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The Molecular Genetics of Sry and its Role in Mammalian Sex Determination [and Discussion]

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The molecular genetics of *Sry* and its role in mammalian sex determination

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SUMMARY

The process of sex determination, by which is meant the decision as to whether an embryo develops as a male or a female, is considered as a paradigm of how gene action can influence developmental fate. In mammals the decision is dependent on the action of the testis determining gene present on the Y chromosome, now known to be the gene *Sry*. *Sry* is expressed for only a brief period in the mouse embryo and must act to initiate rather than maintain the pathway of gene activity required for testis differentiation. It probably acts within cells of the supporting cell lineage to direct their differentiation into Sertoli cells, rather than the granulosa cells characteristic of the ovary. Other lineages in the gonad then follow the male pathway. The nature of the *Sry* transcript in the genital ridge of mice has been determined and compared with that from the human gene which is dramatically different. The expression of *Sry* has been carefully examined during the critical stages of genital ridge development and compared to the expression of a number of other genes involved in gonadal development and male development such as that for anti-Mullerian hormone. This has defined the period in which *Sry* must act to between 11 and 11.5 days *post coitum*. The expression of *Sry* has also been examined in cases of sex reversal in the mouse. There is a dependence on level of expression and extent of testicular differentiation that suggests thresholds for both the amount of SRY per cell and the number of cells expressing the gene. The SRY protein interacts with DNA through an HMG box type of DNA binding domain, however at present no definite target genes have been found. Progress on strategies to find such genes is discussed.

1. INTRODUCTION

It is a common theme in developmental biology today for there to be common themes. For example, genes found to be important in a specific process in *Drosophila* are found to be involved in a very similar process in mammals. Positional information along the anterior–posterior body axis appears to be dependent on *Hox* genes, eye development depends on a specific paired box gene (*eyeless/Pax6*), many induction events depend on *hedgehog* and *wingless* related genes (e.g. *Dhh* and *Wnt1*), (Krumlauf 1994; Halder *et al.* 1995; Ingham 1995).

Naively one would think that mechanisms determining sex should be as well conserved: it seems such an important and basic mechanism. However, this assumption has been shown to be false. *Drosophila melanogaster* and *Caenorhabditis elegans* are the two organisms we understand the most with respect to the gene hierarchies determining sex (Hodgkin 1990; Ryner & Swain 1995). The primary signal in both cases is the number of X chromosomes, however, there is very little else in common between the two pathways and the types of genes involved. It is our aim to understand in similar detail the genetic pathways leading to sex determination in mammals. However, the differences between *C. elegans* and *Drosophila* already tell us that we can not take anything for granted and rely on comparisons with other organisms.

To begin with, in mammalian sex determination the Y chromosome acts as a dominant testis determinant. Thus it does not matter how many X chromosomes are present, if there is a normal Y chromosome then the individual will develop as a male, if there is no Y, the individual develops as a female (Ford *et al.* 1959; Jacobs & Strong 1959; Welshons & Russell 1959).

It follows that the Y chromosome carries a gene or genes that determine maleness and that genetic sex is established at fertilization with the inheritance of either an X chromosome or a Y chromosome from the father. However, phenotypic sex is not established until much later, with the first differences between males and females visible in the developing gonads at about six weeks *post coitum* in humans or about 12.5 days *post coitum* (DPC) in mice. The gonads develop from about 9.5–10.0 DPC in mice as a ridge of distinct cells that forms on the medio-lateral surface of the mesonephros, part of the embryonic kidney system (see figure 1) (Everett 1943; Torrey 1945; Witschi 1948). Up until about 11.5 DPC the mesonephros is thought to contribute mostly two somatic cell types to the genital ridge, the supporting cell precursors and the steroidigenic cell precursors. After 11.5 DPC various connective tissue cells also enter the genital ridge (Buehr *et al.* 1993*a*; Capel & Lovell-Badge 1993 and see chapter by Capel, this volume). The primordial germ cells have also colonized the genital ridge by 11 DPC, having migrated from the base of the allantois at 7.5 DPC,

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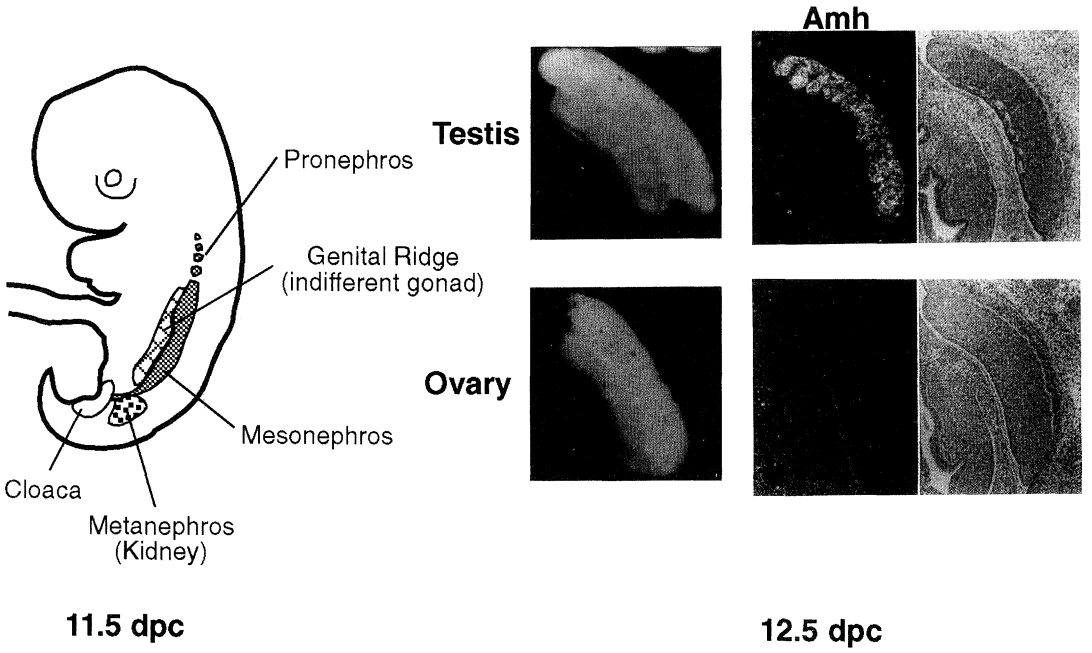

