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Review Article

MOLECULAR BASIS GOVERNING PRIMARY SEX IN MAMMALS

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Summary The function of *Sry* for inducing a male gonad was identified due to a development of a transgenic XX male mouse with testes by introducing a single gene into an embryo. The intronless *Sry* encodes a putative transcriptional protein harboring an HMG motif. The sequence similarity within the HMG motif has been highly conserved despite less conservation in other domains. Hence, the HMG motif must play a critical role in the transcriptional regulation, leading to the development of a male gonad. However, a non HMG box C terminal domain of *Sry* protein may also be indispensable for inducing normal testicular development. Further, several autosomal genes, such as *SF1*, *WT1*, *SOX* and *MIS*, as well as a unique X chromosomal *DAX1* were suggested to be associated with the development of gonadal sex in mammals. Therefore, the significance on the involvement of these genes in the molecular mechanism of mammalian sex determination should be also considered.

Key Words sex determining gene, primary sex determination, mammalian sex

Introduction

The clarification and understanding of the molecular mechanism responsible for mammalian sex determination is very interesting, because the presences of male and female sexes are not only surprising in its mysterious manifestations and graceful in its conception but also absolute benefits. In a mammalian system, the appearance of gonadal sex in a lineage of sex differentiation is most exciting, yet is still not sufficiently understood. In *Drosophila melanogaster*, molecular mechanism of sex determination with implicated genes and their functions has been well understood: the Y chromosome (Chr) is not important but the ratio of the number of X Chr to that of sets of autosomes (A) as the X/A ratio, and this X/A signal activates a key regulatory gene, sex lethal (*Sxl*) on the X Chr, that in turn regulates

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the choice between male and female sex developmental pathways (Parkhurst and Meneely, 1994). In mammals, the existence of males and females contain two processes, such as sex determination and differentiation. During embryogenesis, primary (gonadal) sex are determined as developing testes with regression of Müllerian ducts, or ovarian development with regression of Wolffian ducts from undifferentiated gonad. Following sex differentiation must be controlled by sexual hormones whereas this is not in *Drosophila*. This review considers not sex-differentiation but primary, namely gonadal sex determination in mammals with special reference to molecular basis based on associated genes and their putative functions.

Since the 1950s, it has been known that the male has a Y Chr but the female does not, namely XY offsprings develop testes and XX offsprings develop ovaries (Jacobs and Strong, 1959; Welshons and Russell, 1959). The development of mammalian male gonad from an embryo was inferred to be caused by factors on the short arm of the Y Chr (Welshons and Russell, 1959; Jacobs and Ross, 1966). Some possible candidate factors for primary sex determination were proposed (Wachtel *et al.*, 1975; Page *et al.*, 1987; Mardon and Page, 1989), however these have not been confirmed (McLauren *et al.*, 1984; Palmer *et al.*, 1989; Koopman *et al.*, 1989). A well known male specific histocompatibility-Y (H-Y) antigen was postulated as a primary testis inducer (Wachtel *et al.*, 1975), but this was not verified due to a lack of the H-Y antigen in a mouse with testes (McLauren *et al.*, 1984). However, cumulative karyotypic analysis on several abnormal sex patients revealed a functional region for sex determination on the Y Chr (McLauren, 1990). Further, the proposition of the genetic switch model for primary sex determination with the testis determining gene (factor) *TDF/Tdy* on the Y Chr in humans and mice is credible (Eicher *et al.*, 1982; Eicher and Washburn, 1983). This prediction seems to be valuable for promoting the study on the molecular mechanism of mammalian sex determination.

In 1990, the sex determining region Y genes of humans and mice (*SRY/Sry*) as a candidate for *TDF/Tdy* were proposed in stead of the previous best likely candidate zinc finger Y gene *ZFY/Zfy* (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990). The *Sry* gene is located close to pseudoautosomal region (PAR) on the short arm of the Y Chr (Palmer *et al.*, 1989; Sinclair *et al.*, 1990). Subsequently in 1991, the development of a transgenic XX male mouse with testes by the introduction of only a *Sry* gene into an embryo was reported (Koopman *et al.*, 1991). Hence, the *Sry* was identified as a strong candidate gene for the *Tdy*. Nevertheless, the transduction of a human *SRY* into a mouse XX embryo failed to induce testicular development leading to maleness, in spite of detecting abundant transcript. In the following years, several *Sry* with encoding proteins from various mammals were reported (Whitfield *et al.*, 1993; Tucker and Lundrigan, 1993; Su and Lau, 1993). Moreover, Coward *et al.* (1994) found a novel polymorphic CAG repeat within mice *Sry* correlating with B6 sex-reversions. Therefore, its significance in primary sex determination should be further discussed. Aside from *SRY/Sry*, several

autosomal genes such as the *SF1*, *WT1*, *MIS* and *SOXs*, and a unique gene *DAX1* on the X Chr were recently suggested to be involved in primary sex determination. Some abnormal gonadal sex owing to defectiveness or mutations harbored in these genes were reported despite unknown functional relationships among them (Luo and Parker, 1994; Polletier *et al.*, 1991; Kreidberg *et al.*, 1993; Haqq *et al.*, 1993; Foster *et al.*, 1994; Zanaria *et al.*, 1994). Thus, possible implications of these genes in primary determination of the mammalian sex should be considered. Therefore, taken all together, these genes with their presumed functions should be summarized for further understanding the molecular base governing the development of gonadal sex in mammals.

Genetic Switch Model for Mammalian Primary Sex Determination

In 1982, Eicher *et al.* proposed a genetic model for mammalian primary sex determination (Eicher *et al.*, 1982; Eicher and Washburn, 1983). In the model, they predicted the presence of a testis determining gene *Tdy* on the Y Chr and an ovary determining gene *Od* on the X Chr or PAR. This putative model was derived from genetic analysis by employing inbred mice. Owing to the valuable contribution to the studies on the sex determination, it is worth giving an outline of it. However, to eliminate confusion, the phylogeny of laboratory mouse strains employed in genetic analysis should be summarized. The wild house mouse *Mus musculus* (*M. m.*) is recognized as a polytypic species having diverged mainly into four subspecies within the last 0.6×10^6 years (Nagamine *et al.*, 1992). For example, the subspecies, *M. m. domesticus* (DOM) being indigenous to Europe, migrated to all the Americans, Australia and various atlantic and pacific islands. *M. m. musculus* (MUS) occupies a part of Europe and almost Asia. *M. m. bacterianus* (BAC) inhabits between Iran and Burma, and *M. m. castaneus* (CAS) occupies southeastern Asia. *M. m. molossinus* (MOL) developed from spontaneous hybridization between the MUS and CAS, inhabits Japan, Korea and a part of mainland China. In an ordinary classification based on the chemical markers or mitochondrial DNA-probe (Bishop *et al.*, 1985), inbred mice C57BL/6J (B6) and 129 are related to DOM. However, by means of Y Chr specific DNA probe, B6 and 129 are not related to DOM, but to MUS. This paper follows latter classification. Further, the male MOL as pet mice originated in Japan, were exported to eastern Europe and then established as inbred mice such as B6, or 129 (Nagamine *et al.*, 1992). As a result, these male B6 and 129 are classified into MUS bearing MOL type Y Chr DNA. Recently, Coward *et al.* (1994) reported a polymorphic CAG repeat correlating with various XY females, within the *Sry* from inbred strains of DOMs (see below).

