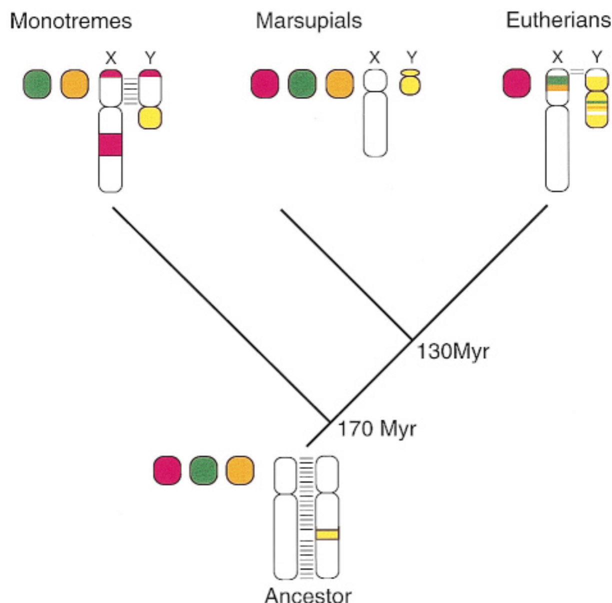

Figure 2. Addition/attrition hypothesis. Yellow denotes the initial testis-determining gene acquired by the proto Y chromosome. Coloured blocks represent three different autosomal regions. Two have been added to the eutherian X and Y, and one to the monotreme X and Y.

The marsupial Y chromosome shares at least four genes with the Y of humans and/or mice. *SRY*, *RBM*1, *SMCY* and *UBE1Y* have been cloned and mapped to the marsupial Y [9–12; B. Duffy, unpublished observations]. However, a number of genes within the recently added region which have homologues on the human X and Y (including genes on the human and mouse PAR) detect only autosomal sequences in marsupials [13]. This implies that the region was recently added, not only to the eutherian X but also to the eutherian Y.

The observations that most or all of mammalian Y-borne genes have X-linked homologues, and that the eutherian X and Y share a homologous PAR, confirm that the X and Y evolved from a pair of autosomes in an ancestral mammal. This hypothesis was proposed long ago by analogy to snake sex chromosomes, which are thought to represent a series of states intermediate between undifferentiated and strongly differentiated Z and W chromosomes [14]. It receives support from comparative studies of mammal and bird sex chromosomes. Limited mapping of genes homologous between birds and mammals reveals none shared between the conserved mammal X and the bird Z. Mammalian X-linked genes are scattered among bird autosomes, and most bird Z-linked genes map to human chromosome 9 [15]. This implies that the two sex chromosome systems evolved independently from different autosomal pairs. It is not possible to deduce the sex-determining system of the common reptilian ancestor, because other vertebrate classes show a wide variety of genetic and environmental sex determination.

It is thought that the original Y was first differentiated when an allele took on a dominant male-determining function. Other genes with a male-specific function, or conferring a male advantage, accumulated near it in a region of low recombination. Once this region became genetically isolated from its homologue, mutations, insertions and deletions accumulated [16]. However, this rapid degeneration of the Y was offset by cycles of addition to the eutherian X and Y. An 'addition-attrition' hypothesis has been presented in which autosomal regions have been progressively added to the X above the PAR, recombined onto the Y by exchange within the PAR, extending the PAR in stages [8] (fig. 2). The initial addition could equally well have been to the Y, with recombination onto the X. One way or the other, the X and the Y have grown incrementally during eutherian, but not marsupial, evolution. Independent additions have been made to the X and Y in monotremes, and in one group of marsupials [5]. The autosomal regions added to the mammalian Y seem to have suffered a fate similar to that of the original Y, though more recently, so that proportionately more genes in the recently added regions of the X still have homologues on the Y.

The continued inactivation and loss of alleles from the Y chromosome would impose gene-dosage differences between males and females were it not for the spread of inactivation along the X which more or less kept pace with Y attrition [17]. Exceptions are the several human X-Y shared genes which escape from inactivation, and several genes whose activity states show that Y attrition and X inactivation is sometimes out of step.