Figure 1. Gonadal development and sex determination. The genital ridge develops in association with the mesonephros and remains morphologically indistinguishable between XX and XY embryos until after 11.5 DPC. By 12.5 DPC, many differences are apparent between testis and ovary in cell type, their organization and in gene expression, as shown by *in situ* hybridization for *Amh* in 12.5 DPC testis (top) and ovary.

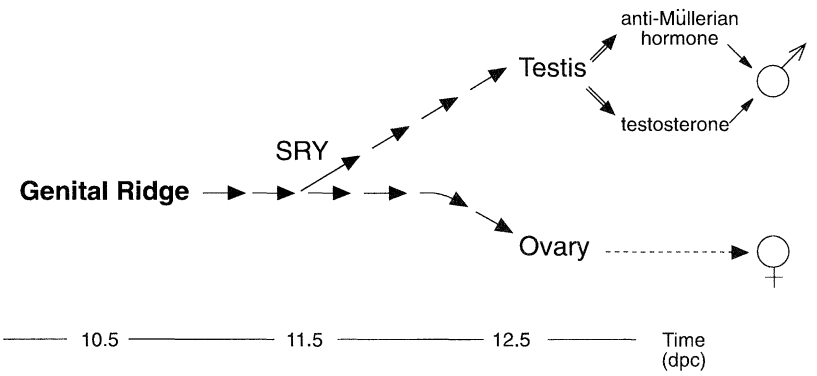

Figure 2. Sex determination viewed as a choice of genetic pathway. *Sry* acts to divert the gonadal development pathway from that of ovary to testis. This results in the production of anti-Mullerian hormone and testosterone which effectively export the male determining signal to the rest of the embryo. SRY could act as an activator of secondary testis determining genes, a repressor of ovarian determining genes or both.

moving through the hind gut, dorsal mesentery and mesonephros (Ginsburg *et al.* 1990, see chapter by A. McLaren, this volume). Up to 11.5 DPC the genital ridges in XX and XY embryos look identical, and are referred to as indifferent gonads. However, over a period of about 24 h, between 11.5 and 12.5 DPC, there are many changes in morphology and gene expression that occur in the male gonad to distinguish it from the female gonad. Most noticeably, the testis takes on a characteristic stripey pattern caused by the differentiation and alignment of Sertoli cells into epithelial testis cords (Satoh 1985; Kanai *et al.* 1989; Merchant-Larios & Taketo 1991). Blood vessels rapidly appear, myoid cells differentiate around the Sertoli cell cords and several gene products characteristic of Sertoli and Leydig cells begin to be expressed at high levels, such as anti-Mullerian hormone and 3βHSD (Münsterberg & Lovell-Badge 1991; Moreno-Mendoza *et al.* 1995; K. Nordqvist & R. Lovell-Badge, unpublished results).

In genetic terms, the process of sex determination can be viewed as a choice of two pathways (see figure 2). The ovarian pathway can be considered as the normal or default pathway (or ground state). This is overidden by the action of the Y chromosome which triggers development of the indifferent gonad along the testicular pathway. The developing testis produces two factors that effectively export the male determining signal to the rest of the embryo (Jost 1953). These are Anti-Mullerian hormone (AMH, otherwise known as Mullerian Inhibiting substance or MIS) and testosterone. AMH is secreted by Sertoli cells and eliminates the anlagen of the female reproductive tract,

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the Mullerian (or paramesonephric) ducts, which would otherwise give oviducts and uterus. Testosterone is secreted by Leydig cells and stimulates development of the male reproductive tract, from the Wolffian (or mesonephric) duct system, and male external genitalia, etc. (see chapter by Behringer, this volume). The developing ovary apparently produces no special factors. The Mullerian ducts develop and Wolffian ducts degenerate in the absence of gonads in the same way as when ovaries are present (Jost 1953; Behringer *et al.* 1990, 1994; Luo *et al.* 1994 and see chapters by Behringer and by Parker, this volume). These observations reduce the problem of sex determination in mammals (at least in eutheria; see chapter by Renfree, this volume, for discussion on metatheria) to whether a testis develops or not and this in turn depends on the activity of a gene on the Y chromosome that was traditionally referred to as *Tdy* (testis determining Y gene) or, in humans, *TDF* (testis determining factor) (Goodfellow & Lovell-Badge 1993).

SRY was isolated by a positional cloning strategy from the minimum testis determining region of the human Y chromosome and it is now well established that *SRY*/*Sry* is genetically and functionally equivalent to *TDF*/*Tdy* from observations and experiments in mouse and man (Gubbay *et al.* 1990; Page *et al.* 1990; Sinclair *et al.* 1990; Koopman *et al.* 1991). Moreover, there is an *Sry* like gene on the Y chromosome of almost all mammals tested (Gubbay *et al.* 1990; Sinclair *et al.* 1990; Foster *et al.* 1992). *Sry* genes are characterized by the presence of an HMG box type of DNA binding domain. Related domains are found in a large number of DNA binding proteins, with properties of transcription factors or architectural components of chromatin or both (see Pontiggia *et al.* 1994 and references therein). *SRY* falls into a large class of probable transcription factors, termed *SOX* proteins, related by very similar HMG box domains. (The arbitrary definition is that they show greater than 50% identity at the amino acid level within the HMG box to human or mouse *SRY*.) There are more than 20 mammalian *Sox* genes known (Collignon 1992; Laudet *et al.* 1993; van de Wetering & Clevers 1993; Wright *et al.* 1993). Most of these probably play no role in sex determination, although *SOX9* clearly does (Foster *et al.* 1994; Wagner *et al.* 1994 and see chapter by Schafer, this volume) and *SOX3* may be the ancestral gene from which *SRY* evolved (Collignon 1992; Stevanovic *et al.* 1993; Foster & Graves 1994; Collignon *et al.* 1995 and see chapter by Graves, this volume). However, many of the *Sox* genes are implicated in different aspects of embryogenesis (Collignon 1992; van de Wetering *et al.* 1993; Kamachi *et al.* 1995; Wright *et al.* 1995).