The genetic model for primary sex determination was originally based on valuable genetic analyses such as mating a male POS (*Mus Poschiavinus/Zalende*) classified as a DOM with female B6 (Eicher *et al.*, 1982; Eicher and Washburn, 1983). Agreeably, in addition to the comparative sex ratio in the F_1 offsprings, all

XY individuals developed as normal male mice. However, subsequent backcrossing of F_1 XY mice to female B6 developed XY female features or hermaphrodites out of approximately 50% of N_2 XY offsprings. Moreover, additional backcrossing of the N_2 male mice to female B6 gives higher ratios of both XX and XY females or hermaphrodites. Conversely, mating male B6 with female POS or other strains of the DOM result in normal sex differentiation. Hence, apart from the *Tdy*, the presences of testis determining autosomal genes *Tda-1* and *Tda-2* were infered. Likewise, an ovary determining gene *Od* (and *od-1*) on the X-Chr or PAR was hypothesized.

The undifferentiated gonad is a bipotential organ capable of developing either ovarian or testicular tissues. According to the switch model, the *Tdy* must induce to differentiate the bipotential primordial gonad into the male gonad. Normally functioning *Tdy* initially inactivate the *Od*, inducing subsequently to differentiate the supporting cell into the sertoli cell at the gonadal primordia. Additionally, these are functionally supported by the *tda-1* and *tda-2*, thereby the testicular development in an XY embryo are further guaranteed. The postulation further says that the expression of *Tdy* starts earlier than that of *Od*. Thereby, it must inactivate the *Od* gene. On the other hand, an *Od* in an XX embryo lacking *Tdy*, initiates expression to differentiate the supporting cells into the follicle cells, leading to develop ovarian tissues. These presumed functions readily allow us to characterize genes responsible for abnormal sex differentiation displaying B6 sex reversal (B6.Y^{POS} or B6.Y^{DOM}). Namely, Tdy^{B6} being the *Tdy* of B6 is recognized to be normal but initiates expression earlier than Tdy^{DOM} being the *Tdy* of DOM, which is thought to function abnormally within B6.Y^{DOM}. In addition, the recessive autosomal $Tda\text{-}1^{B6}$ being *tda-1* of B6 assumedly initiates expression earlier than its allelic dominant gene $Tda\text{-}1^{DOM}$, *tda-1* of DOM.

Otherwise, the involvement of another autosomal gene *Tas* (T-associated sex reversal gene, where T is the locus for the hairpin tail) in the primary sex determination was described. The *Tas* was deduced from the genetic linkages of the sex reversed XY female with the T locus and the recessive autosomal gene *qk* (locus for quaking) on the 17 Chr (Washburn and Eicher, 1983, 1989). Through the interaction with the *Tdy*, the *Tas* was deduced to be implicated in the suppression of ovarian development. In an XX embryo, the *Tas* induces only ovarian formation due to a lack of interaction with the *Tdy*. Nonetheless, if the *Tas* in an XY embryo harbors some deletions or mutations within a functional region, an abnormal interaction with the *Tdy* may cause the expression of the *Od*. This would induce ovarian tissues, leading to develop XY females or hermaphrodites. Therefore, the *Tas* was presumed to be a candidate gene for *Od* despite no verification. Consequently, gonadal sex-determination seems to possibly involve the network among respective gene expressions.

Identification of Primary Sex Determining Gene Sry

Palmer *et al.* (1989) and Koopman *et al.* (1989) indicated that *ZFY/Zfy* was not a candidate for *TDF/Tdy*, because several patients such as three XX males and one XX inter sex who lacked *ZFY* were found. Moreover, mutant mice W^e/W^e with testes lacking germ cells, were defective in expressing a *Zfy*. Subsequently, the human *SRY* and mice *Sry* localizing close to PAR on the short arm of the Y Chr as a strong candidate gene for *TDF/Tdy* were reported (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990). As a result, the development of a transgenic XX male mouse with testes due to the introduction of a single *Sry* gene harbored in the 14 kb genomic DNA but without *Zfy* into an embryo, was reported (Koopman *et al.*, 1991). Thereafter, the overall sequence of Sry^{MOL} from a inbred mouse 129 bearing MOL type Y Chr was reported (Gubbay *et al.*, 1992). The Sry^{MOL} contains three kinds of an intronless open reading frame (ORF) in which the longest ORF encodes 395 amino acid residues harbouring a high mobility group (HMG) motif composed of 80 amino acids at the N-terminus.

The putative target sequences for DNA binding HMG motif, such as AACAAAG or CTTTGTT in the 5′ upstream and AACAAAG also in the 3′ downstream of ORF have been found respectively. We confirmed a similar sequence apart from several variations including a notable premature stop codon in Sry^{DOM} (Coward *et al.*, 1994). By reason of the presence of HMG motif, this *Sry* protein is capable not only to bind, but also to bend this DNA by 80° (Ferrari *et al.*, 1992). These molecular characterization suggests that *Sry* protein seems to be identical with known transcription factors (Mitchell and Tijian, 1989; Jantzen *et al.*, 1990). Hence, this may predict that the transcription of the *Sry* gene primarily requires an interaction with an upstream unknown factor harboring an HMG box, leading to the transcription and the production of the *Sry* protein factor. Successively, this *Sry* protein may interact with the downstream target gene for their expression. Nevertheless, a putative target sequence AACAAAG have not yet been finally identified, but AACAAT was also proposed (Dubin and Oster, 1994). Further, the TATTAAA (TATA box) at 298 bp and the transcriptional initiation site at 278 bp from HMG-box in the 5′-flanking region as well as linear transcripts were identified (Jeske *et al.*, 1995).

