DEVELOPMENT AND DIFFERENTIATION OF THE EMBRYONIC CHICK GONAD: A MORPHOLOGICAL AND MOLECULAR STUDY.

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CONTENTS.

ABSTRACT.

DEVELOPMENT AND DIFFERENTIATION OF THE EMBRYONIC CHICK GONAD: A MORPHOLOGICAL AND MOLECULAR STUDY.

The testis and the ovary are two structurally distinct organs that can differentiate from the same tissue primordia. In avian species, the gene interactions that determine and maintain which developmental pathway is followed are poorly understood and/or unknown. In mammals, the timing of sex determination and a small number of the genes involved in gonadal development have been established. However, other so far unidentified genes are clearly involved in gonadal development, in avian and other species. Chick gonadal development was investigated by studying the morphology of the developing gonads and by attempting to gain a better understanding of the genes involved in the initial stages of this developmental process. The first approach was a histological analysis of chick gonadal development over the period from the indifferent gonad stage to the initial stages of ovary and testis differentiation. Secondly, we analyzed the expression of chick homologues to genes involved in mammalian gonadal development, in an attempt to compare gene regulation and timing of gonadal development between mammals and avians. Finally, we used the technique of differential display to both ascertain the feasability of this approach in the identification of transcripts in a complex developmental system and to isolate novel genes involved in chick gonadal development. As a result of this study we were able to predict that the sex determination event in chicks occurred earlier in embryogenesis than previously documented. We also established that genes involved in mammalian gonadal development were expressed with similar expression profiles in the developing chick gonads. Finally, twelve candidate clones were isolated by differential display, five of which represented novel sequences. Expression profiles, during chick gonadal development and differentiation, were analyzed by Northern analysis and whole mount *in situ* hybridization to confirm differential expression.

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Declaration.

The completion of this thesis and experiments described are my own work, unless specifically stated in the text. No part of this work has been, or will be, submitted for any other degree, diploma or qualification.

ABBREVIATIONS.

aa	- amino acids
ATP	- adenosine 5' triphosphate
bp	- base pairs
BSA	- bovine serum albumin
cDNA	- complementary DNA
d	- days of incubation (chick)
DNA	- deoxyribonucleic acid
dNTP	- deoxynucleotide triphosphate
dpc	- days *post coitum*
DTT	- dithiothreitol
EGTA	- ethylene glycol bis(-amino-ethyl-ether)-N,N,N'-tetra-acetic acid
EtBr	- ethidium bromide
kb	- kilobase pairs
M	- Molar
ml	- millilitre
µl	- microlitre
mM	- milliMolar
µM	- microMolar
mRNA	- messenger ribonucleic acid
U.V.	- ultra-violet light
wmISH	- whole mount *in situ* hybridisation
5'	- 5' terminal phosphate of DNA/RNA molecule
3'	- 3' terminal phosphate of DNA/RNA molecule

Additional abbreviations are explained within the text.

Chapter 1

Introduction.

1.1 General Introduction.

Sexual development can be considered as three separate processes. Firstly there is the establishment of genotypic sex at fertilisation. This is followed by gonadal development which includes a sex determination event directing the indifferent gonads down either a testicular or ovarian developmental pathway. Finally, differentiation of the accessory ducts and male and female phenotype by endocrine function of the testis or ovary. Ultimately, sex chromosome content determines the sexual phenotype of an embryo in mammals and birds. If a Y chromosome is present in mammals, a testis will form ultimately leading to a mature male sexual phenotype. It is the presence of a testis that ensures (under the influence of hormone action) that the internal and external male genitalia develop correctly. If a testis is not present, female internal genitalia will form regardless of whether an ovary has developed or not. Although, in mammals, a gene on the Y chromosome acts as a dominant genetic switch resulting in testicular development, there is evidence that genes on the X chromosome and the autosomes are required for correct gonadal development.

In avian species the female is the heterogametic sex (ZW) and the mechanism of sex determination is unknown. Not only is the nature of the switch that causes testicular or ovarian development unknown, but regulatory genes involved in indifferent gonad formation, and downstream genes that influence and control ovarian or testicular differentiation are also unknown.

The mechanisms responsible for sex determination are not conserved between different classes. For example the gonadal development system in *Drosophila* and *C. elegans,* relies upon the X chromosome to autosome ratio as the master sex determination switch (Parkhurst and Meenely, 1994), and in some species of reptile the sex determining mechanism is based on the incubation temperature of the eggs.

Although a few genes have been assigned putative roles in mammalian gonadal development, it remains a complex process which is still poorly understood with regard to origin of cell precursors and gene regulation of cell differentiation, in both mammalian and avian species.

1.2 Gross morphology of vertebrate gonadal development.

1.2.1. Initial development

The urogenital ridge arises from the intermediate mesoderm in mammals and birds. The intermediate mesoderm gives rise to three kidney systems in mammals and birds: the pronephros, mesonephros and metanephros (Kaufman, 1992; Capel and Lovell-Badge, 1993; Capel, 1996). This urogenital ridge system arises in mice at 9 days *post coitum* (dpc) (Kaufman, 1992; Capel, 1996) and between 3.5 to 4 days of incubation (d) in the chick (Romanoff, 1960; Swift, 1915; Venzke, 1954a). On the

ventral side of the pronephros, segmented mesoderm fuses to form the mesonephric (Wolffian) duct (Capel and Lovell-Badge, 1993), and the mesonephros forms posterior to the pronephros. Invagination of the epithelium covering the urogenital ridge gives rise to the Müllerian duct, and runs parallel with the mesonephric duct (Capel and Lovell-Badge, 1993; Langman, 1975). The genital ridge develops on the surface of the mesonephros, and is visible in mice at 10.5dpc (Capel, 1996; Capel and Lovell-Badge, 1993) (fig 1). At this stage of development there is no difference between the male and female genital ridge and it is referred to as the indifferent or bipotential gonad. Posterior to the mesonephros, the metanephros forms the definitive kidney (Nelsen, 1953; Capel, 1996).

1.2.2. Development through the indifferent stage.

1.2.2.1. General morphology.

Mice have a gestation period of 21 days while the chick incubates for 21 days before hatching. Development of the indifferent gonad proceeds from 10.5dpc through to 12.5dpc in the mammal (Capel and Lovell-Badge, 1993). In the chick there is contention regarding the length of time of indifferent gonad development; ranging from 3.5d to 5d through to 3.5d to 8d (Romanoff, 1960). In chicks and mammals, the indifferent gonad initially consists of one layer of cells, called the germinal epithelium, covering mesenchymal connective tissue (Venzke, 1954a). During development, the germinal epithelium becomes composed of several cell layers and the mesenchyme condenses to form the stroma of the gonad (gonadal blastema) (Nelsen, 1953; Venzke, 1954a; Romanoff, 1960; Torrey, 1966; Byskov, 1986).

At the indifferent stage of gonadal development in mammals and birds the majority of primordial germ cells (PGCs) migrate into the germinal epithelium of the gonads (Venzke, 1954a, Romanoff, 1960; Nelsen, 1953; Kaufman, 1992; McLaren, 1995) (fig 1B). PGCs are distributed evenly in the germinal epithelium of the indifferent gonad before 4d (Romanoff, 1960). After 4d, the PGCs are asymmetrically distributed between the left and right gonad, with approximately 70% PGCs in the left gonad and 30% in the right gonad in both sexes (Swift, 1915; Witschi, 1935; Venzke, 1954a; Romanoff, 1960). Active migration of PGCs from the right to the left gonad has been observed (Witschi, 1935; Thorne, 1995).

During the period of PGC invasion, induction of the primary sex cords begins in mammals followed by or simultaneous with rete tissue formation (figure 1B and 2A &B). In birds primary sex cords develop after the rete cords (Romanoff, 1960; Byskov, 1966; Thorne, 1995), and are derived from the germinal epithelium and appear as protrusions migrating into the mesenchymal region of the gonad. Primary

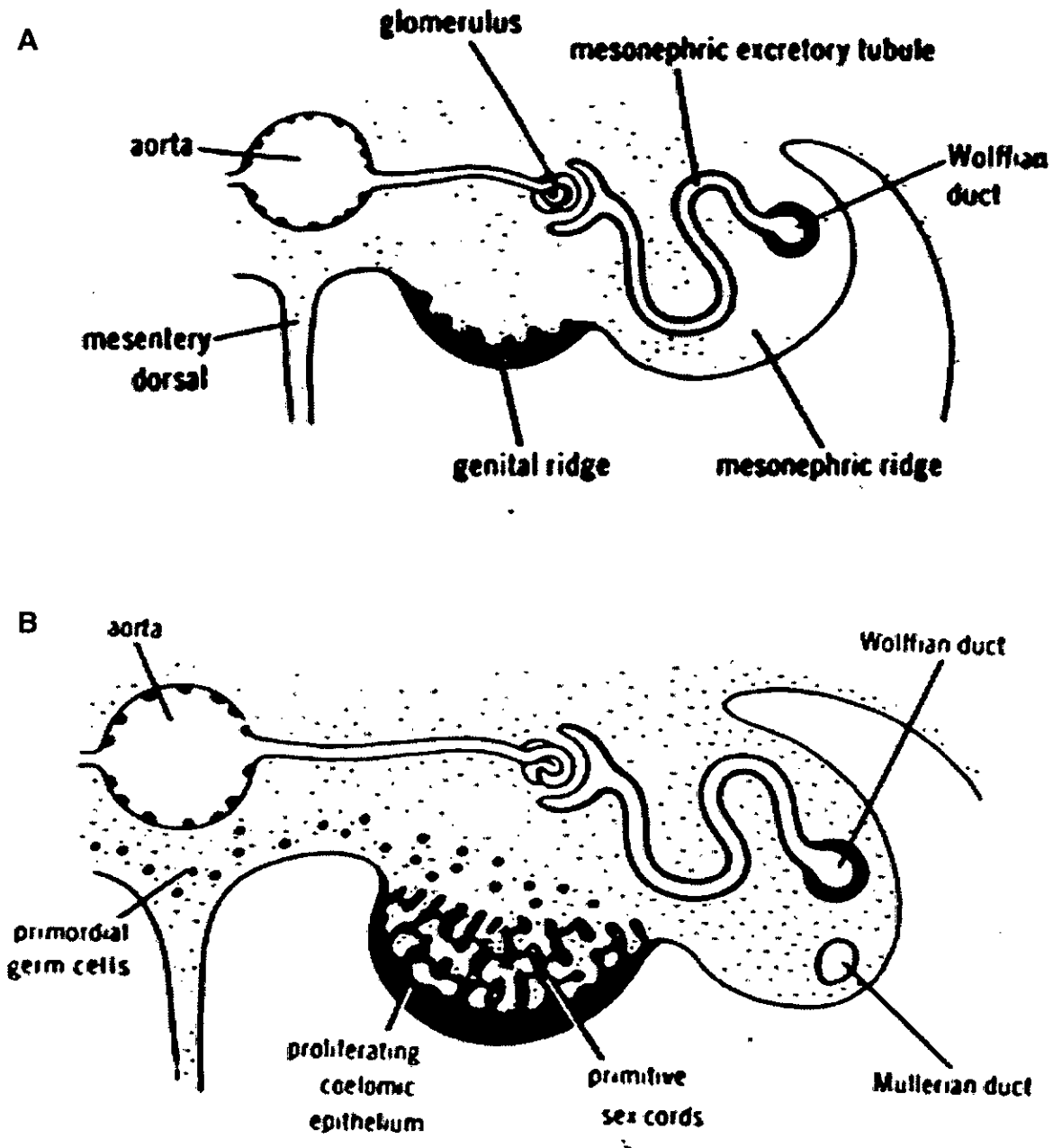


Figure 1: A, Schematic representation of the development of the mammalian genital ridge and its position in relation to the mesonephros. B, Schematic representation of the mammalian indifferent gonad, illustrating the development of the primary sex cords and the migration of the primordial germ cells. Figure from Langman, 1975.

sex cords arise between 5d and 5.5d (Lillie, 1908; Venzke, 1954a; Romanoff, 1960). Rete cords arise in the indifferent gonad of the mammal through cell migration from the mesonephric tubules, followed by condensation of these cells to form connections between the primary sex cords and the mesonephric duct (Byskov, 1978; Torrey, 1966). In the chick, rete cords may arise directly from the mesonephric tubules (Byskov, 1978) or by condensation of the mesenchyme to form a branching network in the anterior of the gonad (Romanoff, 1960; Witschi, 1935).

1.2.3. Development after sex determination.

Testis determination in mammals is dependent on the expression of a gene on the Y chromosome between 10.5 and 12.5 dpc (Hacker *et al.*, 1995), which results in the indifferent gonad following a testicular pathway of development. In the chick, the timing of sex determination and the switch causing male or female differentiation is unknown, and reports on the timing of the sex determination switch range from 5d to 8d (Romanoff, 1960; Hutson *et al.*, 1983).

1.2.3.1. Differentiation of the Testis.

Differentiation of the testis is characterised by the continued proliferation of the primary sex cords into seminiferous tubules (testicular cords) (figure 2C) (Nelsen, 1953; Romanoff, 1960; Langman, 1975; Satoh, 1985; Thorne, 1995). Testicular cord formation is discernible in the chick from 7.5d (Venzke, 1954b) and apparent by 13 dpc in the mammal (Kaufman, 1992). The developing seminiferous cords consist of an outer layer of Sertoli (supporting) cells surrounding the PGCs (Venzke, 1954b; Romanoff, 1960; Torrey, 1966; McCarrey and Abbott, 1979; Byskov, 1986).

The germinal epithelium of the developing testis becomes a flattened membrane of one cell thickness (Nelsen, 1953; Venzke, 1954a; Venzke, 1954b). The testicular cords initially remain attached to the germinal epithelium, however, an intact basal lamina forms along this germinal epithelium and the cords detach and become separated from this epithelium by a layer of fibrous connective tissue called the tunica albuginea; a characteristic structure of the testis (Nelsen, 1953; Venzke, 1954b) (fig 2C).

The testicular rete cords develop and differentiate into rete tubules (Romanoff, 1960; Langman, 1975). The rete tubule is composed of a central lumen surrounded by a layer of flattened epithelial cells (Romanoff, 1960). Rete tubules become continuous with the semniferous tubules and connect these tubules to the Wolffian duct. These tubules ultimately become the efferent ductules of the epididymis (Nelsen, 1953; Romanoff, 1960; Langman, 1975; McCarrey and Abbott, 1979).

After initial testicular cord development, the connective and interstitial tissue of the stroma begins to proliferate to form Leydig cells and blood vessels, developing between these seminiferous tubules. It is the proliferation of the stroma that causes an increase in the size of the testis (Venzke, 1954b), and forms the basement membrane of the seminiferous tubules (Nelsen, 1953; Venzke, 1954b; Langman, 1975). The Leydig cells are the steriod producing cells of the testis and produce testosterone, the hormone essential for the development of the Wolffian duct into male accessory structures (Romanoff, 1960; Byskov, 1986). Leydig cells have been identified in the testis of the chick from 10d (Carlon and Erickson, 1978).

By 13d in the chick, the PGCs begin to actively divide (Romanoff, 1960b). In the mouse, germ cells continue to proliferate for a few days after they have entered the genital ridge (McLaren, 1991; McLaren, 1995). This proliferation continues for approximately 4 days, after which time the cells are arrested in the G1 phase of the cell cycle (13.5 dpc in the mouse), and are designated spermatogonia (Venzke, 1954b; Romanoff, 1960a; McLaren, 1995). Once a lumen has formed in the seminiferous tubules they have a characteristc appearance whereby successive stages of spermatogenesis can be observed; with the spermatogonia at the periphery of the tubule and spermatids in the lumen of the tubule (Romanoff, 1960).

Initially there are two sets of ducts, the Wolffian (mesonephric) and Müllerian ducts. In male reptiles, amphibia, birds and mammals it is the Wolffian ducts that develop, while the Müllerian ducts regress (Nelsen, 1953; Romanoff, 1960). This regression in the chick begins on 8d and is complete by 12d (Romanoff, 1960). The Wolffian duct develops into the vas deferens and the mesonephric tubules linking it to the testis are called the vasa efferentia (Nelsen, 1953; Torrey, 1966; Langman, 1975).