Studies on *Sry* expression in the mouse using RT-PCR assays and *in situ* hybridization showed that transcripts are present specifically within cells of the genital ridge during indifferent stages from about 10.5 DPC (Koopman *et al.* 1990). Once testis cords become visible at about 12.5 DPC, *Sry* transcripts are no longer detected. *Sry* therefore acts to initiate testis determination and it is not required to maintain activity of any testis specific genes.

Our working hypothesis with respect to *Sry* action considers that there are essentially four bipotential cell lineages within the developing gonad: germ cells, supporting cells, steroidigenic cells and connective tissue cells. The germ cells, which enter meiosis early in the ovary but go into mitotic arrest in the testis, are irrelevant for testis determination as testes differentiate in their absence (Buehr *et al.* 1993*b*). Moreover, *Sry* is clearly expressed by one or more of the somatic cell types (Koopman *et al.* 1990). It is most likely that *Sry* acts within the supporting cell precursors, which give either follicle (granulosa) cells in an ovary or Sertoli cells in a testis. The strongest evidence for this comes from chimaera experiments by Burgoyne and colleagues (Burgoyne *et al.* 1988*a*; Palmer & Burgoyne 1991*a*). If more than 25% of cells in an XX↔XY chimaera are XY then the animal develops as a male. It was found that there was no bias in any cell type except the Sertoli cells which were almost exclusively XY. This result indicates that the testis determining gene must act cell autonomously within this lineage. *SRY* is a DNA binding protein therefore it is logical that it would do so. The differentiating Sertoli cells would then signal to the other cell types, causing the steroidigenic lineage to differentiate as Leydig rather than theca cells and causing connective tissue to give rise to myoid cells and generally to organize in the characteristic testis specific pattern.

However, *Sry* is not acting to force the supporting cell precursors to differentiate as Sertoli cells. In an XX↔XY chimaera with less than 25% XY cells an ovary may differentiate. However, there is no bias in the distribution of XY cells: follicle cells can be either XX or XY (Burgoyne *et al.* 1988*b*; Palmer & Burgoyne 1991*c*). The XY cells must have expressed *Sry*, but despite this they have neither differentiated as Sertoli cells nor have they died. This suggests that *Sry* is involved in competence rather than commitment. On the other hand, the requirement for the proportion of XY cells to be above a critical threshold level before Sertoli cells are seen could be explained if SRY allows expression of an extracellular signalling molecule that must be present at sufficiently high concentration to permit their differentiation. A simple way to reconcile the threshold and competence mechanisms would be if SRY regulates both the signal and its receptor. (Of course, the latter could be any part of the signal transduction pathway from cell surface to nucleus.)

Apart from not being sufficient in some cases, *Sry* is also not absolutely required for Sertoli cell differentiation as evidenced naturally by Freemartin cattle or experimentally by grafting fetal ovaries to the kidney capsule of female mice, etc. (Jost *et al.* 1975; Taketo & Merchant-Larios 1986). It is necessary to conclude that *Sry* merely influences the choice between follicle or Sertoli cell differentiation and the problem of sex determination can effectively be reduced to this decision. *Sry* is normally responsible for this decision in the indifferent gonad, as shown by mutation and transgenic studies which prove that the correct expression and functioning of SRY protein is critical, but there are many other factors involved. Clearly cell–cell interactions are important. However, there

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will also be other genes involved in the testis determining pathway within the supporting cell precursor lineage. These will include regulators of *Sry* expression, i.e. upstream factors, factors interacting with SRY protein and the downstream targets of SRY.

2. *SRY* GENES AND THEIR TRANSCRIPTS

Before we can begin to unravel the pathway in which SRY acts, it is necessary to know more about the gene itself. However, this is a problem because the gene is very different between species. This becomes very evident when comparing the transcription units between mouse *Sry* and human *SRY*.