It is mentionable that the ORF of mouse *Sry* gene encodes various polyglutamine repeats flanked by the sequences Phe-His-Asp-His-His except for a few variations. The *domesticus*-type *Sry* gene Sry^{DOM} from B6.Y^{DOM} displaying B6 sex reversal conserves similar traits to that of MOL with some variations (see below). However, human *SRY* and other mammalian *Sry* lack these repeats, thus this seems to be specific to mice. Recently, these polyglutamine repeats were suggested to activate the transcription by GAL-4 responsive reporter gene assay (Dubin and Oster, 1994) and by means of CAT assay (Koopman *et al.*, unpublished data, 1995).

In 1993, Su and Lau clarified the overall sequence with the organized structure

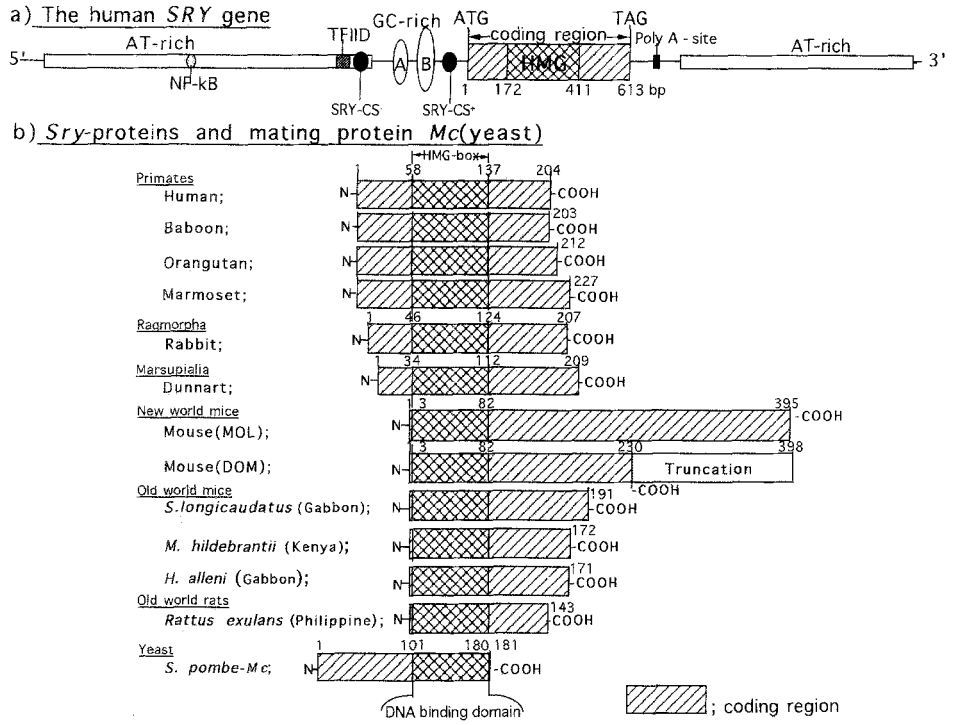

Fig. 1. The structure of *Sry* genes and encoded proteins. (a) The structural organization of the human *SRY* gene; The *SRY* contains two intronless ORFs, of these, longer one encodes a protein composed of 204 amino acids, harboring an HMG motif containing 80 amino acid residues. The promoter region in 5′ flanking sequence such as TFIID binding site as TATA box, NF-$\varkappa$B like element as an enhancer, HMG binding site as *SRY*-CS$^+$ (AACAAAG)/ *SRY*-CS$^-$ (CTTTGTT), AT-rich and GC-rich region (A and B) are indicated, respectively. The transcription initiation site is identified at -310 bp (not shown). The poly A site was identified at $+728$ bp in the 3′ flanking region. The AT-rich region was identified also in the 3′ flanking region. (b) The encoded mammalian *Sry* proteins and mating protein Mc of yeats; N and COOH indicate amino and carboxyl terminus of each protein. The numerical orders refers to the number of the amino acid residues. The shaded and cross shaded area indicate the coding region and HMG motif, respectively. *Sry* proteins of gorilla, chimpanzee, pygmy chimpanzee and gibbon are composed of 204 amino acids as similar to human (not shown).

of the human sex determining gene *SRY* (Fig. 1a) (Su and Lau, 1993). The human *SRY* encodes two kinds of intronless ORF. A longer ORF encodes 204 amino acid residues harboring an HMG-motif being composed of 80 amino acids similar to the mice *Sry* (Figs. 1 and 2). Moreover, the 1.3 kb transcriptional product was also identified in both adult human testis and the recombinant *SRY*-DNA transfected cells derived from sertoli cells (Su and Lau, 1993). The organized structure of the

human *SRY* gene is shown in Fig. 1a. In the 5′ upstream promoter region, TFIID binding site, transcriptional initiation site at −310 bp, AT-rich and GC-rich domains, the putative target sequence AACAAAG and CTTTGTT for HMG motif and the enhancer were identified, respectively. The identification of the structure of other mammalian *Sry* are insufficient. However, these are intronless genes coding putative transcriptional protein factor harboring HMG motif similar to that of human *SRY* except their sizes.

The *Sry*-protein sizes in terms of amino acid residues varies widely depending on species as shown in Fig. 1b. Only within primates are homogeneous such as 203/baboon-227/marmoset, but those in mice *Sry* indicate larger variation such as 171/*H. alleni* (old world)-230/DOM and 395/MOL (new world). Interestingly, the sequence identity of HMG domain between the human *SRY* and the mice as well as other mammalian *Sry* has been highly conserved (63-80%) despite no similarity in other domains. It is noticeable that the HMG motif harbored in mating protein Mc of yeast (*Schizosaccharomyces pombe*) has 30% sequence similarity with human *SRY* protein. Thus, the HMG motif must have been preserved due to its functional importance in primary sex determination. Several mutant *SRY* from patients with abnormal sex differentiation displaying XY females were described (Müller *et al.*, 1992; Hawkins *et al.*, 1992; Harley *et al.*, 1992). Actually, approximately 15% of XY females have mutations in *SRY*

