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Sex determining gene expression during embryogenesis

ROBIN LOVELL-BADGE

Laboratory of Eukaryotic Molecular Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

SUMMARY

The Y-linked gene *Sry* acts during a critical period of gonadal differentiation to divert the normal or default pathway of gene activity that would otherwise lead to the development of ovaries into one that leads to the development of testes. It acts cell autonomously, probably within the cell lineage that gives rise to Sertoli cells in the testis or follicle cells in the ovary. The remaining cell types within the gonad, each of which has a developmental choice, then become fated to follow the testicular pathway. This process must depend on cell–cell interactions as *Sry* is not required within these other cell types for their differentiation. Subsequent male development of the animal as a whole is dependent on the production of testosterone and other factors by the testis.

Sry encodes a DNA binding protein of the HMG box class, and presumably acts to regulate the expression of other genes which then confer cellular phenotype. However, rather than operating like other classes of transcription factor, it has been shown to induce a dramatic bend in its DNA binding sites, and may not directly affect transcription of target genes. Instead, it may permit other factors to interact, which in turn either activate or repress transcription. Sequence comparisons between *Sry* genes from various species suggest that the HMG box is the only functional part of the protein. This part is responsible for DNA binding, and both mouse and human SRY bind the same consensus sequence at high affinity *in vitro*. However, the human gene fails to cause female to male sex reversal in transgenic mice. Possible reasons for this are discussed. There is also much evidence, including transgenic data, to suggest that the level of expression of *Sry* is critical to its function. On top of this, the gene can only successfully induce testis differentiation if the correct cell–cell interactions occur within the developing gonad. Despite knowing the identity of the testis determining gene, we are therefore still a long way from understanding how it achieves its function.

1. INTRODUCTION

For those interested in the mechanisms underlying decisions of cell fate, sex determination provides an obvious subject for study. Most species exhibit an extensive range of dimorphic characteristics that distinguish the two sexes, and the genetic pathways underlying the differences can often be studied with relatively few problems of lethality as mutations tend to lead to transformation of sex. However, it is clear that widely differing mechanisms operate to control sex determination and differentiation among different animal species. This is evident in the type of switch used to initiate either the program of male or of female development. Thus, the switch can be environmental as in many reptiles and fishes, or it may correspond to the number of X chromosomes, as in *Drosophila* and *C. elegans*, or it may be a dominant genetic switch as in mammals where the inheritance of a Y chromosome at fertilization determines maleness (Ford *et al.* 1959; Jacobs & Strong 1959; Welshons & Russell 1959).

There are many other fundamental differences between mammalian sex determination and that of *Drosophila* and *C. elegans*, the two species for which we know the most (Hodgkin 1992). In the latter two species, because sex determination is linked to the X chromosome dosage compensation mechanism, it has to occur early. In *Drosophila*, the pathway appears to act entirely cell autonomously, with many of the steps involving post-transcriptional control of gene activity. In *C. elegans*, it is also initiated in all cells in a cell autonomous manner, although the pathway includes a short range extracellular step, which may serve to ensure that no mistakes are made (Hunter & Wood 1992). In mammals it is not linked to dosage compensation, as the number of X chromosomes is irrelevant to the resulting sex of an individual. Also, even though genetic sex is established at fertilization, the decision as to which pathway to follow operates much later on in development, during the period of organogenesis. This involves the differentiation of the gonadal primordia, the genital ridges, into testes rather than ovaries if the Y-linked testis determining gene is present (see Capel & Lovell-Badge 1993). The male determining signal is then effectively exported, potentially to all cells of the body, through the production of two factors by the testis, anti-Müllerian hormone (AMH, otherwise known as Müllerian inhibiting substance or MIS) and testosterone (Münsterberg & Lovell-Badge 1991; Capel & Lovell-Badge 1993). It is the action of these factors that leads to the dramatic differences between males and females in anatomy, physiology and behaviour. The female pathway can be considered the default pathway, as it will occur in

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the absence of gonads, whether they are removed from XX or XY embryos (Jost 1947; Jost *et al.* 1973).

The critical event in sex determination in mammals, therefore, is the action of the Y-linked testis determining gene. This switches the pathway of gene activity which leads to the differentiation of ovaries into one which leads to the differentiation of testes. The central problem is to understand how this switch is brought about.

