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Review

Whatever happened to *SRY*?

M. J. O'Neill and R. J. W. O'Neill*

Department of Molecular and Cell Biology, University of Connecticut, Storrs (Connecticut 06269, USA)

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Abstract. The mammalian sex-determining gene, *SRY*, was identified by positional cloning approximately 10 years ago. Since its discovery, intense research into this gene has been directed on two main fronts: elucidation of its function in development of the testis and examination of its singular evolutionary history. The role or *SRY* as the testis-determining factor (*TDF*) places it at a crucial point in the highly conserved morphogenetic process of vertebrate gonadogenesis. None of the genes that directly activate *SRY* nor any of its immediate downstream targets have yet been positively identified. Several genes, however, such as *SF1*, *DAX1*, and *SOX9*, whose spatial and temporal expression profiles overlap with that of *SRY*, are strongly implicated as co-regulators of gonadogenesis. Molecular genetic manipulation of these genes in mice has shown that they are indispensable to sexual development. Remarkably, its key position in this cascade of gene action has not protected *SRY* from strong yet poorly understood selective forces that have caused it to evolve rapidly in mammals. The evolution of *SRY* has been characterized not only by rapid sequence divergence within mammals, but also by structural changes such as intron insertion, gene amplification, and deletion.

Key words. *SRY*; sex determination; *SOX*; HMG box; Y chromosome; rapid sequence evolution.

What of *SRY*? It emerged at the beginning of the decade from the mysterious genetic wasteland of the mammalian Y chromosome amid great fanfare. The cloning of *SRY* promised to fill new arenas of molecular genetic research and to enlighten that long pedigree of biologists whose mantra for decades had been: 'Why sex, how sex?' Is the story of *SRY* the story of a meteoric rise and a gradual fade into obscurity? Most definitely not, as attested to by even a cursory search of med-line. The story of *SRY* is the story of a field of molecular genetic research that has matured gracefully from the early days of stir surrounding its positional cloning into a productive middle-age.

Research involving *SRY* continues to contribute to our understanding of the elaboration of a key developmental program, gonadogenesis, and to our emerging vision chromatin structure. What follows is a behind-the-gene look at *SRY*: its evolutionary origins from a family of genes fundamentally important to several developmental pathways, its history of mutation and functional change, and how it was recruited into its role as the mammalian testis-determining factor. The story of *SRY* is the story of the mammals that rely on its still enigmatic function to go about the business of reproduction and the few that have shrugged off its influence and seem to get along without it. It is the story of how this strange protein with a DNA-binding motif but little else of apparent use, despite its beleaguered home on the Y chromosome, can instigate the commitment of sexually indifferent cells to a testicular fate. To present the current synthesis of knowledge surrounding this gene clearly, our review is divided into two main parts: the first addresses the function of *SRY* as a regulator of testicular development and the second, our understanding of the evolution of *SRY*.

* Corresponding author.

SRY: the testis-determining factor

Identification of *TDF*

SRY is the major switch gene of mammalian gonadal development. It has been shown to be both necessary and sufficient to initiate testicular development in mice and humans [1, 2]. Because of this and because of its location on the Y chromosome of both placental mammals and marsupials [3], *SRY* has been identified, unequivocally, as the mammalian testis-determining factor (*TDF*). The first clues to the existence of *TDF* came from the work of Jost in 1947 [4]. Jost castrated embryonic rabbits in utero and observed that they all developed as females. In effect then, development as a male proceeds from development of the male gonad, the testis. Subsequent to the work of Jost, Ford et al. [5] discovered the requirement for the Y chromosome for male development when they examined the karyotypes of patients with Turner's syndrome and found them to be XO (hemizygous for X and no Y). Individuals affected by Turner's syndrome invariably develop as phenotypic females. At about the same time, Jacob and Strong [6] observed that individuals affected by Klinefelter's syndrome, who have oligosomies for the X chromosome with only one Y, are invariably phenotypic males. Together, these observations led to the consensus that a dominant testis-determining gene exists on the mammalian Y chromosome: *TDF*.

Through the meticulous efforts of clinical and human geneticists spanning the years since the discoveries of Jost, the minimal portion of the Y chromosome harboring *TDF* was eventually narrowed to a 35-kb region [1]. As the minimal sex-determining segment of the Y chromosome was narrowed down, several candidates for *TDF*, like the *H-Y* antigen [7], *BKM* repeats [8], and *ZFY* [9, 10], were excluded. A select sample of sex-reversed XX human males was eventually identified which had only a small segment of the Y chromosome attached to one of their Xs. Only one transcribed sequence was found in this small sex-reversing portion of the Y chromosome: the small, single-exon-encoded gene *SRY* (Sex-determining region of the Y chromosome) [1]. In the flurry of experimentation that followed its identification, mutations in *SRY* were found in sex-reversed XY human females [11, 12], and XX mice carrying an *SRY* transgene were observed to develop as males [2]. Furthermore, Y-linked copies of *SRY* were found in several eutherian (placental) mammals [1] and in marsupials [3]. *SRY*, therefore, was unequivocally identified as the mammalian sex-determining gene, TDF.