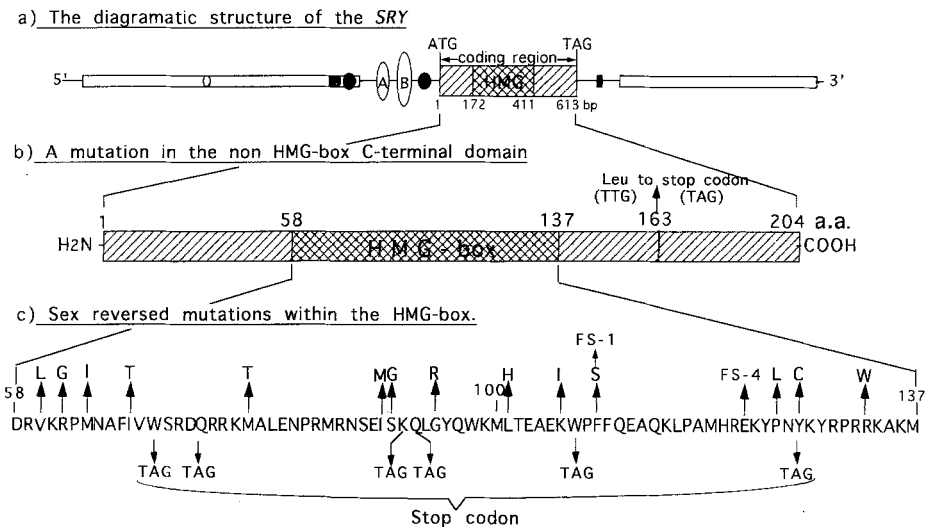

Fig. 2. The mutant *SRY* causing sex reversions. (a) The diagrammatic structure of human *SRY* gene as that in Fig. 1a. (b) A mutation, Ile at 163 changes to the stop codon, in the non HMG-box C-terminal domain is indicated. The shaded and cross shaded box represented the coding region and HMG-box, respectively. (c) The sex reversed mutations occurred within an HMG-box are shown. The FS with numbers indicate frameshift due to base pair deletions 1 and 4.

(Goodfellow, 1993; Hawkins, 1995). Mentionably, these mutations mostly occur within the HMG box except one in C terminal region of *SRY* protein (Fig. 2b and c). This suggests that the HMG box must be an essential domain correlating with molecular functions of *SRY* responsible for inducing testicular formation. Consequently, the HMG domain of *Sry* protein must play a critical role for the expression of the target genes in the down stream, leading to induce a normal testicular development. Recently, this was partially verified in the three-dimensional structural analysis on the SRY-HMG protein-GCACAAAC complex: the binding of HMG protein to its target site occurs in the minor groove and induces a large comformational change in the DNA, including an overall 70–80° bending (Werner *et al.*, 1995). However, a premature stop codon due to a mutation outside of the HMG-box toward C-terminal region of the SRY protein resulted in XY female-sex reversion (Tajima *et al.*, 1994). Therefore, non HMG-box C-terminal domain may also be indispensable for inducing normal gonadal sex. Similarly, a required role of non HMG box C-terminal domain of mice Sry-protein for the determination of primary sex was also reported.

Polymorphic CAG Repeat within Sry^{DOM} Correlates with XY Females

B6 sex reversion displaying XY females (B6.Y^{DOM}) resulting from back-crossing F_1 XY offsprings with B6 females as described above, varies phenotypically depending on the employed strains of inbred mice DOM (Biddle and Nishioka, 1988; Biddle *et al.*, 1991). (1) The POS and DOM display complete sex reversion in developing XY females or true hermaphrodites with ovaries or ovotestes in one half of XY offspring. (2) Incomplete sex reversal by AKR/J, RF/J, MA/Mn/J, PL/J; XY fetus has ovaries, but only normal testes appear in adults. (3) Normal sex differentiation without sex reversion in such as FVB/N, SJL/J, SWR/J, Bub/Bn/J, ST/bJ; XY offsprings with only normal testes never develop an ovary. These findings predict the presence of structural variations correlating with varying functions responsible for the sex determining region on the Y Chr, however no verification. Otherwise, it has been reported that the HMG motif of Sry^{DOM} harbored the mutation of the Ile63Thr despite normal sex differentiation (Kunieda and Toyoda, 1992; Graves and Erickson, 1992). Meanwhile, the overall sequence and structural organization of Sry^{DOM} was unknown. Then, Lau's collaborators including this author, clarified these provocative questions, and revealed quite unusual findings as follows.

Consequently, the primary structure of Sry^{DOM} contains 1200-nucleotide intronless ORF and respective nucleotides of 703 in upstream and 312 in downstream. According to the 14.6 kb Sry^{MOL} genomic sequence from the laboratory mouse strain 129 retrieved from GeneBank [Fig. 3(1)], the longest ORF of Sry^{MOL} gene encodes a protein of 395 amino acids with a calculated molecular weight of 49.5 kD. The conserved DNA binding domain namely HMG motif contained 80 amino acids, is present within the first 100 amino acids, being followed by 20 kinds

of poly Gln (CAG repeats). More than half of these tracts seem to be dispensable due to the fact of truncation found in Sry^{DOM}. After all, this repetitive nature was suggested to contribute to the transcriptional activation as described above. As expected previously, this suggestion may be reasonable owing to an analogy to known DNA binding transcription factors with Gln-rich domains that interact with the other protein factors and activate it (Mitchell and Tijian, 1989; Jantzen *et al.*, 1990).

In spite of being highly similar to the Sry^{MOL} sequence, a striking change of C to T substitution at codon 231 that turns a CAG (Gln) codon into a TAG stop codon truncates the ORF of Sry^{DOM} prematurely. In addition to several single-base changes causing amino acids substitutions and deletions, the truncated ORF of Sry^{DOM} encodes 230 amino acid protein with a predicted MW of 28.4 kD as approximately 20 kD (165 amino acids) smaller than that of Sry^{MOL}. Besides these, there are three additional single-base substitutions in the upstream of ORF, but their effects on gene expression are unknown.

The appearance of stop codon TAG at codon 231 truncating the ORF of

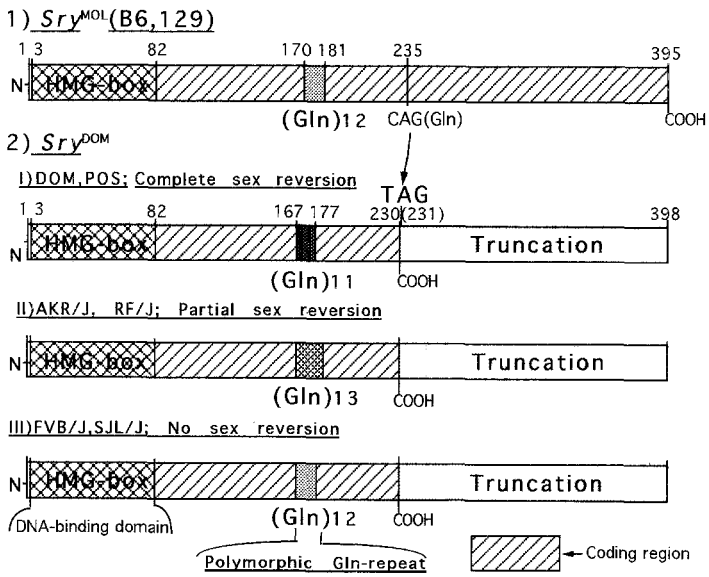

Fig. 3. The polymorphic polyglutamine within *Sry* proteins of mice. The *Sry* proteins of MOL contain 20 kinds of polyglutamine but, a half of them are truncated in DOM. One of these polyglutamine aligned at the 170–181 amino acids with respect to Sry^{MOL} protein has a polymorphism correlating with sex reversions. The shaded and cross shaded area indicate coding region and the HMG motif, respectively. The N and COOH show amino and carboxyl terminus of the protein. (1) The Sry^{MOL} protein from inbred mice such as B6 or 129. (2) The Sry^{DOM} proteins of inbred mice bearing DOM-type Y Chr, causing varying B6 sex reversions.