Of the thousands of genes originally present on the proto-Y, only a few remain, and many of these are inactive or partially active copies of genes on the X. What selective forces operated to retain active genes on the Y against this inexorable degradation? Conceivably, a gene might be so dosage-sensitive that haploin sufficiency is deleterious or lethal. However, we know of no X-Y shared genes outside the PAR with identical functions and activity. It seems more likely, then, that a Y-specific gene is retained for long periods because it acquires a unique function, presumably a male-specific one that is critical for male determination or differentiation. It seems certain, then, that genes originally with a general function in both sexes progressively took on male-specific functions in sex determination and male differentiation, whereas their X-linked partners retained the original ubiquitous function in both sexes.

Thus genes on the Y chromosome in different mammals seem to represent small, nonidentical but overlapping

subsets of genes on the X. Some, evidently expendable, genes seem to be dead or dying, whereas others appear to serve a male-specific function which ensures their survival over long periods of evolutionary time. The cloning and characterization of the *SRY* gene, and the investigation of related genes, suggest that the *SRY* gene itself acquired its testis-determining function in this haphazard manner.

Comparative mapping in the hunt for the testis-determining factor

The formation of a testis in the mammalian embryo is the primary sex-determining event, leading to the expression of male hormones, which in turn induce embryo masculinization. In the absence of the Y chromosome, the embryo follows the female development pathway. The Y must therefore contain a testis-determining factor (TDF for testis-determining factor in humans and Tdy for testis-determining gene on the Y in mouse). A positional cloning approach, relying on deletion analysis in human and mouse, was taken to pinpoint and clone the TDF gene. Comparative mapping and analysis of candidate genes in other species played a critical part in the correct identification of the mammalian TDF.

The first gene which was identified within a critical region was *ZFY* (zinc-finger protein on Y chromosome) [18], which encodes a putative DNA-binding protein which could function as a transcriptional regulator and trigger a pathway for testis differentiation. *ZFY* mapped to the Y chromosome in all eutherian mammals tested, and detected a homologous sequence on the short arm of the X.

The first indication that *ZFY* was not the sex-determining gene came with the finding that *ZFY* homologues mapped to autosomes in several marsupial species [19], colocalizing with other genes within the recently added region. As marsupial mammals have an XY sex-determining mechanism and share at least part of the Y with eutherians, and the marsupial Y is testis determining (if not entirely responsible for the male phenotype), we would assume that the same TDF gene would be on the Y in marsupials as well as eutherian mammals. The autosomal location of *ZFY* in marsupials means that it could not act as a universal male-determining switch gene, contradicting the hypothesis that *ZFY* could be a conserved therian TDF. This conclusion was confirmed by expression studies [20] and by the observation of XX males that lack the *ZFY* gene [21]. Another gene or genes on the Y chromosome must therefore be responsible for sex determination.

The *SRY* gene (*sex-determining region on the Y chromosome*) was isolated from the newly defined critical sex-determining region on the human Y and detected male-specific homologues in all eutherian mammals tested [22]. *SRY* is a member of a large gene family. The human *SRY* gene encodes a protein that contains a 79-amino acid conserved high mobility group (HMG) box which can bind to and bend DNA. Mutation analysis showed that XY females have mutations within *SRY* [23].

Critical to acceptance of *SRY* as the mammalian testis-determining gene came from the demonstration that the mouse homologue (*Sry*) mapped within the critical sex-determining region of the mouse Y chromosome [24]. Furthermore, *Sry* had an appropriate expression profile in the developing testis, and promoted male development of XX transgenic mice [25]. Finally, to everyone's relief, a marsupial *SRY* homologue was cloned and mapped to the Y chromosome [26]. In the absence of mutation analysis or the prospect of transgenesis, it has not been possible to confirm that *SRY* is testis determining in marsupials, but its long association with the mammalian testis-determining chromosome has been confirmed.

Comparative analysis of SRY in other mammals

The testis-determining gene, thought to code for a transcription factor critical for reproduction, was expected to be highly conserved between species. It therefore came as a surprise to find that *SRY* sequences of human, mouse and marsupial species are poorly conserved even within the HMG box [27]. *SRY* is even interrupted by a de novo intron in one marsupial family [28], and has been amplified in several species of old-world mice.

Outside the HMG box, sequences cannot even be aligned. A potential transactivating domain in the 3′ region of the mouse SRY protein [29] is not conserved in human or marsupial SRY. In addition, the unique C-terminal domain of human SRY that can bind nuclear proteins containing two PDZ domains such as *SIP*1 [30] is absent from mouse and marsupial SRY. This suggests that all the conserved sex-determining activity of SRY is in the HMG box, a conclusion reinforced by the finding that almost all of the known amino acid substitutions found in mutant SRY proteins from XY females are within this region [23].