Once it became clear that *SRY* was *TDF*, the next order of business was to determine how *SRY* functioned as *TDF*. Two vital clues surfaced with the cloning of *SRY*. The first was revealed when sequence analysis of the *SRY* gene showed that it encoded a protein that resembled a group of DNA-binding factors known as the high-mobility group (HMG) proteins [1]. HMG proteins are thought to serve as chromatin architectural factors, and it has been in this arena, i.e., the study of the structural dynamics of DNA/protein interactions, that *SRY* has been most useful. The similarity of SRY to HMG proteins is restricted to a 79-amino-acid 'HMG box,' which is highly conserved between mouse and human [1] (fig. 1a, b). Interestingly, the *SRY* sequence outside the HMG box is highly divergent in all the mammalian species for which *SRY* has been sequenced (discussed in detail below)

The second clue emerged when *SRY* cDNA was hybridized to Southern blots of genomic DNA from a variety of mammals of both sexes. Strongly cross-hybridizing bands were revealed, indicative of genes closely related to *SRY* that were not on the Y chromosome [1]. Many of these cross-hybridizing genes were cloned and, indeed, found to be very similar in sequence to *SRY*, especially in the HMG box. These genes have become known as the *SOX* (*SRY*-like box) genes. As will be discussed below, the study of one *SOX* family member, *SOX9*, has greatly enhanced our understanding of the early events of gonadogenesis.

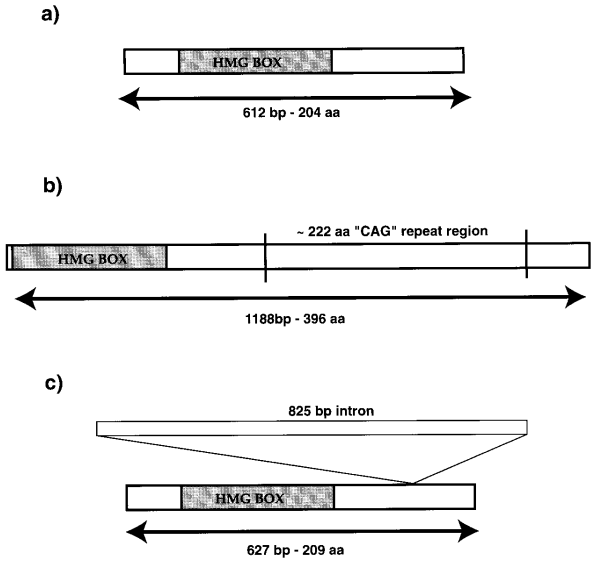

Figure 1. Schematic structures of the *SRY* coding regions from human (*a*), mouse (*Mus musculus*) (*b*), and a dasyurid marsupial (*Sminthopsis macroura*) (*c*).

Table 1. Summary of sex-reversing mutations in human *SRY*.

Region	Mutation
5′ noncoding	3 [16, 17]
5′ coding	3 [18–20]
HMG box	28 [reviewed in 20, 21]
3′ coding	1 [22]
3′ noncoding	1 [23]

SRY as a transcription factor

The HMG box imbues SRY and its relatives, the SOX proteins, with the ability to bind DNA in a sequence-specific manner [13]. Subsequent to its identification as a DNA-binding protein, a consensus binding site for SRY, A/TAACAAT/A, was identified [14]. Not only does SRY bind DNA, it bends it through a characteristic angle [13] which may serve to bring together spatially separated *cis*-acting controlling elements or chromatin domains. Interestingly, the angle of bending differs slightly between the mouse Sry protein and the human version [13]. Most importantly, in terms of its role as *TDF*, it was shown that sex-reversing mutations in human *SRY* either abolished its ability to bind to DNA or altered the angle to which it bends DNA once bound [15].

In the years since its discovery, most of the sex-reversing mutations found in the coding sequence of *SRY* have fallen in the HMG box (table 1). Of the four mutations identified in the coding region outside the HMG box, two are mutations 5′ of the HMG box that result in premature termination [18, 22]. A recent report has identified a non-HMG box mutation resulting in a missense amino acid substitution. Interestingly, this mutation was identified in a partially sex-reversed patient whose father and brother also carried the mutation but were normal [19]. Three sex-reversing mutations have also been recently reported in the 5′ noncoding region flanking the gene [16, 17]. The possibility of sex-reversing mutations affecting the level of *SRY* expression is intriguing in light of critical threshold effects, observed in mice, on the initiation of testicular development (see below).

The fact that SRY performs its function by binding to DNA and perhaps altering local chromatin architecture suggests that it acts as a transcription factor. But whether SRY activates the transcription of downstream genes promoting testicular development or represses genes promoting ovarian development is not known. Some circumstantial evidence suggested SRY could act as a transcriptional activator. The consensus binding site for SRY has been identified in the promoters of several genes expressed in testicular development, and two of these genes, *AMH* (Anti-Mullerian Hormone) and *Fra-1* (Fos-related antigen 1) are activated by SRY in transfected cell lines [24, 25]. However, the relatively brief expression of *SRY* (see below) and the appearance, in vivo, of the transcripts of *AMH* and *Fra-1* well after *SRY* expression has switched off excludes them as direct targets of *SRY* [26].