Sry^{DOM} was then supposed to cause sex reversion displayed in B6.Y^{DOM} due to its shortened protein. This might be supported by the finding that a mutational stop codon appeared in ORF but within the HMG box of human *SRY* caused a sex reversed XY female (Hawkins *et al.*, 1992; Harley *et al.*, 1992). However, it is hard to interpret normal sex differentiation displayed in offspring developed from inbreeding within DOM subspecies. Hence, this led to analyze the primary structures of other *Sry* alleles from other strains of DOM mice manifesting various B6 sex reversals. Consequently, the molecular weight of the Sry^{DOM} protein was estimated as 28.5 kD, approximately 20 kD smaller than that of $Sry^{Mol(129)}$, as predicted by their respective ORFs.

The Sry^{B6} protein translated from *Sry* of B6 bearing MOL-type Y Chr, is identical to the MW about 48.5 kD with $Sry^{Mol(129)}$ protein. However, all *Sry* proteins from other strains of DOM such as AKR/J, FVB/N, SJL/J or SWR/J mice have a similar MW (28.5 kD) to that of Sry^{DOM}. This means that the TAG stop codon must be functional and suggests that it may be present in all *domesticus* derived *Sry* alleles. Unexpectedly, the stop codon presenting in all *Sry* alleles from various strains of DOM does not correlate directly with B6 sex reversions.

Thus, this led to the search for the specific sequences correlating with various B6 sex reversals caused by *domesticus* type *Sry*. Finally, a single polymorphic CAG (Gln) repeat from codon 170 to 181 with respect to the Sry^{MOL} sequence coding 12 repeats at this site was found [Fig. 3(1) and (2)]. The *Sry* from FVB/N, SJL/J, SWR/J, BUB/Bn/J and St/bJ mice code for 12-Gln similar to Sry^{MOL} of 129 and B6 displaying normal sex differentiation. Interestingly, Sry^{DOM} from B6.Y^{DOM} and the *Sry* allele from the POS displaying complete B6 sex reversal encodes 11 glutamine residues. Nevertheless, other strains of DOM mice displaying partial B6 sex reversal, such as Sry^{AKR} from B6.Y^{AKR}, and the *Sry* allele from RF/J, MA/Mn/J, PL/J encodes 13 residues of glutamine at this site.

These unusual results help fuel speculation on the functional variations originating on the polymorphic structures. The fact that transcriptional activity of *Sry* may vary depending on in frame CAG repeats at a C-terminal region of encoded proteins, in analogy to the Gln-rich region interacting with other factors, is thought to be due to the activation domain of DNA-binding transcription factors (Mitchell and Tijian, 1989; Jantzen *et al.*, 1990). This was recently supported partially as described above. This functional disturbance which originated on polymorphic CAG repeats may lead to the development of various abnormal gonads in XY offsprings. In support of this discussion, it should be mentioned that human genetic disorders associated with some polymorphic trinucleotide repeats such as $(CAG)_n$ or $(CTG)_n$ occur in myotonic dystrophy, spinal and bulbal muscular atrophy, Huntington's disease, spino cerebellar ataxia and recently, dentator and pallidoluysian atrophy (Brook *et al.*, 1992; La Spada *et al.*, 1991; The Huntington's Disease Collaborative Research Group, 1993). In the general population, the number of repeats within affected genes are polymorphic and

increases dramatically in patients suggesting significant genetic instability.

The Role of SF1, WT1, SOX, MIS and DAX1 in Primary Sex-Determination

More than 80% of XY female patients have normal *SRY*, thereby possible involvement of several autosomal or X Chr genes in the determination of gonadal sex has been suggested. Eicher and Washburn (1986) had previously supposed to be the implications of autosomal *tda-1*, *tda-2*, *Tas* and *Od* on the X-Chr or PAR in the determination of mouse gonadal sex despite less identification. Besides the *SRY/Sry*, possible association of several genes with the determination of mam-

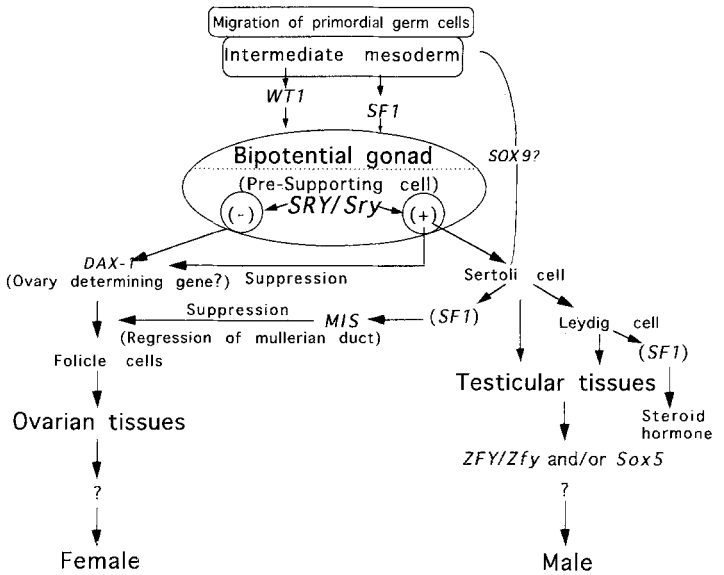

Fig. 4. Genes associated with the determination of primary mammalian sex. The possible relationships among proposed genes involved in the primary determination of mammalian sex is schematically shown. The *WT1* and *SF1* may initiate before expression of *Sry* and may also contribute to give rise to the bipotential gonad from intermediate mesoderm. Human *SOX9* may be associated with this pathway, but no identifications. If a bipotential gonad harbors the *Sry*, supporting cell precursors differentiate into sertoli cells and leydig cells, leading to the development of testis. Otherwise, the *SF1* probably acts on the *MIS* gene. Fully differentiated sertoli cells express the *MIS*, resulting in the regression of the Müllerian duct. The human *ZFY*, mice *Zfy* and/or the *Sox5* may also be associated with spermatogenesis. In an embryo lacking the *Sry*, gene responsible for the ovarian development, such as DAX1 may be initiated. Abnormal functions of respective genes such as the *SF1*, *WT1*, *SRY/Sry*, *SOX9* or *MIS* within XY embryos possibly allow the *DAX1* to express, inducing to the development of femaleness to varying degrees. If the *DAX1* is functionally disorders or defective, testicular tissues may be formed despite a lack of *Sry*, developing an XX male.

malian gonadal sex have been describing (Fig. 4). Although detailed interrelationships among them are unknown, molecular biological as well as genetic studies may clarify the molecular mechanism with additional factors soon. Therefore, it is worth summarising these genes with their putative functional relationships for future studies.