(*a*) *Mouse* Sry

The mouse *Sry* gene is located in the proximal part of the small short arm of the Y (Capel *et al.* 1993*a*). The locus has a very unusual structure in that it consists of a small unique region of 2739 b.p., containing a long open reading frame (ORF), flanked by a large inverted repeat of at least 17 kb (Gubbay *et al.* 1992). The sequences of the two arms of the inverted repeat are almost identical which makes analysis of the gene complex and has had at least one odd consequence. *Sry* transcripts are present in low abundance in the genital ridge; we have yet to see a convincing signal by northern analysis. However, they are also present at a much higher level in adult testis where a clear band of 1.3 kb is seen on northerns (Capel *et al.* 1993*b*). In contrast to the genital ridge where *Sry* is expressed by somatic cells, in the testis it is expressed within germ cells, most notably round spermatids (Guerrier *et al.* 1990; Capel *et al.* 1993*b*). The 1.3 kb transcript is, however, very unusual in that it is a circular RNA (Capel *et al.* 1993*b*). This is thought to arise from splicing of a long primary transcript which begins within the 5′ arm of the inverted repeat, extends through the unique region and finishes within the 3′ arm of the repeat. Because the sequences of the two arms of the repeat are so similar, the two ends of this long transcript will be complementary and should hybridize to form a stem of double stranded RNA. The unique region contains a splice donor site 3′ to an efficient splice acceptor site (see figure 3), but as it will form a loop of single stranded RNA the splicing machinery is 'misled' and treats it as if the donor is 5′ to the acceptor. A normal splicing reaction would then generate the 1.3 kb circular 'exon' and an 'intron' which apart from being branched as normal, is also potentially capped and polyadenlylated. However, we have not detected any sign of the latter; perhaps it is very short lived as it could be mostly double stranded. The circular RNA, on the other hand appears very stable. Suprisingly, it is located within the cytoplasm despite the lack of a cap and polyadenylation. However, because it is not obviously associated with polysomes it appears unlikely that it is translated, even though it contains much of the ORF including the translational initiation site (Capel *et al.* 1993*b*). There is also evidence to suggest that *Sry* has no critical function in spermatogenesis as sperm carrying a Y chromosome lacking *Sry* in a chimaera gave rise to XY female offspring (Lovell-Badge & Robertson 1990). Although it is possible to detect a low level of transcript that is presumably unspliced and which extends well into the 5′ arm of the repeat, the precise location of the spermatid promoter is not known. The circle is made in transgenic mice from a 14 kb *Sry* genomic fragment, suggesting the promoter is included within this (Hacker *et al.* 1995). It is also possible that there is no specific promoter, but simply a low level of aberrant transcription through the locus (or from a gene at the site of integration in the transgenics). The circle could accumulate to easily detectable levels because of its stability.

The genital ridge transcript has been difficult to characterize because of its low abundance combined with an apparent poor cloning efficiency of sequences throughout the region (Gubbay *et al.* 1992). The approach we adopted to determine the nature of the transcript was to use RNase protection assays. These are both sensitive and quantitative and do not rely on any amplification or cloning steps: but they are very laborious! Using probes spanning the *Sry* locus it was possible to define a normal linear single exon transcript of just under 5 kb (Hacker *et al.* 1995). The 5′ and 3′ ends of this have been confirmed by RACE-PCR. A cluster of three or four transcriptional initiation sites are used, and importantly these are located within the unique region close to the ORF. This is consistent with alternative promoter usage. One or more promoters within the 5′ inverted repeat which lead to formation of the circular RNA in spermatids in the adult testis, and one in the unique region which leads to the functional linear transcript within the genital ridge. There have been reports of *Sry* transcripts also being present in preimplantation embryos (Gaudette *et al.* 1993; Zwingman *et al.* 1993; Boyer & Erickson 1994; Cao *et al.* 1995). It is possible that these transcripts are also circular and no function for them has yet been demonstrated (see chapter by Burgoyne, this volume).

Many features of the mouse *Sry* gene seem to be designed for maximum inefficiency (see figure 3). There are multiple transcription initiation sites. There is a simple microsatellite repeat sequence (TCTG) within the 5′ untranslated region that could affect translation. Before the start of the long ORF, there are two short reading frames preceded by methionines, at least the first of which is in a reasonable Kozak consensus sequence. These short ORFs could also serve to reduce translation efficiency. The protein predicted to be translated from the long ORF consists of a two amino acid, N-terminal domain, followed by the 79 amino acid HMG box domain and then a long 314 amino acid C-terminal domain. The latter is largely composed of a highly repetitive glutamine and histidine rich region based on a degenerate CAG trinucleotide repeat sequence. In the beginning of the 3′ untranslated region there is a group of four ATTTA sequences, that are often associated with message instability (Shaw & Kamen 1986; Wilson & Treisman 1988). There is also a peculiar choice of polyadenlyation site, which may suggest further controls on translation or message stability by sequences within the 3′ UTR. The first two

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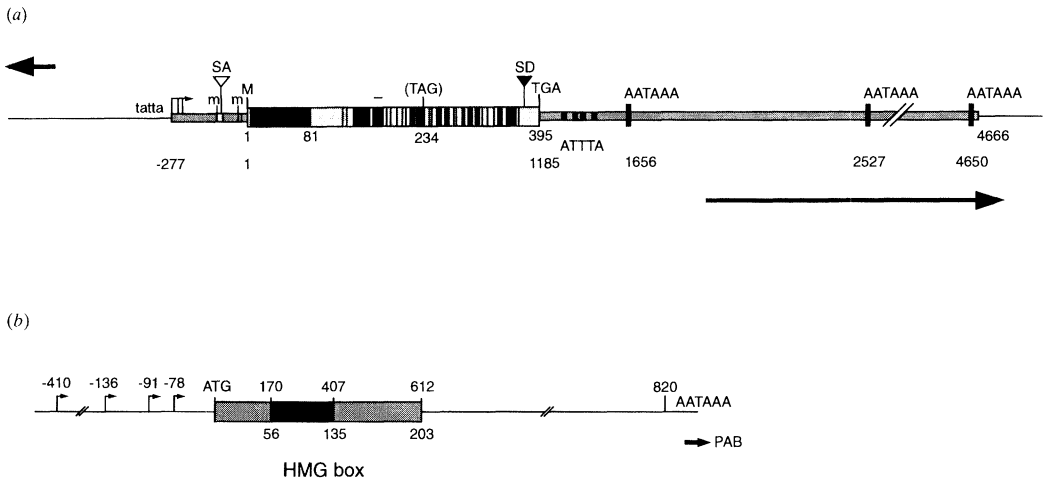

Figure 3. Comparison between the mouse *Sry* and human *SRY* loci, transcripts and predicted protein products. The mouse *Sry* gene is located within a large inverted repeat (thick arrows). The human gene is just 5 kb from the pseudoautosomal boundary (PAB). The numbers indicate nucleotide or amino acid position. SA and SD: splice donor and acceptor sites that give rise to the circular *Sry* transcript in adult mouse testis. TGA and TAG: stop codons found in *musculus* and *domesticus Sry* alleles respectively. ATTTA: putative message instability sequences. →: transcription initiation sites (see text). Within the proteins, the large (79 amino acid) shaded block is the HMG box. The smaller shaded blocks in the mouse protein represent glutamine repeats.

potential consensus sites, both AATAAA, are not used at all. There is a minor, less conventional site (AATATA), used by about 5% of transcripts and then, eventually a third AATAAA site is used by about 70% of transcripts. This gives an RNA of about 4943 bases in length. The remainder of the transcripts appear not to extend beyond the next consensus polyadenylation signal (A. H. Hacker, unpublished results).