While the actual targets of *SRY* action remain a mystery we have gained considerable knowledge as to how its action on its targets may be mediated. Perhaps the most unusual feature of SRY as a transcription factor is that it is a DNA-binding protein with no apparent activation domains. The fact that sequences flanking the HMG box have evolved so rapidly has led some to argue that they are under no functional constraint and that the SRY DNA-binding domain is its only raison d'être (see detailed discussion in the section on evolution). It appears, however, that the HMG box is not there simply to bind DNA. It has been shown that the SRY HMG box can bind calmodulin, a property it shares with at least one other HMG box protein, HMG 1 [27]. By binding the HMG box of these proteins, thereby blocking the interaction of the HMG box with DNA, calmodulin may regulate the activity of HMG proteins like SRY. Another feature of SRY and its relative, SOX9, that may influence their ability to act in the nucleus are two nuclear localization signals (NLS), also present within their HMG boxes [28].

While the HMG box has been shown to be necessary for *SRY* function, only recently has evidence been found for function outside the HMG box of *SRY*. Poulat et al. [29] found that the C-terminal seven amino acids of human SRY interact with the PDZ domain of human SIP-1. They hypothesize that SIP-1 may act as a bridge between SRY and other transcription factors. Desclozeaux et al. [30] found that SRY protein is phosphorylated on serine residues near the N terminus by cyclic-AMP-dependent protein kinase A (PKA) both in vitro and in vivo. They also observed that phosphorylation of this motif enhanced the DNA-binding activity of human SRY.

As our understanding of the interaction of SRY with DNA and other DNA-associated factors improves, it is hoped that the direct targets of *SRY* action will be identified. In the meantime, much has been learned about the molecular genetic events happening just after *SRY* initiates testicular development.

SRY and the big picture of gonadal development

Our best understanding of how *SRY* functions as the testis-determining factor comes from experiments in the laboratory mouse, *Mus musculus*. *Sry* expression spans a short window of development beginning at 10.5 days postcoitum (d.p.c.) becoming undetectable by 12.5 d.p.c. [31]. *Sry* transcripts are also detected later, in

adult mouse testis. Strikingly, the bulk of *Sry* transcripts in adult testis are circular, and appear to originate from a larger transcription unit encompassed within an inverted repeat [32]. Complementarity at the 5′ and 3′ ends of these transcripts may promote hairpin-loop formation and splicing of the transcript into a circular form. These circular transcripts appear not to be translated.

The site of *Sry* expression in the embryo is in the sexually indifferent genital ridges, which arise as epithelial folds off the underlying embryonic kidney, the mesonephros. In one of the earliest cellular events of gonadogenesis, the somatic lineage within the genital ridges, fated to give rise to the supporting cells of the gonad, differentiates into either granulosa cells in the ovary or Sertoli cells in the testis [33]. It is thought that a critical threshold in the commitment of this supporting lineage to a Sertoli cell fate tilts the indifferent gonad in the direction of testicular development. *Sry* is thought to act in these pre-Sertoli cells by establishing a critical threshold of gene expression tilting the cell to a Sertoli fate. The events immediately following the initiation of *SRY* expression in the developing testis are largely unknown but a better picture is starting to emerge through analysis of the genes acting downstream of *SRY*.

The search for target genes of *SRY* was primarily confounded by the fact that the only thing known about them was their potential to contain the consensus binding site for SRY. This is almost never a good starting point to search for target genes of a known transcription factor. In the case of *SRY*, the problem was exacerbated by the fact that many of the identified SOX proteins bound to the SRY consensus site with the same affinity as SRY itself [34, 35]. For this reason, the task of filling in the gaps in the cascade of gene action leading to testicular or ovarian development once again fell to positional cloning approaches. Once particular sex-reversing loci had been mapped in human patients, candidate genes from those regions could be subjected to molecular genetic manipulation in laboratory mice. This approach has been extremely fruitful for our attempts to understand the role of two genes critical to the early differentiation of the gonad: *SOX9* and *DAX1*.

Mutations in *SOX9* have been identified as the genetic defect underlying the human disease campomelic dysplasia (CD) [36, 37]. CD is an autosomal dominant congenital disease mapping to human 17q, characterized by gross defects in bone development and by a propensity for sex reversal of XY individuals. Predictably, *Sox9* transcripts were found in chondrocytes and primordial gonads in mice [38]. *Sox9* is initially expressed at low levels in sexually indifferent gonads in mice. Immediately following the expression of *Sry* in males, *Sox9* expression is upregulated and persists throughout testis development [39, 40]. Conversely, in genetic females, in the absence of *Sry* expression, *Sox9* expression is switched off.

CD is, as stated above, an autosomal dominant disorder, meaning mutation of only one allele of the gene is sufficient to cause the disease. It is clear, however, that the effect of a mutated *SOX9* gene is not one of a dominant gain-of-function, but rather an effect of haploinsufficiency when one copy of the gene is rendered nonfunctional [36]. Haploinsufficiency leading to sex reversal in CD gives us our first clue that gene dosage is critical in the early steps of cell fate determination in the gonad.

