Sexy splicing: regulatory interplays governing sex determination from *Drosophila* to mammals

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Summary

A remarkable array of strategies is used to produce sexual differentiation in different species. Complex gene hierarchies govern sex determination pathways, as exemplified by the classic *D. melanogaster* paradigm, where an interplay of transcriptional, splicing and translational mechanisms operate. Molecular studies support the hypothesis that genetic sex determination pathways evolved in reverse order, from downstream to upstream genes, in the cascade. The recent identification of a role for the key regulatory factors SRY and WT1(+KTS) in pre-mRNA splicing indicates that important steps in the mammalian sex determination process are likely to operate at the post-transcriptional level.

Key words: Sex determination, Splicing factors, Developmental pathways, Sox factor

Introduction

Sex is always a risk because of the danger of altering genomes that are well adapted to the existing environment. Nonetheless, sexual reproduction has emerged as the rule in the vast majority of species. To this end, repartition of individuals into two morphologically distinct genders is accomplished by a developmental process termed sex determination. During this process a germ-cell-containing, undifferentiated gonad is converted into either a male or a female specialized reproductive organ. Concomitantly, all necessary additional structures permitting copulation are formed. Here, we discuss the molecular mechanisms acting in the sex determination process, emphasising the role of pre-mRNA splicing regulators.

A cornucopia of sexual strategies

A surprising variety of mechanisms is used in a number of organisms to produce differentiation into two sexes (Zarkower, 2001). Sex can be genetically determined or controlled by environmental conditions. Different sex determination mechanisms are even used within the same species, and in some cases individuals can belong sequentially first to one sex and then to the other. In addition, in several species animals are hermaphrodites, bearing both a male and a female gonad. Why has such a variety of strategies evolved that elicit the same result, production of two types of sexually distinct individuals? Marin and Baker have argued that this diversity is not specific to sex determination but only easier to detect compared with other developmental processes (Marin and Baker, 1998). Nevertheless, we are beginning to appreciate how related genes may play conserved roles in the most downstream steps of the sex determination pathway in different species.

Alternative splicing, sex and death in *Drosophila melanogaster*

One of the organisms in which the sex determination pathway has been elucidated in greatest detail is *Drosophila melanogaster* (Schütt and Nöthiger, 2000). Although the molecular mechanisms governing sex determination in this species do not constitute a universal paradigm, they represent a revealing example of the complexity of the sex determination cascade.

In *D. melanogaster* a ratio of X chromosomes to autosomes equal to 1 produces females; a ratio of 0.5 produces males. X-linked genes are dosage compensated in males by transcriptional upregulation. The X-to-autosome ratio signal impinges upon the expression of the *sex-lethal* (*Sxl*) gene, which encodes an RNA-binding protein endowed with two RNP-type RNA-binding domains that have affinity to uridine-rich sequences. In females a high level of SXL prevents assembly of spliceosomes on the male-specific exon 3 within its own transcript. SXL also represses translation of MSL-2, a component of the dosage compensation complex, by binding to untranslated regions of its mRNA. In the absence of *Sxl*, overexpression of X-linked genes in female flies is lethal. SXL facilitates the use of a downstream alternative 3′ splice site in the *transformer* (*tra*) pre-mRNA, binding to a sequence next to a stronger upstream 3′ splice site and blocking access of U2AF. Alternative splicing allows the production of a full-length TRA protein in females but not in males. TRA, which contains an RS domain, heterodimerizes with the product of the *transformer2* (*tra2*) gene, which encodes an RNA-binding protein endowed with one RNP-type RNA-binding domain and two RS domains. TRA/TRA2 heterodimers bind to a splicing enhancer situated within the female-specific exon 4 of the *doublesex* (*dsx*) gene and stimulate the use of the 3′ splice site of this exon, through formation of a complex containing SR

proteins and RS-domain-mediated interactions with the U2AF small subunit. This triggers expression of a short isoform of the *dsx* gene in females (DSX^F), whereas males express a longer isoform (DSX^M). Both isoforms are transcription factors, binding sequences present in the promoters of genes involved in sexual differentiation through an N-terminal DNA-binding domain, the DM domain (Raymond et al., 1998). Specificity in the effects of DSX^F compared with DSX^M is obtained through the sex-specific protein C-terminus. Sexual differentiation in the nervous system is also under the control of the TRA-TRA2 complex, which activates a female-specific 5′ splice site in the *fruitless* gene and regulates production of sex-specific isoforms (FRU^F and FRU^M). *Sxl* is also required for oogenesis in the germline, whereas *tra2* plays a role in spermatogenesis.