Equally as puzzling are the inconsistent expression patterns of *SRY* in different species. In mouse, *Sry* is expressed appropriately in the developing mouse gonadal ridge in males at day 11.5 p.c. (post coitum), the time at which the first histological signs of testis differentiation are noted [31]. *Sry* is also expressed in the adult testis as a unique nontranslated circular transcript [32]. Human *SRY* is transcribed at a low level in many

embryonic tissues, but is limited to the testis in adults. The finding of XY females with a mutations 5′ to a normal *SRY* gene [33] suggests that 5′ regulatory elements control the time and location of *SRY* expression. In contrast to the restricted expression patterns in mouse and human, *SRY* is expressed virtually ubiquitously in marsupials. In the tammar wallaby, *SRY* is transcribed in the embryo at every stage sampled, as well as in a wide range of adult tissues [34]. The significance of *SRY* transcripts in developing tissues other than testis is unclear. Does *SRY* have a function other than testis determination in marsupials?

The action of SRY is thought to depend on the properties of the HMG box, which binds to a 6-bp target sequence, and bends DNA through a specific angle [35, 36], which may promote association of distant regulatory elements into a complex that can control the activity of other genes. The angle of bending is very specific, and recombinant products of some mutant human *SRY* genes are deficient in bending. It is puzzling, therefore, that the HMG box bends DNA through quite different angles in different species.

The mechanism by which *SRY* acts to initiate testis determination is elusive. Because of the positive action of the testis-determining factor in promoting male development, it has always been expected that the testis-determining gene *SRY* would prove to function by transcriptional activation of testis-differentiating genes. Indeed, the products of other HMG box-containing genes are transcriptional activators, and SRY does show transcriptional activity in vitro [37].

However, the testis-determining factor could operate equally well by repressing a gene (ovary differentiating?) which overrides *SRY* [38]. An inhibitory role for *SRY* was previously suggested to account for the puzzling cases of XX males who lack *SRY* [39]. In fact, there are entire species of rodents that lack *SRY*. The mole voles *Ellobius lutescens* and *E. tancrei* undergo apparently normal sex determination, although they lack a Y chromosome and have no *SRY* gene homologue [40]. Evidently some other gene has taken over the primary sex-determining function in triggering the male developmental pathway. These observations suggest that *SRY* acts only as a switch to initiate the male developmental pathway and contributes little to male differentiation itself.

Other genes must therefore act in a sex-determining pathway. Comparative analysis has also been helpful in assessing the credentials of candidate genes.

Comparative analysis of genes in the sex-determining pathway

The next step in understanding how the *SRY* gene functions in male determination was therefore to look for genes which lie up- or downstream of *SRY* in the testis-determining pathway. Three approaches have been taken: to search for proteins that interact with *SRY*, to examine genes which code for testis-specific products such as AMH, and genes which are involved in sex-reversal syndromes, such as *SOX*9 and *DAX*-1.

SRY-interacting factors

The search for sequences to which *SRY* directly binds, or proteins that interact directly with *SRY*, has been frustrating. The 6-bp target site for binding of the HMG box of *SRY* is present in many genes and is shared with many other HMG box-containing proteins. The HMG box also contains a calmodulin binding domain [41], also shared with other HMG box-containing proteins. Calmodulin binding may facilitate a conformational change in SRY protein that affects its ability to bind to its target sequence and thereby regulates its activity. In addition, *SIP*1 (*SRY-interacting protein* 1) has been identified by its ability to bind and interact with the C-terminus of the human SRY protein [30] and may facilitate *SRY* binding or bending its target site.

AMH

The search for genes downstream of *SRY* in the sex-determining pathway began with the first substance secreted from the Sertoli cells in the developing testis. AMH (anti-Müllerian hormone) induces regression of the female Müllerian tubules in the male embryo (reviewed in [42]). *AMH* is an obvious potential target for initiation by *SRY*. Indeed, the SRY protein has been shown to bind to the *AMH* promoter, suggesting that *SRY* acts as a transcriptional regulator of *AMH* [43]. However, the possession of the target site may be fortuitous, and in vitro transcription assays misleading. A direct interaction between *SRY* and *AMH* is unlikely in mouse, where *AMH* is expressed long after *SRY* has ceased transcription in the developing testis.