There is now substantial evidence, both circumstantial and direct, to prove that *SRY* (Sex determining region, Y gene) is the testis determining gene. *SRY* was found through a positional cloning strategy which involved looking for conserved genic sequences within the smallest region of the human Y chromosome found in XX males (Sinclair *et al.* 1990). The mouse homologue, *Sry*, was also found to map to the minimum portion of the Y chromosome known to be sex determining in this species (Gubbay *et al.* 1990). In addition there appears to be an *SRY* homologue on the Y chromosome of all mammals with a Y chromosomal sex determining mechanism. This includes metatheria (marsupials) as well as eutheria (Sinclair *et al.* 1990; Foster *et al.* 1992). The gene was found to be expressed at the right time in the right tissue for it to be sex determining (see below) (Koopman *et al.* 1990). It was also found to encode a DNA binding protein consistent both with the presumed regulatory role of the testis determining gene and with the prediction that it acts cell autonomously (Gubbay *et al.* 1990; Sinclair *et al.* 1990). Direct evidence that *SRY*/*Sry* is the Y encoded testis determining gene comes from two sources. First, the association of mutations within the DNA binding domain of SRY with male to female sex reversal in humans (Harley *et al.* 1992), and secondly, female to male sex reversal found when a small genomic fragment carrying the mouse *Sry* gene was introduced as a transgene into XX embryos (Koopman *et al.* 1991). This latter experiment showed that *Sry* was not only involved in testis determination, but that it is the only gene from the Y chromosome that is required for this process.

The purpose of this report is to discuss data relevant to how *SRY*/*Sry* might function. It is first necessary to consider how the gonads develop and how *Sry* is expressed.

2. GONADAL DEVELOPMENT AND *SRY* EXPRESSION

The development of the gonads is intimately linked with that of the mesonephros, the primitive kidney, which itself develops from unsegmented, intermediate lateral plate mesoderm (see Capel & Lovell-Badge 1993). The genital ridges arise as a thickening on the ventral–medial surface of each mesonephros, from about 10 dpc in the mouse. This appears to involve the ingression of mesenchymal cells from the coelomic epithelium, or the epithelium of mesonephric or Wolffian ducts. The genital ridges increase in volume but remain indifferent, i.e. morphologically identical between the two sexes, until 11.5 dpc. Between 11.5 and 12.5 dpc the developing testis takes on a characteristic stripey appearance, due to the differentiation and alignment of Sertoli cells into epithelial testis cords. No overt sign of differentiation occurs in the developing ovary until several days later when follicle cells organize around oocytes. However, there may be earlier changes in the state of the cells as there is some evidence to suggest that competence to form a testis exists for only a limited period (Palmer & Burgoyne 1991*c*).

Experiments to detect *Sry* transcripts, mainly using the reverse transcriptase–polymerase chain reaction (RT–PCR) method, but backed up by *in situ* hybridization data, show that the gene is expressed for a limited period between 10.5 and 12.0 dpc (Koopman *et al.* 1990). This expression is in cells of the genital ridge as it develops, with no sign of expression beforehand at 9.5 dpc and only traces sometimes present at 12.5 dpc when testis cord formation is apparent. This expression for a brief period only, suggests that *Sry* initiates testis development and that it is not required for long term maintenance of testis specific gene expression.

Our current working hypothesis assumes that there are four cell lineages that make up the developing mammalian gonad, each of which is effectively bipotential (see, for example, Lovell-Badge 1992). These comprise the germ cells, which migrate into the genital ridge between the tenth and eleventh day of development, and three somatic cell types: (i) the supporting cell precursors, which give rise to Sertoli cells in the male and follicle cells in the female; (ii) steroid-producing cell precursors, which give Leydig cells in the male, theca or interstitial cells in the female; and (iii) connective tissue cells, which include those that give vascular tissue (particularly prominent in testis), tunica and peritubular myoid cells in the testis, and tunica and stromal cells in the ovary.

Testes develop normally in embryos homozygous for mutations at the *W* (or *white spotting*) locus, which compromise the ability of germ cells to proliferate and colonize the genital ridges (Mintz & Russell 1957). The germ cells are therefore irrelevant to testis determination. Expression of *Sry* is unaffected in *W*/*W* mutant embryos, confirming that *Sry* is expressed by one or more of the somatic cell types (Koopman *et al.* 1990). On the other hand, germ cells are influenced by their environment (McLaren 1985). In the fetal testis they enter mitotic arrest, resuming mitosis and entering meiosis after birth. In an ovary, or in ectopic sites, they enter meiosis and arrest in the first meiotic prophase. In both cases, their differentiation is independent of their sex chromosome constitution.