1.2.3.2. Differentiation of the ovary.

During initial ovarian development, the germinal epithelium continues to proliferate (fig 2D) (Swift, 1915; Nelsen, 1953; Venzke, 1954a; Romanoff, 1960; Torrey, 1966). This thickening begins at approximately 7d in the chick, a similar time to documented testicular cord development (Venzke, 1954a). In mammalian species, ovarian development is reported to begin after testicular development (Nelsen, 1953). PGCs in birds are evenly distributed within this proliferating germinal epithelium (Romanoff, 1960), and begin dividing by 8d to form oogonia (Swift, 1915; Romanoff, 1960). In the female mice, germ cells enter meiotic prophase at 12.5 dpc (McLaren, 1995).

Characteristic to the female, there is a second proliferation of sex cords (fig 2D).

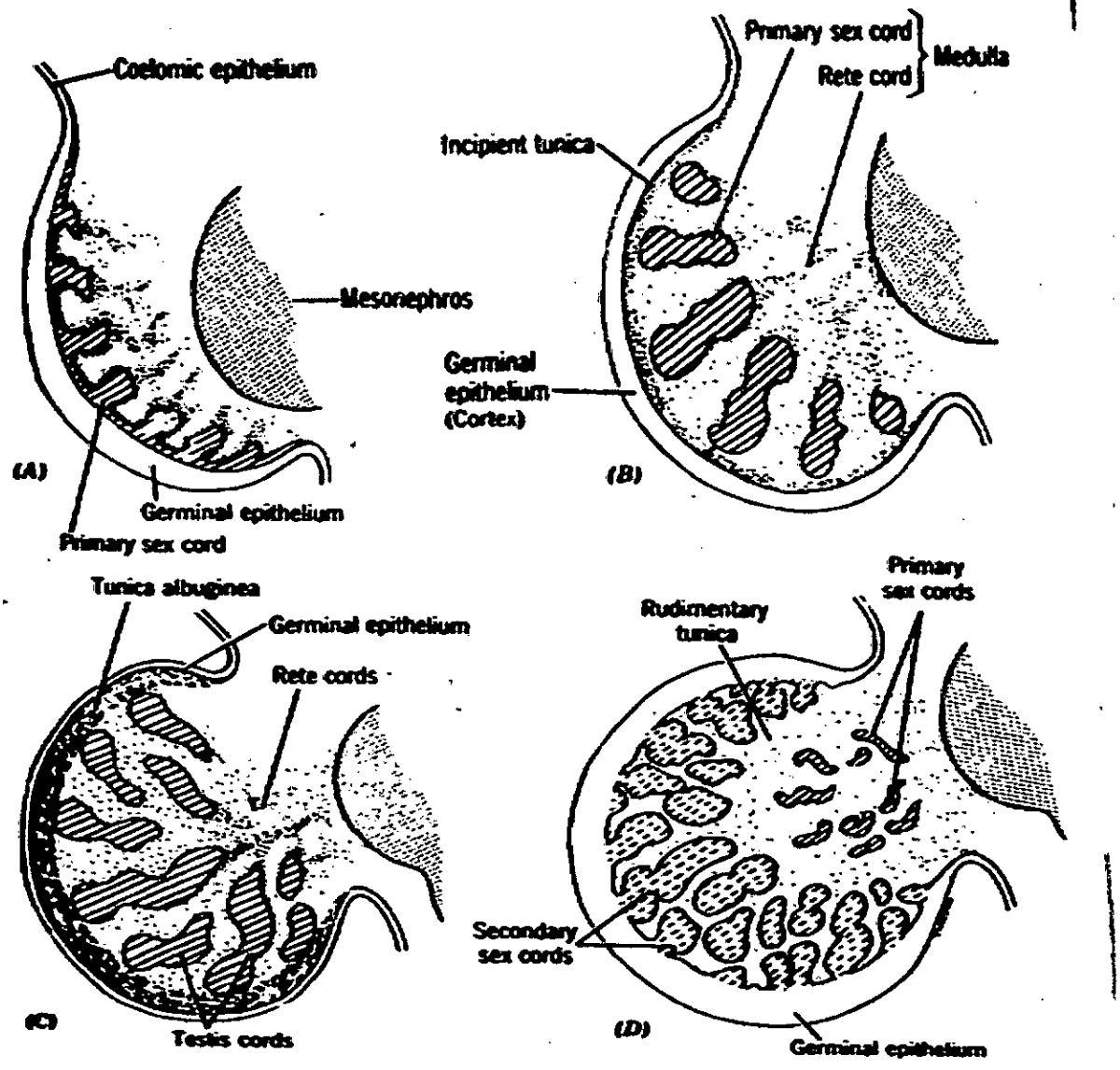


Figure 2: Schematic representation of the developing mammalian gonads. A & B, Development of the indifferent gonad, showing the medullary and cortical components. C, Developing embryonic testis. D, Developing embryonic ovary. Figure from Torrey, 1966.

Several oogonia and a small number of germinal epithelial cells cluster together and form cortical cords, which penetrate inwards to the medulla and eventually become ovarian follicles (Nelsen, 1953; Romanoff, 1960; Langman, 1975; Thorne, 1995). Initially, the medulla and the cortex of the ovary are separated by a thin layer of the tunica albuginea. However, after cortical cord formation, a connective tissue stroma develops between the cortical cords; called the ovarian albuginea, separating cords from one another and from the medulla (Nelsen, 1953; Romanoff, 1960; Langman, 1975).

The medullary (primary) cords enlarge and distend, causing an increase in ovarian size in the chick (Romanoff, 1960; Swift, 1915). These cords can contain isolated PGCs or consist of clear or fat-laden cells (Romanoff, 1960). In the mammal, the medullary cords degenerate and become pressed inwards, due to the invasion of the cortical cords, and form the primary medulla. Later in development, a secondary medulla forms composed of connective tissue and blood vessels (Nelsen, 1953).

Interstitial cells, involved in steroid production, are present in human ovaries during foetal development (Byskov, 1986) and are thought to differentiate at the beginning of follicular development. In the chick these cells have been reported to populate the ovary after hatching (Romanoff, 1960). However, Narbaitz and Adler (1966) detected cells in the medulla with large vesicles containing lipid deposits, characteristic of steroid-producing cells, after 8d.

Asymmetrical ovarian development is a feature in the chick that is not present in the mammal (Witschi, 1935; Nelsen, 1953). The right gonad in the female does not develop as extensively as the left gonad, and is considered a rudimentary structure (Romanoff, 1960). The medulla does distend, but is much thinner and the cortex consists of a few cell layers that eventually degenerate (Romanoff, 1960; Thorne, 1995). Witschi (1935) suggested that the right ovary does not develop because of limited space within the body cavity. The oviduct size is dependent on the type of egg produced and considering the size of the large avian egg, the oviduct can easily fill the whole of the body cavity (Witschi, 1935; McCarrey and Abbott, 1979).

The ductal system in female avian species is also asymmetric. The right Müllerian duct in the female chick does not develop after 8d and degenerates between 9d and 16d. The left Müllerian duct develops to form the oviduct (Romanoff, 1960). The Wolffian ducts regress as it requires the production of testosterone for its maintenance (Capel and Lovell-Badge, 1993).

1.2.4. Cell contribution to the somatic cell component of the indifferent gonad.

Contributions to the somatic cell component of the indifferent gonad are not fully understood. It is thought that cells from the coelomic epithelium, the tubules of the mesonephros and the mesenchyne of the mesonephros could all contribute, although these contributions are not thought to be sex specific at the indifferent stage of development (Capel, 1996). The mesenchyme of the gonad is continuous with the mesenchyme of the mesonephros (Venzke, 1954a), suggesting cells from the mesonephros could migrate and populate the indifferent gonad.

Rodemer-Lenz (1989) carried out dye labelling and quail-chick chimera experiments which showed that the surface epithelium contributes cells to the indifferent gonad. The mesonephros does not contribute cells until between 6d and 7d (Rodemer-Lenz, 1989), suggesting the mesonephros is important, but not before the point of sex determination. Studies carried out in mice by Karl and Capel (1995) and Martineau *et al.* (1997), support the theory that the mesonephric contribution occurs at the time of and after sex determination. However, these experiments study a narrow window of development, so earlier cell migration from the mesonephros cannot be discounted. Popova and Scheib (1981), using similar experimental methods, have shown gonadal development occurs in the absence of the mesonephros, although development is abnormal. The experiments carried out by these different groups all show abnormal gonadal development occurs, but the conclusions drawn do not confirm the mesonephros is essential in this process.

Other evidence suggests the coelomic epithelium invaginates and cells enter to populate the somatic component of the gonad (Smith and MacKay, 1991; Capel and Lovell-Badge, 1993) and form the medulla of the indifferent gonad (Jordanov *et al.,* 1978; Byskov, 1986). However, some researchers define the somatic supporting cells of the indifferent gonad as originating from both the mesonephros and coelomic epithelium (Wartenberg, 1983; Smith and McKay, 1991; Kanai *et al.,* 1989).

1.2.5. Cell Contribution to the developing gonads.

The majority of work carried out to study the cell contribution to the developing gonads has been performed in mammals. Karl and Capel (1995) have shown by electron microscopy that cellular bridges extend from the mesonephric tubules to the gonad in both male and female mice. Initial development of these bridges is unknown but they disappear by 12dpc in the mouse, suggesting mesonephric cells contribute to male and female gonads (Karl and Capel, 1995). Gonads from 11.5 dpc, cultured separately from the mesonephros failed to develop testicular cords, indicating the

importance of the mesonephros (Buehr *et al.*, 1993; Merchant-Larios *et al.*, 1993). In females, contribution to the gonad after the sex determination event is poorly documented. McLaren and Buehr (1990) reported normal, but small ovaries when the 11.5 dpc female genital ridge was separated from the mesonephros. It is possible the mesonephros is not required in mammalian female sexual development because an efferent duct system is not required to transport the germ cells to the outside.

Recent experiments have been carried out where an 11.5 dpc XX or XY genital ridge is grafted onto a mesonephros (either XX or XY) that constitutively expressed β-galactosidase. Blue cells migrated into the XY genital ridge but not the XX genital ridge, suggesting male-specific cell migration from the mesonephros contributing to the XY gonad. Cells from the mesonephros gave rise to three types of cells. Firstly, the myoid cells that partition the seminiferous cords from the interstitial cells, the myoepithelial cells and endothelial cells which are involved in blood vessel formation (Martineau *et al.*, 1997). A piece of an XY genital ridge was grafted on top of an XX genital ridge (both 11.5 dpc), and this was grafted onto a constitutively expressing β-galactosidase mesonephros. Blue cells migrated through the XX genital ridge into the XY genital ridge, suggesting a diffusible factor from the XY genital ridge induces this migration from either an XX or XY mesonephros (Martineau *et al.*, 1997). In contrast, in chick embryos from 7d to 10d in which a quail-chick chimeric mesonephros had been grafted onto a chick gonad (Rodemer *et al.*, 1986), quail cells were observed in the stroma of the ovarian medulla. In male embryos, only a few groups of quail cells were reported to colonize the gonad.

1.3. Mechanisms of sex determination.

The mechanism for sex determination, employed by different species, can be extremely varied. They include: behavioural, environmental and chromosomal sex determination mechanisms. Behavioural sex determination is dependent upon social structure and interactions in a population to induce sex reversal in an adult. Certain species of fish exhibit behavioural sex determination and it is thought changes along the hypothalamo-pituitary-gonadal axis are required for structural reorganisation of the gonad (Larson, 1997).

1.3.1. Temperature dependent sex determination.

Temperature dependent sex determination (TSD) results in gonadal sexual differentiation being sensitive to the incubation temperature of eggs at a certain time

period during embryonic development (Bull, 1980), and is associated with species lacking heteromorphic sex chromosomes e.g. crocodile (Johnston *et al.,* 1995).

Three different patterns of TSD have been documented (Ewert *et al.,* 1994; Pieau, 1996). The first pattern shows that a low temperature yields 100% or predominantly males and a high temperature produces 100% or predominantly females. The second pattern is the reverse situation of the first. Both of the first two patterns have intermediate temperatures resulting in both sexes and sometimes intersexes developing. The third pattern shows both low and high temperatures yielding 100% or predominantly females. Intermediate temperatures produce various ratios of males (Pieau, 1996).

During embryonic development of species with TSD, there is a thermosensitive period (TSP), at which point temperature determines which sexual phenotype will develop (Johnston *et al.,* 1995). Shifting embryos from male producing to female producing temperatures, and vice versa, has defined the TSP in different species (Deeming and Ferguson, 1989). The TSP generally encompasses the middle one third to one half of embryonic development (Wibbels *et al.,* 1994). The TSP is not sufficient in itself for determining sex, but requires the accumulation of a factor or factors over a period of time (Deeming and Ferguson, 1989). It indicates the earliest and latest times the sex of an embryo can be altered (Deeming and Ferguson, 1989). Due to intermediate temperatures producing a mixed ratio of males and females, it supports the theory that temperature does not act as a primary switch. However, it is possible temperature could effect a variety of factors involved in sex determination, such as growth factors and hormones (Wibbels *et al.,* 1994; Johnston *et al.,* 1995).

Recent studies have focused on the activity of aromatase at male and female producing temperatures (Wibbels *et al.,* 1994). Initially, aromatase levels are low at the beginning of the TSP. However, there is an increase in aromatase activity at the end of the TSP, at female producing temperatures (Desvages and Pieau, 1992; Pieau *et al.,* 1994). This increase was due to increased synthesis as opposed to an increase in activity (Desvages and Pieau, 1992). This suggested temperature could affect aromatase synthesis which in turn would affect the levels of estrogen concentration. Overall, it appears estrogens are required for ovarian development, but androgens do not play a major role in testis development (Pieau, 1996). This theory was supported by treatment of embryos with aromatase inhibitors at female producing temperatures, which resulted in varying degrees of masculinization (Pieau *et al.*, 1994).

1.3.2. Dosage mechanism for sex determination.

In *Drosophila* and *C. elegans*, the primary sex determinant is the ratio of X chromosomes to the number of sets of autosomes (X/A ratio) (Parkhurst and Meneely, 1994; Charlesworth, 1996). Normally, *Drosphila* females have an X/A ratio of 1.0, because they have two X chromosomes and two sets of autosomes. This ratio results in hermaphrodites in *C. elegans*. Males have an X/A ratio of 0.5, in both *Drosphila* and *C. elegans*, as they only have one X chromosome. If there is an intermediate sex ratio of 0.67 (e.g. XX AAA), then flies develop as intersexes and worms develop as males (McCarrey and Abbott, 1979; Parkhurst and Meneely, 1994; Marx, 1995; Cline and Meyer, 1996).

The primary sex determining mechanism depends upon "counter" loci on the X chromosome and the autosomes to determine the sexual fate of the embryo (Cline and Meyer, 1996; Charlesworth, 1996). In *Drosphila*, the gene that is the immediate target of the X/A ratio and the master sex determination switch is called *sex-lethal* (*sxl*) (Cline, 1984; Parkhurst and Meneely, 1994; Cline and Meyer, 1996). *Drosophila* female sexual development requires continuous activity of *Sxl* while male sexual development does not require *Sxl* activity (Parkhurst and Meneely, 1994).

Initial expression of full-length, active *Sxl* in females is activated from an establishment promoter $Sxl_{Pe,}$ and is maintained from a maintenance promoter Sxl_{Pm} (Parkhurst and Meneely, 1994; Cline and Meyer, 1996). Transcripts from this promoter contain an exon with an in-frame stop codon. In the female, full length *Sxl* that has already accumulated is able to splice out this exon, so active Sxl protein is continuously produced. In the male, where no *Sxl* has accumulated, the transcript retains this exon resulting in a truncated, inactive Sxl protein (Parkhurst and Meneely, 1994; Cline and Meyer, 1996). *Sxl* encodes an RNA binding protein, which regulates downstream expression of *transformer (tra)*, by RNA splicing (Parkhurst and Meneely, 1994). This results in two different Dsx (doublesex) proteins being produced, dependent upon the presence or absence of a functional Tra protein. Different Dsx proteins determine which sexual development pathway is followed (fig 3) (Marx, 1995; Ryner and Swain, 1995; Cline and Meyer, 1996).