There is some evidence that *AMH* expression may actually be an early event, at least in other vertebrates, since transcription coincides with the earliest signs of histological differentiation in chicken [56]. It may be in a parallel pathway, or even have some upstream effect on testis determination, as suggested by freemartin cattle, where sex reversal in female twins is induced by *AMH* in shared circulation with a male twin embryo [44].

*SF*1

Study of sex reversal in human patients and mouse mutants has yielded genes that could be part of the

mammalian sex-determining pathway. For instance, mutations in the *SF*1 (*steroidogenic factor* 1) gene cause complete dysgenesis of the developing gonads in both sexes [45], placing it upstream of *SRY* in the sex-determining pathway, in the formation of the indifferent gonad. *SF*1 codes for an orphan nuclear receptor which is responsible for regulating steroid hydroxylases. In humans, it is expressed throughout the reproductive system, hypothalamus, pituitary, adrenal cortex and gonads. *SF*1 has been shown to regulate AMH directly [46], but seems to have no direct interaction with *SRY*.

*SOX*9

SRY belongs to a family of HMG box-containing (*SOX*) genes. *SOX*9 was discovered in an investigation of campomelic dysplasia (CD), an autosomal dominant bone and cartilage disorder frequently accompanied by sex reversal. CD was localized to human chromosome 17q [47]. A SOX gene was isolated from the critical region and was demonstrated by mutational analysis to be the gene responsible for both CD and XY sex reversal [48, 49]. This *SOX*9 gene was already known to be involved in chondrogenesis in mouse [50], and acts by inducing the expression of a collagen gene involved in forming the framework of bone [51, 52]. *SOX*9 appears to be a transcription factor, containing an HMG box which can bind to the same consensus sequence as *SRY*, and a transactivation domain in the C-terminus [53]. The sequence is highly conserved between human *SOX*9 and its mouse homologue *Sox*9, and even chicken, alligator and fish homologues [54–56].

Expression patterns of *SOX*9 in humans and mouse suggest that this gene has a conserved role in normal testis determination. Human *SOX*9 showed high levels of expression in the testis [48], and the murine homologue *Sox*9 showed specific expression in the developing male, but not the female, gonadal ridge, being upregulated in the developing sex cords and the Sertoli cells at the time of gonadal differentiation [54, 57]. Its induction coincides with *SRY* expression, but *Sox*9 remains active in embryonic testis long after *SRY* expression has ceased. Thus *SRY* may act by turning on *SOX*9, or may act with *SOX*9 in the developing testis to bring about Sertoli cell differentiation, but is not required for *Sox*9 maintenance.

A strong indication that *SOX*9 has a conserved role in sex determination comes from expression studies in nonmammalian vertebrates, in which there is no sex-specific *SRY* gene. The chicken homologue c*SOX*9 showed male-specific genital ridge expression [54], despite fundamental differences in chromosomal sex determination in birds, in which females are the heterogametic sex. Even in the alligator, which has temperature-dependent sex determination, *SOX*9 is upregulated in hatchlings incubated at the male, but not the female-determining temperature (P. Western, unpublished observations). *SOX*9 homologues in rainbow trout are also expressed predominantly in the adult brain and testis [55]. *SOX*9 therefore has an essential and critical role in the development of the vertebrate testis which need not depend on *SRY*. Thus, *SRY* appears to be a mammal-specific switch operating on a highly conserved underlying developmental pathway involving *SOX*9.

However, it is significant that *SOX*9 expression in alligator and chicken begins well after pre-Sertoli cell differentiation and AMH expression, the first signs of testis differentiation [54, 56; P. Western, unpublished observations]. This would suggest that *SOX*9 is not, after all, the first gene in the conserved testis differentiation pathway and may be involved in Sertoli cell organization, rather than determination. If *SRY* has its effect in regulating *SOX*9 activity, there is a real question about what steps precede it in the pathway.

*DAX*1

In mammals, female development occurs by default in the absence of the testis-determining gene, presumably by activation of genes that control ovary differentiation. The possibility that male determination occurs, not by activation of male-gonadogenesis genes but suppression of female-gonadogenesis genes, was revisited with the discovery of XY females with an intact *SRY* gene, but with small duplications of the short arm of the X chromosome. An extra copy of a gene within this region (*DSS* for *dosage-sensitive sex reversal*) can evidently override the male-determining signal from *SRY* and cause female development [58]. Deletions of part of the DSS critical region may cause X-linked adrenal hypoplasia congenita (AHC), which is characterized by a primary insufficiency of the adrenal glands, as well as impaired male gonadal differentiation [59]. A gene *DAX*1 (*DSS/AHC critical region on the X chromosome*), isolated from the DSS critical region, was shown to be responsible for both AHC and sex reversal [60]. A mouse homologue *Dax*1, sharing 75.5% nucleotide sequence identity with human *DAX*1, lies on the mouse X [62].