The *SF1* (Steroidogenic factor 1, or *AD4BP*; Adrenal 4 binding protein) was originally isolated as a transcriptional regulation factor of steroid cytochrome hydroxylase required for steroid hormone synthesis. The expression of *SF1* was detected in both male and female genital ridges at 9–12.5 dpc, overlapping with that of *Sry*, following by only males (Luo *et al.*, 1994). The *SF1* is expressed in both sertoli and leydig cells however, its expression pattern chronologically is coincides with that of the gene for *MIS* (Müllerian inhibiting substance; see below). Furthermore, the disruption of the *SF1* (knock out XY mouse with normal *Sry*) resulted in the development of ovarian tissues as an XY female without inducing a testicular tissue. It is putatively proposed that *SF1* may initiate expression earlier than *Sry* in both male and female, and is maintained longer only in males. Namely, *SF1* gene may start expression before giving rise to bipotential gonad from the intermediate mesoderm. Thus, the *SF1* seems likely to be required for normal development of gonadal sex, despite unknown correlations with the *Sry* and/or *MIS*, being expected to be verified.

The WT1 located on the short arm of chromosome 11 (11p13) is identified as a tumor suppresser gene for Wilms' tumor (Polletier *et al.*, 1991). This gene has been characterized as being highly expressed in the glomeruli of the kidney, as well as the gonadal ridge of the developing gonad, the sertoli cells of the testis and the epithelial and granulosa cells of the ovary. Thereby, the *WT1* was suggested to play a developmental role in the genital system as well as in kidney (Kreidberg *et al.*, 1993). A patient harboring a deletion at exon 4 in the *WT1* gene, had bilateral Wilms' tumor, hypospadias, and an undescended left testicle. In another Wilms' tumor bearing patient with hypospadias and bilateral cryptorchidism, a single nucleotide deletion within exon 6, causing a truncation was revealed. Further, gonadal development was arrested at an early stage due to the disruption of the *WT1* in the homozygous mutant embryo despite normal *Sry*. The *WT1* may cause intermediate mesoderm thickens to give rise to the bipotential gonad (Fig. 4) (Bogan and Page, 1994). Nonetheless, its expression is maintained for longer in both males and females. Therefore, the *WT1* was thought to play a critical role in early urogenital development (Kreidberg *et al.*, 1993), though further detailed correlations with the *Sry* gene is expected to be elucidated.

The *Sry* was suggested to possibly induce the bipotential supporting cell precursors to differentiate into the precursor of the sertoli cells, leading to fully differentiated sertoli cells (Goodfellow, 1993). These sertoli cells produce the *MIS*, inducing the regression of the Müllerian ducts, which would otherwise develop into the uterus, cervix, fallopian tubes, and part of the vagina. Several findings

suggested that the *MIS* must be responsible for gonadal sex differentiation in mammals. The *Sry* protein binds slightly to the promoter region of the *MIS* gene, and this led to the proposal that the *MIS* is the target down stream gene of the *Sry* (Haqq *et al.*, 1993). Additionally, possible regulation against to the expression of the *MIS* due to the action of the *SF1*, was proposed however, further verifications are required.

The *Sox* (*Sry* related HMG box) genes were isolated originally by screening a genomic library from mouse with *Sry*-derived probes containing HMG-box with its flanking regions. These *Soxs* are defined as genes that have more than 60% sequence similarity with the HMG box harbored in the *Sry*. Several *SOX* genes (*SOX1-13*) in humans and more than 20 *Sox* genes in mice have been detected however, these are functionally unknown.

The human *SOX9* located at the long arm of the autosomal 17 Chr, was described to be involved in both bone formation and control of testicular development (Foster *et al.*, 1994). In analyzing the mutant DNA from a patient displaying both sex reversed XY female and campomelic dysplasia with abnormal bone formation, the mutations within the *SOX9* was found. Whether the gene product of the *SOX9* interacts directly with the *Sry* or not, has been questioning. The mouse *Sox9** located at the distal long arm of chromosome 11 is suggested to be involved in the chondrogenesis however, its association with gonadal sex determination is unknown (Wright *et al.*, 1995). Remained *SOX/Sox* genes are supposed to be less responsibility for primary sex determination except mouse *Sox5*. Namely, the *Sox5* and/or *ZFY/Zfy* may be implicated with spermatogenesis, despite no verification (Denny *et al.*, 1992).

The inactivation of X Chr has been considering to be regulationally associated with primary sex determination. This was led from the fact that the duplication of Xp21-22 on the short arm cause the sex reversed XY female, thereby the presence of dosage sensitive gene (*DSS*) within 160 kb nucleotide size correlating with gonadal sex determination was predicted. Then, a unique candidate gene for *DSS* was proposed to call as *DAX-1* (*DSS-AHC* critical region on the X-Chromosomal gene-1, where the *AHC* refers to X-linked adrenal hypoplasia congenita), having recently been cloned from X-chromosome (Zanaria *et al.*, 1994). The *DAX1* encodes a protein being composed of 470 amino acids, of which C-terminal sequence of 250 residues conserves 50% homology with that of the ligand of nuclear steroid hormone receptor super family. The mutations within the *DAX-1* was suggested to cause an Adrenal hypoplasia (Congenita), leading to abnormal gonadal sex. Accordingly, the *DSS* is supposed to be indispensable gene for inducing femaleness with the development of ovaries rather than maleness with testes. Hence, even if the *SRY* is absent in an embryo while, a critical mutations harbored in *DSS* are predicted to cause a sex reversed XX male. So that, if a sex reversion caused by only a duplication of *DAX1* is found, this gene must be true *DSS*. Thus, the *DAX1* seems likely to be the ovary determining gene *Od*, having

been previously hypothesized by Eicher and Washburn (1983). Taken all together, in early development of gonadal sex within XY embryo, the *SRY* or its product initially may inactivate the *DAX1* for preventing the development of ovaries, leading to induce normal testicular development and hence, the male sex: thus, the *SRY* may be repressor gene. Differently, in an XX embryo, initial expression of the *DAX1* due to lack of the *Sry* allow the induction of ovarian formation, leading to develop a normal femaleness.