A different sex chromosome complement in male and female results in one or two copies of X chromosome genes present in the somatic cells of flies and worms respectively. This problem is overcome by a dosage compensation mechanism. Sxl protein regulates dosage compensation by regulating splicing of the *msl2* (*male-specific-lethal*) gene (Ryner and Swain, 1995; Cline and Meyer, 1996). If Sxl is present (female), a splice event is blocked in the *msl2* gene (Kelley *et al.*, 1995). In the male, the splice event occurs, removing a 5' untranslated region which normally interferes

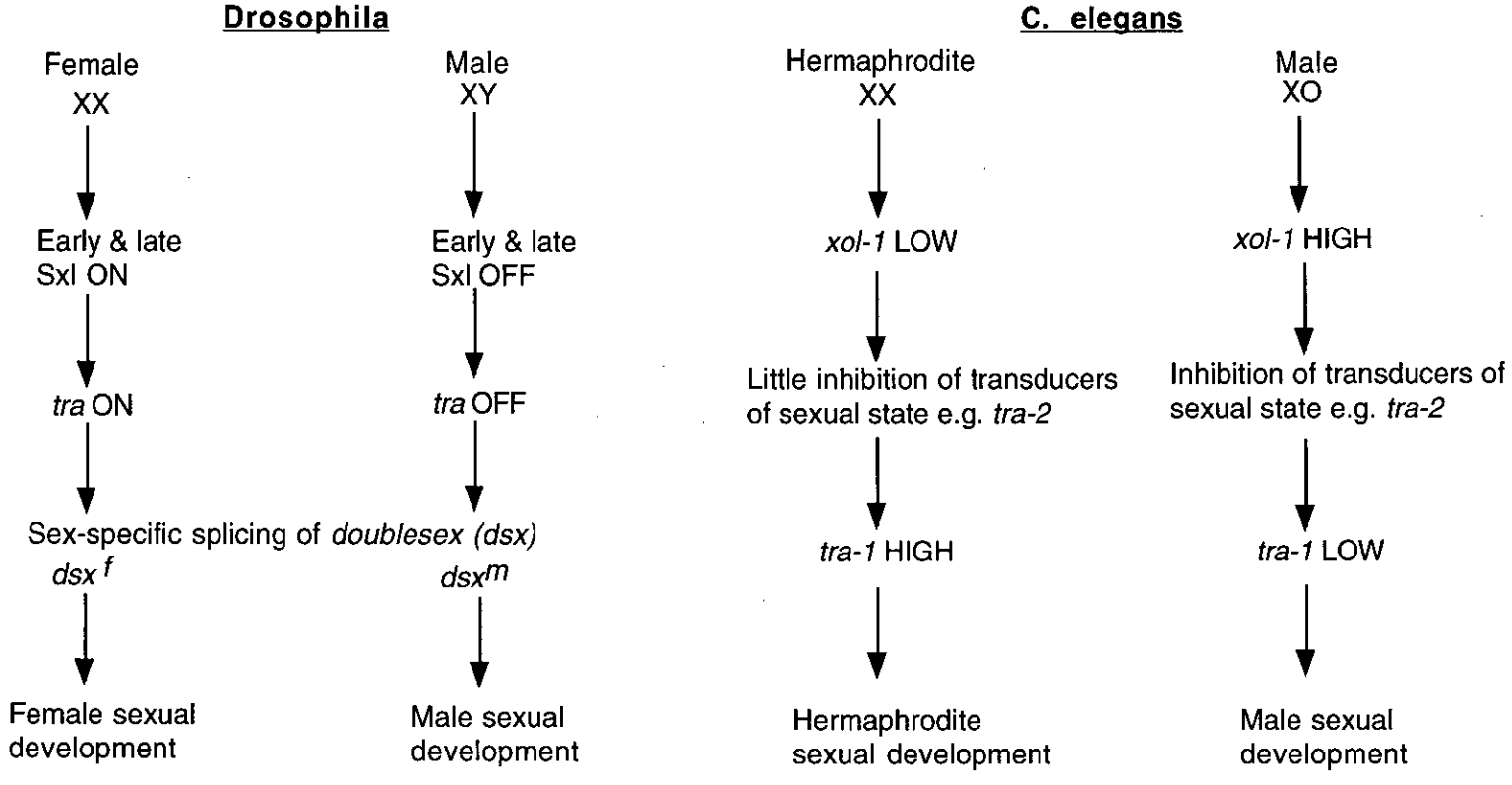


Figure 3: Schematic representation of somatic sex determination in *Drosophila* and *C. elegans*. A more detailed account is given in the text. Figure adapted from Wolpert *et al.*, 1998.

with translation. This results in a functional protein being produced which interacts with other proteins and forms an Msl complex, which assembles on the male X chromosome and induces hyperactivation of gene expression, to compensate for two X chromosomes in the female (Cline and Meyer, 1996).

In *C. elegans*, the primary mechanism for sex determination is the X/A ratio, and the earliest gene in the cascade is *XO lethal* (*xol-1*) (Parkhurst and Meneely, 1994; Cline and Meyer, 1996). The *xol-1* gene is similar to *Sxl* in that it controls sex determination and dosage compensation via the X/A ratio signal (Cline and Meyer, 1996). Two X chromosomes (hermaphrodites) resulted in inactivation or low levels of *xol-1* and one X chromosome (male) activated *xol-1* (Cline and Meyer, 1996). In the XO male, signal transducers for the high *xol-1* activity, ultimately leads to negative regulation or inactivation of the *tra-1* gene, resulting in male sexual development (Parkhurst and Meenely, 1994; Ryner and Swain, 1995). If *tra-1* is active, hermaphrodites develop (fig 3).

The *xol-1* gene controls dosage compensation, by regulating expression of the *sdc2* (Sex and Dosage Compensation) gene. In XO males, *xol-1* inactivates *sdc2* resulting in full expression from the one X chromosome in the male. In hermaphrodites, *xol-1* levels are low, resulting in the *sdc2* gene being active. This results in a heterodimeric protein complex forming and reducing expression from both X chromosomes to compensate for the one X chromosome in the male (Cline and Meyer, 1996).

1.3.3. Dominant genetic switch mechanism.

The chromosomal sex determination mechanism utilised by mammals is a dominant genetic switch on the Y chromosome. Eutherian mammals with a Y chromosome develop a testis, regardless of the number of X chromosomes present (Graves and Reed, 1993). On the Y chromosome of eutherian mammals, it was assumed there was a gene that acted as a master switch, causing development along a testis determining pathway. The gene was called the testis-determining factor (TDF) in humans and the testis-determining Y gene (Tdy) in mice (Goodfellow and Lovell-Badge, 1993).

The Y chromosome is small (~2% of the haploid genome), is constitutively heterochromatic and mainly consists of repetitive sequences (Watson *et al.*, 1993; Graves, 1995). The Y chromosome consists of a pseudoautosomal region (PAR) and a Y-specific region. The PAR is homologous to a region on the X chromosome and is where X and Y pair. The TDF is located in the Y-specific region and does not normally recombine with the X chromosome. However, the majority of XX males have inherited

Y-specific sequences and so an abnormal recombination event must have occurred (Goodfellow and Lovell-Badge, 1993; Fechner, 1996).

Various candidate TDF genes have been proposed e.g. male specific H-Y histocompatibility antigen, banded krait minor (Bkm) (Wachtel and Tiersch, 1994) and ZFY (Zinc Finger containing region on Y) (Wachtel *et al.*, 1975; Page *et al.*, 1987). H-Y antigen was originally identified as a transplantation antigen that caused females from a particular strain to reject skin transplantations from males of the same strain (Simpson, 1986). Its role as a candidate in sex determination was rejected after mice without H-Y antigen developed as males (Wiener *et al.*, 1996). Banded krait minor (Bkm) satellite repeat sequence was isolated from the W chromosome of snakes (Singh *et al.*, 1980) and correlated with the amount of heterochromatin on the W chromosome (Wachtel and Tiersch, 1994). Bkm was identified in mammals, birds, reptiles and insects (Wachtel and Tiersch, 1994). In mice, the high concentration of Bkm on the Y chromosome lead to the hypothesis that Bkm was involved in sex determiation (Jones and Singh, 1981). However, the presence of Bkm throughout the genome argued against a major role in sex determination (Wachtel and Tiersch, 1994).

Initially, the TDF gene was isolated to a 140kb region on the short arm of the Y chromosome (Page *et al.*, 1987). A gene isolated from this region was called ZFY. However, a homologue to ZFY was isolated on the X chromosome (Scheinder-Gadicke *et al.*, 1989). Palmer *et al.* (1989) identified four XX males that had 60kb of Y chromosome sequence adjacent to the PAR boundary, but ZFY was not contained in this region, indicating ZFY was not the sex determining gene. Finally, from a region of 35kb, the *SRY/Sry* (Sex determining Region Y) gene was isolated in humans and mice and shown to be the TDF (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990).

1.3.4. Avian sex determination.

1.3.4.1. The avian sex chromosomes.

As the female chick possesses a heteromorphic pair of sex chromosomes (ZW), it was assumed that chicks employed a chromosomally based sex determination mechanism (Thorne, 1995). Graves (1995b) and Stevens (1997) reported no obvious homology between the mammalian X chromosome and the avian Z chromosome. This indicated the chromosomal sex determination mechanism employed by the XX:XY system and the ZZ:ZW system evolved separately and resulted in different sex determining genes in birds and mammals (Graves, 1995b). A difference in the sex determination mechanism is supported by the failure to isolate a *SRY* homologue in birds (Griffiths, 1991; Tiersch *et al.*, 1991; McBride *et al.*, 1997).

The Z chromosome is a macrochromosome, euchromatic and contributes approximately 7% of the haploid genome (Schmid *et al.,* 1989; Thorne, 1995; Stevens, 1997). The W chromosome is much smaller, the size of a microchromosome and contributes approximately 1.4% of the haploid genome (Thorne, 1995). The Z chromosome consists of three regions. There is a pseudoautosomal region (PAR) at the terminal end of the short arm. No crossing-over occurs in this region between the Z and the W chromosome, unlike the X and Y chromosomes (Solari *et al.,* 1988). The second region is approximately four times as large as the PAR (Solari *et al.,* 1988), and contains a single recombination nodule which is localized near the pairing end of the ZW bivalent (Rahn and Solari, 1986). This region is said to be partially sex-linked because the genes will remain on the Z or the W chromosome unless recombination occurs (Thorne, 1995). Finally, a region specific to the Z chromosome is contained within the long arm of the chromosome and the majority of the short arm of the chromosome (Thorne, 1995). Genes in this region are sex-linked because no homologous recombination occurs between the Z and W chromosomes (Thorne, 1995).

The W chromosome is highly heterochromatic, with approximately 75% of the chromosome made up of two repetitive DNA sequences called the EcoRI family and the XhoI family (Saitoh *et al.,* 1991; Thorne, 1995; Stevens, 1997). Only a few genes have been located on the W chromosome. The first gene isolated from this chromosome was the CHD 1 (chromo-helicase DNA binding domain 1) gene (Griffiths and Tiwari, 1995). This gene is considered to have a regulatory role in chromatin architecture (Griffiths and Korn, 1997; Stevens, 1997). A homologue to this gene has been located on the Z chromosome but it contains an extra 88 amino acid hydrophilic domain (Griffiths and Korn, 1997). A second gene has been identified (DZWM1) and shown to be located at different loci on the Z and the W chromosomes. At present no function has been assigned to this gene (Dvorák *et al.,* 1992). A third gene has also been identified which is specific to the W chromosome. This gene is called ASW and is expressed only in females (Sinclair, 1997).

Only a small number of genes have been mapped to the Z chromosome (for a review of these genes see Thorne, 1995; Stevens, 1997). Genes located on the Z chromosome are generally not located on the W chromosome (Stevens, 1997). This leads to sex-linked patterns of gene expression. A mammal overcomes this problem by inactivating one of these X chromosomes, thus regulating gene dosage (Migeon, 1994). In birds, neither asynchronous replication between the euchromatic bands on the Z chromosomes (Schmid *et al.,* 1989), nor Barr bodies, characteristic of a cell containing an inactivated X chromosome (Migeon, 1994), were observed in males (ZZ). Also, a two-fold increase of transcription from the one Z chromosome in the female has not

been observed (Baverstock *et al.*, 1982). The apparent lack of Z chromosome inactivation and the lack of increased transcription indicated that the Z chromosomes in birds do not have a dosage compensation mechanism (Bloom, 1974; Thorne, 1995; Stevens, 1997).

1.3.4.2. Mechanisms of sex determination in avians.

Due to the heteromorphic sex chromosomes in birds, it has been suggested that the sex determination mechanism is either a dominant genetic switch as employed by the Y chromosome in mammals (Bitgood and Shoffner, 1990), or a dosage mechanism as typified by *Drosophila* and *C. elegans* (McCarrey and Abbott, 1979; Halverson and Dvorak, 1993). In birds the female is the heterogametic sex (ZW), therefore, the dominant genetic switch mechanism would result in a dominant gene on the W chromosome initiating ovary development (Bitgood and Shoffner, 1990; Thorne, 1995).

Triploid and aneuploid birds have been studied in an attempt to elucidate which mechanism is used to determine sex (reviewed in McCarrey, 1979; Halverson and Dvorak, 1993; Thorne, 1995; Stevens, 1997) (table 1). The two genotypes that would provide the most information for the sex determination mechanism in birds would be 2A ZO and 2A ZZW (McCarrey and Abbott, 1979; Halverson and Dvorak, 1993; Thorne, 1995; Stevens, 1997). No ZO aneuploids have been reported (Kagami *et al.*, 1995), but they would be female if the genic balance mechanism is correct and male if the dominant W chromosome theory is correct. Only one ZZW aneuploid has been reported (Crew, 1933). If the genic balance mechanism is correct the ZZW aneuploid would be male, but female if the dominant W chromosome mechanism is correct. The ZZW aneuploid was male but exhibited female sex-linked plummage. Crew (1933) concluded the male chick had the sex chromosome constitution of ZZW on the basis of phenotype and cytology of its progeny, and thus favoured the genic balance sex determination mechanism. Thorne (1995) argued that at the time this ZZW aneuploid was identified, cytological techniques were poor and could not always resolve the presence of a W chromosome. In addition, it was claimed Crew incorrectly identified the Z chromosome as being the largest macrochromosme as opposed to the fifth largest macrochromosome in size (Thorne, 1995). Overall, as there has only been one observation reported and this observation is unreliable, it is impossible to confirm that the male phenotype assigned to this chicken had a ZZW genotype.

Triploid studies have been more successful, but less informative. The triploid ZZW chickens are classed as intersexes, with two developing gonads, the left being an ovotestis and the right a testis (Thorne *et al.*, 1988; Thorne and Sheldon, 1993;

Genotype	Mammals	*Drosophila*	Birds	Ratio X(Z): autosome sets
2A: XY (ZW)	Male	Male	Female	1:2
2A: XX (ZZ)	Female	Female	Male	1:1
2A: XO (ZO)	Female	Male	?	1:2
2A: XXY (ZZW)	Male	Female	Male??	1:1
2A: XXX (ZZZ)	Female	Female	Male	3:2
3A: XXX (ZZZ)	Female	Female	Male	1:1
3A: XXY (ZZW)	Male	Intersex	Intersex	2:3
3A: XYY (ZWW)	-	Male	Female? (not normally viable)	1:3

Table 1: A comparison of the diploid, triploid and aneuploid genotypes in relation to their phenotypes in mammals, *Drosophila* and birds. Table modified from Stevens, 1997.

Thorne, 1995. At hatching, the left gonad resembled a normal ovary (Thorne *et al.*, 1988) or an ovotestis (Lin *et al.*, 1995). However, the gonads gradually became masculinized; abnormal spermatozoa being produced from both gonads (Lin *et al.*, 1995; Thorne *et al.*, 1988; Lin *et al.*, 1995). McCarrey and Abbott (1979) suggested that the genic balance mechanism was favoured because a 1:1 ratio of Z chromosomes to autosomes resulted in a male phenotype (e.g. 2A:ZZ and 3A:ZZZ). A female phenotype results if the Z:A ratio is 1:2 (e.g. 2A:ZW). Finally, an intermediate Z:A ratio results in an intersex developing (e.g. 3A:ZZW). These observations do not take into account the 3:2 ratio in 2A:ZZZ male embryos or the 1:3 ratio in 3A:ZWW embryos, which have tentatively been classed as females (Bonaminio and Fechheimer, 1993).