(*b*) *Human* SRY

The differences between the mouse and human *SRY* genes are dramatic (see figure 3). Although the mouse gene is located in a precarious position within an inverted repeat and close to the centromere (see below), the human gene is situated in another precarious position just 5 kb from the boundary with the pseudoautosomal region (Sinclair *et al.* 1990). This is presumably responsible for the relatively high frequency of XX males that occur as a result of abnormal X-Y interchange during male meiosis (Guellaen *et al.* 1984; Page 1986). There is no inverted repeat flanking human *SRY*. With respect to its expression, the human gene is far more promiscuous than the mouse gene. Although most data comes from non-quantitative and very sensitive RT-PCR assays, human *SRY* apperas to be expressed in many tissues of embryonic, adult and tumour origin (Clépet *et al.* 1993). Figure 3 illustrates some of the features of the transcript and it can be seen that it is almost completely different from that of the mouse gene. There are some problems in that it has not been possible to define the nature of the transcript in human genital ridge, and there is some discrepancy between published reports on the position of the transcriptional initiation site(s). Four are illustrated in figure 3, but these have been found using different assays and may not all be used *in vivo* (Vilain *et al.* 1992; Behlke *et al.* 1993; Clépet *et al.* 1993; Su & Lau 1993). There is however, only a single methionine preceeding the long open reading frame. This lies in a non-optimal sequence context which might serve to reduce translational efficiency, but there is no microsatellite or alternative translational initiation site. The predicted protein comprises a 56 amino acid N-terminal domain, the 79 amino acid HMG box and a 68 amino acid C-terminal domain. This structure looks very different from that of the mouse protein. The only region of homology between the mouse and human SRY proteins is in the HMG box. There is no similarity at all between human and mouse N- and C-terminal domains and moreover there is no glutamine-rich region in the human protein. The 5′ and 3′ UTRs and sequences flanking the two genes also show no significant homology apart from two small regions 5′ which could denote regulatory motifs (see Hacker *et al.* 1995). If it was not for location on the Y chromosome and functional data to say that both were testis determining, it would be natural to conclude that mouse and human *Sry*/*SRY* were different genes. They seem no more related to each other than two completely different *Sox* genes. Indeed, it is not surprising that the human gene fails to give sex reversal in transgenic mice (Koopman *et al.* 1991). A variety of tests suggest that *Sry* is one of the fastest evolving genes yet found (Tucker & Lundrigan 1993; Whitfield *et al.* 1993). Possible reasons are discussed in the chapters by Tucker and by Graves, this volume.

3. SEX REVERSAL AND SRY

We now understand many cases of sex reversal in both man and mouse where *SRY*/*Sry* expression or action is affected. Mutations within the HMG box which compromise DNA binding properties of human SRY are discussed in the chapter by Bianchi, this volume (Nasrin *et al.* 1991; Harley *et al.* 1992; Jäger *et al.* 1992; Pontiggia *et al.* 1994; Poulat *et al.* 1994). Table 1 lists cases of sex reversal in the mouse where *Sry* is clearly implicated. These can be 'trivial', as in the

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Table 1. *Cases of sex reversal in the mouse where* Sry *is implicated*

genotype	phenotype	defect in *Sry*	other causes
XY^{tdym1}	i♀ (fertile)	11 kb deletion	–
XY^{pos}/outbred	♂, 14 h delay in cord formation	structure?	–
		regulation?	
XY^{pos}/C57BL/6J	♀, ♀	structure?	*tda-1*, *tda-2*, etc.
		regulation?	
$XXSxr^{a}$	♂	none	
T16X:$XSxr^{a}$	♀, ♀, ♂	expression	X-inactivation
XX + 14 kb *Sry*	30% ♂		
transgene	70% ♂	expression	integration site/genetic background
XY^{d1}, XY^{d2}, etc.	♀, ♀	expression	deletion of *Sx1* repeat region

case of *tdym*1 where an 11 kb deletion of *Sry* resulted in XY females (Lovell-Badge & Robertson 1990; Gubbay *et al.* 1992), or complex as with the poschiavinus Y chromosome where sex reversal depends on the genetic background as well as a deficiency in *Sry* (Eicher & Washburn 1986; Palmer & Burgoyne 1991*b*, and see chapter by Eicher, this volume). There are also several cases where level of *Sry* expression is affected leading to female or hermaphrodite development. These include long range effects on *Sry*. For example, *Sry* appears to be inactivated along with the X chromosome in T16X:XSxr mice (McLaren & Monk 1982). Also, there are several cases where copies of a repetitive sequence, *Sx1*, which maps between *Sry* and the centromere, have been deleted (Capel *et al.* 1993*a*). XY^{d1} female mice are the most extreme of these cases where no *Sry* transcripts are detected and most *Sx1* repeats have been deleted. The simplest explanation is that the loss of these sequences has allowed the spread of centromeric heterochromatin into the *Sry* locus, such that it now transcriptionally inactive.

