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Sex differentiation – gonadogenesis and novel genes

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ABSTRACT During embryogenesis, most organ rudiments differentiate into only one type of organ and functional mutations are normally lethal for the embryo. However, the indifferent gonad has two options, to form either a testis or an ovary, and mutations of this tissue usually produce sex reversal or sterility which is not lethal for the individual. Therefore, gonadal development serves as an excellent model system for investigating questions of cell fate and organogenesis. The studies of human patients showing different types of sex reversal, in combination with the use of transgenic mice and/or gene targeting disruption, have led to the isolation of several genes important for sex development. These include *SRY/Sry,* encoding the testis-determining factor, *Ftz-F1* encoding steroidogenic factor 1 (SF-1) and Wilms' tumor gene *(WT-1).* However, the mammalian sex differentiation pathway requires the function of a number of additional genes which we are now trying to identify with the help of the mRNA differential display technique.

KEY WORDS: *sex differentiation, testis determination, gonads, mRNA differential display*

Introduction

The mechanism by which an individual's sex is determined has been one of the great questions of embryology since antiquity. Aristotle, who investigated how embryos develop, claimed that sex was determined by the heat of the male partner during intercourse. The more heated the passion, the greater the probability of male offspring. He therefore counseled elderly men to conceive in the summer if they wished to have sons.

Today we know that the environment has little to do with sex determination in mammals; instead, it is chromosomally based: females have two X chromosomes and males have an X and a Y chromosome. Independent of the number of X chromosomes present, inheritance of a Y chromosome results in male development. For example, in the Klinefelter syndrome, which is caused by the sex chromosome aneuploidy XXY, the phenotype is male (reviewed by Grumbach and Conte, 1992). This is in contrast to, for example *Drosophila melanogaster*, where sex determination depends on the X chromosome to autosome ratio (reviewed by Hodgkin, 1992). Thus, the mammalian Y chromosome acts to divert development of the indifferent gonads along the testicular pathway and therefore the testis-determining gene must be present on the Y chromosome. In 1990, a gene named *Sry* (sex determining region, y gene; *Sry* in mouse and *SRY* in human) was located to the sex determining region on the Y chromosome in mouse and human and it is now clear that the presence or absence of *SRY/Sry* determines whether male or female gonadal differentiation takes place (Gubbay *et al.,* 1990; Sinclair *et al.,* 1990).

The aim of this article is to give an overview of testicular and ovary development, how organogenesis takes place and what kind of genes are known to be involved in these processes. In the final part, I will also give a brief description of some approaches used to identify novel genes involved in sex differentiation.

Organogenesis of the testis and the ovary

In a classical experiment by Alfred Jost, early differentiating testes or ovaries were removed from developing rabbit fetuses *in utero.* When these animals developed to term, their internal and external genitalia showed female characteristics. Therefore, female differentiation is considered as the default pathway, which is overridden if a testis differentiates and produces factors that masculinize the embryo (Jost, 1947).

The adult gonads, ovary and testis, emerge from precursor tissues initially common to both females and males: the indifferent gonad. This anlagen appears as a narrow band of tissue along the mesonephros, which is located on the dorsal mesentery, on either side of the dorsal aorta and neural tube in the embryo, and develops into either testis or ovary depending on the sex chromosomes (Fig. 1) (Capel and Lovell-Badge, 1993). The indifferent gonad (also called genital ridge) and the mesonephros are together named the uro-genital ridge, and in the mouse this rudiment can be recognised at 10.5 days post coitum (dpc, all time points refer to mouse development unless stated otherwise). Within the mesonephric part of the uro-genital ridge two tubules are located, the Müllerian duct and the Wolffian duct. In males, through the induction of testosterone, the Wolffian duct will develop into the internal genitalia as epi-

Abbreviations used in this paper: dpc, days post coitum.

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didymis, vas deferens and seminal vesicles. Later on, dihydrotestosterone promotes formation of the male urethra and prostate as well as the penis and scrotum. By producing Müllerian inhibiting substance, MIS (also known as anti-Müllerian hormone, AMH), the Müllerian duct degenerates in males. In females, who are devoid of MIS production, the Müllerian ducts differentiates into fallopian tubes, uterus and upper vagina while the Wolffian duct system regresses due to the lack of testosterone (reviewed by Josso *et al.*, 1993).

Four different cell lineages are thought to build up the developing gonad and these are the supporting cells, the steroid-producing cells, the connective tissue cells and the germ cells (Fig. 2). The supporting cells, which differentiate into Sertoli cells in the testis and into follicle cells in the ovary, are thought to derive from common progenitors as these cells have similar functions and can also switch from one cell type to the other (reviewed by McLaren, 1991). The steroid-producing cell lineage is thought to develop as Leydig cells in testis and Theca cells in the ovary. A common precursor cell of these cell types has not yet been found, although it is an attractive hypothesis since androgens and estrogens are products from the same steroid biosynthetic pathway. Connective tissue cells include the peritubular myoid cells as well as endothelial cells which form blood vessels, necessary for transport of testosterone to target tissues. In the fetal testis, the germ cells develop into prospermatogonia and arrest in mitosis around 13.5 dpc while in the fetal ovary at the same time point, the germ cells progress into meiosis and form oocytes (reviewed by McLaren, 1988). However, germ cells are not necessary for testis development since mouse mutants such as dominant white spotting (W^e) or steel (Sl^d) which lack germ cells, still show normal testis cord formation. In contrast, females from these mouse mutants show streak gonads which lack pre-follicle cells, only containing stromal tissue, suggesting oocytes being essential for the establishment or maintenance of primordial follicles (Mintz and Russell, 1957; Merchant-Larios and Centeno, 1981; McCoshen, 1982).