Halverson and Dvorak (1993) favour the genic balance mechanism on the basis of gynandromorph data. Gynandromorphs are chimeric with one half of the body being male and the other being female. One gynandromorph was a parrot, which had male green plumage on the on the left side and female red plumage on the right side (Halverson and Dvorak, 1993). The gonads of this gynandromorph had testes on both sides of the embryo. It was assumed that plumage colour was determined cell autonomously and gonadal development was determined by hormones or factors secreted from the male side of the embryo (Halverson and Dvorak, 1993). With the female side of the gynandromorph parrot being on the right; the gonad normally regresses in females on this side and so testicular hormones might initiate testicular development on this side by default. Thorne (1995) studied a chicken with female plumage on the left side and the male plumage on the right side. The chromosomes were analyzed cytogenetically and found to be ZZ/ZW. When the gonads were studied, there was a right testis and an atretic left ovary and left and right oviducts. Therefore, the effect of a dominant W chromosome can not be discounted (Thorne, 1995).

1.4. Mammalian gonadal development and genetic regulation.

1.4.1. SRY (Sex-determining Region Y).

The gene responsible for testis determination on the Y chromosome is called the testis-determining factor (TDF) in humans or testis determining Y gene in mice (Tdy) (Goodfellow and Lovell-Badge, 1993). The TDF has been isolated and called *SRY* (Sex-determining Region Y) (Sinclair *et al.*, 1990). The gene has also been isolated in mice and termed *Sry* (Gubbay *et al.*, 1990). This gene was shown to encode the transcript responsible for testis determination through mutational studies (Berta *et al.*, 1990; Jäger *et al.*, 1990) and transgenic studies, which involved the insertion of a 14kb

genomic fragment containing *Sry* into female fertilised eggs resulting in normal testis development (Koopman *et al.*, 1991).

The human *SRY* gene consists of 1 exon encoding a protein which contains a 79 amino acid motif called the HMG (high mobility group) domain; a conserved region identified in other proteins such as HMG1 (Sinclair *et al.*, 1990; Clépet *et al.*, 1993) (fig 4), that mediates DNA binding in a sequence-specific manner (Koopman, 1995). The human *SRY* gene also has a 56 amino acid N-terminal and a 68 amino acid C-terminal region (Lovell-Badge and Hacker, 1995; Koopman, 1995). If the human gene is compared with the mouse *Sry* gene, the HMG domain is the only region of homology (Lovell-Badge and Hacker, 1995; Koopman, 1995). The mouse protein consists of a 2 amino acid N-terminal and a 314 amino acid C-terminal domain; the C-terminal domain being composed of a repetitive glutamine/histidine-rich region which could potentially function as a transactivator (Lovell-Badge and Hacker, 1995; Koopman, 1995).

In the mouse and human *Sry/SRY* transcripts are expressed at a very low level in the genital ridge, and have only been detected by RT-PCR, RNase protection assay and *in situ* hybridization (Hacker *et al.*, 1995; Koopman *et al.*, 1990; Lovell-Badge and Hacker, 1995). In mouse, *Sry* is first detected at 10.5dpc (Koopman *et al.*, 1990; Hacker *et al.*, 1995). Levels peak at 11.5dpc and decline rapidly so that *Sry* cannot be detected at 12.5dpc (Hacker *et al.*, 1995) (fig 5). This short window of expression suggests the gene acts as a switch directing development down a certain pathway and is not required for maintenance of this pathway.

In situ hybridization of *Sry* in embryos lacking germ cells, showed that the transcript is located in the somatic component of the genital ridge (Koopman *et al.*, 1990), assumed to be the Sertoli cells, as these are the first cells to differentiate in the male genital ridge (Koopman, 1995). This hypothesis was supported by experiments involving chimaeric mouse embryos. If 25% of cells in an XX-XY chimaera are XY, male development occurs. The only cell type to exhibit bias are the Sertoli cells, where the majority are XY (Burgoyne *et al.*, 1988), suggesting *Sry* is required for initial Sertoli cell differentiation and this allows signalling pathways to be activated and differentiation of other testicular cell types to occur.

As the HMG box is the only domain that is conserved between different species and no mutations outside the HMG box have been detected in sex reversal patients, it is assumed this region is of functional importance (Koopman, 1995; Goodfellow and Lovell-Badge, 1993). The HMG box of *SRY* is known to bind DNA sequence specifically and induce a bend in linear DNA of approximately 76° (Rimini *et al.*, 1995; Greenfield and Koopman, 1996). This enables *SRY* to bind DNA directly and activate

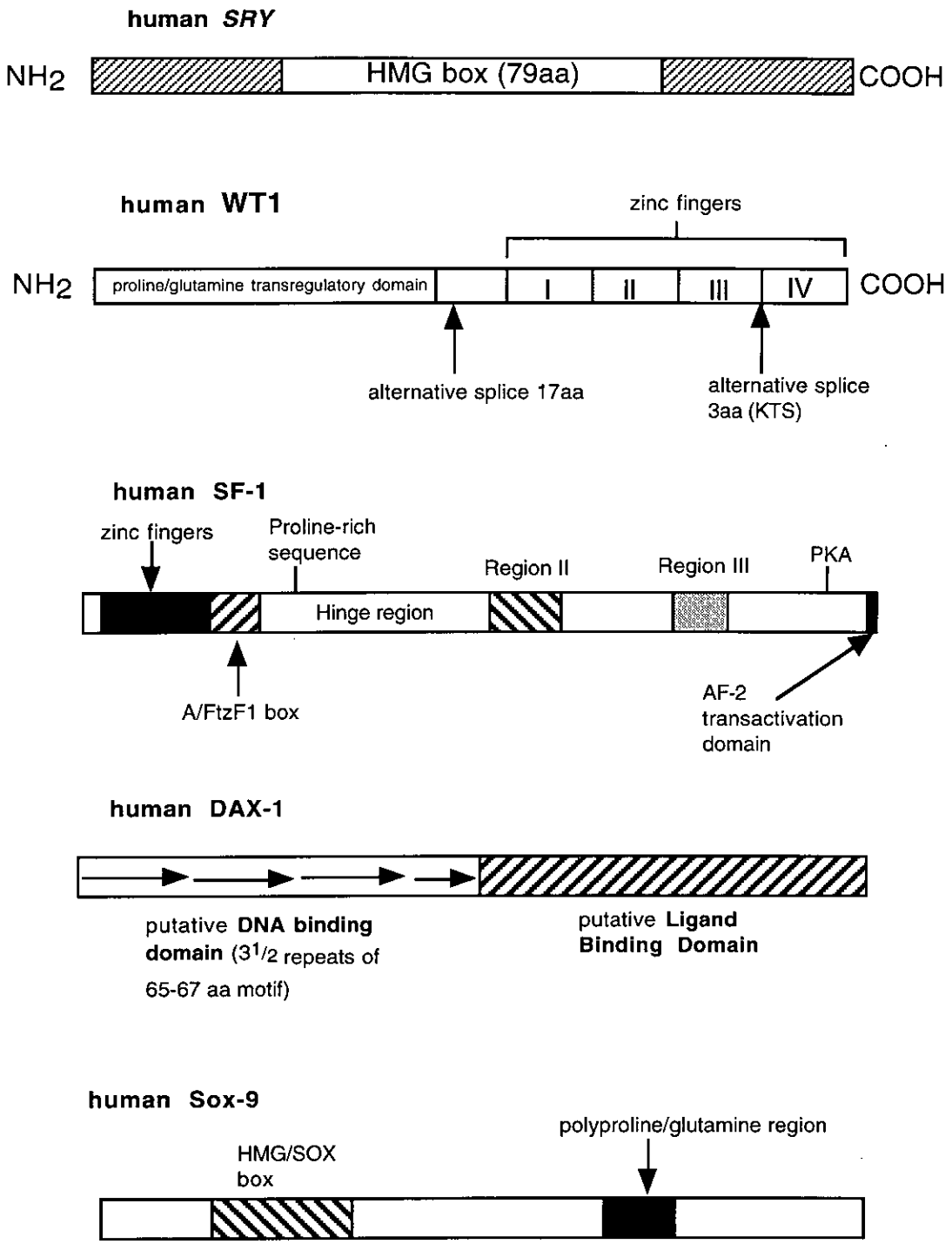


Figure 4: Schematic representation of the structure of SRY, WT1, SF-1, DAX-1 and SOX-9 proteins. A full description is given in the text. Figures adapted from: Nagai, 1996; Hastie, 1993; Parker and Schimmer, 1997; Swain *et al.*, 1996; Foster, 1996.

or repress transcription either by competing with a transcription factor for a binding site or possibly bringing transcription factors together via the bending of DNA (Koopman, 1995; Capel, 1996). One theory is that *SRY* negatively regulates a gene that normally inhibits a cascade of genes involved in testis differentiation (McElreavey *et al.,* 1993). The actual mechanism of how SRY protein switches development along the testis determining pathway is unknown. Also, genes that regulate or are regulated by *SRY* are yet to be identified.

Numerous groups have attempted to isolate *SRY* from non-mammalian species (Tiersch *et al.,* 1991; Griffiths, 1991; McBride *et al.,* 1997). *SRY* has never been isolated from birds, reptiles and lower vertebrates (Koopman, 1995), suggesting a different mechanism is used as the switch to initiate testis development in these species.

1.4.2. WT1 (Wilms' Tumour 1).

WT1 encodes a putative transcription factor, that localizes to the nucleus (Call *et al.*, 1990; Gessler *et al.*, 1990). Mutations in the tumour suppressor gene WT1 predisposes individuals to Wilms' tumour, an embryonal malignancy affecting the kidney (Matsunaga, 1981). The WT1 protein has four zinc finger domains, of the Kruppel Cys_2 - Hys_2 class, at its carboxy-terminus. At its amino-terminus there is a proline and glutamine rich transregulatory region (Kent *et al.*, 1995; Rauscher, 1993; Call *et al.*, 1990; Gessler *et al.*, 1990; Van Heyningen and Hastie, 1992; Hastie 1994). Comparison of the WT1 protein with other species indicates a high level of similarity at the nucleotide and amino acid level; although the zinc finger domain is more conserved than the transregulatory region (Kent *et al.*, 1995) (fig 4).

The mammalian *WT1* gene is able to produce four different isoforms through alternative splicing (Haber *et al.*, 1991). One splicing difference can result in the insertion of 17 amino acids downstream of the proline - glutamine region. The other leads to the insertion of three amino acids KTS (lysine, threonine and serine) between zinc fingers III and IV. The mRNA containing both insertions is the most common form in mammals (Hastie, 1992; Hastie, 1993). In other species, only the KTS alternative splice is conserved (Kent *et al*, 1995). WT1 is considered to be a transcriptional repressor which is mediated through the proline - glutamine region (Madden *et al.*, 1991; Lee and Kim, 1996). WT1 also binds to TCC nucleotide repeats, leading to transcriptional activation (Wang *et al.,* 1993a; Wang *et al.*, 1993b). Therefore, interactions with other proteins in addition to alternative binding sites and different isoforms may determine whether WT1 activates or represses transcription.

Expression profiles of WT1 indicate that it has an important role in normal urogenital development (fig 5). WT1 is initially expressed in the mouse at 9 dpc in the

intermediate mesoderm and at 9.5 dpc in the urogenital ridge (Armstrong *et al.*, 1992). By 10 dpc, just prior to sex determination, WT1 expression is observed in the genital ridge, mesonephros and mesothelium (a layer of cells that surround the body cavity and thoracic organs). By 12 dpc, there is strong expression in the mesothelium, the developing podocytes of the glomerulus in the metanephros and the developing gonads. Expression of WT1 in the gonads is restricted to the Sertoli cells in the testis and the granulosa cells in the ovary, in addition to the mesenchymal cells which will differentiate into these epithelial cells. The Wolffian duct, Mullerian duct and germ cells are all negative for WT1 expression (Armstrong *et al.*, 1992; Kent *et al.*, 1995; Pelletier *et al.*, 1991a; Rackley *et al.*, 1993; Rauscher, 1993; Hastie, 1992; Pritchard-Jones *et al.*, 1990).

In the chick and alligator, WT1 is expressed from the earliest stages of urogenital ridge development (Kent *et al.*, 1995). Expression is also observed in the mesothelium and somites. These studies concentrated on mesonephros expression, which is functional in the embryonic chick and alligator and its expression profile closely resembles the mammalian metanephros profile (Kent *et al.*, 1995; Armstrong *et al.*, 1992).

In an attempt to elucidate the function of WT1 during development, mice homozygous for a mutated *wt-1* allele were generated (Kreidberg *et al.*, 1993). These mice died at mid-gestation, and gonadal and metanephric development was arrested at a very early stage. The blastemal cells of the metanephros underwent apoptosis and the mesenchymal cells failed to differentiate. These findings suggested WT1 had an important role in urogenital development (Kreidberg *et al.*, 1993; Hastie, 1994).

The role of WT1 in urogenital development has also been studied by examining mutations in the WT1 gene in children with Denys-Drash Syndrome (DSS) (Pelletier *et al.*, 1991b). The gonadal phenotype can range from streak gonads (undifferentiated mesenchyme) to mild pseudohermaphroditism. The tissues that are affected by abnormal development correspond to those tissues that express WT1 during development (Hastie, 1993; Hastie, 1994).

There are two proposed functions for WT1. The first is regulating transcription of genes required in urogenital development by repressing genes that maintain cellular proliferation, or by activation of genes involved in terminal differentiation (Hastie, 1992; Madden *et al.*, 1991; Lee and Kim, 1996; Wang *et al.*, 1993a). At present no target genes of WT1 have been identified (Lamond, 1995). However, WT1 protein has been shown to repress, *in vitro*, the IGFII (insulin growth factor II) promoter (Lee and Kim, 1996; Drummond *et al.*, 1992; Ward *et al.*, 1995).

The second proposed function for WT1 is RNA processing (Lamond, 1995; Larsson *et al.*, 1995). Larsson and collegues (1995) have shown that WT1 co-localizes with splicing factors in the embryonic kidney, gonads and cell lines expressing WT1. Localisation of WT1 with splicing factors or transcription domains appears to be dependent upon the alternative splice forms generated in the zinc finger domain (+KTS or -KTS).

1.4.3. SF-1 (Steroidogenic factor 1).

SF-1 (steroidogenic factor 1) or Ad4BP (adrenal 4 binding protein) was identified as a mammalian homologue of the *Drosophila Ftz-F1* gene, which regulates the expression of the *ftz* (*fushi tarazu*) gene during development (Lala *et al.*, 1992; Honda *et al.*, 1993). The SF-1 protein is a member of the nuclear hormone receptor family, which mediates transcriptional activation by various steroid hormones (Parker and Schimmer, 1997). This protein, which was only present in steroidogenic cells, bound to a regulatory element (Ad4) in P450 steroid hydroxylases and was thought to be responsible for the tissue-specific and developmentally regulated expression of the enzymes (Omura and Morohashi, 1995; Parker and Schimmer, 1997).

Ftz-F1 encodes two isoforms that are differentially regulated during *Drosophila* development (Ikeda *et al.*, 1993; Parker and Schimmer, 1994; Parker and Schimmer, 1997). In comparison, the gene encoding SF-1 also encodes an additional three transcripts called embryonal long terminal repeat-binding protein (ELP 1-3), which bind a negative regulatory element in retroviral long terminal repeats (Parker and Schimmer, 1997). The three ELP transcript and SF-1 differ from one another due to different promoter usage and alternative 3' splicing (Omura and Morohashi, 1995; Parker and Schimmer, 1997).

SF-1 has, at the amino-terminus, two zinc fingers (region I) that mediate binding to DNA hormone response elements (Honda *et al.*, 1993) (fig 4). Immediately adjacent to region I is the FTZ-F1 or A box, which recognise bases 5' of the Ad4 binding site and stabilises binding of SF-1 (Wong *et al.*, 1996; Parker and Schimmer, 1997). The hinge region is 3' to the A box and is putatively involved in transcriptional activation (Wong *et al.*, 1996; Parker and Schimmer, 1997). Region II and III, in the carboxy-terminus, comprise the ligand-binding and dimerization domains (Honda *et al.*, 1993; Wong *et al.*, 1996). Next to these domains is a potential motif for phosphorylation by cAMP-dependent protein kinase (Honda *et al.*, 1993; Wong *et al.*, 1996). Finally, at the most 3' terminus of the protein there is an AF-2 domain essential for transcriptional activation (Wong *et al.*, 1996; Parker and Schimmer, 1997).