Patterns of mammalian sex differentiation

Compared with the neatly defined *D. melanogaster* paradigm, our understanding of the molecular mechanisms involved in mammalian sex determination is still at an early stage. However, cloning of genes responsible for human sex reversal syndromes and studies of mouse mutants have helped to identify at least some of the key players in the mammalian sex determination cascade (reviewed in Swain and Lovell-Badge, 1999).

In mammals, an undifferentiated gonad forms from the intermediate mesoderm. During this bipotential stage, two ductal structures are present: the Wolffian duct, which will differentiate into the epidydimis and vas deferens in the male; and the Müllerian duct, which is the progenitor of the oviducts, uterus and upper vagina in the female. A few genes are known to play a master role in gonadogenesis, namely Wilms' tumor (*Wt1*), the orphan nuclear receptor *Sf1*, the LIM homeobox *Lhx1* and *Lhx9*, the homeobox *Emx2* and the Polycomb group *M33*. Once the bipotential gonad is formed, the critical event in sex determination is the formation of Sertoli cells rather than ovarian follicle cells. Sertoli cells release the Müllerian-inhibiting substance (MIS), which triggers the regression of Müllerian ducts in males. In the absence of MIS production, the default female differentiation program is activated. This critical stage is regulated by the testis-determining factor SRY, whose gene is present on the Y chromosome. SRY is the founder member of the SOX family, whose members share similarity in their HMG domains (see below). Another SOX factor, SOX9, is also required for testis differentiation. In the absence of SRY, ovaries are developed. Another important determinant of testis differentiation is the FGF9 growth factor, which appears to function downstream of SRY to stimulate mesenchymal proliferation, cell migration and Sertoli cell differentiation. Finally, once gonadal sex has been determined, gonad differentiation ensues. In males, genes involved in testis differentiation include *Wt1*, *Sf1*, *Gata4*, *Dhh*, *Sox9* and *Dmrt1*. Conversely, the *Wnt4* signalling molecule suppresses Leydig cell differentiation in the ovary.

Conservation of downstream effectors

Wilkins has put forward a stimulating hypothesis for evolution of the sex determination pathway of *Caenorhabditis elegans*, and possibly other genetic sex determination cascades, in which he suggested that the pathway evolved in reverse order from the final steps up to the first in the cascade (Wilkins, 1995). This hypothesis predicts that evolutionary conservation should exist among the most downstream genes in the cascade. The cloning of the male *C. elegans* sexual regulatory gene *mab-3*, which encodes a protein that has a domain similar to the DNA-binding domain of DSX in the fly (for this reason named the DM domain) has provided evidence for Wilkins' hypothesis. Ectopically expressed DSX^M can restore differentiation of *C. elegans* male-specific phenotypic characters in *mab-3* mutant animals (Raymond et al., 1998). Moreover, at least three DM-motif-containing proteins are encoded by genes on human distal chromosome 9p, a locus associated with XY sex reversal (Ottolenghi and McElreavey, 2000). However, male mice lacking the *mab-3* homologue *Dmrt1* are not sex reversed, but have hypoplastic testes with disorganized seminiferous tubules (Raymond et al., 2000). It is possible that, in mammals in which the DM domain family has diverged to yield several members, multiple genes have acquired the function of *dsx* in *D. melanogaster* and *mab-3* in *C. elegans*. This issue will probably be clarified when mice lacking multiple DM domain genes become available.

How does SRY work?