*DAX*1 codes for a member of the nuclear hormone receptor superfamily and can repress steroid-producing genes [59, 61]. Human *DAX*1 is expressed in adrenal glands and gonadal tissue, consistent with the disease phenotypes. In mouse, *Dax*1 is expressed in the adrenals throughout development. *Dax*1 is initially expressed in both male and female genital ridges, but

rapidly decreases in males to an almost undetectable level by day 12.5 p.c. [62, 63]. This is the same time at which *Sry* expression ceases in the genital ridge, suggesting that *SRY* might work by interfering with *DAX*1 function to induce testis development. Perhaps an extra active copy of the *DAX*1 gene in XdupY females is sufficient to repress *SRY* action and prevent male sex determination.

In both species, the expression pattern of *DAX*1 is almost identical to that of *SF*1, and this, with the presence of a conserved *SF*1 response element in the *DAX*1 promoter region, suggests that the *SF*1 gene directly regulates *DAX*1. However, *Dax*1 expression does not depend on the presence of *Sf*1, since *Dax*1 expression is unaffected in *Sf*1 knockout mice [64] and also occurs in the absence of *Sf*1 in the foetal rat testis [65]. This would suggest that that *SF*1 and *DAX*1 do not directly interact in the sex-determining pathway, but may act together in activating and regulating steroid hormones during embryogenesis.

Several other sex-reversal syndromes have been observed in human patients, but the genes responsible have not yet been identified. One of particular interest has been located to human chromosome 9, which may be significant because this chromosome bears a large portion of the genes on the chicken Z chromosome [66]. Several autosomal genes also appear to be involved in sex reversal in interspecies mouse back-crosses [67]. The sex-determining pathway is obviously much more complex than was first appreciated [68]. Further clues to the role of these and other genes in the pathway may be gained by considering how the sex-determining pathway evolved in mammals.

Evolution of genes in the sex-determination pathway

Our knowledge of the evolution of mammalian sex chromosomes predicts that genes on the X and Y chromosomes were originally autosomal. Genes such as *SRY*, which are shared by the marsupial Y, must have remained on the Y for at least the 130 MYrBP since marsupials and eutherians diverged, making it likely that they acquired a male-specific function early in mammalian evolution. But genes such as *ZFY*, which are autosomal in marsupials, acquired this role more recently. The origin and relationships of putative sex-determining and -differentiation genes can be tested by cloning, sequencing and mapping them in marsupials. The roles of SRY and its relatives, and of DAX1, have been reevaluated from an evolutionary standpoint.

The evolution of *SRY*

The male-dominant action of the mammalian testis-determining factor led to the expectation that this gene is a critical and integral part of the sex-determining pathway. However, the absence of a sex-specific SRY from nonmammalian vertebrates implies that SRY has no conserved role in vertebrate sex. Nor is there any evidence for a sex-specific *SRY* in monotremes (A. Pask and P. Western, unpublished observations). At the earliest, then, *SRY* evolved after the monotreme-therian divergence about 170 million years ago. This suggests that *SRY* evolved a control function relatively recently, and is incidental in the sexual development pathway.

Its sex determining function may be even more recent. The discovery of a Y-borne *SRY* in marsupials suggested that this gene is the testis-determining factor in all therian mammals, although testis determination in marsupials does not determine all other sexual dimorphisms as it does in eutherians. While the ubiquitous expression of marsupial *SRY* does not disqualify it from acting as a testis determinant, in the absence of mutation analysis or transgenesis we cannot be certain that *SRY* is sex determining in marsupials. The sequence of marsupial *SRY* is too poorly conserved outside the HMG box to be sure that the gene serves the same function, and its ubiquitous expression suggests it lacks the regulatory elements present in eutherian *SRY*. Perhaps marsupial *SRY* retains a more primitive form of regulation that has come under tighter control in eutherian mammals, or perhaps it performs some other more general function. It may not, after all, even be sex determining in this group of mammals. Thus *SRY* may have evolved its function a mere 80 MYrBP.