SF-1 expression has been detected in primary steroidogenic tissues: testis, ovary and adrenal in addition to certain areas of the brain, placenta and spleen (Morohashi and Omura, 1996). SF-1 is detectable in the urogenital ridge when it first arises at 9 dpc in the mouse (fig 5) (Ikeda *et al.*, 1994). By 9.5 to 10.5 dpc, in the mouse, there are two discrete cell populations expressing SF-1, the adrenocortical precursors and the gonadal precursors (Ikeda *et al.*, 1994; Ikeda, 1996; Parker and Schimmer, 1997). By 12.5 dpc, SF-1 is dectected in the Leydig cells and the Sertoli cells (Ikeda *et al.*, 1994; Hatano *et al.*, 1994). This expression is maintained in the male throughout fetal and adult life in Leydig cells, but is down-regulated in adult Sertoli cells (Morohashi and Omura, 1996; Parker and Schimmer, 1997). In contrast, at the time of ovarian differentiation there is a down-regulation in SF-1 transcripts and protein (Ikeda *et al.*, 1994; Hatano *et al.*, 1994). This low or undetectable level of SF-1 expression in the ovary is maintained until follicular development begins after birth, when SF-1 is expressed in granulosa and theca cells (Ikeda *et al.*, 1994; Hatano *et al.*, 1994). SF-1 expression was also detected in the ventromedial hypothalamic nucleus (VMH) and the anterior pituitary gland in the brain (Ikeda *et al.*, 1995; Parker and Schimmer, 1997). VMH and the pituitary gland do not express steroid P450 genes, therefore SF-1 must interact with other target genes (Morohashi and Omura, 1996).

In order to elucidate the function of SF-1, targeted disruption of the mouse *Ftz-F1* gene was carried out. *Ftz-F1* knockout mice died by 8 days after birth, due to adrenocortical insufficiency (Luo *et al.*, 1994; Luo *et al.*, 1995). Externally all knockout mice had female genitalia, but internally lacked adrenal glands and gonads (Luo *et al.*, 1995). At 10.5 dpc mesenchymal thickening is observed, where the genital ridge normally forms, suggesting SF-1 is not required for initiation of urogenital ridge development (Luo *et al.*, 1994). By 12 dpc, cells in the genital ridge region of knockout mice exhibited features of apoptosis. By 12.5 dpc, the gonads were almost absent. This abnormal development of the adrenal glands and gonads coincides with the stage of sexual differentiation (Luo *et al.*, 1994). The pituitaries of *Ftz-F1* knockout mice also lacked specific gonadotrope proteins, such as: α-glycoprotein hormone subunit (α-GSU) (Ingraham *et al.*, 1994). VMH structure had decreased cellular organisation and was almost ablated in knockout mice, by postnatal (P) day 1 (Ikeda *et al.*, 1995).

Another approach to elucidate the function of SF-1 is to identify target genes. One potential downstream target of SF-1 is Steroidogenic Acute Regulatory protein (StAR) (Caron *et al.*, 1997). StAR is required for acute induction of steroid hormones

(Clark *et al.*, 1995; Parker and Schimmer, 1997). The StAR 5' flanking region contains a binding site for SF-1 (Caron *et al.*, 1997). StAR is located in the steroidogenic cells of the ovary, testis and adrenal, and is not detectable until after the onset of SF-1 expression (Clark *et al.*, 1995).

Anti-Müllerian hormone (AMH) is another potential downstream target of SF-1. The 5' flanking region of AMH contains a motif matching the sequence that SF-1 binds, called MIS-RE-1 (Shen *et al.*, 1994). Disruption of this motif reduced the activity of AMH. However, when transactivation experiments were carried out AMH activation by SF-1 required the removal of the SF-1 ligand binding domain, suggesting a Sertoli cell specific co-factor was required for activation (Shen *et al.*, 1994). Giuili *et al.* (1997a) have studied the 180bp 5' flanking region of AMH in transgenic mice. An SF-1 point-mutated 180bp proximal AMH promoter was fused to the human growth hormone gene (hGH) and expression of the transgene was abolished, suggesting the SF-1 binding site is essential for AMH expression (Giuili *et al.*, 1997a). Other potential targets of SF-1 include: aromatase (Carlone and Richards, 1997); α-GSU (Parker and Schimmer, 1997) and LHβ (Halvorson *et al.*, 1996; Keri and Nilson, 1996). Two other genes that were putative targets or regulators of SF-1 are *SRY* and WT1 (Parker and Schimmer, 1997; Ikeda, 1996). *Dax-1* is another gene that could potentially interact with SF-1.

Regulation of SF-1 has also been studied. Three motifs in the promoter of SF-1 have been identified: an E box, a CAAT binding factor and a GA-rich element (Woodson *et al.*, 1997; Nomura *et al.*, 1995). The E box is the binding site for basic helix-loop-helix (BHLH) proteins (Woodson *et al.*, 1997; Nomura *et al.*, 1995). The CAAT binding site binds the CAAT binding factor (CBF) and the GA-rich element binds the Sp1 transcription factor (Woodson *et al.*, 1997). Deletion analysis of the promoter has shown that all three elements are required for SF-1 expression, but the E box is essential (Woodson *et al.*, 1997).

Until recently no potential ligands for SF-1 had been identified. Lala *et al.* (1997) have implicated oxysterols as ligand activators of SF-1. Oxysterols inhibit cholesterol biosynthesis but are thought to enhance SF-1 activity. Activation of SF-1 is dependent on the presence of the AF-2 transactivation domain at the C-terminus of the protein. Oxysterols for SF-1 are unable to activate other members of the nuclear hormone receptor family. At present it is unknown whether oxysterols bind directly to SF-1 or if an oxysterol metabolite is the ligand. Alternatively, oxysterols may recruit other proteins that specifically interact with SF-1 (Lala *et al.*, 1997).

1.4.4. DSS (Dosage Sensitive Sex reversal) locus.

It is well documented that partial duplications on the short arm of the X chromosome (Xp) in males with a functional copy of *SRY* leads to variable phenotypes of gonadal dysgenesis (Ogata and Matsuo, 1996; Zanaria *et al.*, 1995)., The sex reversal phenotype is observed if part of Xp is duplicated in males which is not subject to X-inactivation. This suggested that there were gene(s) on the X chromosome important for the determination of gonadal sex (Zanaria *et al.*, 1995). The region critical for this sex reversal termed DSS (Dosage Sensitive Sex reversal), and shown to partially overlap the AHC (Adrenal Hypoplasia Congenita) locus (Bardoni *et al.*, 1994), suggesting that the gene responsible for AHC could also be responsible for DSS.

XY individuals that are deleted for the DSS critical region develop normal male genitalia , indicating the DSS locus does not have an important role in testis differentiation (Bardoni *et al.*, 1994). Two active copies of DSS in combination with *SRY* prevents testis differentiation. However, one copy of DSS in the presence of *SRY* results in normal male development. One hypothesis states there could be an interaction between *SRY* and DSS which determines if testicular or ovarian development occurs. In normal males *SRY* could represses DSS, which in turn leads to testis differentiating genes being released from suppression by DSS. In females, lack of *SRY* means that DSS continues to repress testis determining genes, allowing ovarian development to proceed (fig 5). In males with a duplication of DSS, *SRY* may not sufficiently repress DSS. Testis determining genes would, therefore, be suppressed leading to gonadal dysgenesis (Zanaria *et al.*, 1995; Ogata and Matsuo, 1996).

Candidate genes have been isolated within the DSS critical region. These are: DAM 6, DAM 10, *MAGE-Xp* and *Dax-1*. Dam genes (DSS/AHC critical interval belonging to the MAGE superfamily) and *MAGE-Xp* (melanoma antigen gene) belong to the MAGE superfamily, which have so far been located on the long arm of the X chromosome (Xq27-qter). The biological function of the MAGE or DAM genes is unknown and there is no evidence, at present, to support *MAGE-Xp* or either DAM gene as the critical gene in the DSS locus (Muscatelli *et al.*, 1995; Dabovic *et al.*, 1995; Zanaria *et al.*, 1995).

Deletions and mutations in *Dax-1* (DSS-AHC critical region on the X, gene 1) enabled it to be isolated as the gene responsible for the X-linked form of adrenal hypoplasia congenita (AHC) (Zanaria *et al.*, 1994; Muscatelli *et al.*, 1994). AHC is characterised by a general structural disorganisation of the adrenal cortex, resulting in glucocorticoid and mineralocorticoid insufficiency (Burris *et al.*, 1996). The AHC

locus partially overlaps the DSS critical region and males with X-linked AHC often exhibit abnormalities of the genitourinary system, suggesting that *Dax-1* may also be the critical gene in the DSS locus (Burris *et al.,* 1996).

Human *Dax-1* encodes a member of the nuclear hormone receptor superfamily (Zanaria *et al.,* 1994; Swain *et al.,* 1996). The highest homology is to the E box domain of the retinoid X receptor (RXR) subfamily (Zanaria *et al.,* 1994). The ligand binding domain, in the C-terminus, is important for hormone binding, dimerization and transactivation (Zanaria *et al.,* 1994; Burris *et al.,* 1996). The N-terminal portion of the protein consists of three and a half repeats of an alanine- and glycine-rich 65 - 67 aa motif (Zanaria *et al.,* 1994; Swain *et al.,* 1996; Burris *et al.,* 1996) and is thought to encode a novel DNA binding domain, consisting of two zinc fingers (Zanaria *et al.,* 1994) (fig 4).

Dax-1 is first expressed at 10.5 to 11 dpc in mouse, in the somatic cells of the genital ridge of both sexes (fig 5). Expression of *Dax-1* is down-regulated in males at 12 dpc. Expression is maintained in the female gonad up to 14.5 dpc, when it becomes restricted to a region of the ovary proximal to the mesonephros (Swain *et al.,* 1996). *Dax-1* expression is not restricted to the steroidogenic cells in the gonads (Ikeda *et al.,* 1996; Tamai *et al.,* 1996). If *Dax-1* is DSS, it is unlikely *SRY* would repress it directly because *Dax-1* expression is observed in males and females at 11.5 dpc, when *SRY* is being maximally expressed. However, *Dax-1* activity could be repressed by genes downstream of *SRY* in the testis-determining pathway (Swain *et al.,* 1996). Expression of *Dax-1* is also observed in the hypothalamus and anterior pituitary, adrenal, heart, thymus, spleen and lung (Zanaria *et al.,* 1994; Swain *et al.,* 1996; Bae *et al.,* 1996; Guo *et al.,* 1995).

Due to a similar tissue type and developmental pattern of expression between SF-1 and *Dax-1* and the identification of an SF-1 site in the *Dax-1* promoter; the possibility of an interaction or transcriptional regulation which might affect a common developmental pathway was studied (Burris *et al.,* 1995; Ikeda *et al.,* 1996). Parker and colleagues studied the co-localization of *Dax-1* and SF-1 transcripts. SF-1 initially precedes expression of *Dax-1* in the genital ridge by 1 - 1½ days of development; but co-localization of the transcripts is detected in the adrenal gland and the gonads from 11 dpc to 12.5 dpc (Ikeda *et al.,* 1996). In the ovary, SF-1 and *Dax-1* can be detected at 12.5 dpc, but are down-regulated by 14.5 dpc (Ikeda *et al.,* 1996). Majdic and Saunders (1996) studied the localisation of SF-1 and *Dax-1* proteins in rat fetal testis. They found that *Dax-1* protein did not exclusively immunolocalize with cells expressing the SF-1 protein, indicating *Dax-1* does not require SF-1 for expression.

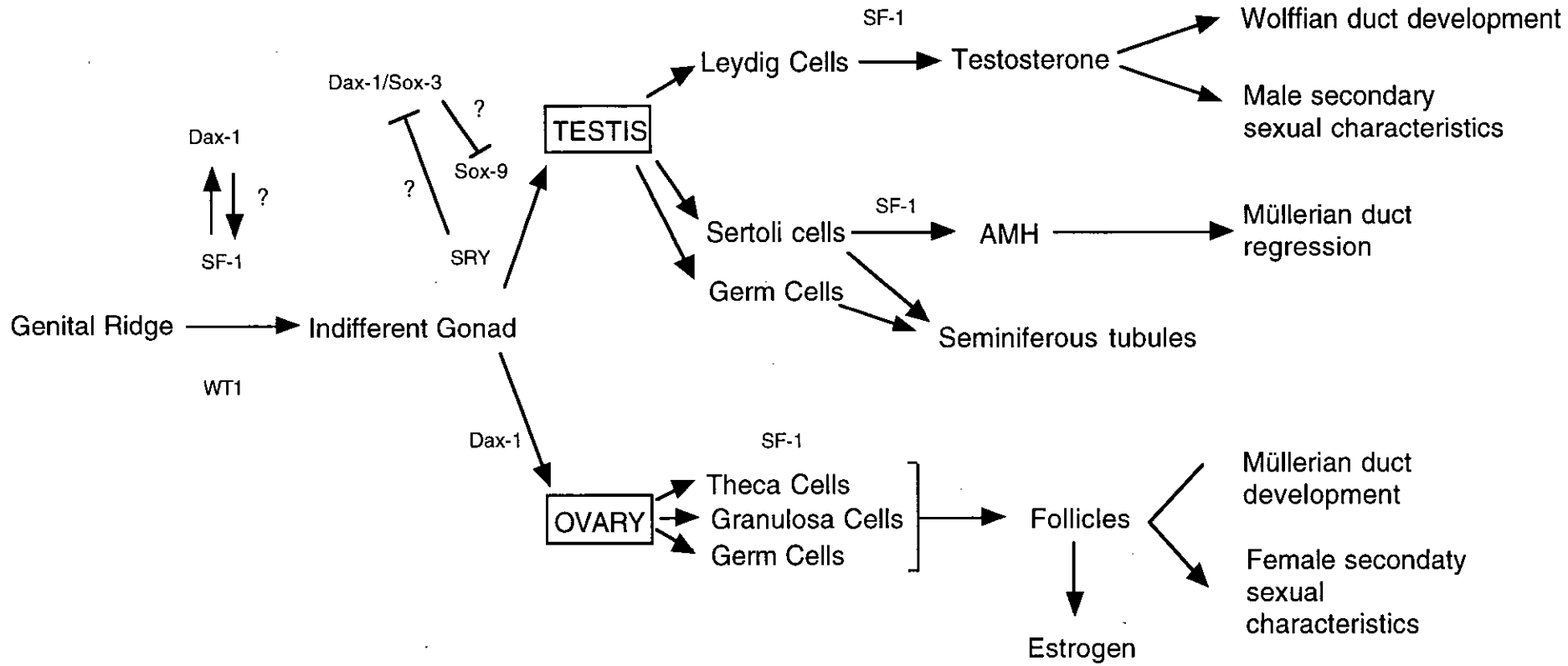


Figure 5: Schematic representation of the initial stages of mammalian sex determination and gonadal development.Modified from Ramkissoon and Goodfellow, 1996.

If *Dax-1* is regulated by SF-1, its expression should be affected in *Ftz-F1* knockout mice. However, *Dax-1* was detected in the gonad at 11.5 dpc and in the VMH in the developing hypothalamus, which degenerate, in the knockout mouse (Ikeda *et al.*, 1996). Finally, one other possibility is that *Dax-1* and SF-1 interact directly as heterodimers, as the proteins do co-localize in some cells (Vilain *et al.*, 1997; Majdic and Saunders, 1996) and *Dax-1* has been shown to inhibit SF-1 mediated transactivation *in vitro* (Ito *et al.*, 1997).

Dax-1 expression profile correlates well with the potential DSS location and its role in sex determination and sex reversal. At present, however, there is no conclusive evidence that *Dax-1* is DSS.