One other case of sex reversal that deserves brief mention here is that of mice transgenic for *Sry*. In about 30% of cases (Koopman *et al.* 1991 and R. Lovell-Badge, N. Vivian & A. Hacker, unpublished results) chromosomally female mice transgenic for a 14.5 kb genomic fragment of *Sry* develop as males. The failure of the remainder to show sex reversal is simply the result of site of integration influencing expression of the *Sry* transgene. This is illustrated by the study of one line, 32.10, where the founder animal was a female. Sex reversal was not seen amongst her offspring, but when these were crossed together, XX transgenic males were found. This is the result of a genetic background effect (N. Vivian, A. Hacker & R. Lovell-Badge, personal observations). When the *Sry* transgene is on an MF1 outbred background essentially no XX males are found. On a C57BL/6 background a low level of sex reversal is seen, but on a CBA background approximately 60% of XX animals are male. RNAse protection assays to look at *Sry* expression show that transcripts are almost undetectable in genital ridges of XX transgenics with an MF1 background, but they are at approximately 50% of normal with a CBA background. This suggests that the level of *Sry* transcription is close to a functional threshold on the CBA background (Hacker *et al.* 1995). This genetic background effect is also clearly different from that seen with the *poschiavinus* Y chromosome, which would not show any XY female development on either CBA or MF1 backgrounds.

4. THE QUEST FOR TARGET GENES

If we are to understand how SRY functions to initiate testis differentiation we need to identify the target genes it regulates. One problem is that we do not know whether SRY acts as an activator, repressor or both. Unfortunately the structure and properties of SRY proteins give us few clues. The glutamine rich region of the mouse SRY protein could act as a transcriptional activation domain, and there is some evidence to support this (Dubin & Ostrer 1994). However, this region varies dramatically in length between mouse subspecies and there is no such sequence at all in the human SRY protein (Miller *et al.* 1994). Human SRY could depend on an interacting protein which supplies such a domain, or it is only the HMG box that is important in both mouse and human SRY. In this case, it could still act as an activator through its ability to bend DNA. For example, this could allow interaction of other transcription factors bound at adjacent sites. Alternatively, it could act as a repressor simply by occupying critical regulatory sites otherwise bound by activators. At this point we are therefore restricted to guesswork, searching for genes showing differential expression or using genetics to find other genes responsible for cases of sex reversal.

To be a target, a candidate gene it would have to satisfy the following criteria.

1. It needs to be expressed in the same cell lineage as *Sry*.
2. It needs to be expressed differently between males and females at a time when SRY protein is present (because of lack of good antibodies we do not know exactly when this is but assume it closely follows the mRNA). However, the target gene could be turned on or off in males depending on whether SRY is an activator or repressor.
3. There needs to be a good binding site for SRY. Random oligo selection experiments *in vitro* have defined this as AACAAT, although it will also bind some similar sequences with reasonable affinities (Denny *et al.* 1992; Harley *et al.* 1992, 1994).

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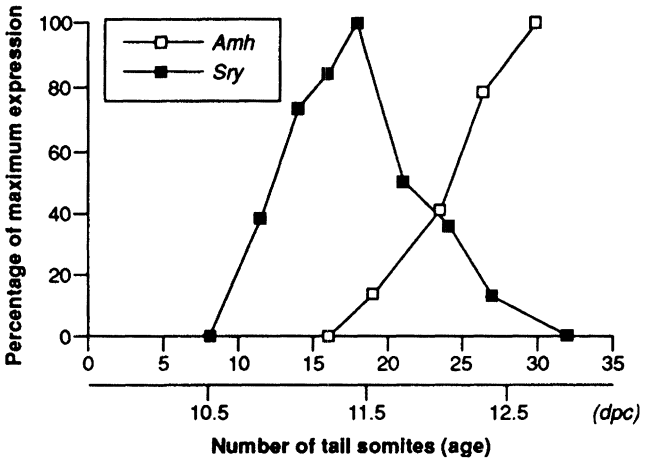

Figure 4. *Sry* and *Amh* expression during gonadal development. Expression was analysed by RNAse protection assays and measured using a phosphoimager. (See Hacker *et al.* 1995, from which this figure is taken, for details.)

4. It also seems logical (although this is not an absolute requirement) that the candidate target gene should itself be important for testis differentiation: either promoting or inhibiting it.

As discussed above, because differentiating Sertoli cells seem to rapidly induce the differentiation of both themselves and the other lineages that make up the testis, it would seem likely that genes encoding extracellular signalling molecules are good candidates. Anti-Mullerian hormone, which is a signalling molecule, has been suggested as a target gene, partly because it is expressed very early on by differentiating Sertoli cells, but also because of its involvement in development of the male phenotype. We and others have looked at *Amh* as a possible target gene, but although there is some discrepancy, this possibility seems increasingly unlikely.

Although it satisfies the first of the above criteria, in that it is expressed in the same lineage as *Sry*, there was a question as to whether the expression overlapped. It was first necessary to know in more detail how *Sry* is expressed. RNAse protection assays were used to look at a detailed time course of genital ridge development (Hacker *et al.* 1995). The results were in broad agreement with those obtained earlier by RT-PCR, but they were more accurate and quantitative. *Sry* transcripts were first detected at 10.5 DPC, increased to a sharp peak at 11.5 DPC and then declined such that there was no detecteble signal by 12.5 DPC. Using the same samples, *Amh* expression was also ascertained, and it was found to initiate at 11.5 DPC and then increase rapidly to a maximum at about 13 DPC which was maintained throughout fetal life (Hacker *et al.* 1995). This result makes it possible that *Amh* is a direct target gene as its expression overlaps with *Sry*, indeed it is coming on when *Sry* transcripts are at their peak. However, there is a significant gap of about 22 h between the onset of *Sry* expression and that of *Amh*; room for at least one other regulatory step (see figure 4).