An important step in our understanding of mammalian sex determination was the identification of *SRY* (Sinclair et al., 1990). The protein encoded by *SRY* contains an HMG domain, a DNA-binding domain present in some chromatin-associated proteins of the high mobility group family and some transcription factors. The HMG domain is the only domain common to Sry proteins from different species. In addition, a large family of genes (Sox genes) that encode proteins similar to SRY only in their HMG domain (Sry box) has been cloned. On the basis of these similarities, SRY, and the related Sox proteins, are considered to work by binding to specific DNA sequences and modulating transcription of target genes (reviewed in Wegner, 1999; Kamachi et al., 2000). In vitro binding site selection has identified the A/TAACAAT/A sequence as the preferred SRY-recognition site on linear DNA (Harley et al., 1994). However, SRY is a fairly atypical transcription factor. It has a relatively high binding affinity for nonspecific DNA sequences, and mutations in its binding site are often well tolerated (Ferrari et al., 1992; Harley et al., 1992). Similarly to other HMG domain factors, SRY bends DNA by partial intercalation in the minor groove and can also bind to four-way junctions and to cisplatin-modified DNA (Ferrari et al., 1992; Pontiggia et al., 1994; Trimmer et al., 1998). In addition, mouse Sry contains a C-terminal domain endowed with a glutamine-rich region that acts as a transcriptional activation domain. This domain is required for male sex determination in transgenic mice (Bowles et al., 1999) but is absent from human SRY and even from some mouse species.

SRY is thought to work as an architectural factor modulating local chromatin structure in the vicinity of target genes to favour the assembly of the transcriptional machinery (Wegner, 1999; Kamachi et al., 2000). However, target genes for SRY remain unknown. One idea is that SRY directly activates the MIS gene promoter (Haqq et al., 1994), but the time lag between *SRY* and *MIS* expression in the male gonad and the

absence of transactivation properties in human SRY suggest that other genes directly regulated by SRY mediate its effects. One of these is probably SOX9. Conversely, the existence of phenotypically male XX individuals lacking SRY has led to the hypothesis that SRY works as a negative regulator of a gene (*Z*) that in turn negatively regulates the male sex determination pathway (McElreavey et al., 1993). According to this hypothesis, XX males lacking SRY harbor mutations that inactivate the *Z* gene. Recessive mutations causing female-to-male sex reversal are known in a variety of species (Vaiman and Pailhoux, 2000). In the goat, the polled intersex syndrome (PIS) associates polledness and female-to-male sex reversal. A chromosomal deletion responsible for PIS in goats alters the expression of two transcripts: PISRT1, a non-coding RNA; and FOXL2, a putative forkhead transcription factor (Pailhoux et al., 2001). These two are potential candidates for Z, together with WNT4, which represses Leydig cell differentiation in the ovary (Vainio et al., 1999) and the products of genes present on human distal chromosome 9p (Ottolenghi and McElreavey, 2000).

Recent studies showing that the HMG domains of two different Sox proteins, Sox3 and Sox9, can substitute for the Sry HMG domain and trigger male sex determination in transgenic mice further complicate matters (Bergstrom et al., 2000). Since multiple Sox genes, including *Sox3*, are expressed in the developing XX and XY gonads during the period critical for sex determination (Collignon et al., 1996), it is not clear what makes SRY act in such a specific way to trigger the male differentiation pathway.

Splicing regulators in mammalian sex determination

Despite the lack of direct evidence that human SRY functions as a transcription factor, transcriptional regulation is commonly evoked when people speculate about its mechanism of action (reviewed in Clarkson and Harley, 2002). Recent studies, however, have shown that the SRY and other SOX factors play a role in pre-mRNA splicing in mammalian cells. In fact, nuclear extracts depleted of SOX6 have an impaired splicing activity in vitro. Splicing activity of SOX6-depleted nuclear extracts can be restored not only by addition of the recombinant HMG domain of SOX6 but also by SRY and the SOX9 HMG domain (Ohe et al., 2002). SRY and SOX6 are localized in splicing factor compartments in the nucleus (Misteli, 2000). Furthermore, blockage of splicing in living cells causes redistribution of SRY and SOX6 into enlarged nuclear speckles (Ohe et al., 2002). At this stage, the most likely mechanism for SRY and other SOX proteins function in pre-mRNA splicing is through interaction with RNA. Veretnik and Gribskov have identified similarity between the SRY HMG domain and the RNA-binding domain of the hepatitis delta small antigen (Veretnik and Gribskov, 1999). Indeed, we have confirmed that SRY and SOX6 can bind RNA (E.L., K.O. and P.S.-C., unpublished). By binding to RNA in spliceosomes, SRY and SOX proteins might induce its bending and thereby modulate protein-RNA and RNA-RNA interactions that allow spliceosome rearrangements during splicing (Steitz, 1992; Staley and Guthrie, 1998). Another possibility is that SRY and SOX proteins regulate alternative splicing of specific pre-mRNAs by binding to sequences adjacent to splice sites and thereby inhibiting or stimulating their use in a manner similar to the splicing regulators SXL and TRA in the fly (Fig. 1).