Another human sex-reversing condition, DSS (dosage-sensitive sex reversal), maps to the human X chromosome [41]. XY individuals with a duplication of a minimal 160-kilobase region of the short arm of the X chromosome show male-to-female sex reversal. Several genes map to this 160-kilobase region, but only one has been shown by mutational analysis in humans and by genetic manipulation in mice to play a role in gonadogenesis: *DAX1* [42–44]. *DAX1* is a member of the nuclear hormone receptor family, but so far no ligand for it has been identified. In mice, *Dax1* has been shown by one group to have an expression profile that is complementary to that of *Sox9* [45]. Swain et al. [44] showed by whole mount in situ hybridization that *Dax1* is expressed in the gonads of both sexes prior to the initiation of *Sry* expression. In the developing ovary, where *Sry* is absent, *Dax1* expression is maintained at high levels. Following *Sry* expression in the developing testis, with the upregulation of *Sox9*, *Dax1*, as shown by whole mount in situ, is rapidly switched off. Interestingly, Nachtigal et al. [46] showed by RNase protection assays equal expression of *Dax1* in developing testis and ovary from indifferent stages through 18.5 d.p.c. While the expression profile of *Dax1* remains a subject of controversy, functional studies using transgenic mouse models have yielded fascinating results.

In a series of elegant experiments by Swain et al. [45], where the dosage and timing of expression of *Sry*, *Dax1*, and *Sox9* were altered in transgenic mice, an intriguing picture of the interactions of these genes has emerged. Again, it was thought that the critical arena for the action of these genes is the population of cells that gives rise to the Sertoli cells. These studies demonstrated that, in fact, *Dax1* and *Sry* must be operating in the same cells because when the *Sry* coding sequence was put under the control of the *Dax1* promoter, this transgene induced testicular development in four of six transgenic XX mice carrying the construct. Therefore, *Dax1* and *Sry* must not only be working in the same cell type, but also at roughly the same time. A detailed developmental time course of the activity of the *Dax1* promoter, driving a *lacZ* transgene, indicated that it is activated slightly later than *Sry*.

To address the question whether these two genes may antagonize the action of the other, Swain et al. [45] created transgenic lines over-expressing *Dax1*. They found that lines expressing *Dax1* at ten times normal levels showed only slight adverse effects on testicular development in XY individuals. However, when they put this over-expressing *Dax1* transgene on a background carrying the weak *M. m. poschiavinus* Y chromosome (Y^{pos}) they created several completely sex-reversed XY females. When Y^{pos} males are mated to females of the C57BL/6J inbred line, XY offspring are invariably sex reversed or hermaphroditic [47]. The weakly acting Y^{pos} is probably due to lower than normal expression of *Sry* from these males. When the *Dax1* tenfold-over-expressing transgenic line is crossed with *M. m. poschiavinus* males, normal testicular development is disrupted [45]. The over-expression of *Dax1* in these mice presumably overwhelms the low level of *Sry* activity, keeping it below the threshold necessary for initiating testicular development. In summation then, it appears that *Dax1* and *Sry* may be competing at the molecular level in pre-Sertoli cells, but the nature of this competition remains unclear.

There is some evidence that the countervailing influences of *Dax1* and *Sry* may be mediated at the level of *Sox9* activity. As stated previously, *Sox9* expression is upregulated in the mouse male gonad at about 11.5 d.p.c., soon after *Sry* expression is initiated, and it is switched off in the female gonad coincidentally with the upregulation of *Dax1* [39, 40]. Soon after *Sox9* upregulation in early testis, *Amh* expression is activated [45]. Two other genes show testis-specific upregulation concomitant with *Sox9*: *Wt1* (Wilm's-tumor-associated gene 1) [48] and *Sf1* (steroidogenic factor 1) [49]. Like *Sox9*, these two genes are also initially expressed in both sexes in the sexually indifferent primordial gonads. *Wt1* is essential for development of the kidney and *Sf1* plays a key role in development of the adrenal gland and hypothalamus. Interestingly, mice deficient for either *Wt1* or *Sf1* lack gonads entirely, so it is clear they are essential to gonadal development as well.

Expression profiles, sequence analysis of the promoters, and transcriptional studies of some of these genes in tissue culture hint at possible scenarios in which these genes interact. The *AMH* gene, for example, has consensus binding sites for both *SF1* and *SRY*/*SOXs* [25]. In vitro studies suggest that *SF1* and *SOX9* can act co-operatively to activate *AMH* transcription [50]. Other in vitro studies have shown that *DAX1* can antagonize transcriptional activation by *SF1* [51]. A possible scenario is that early expression of *SF1* could activate *SRY* in males, which in turn elevates *SF1* and *SOX9* expression to a critical threshold in pre-Sertoli cells leading to testicular development. In females, high levels of *DAX1* would antagonize *SF1* action eventually leading to the switching off of *SF1* and *SOX9*, thereby promoting ovarian development. Figure 2 shows a hypothetical cascade of gene expression in the supporting cell lineage of the developing gonad. Representations of the expression profiles of the genes involved are shown, but their actual regulatory interactions remain to be characterized.