Considerable efforts have been made to understand from where different cells in the developing gonads originate. However, this subject is still controversial. Results obtained from different species are especially conflicting despite it seeming likely that closely related species would have the same basic mechanism. However, it appears that sex differentiation is one of the most rapidly evolving systems in the organism and therefore variations between connected species could occur (see below on *Sry*). Both the mesonephros and the coelomic epithelium have been suggested as possible sources for gonadal cells. In a species with slow maturing gonads such as man, cattle or sheep, the major portion of gonad cells are thought to derive from the mesonephros (Satoh, 1991 and references therein). In rabbit, on the other hand, the coelomic epithelium may play a more active role, and in combination with mesonephric cells, form a mixed blastema from which Sertoli cells or follicle cells are thought to derive (Wartenberg *et al.*, 1991).

Gonadal differentiation in rat and mouse begins with condensation of somatic cells and deposition of basal laminar components around clusters of epithelia-like cells (pre-Sertoli or

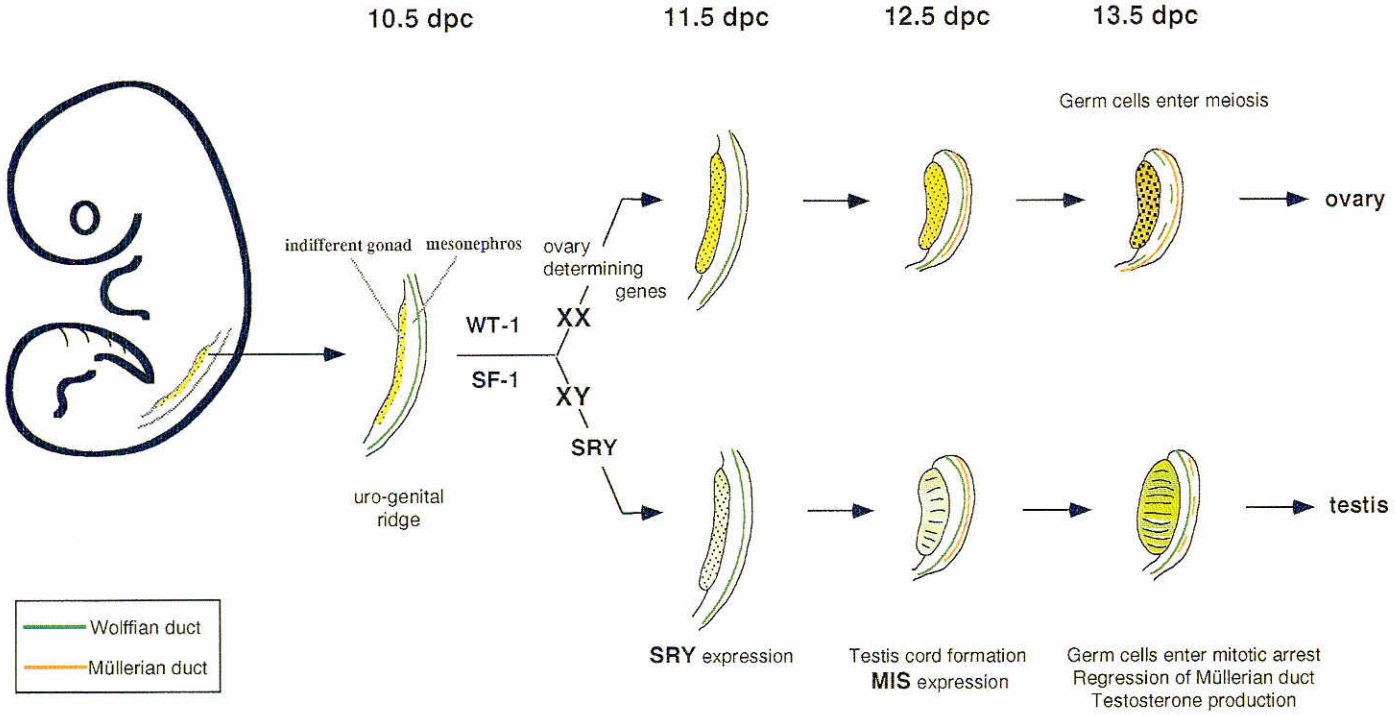

Fig. 1. Ovary and testis development in mammalian fetus. *Both WT-1 and SF-1 are necessary for development of the indifferent gonad and may be important for later stages as well. SRY directs XY gonads into the testis developing pathway, while postulated and so far unknown, ovary determining genes are important for ovary development. The first sign of ovary development is germ cells entering meiosis. Testis development is recognized by the formation of testis cords and the regression of the Müllerian ducts. Further development of male characteristics is dependent on testosterone production. Approximate times during mouse development are shown at the top.*