Of the three somatic cell lineages the supporting cells would appear to be the most critical for testis determination. Burgoyne and colleagues (Burgoyne *et al.* 1988; Palmer & Burgoyne 1991*a*) looked at the sex chromosome constitution of cells within the testes of XX↔XY chimaeras, and found that Sertoli cells were almost exclusively XY, whereas the proportion of XX and XY cells in the other gonadal lineages was the same as that found elsewhere in the embryo. This suggested that the testis determining gene acts cell autonomously within the supporting cell precursors. We assume these are the cells that express *Sry*, and

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that this expression is responsible for their differentiation along the Sertoli cell pathway. However, recent experiments by Buehr *et al.* (1992) show that 11.5 dpc male genital ridges, isolated from their mesonephros, fail to organize to give testis cords. This suggests additional factors are required, in addition to *Sry* expression, for Sertoli cells to complete their differentiation. When the genital ridges were explanted together with mesonephros, testis cords developed normally. By using mesonephros isolated from embryos carrying a transgene marker, Buehr and colleagues were then able to demonstrate that a population of cells migrate into the developing testis from the mesonephros after 11.5 dpc (i.e. when *Sry* transcription is declining). These give rise to the peritubular myoid cells. These would appear to be essential for the organization of pre-Sertoli cells into testis cords, and therefore for their proper differentiation. This must be due to either a short range paracrine effect or direct cell–cell contact.

The migration of these mesonephros-derived cells, and their differentiation into peritubular myoid cells, must in turn be due to an interaction with the pre-Sertoli cells. Other signals from the differentiating Sertoli cells must also be responsible for the entry of germ cells into mitotic arrest and the differentiation of Leydig cells, etc.

From the above it would appear that the role of *Sry* is not to irreversibly commit supporting cell precursors to the Sertoli cell pathway. Other data from XX↔XY chimaeras or mosaics would agree with this. When the proportion of XY cells is less than about 25% (depending on strain combination) these embryos develop as females. However, perhaps contrary to expectation, there is no bias in the formation of XY follicle cells. These are found in the same proportions as XY cells in other tissues (Palmer & Burgoyne 1991*b*). It would appear that *Sry*-expressing pre-Sertoli cells are required to induce their own differentiation, perhaps by an autocrine effect, and that they fail to do so when present in too low a number. But, from the experiments of Buehr *et al.* (1992), it is more likely that there is a failure to induce myoid cells and in the absence of the return signal from these cells, the Sertoli cell pathway is blocked and the supporting cells revert to the follicle cell pathway.

Sry is also not essential for Sertoli cell differentiation. There are a number of natural or experimental conditions where XX follicle cells take on the appearance and many of the biochemical properties of Sertoli cells. These cases include Freemartin cattle (Jost *et al.* 1975), grafts of foetal mouse ovaries under the kidney capsule of newborns (Taketo-Hosotani 1987), and female mice expressing high ectopic levels of an AMH transgene (Behringer *et al.* 1990). In all cases, the transition to Sertoli cell phenotype is associated with loss of oocytes from the ovaries. This implies a tendency for supporting cells to make Sertoli cells unless prevented from doing so by an oocyte-derived factor. Any such factor could not play a role in the normal sex determination process, as it would be absent from XX embryos lacking germ cells due to mutations at *W*, and these have only a streak gonad without any sign of Sertoli cell differentiation.

3. THE INTERACTION OF SRY PROTEIN WITH DNA

Our thinking has perhaps been constrained by the notion of master regulatory genes acting to direct cellular differentiation down specific pathways. For example, *MyoD* was thought to operate in this way, giving muscle cell differentiation when introduced by itself into a variety of different cell types (Weintraub *et al.* 1991; Rudnicki *et al.* 1992). However, *Sry* can not possibly act in this way. Although it functions as the normal switch to induce Sertoli cell differentiation, in some circumstances it is clearly not sufficient or even necessary. Instead, the gene appears to influence cell fate rather than direct it. It is worth considering the molecular properties of SRY as this may provide clues to its mode of action.

SRY was one of the first members of what is now a very large and still expanding group of DNA binding proteins united by possession of a domain referred to as an HMG box. The latter was first described as a region of homology between the high mobility group non-histone protein HMG1, and hUBF, a cofactor of RNA polymerase 1 required for ribosomal RNA transcription (Jantzen *et al.* 1990). HMG1 is a highly abundant and ubiquitous chromatin associated protein which binds linear DNA with low affinity and without apparent sequence specificity (Bianchi *et al.* 1992). Despite being associated with its target genes, hUBF has no clear consensus DNA sequence motif to which it binds, and its interaction with DNA is fairly weak (Jantzen *et al.* 1990). There are a number of other DNA binding proteins of this sort which can be loosely grouped together by their property of having little or no sequence specificity, being expressed ubiquitously and tending to have more than one HMG box. SRY falls into a second class, however, the members of which often show highly restricted tissue distribution, bind to specific sequences at high affinities and only have a single HMG box (Ner 1992). The two best characterized members of this class, apart from SRY itself, are TCF1 and LEF1 (or TCF1α) which are expressed in lymphoid cells and are involved in transcriptional regulation of, for example, T-cell specific genes such as CD3ε and TCRα (Travis *et al.* 1991; van de Wetering *et al.* 1991).