Conclusively, in addition to the *Sry* gene, several other autosomal genes in upstream and downstream as an organized net work, seem to be possibly associated with normal testicular development. Further, the *Sry* must play a key role in the network of these gene expressions. Moreover, X-linked gene must also be associated with normal development of gonadal sex in mammals. In contrast to mammalian system, the molecular mechanism of sex-determination in flies, *Drosophila*, has been well clarified. In XX embryos with X/A=1, the transcriptional switch of the *Sxl* on the X Chr is "on," leading to female-specific alternative splicing of the transcripts of the downstream gene, *transformer* (*tra*), *transformer-2* (*tra-2*) and *doublesex* (*dsx*), resulting in females. Whereas the *Sxl* is inactive in XY embryos with X/A=0.5, thereby no transcription of the *tra*, resulting in the male development (Inoue *et al.*, 1990; Hoshijima *et al.*, 1991; Parkhurst and Meneely, 1994). In the mammalian sex determination, a striking difference from flies is the presence of *Sry* on the Y-Chr and its molecular functions despite insufficient identification. The dosage compensation being regulated by *Sxl* in flies is not obvious in mammals. However, the X/A ratio seems unlikely critical factor in mammalian sex determination such as XXY and XXXY are phenotypically male, but the presence or absence of the *Sry* on the Y Chr in an embryo is an essential molecular determinant. The role of the *DAX1* seems also to be uniqueness in mammalian sex development. Thus, the direct target genes either in upstream and downstream for both *Sry* and *DAX1* should be elucidated, thereby the functional interrelationships among these genes must be further clarified, yet are still puzzling. For better understanding the molecular mechanism governing the testicular as well as ovarian developments in mammals, more investigations are required.

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REFERENCES

Biddle FG, Nishioka Y (1988): Assays of testis development in the mouse distinguish three classes of *domesticus*-type Y chromosome. Genome **30**: 870–878

Biddle FG, Eales A, Nishioka Y (1991): A DNA polymorphism from five inbred strains of the mouse identifies a functional class of *domesticus*-type Y chromosome that produces the same

phenotypic distribution of gonadal hermaphrodites. Genome **34**: 96–100

Bishop CE, Boursot P, Baron B, Bonhomme F, Hatat D (1985): Most classical *Mus musculus domesticus* laboratory mouse strains carry a *Mus musculus musculus* Y-chromosome. Nature **315**: 70–72

Bogan JS, Page DC (1994): Ovary? Testis?—Mammalian dilemma Cell **76**: 603–607

Brook JD, McCurrach ME, Harley HG, Bckler AJ, Church D, Aburatani H, Hunter K, Stanton VP, Thirion J-P, Hudson T, Sohn R, Zemelman B, Snell RG, Rundle SA, Crow S, Davies J, Shelbourne P, Buxton J, Jones C, Juvonen V, Johnson K, Harper PS, Shaw DJ, Housman DE (1992): Molecular basis of myotonic dystrophy: Expansion of trinucleotide (CTG) repeat at 3′ end of a transcript encoding a protein kinase family member. Cell **68**: 799–808

Coward P, Nagai K, Chen D, Thomas HD, Nagamine CM, Lau Y-FC (1994): Polymorphism of CAG trinucleotide repeat within *Sry* correlates with B6.Y^{Dom} sex reversal. Nature Genet **6**: 245–250

da Silva SM, Hacker A, Harley V, Goodfellow P, Swain A, Lovell-Badge R (1996): *Sox9* expression during gonadal development implies a conserved role for the gene in testies differentiation in mammals and birds. Nature Genet **14**: 62–68

Denny P, Swift S, Connor F, Ashworth A (1992): An *SRY*-related gene expressed during spermatogenesis in the mouse encodes a sequence-specific DNA-binding protein. EMBO J **11**: 3705–3712

Dubin RA, Oster H (1994): *Sry* is a transcriptional activator. Molec Endocrinol **8**: 1182–1192

Eicher EM, Washburn LL, Whitney JB III, Morrow KE (1982): *Mus poshiavinus* Y-chromosome in C57BL/6J murine genome causes sex reversal. Science **217**: 535–537

Eicher EM, Washburn LL (1983): Inherited sex reversal in mice: Identification of new primary sex-determining gene. J Exp Zool **228**: 297–304

Eicher EM, Washburn LL (1986): Genetic control of primary sex determination in mice. Ann Rev Genet **20**: 327–360

Ferrari S, Harley VR, Pontiggia A, Goodfellow PN, Lovell-Badge R, Bianchi ME (1992): *SRY*, like HMG1, recognizes sharp angles in DNA. EMBO J **11**: 4497–4506

Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, Brook JD, Schafer AJ (1994): Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. Nature **372**: 525–530

Goodfellow PN (1993): *SRY* and sex determination in mammals. Annu Rev Genet **27**: 71–92

Graves PE, Erickson RP (1992): An amino acid change in the DNA-binding region of *Sry*, found in *Mus musculus domesticus* and other species, does not explain C57BL/6J-Y^{DOM} sex-reversal. Biochem Biophys Res Commun **185**: 310–316

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow PN, Lovell-Badge R (1990): A gene mapping to the sex determining region of the Y chromosome is a member of a novel family of embryonically expressed genes. Nature **346**: 245–250

Gubbay J, Vivian N, Economou A, Jackson D, Goodfellow P, Lovell-Badge R (1992): Inverted repeat structure of the *Sry* locus in mice. Proc Natl Acad Sci USA **89**: 7953–7957

Haqq CM, King CM, Donahoe PK, Weiss MA (1993): *SRY* recognizes conserved DNA sites in sex specific promoters. Proc Natl Acad Sci USA **90**: 1097–1101

Harley VR, Jackson DI, Hextall PJ, Hawkins JR, Berkobitz GD, Sockanathan S, Lovell-Badge R, Goodfellow PN (1992): DNA binding activity of recombinant *SRY* from normal males and XY females. Science **255**: 453–456

Hawkins JR, Taylor A, Berta P, Levilliers J, Van der Auwera B, Goodfellow PN (1992): Mutational analysis of *SRY*: nonsense and missense mutations in XY sex reversal. Hum Genet **88**: 471–474