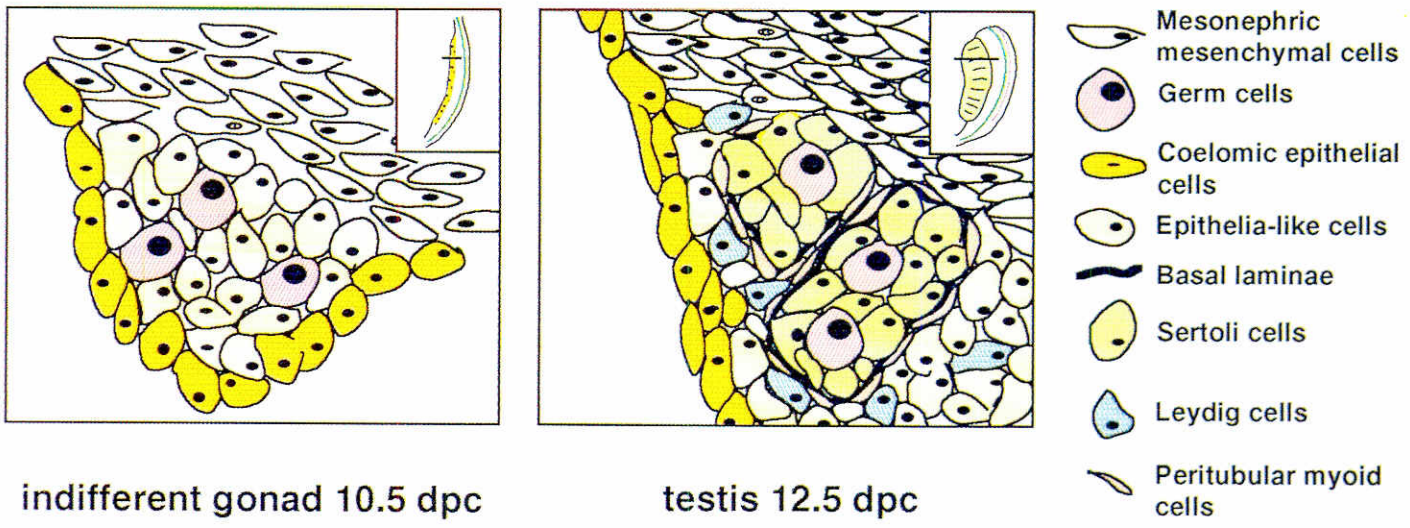

Fig. 2. Schematic picture of different cell types present during testis cord formation. *The horizontal line across the gonads in the small boxes indicates the level of the transverse sections shown in the big boxes.*

pre-follicle cells?). This occurs both in male and female gonad and is referred to as gonadal cord formation (Merchant-Larios and Taketo, 1991; Smith and MacKay, 1991). At 10.5 dpc, the testis determining gene *Sry* is turned on in the male mouse embryo and at 12.5 dpc there is an obvious difference in gonadal morphology when compared to female (Fig. 2) (Koopman *et al.,* 1990). In the male gonad, pre-Sertoli cells have differentiated by 12.5 dpc, as identified by the development of cells with rough endoplasmic reticulum and expression of MIS (Merchant-Larios, 1976; Münsterberg and Lovell-Badge, 1991). Testis cords have formed containing Sertoli cells and primordial germ cells, which enter the indifferent gonad after migrating from the base of the allantois through the hindgut and dorsal mesentery between 10-12 dpc (Ginsburg *et al.,* 1990). Each testis cord is surrounded by a basal laminae which is at least partly made by myoid cells (Tung *et al.,* 1984). These cells are attached to the basal laminae outside the testis cords, in the stromal compartment. Other stromal cells are the Leydig cells, which begin to produce testosterone at 12.5 dpc, and endothelial cells forming blood vessels (Merchant-Larios and Taketo, 1991; Taketo *et al.,* 1991). As a result of this, the male gonad is distinguished from a female by testis cord formation but also by its higher vascularization at 12.5 dpc. The first indication of female gonad development is the entry of germ cells into meiosis which starts around 13.5 dpc (reviewed by McLaren, 1988). Otherwise the female gonad morphology remains similar to the indifferent gonad until the onset of folliculogenesis which occurs around birth. However, this does not exclude the possibility of an ovarian determination step occurring at these early stages. When the Y chromosome of *Mus musculus domesticus* (Y^{DOM}) is placed onto the C57BL/6J mouse background, the XY progeny (B6.Y^{DOM}) develop either ovaries or ovotestis (Eicher *et al.,* 1982; Nagamine *et al.,* 1987). In these ovotestes, seminiferous cords and ovarian cords are often enveloped by common basal laminae, suggesting that both structures share an embryonic origin (Merchant-Larios and Taketo, 1991).

The majority of the studies on gonadal morphogenesis have been based on the use of light and electron microscopy. Recently, a more direct way of assaying cell origins was established by using *in vitro* organ cultures (Buehr *et al.,* 1993; Merchant-Larios *et al.,* 1993). By culturing 11.5 dpc XY genital ridges *in vitro*, with or without mesonephros, it was shown that the mesonephros are necessary for testis cord formation. However, testis cord formation was not necessary for Sertoli cell and Leydig cell differentiation since genital ridges without mesonephros produced both MIS and testosterone. When testes were cultured together with mesonephros, where the latter was either labeled with 3H-thymidine or taken from mice carrying a transgenic marker, the label/marker was found in peritubular myoid cells and endothelial cells in the stromal compartment. These results confirm that pre-Sertoli cells, but also pre-Leydig cells, are already located in the genital ridge prior to 11.5 dpc and the origin of these cell types still remains an open question.