Since *SRY* has evolved and been recruited into the sex-determining pathway only 170 MYrBP at most, we may be able to learn about its possible function by examining the gene from which it evolved. The *SOX*3 gene was found to be on the X in marsupials, and subsequently in all therian mammals, so it must have been on the X in a mammalian common ancestor. Of all its relatives in the SOX gene family, *SRY* shows the most sequence similarity to *SOX*3 within the HMG box—in fact, *SRY* genes from different species are more similar to *SOX*3 than they are to each other. This suggests that *SRY* evolved from *SOX*3 [69]. *SRY* seems to be essentially a truncated *SOX*3. Like other genes on the Y chromosome, it has been mutated and deleted—but has been retained because it found a male-specific function.

*SOX*3 is highly conserved between species, suggesting that it has a critical function in mammalian development [69]. Expression analysis of *SOX*3 in human embryos detected transcripts in the developing brain, spinal cord, thymus and heart, and *SOX*3 transcripts were detected in several adult tissues including testis [70]. Mouse *Sox*3 is expressed in the developing central nervous system and the indifferent gonadal ridge at low levels comparable to those of *SRY* [71]. Expression of a chicken homologue c*SOX*3 is also restricted to the central nervous system.

An amphibian homologue, X*SOX*3 from *Xenopus*, is expressed only in the ovary, and shows highest expression early in oocyte development [72, 73].

The expression of *SOX*3 in developing gonads in different vertebrates suggests a conserved role in gonadal differentiation in mammals, as well as a role in the differentiation of central nervous system. However, two mentally retarded boys with *SOX*3 deletions show testicular development, excluding *SOX*3 from a role in male sex determination [70]. Perhaps, then, *SOX*3 is involved in ovarian development, as in *Xenopus*, or perhaps it acts as an inhibitor of testis determination.

It has been postulated that *SRY*, *SOX*3 and *SOX*9 interact to determine testis [74]. In females, in the absence of *SRY*, *SOX*3 inhibits *SOX*9 and no testis forms. In males, *SRY* inhibits *SOX*3, permitting *SOX*9 to enact its testis-determining role (fig. 3). This hypothesis requires that *SRY* and *SOX*3 be expressed in the same tissue type at the same time. In mouse, *SOX*3 and *SRY* are both expressed in the indifferent gonad around day 11.5 p.c., but there is no information on expression in other mammals. The hypothesis provides a good explanation of sex-reversing mutations in humans, particularly of *SRY*-negative XX males, who may have a mutation in *SOX*3.

The hypothesis that *SOX*3 is involved in the sex-determination circuitry also lends itself to a sensible account of the evolution of the male-dominant testis determination by *SRY* from an earlier dosage-dependent system. It has been proposed that, in an ancestral mammal, homozygotes for wild-type *SOX*3 were female, whereas heteroygotes for a null allele were male; the 2:1 dosage difference determined sex via a differential effect on *SOX*9 activity. In *Xenopus*, *SOX*3 may act to inhibit *SOX*9 and permit ovary determination. Since *SOX*3 is subject to X inactivation in eutherian mammals, this dosage-sensitive system must have been supplanted; but perhaps in marsupials, which show incomplete X inactivation, *SOX*3 dosage could still determine sex. Further investigations into *SOX*3 function and expression in other mammals will shed light on its role in sex determination and its interaction with *SRY* and *SOX*9.

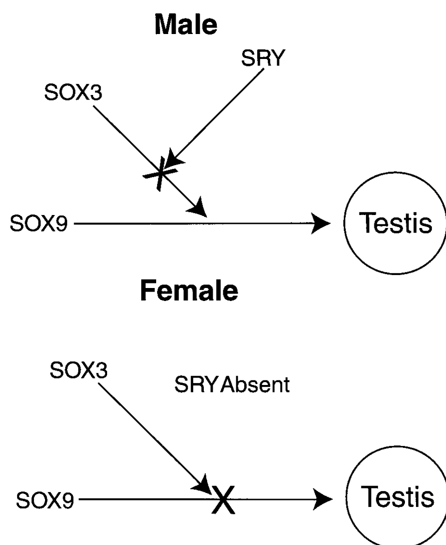

Figure 3. Proposed mechanism of *SOX*3, *SOX*9 and *SRY* in sex determination. In the presence of *SRY*, *SOX*3 is prevented from repressing *SOX*9, which in turn results in the development of a testis. In the absence of *SRY*, *SOX*3 can inhibit *SOX*9, which in turn prevents the formation of a testis, leading to female development.