1.4.5. Sox-9 (SRY-related HMG box containing gene 9).

The discovery that there is male to female sex reversal in 46 XY individuals, with an intact *SRY* gene, and there are XX males not resulting from the presence of *SRY* lead to the conclusion that other genes on the X chromosome and autosomes were required for sex determination regulation (Schafer *et al.*, 1995; Foster, 1996; Wagner *et al.*, 1994). One such locus is the autosomal sex reversal locus (SRA1) located on chromosome 17q. Sex reversal phenotypes range from partial testis development to gonads with some ovarian development. This sex reversing locus is associated with campomelic dysplasia (CD); a skeletal malformation syndrome (Sinclair, 1997; Foster, 1996; Schafer *et al.*, 1995). As XX CD patients did not express any ovarian degeneration, it was assumed the gene at this locus was required for correct testis differentiation (Capel, 1995).

SOX9 belongs to the SOX (SRY-like HMG box containing) family. These genes exhibit at least 60% similarity to the *SRY* HMG domain (Goodfellow and Lovell-Badge, 1993). *SOX9* exhibits 71% homology at the amino acid level to this potential DNA binding domain (Foster *et al.*, 1994). The other characteristic of *SOX9* is the proline/glutamine rich region in the carboxy-terminus of the protein (Foster *et al.*, 1994; Wagner *et al.*, 1994; Wright *et al.*, 1995) (fig 4).

Mutational studies were carried out on patients with CD and XY sex reversal to elucidate whether *SOX9* caused this phenotype. Mutations were identified that resulted in premature stop codons, amino acid substitutions and frameshift mutations, all affecting a single allele (Foster *et al.*, 1994; Wagner *et al.*, 1994; Kwok *et al.*, 1995; Meyer *et al.*, 1997). In fact, as mutations appeared to affect one allele, haploinsufficiency was proposed as the cause of this phenotype and *SOX9* was considered to play a role in skeletal and testis development (Foster *et al.*, 1994; Wagner *et al.*, 1994; Foster, 1996; Schafer *et al.*, 1995). Mutations in the gene have

a more severe phenotype than translocation breakpoints upstream of the gene (Wagner *et al.*, 1994). The relationship between *SOX9* and the translocation breakpoints, which can be upto 100kb upstream of the gene, is unknown. The composite transcript of *SOX9* was found to be 3934bp in length. However, by Northern analysis a transcript of ~4.3kb is detected (Foster *et al.*, 1994; Wagner *et al.*, 1994). One explanation would be that there are exons 5' to the already existing exons and this 5' exon is disrupted by the translocation (Foster, 1996; Schafer *et al.*, 1995).

Expression profiles of *Sox9* in mouse and chicken were studied. Wright *et al.* (1995) demonstrated that *Sox9* in mouse is expressed throughout chondrogenesis. Chondrogenesis is the process whereby mesenchymal cells condense to the shape of bone, resulting in hyaline cartilage differentiation and subsequent skeletal development (Wright *et al.*, 1995).

Due to the sex reversal phenotype, expression of *Sox9* was also analyzed by *in situ* hybridization in the gonads of mouse and chick. *Sox9* transcripts are detectable at low levels in mouse in both sexes (10.5 dpc) (Kent *et al.*, 1996; da Silva *et al.*, 1996) (fig 5) and in the male and female chick (5.5d) (da Silva *et al.*, 1996). By 11.5 dpc in the mouse and 6.5d to 7.5d in the chick *Sox9* expression is detected at higher levels in the gonads of male embryos. Low levels (da Silva *et al.*, 1996) to undetectable levels of *Sox9* expression (Kent *et al.*, 1996) were reported in female chick gonads. No *Sox9* transcripts are detectable in female mouse gonads at this stage (Kent *et al.*, 1996; da Silva *et al.*, 1996).

By 12.5 to 13.5 dpc in the mouse, *Sox9* is localized to the Sertoli cells of the sex cords (da Silva *et al.*, 1996; Kent *et al.*, 1996). Localisation of SOX9 protein is initially cytoplasmic, before 11.5 dpc. It is only detected in the nucleus after 11.5 dpc (da Silva *et al.*, 1996), coincident with high levels of *SRY* expression (Harley *et al.*, 1997), indicating a possible interaction between SRY and SOX9. Recently, two nuclear localisation signals have been identified, located in the HMG domain of Sry and SOX9 proteins (Südbeck and Scherer, 1997). The timing of *SOX9* expression in relation to *SRY* expression suggests there could be an interaction between the two genes. However, the postulated role for *SRY* is as an inhibitor, and as *SOX9* and *SRY* are both involved in testis determination it is unlikely *SRY* would directly regulate *SOX9* (Graves, 1997a). It is possible that *SRY* could regulate the sexually dimorphic expression of *SOX9* by inhibiting a repressor of *SOX9* allowing testis development to occur (Graves, 1997b; Swain *et al.*, 1998).

1.5. Hormonal regulation of gonadal development.

1.5.1. Background to hormonal studies.

Chick embryos were castrated between day 3 and day 4 of incubation by localized X-irradiation and characterised on the basis of the development of three structures: the syrinx, genital tubercle and Müllerian ducts. Castrated male and female embryos developed as neutral types. The neutral type has a syrinx and genital tubercle identical to the male. However, the Müllerian ducts developed equally on both the left and right side, unlike males or females (Wolff and Wolff, 1951), which suggested the male phenotype was the default pathway. In mammals, early castration of mammalian embryos (e.g. rabbit) resulted in feminization of the genital tract occurred (Jost, 1953; Jost, 1960; Jost 1965). This lead to the theory that mammalian ovarian development is the default pathway (Jost, 1953). Together these observations lead to the hypothesis that in the mammalian embryo, during gonadal development, a masculinizing hormone is predominant whereas during gonadal development in the avian embryo a feminizing hormone is predominant (Jost, 1965; Wolff and Wolff, 1951).

The dominance of the male phenotype over the female phenotype was observed in "freemartins" of cattle (Lillie 1917; Jost, 1965). The term "freemartin" applies to the female masculinized in the presence of a male co-twin. The cortical development of the ovary is inhibited, tubules resembling seminiferous tubules are observed in the medulla, the Müllerian ducts are inhibited and the Wolffian ducts may persist (Jost, 1965; Willier, 1921). However, masculinization is not complete especially as development of external structures is slight, if at all (Hunter, 1995). It was suggested the hormone essential for masculinizing the embryo reached the "freemartin" in limited amounts, that the hormone acted locally, or that other factors were involved (Jost, 1960; Jost, 1965). The mammalian testis is essential for inhibiting development of female structures derived from the Müllerian ducts, and inducing differentiation of male structures from the Wolffian ducts (Jost, 1953).

An experiment was carried out in rabbits, where a crystal of testosterone was placed near one ovary, or a foetal testis was grafted onto an ovary. Both the foetal testis and the crystal of testosterone stimulated Wolffian duct differentiation . The foetal testis, however, also inhibited Müllerian duct development locally, whereas the crystal containing testosterone did not (Jost , 1953). Therefore, it appeared one hormone did not possess both activities and so two different hormones were proposed to be required for development of the complete masculine phenotype. One hormone to inhibit Müllerian duct development and an androgen type hormone to stimulate male organogenesis (Jost, 1960).

1.5.2. AMH (anti-Müllerian hormone).

AMH is a hormone secreted by the testis, and causes regression of the Müllerian ducts (Vigier *et al.*, 1984; Jost, 1960). This hormone has a variety of other names: Müllerian-inhibiting factor (MIF), Müllerian-inhibiting substance (MIS) or Müllerian inhibitor (Josso and Picard, 1986).

The bovine, murine, rat and human genes for AMH have been isolated (Cate *et al.*, 1986; Münsterberg and Lovell-Badge, 1991; Haqq *et al.*, 1992) and have homology to the transforming growth factor-β family of genes (TGF-β) (Cate *et al.*, 1986). The mature AMH protein is 140kD glycoprotein dimer that is linked by disulphide bonds (Picard *et al.*, 1978; Lee and Donahoe, 1993). The role of the N-terminus is unknown, but it has been proposed to be required for folding and secretion of the C-terminal region (Wilson *et al.*, 1993). The regression and antiproliferative activity of the protein is located in the C-terminus of the protein (Lee and Donahoe, 1993; Josso, 1992). Wilson *et al.* (1993) utilised a C-terminal fragment of AMH to cause regression of the Müllerian ducts, indicating the receptor binding domain is present in this region. However, addition of the N-terminal region increased the activity of the C-terminal region. The chick AMH gene has also been isolated and cloned (Eusèbe *et al.*, 1996; Neeper *et al.*, 1996). Homology between human and chick AMH is reported to be 27% identity (Eusèbe *et al.*, 1996) or 42% identity along its length (Neeper *et al.*, 1996).

AMH is the earliest Sertoli cell specific marker in the mammal (Münsterberg and Lovell-Badge, 1991). In the mouse, AMH is first detected in the male at 11.5 dpc, coincident with the highest levels of expression of *Sry* (Hacker *et al.*, 1995) and is expressed as a result of sex determination. Expression of AMH, in the mouse, peaks at 13 dpc and levels out thereafter (Hacker *et al.*, 1995). Suppression of testicular expression of AMH coincides with the beginning of meiosis in spermatids (Münsterberg and Lovell-Badge, 1991). AMH expression is also detected in the female postnatally, from day 6 onwards. Expression is only observed in the granulosa cells, and is possibly involved in oocyte maturation and follicle development (Münsterberg and Lovell-Badge, 1991). These results suggest AMH expression prevents germ cell development through meiosis.

In contrast to mammalian AMH expression, AMH in the chick is expressed in male and female gonads, although at much higher levels in the male (Teng, 1987; Eusèbe *et al.*, 1996). Expression was detected at 8d and peaked at 10d in the male (Eusébe *et al.*, 1996). This contradicts work carried out by Teng (1987), who identified three stages when AMH content is high in the testis. These are 6d to 8d,

14d to 20d and 5 to 8 weeks after hatching. Expression was high in the left and right ovary at 8d, but after 10d higher expression was detected in the left ovary compared to the right ovary (Eusèbe *et al.,* 1996). In contrast, Teng (1987) detected low levels of AMH protein in left and right gonads from 6d to 12d. Highest AMH levels, in the combined left and right ovaries, was detected between 9d and 14d. AMH was detected in the future and definitive Sertoli cells of the testis and in the region between the cortex and the medulla of the left ovary (Eusèbe *et al.,* 1996).

The Müllerian ducts are responsive to AMH for only a short time during embryonic development in all species studied (Josso *et al.,* 1977; Rey and Josso, 1996). Chronic overexpression of AMH in females, resulted in the Müllerian ducts and ovaries degenerating, with few germ cells that were eventually lost. The somatic cell organisation of the degenerating ovary resembled seminiferous tubules (Behringer *et al.,* 1990). This phenotype correlated with the female bovine freemartin (Behringer, 1995). The majority of males chronically expressing AMH exhibited normal development. However, those males expressing the highest levels of AMH exhibited feminization of external genitalia, incomplete Wolffian duct development and undescended testes (Behringer *et al.,* 1990). This indicates the potential developmental processes in which AMH is involved and the possibility of very high levels of AMH leading to incorrect interactions with its receptor (Behringer, 1995).

Interestingly, overexpression of AMH in rat, turtle and chick females leads to increased levels of testosterone. This is due to AMH reducing the activity of aromatase (di Clemente *et al.,* 1992; Vigier *et al.,* 1989), the enzyme essential for conversion of androgens to oestrogens (Josso, 1992).

The specificity of AMH between different species has also been examined. Chick Müllerian ducts regressed only in the presence of chick AMH (Weniger *et al.,* 1991; Tran and Josso, 1977). However, rat Müllerian ducts regressed in the presence of rat or chick AMH (Tran and Josso, 1977). As chick AMH is active in the rat but rat AMH is not active in the chick, it is possible the rat receptor does not have specific binding requirements compared with the chick receptor (di Clemente *et al.,* 1992; Weniger *et al.,* 1991). Alternatively, as cleavage of mammalian AMH is required for an active molecule, it is possible the chick embryo does not produce the correct protease for this cleavage (di Clemente *et al.,* 1992; Weniger *et al.,* 1991).

AMH loss-of-function studies were also carried out in order to better study the function of AMH during development (Behringer *et al.,* 1994). Male mice lacking functional AMH have fully descended, normal-sized testes and produced functional sperm. However, female reproductive organs also developed, which interfered with sperm transfer, and the mice were infertile (Behringer *et al.,* 1994; Behringer, 1995).

In addition, Leydig cell hyperplasia was observed in some cases, indicating the potential role of AMH in regulation of Leydig cell proliferation (Behringer *et al.*, 1994; Behringer, 1995).

The AMH gene must be tightly regulated to ensure expression begins at the correct developmental stage and in the correct tissue type to ensure Müllerian duct regression and potentially regulate spermatogenesis, oogenesis and other developmental processes. Guerrier *et al.* (1990) identified multiple transcription initiation sites in the AMH promoter, with one major start site and at least three minor start sites. In the rat AMH promoter, a sequence corresponding to the binding site for SF-1 was identified (Lee and Donahoe, 1993). Finally, a 13bp palindromic sequence, with identity to the oestrogen response element (ERE) has been identified in the promoter region of the human AMH gene, suggesting estrogen could regulate AMH expression (Guerrier *et al.*, 1990; Lee and Donahoe, 1993).

The receptor for AMH has been isolated in rabbit, rat and human (di Clemente *et al.*, 1994; Baarends *et al.*, 1994; Imbeaud *et al.*, 1995). The receptor for AMH shows homology to the type II membrane-bound serine/threonine kinase receptor of the TGF-β receptor family (Behringer, 1995; di Clemente *et al.*, 1994). Two transcripts of the AMH type II receptor were identified, generated by alternative splicing (di Clemente *et al.*, 1994). Both isoforms are expressed in AMH target tissues, although there are slightly higher levels of the longer isoform (di Clemente *et al.*, 1994). Binding studies carried out using these two different isoforms indicated the long receptor isoform allowed AMH binding, whereas the shorter isoform did not (di Clemente *et al.*, 1994).

Expression of the AMH receptor was detected in the Sertoli cells of the foetal testis and in the foetal ovary. It is also detected in the granulosa cells of small and medium follicles. In addition, the AMH receptor is detected in the mesenchymal cells surrounding the Müllerian ducts (di Clemente *et al.*, 1994; Baarends *et al.*, 1994).

In an attempt to establish that signalling through the AMH receptor is essential for Müllerian duct regression, receptor mutant mice were generated by gene targeting (Mishina *et al.*, 1996). Female mice homozygous for the receptor mutation developed normally and were fertile (Mishina *et al.*, 1996), corresponding to the phenotype of AMH knockout female mice (Behringer *et al.*, 1994). Male AMH receptor knockout mice developed as internal pseudohermaphrodites. They had fully descended, normal-sized testes and the Wolffian ducts had developed normally. However, the Müllerian ducts had not degenerated and so female reproductive organs had also developed (Mishina *et al.*, 1996), again showing the same phenotype observed with male AMH knockout mice (Behringer *et al.*, 1994). The receptor deficient male mice exhibited

normal levels of AMH expression, indicating the reason the Müllerian ducts did not regress was caused by a lack of AMH signal transduction (Mishina *et al.*, 1996).

Double mutant mice for AMH and the AMH receptor were also generated. The double mutant phenotype was the same as for each of the signal mutant phenotypes (Mishina *et al.*, 1996). These results suggest no other ligands signal through the AMH receptor and AMH does not utilize any other receptor for signal transduction (Mishina *et al.*, 1996; Josso and di Clemente, 1997).

1.5.3. Testosterone.

Testosterone is required during gonadal development for maintenance of, and differentiation of, the Wolffian ducts into the epididymis, vas deferens, seminal vesicles and ejaculatory ducts and possibly has a role in regulating spermatogenesis (Siiteri and Wilson, 1974; Josso, 1981; Wilson *et al.*, 1993). Testosterone is secreted by the Leydig cells of the foetal and adult testis (Josso, 1981; Josso, 1992; Wilson *et al.*, 1993), and is the only androgen produced by the testis at the time of male sexual differentiation (Wilson and Siiteri, 1973).