By combining results from EM studies and *in vitro* organ culture experiments, a highly schematic picture on early gonadal organization can be envisioned. First, cells from the mesonephros and/or the coelomic epithelium are reprogrammed to form epithelia-like cells (pre-Sertoli/pre-follicle cells?) which constitute the indifferent gonad. These cells could also be the source of pre-Leydig cells. The origin of pre-Sertoli/pre-follicle and pre-Leydig cells could be revealed by dye-injections of single cells and subsequent tracing of their movements in *in vitro* organ cultures. Second, gonadal cords, containing primordial germ cells and epithelia-like cells are formed in both male and female gonads. In the ovary, these gonadal cords remain relatively unchanged until the start of folliculogenesis where the stromal region increases due to invasion of mesonephric cells (Merchant-Larios and Chimal-Monroy, 1989). Third, the onset of *Sry* triggers testis differentiation. Pre-Sertoli cells begin to differentiate and mesonephric cells move into the gonad, enlarging the stromal compartment by contributing endothelia and myoid cells. Testis cords are formed, and as a result of Leydig cell differentiation, testosterone is secreted.

At least two different signals must be present during this early gonad differentiation. One which is sex independent and reprograms mesonephric and/or coelomic epithelia cells to form epithelia-like cells, and one which is sex specific and promotes migration of mesonephric cells into the stromal compartment of the male gonad. However, the sex of the cells responding to the sex specific signal is unimportant since cells from female mesonephros can participate in testis cord formation (Buehr *et al.*, 1993). The only signals characterized so far to be expressed during early gonadal differentiation are MIS and testosterone. However, expression of these hormones starts around 12.5 dpc, which is inconsistent with involvement in stromal cell invasion which occurs around 12 dpc. In addition, MIS is probably not necessary for testis development since men with MIS mutations and persistent Müllerian Duct syndrome, still form normal testes. Therefore, other signal molecules, which have not yet been found, must exist.

Gene products important for sex development

During the last 30 years, our knowledge about sex determination and differentiation has increased remarkably. This is largely due to the studies of human patients with abnormalities of sexual differentiation, originally with the use of biochemistry but now mainly with molecular biology. The identification and cloning of *Sry/SRY* in 1990 opened up a new era in sex differentiation research which has led to the isolation and characterization of additional genes which are also of importance for gonadal development. A whole set of novel genes have emerged which are expressed in differentiating gonads; these include Pax-2, Hox D.2, D.3 D.4 and many more. However, as their function in gonadal development has not been proven, neither these genes nor genes involved in primordial germ cell migration and proliferation will be considered in this review. I will instead concentrate on genes which, in addition to showing a gonadal expression pattern, have been proven to have functional roles in development of the gonads.

SRY is the testis determining factor in mammals

In 1990, analysis of a Y-specific 35 kb DNA fragment in human XX males led to the isolation of *SRY*, which was shown to be conserved on the Y chromosome among all mammals tested (Sinclair, *et al.*, 1990). In cases of sex reversal in both humans and mice, the presence or absence of this gene correlated exactly with male or female phenotype (reviewed by Goodfellow and Lovell-Badge, 1993). In addition, XX transgenic mice carrying a 14 kb DNA fragment encoding mouse Sry showed normal male internal and external genitalia as well as normal male mating behavior (Koopman *et al.*, 1991). Thus, *Sry* is the only gene on the Y chromosome required for testis formation and it can promote sex reversal when located on an XX background. Taken together, all these data lead to the conclusion that *SRY/Sry* encodes the Y-linked testis determining factor.

In the mouse, the *Sry* transcript, which consists of a single exon, is expressed during a rather short interval between 10.5-12.5 dpc (Koopman, *et al.*, 1990; Hacker *et al.*, 1995; Jeske *et al.*, 1995). The location of *Sry* expression is probably pre-Sertoli cells since these cells are the first male specific cells to differentiate in the fetal testis. In experiments involving XX-XY chimeric mice, the Sertoli cell population is the only cell lineage which is almost exclusively composed of XY cells. This suggests direct action of the Y chromosome, and therefore *Sry*, in Sertoli cells (Palmer and Burgoyne, 1991; Patek *et al.*, 1991).

The length and constitution of the *Sry* open reading frame (ORF) varies between, but also within, different species, suggesting a rapid evolution of the *Sry* gene (Tucker and Lundrigan, 1993; Whitfield *et al.*, 1993; Coward *et al.*, 1994). The only conserved region is an HMG-box DNA binding domain of 79 amino acids. HMG-boxes have been found in many different proteins with DNA-binding properties and in agreement with this, SRY protein has been shown to bind DNA in a sequence specific manner *in vitro* (Nasrin *et al.*, 1991; Harley *et al.*, 1992, 1994; Ner, 1992). Upon binding, SRY induces a bend in the DNA (reviewed by Lovell-Badge, 1992). The significance of the DNA bending is unknown but has been suggested to effect transcriptional control by bringing different regulatory elements closer together (Pontiggia *et al.*, 1994). The carboxy terminus of mouse SRY is greatly extended when compared to human SRY. This region features a polyglutamine repeat which may be important for testis development since deletion or addition of an extra glutamine residue at this site correlates with sex reversal (Coward *et al.*, 1994). Polyglutamine repeat stretches are primarily found in transcription factors and the polyglutamine repeat found in mouse SRY has been shown to activate transcription when fused to the DNA binding domain of GAL4 (Dubin and Ostrer, 1994). The absence of polyglutamine repeats in human SRY could also be an explanation to why transgenic mice containing the human *SRY* gene do not show any sex reversed phenotype although the human *SRY* transcript is expressed in the genital ridge at 11.5 dpc (Koopman *et al.*, 1991). Maybe the mouse SRY glutamine repeat is important for protein-protein interactions and thereby promotes activation or repression of downstream genes. If this idea is true, humans must encode an additional factor which functions as a link between the DNA-binding SRY molecule and downstream target genes. Another possibility is that the variation in the CAG repeats only reflects the rapid evolution which occurs in *SRY/Sry* and the sole mechanism by which SRY acts may be to bring distant DNA sequences together by bending the DNA. So far, no specific DNA binding site or down stream target gene/s have been found *in vivo*.