To test the third criterion it is necessary to look at the regulation of *Amh*, in particular to determine the regulatory sequences required for early testis specific expression. Towards this aim we have characterized the sequences flanking the gene in some detail. Comparisons between 5′ flanking sequences of human and mouse *Amh* revealed the presence of another gene located just upstream (Dresser *et al.* 1995). This is a gene termed *Sap62* which encodes a spliceosome protein. In the mouse the polyadenylation site used by the majority of *Sap62* transcripts lies just 328 b.p. 5′ of the *Amh* transcriptional initiation site. However, a significant proportion of transcripts fail to terminate in the intragenic region and continue into the *Amh* gene. *Sap62* is ubiquitously expressed, so its location so close to a gene which needs to be tightly regulated seems odd, especially as the readthrough transcripts imply that *Amh* has an open chromatin conformation in all cells of both sexes.

These observations imply that critical regulatory regions for *Amh* expression may be located very close to the gene. Preliminary observations suggest that the short intragenic region is indeed sufficient to initiate testis specific expression of a reporter gene in transgenic mice (C. Beau, N. Vivian, D. Guerrier & R. Lovell-Badge, unpublished results). Close inspection of the DNA sequence within this region fails to reveal any consensus binding site for SRY. We and others have also been unable to convincingly demonstrate SRY binding to this region (Harley *et al.* 1994; Shen *et al.* 1994). Although there are some results that seem to contradict this, recent cotransfection experiments suggest that if there is an interaction between SRY and *Amh*, then it is indirect (Haqq *et al.* 1994).

Finally, *Amh* appears to have no critical function in testis development as revealed by mutation studies where normal testes develop in mice lacking the gene (Behringer *et al.* 1994). *Amh* therefore fails to satisfy the last of the above criteria. However, it is still important to study the gene's regulation. Within the minimal promoter region there are several conserved sequence motifs. One of these is for steroidigenic factor 1 (SF1) and it seems likely that this is required for *Amh* expression (Luo *et al.* 1994; Shen *et al.* 1994 and see chapter by Parker, this volume). However, SF1 RNA and protein are present before sex determination and the levels appear not to differ between male and female genital ridges at 11.5–12.5 DPC at a time when *Amh* transcripts are first detected. The factor responsible for testis specific initiation of *Amh* transcription has yet to be found, but it is likely to be informative as it could itself be directly regulated by SRY.

5. CONCLUSIONS

Five years after the cloning of *Sry* we know a considerable amount about the gene in mouse and human and some details from other species. Much intriguing biology has been revealed and a whole new set of questions can be formulated. We have a reasonable understanding of how the protein functions at a molecular level and a picture is gradually developing to suggest how *Sry* impinges on gonadal development at a cellular level. Considerable progress has also been made over the last year in the

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identification of other genes involved in gonadal development and sex determination. However, probably the most important gap in our knowledge is the identity of any true SRY target gene. Working upstream from genes such as *AMH* can possibly lead to this, but other strategies are required. Differential screening or subtraction of cDNA libraries made from critical stages of genital ridge development offers one sort of approach. Having shown that there is just a 22 h window between the first appearance of *Sry* transcripts and the onset of *Amh* expression pinpoints the time when differences in target gene expression should first become apparent (Hacker *et al.* 1995). Also, with the relentless progress in genome mapping projects and the increasing ease with which genes may be cloned by position, etc. it is clear that genetics will continue to be a very powerful tool in unravelling the sex determination pathway in mammals.

We thank all our colleagues who have contributed to the work discussed here, particularly those who have allowed us to mention their unpublished data: Charlotte Beau, Nigel Vivian, Daniel Guerrier and Katarina Nordqvist. We are also indebted to Larysa Pevny for critical reading of the manuscript.

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Discussion

U. Mittwoch (*Department of Anatomy, Queen Mary & Westfield College, London*). Can Dr Lovell-Badge explain why in chimaeras in which the overall proportion of cells containing a Y chromosome is 25%, the supporting cells in the gonad contain a Y chromosome in 90% of their cells. Also, why isn't there a larger proportion of gonads with fewer Y chromosomes in their supporting cells resulting in ovary formation.

R. Lovell-Badge. I assume that if the supporting cells carrying a Y produce an extracellular signalling molecule present at levels above a critical threshold then this drives differentation of XY cells towards Sertoli cells. A few XX cells also form Sertoli cells, however what happens to the rest is a very interesting question. They could begin to form Sertoli cells and then die, or they could be recruited into different lineages, such as that giving rise to Leydig cells. However, as far as I am aware, no bias was seen in favour of XX cells in the other lineages in the experiments of Paul Burgoyne and colleagues.

M. Fellows (*Pasteur Institute, 25 Rue de Roux 75724, Paris*). Can Dr Lovell-Badge explain why no-one has described cases of XX sex reversal which are *Sry* negative whereas in man all familial cases of XX sex several are *Sry* negative?

R. Lovell-Badge. Perhaps they exist, but they have not been found. Such mice would be sterile males which would usually just get discarded from a colony without proper analysis. However, they ought to have been noticed in crosses involving mice carrying different X-linked markers. XX *Sry* negative sex reversal has been seen in other animals such as dogs (Meyers-Wallen *et al.* 1995). V. N. Meyers-Wallen, V. L. Palmer, G. M. Acland, B. Hershfield 1995 *Sry* negative XX sex reversal in the American cocker spaniel dog. *Molec. Reprod. Dev.* **41**, 300–305.

N. Josso (*Unité de Recherches sur l'Endocrinologie du Développement, Ecole Normale Supérieure, Département de Biologie, France*). The statement that AMH is able to 'turn an ovary into a testis' is not accurate. AMH is able to induce the formation of testicular tubules in freemartin ovaries and in the ovaries of MT-hAMH transgenic mice (Behringer *et al.* 1990 *Nature, Lond.* **345**, 167) but this does not affect the whole ovary and the proportion of masculinized ovaries is only 50% in freemartins and much lower in transgenic mice.

R. Lovell Badge. I am sorry, you are quite right. I was only tring to make the point that testicular tissues can differentiate in the absence of *Sry*, not that normal testes develop.