Another gene that plays a crucial role in mammalian sex determination and encodes splicing-regulating proteins is *Wt1*. The WT1 isoforms that include a lysine-threonine-serine (KTS) sequence at the end of exon 9 localize to nuclear speckles, interact with the U2AF65 splicing factor and associate with spliceosomes (Larsson et al., 1995; Davies et al., 1998). WT1 also interacts with WTAP, the mammalian homologue of *D. melanogaster* female-lethal(2)d, which is required for the female-specific splicing of the *sxl* and *tra* pre-mRNAs (Little et al., 2000). Interestingly, WTAP has been isolated as a component of active human splice complexes (Zhou et al., 2002).

Dominant donor splice site mutations impairing WT1(+KTS) expression produce Frasier syndrome, which is characterized by progressive glomerulopathy and male-to-female sex reversal (Barbaux et al., 1997). Hammes et al. have

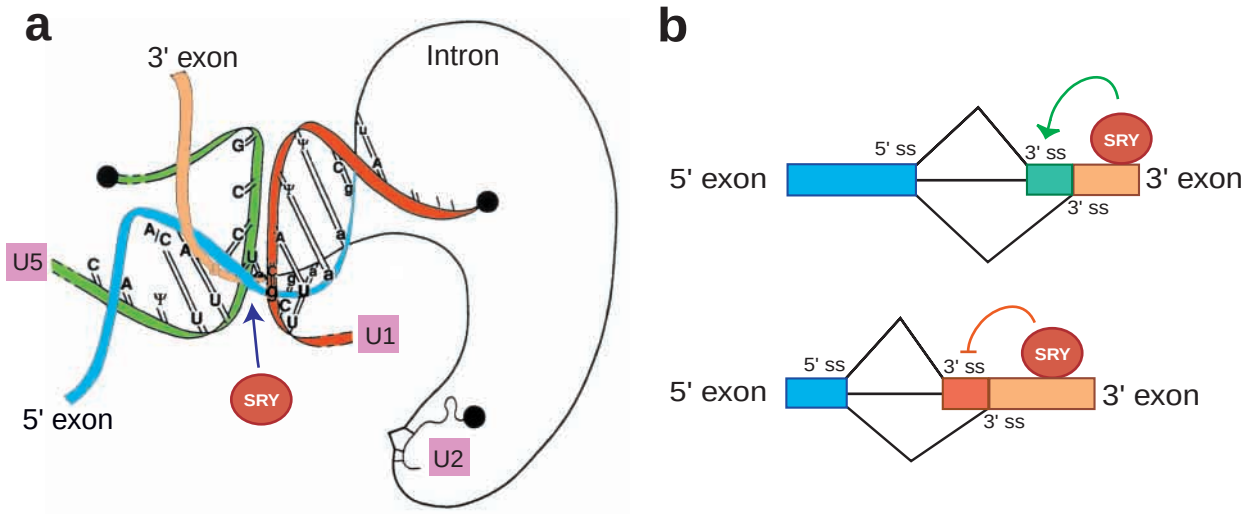

Fig. 1. Mechanisms for SRY and SOX protein function in pre-mRNA splicing. (a) Possible interaction with structures mimicking a four-way junction that favors spliceosome rearrangements during splicing (redrawn from Steitz, 1992). (b) Activation (top) or repression (bottom) of alternative splice site usage through binding to specific RNA sequences.