Wt-1 is necessary for formation of the indifferent gonad

Wilms' tumor is a childhood kidney malignancy which has been associated with loss of a tumor suppressor gene (reviewed by Haber and Housman, 1992). In 1990 a candidate gene *(WT-1)* was cloned and the polypeptide sequence revealed the presence of four Zinc finger domains and a region rich in prolines and glutamines, suggesting that WT-1 is a transcription factor (Call *et al.*, 1990; Gessler *et al.*, 1990).

The evidence that WT-1 is important for gonadal development is compelling. First, individuals heterozygous for mutations in the *WT-1* gene, as in the WAGR or Denys-Drash syndrome, show genital malformations (reviewed by Hastie, 1992). Second, the predominant site for *WT-1* expression in the embryo is in the developing kidney and gonad (Pritchard-Jones *et al.*, 1990; Buckler *et al.*, 1991; Pelletier *et al.*, 1991; Armstrong *et al.*, 1992). Transcription is first detected at 9 dpc, shows very strong, uniform, expression in the genital ridge at 12 dpc and is confined

mRNA differential display

1. Reverse transcription:

   With one of four different primers:

   T(11)CA T(11)GC T(11)AG T(11)CT

   ----------TGAAAAAAAAAAAAAAAAAAAAAAAA
   ----------ACTTTTTTTTTTT

2. PCR amplification:

   3' primer - the same as used for reverse transcription
   5' primer - one from a set of 20 different arbitrary 10-mers

   ^{33}P-dATP is used in the PCR to label the amplified products. Theoretically, the combination of four different 3' primers and 20 different 5' primers should display all mRNAs expressed in a cell.

3. Denaturing polyacrylamid gel

4. Clone cDNA fragments from gel

5. Select correct clones by Northern hybridisation and sequence insert

6. In situ hybridisation on sections or whole mounts

7. Screen conventional cDNA and genomic libraries

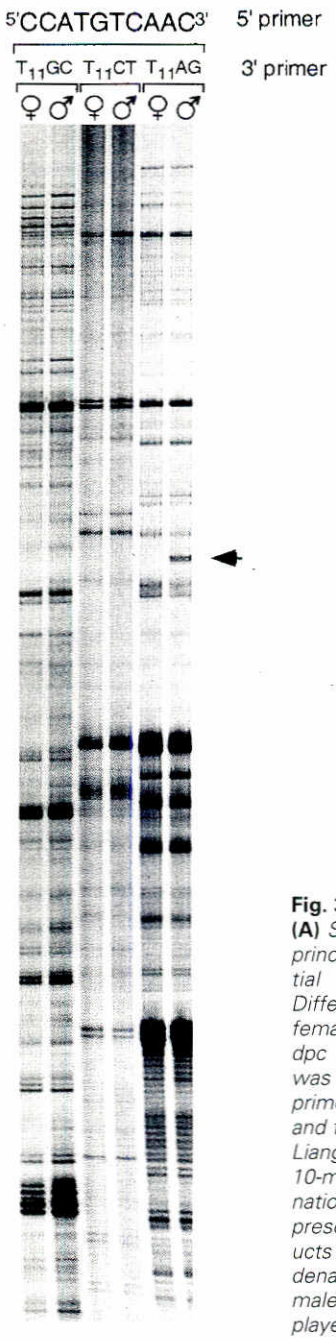

Fig. 3. mRNA differential display. **(A)** *Schematic picture showing the principle with the mRNA differential display technique.* **(B)** *Differential display of mRNA from female (♀) versus male (♂) 13.5 dpc genital ridge. Purified mRNA was reversed transcribed with a 3' primer ($T_{(11)}AG$ or $T_{(11)}CT$ or $T_{(11)}GC$) and then amplified as described by Liang and Pardee (1992) by using a 10-mer (CCATGTCACC) in combination with the 3' primer and in the presence of ^{33}P-dATP. PCR products were then separated on a 5% denaturing polyacrylamide gel. One male specific cDNA band is displayed (arrowhead).*

to Sertoli cells in adult testis and to follicle cells in the ovary. Third, targeted disruption of the murine *Wt-1* gene leads to failure of kidney and gonad development in embryos homozygous for the mutation (Kreidberg *et al.*, 1993). These embryos also have other severe defects, such as abnormalities in cardiac development and they became edematous at 12.5 dpc and die between days 13 and 15 of gestation. Gonadal development is arrested at an early stage in homozygous mutants, and by 12.5 dpc almost no gonadal tissue can be detected. Thus, *Wt-1* expression must be necessary for early commitment and maintenance of the gonadal tissue.