The rabbit has a gestation period of 30 to 32 days (McLaren, 1972). The sexual differentiation of the gonads in rabbit occurs from day 15 (Jost, 1953) and phenotypic differentiation of the gonads does not occur until approximately day 17 or day 18 (Wilson *et al.*, 1980). In all mammalin species studied thus far, testosterone synthesis does not begin until just before the onset of male phenotypic differentiation (Wilson *et al.*, 1980). Leydig cells are the steroid producing cells and differentiation of these cells occurs after the initial stages of sexual differentiation at day 18 (Wilson and Siiteri, 1973).

Wilson and Siiteri (1973) reported negligible amounts of testosterone in the testis at day 17, but by day 19 testosterone levels had increased. Levels of testosterone continued to increase to day 23, after which point levels remained high in the testis. Testosterone production was not detected in the ovaries, between day 23 to 3 days after birth. The results of testosterone synthesis appears to correlate with Leydig cell differentiation.

Androgen synthesis by chick gonads has also been studied. Androgens were first detected in the genital ridge of both sexes at 3.5d, in interstitial cells (Woods and Podczaski, 1974). However, it is considered that interstitial cells differentiate after sex determination, which does not occur until later in development (Romanoff, 1960). The amount of androgens produced were similar in both testes and ovaries between 3.5d and 4.5d. Initially, androgen levels increased slightly in the right ovary, up to day 6.5 of incubation, then levels decreased. In the left ovary, levels of androgen

synthesis only increased very slightly to 19.5d. In the testis, there was an overall steady increase in the levels of androgen production (Woods and Podczaski, 1974). However, these results are contradicted by recent work carried out by McBride *et al.* (unpublished observations), in which testosterone levels remain low in both male and female genital ridges from 3.5d to 12.5d.

Testosterone is secreted by the testis and enters its target tissues by a passive diffusion mechanism (Wilson *et al.,* 1980; Wilson *et al.,* 1993). Once inside its target cell, testosterone can be converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase (Wilson *et al.,* 1993; Josso, 1981), or it can interact with the androgen receptor protein (Wilson *et al.,* 1980). The undifferentiated Wolffian ducts require testosterone for differentiation of these ducts (Josso, 1981). DHT is required for masculinization of the urogenital sinus and external genitalia during embryogenesis, and at puberty for male sexual maturation (Josso, 1981; Wilson *et al.,* 1993).

Testosterone and DHT bind to the same receptor, but with different affinities and different resulting functions in gonadal development. Conversion of testosterone to DHT amplifies the androgen signal in several ways. Firstly, DHT is unable to be aromatized to estrogen. Secondly, DHT binds the androgen receptor with greater affinity and with more stability compared to testosterone (Josso, 1992). Finally the DHT-receptor complex is more rapidly transformed into a DNA-binding state (Wilson *et al.,* 1993). Testosterone and DHT bind to the androgen receptor (AR). Once bound to, an active androgen-AR complex is formed which is then located predominantly in the nucleus and can regulate transcription (Jenster *et al.,* 1991; Batch *et al.,* 1992; Pinsky *et al.,* 1994).

The androgen receptor is a member of the steroid hormone receptor family (Lubahn *et al.,* 1988; Tilley *et al.,* 1989). The receptor is composed of three distinct functional domains (Batch *et al.,* 1992; Lubahn *et al.,* 1988). The amino-terminal domain has a putative transcriptional activation function (Lubahn *et al.,* 1988). The second domain is the central region which forms two zinc fingers and is involved in DNA binding (Lubahn *et al.,* 1988; Batch *et al.,* 1994). The carboxy-terminus is the domain responsible for androgen binding (Lubahn *et al.,* 1988; Batch *et al.,* 1992).

In both mammals and birds, exogenous testosterone in a genetic female causes masculinization of the genital tract, although regression of the Müllerian duct does not occur (Grumbach and Ducharme,1959; Jordanov and Angelova, 1984). In 7.5d female chick gonads treated with testosterone, the cortex does not thicken significantly. However, it has been noted that there are varying effects on the cortex of the gonad by testosterone. The medulla enlarges with tubular structures forming,

similar to those observed in the testis. Interstitial cells also had an appearance similar to those observed in testes (Jordanov and Angelova, 1984).

1.5.4. Oestrogens.

Due to the fact that early castration of rabbits results in development of female characteristics (Jost, 1953), it was suggested steroid hormone synthesis was not essential for female development and differentiation. However, oestrogen production was first detected in the foetal ovaries of rabbits between day 17 and 17.5, and increased up to day 25 (Milewich *et al.*, 1977; George *et al.*, 1978; Wilson *et al.*, 1980). The onset of oestrogen production occurs at the same time as testosterone production in the testis, in both rabbits and humans (Wilson *et al.*, 1980). Oestrogen synthesis was detected in testis at day 19, but at much lower levels compared with the foetal ovary (George *et al.*, 1978).

In the chick, if gonads were not present male characteristics developed. Therefore, it is possible oestrogen is the essential steroid hormone in chick gonadal development needed for feminization of the genital tract (Jost, 1965). Woods and Erton (1978) reported detection of oestrone and 17β-oestradiol in the indifferent gonads at 3.5d. Estrogen synthesis was approximately equal between the left ovary, right ovary and the testes up to 5.5d. By 6.5d highest levels of oestrogen was in the left ovary compared to the right ovary and testes (Woods and Erton, 1978). Oestrogen synthesis increased to up to 13.5d and then plateaued, in the left ovary. Oestrogen was detected in the medullary interstitial cells between 3.5d and 12.5d, and from 13.5d onwards, in the cortical cords (Woods and Erton, 1978). In the right ovary, oestrogen production decreased to almost undetectable levels by 13.5d. In the testis, oestrogen synthesis exhibited a gradual increase, but at much lower levels compared to the left ovary (Woods and Erton, 1978). This study was contradicted by recent radioimmunoassay studies carried out by McBride *et al.* (unpublished observations), which indicated oestrogen synthesis was not detectable in ovaries until 6.5d.

As the rate-limiting enzyme for oestrogen production is aromatase (Josso, 1992), it is possible the expression profile of aromatase might clarify which report on oestrogen production is correct. In rabbits, oestrogen synthesis via aromatase activity is detected in the ovaries between day 18 and 29, and there is low aromatase expression in the testis (Weniger, 1990).

Aromatase expression has also been analyzed during chick gonadal development. Aromatase expression in the female gonad was detected from 6.5d (Yoshida *et al.*, 1996; Smith *et al.*, 1997), which corresponds to the initial detection of oestrogen at the same stage, as reported by McBride *et al.* (unpublished

observations). Aromatase was not detected in the male gonad at any stage examined (Yoshida *et al.*, 1996; Smith *et al.*, 1997), indicating only females are able to produce oestrogens. Aromatase expression was observed in the medullary cords of the female gonad (Yoshida *et al.*, 1996).

If chick embryos from preincubation to day 7 of incubation are treated with an aromatase inhibitor, varying degrees of male development are observed (Elbrecht and Smith, 1992; Wartenberg *et al.*, 1992; Shimada, 1997). In the left (genetic female) chick gonad, the cortex can be reduced, a tunica albuginea may develop, reduced numbers of germ cells can be observed in the cortex and there can be an increase in the volume of the medulla (Wartenberg *et al.*, 1992). Of the treated females that developed as phenotypic males at hatching, only 50% continued to develop to maturity as phenotypic males (Elbrecht and Smith, 1992). No effect was ever observed in the male. Sex reversed females were able to mate with hens, but had a low sperm count and were often infertile (Shimada, 1997). Addition of extra oestrogen was able to reverse the effects of the aromatase inhibitor, indicating the aromatase inhibitor was preventing oestrogen production (Elbrecht and Smith, 1992). This indicates the importance of oestrogen in feminization of the genital tract.

A situation analagous to the bovine freemartin is the double yolk egg, where a female and male embryo develop in the same egg (Hutson *et al.*, 1983). In the female there was partial regression of the Müllerian ducts and in the male there was partial feminization of the gonads (Hutson *et al.*, 1983). This observation indicated the importance of oestrogen in the development of the gonads, although the presence of oestrogens did not fully feminize the male gonads. As addition of testosterone to the female chick gonad has a masculinizing effect and addition of oestrogens to the male gonad induces feminization of the genital tract (Jordanov and Angelova, 1984), it has been hypothesised that the ratio of androgens to oestrogens is critical for mediating gonadal development in the chick (MacLaughlin *et al.*, 1983; Jordanov and Angelova, 1984).

Oestrogen receptor (*cER*) expression has been analyzed in the chick (Smith *et al.*, 1997; Andrews *et al.*, 1997). Expression of *cER* was detected at 3.5d in the female genital ridge, and by 4.5d it was detected in the indifferent gonads and the anterior tips of the Müllerian ducts of both sexes. By 7.5d, expression was detected in the gonads and Müllerian ducts of both sexes (Andrews *et al.*, 1997, Smith *et al.*, 1997). However, the cER protein was only detected in the left and right ovaries at 7.5d (Andrews *et al.*, 1997), suggesting cER is not functional in the male. However, addition of exogenous oestrogens (at doses similar to physiological levels) to a 7.5d male chick embryo resulted in feminization of the genital tract, with formation of a

thicker cortex and some disorganisation of the seminiferous cords, although this feminization of the gonads was transitory (Scheib, 1983; Jordanov and Angelova, 1984; Stoll *et al.*, 1993).

It is documented that AMH may regulate gonadal steroids by decreasing aromatase biosynthesis (Vigier *et al.*, 1989). In male mammals, AMH is secreted during embryonic development but only postnatally in females. It follows, therefore, that in female mammals delayed expression of AMH would allow aromatisation of androgens to occur. The oestrogens that are produced would be able to protect against the effects of AMH, as has been predicted (Hutson *et al.*, 1982). Localisation of oestrogen receptors to the Müllerian ducts (Andrews *et al.*, 1997) support this theory. Isolation of oestradiol-specific nuclear type II receptor, which exhibited higher expression levels in the left Müllerian ducts of chick embryos (MacLaughlin *et al.*, 1983), could be related to the preservation of the left Müllerian duct in female chicks.

If a male (chick or mammal) gonad is treated with an oestrogen, it becomes feminized and Müllerian ducts are retained, although production of AMH still occurs. These studies supported the hypothesis that oestrogen does not cause retention of the Müllerian ducts by suppressing AMH production, but by interacting with AMH at the Müllerian ducts or by protecting the Müllerian ducts from AMH action (Hutson *et al.*, 1982; Newbold *et al.*, 1984; Doi and Hutson, 1988).

1.5.5. Summary of AMH, oestrogen and testosterone action.

1. AMH is required for Müllerian duct regression in mammalian males and avian males. It is also required for regression of the female right Müllerian duct in avians.
2. Rat AMH is not active in chick embryos, but chick AMH is active in rat embryos, suggesting differences in specificity between AMH from different species.
3. Chronic overexpression of AMH in a female mouse results in degeneration of the Müllerian ducts and the somatic component of the ovary resembles seminiferous tubules. This result correlated with the bovine freemartin phenotype.
4. Loss-of-function studies of AMH in male mice resulted in normal testis development with functional sperm, but female structures also developed because the Müllerian ducts did not regress, thus interfering with sperm transfer. The same phenotype was observed in genetic males if the AMH receptor was knocked out. Female mice without functional AMH or AMH receptor were morphologically normal.
5. Testosterone is secreted by the Leydig cells and is required for the maintenance and differentiation of the Wolffian ducts in male mammals.
6. Testosterone was detected in the male rabbit testis just after sex determination. No testosterone was detected in the ovary at the same stage of development.

7. Woods and Podczaski (1978) detected testosterone in the urogenital ridge at day 3.5 of incubation in male and female chick embryos. Testosterone levels were higher in the testes compared to either ovary, after day 4.5 of incubation. However, this is contradicted by McBride *et al.* (unpublished observations), where testosterone levels remained low in males and females from day 3.5 to day 12.5 of incubation, indicating testosterone is not required for initial testis development in the chick.

8. The androgen receptor (AR) has been isolated in mammals. A mutation in the AR results in androgen insensitivity, even if testosterone and DHT levels are normal.

9. In mammals and birds, exogenous testosterone in a genetic female results in masculinization of the genital tract, but without regression of the Müllerian ducts. However, in birds, the effect of testosterone on the cortex of the gonad can vary and can be transitory.

10. Oestrogens are not considered essential for mammalian female sexual development, because castration of male and female rabbits resulted in the development of female characteristics.

11. Castration of female chick embryos resulted in male characteristics developing, although the Müllerian ducts did not regress. This indicated oestrogens were required for chick female gonadal development.

12. Oestrogen has been detected as early as day 3.5 of incubation in chick embryos (Woods and Erton, 1978). However, McBride *et al.* (unpublished observations) did not detect estrogen until day 6.5 of incubation in chick ovaries. Oestrogen was not detected above background levels in the testes.

13. Aromatase is the rate limiting step for oestrogen production in mammals and birds. Aromatase biosynthesis can be regulated by AMH.

14. Genetically female chick embryos treated with an aromatase inhibitor develop as phenotypic males or exhibit intermediate stages of sex reversal. However, this sex reversal is transitory for 50% of the embryos.

15. The role of testosterone and oestrogen in mammals is fairly well understood. However, in chicks sex reversal is observed by addition of oestrogens or androgens (Thorne, 1995). The various range of effects on gonadal development, in the chick, by steroid hormones, and the fact that most of these effects are transitory suggest that hormonal control is not the primary factor determining sex.

Chapter 2

Aims and Objectives.

Although structurally distinct, the vertebrate testis and ovary develop from the same tissue primordia during embryogenesis. The exact mechanism which allows two apparently different developmental outcomes is unknown. By far the best understood vertebrate gonadal development system is the mammalian system, where the exact timing and a number of the genes involved in this process have been described. However, even in the mammalian gonadal system, interactions between these genes have not been fully established. There are clearly other genes involved in this process that have not as yet been identified, also the origins of the cells contributing to the indifferent gonads and the developing ovary and testis remains controversial.

This investigation is concerned specifically with gonadal development in the chick. At present the mechanism for determining whether the indifferent gonad follows the ovarian or testicular developmental pathway is unknown, although it is assumed to be unlike the mammalian mechanism as the female is the heterogametic sex (ZW) in birds, and the male is the heterogametic sex (XY) in mammals. A number of studies have failed to identify a homologue to the gene which acts as the sex determining switch in mammals (Sinclair *et al.*, 1990; Griffiths, 1991; Tiersch *et al.*, 1991; McBride *et al.*, 1997). Gonadal differentiation in birds is assumed to follow the same pattern as in mammals, with regard to morphology and genetic regulation. However, asymmetry in the female chick reproductive system and the fact that only piecemeal studies have been carried out on the expression of chick homologues to mammalian genes, means further work needs to be done before this assumption can be confirmed. The aims of this project are to document the morphology of chick gonadal development and analyse gene expression, of both novel and previously identified genes, during the entire period of chick gonadal development; i.e. from the stage of genital ridge formation and development through to the early stages of ovary and testis formation. This study will attempt to establish a) whether the gonadal development pathway between avians and mammals is conserved, b) the timing of sex determination in chicks more accurately than at present, and c) identify novel genes involved in gonadal development and study their expression profile.

In the mammal, histological analysis of gonadal development is well documented (Kaufman, 1992). However, in the chick, despite numerous descriptive accounts of gonadal development (e.g. Romanoff, 1960; Venzke, 1954a; Venzke, 1954b), a histological analysis detailing gonadal development has not been well documented. The descriptive accounts of chick gonadal development are detailed, but often conflicting observations are recorded regarding development and the timing of

this development. This results in an unclear pattern and timing of chick gonadal development.

The fact that the sex determination mechanism and, therefore, the exact timing of sex determination are unknown in the chick, leads to difficulties in establishing whether certain genes are expressed prior to sex determination, are involved in the sex determination switch or are expressed as a consequence of sex determination. Studies that narrow the time period for the sex determining mechanism will lead to a better understanding of the genes involved in the various stages of chick gonadal development.

A great deal of information can be gained by studying chick homologues to genes involved in mammalian gonadal development, to elucidate whether they are likely to have the same roles in chicks. Such a study of chick homologues, while informative is unlikely to provide complete details due to differences between mammalian and chick gonadogenesis. Therefore, identification of novel transcripts involved in the many aspects of gonadal differentiation and development in the chick is required.