Dosage-sensitive action of *DAX*1

Gene-dosage differences seem to be a recurring theme in sex-determination systems in a wide variety of animal species, and they are well characterized in *Drosophila* and *Caenorhabditis elegans*. It was proposed that X chromosome inactivation evolved in mammals as a sex determination, rather than a dosage-compensation system [75]. The effects of X chromosome dosage on the sexual phenotype in XO and XXY marsupials also suggested that a dosage-sensitive gene is involved in sexual differentiation, at least in this mammal group.

This intriguing idea was put to one side in the excitement of cloning the testis-determining factor, but the identification of the dosage-sensitive sex-reversing gene *DAX*1 on the X led to speculation that this gene might regulate ovarian development in mammals by virtue of its dosage on the X in males and females. Two copies of this gene could determine female development, and one male development. This could be the case in marsupials, in which X inactivation is incomplete; however, *DAX*1 must be subject to inactivation in humans and mice, since XXY are male in both species, and the dosage of *DAX*1 is equal in males and females.

It is still possible that *DAX*1 represented an ancestral dosage-sensitive gene that functioned in sex determination in a mammalian ancestor before this locus was recruited into the X inactivation system. If *DAX*1 were involved in an ancestral mammalian DSS mechanism, it should lie on the conserved region of the X chromosome present in all mammalian groups. *DAX*1 is also a good candidate for the X-linked dosage-sensitive sex differentiation locus found on the marsupial X chromosome, which is responsible for the intersexual phenotypes of XO and XXY marsupials.

Both hypotheses predict that *DAX*1 should lie on the marsupial X chromosome. A marsupial *DAX*1 homologue was isolated and shown to share 73% amino acid identity to human *DAX*1 within the ligand-binding do-

main. This gene was mapped by fluorescence in situ hybridization to chromosome 5 in the tammar wallaby, colocalizing with several other genes on the short arm of the human X [76]. This implies that *DAX*1 was not present on the X chromosome in the mammalian common ancestor, but is part of a recent addition to the eutherian X. An autosomal *DAX*1 gene could never have functioned in a dosage-sensitive role in marsupials, and cannot be the X-linked dosage-sensitive sex differentiation gene on the marsupial X chromosome.

This does not necessarily exclude *DAX*1 from a dosage-sensitive role in eutherian sex determination, but it would mean that this role must have been confined to a time after the marsupial-eutherian divergence 130 MyrBP, and the recruitment of *DAX*1 into the X inactivation system. It is difficult to see why a gene recruited to the X chromosome would take on a dosage-sensitive sex-determining function, which has the ability to override the male-determining signal from the *SRY* gene. It seems more likely that *DAX*1 has a role in gonad determination that is independent of its position. The level of *DAX*1 expression in human gonads must be critical, as two active copies can override the male-determining signal from *SRY*. It will be interesting to define the role of *DAX*1 in the gonads of marsupials, in which there are two active (autosomal) loci. This may help to explain how it became dosage-sensitive.

Conclusion

Although testis determination seems to be very similar at the histological level in all vertebrates, the control of the sex-determining pathway has evidently changed radically in evolution. Comparisons of the location, sequence and expression of sex-determining genes in other vertebrates, and particularly between the three extant mammalian groups, has provided many insights into their importance and role in the vertebrate sex-determining pathway.

Comparative mapping has allowed us to assess the conserved role of gene in the sex-determining pathway between distantly related groups. Comparisons of the candidate mammalian sex-determining genes revealed that *ZFY*, the first candidate TDF, is a recent addition to the eutherian sex chromosomes and therefore could not be the universal mammalian testis-determining factor. In contrast, *SRY* was found to lie on the Y chromosome in marsupials as well as eutherians.

There is no evidence for a sex-specific *SRY* gene outside of Class Mammalia, indicating that this gene has only recently acquired its male-determining switch role in therian mammals, whereas other vertebrates use different mechanisms for triggering the male sexual development pathway.

At what time in mammalian evolution SRY acquired its male-determining role is undetermined. As yet monotreme mammals have not been demonstrated to possess a sex-specific *SRY* gene, suggesting that *SRY* evolved its male-determining function after the divergence of monotremes and therian mammals about 170 million years ago. However, this figure could be much more recent, as in marsupials it is yet to be demonstrated that *SRY* is sex determining, and it is still possible that in this mammal group testis determination is triggered by some other gene. It is clear, however, that *SRY* appears to be no more than a trigger of the male developmental pathway, and its action may be very indirect.