What is the function of WT-1 during gonadogenesis? In kidney development, the ureteric bud, which is an outgrowth of the Wolffian duct, induces the transformation of mesenchymal cells into epithelial cells in the metanephros (Grobstein, 1953). In mice lacking *Wt-1*, the ureteric bud is absent (Kreidberg, *et al.*, 1993). However, in wild-type embryos, *Wt-1* is expressed in the mesenchyme, and not in the Wolffian duct. WT-1 therefore conceivably acts via a signal to induce ureteric bud formation and subsequent epithelialization of mesenchymal cells. In genital ridge, it is not known whether WT-1 acts via a signal mechanism or not, but it seems likely that WT-1 is involved in some way in the transformation of mesenchymal cells into epithelia-like cells, which may later become pre-Sertoli cells. So far, no connection has

been made between WT-1 and *Sry*. The possibility exists that WT-1 acts as a transcription factor regulating the *Sry* promoter. However, the early expression of *Wt-1* at 9 dpc and its wider distribution shows that other genes would also be required.

SF-1 is an orphan nuclear receptor with dual functions in sex development

Steroidogenic factor 1, SF-1 (or adrenal 4-binding protein, Ad4BP) is an orphan member of the nuclear hormone receptor family and was initially isolated as a regulator of steroid hydroxylase expression in adrenal cortical cells, Leydig cells and Theca cells (Lala *et al.,* 1992; Honda *et al.,* 1993). Recent findings suggest that SF-1 is also active in Sertoli cells where it binds to a conserved element in the MIS promoter, thereby stimulating MIS expression (Shen *et al.,* 1994). However, SF-1 expression is detected as early as 9-9.5 dpc in the urogenital ridge, in contrast to MIS which is turned on around 12.5, suggesting additional roles for SF-1 (Münsterberg and Lovell-Badge, 1991; Ikeda *et al.,* 1994).

The gene expressing SF-1, which is called *Ftz-F1* because of its homology to the *Drosophila* orphan nuclear receptor *fushi tarazu* factor 1, has been subjected to targeted gene disruption (Luo *et al.,* 1994). Independent of the presence or absence of a Y chromosome, all SF-1 deficient mice showed female internal and external genitalia, no gonad tissue or adrenal glands, and they died around day 8 after birth due to adrenocortical insufficiency. The characteristics of gonadogenesis in SF-1 deficient mice closely resemble the phenotype of the *Wt-1* disrupted mice (Kreidberg, *et al.,* 1993). The urogenital ridge showed nearly normal appearance at 10.5 dpc, although the gonadal portion was slightly smaller when compared to wild-type. At 12.5 dpc almost no gonadal tissue could be found and the few cells remaining showed characteristics of undergoing programmed cell death. Thus, in addition to MIS and the steroid hydroxylase genes, SF-1 must activate other genes which are of importance for early gonadal development.

Most nuclear receptor ligands known are small molecules such as steroids and retinoic acid (Trai and O'Malley, 1994). Experiments where the ligand binding domain of SF-1 has been deleted, suggest the presence of a Sertoli cell specific ligand (Shen *et al.,* 1994). A steroid hormone could be one ligand candidate since Sertoli cells are located close to the steroid-producing Leydig cells within the developing testis, but also because SF-1 is mainly being expressed in steroid-producing cells. Not much is known about steroid production during early gonadal development and in patients with mutations in the known steroid hydroxylase genes, the testes appear normal which argues against this idea (reviewed by Grumbach and Conte, 1992). However, there is a small possibility that novel, fetal specific, steroid hydroxylase genes exist which are responsible for steroid production in developing gonads.

All three factors mentioned above are potential transcription factors. SRY with an HMG box, WT-1 containing zinc fingers and proline/glutamine rich domain, and SF-1 with its close resemblance to nuclear receptors. The only known molecules acting downstream of these factors in the male sex development pathway are two hormones, MIS and testosterone. MIS is a member of the transforming growth factor ß (TGF-ß) family and it is the first product to be secreted from developing Sertoli cells (reviewed in Josso *et al.,* 1993). Patients with mutations in MIS, with persistent Müllerian duct syndrome, show normal testis phenotype, suggesting that MIS is not required for testis development as such. Therefore, another signaling molecule must exist to link SRY expression and Sertoli cell development with Leydig cell differentiation and testosterone production.

Testosterone is a steroid hormone which is produced from cholesterol through the steroid biosynthetic pathway involving four different enzymes: P-450scc, P-450c17, 3ß-HSD and 17ß-HSD (Miller, 1988; Nelson *et al.,* 1993). Functional disruption of any of the genes encoding these enzymes give rise to male pseudohermaphroditism with incompletely masculinized genital ducts and/or external genitalia. However, the gonads in patients with these conditions are always testes. Thus, testosterone, in addition to MIS, is not required for testis development.

So far, the only well characterized interactions between proteins and promoters in the sex development pathway are the ones between SF-1 and the *MIS* promoter, and SF-1 and the promoter regions of genes encoding steroid hydroxylases as P-450scc, P-450c17 and P-450 aromatase (Morohashi *et al.,* 1992; Lynch *et al.,* 1993; Shen *et al.,* 1994). SRY has also been suggested to bind to upstream sequences of both the P-450 aromatase promoter and the *MIS* promoter, although these data are conflicting (Haqq *et al.,* 1993, 1994; Shen *et al.,* 1994). It remains possible that SRY is involved in initial activation of these promoters and that SF-1 is subsequently required for maintenance of P-450 aromatase and MIS expression.