Elucidating the heirarchical relationships between the genes involved in sexual differentiation is, clearly, a daunting task for developmental geneticists. By continuing the approach of positionally cloning human sex-reversing mutations, and subsequent genetic manipulation of these genes in mice, the big picture of gonadogenesis will be gradually filled in. Another promising approach is to study this process in nonmammalian vertebrates. While sex-determining mechanisms show surprising diversity in vertebrates, the morphogenetic process of gonadogenesis is highly conserved. We now know that some of the genes important to gonadal development in mammals show similar patterns of expression in other animals. The central role of *SOX9* in the initiation of testicular development is again supported by studies of the gonadal expression profile of this gene in chickens

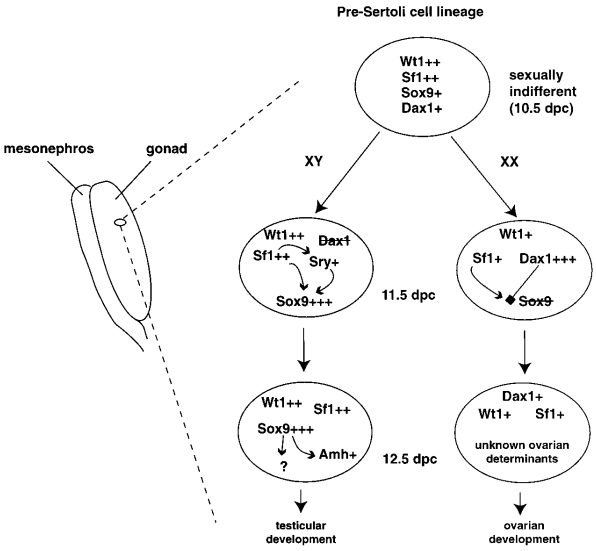

Figure 2. Schematic representation of the development of the supporting cell lineage of the mouse gonad from 10.5 to 12.5 d.p.c. Relative levels of transcript expression are represented by the + signs. Hypothetical interactions between several factors are also shown (see text). Differentiation of the supporting cell lineage is accompanied by differentiation of steroid-hormone-producing cells (Leydig cells in males, theca cells in females), connective tissue cells, and germ cells. Connective tissue cells that give rise to the peritubular myoid cells, which form the testicular cords, appear to migrate from the underlying mesonephros into the developing testis [75]. This migration is not seen in females.

and alligators. *SOX9* expression is dramatically upregulated in developing testis when sexual dimorphism in the gonad is first recognized in the chicken [39, 40]. Likewise alligators, which determine sex by egg incubation temperature, show upregulation of *SOX9* in the critical sex-determining stages only at male-promoting temperatures [52]. It should be noted that sex determination in birds and reptiles does not involve *SRY*. Attempts to find *SRY* homologs in nonmammalian animals have been unsuccessful (see below).

One of the enduring mysteries of biology is how the key developmental switches determining sex can be prone to such diversity. Given the repercussions of improper or diminished functioning of reproductive organs, it is remarkable that there has been such lability in the primary signalling event among even closely related taxa. How *SRY* may have come to be the sex-determining signal in mammals, and how its role as the sex-determining gene may have affected its own subsequent evolution is discussed in detail below.

Evolution of *SRY*

Conservation and evolution of *SRY* in vertebrates

Conservation of the molecular genetic pathway leading to testicular or ovarian development contrasts with the extreme diversity of mechanisms that initiate the commitment to one or other of these pathways, i.e., sex-determining signals. Vertebrate sex-determining mechanisms fall broadly into two classes: environmental sex determination (ESD) and genetic sex determination (GSD). Some species of reptiles exhibit GSD (e.g., squamates) while others exhibit ESD (e.g., crocodilians) [53]. In birds and mammals, sex is determined genetically. In mammals, males are the heterogametic sex (XY) with the Y chromosome acting as a dominant determiner of sex due to the action of *SRY*. In birds, females are the heterogametic sex (ZW); however, it is not known if the W chromosome carries a dominant ovary-determining gene, or whether Z chromosome dosage determines sex. It should be noted that the mammalian X and the avian Z chromosomes are not homologous.

The fact that these different animals (reptiles, birds, and mammals) have completely different sex-determining mechanisms implies that *SRY* is a relative latecomer to the process. *SRY*, in fact, may have imposed itself on a pre-existing sex-determining pathway in an ancestral mammal. Attempts to identify homologs of *SRY* in reptiles and birds have invariably resulted in the isolation of the closest relative to *SRY*, *SOX3*. There is strong evidence that *SOX3* may be the progenitor of *SRY*. First, the HMG box of *SRY* is more closely related to the HMG box of *SOX3* than to that of any other member of the *SOX* family [54]. Second, *SOX3* is located on the X chromosome [55]. Foster and Graves [54] proposed that once *SRY* attained its function as the testis-determining factor, it diverged from *SOX3* as the incipient Y chromosome became recombinationally isolated from its partner, the X chromosome.

While the function of *SRY* as the testis-determining factor is conserved across mammalian orders, its sequence shows a surprising amount of variability between species (fig. 3a, b). The HMG box shows the highest amount of conservation, sharing 80% identity between human and mouse [1], and 65% between human and marsupial (*Sminthopsis macroura*) [3]. However, the region 3′ to the HMG box exhibits a striking level of variability. Alignments between this region of human and mouse *SRY* are made difficult by a large section of repeats in the mouse gene [56] (fig. 1b). In fact, the low level of similarity of sequences flanking the HMG box of *SRY* from several mammalian species indicates that the gene is evolving rapidly. This observation prompted a series of in-depth analyses to define and explain the seemingly unconstrained evolution displayed by this gene.