The poor sequence conservation of SRY between species both within and flanking the HMG box suggests that *SRY* may encode a represser rather than a transcriptional activator. This is in direct contrast to the related SOX9 gene that is highly conserved, and includes a transactivating domain suggesting its action is much more direct in sex determination. An inadequate dose of the *SOX*9 gene is sex reversing in mammals, and its male-specific transcriptional upregulation in the gonad of all mammals, reptiles and birds makes it a candidate for an important role in testis organization, if not testis determination.

Little attention has been paid to the potential role of *SOX*3 in sex determination, despite its close evolutionary relationship with *SRY* and *SOX*9. Furthermore, its expression in developing gonads and ability to bind to the same target site as SRY raises the question of its interaction with SRY in sex determination. *SOX*3 is highly conserved among eutherian and marsupial mammals, and is expressed in the testis in humans and mouse. However, bird *SOX*3 is not expressed in developing gonads, and in amphibians expression is detected in the developing ovary, and appears to be involved in oocyte development. This gene may have originally had a mammalian dosage-sensitive role in female determination which was taken over in therian mammals when *SRY* was differentiated on the Y chromosome.

*DAX*1 is of great interest because of its dosage-sensitive effect on sex reversal. Although X inactivation ensures equal dosage between the sexes in human and mouse, there might still be an effect in marsupials, which have an incomplete dosage compensation, and it may have acted as the sex-determining switch in an ancestral mammal prior to the differentiation of *SRY*. However, the autosomal location of *DAX*1 in marsupials suggests that it was not present on the ancestral sex chromosome but rather is a recent addition to the eutherian X. Why and how *DAX*1 acquired this dosage-sensitive function is still unknown, as is the mechanism by which it triggers female development. Analysis of autosomal

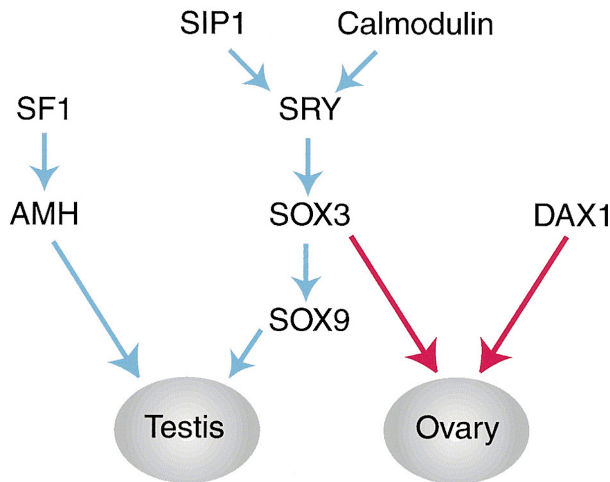

Figure 4. Proposed interactions among the sex-determining genes. The exact nature of each interaction is still unknown. Blue arrows indicate the male developmental pathway. Pink arrows indicate the female developmental pathway.

*DAX*1 expression in marsupials may help to define its role in mammalian sexual development.

As it currently stands we are still lacking a vast amount of knowledge on the role of each gene in sex determination. The action of SRY is dependent on regulatory sequences and could also be affected by interactions with SIP1 and calmodulin. The coexpression of SOX3 and SRY in the gonadal ridge would suggest that the two genes compete for the same binding site. It is suggested that SRY binding would block SOX3 from initiating female development, and could possibly lead to the activation of SOX9, essential for the organization of the testis. SF1 is responsible for activation of AMH expression from the pre-Sertoli cells, leading to Müllerian duct regression and the masculinization of the gonads. In females the story is more vague, with DAX1 being the only gene identified with a role in ovary determination. SOX3, as indicated above, could also play a role in female development. At what level each of the interactions described above occur is still undetermined (fig. 4).

Comparative studies of the genes involved in sex determination have therefore allowed us to determine that some genes, such as *SOX*9, have critical conserved functions in sex determination, whereas others, such as *SRY*, have recently evolved a role in the sex-determining pathway of one vertebrate lineage. Obviously the pathway is more complex and more circuitous than was originally envisaged, and there are many genes and more question marks (fig. 4). Further comparative studies may assist our understanding of the role of genes associated with the pathway, and may ultimately help us to construct the entire mammalian sex-determining pathway and identify genes which have functions in common with other pathways of vertebrate organogenesis.

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