Hunting for novel genes

One of the more successful ways of finding genes important for sexual development has been through the study of humans showing different types of sex reversal. *SRY* and the majority of the steroid hydroxylase genes were identified in this way. In addition, male to female sex reversal has been found in Campomelic dysplasia (CD) which is a disorder characterized by an array of skeletal and extraskeletal defects. Both CD and sex reversal coupled to CD have been shown to be caused by mutations in an *SRY*-related gene, *Sox*9 (Foster *et al.,* 1994; Wagner *et al.,* 1994). Another form of male to female sex reversal shows X-linked inheritance. The locus has been mapped to Xp21 and named DSS (Dosage Sensitive Sex reversal) as two active copies of this locus are thought to cause sex reversal in 46XY individuals (Bardoni *et al.,* 1994). In late 1994, a novel gene named *DAX-1* (DSS-AHC critical region on the X gene 1) was isolated from the DSS locus and found to encode a new member of the nuclear hormone receptor family (Muscatelli *et al.,* 1994). Since males deleted for *Dax-1* show normal testes, it has been suggested that *Dax-1* has a role in ovarian development and/or functions as a link between ovary and testis formation. However, there still exist some novel genes to hunt for. For example, a small percentage of 46XX males possess no Y sequence, implying the presence of mutations in genes downstream of *SRY*.

An alternative way of finding novel genes important for sex differentiation is to compare mRNA populations from developing male and female gonads. Current methods to distinguish mRNA from different organs, developmental stages etc., have so far relied largely on the use of the subtractive hybridization technique. This technique is rather difficult to establish and requires