Rapid evolution of *SRY* sequences

The rate of nucleotide mutation has traditionally been considered uniform over the entire genome, and any variations in substitution rate are deemed functions of differential selective constraints [57]. Positive directional selection, in contrast to neutral evolution, selects nonsynonymous substitutions that cause adaptive amino acid change rather than synonymous substitutions, which remain silent. In discussions of rates of divergence between two sequences, the term K_A is defined as the rate of nonsynonymous substitutions per nonsynonymous site and the term K_S is defined as the rate of synonymous substitutions per synonymous site. Neutral evolution would predict a K_A/K_S value of 1 [58, 59]. Most genes, however, are under some form of selective constraint and the estimated average K_A/K_S value for 363 genes compared between mouse and rat was equal to 0.14 [57].

To examine the relative rates of evolution of the *SRY* gene within pairwise comparisons between human and seven primate species [60], and between *M. musculus* and six species of Old World mice and rats [56], K_A/K_S values were determined for the gene. In the primate study they ranged from 0.47 to 1.88 [60] and values in the mouse study ranged from 0.33 to 0.45 [56]. Both deviate from the average (0.14) and were deemed evidence of selective drive upon the sequence, providing the first indication that positive directional selection may be acting upon this gene.

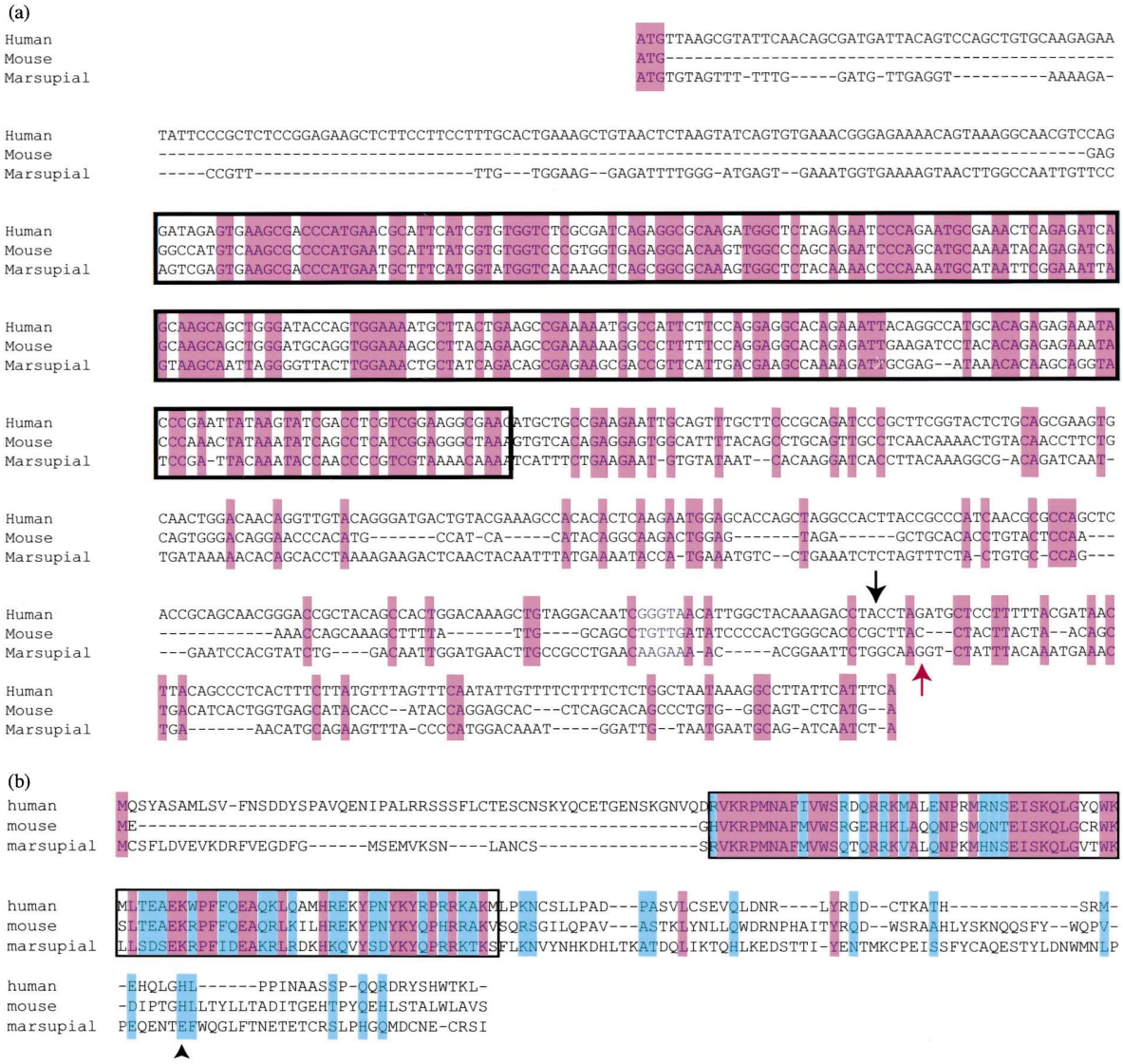

Figure 3. Alignment of the coding nucleotide sequence (*a*) and amino acid sequence (*b*) of *SRY* from human (accession L10101), mouse (*Mus musculus*) (accession X67204 [65]) and dasyurid marsupial (*Sminthopsis macroura*) (accession S46279) using Clustal V. Regions of identity between all three sequences are shaded pink. Conservative amino acid changes between all three sequences are shaded aqua. The HMG box is outlined in black. The CAG repeat in the mouse *Sry* sequence has been left out of both alignments. The position of this repeat has been marked by a black arrow. The position of the intron in the marsupial sequence is marked by a red arrow.