Hawkins JR (1995): Genetic of XY sex reversal. J Endocrinol **147**: 183–187

Hoshijima K, Inoue K, Higuchi I, Sakamoto H, Shimura Y (1991): Control of *doublesex* alternative splicing by *transformer* and *transformer-2* in *Drosophila*. Science **252**: 833–836

Inoue K, Hoshijima K, Sakamoto H, Shimura Y (1990): Binding of the *Drosophila Sex-lethal* gene product to the alternative splice site of transformer primary transcript. Nature **344**: 461–463

Jacobs PA, Strong JA (1959): A case of human intersexuality having a possible XXY sex-determining mechanism. Nature **183**: 302–303

Jacobs PA, Ross A (1966): Structural abnormalities of the Y chromosomes in man. Nature **210**: 352–354

Jantzen H-M, Admon A, Bell SP, Tijian R (1990): Nucleolar transcription factor *hUBF* contains a DNA-binding motif with homology to HMG proteins. Nature **344**: 830–836

Jeske YWA, Bowles J, Greenfield A, Koopman P (1995): Expression of a linear *Sry* transcript in the mouse genital ridge. Nature Genet **10**: 480–482

Koopman P, Gubbay J, Collignon J, Lovell-Badge R (1989): *Zfy* gene expression patterns are not compatible with a primary role in mouse sex determination. Nature **342**: 940–942

Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R (1991): Male development of chromosomally female mice transgenic for *Sry*. Nature **351**: 117–121

Kreidberg JA, Saliola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R (1993): *WT1* is required for early kidney development. Cell **74**: 679–691

Kunieda Y, Toyoda Y (1992): Nucleotide sequence of mouse *Sry* gene is different between Y chromosomes originating from *Mus musculus musculus* and *Mus musculus domesticus*. Genomics **13**: 236–237

La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH (1991): Androgen receptor gene mutations in X linked spinal and bulbar muscular atrophy. Nature **352**: 77–79

Luo X, Ikeda Y, Parker KL (1994): A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell **77**: 481–490

Mardon G, Page DC (1989): The sex-determining region of the mouse Y-chromosome encodes a protein with a highly acidic domain and 13 zinc fingers. Cell **56**: 765–770

McLauren A, Simpson E, Tomonari K, Chandler P, Hogg H (1984): Male sexual differentiation in mice lacking H-Y antigen. Nature **312**: 552–555

McLauren A (1990): What makes a man a man? Nature **346**: 216–217

Mitchell PJ, Tijian R (1989): Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**: 371–378

Müller J, Schwartz M, Skakkebaek NS (1992): Analysis of sex-determining region of the Y chromosome (*SRY*) in sex reversed patients: Point-mutation in *SRY* causing sex-reversion in a 46,XY female. J Clin Endocrinol Metab **75**: 331–333

Nagamine CM, Nishioka Y, Moriwaki K, Boursot P, Bonhomme F, Lau Y-FC (1992): The *musculus*-type Y chromosome of laboratory mouse is of Asian origin. Mammalian Genome **3**: 84–91

Page DC, Mosher R, Shimpson EM, Fisher EMC, Mardon G, Pollack J, MacGillivary B, de la Chapelle A, Brown LG (1987): The sex-determining region of the human Y-chromosome encodes a finger protein. Cell **51**: 1091–1104

Palmer MS, Sinclair AH, Berta P, Ellis NA, Goodfellow PN, Abbas NE, Fellous M (1989): Genetic evidence that *ZFY* is not the testis-determining factor. Nature **342**: 937–939

Parkhurst SM, Meneely PM (1994): Sex determination and dosage compensation: Lessons from flies and worm. Science **264**: 924–932

Polletier J, Bruening W, Li FP, Hober DA, Glasser T, Housman DH (1991): *WT1* mutation contribute to abnormal genital system development and heriditary Willms' tumour. Nature **353**: 431–434

Sinclair AH, Berta P, Palmer MS, Howkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf A-M, Lovell-Badge R, Goodfellow PN (1990): A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature **346**: 240–244

Su H, Lau Y-FC (1993): Identification of the transcription unit, structural organization, and promoter sequence of the human sex-determining region Y (*SRY*) gene, using a reverse

genetic approach. Am J Hum Genet **52**: 24–38

Tajima T, Nakae J, Shinohara N, Fujieda K (1994): A novel mutation localized in the 3′ non-HMG box region of the *SRY* gene in 46,XY gonadal dysgenesis. Human Molec Genet **3**: 1187–1189

The Huntington's Disease Collaborative Research Group (1993): A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell **72**: 971–983

Tucker PK, Lundrigan BL (1993): Rapid evolution of the sex determining locus in old world mice and rats. Nature **364**: 715–717

Wachtel SS, Ohno S, Koo GC, Boyse EA (1975): A possible role for H-Y antigen in the primary determination of sex. Nature **257**: 235–236

Washburn LL, Eicher EM (1983): Sex reversal in XY mice caused by dominant mutation on chromosome 17. Nature **303**: 338–340

Washburn LL, Eicher EM (1989): Normal testis determination in the mouse depends on genetic interaction of locus on chromosome 17 and the Y chromosome. Genetics **123**: 173–179

Welshons WJ, Russell LB (1959): The Y-chromosome as the bearer of male determining factors in the mouse. Proc Natl Acad Sci USA **45**: 560–566

Werner MH, Huth JR, Gronenborn AM, Clore M (1995): Molecular basis of human 46X,Y sex reversal revealed from the three-dimensional solution structure of the human *SRY*-DNA complex. Cell **81**: 705–714

Whitfield LS, Lovell-Badge R, Goodfellow PN (1993): Rapid sequence evolution of the mammalian sex-determining gene *SRY*. Nature **364**: 713–715

Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, Gangadharan U, Greenfield A, Koopman P (1995): The *Sry*-related gene *Sox9* is expressed during chondrogenesis in mouse embryos. Nature Genet **9**: 15–20

Zanaria E, Muscatelli F, Bardoni B, Strom TM, Guioli S, Guo W, Lalli E, Walker AP, McCabe ERB, Meitinger T, Monaco AP, Sassone-Corsi P, Camerino G (1994): An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature **372**: 635–641

*Recently (da Silva *et al.*, Sept. 1996), *Sox9* was suggested to play an essential role in sex determination, possibly immediately downstream of *Sry* in mammals, and that it functions as a critical Sertoli cell differentiation factor.
Further, *SOX9* rather than *SRY* was proposed to possibly regulate downstream *Amh* gene.