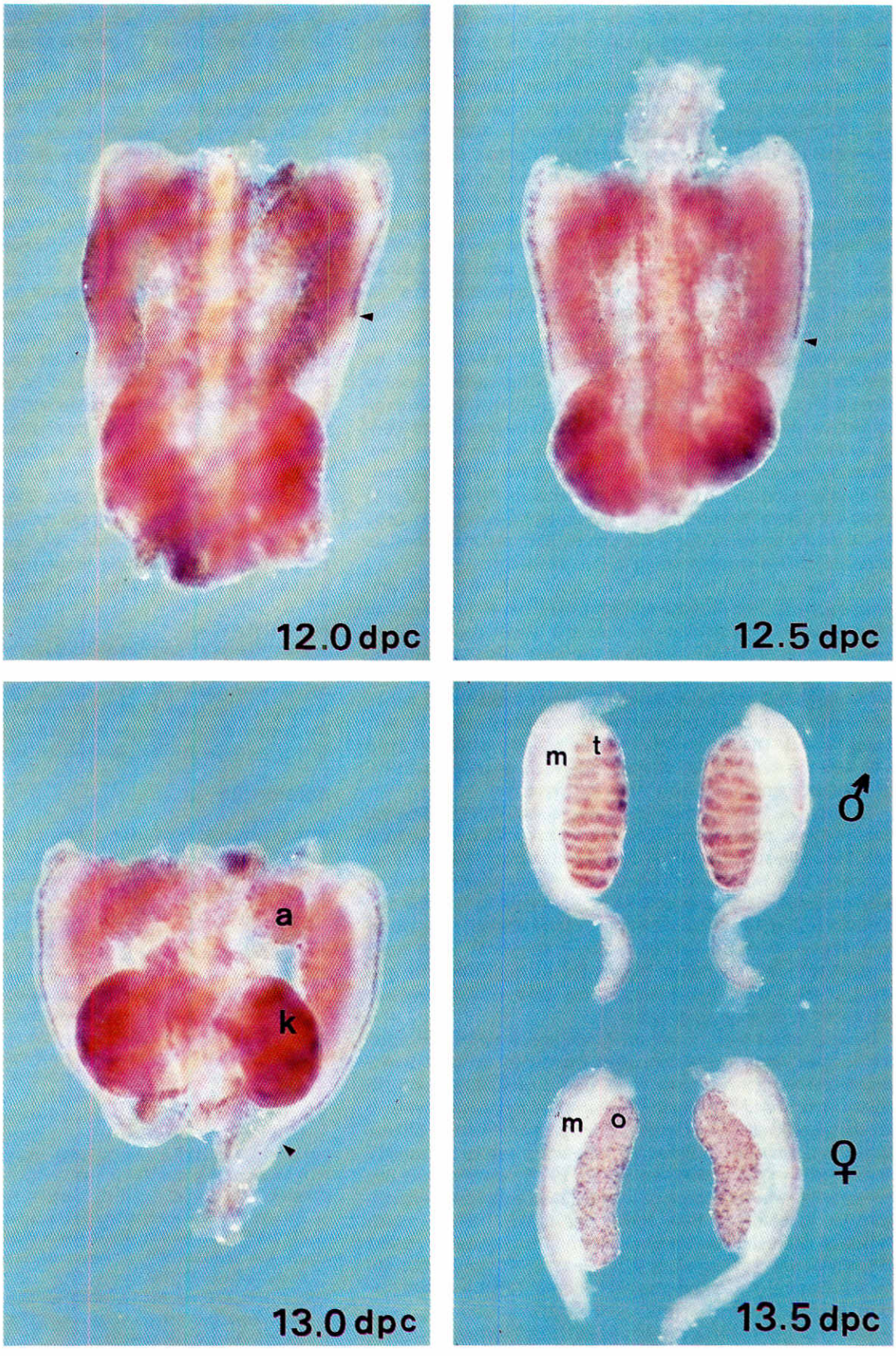

Fig. 4. Localization of TSG67 mRNA in the uro-genital tract, from 12.0, 12.5, 13.0 and 13.5 (gonads only) dpc old embryos, determined by whole-mount *in situ* hybridization (Wilkinson, 1992). *TSG67 expression (shown in purple-brown) is present in progressing Müllerian ducts (arrowheads), developing kidneys (k), adrenals(a), testis (t) and ovary (o), but not in mesonephric mesenchyme (m) or Wolffian ducts (shown on sections, data not shown).*

large amounts of RNA. In 1992 a new method to isolate differentially expressed genes was presented by Liang and Pardee, named mRNA differential display (Liang and Pardee, 1992). The basic principle with this technique is to display subsets of mRNAs with the help of reverse transcription and polymerase chain reaction (PCR) (reviewed by Watson and Margulies, 1993). Two different kind of primers are used. One oligo-dT primer consisting of 11 T's plus two additional 3' bases to provide specificity. This primer anneals to the beginning of the polyadenylated tail of a subpopulation of mRNAs and is used for the reversed transcription reaction as well as the PCR. The second primer, which is used in the PCR, is a decamer with an arbitrarily defined sequence so that it anneals to different positions upstream of the oligo-dT primer. After the PCR, cDNA fragments representing the 3' termini of mRNAs, are separated by size on a denaturing polyacrylamide gel. Different subpopulations of mRNAs can be detected and displayed on the gel by using various sets of primers (Fig. 3). By comparing cDNA populations from developing female and male genital ridges, it should be possible to identify both female and male specific cDNAs at the same time. There are several advantages in the use of the mRNA differential display technique. It allows for simultaneous detection of both groups of differentially expressed genes and it is rapid. In addition, it is very sensitive due to the use of PCR, making it possible to isolate cDNAs corresponding to rare transcripts.

We have chosen to use mRNA from 13.5 dpc male and female gonads. The reasons for using this timepoint are several. It is easy to distinguish between male and female gonads. Thus, contamination of female mRNAs in the male mRNA population, and vice versa, will be reduced to a minimum. Also, at 13.5 dpc, the male gonad has been developing for 1-2 days and genes involved in this process will most likely be expressed (as for *WT-1* and *Ftz-F1*). The first event of female development, i.e., germ cells entering meiosis, has started and this gives us the possibility to isolate genes important for ovary formation.

To date, we have cloned four sex-specific cDNA fragments. Two of these, cDNA4 and cDNA80, which both were isolated from the male-specific mRNA differential display, show perfect homology with two steroid hydroxylase genes when compared with the EMBL data base. cDNA4 is equivalent to 3ß-HSD and cDNA80 to P-450c17 (Nordqvist and Lovell-Badge, in preparation). These results are very encouraging since they show that it is possible to use the mRNA differential display method for isolating at least male but probably also female specific genes expressed in developing gonads.

Several genes such as *Sry, Wt-1, MIS* and *Ftz-F1* are, in addition to their fetal expression, also expressed in adult testis. Based on this fact, we have developed a second approach to isolate male specific genes involved in gonadal development. In collaboration with Dr. Christer Höög, Karolinska Institute, Stockholm, who has isolated 52 different adult testis specific clones (Höög, 1991; Starborg *et al.,* 1992), we are currently investigating whether some of these clones show fetal expression. Up to date, we have found one cDNA, TSG67, which shows an interesting expression pattern in fetal gonads. TSG67 shows a low level of homology to the yeast SRP1 gene, although the significance of this homology is not clear. SRP1 was first isolated as an extragenic suppressor of mutations in the large subunit of RNA polymerase I (Yano *et al.,* 1992). When analyzing the mRNA expression pattern of TSG67 in developing uro-genital tract, it seems that the gene is expressed in proliferating cells, in both the kidney and the genital ridge (Fig. 4). In the mesonephros, TSG67 mRNA expression is restricted to the developing Müllerian duct. Thus, TSG67 could be used as a marker for Müllerian duct development. At 13.5 dpc, the TSG67 mRNA is expressed in both the testis and the ovary. In the testis, expression is confirmed to the testis cords, but the cell type remains unclear. TSG67 mRNA expression is down-regulated in both the female and the male gonad around 14.5 dpc. We are now investigating whether this decrease in expression is coupled to meiotic and mitotic arrest of germ cells.

With the help of the experimental approaches outlined above we hope to find some novel genes important for sex differentiation. Theoretically, there are still some predicted molecules/proteins missing and worth hunting for. As mentioned previously, at least two different signals could be envisioned which are of importance for cell differentiation and migration in the indifferent gonad. There must also be downstream genes acting directly after SRY which are involved in Sertoli cell differentiation. In addition, a signal between Sertoli cells and pre-Leydig cells must be present to tell pre-Leydig cells when to start to differentiate. There must also exist Leydig cell specific proteins which, in combination with SF-1, promote cell specific expression of the steroid hydroxylases. Lastly, there must be at least some specific genes involved in ovarian development.

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