The data for primates and mice were subsequently reanalyzed using an independent method for calculating K_A/K_S values, and data for bovids were included [61]. In addition, data were generated for the HMG box and terminal regions based on the total lengths of the respective phylogenetic trees for mice, primates, and bovids. This reanalysis confirmed and strengthened the conclusion that the *SRY* genes have high K_A/K_S ratios indicative of rapid evolution, and demonstrated that the ratio is particularly high in the terminal sequences. This is in contrast to the severely constrained rates found in both the HMG box and terminal regions for other members of the *SOX* family (e.g., *SOX2* and *SOX3*) (fig. 4).

The seemingly high rates of evolution among *SRY* genes in primates and mice led several authors to propose that the rapid evolution of the gene may result from species-specific adaptive divergence due to positive

directional selection [56, 60]. Since it initiates the cascade of male sexual development, it has been postulated that species specificity for the *SRY* gene may be involved, to some degree, in reproductive isolation [56, 62]. By acting in testis development, and possibly in spermatogenesis, species specificity of *SRY* action imbued by sequence divergence could result in incompatibilities between *SRY* and other genes in the pathway in a conspecific background [63]. A good example of the incompatibility of *SRY* on a hybrid background is the sex reversal observed in C57BL/6J mice carrying a *M. m. poschiavinus* Y chromosome (Y^{pos}) as discussed above [47]. Failure of cross-species interactions between *SRY* and other genes in the developmental pathway may lead to failure of testis differentiation or create sterile males with impaired spermatogenesis within a hybrid zone between two incipient species [63].

The hypothesis of positive directional selection on *SRY* in recently diverged species suggests a possible explanation for the observed rapid evolution of *SRY*. However, the primate and mouse species used in the aforementioned studies diverged several million years ago. Consequently, any selective constraint, or lack thereof, upon the *SRY* sequences may have been masked by other nonselective or episodic events. To test for positive directional selection on *SRY*, O'Neill et al. [62] compared the nucleic acid and amino acid sequences and the substitutions rates of *SRY* within a recently diverged and rapidly speciating group of mammals, rock wallabies (*Marsupialia*, *Macropodidae*). Previous studies have shown that if a gene undergoes positive directional selection, recently diverged species have K_A/K_S values significantly above the neutral line of evolution ($K_A/K_S = 1$) [59].

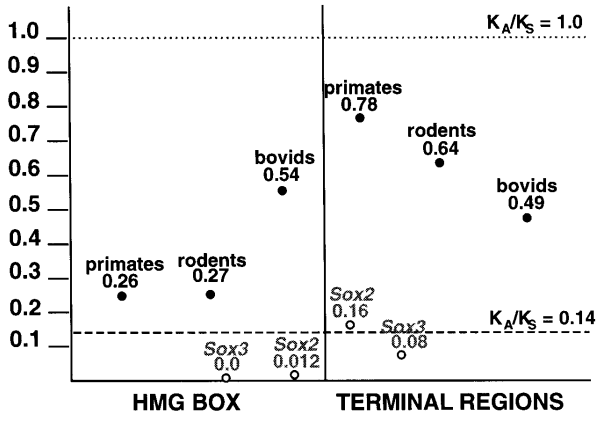

Figure 4. Graphic representation of the K_A/K_S values for *SRY* obtained by Pamilo and O'Neill [61]. The X-axis delineates the two regions of *SRY*: the HMG box and the terminal regions (C and N terminus). The Y-axis represents K_A/K_S values. $K_A/K_S = 1$ (i.e., neutral evolution) is shown as a gray dotted line and $K_A/K_S = 0.14$ (the average value obtained by Wolfe and Sharp [57] for 363 genes [see text]) by a black dashed line. The values for the *SRY* gene within rodents, primates, and bovids were calculated from the total lengths of the respective phylogenetic trees [61] and are shown as black dots. The values for *Sox3* and *Sox2* were calculated from pairwise comparisons between human and mouse and are shown as gray dots.

Analysis of the *SRY* gene from 12 species of rock wallaby (*Petrogale*) demonstrated that *SRY* sequences are not under positive selective pressures within short evolutionary time periods [62]. K_A/K_S values were calculated for pairwise comparisons of *Petrogale* taxa, and 71% were less than 0.03, well below the average of 0.14 estimated for most genes. While rock wallabies are found across the Australian continent, their *SRY* sequences are not correspondingly diverse. Neither the nucleic acid sequences nor the amino acid sequences are species specific. Among the 12 *Petrogale* species studied, there are only eight different SRY protein sequences.

This in-depth analysis of *SRY* evolution in rock wallabies provided a clear inconsistency between the rates observed in primates and mice with those found in rock wallabies. The values obtained suggested that *SRY* sequences in the *Petrogale* species complex were not subject to positive directional selection. The rapid sequence evolution of *SRY* found in studies of primate and mouse species, which diverged in the distant past, may therefore indicate a lack of functional constraint on regions surrounding the HMG box and/or may be a result of past episodic selection events or Y-specific events. It seems likely that *SRY* evolution is not rapid over short evolutionary time scales, yet may undergo periods of rapid change through a variety of Y-specific mechanisms, such as genetic drift, chromosomal sweeps through a population, or an unknown mechanism.