All these factors interact with DNA through the HMG box which is approximately 79 amino acids in length. Mutations in the HMG box of SRY have been associated with cases of sex reversal in humans leading to XY females (Harley *et al.* 1992). *In vitro* DNA binding studies have also shown that the boxes alone, from hUBF, LEF-1 and SRY, are sufficient for DNA binding to target sequences (Jantzen *et al.* 1990; Giese *et al.* 1991; Nasrin *et al.* 1991). SRY has been shown to bind the same target sequence as TCF1, AACAAAG, but the sequence AACAAT is more likely to be the highest affinity site for both human and mouse SRY (Denny *et al.* 1992; Giese *et al.* 1992; Harley *et al.* 1992, unpublished results).

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Grosschedl and colleagues (Giese *et al.* 1992), and more recently Bianchi and coworkers (Ferrari *et al.* 1992), have looked at the interaction of HMG box containing proteins with DNA in more detail. LEF-1 and SRY, and probably other members of this class, induce a dramatic bend in target DNA on binding. This bend is in the order of 130°, which is more than any other known transcription factor, although IHF, the *E. coli* Integration Host Factor, bends DNA by 140°. Also, like IHF, SRY and LEF1 contact DNA in the minor groove, which is unusual compared to known transcription factors (Giese *et al.* 1991; van de Wetering *et al.* 1991).

HMG1 binds to four-way DNA junctions and cruciform DNA structures at much higher affinities than it does to linear DNA. These cruciform structures, which may be made artificially by using four synthetic oligomers, also form at inverted repeats and at Holliday junctions during recombination. They have two angles of 60° and two of 120° and HMG1 is thought to bind to the former, which would be equivalent to a bend of 120° in linear DNA (Bianchi *et al.* 1992). Bianchi's recent results also show that SRY will bind the same cruciform structures as HMG1, without sequence specificity and with affinities as high as its binding with AACAAT motifs in linear DNA (Ferrari *et al.* 1992). Probably all HMG boxes have this ability to bind structured DNA, but the binding of linear DNA, and its subsequent bending, is an extra property of the SRY/LEF1 class.

While SRY appears to bind the same structures as HMG1, it seems unlikely that it would do so within the context of the nucleus. HMG1 is such an abundant protein, and can be assumed to occupy all such sites. SRY is therefore likely to bind only its free specific target sequences in linear DNA. This binding will then induce the dramatic bend. Until target genes are identified it is only possible to speculate how this may affect transcription, but a comparison with LEF1 may be pertinent. Co-transformation studies with LEF-1 and a target gene construct shows that it only has weak activation properties by itself (Giese *et al.* 1992). However, the LEF1 binding site is flanked by sites for two other transcription factors. It appears that the bend in DNA induced by LEF1 binding, brings these two sites together, allowing the other factors to interact. This interaction then activates transcription. The distance between the sites for all three factors is critical, as is the degree of bending induced by LEF1. One could equally imagine schemes by which the bending prevented the binding or interaction of other factors, leading to target gene repression.

HMG box containing factors, such as SRY, could therefore facilitate or prevent the action of other specific transcription factors on target gene expression, while having no direct influence by themselves on RNA polymerase activity. This could provide a molecular explanation for why *Sry* is not sufficient to induce Sertoli cell differentiation during normal testis determination, and why it may not be necessary in other circumstances.

4. TRANSGENIC STUDIES RELEVANT TO SRY ACTION

A 14 kb mouse genomic DNA fragment carrying *Sry* was shown to be sufficient to induce testis formation when introduced as a transgene into chromosomally female embryos (Koopman *et al.* 1991). This provided the formal proof that *Sry* is the Y encoded testis determining gene. Moreover, it showed that it was the only gene from the Y chromosome needed for normal male development. The sex reversed mice were sterile, but this is a separate problem, due to the presence of two X chromosomes which are incompatible with spermatogenesis and to the lack of genes involved in this process which are known to map elsewhere on the Y chromosome.