J. A. M. Graves (*La Trobe University, Melbourne, Australia*). First, does the differnce in *Sry* expression pattern between mouse and human suggest *Sry* may have functions other than TDF in humans? Secondly, is it possible that *Sry* has a much more indirect action, e.g. by competitive inhibition of another HMG box-containing gene SOX 9? SOX 3?

R. Lovell Badge. In answer to your first question: it is difficult to know the significance of the widespread expression seen in humans. Although it is conceivable that SRY does have a specific role in other tissues, and perhaps this role has been lost in the mouse or gained in humans, this is going to be hard to demonstrate. However, there is no reason to suppose that it is important. First, the widespread expression has mostly been detected by non-quantitative RT-PCR methods and may represent extremely low abundant transcription. Second, as the human genital ridge transcript has not been characterized and nor has that seen in most other tissues, it is also conceivable that the latter may not be functional. Finally, even assuming this widespread expression does give reasonable levels of SRY protein, it still may not be functional in nongonadal tissues. Perhaps human SRY needs a protein partner with which it interacts. For example, this could provide a transcriptional activation domain to make it more like the mouse SRY protein. This partner could itself be restricted to the genital ridge. Alternatively, the DNA target sites for SRY protein may only be accessible in cells of the genital ridge. We have so far not seen any effect of ectopic expression of *Sry* in transgenic mice, although levels have been very low. In answer to your second question, yes, of course. It could compete for target site binding with other similar HMG box proteins or with any transcription factor that has an overlapping binding site. As SRY bends DNA it could interfere with the action of another factor at some distance. Of course the other protein could itself be an activator or a repressor. This type of mechanism is quite common and is of no less importance than more direct transcriptional activation.

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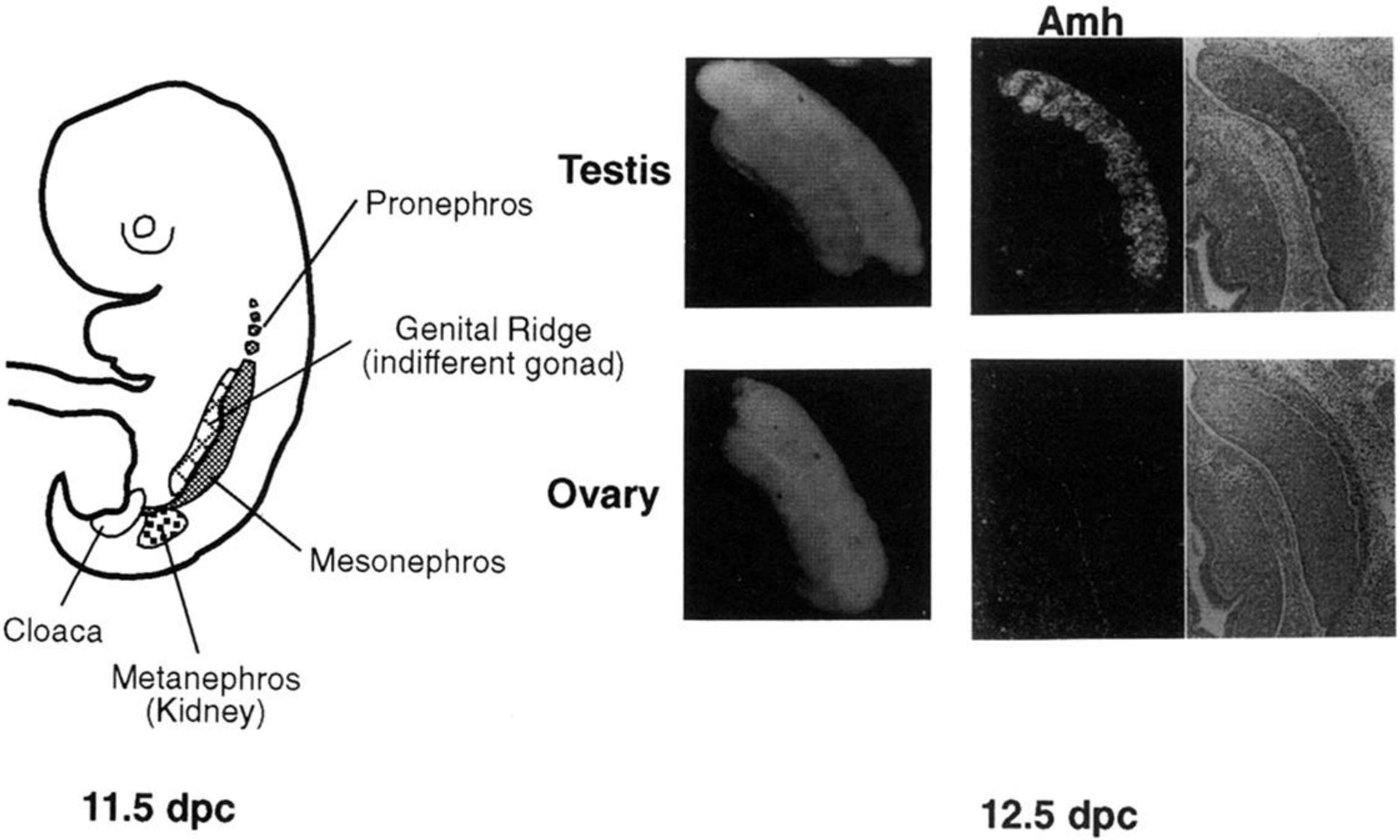

Figure 1. Gonadal development and sex determination. The genital ridge develops in association with the mesonephros and remains morphologically indistinguishable between XX and XY embryos until after 11.5 DPC. By 12.5 DPC, many differences are apparent between testis and ovary in cell type, their organization and in gene expression, as shown by *in situ* hybridization for *Amh* in 12.5 DPC testis (top) and ovary.

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