Many authors have remarked that the function of the SRY protein seems to depend on the HMG box and no function is known for the terminal parts [25, 60, 64]. Based on distance analyses [61], it is clear that the terminal regions of *SRY* are far less conserved among taxa than the HMG box (see figs. 3a, b, 4). If there were a complete lack of functional constraint on the terminal regions, the synonymous and nonsynonymous sites would show approximately similar evolutionary rates, i.e., neutral evolution. The data from analyses of *Sry* from rodents [56] indicate that nonsynonymous sites in the terminal regions display some constraint, and the data from apes [60] also hint at the existence of selection at the protein level. These findings indicate that the terminal sequences may have some function in all mammals. This is interesting in light of the evidence that human SRY C-terminal amino acids interact with the PDZ domain of human SIP-1.

In addition to the remarkable sequence evolution of *SRY* discussed above, there have been sequence changes

on a larger scale that could affect *SRY* function in different mammalian lineages. These large-scale changes, discussed in the next section, include trinucleotide repeat expansions, intron insertion, duplications, and deletions.

Structural modifications of *SRY*

Early studies of *Sry* function in mouse produced the first notable structural eccentricity: a CAG trinucleotide repeat in the region 3′ to the HMG box (amino acids 144–366) [56], subsequently identified in many other rodent species [56, 65]. Sequence analysis of this repeat showed extreme variability in composition and length among Old World mice and rats [56]. For example, *M. musculus Sry* carries a repeat region 666 bp in length, while *Hylomyscus alleni Sry* contains a repeat of only 60 bp in length. Polymorphisms of CAG repeat length may underly the sex reversal produced when certain *Sry* alleles are put on the C57BL/6J inbred background [65], an effect similar to the weakly acting Y^{pos} allele on the same background. Interestingly, this repeat appears to be absent outside the subfamily Murinae.

While it seems that the nucleotide sequence of *SRY* is not well conserved across mammalian orders, one characteristic of this gene is conserved: its existence as a single exon. However, studies of *SRY* evolution in marsupials revealed a surprising discovery in the *SRY* gene of dasyurid marsupials. The *SRY* gene of *S. macroura* (stripe-faced dunnart) was found to contain not one, but two exons [66]. In this species, *SRY* contains a functionally spliced 825-bp intron (fig. 1c). The presence of the intron in dasyurids and its absence in all other eutherian and marsupial clades studied indicates that an insertion occurred de novo after the divergence of macropods (kangaroos) and dasyurids, about 45 MYA. The presence of the intron in all dasyurids [66] implies that it was inserted prior to the dasyurid radiation and has been retained in the lineage. As discussed earlier, *SRY* is most closely related to the X-borne *SOX-3* gene, from which it is thought to have evolved as the sex chromosomes differentiated [54]. *SOX-3*, like most *SOX* genes [67], also lacks introns, implying that a single exon is the ancestral state of the HMG-encoding *SOX* family [see ref. 68 for *SOX* family diversification patterns]. The insertion of an intron into the dasyurid *SRY* gene is one of the few cases illustrating 'late' evolution of introns.

Another curiosity of *Sry* evolution in rodents is the presence in some species of amplified copies of *Sry* on the Y chromosome. Examples of multiple copies of *Sry* have been found in males of several South American *Akodon* (Cricetidae) species [69], African murine species [70], Asian mouse species, as well as several non-*Mus* species, including the laboratory rat [71]. Amplified copy numbers range from two in *M. caroli* to 13 in *Nannomys minutoides* [71]. Sequence analysis of multiple copies of *Sry* in six species of African murines showed long open reading frames and conserved HMG box sequences that led the authors to conclude that all copies identified in each species may be functional [70]. Multiple copies of *Sry* (15) were also found in the vole species *Microtus cabrerae* and, surprisingly, the majority of the copies were found on the X chromosome [72]. However, sequence analysis revealed that only one copy, residing on the Y chromosome, was likely to be functional. Given the indispensable function of *SRY* as the mammalian sex determiner, and its sensitivity in this role to thresholds of gene activity, it is perhaps surprising that the gene has undergone such drastic changes in copy number in several species. Even more remarkable are those species in which *SRY* function has apparently been dispensed with altogether.

The *Sry* gene is missing in two vole species, *Ellobius lutescens* and *E. tancrei*, while their sister taxon, *E. fuscocapillus*, has retained it [73]. This is the result of the total loss of the Y chromosome from these species, yet sex determination remains normal for reasons as yet not understood. In *E. lutescens*, males and females both exhibit a $2n = 17$, XO karyotype, while *E. tancrei* males and females both exhibit a $2n = 32–54$, XX karyotype. *Sry* has also been lost in another XO murid, the spiny rat (*Tokudaia osimensis*) [74]. In these systems, sex determination is fulfilled in the complete absence of the Y chromosome including the *Sry* gene. These observations are undoubtedly a reflection of the rapid nature of the evolution not only of this gene but of the Y chromosome itself.

Gene amplification and gene loss are common features of Y chromosome evolution, itself a prime example of the operation of Muller's ratchet. Once *SRY* became the dominant sex-determining signal in the ancestral mammal, and became recombinationally isolated, it was subject to attritional forces typical of nonrecombining chromosome regions.

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