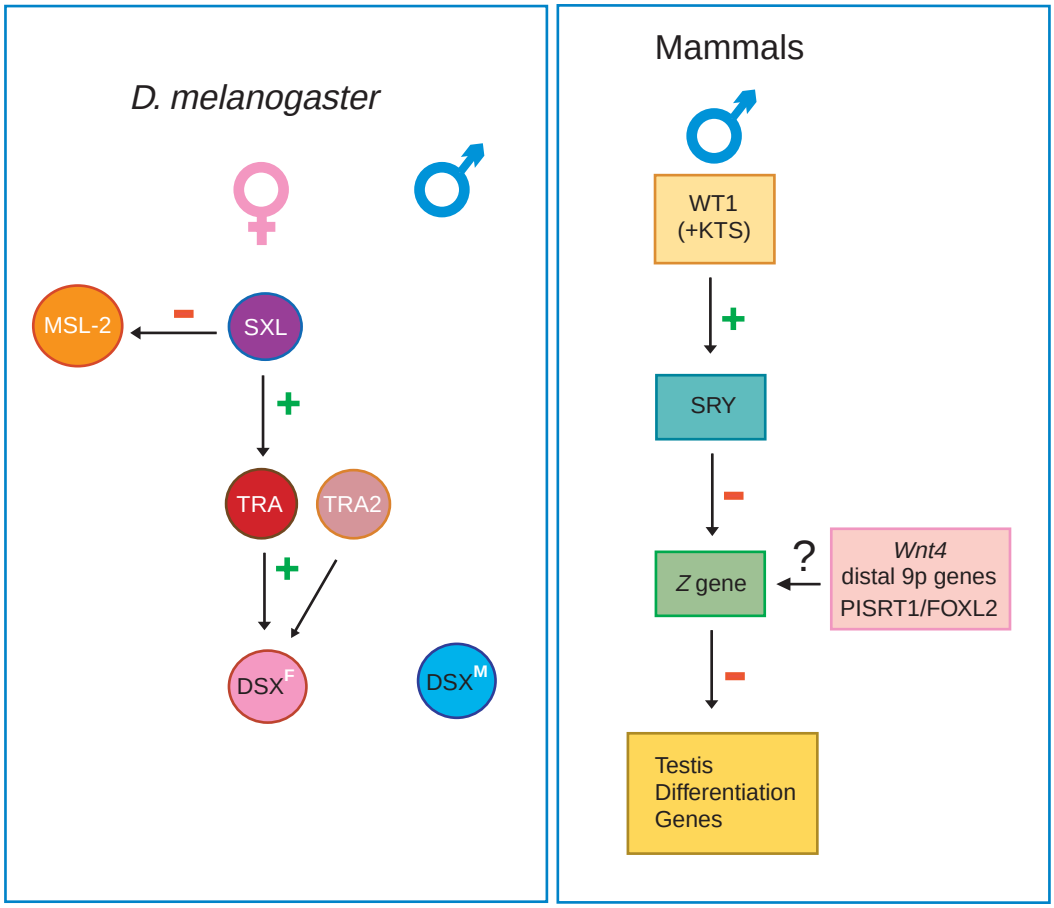

Fig. 2. Regulatory cascades in *D. melanogaster* and mammalian sex determination involve factors acting upon pre-mRNA splicing. In the fly (left) SXL positively regulates TRA expression and downregulates MSL-2 in female flies. TRA-TRA2 heterodimers trigger expression of the female-specific DSX^F isoform. In the absence of SXL, males express DSX^M. In mammals (right), WT1(+KTS) isoforms upregulate Sry in the developing male gonad. Sry, in turn, negatively influences the expression – or the function – of an unknown Z gene, which inhibits testis differentiation genes. Z gene candidates are indicated (pink box).

recently described a mouse model for Frasier syndrome (Hammes et al., 2001). Homozygous XY Frasier mice show, in addition to glomerulosclerosis, significantly reduced *Sry* expression and complete sex reversal. Conversely, XY mice in which the expression of WT1(–KTS) isoforms has been selectively ablated still express male-specific genes in severely hypoplasic gonads. These findings strongly argue for a specific role of WT1 (+KTS) isoforms in the sex determination process.

On the basis of these findings, we suggest that WT1 (+KTS) isoforms and SRY regulate the expression of critical testis differentiation genes at the post-transcriptional level, with WT1 (+KTS) lying upstream in the cascade and also controlling genes required for kidney development. SRY, in turn, would repress the expression of one or more genes acting negatively upon male sex determination. The pathways of sex determination in *D. melanogaster* and mammals, showing the positive and negative effects of gene products upon subsequent steps in the cascade, are shown in parallel in Fig. 2.

Conclusions

Regulation at the transcriptional level is believed to be the essential mechanism governing the mammalian sex determination cascade (Swain and Lovell-Badge, 1999; Clarkson and Harley, 2002). We propose that an important new wrinkle needs to be added to this view: regulation at the level of splicing. Indeed, in addition to notable evolutionary conservation of some genes involved in sex determination, it appears that pre-mRNA splicing regulatory mechanisms constitute a common theme among distant phyla. Further studies are now required to identify genes that play critical roles in the sex determination process and are subject to regulation at the post-transcriptional level.

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