This 14 kb genomic fragment failed to cause sex reversal in all cases. In fact only about 25% of XX transgenic mice develop as males. In some cases this is likely to be due to mosaicism for the presence of the transgene, due to integration after the first cleavage division, such that it is present in less than 25% of supporting cell precursors. Alternatively, it is known that transgenes often show very different levels of expression from one line to another, probably due to the effect of genomic sequences at the site of integration. Since the purpose of our experiments was to assay function and not simply tissue specific expression, the timing or level of expression of the *Sry* transgene may be ineffective in some cases. Evidence for this comes from the study of one particular transgenic line, termed 32.10 (N. Vivian, P. Koopman & R. Lovell-Badge, unpublished data). The founder animal was a fertile female who transmitted the transgene to both normal daughters and sons. This ruled out mosaicism as a possible reason for the failure to sex reverse. However, subsequent breeding of these animals has led to instances of sex reversal, either when they were bred with wild-type (one case) or with other transgenic siblings (five cases). Two intersex offspring have also been obtained from these matings. Preliminary results showed that *Sry* is being expressed in the genital ridge of transgenic XX embryos at 11.5 dpc. Careful measurement of the number of transcripts over a time course of development is required, but the expression of the transgene appeared to be at a lower level than normal. It is likely that *Sry* expression is at a threshold level, and homozygosity for the transgene increases the incidence of sex reversal. However, not all homozygotes are male and some hemizygotes are, suggesting that genetic background effects segregating in these F2(C57BL/10 × CBA) and later generation crosses are affecting the ability of the transgene to induce testis differentiation.

Transgenic mice have also been generated using genomic fragments carrying the human *SRY* gene (Koopman *et al.* 1991; Palmiter, personal communication). However, no evidence of sex reversal has been seen despite apparently normal expression of the transgene. No difference has been seen so far in the interaction of mouse and human SRY protein with DNA *in vitro*, both bind the same target DNA

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sequences, and appear to induce similar degrees of bending. Why then does the human gene not work in mice?

If the simple act of bending the DNA is sufficient to cause an effect on transcription then there is no need to have other domains associated with the HMG box for activation or interaction with other proteins. This is clearly not the case for many of the HMG box containing genes, as they have such putative sequences which are well conserved amongst different species. However, when mouse and human *Sry*/*SRY* sequences are compared there is no homology outside the HMG box. This lack of homology is, in fact, evident when comparing SRY from any two species. On the other hand, although the HMG box clearly represents a conserved domain between SRY proteins from different species, the degree of homology within the box is much less than that found when comparing other HMG box containing proteins. Indeed, if all the known SRY sequences are compared with those of *Sry*-related HMG box containing genes (these so-called *Sox* genes are located on chromosomes other than the Y), no particular amino acid residues are found that are characteristic of the SRY proteins (Foster *et al.* 1992). There are several possibilities to explain the transgenic results: (i) the variation within the HMG box is significant and the HMG boxes of human and mouse SRY interact with target sequences in subtly different ways not detectable *in vitro*; (ii) it is significant, but affects interaction with other proteins and not DNA; or (iii) this variation is not significant, and the failure of the human gene to work in mice is due to the variation in the non-HMG box parts of the protein. Whichever possibility applies it suggests that there is very rapid evolution of protein–DNA or protein–protein interactions that can affect SRY action. Carrying out domain swap experiments between the human and mouse genes in transgenic mice should help to resolve this problem.

5. CONCLUSIONS

Sry clearly acts as the normal switch in mammalian sex determination. However, we do not know how it affects the expression of other genes required for Sertoli cell differentiation. Parallels drawn between SRY and other HMG box containing proteins suggest that it may be a cofactor of transcription that mediates the interaction of other transcription factors. Alternatively, it may alter the accessibility of these other factors to target sites within chromatin. However, we need to identify the target genes. This is difficult, and at present we have no direct information to indicate whether SRY is an activator of genes involved in testis determination, a repressor of genes involved in the ovarian determination pathway, or both.

It is perhaps easier to consider SRY acting as a repressor. The lack of conservation between species suggests that there are few interactions with other proteins, and, simply by bending DNA, SRY could prevent the binding of other transcription factors (but see the discussion above). Some genetic data are also easier to reconcile with this possibility. A significant proportion of human XX males do not have any Y chromosome sequences, let alone *SRY* (Palmer *et al.* 1989). The frequency of these is greater than expected for gain of function mutations, but would be appropriate for loss of function mutations in a downstream gene if SRY is a repressor of this gene. If *Sry* is a repressor, it must act on the female pathway just long enough to allow the male pathway of gene activity to operate. If so, this subtly alters our notion of the former being the default pathway.

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