Three approaches were followed in order to gain a better understanding of chick gonadal development. These were:

1. A histological study of chick gonadal morphology during development.
2. A study of the expression of chick homologues to genes reported to be involved in mammalian gonadal development.
3. The isolation of novel genes involved in chick gonadal development using the technique of differential display.

2.1. Morphology of chick gonadal development.

Both the gross morphology and the histological detail of gonadal development was studied. The developmental period chosen was from day 3.5 to day 8.5 of incubation (stage 21 to 35). This was based on the estimated time of sex determination in the chick as occurring at a stage between day 5 and day 8 of incubation (Romanoff, 1960). The developmental time period chosen would, therefore, cover indifferent gonad formation through to the early stages of ovary and testis development.

This study would provide an easily visualised profile of the morphology of chick gonadal development, possibly resolving the discrepancies recorded and more accurately show the developmental stage when initial differences are observed between males and females. It would also allow a direct comparison with mammalian gonadal development.

2.2. Expression of chick homologues to genes involved in mammalian gonadal development.

Chick homologues to genes reported to be involved in mammalian gonadal development analyzed in this study were: WT-1, Sox-9, aromatase, AMH and SF-1. Expression of these genes was studied by Northern analysis and for three of the genes by whole mount *in situ* hybridization. A number of these genes are expressed as a consequence of sex determination and others are expressed throughout the indifferent period and into ovary and/or testis development, with differences in expression levels between the sexes due to sex determination. A study of these genes in the chick system would provide a comparison at the level of gene regulation between mammals and birds and establish whether chick gonadal development follows a similar gene pathway to mammalian gonadal development. Also, as gene expression can be a consequence of sex determination and alter expression levels, a profile of these genes might more accurately predict the sex determination period in chicks.

2.3. Isolation of novel genes involved in chick gonadal development using the technique of differential display.

In an attempt to identify novel genes involved in chick gonadal development, the technique of differential display was utilised (Liang and Pardee, 1992). This technique allows the comparison of transcripts at different developmental timepoints between different sexes. Each transcript is represented by a fragment of cDNA that can be visualised on a gel by autoradiography. Different sample populations can be visualised side by side, thus allowing differentially expressed transcripts to be identified and the DNA recovered for further analysis.

Alternative techniques that could have been utilised for differential gene expression analysis are: subtractive hybridization and differential screening. Both of these techniques involve the construction of cDNA libraries, a time consuming and labour intensive procedure. Subtractive hybridization involves the enrichment of differentially expressed sequences by subtraction of commonly expressed sequences. As subtractive hybridization is a kinetic reaction (which results in the reaction never reaching completion), not all common transcripts will be removed. This would lead to a high rate of false positives. Differential screening has the disadvantage that duplicate lifts of the cDNA library need to be made; thus equal amounts of cDNA transferred to both filters would be difficult to achieve and lead to false differences in expression being detected. Another disadvantage of both techniques is the large amounts of starting material (poly A+ RNA) required, suggesting low abundance transcripts would be difficult to detect. Finally, the differences in expression are not easily

visualised compared to differential display, where products are separated on a polyacrylamide gel and can be compared in adjacent lanes (Liang and Pardee, 1992; Maser and Calvert, 1995; Watson and Margulies, 1993).

The incubation period of a chick is 21 days. The development of the urogenital ridge through to the initial stages of ovary and testis development occurs from approximately 3.5 to 8 days of incubation (Romanoff, 1960). The differential display analysis was performed on male genital ridge/mesonephric tissue and female genital ridge/mesonephric tissue from day 3.5 to 9.5 of incubation (stage 21 to 36), encompassing these developmental time periods.

The aim of this study was to assess the potential of using this technique in a complex system and if possible to isolate novel genes involved in chick gonadal development.

Chapter 3

Materials and Methods.

3.1. Materials.

3.1.1. Chemicals, reagents and equipment.

Chemicals and reagents were obtained from Sigma Chemical Co., Poole UK and were of analytical grade, with the following exceptions:

Restriction enzymes were obtained from Boehringer Mannheim, UK; Promega, USA; or New England Biolabs, Cambridge UK.

Radio-isotopes ^{32}P and ^{35}S were from Amersham International, Aylesbury, UK.

Propan-2-ol, ethanol, paraformaldehyde, Tween-20, agarose, EDTA (diaminoethanetetra-acetic acid disodium salt), Tris (hydroxymethyl) methylamine, sodium chloride, urea, lead (II) acetate, sodium dihydrogen orthophosphate 1-hydrate, di-sodium hydrogen orthophosphate anhydrous, acetone, chloroform, formaldehyde solution (40% w/v), hydrochloric acid sp. gr. 1.18, acid fuchsin, aniline blue and phosphotungstic acid were all obtained from BDH, Poole UK.

40% (w/v) acrylamide/Bis-acrylamide stock solution (19:1) and ammonium persulphate were obtained from Anachem, Beds UK.

Long Ranger™ Gel solution for DNA sequencing was obtained from Flowgen, Staffs UK.

TEMED ([N,N,N',N'-Tetramethylethylenediamine]), X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thio-galactopyranoside) were obtained from Promega Ltd, Southampton UK.

Proteinase-K solution (RNA grade), phenol:chloroform:isoamyl alcohol (25:24:1 (v/v)) and sonicated salmon sperm were obtained from GibcoBRL Life Technologies™, Scotland.

Sodium acetate (anhydrous), Potassium chloride, D-glucose, Silica gel 4-7 mesh self indicating, magnesium chloride, magnesium sulphate, sucrose, tri-sodium citrate, sodium hydroxide, tetrahydronaphthalene, dimethylformamide, Optiphase "HiSafe" 2

scintillation fluid, methanol and glacial acetic acid were from Fisher Scientific, Loughborough UK.

Phosphate buffered saline tablets (PBS) (Ca^{2+} and Mg^{2+} free) were obtained from ICN Biomedicals Inc., Ohio USA.

Deionized formamide was obtained from AMS Biotechnology, UK.

Tryptone and yeast extract were obtained from Difco Laboratories, USA.

Kodak EPY64 film was obtained from H.A. West, Edinburgh.
Cronex X-ray film was obtained from Tech Photosystems, Cumbernauld UK.
Kodak Biomax MS and Biomax MR film was obtained from Sigma-Aldrich Techware, UK.

Picric acid (2, 4, 6 - Trinitrophenol) and Haematoxilyn were obtained from Hopkin and Williams, Essex UK.

Orange G and Ponceau 2R Xylidine-Rd were obtained from George T Gurr Ltd., London UK.

Eosin (yellowish - water/alcohol soluble) was obtained from Raymond A. Lamb, London UK.

Equipment.

All solutions, glassware and plastics were presterilized by autoclaving and those used in RNA procedures were autoclaved twice. Glassware used in RNA procedures was baked at 180°C for 12 hours before use.

Centrifugation.

Centrifugation of eppendorf tubes (3 000 to 13 000rpm) was performed using a Sorvall® RMC 14 (Sorvall Ltd, UK) and a IEC Micromax® (Hybaid Ltd, Middlesex UK) bench top microfuge. Centrifugation of nalgene tubes (React Scientific, Ayrshire) (6 000g and 15 000g) was performed using a JA20 rotor in a J2-21M/E centrifuge (Beckman, UK).

3.1.2. Chick Material.

ISA-Brown and 101 eggs from commercial hens were incubated at 38°C ± 0.5°C on day of lay. Day of lay was considered day 0 of incubation and all embryos were staged according to Hamburger and Hamilton (1951). All embryos were harvested into a petri dish containing PBS (phosphate buffered saline). The genital ridge and a small portion of the mesonephros were dissected from chick embryos after 3.5d, 4.5d, 5.5d, 6.5d, 7.5d, 8.5d and 9.5d, using standard dissection instruments. The genital ridge was frozen in liquid nitrogen and stored at -70°C.

3.1.3. Bacterial Growth.

Bacteria were grown at 37°C overnight in the following media containing the appropriate antibiotic:

Luria-Bertani (LB) medium (1l)	10g Difco Bacto Tryptone
	5g Difco Bacto Yeast extract
	5g sodium chloride
	pH 7.2
L agar (1l)	10g Difco Bacto Tryptone
	5g Difco Bacto Yeast extract
	10g sodium chloride
	3.75g Difco agar
	pH 7.2
NZY medium (1l)	5g Sodium chloride
	2g magnesium sulphate
	5g Difco Bacto Yeast extract
	10g NZ amine (caesin hydrolysate)
	pH 7.5
NZY agar	1 litre NZY medium + 15g Difco agar
	pH 7.5
NZY top agar	1 litre NZY medium + 0.7% (w/v) agarose

The following bacterial strains were used:

INVαF' TA cloning (Invitrogen, Netherlands).

F' endA1 recA1 hsdR17 (r_k^- m_k^+) supE44 thi-1 gyrA96 relA1 ϕ80 lacZΔM15Δ(lacZYA-argF) U169 deoRλ⁻.

DH5α (GibcoBRL Life Tecnologies™, Scotland).

F' ϕ80d lacZΔM15Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (r_k^-, m_k^+) phoA supE44 λ^- thi-1 gyrA96 relA1.

JM109 (Stratagene Ltd, Cambridge UK).

F' λ^- e14-(mcrA) recA1 endA1 gyrA96 thi-1 hsdR17 (r_k^-, m_k^+) supE44 relA1Δ(lac-proAB) [F' traD36 proAB $lacI^q$ ZΔM15].

XL1-Blue MRF' (Stratagene Ltd, Cambridge UK).

λ^-, Δ(mcrA)183Δ(mcrCB-hsdSMR-mrr)173 end A1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r)].

XLOLR (Stratagene Ltd, Cambridge UK).

Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac[F' proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r)]Su^- λ^r.

The following vectors were used:

pCR™II (Invitrogen, Netherlands) Kan^r Amp^r

pBluescript MS(-) (Stratagene Ltd, Cambridge UK) Amp^r

pBK-CMV (Stratagene Ltd, Cambridge UK) Kan^r Neo^r

3.2. Biochemical techniques.

3.2.1. Phenol extraction

Protein was removed from DNA by extraction with phenol:chloroform:isoamyl alcohol (25:24:1 (v/v)). An equal volume was added to the solution, vortexed and centrifuged at 13 000rpm for 4 mins. The upper aqueous phase was removed and combined with an equal volume of choloroform and mixed. The solution was centrifuged for 2 mins and the upper aqueous phase recovered. The DNA was recovered by precipitation with 2.5 volumes of 100% ethanol and 0.1 volumes of 3M Na OAc (Sambrook *et al.*, 1989).

3.2.2. PCR purification.

PCR products were purified away from primers, nucleotides and proteins using a QIAquick spin column, according to the manufacturers instructions (Qiagen, Germany). PCR product was eluted in 50µl deionized distilled dH_2O and stored at -20°C.

3.2.3 Spectrophotometric analysis

DNA and RNA were quantitated by measuring the optical density at 260nm using a GeneQuant (Pharmacia Biotech, UK) spectrophotometer. At 260nm wavelength, an absorbance reading of 1 is the equivalent of a concentration of 50µg/ml and 40µg/ml for DNA and RNA respectively. Purity of the nucleic acid was calculated by measuring the absorbance ratio at 260/280nm. Pure DNA has a value of 1.8 and RNA a value of 2.0 (Sambrook *et al.*, 1989).

3.2.4. Storage of bacteria

Colonies were stored on inverted L-agar plates containing the appropriate antibiotic and sealed with Parafilm M® (American National Can™, USA) for up to 6 weeks at 4°C. Long term storage was in a 44% glycerol solution, snap frozen on dry ice/ethanol and stored at -70°C. Recovery of the bacteria was carried out by scraping the frozen culture with a toothpick and streaking out on a L-agar containing the appropriate antibiotic (Sambrook *et al.*, 1989).

3.3. Isolation of plasmid DNA.

3.3.1. TENS Preparation.

2ml of plasmid bearing strain was grown at 37°C overnight in LB medium under the appropriate antibiotic conditions. Cells were harvested by centrifugation at 13 000 rpm and resuspended in 50µl supernatant. Cells were lysed by addition of 300µl TENS solution (10mM Tris pH 7.5, 1mM EDTA, 0.1M Na OH, 0.5% SDS). Cell debris and chromosomal DNA were pelleted by centrifugation at 13 000 rpm for 4 mins. Supernatant containing plasmid DNA was removed and recovered by precipitation with 2.5 volumes 100% ethanol and 0.1 volume 3M Na OAc. Plasmid DNA was washed in 70% ethanol and resuspended in 50µl distilled deionized dH_2O containing 20µg/ml RNAse A and stored at -20°C.

3.3.2. "Mini" Preparation of plasmid DNA.

The plasmid bearing bacterial strain was grown in 2ml LB medium containing the appropriate antibiotic at 37°C overnight. The mini preparation of plasmid DNA was carried out following the manufacturers instructions and utilising spin columns provided with the kit (Qiagen, Germany; Hybaid Ltd, Middlesex UK). DNA was eluted in 50 µl distilled deionized dH_2O and stored at -20°C.

3.3.3. "Midi" Preparation of plasmid DNA.

The plasmid bearing bacterial strain was grown in 25ml LB medium containing the appropriate antibiotic at 37°C overnight. The midi preparations were carried out according to the manufacturers instructions, using QIAGEN-tip 100 or Quick flow columns provided with the kit (Plasmid Midi kit, Qiagen, Germany; Quick Flow Midi kit, Hybaid Ltd, Middlesex UK). Plasmid DNA was eluted in 1ml aliquots of elution buffer and recovered by precipitation with propan-2-ol at 13 000 rpm at 4°C for 30 mins. DNA was washed in 70% ethanol and resuspended in 200-400µl distilled deionized dH_2O and stored at 4°C.

3.4. Isolation of chick RNA

Genital ridge tissue from 50 pooled males and 50 pooled females was used for RNA extraction each time. RNA extraction was carried out 7 times. Dissected chick gonadal ridge/mesonephros, was homogenized in 2ml per 100mg of tissue of RNAzol™ B (AMS Biotechnology, Oxon U.K.) using a Polytron homogeniser. Between each homogenisation, the probe was washed 3 times in 0.2M Na OH and 3 times in distilled, deionized water. Total RNA was extracted by addition of chloroform (0.2ml per 2ml) to the homogenate. This was mixed vigorously and centrifuged for 15mins at 12 000g at 4°C. The upper aqueous phase containing total RNA was removed and the RNA recovered by precipitation with propan-2-ol at 4°C. Total RNA was washed in 70% ethanol and resuspended in a small volume of distilled, deionized water. Total RNA was stored as an ethanol precipitate at -70°C.

3.5. Enzymatic reactions

3.5.1. Restriction endonuclease digestion.

DNA restriction digests were routinely carried out at 37°C from 1 hour to overnight using restriction enzymes (see section 3.1.1. for manufacturers) and their recommended buffers. The majority of restriction enzymes were of the concentration 10U/µl, therefore 10U (1µl) of enzyme and 2µl of buffer were used to restrict 5µg of

DNA in a 20µl volume. In larger volumes containing higher concentrations of DNA, the enzyme concentration was always less than 5% of the total reaction volume.

Reactions that required digestion by two enzymes were carried out in the same buffer, if compatible. If two separate buffers were required, the first enzyme was added and incubated for 1 hour before recovering the DNA by phenol extraction and ethanol precipitation, then incubating in the buffer recommended for the second enzyme. Bovine serum albumin (BSA) was added to a final concentration of 1mg/ml. Digestion of genomic DNA required a longer incubation time and a higher concentration of enzyme.

3.5.2. Ligation.

Ligation into linearised and dephosphorylated pBluescript M13(-) (Stratagene) or pCR™II (Invitrogen, Netherlands) was carried out in the presence of T4 DNA ligase at 14°C overnight. A 1:1 or a 1:3 ratio of vector to insert was used in the ligation reaction. The reaction was stored at -20°C.

3.5.3. 5' dephosphorylation by alkaline phosphatase.

Dephosphorylation of a linearised vector was carried out by addition of 1µg/ml calf intestinal alkaline phosphatase (CIAP) and incubation at 37°C for 1 hour followed by addition of a further 1µg/ml CIAP and incubation at 37°C for 1 hour. Incubation at 85°C for 10 mins removed residual phosphatase activity (Sambrook *et al.,* 1989).

3.6. Transformation.

Introduction of a vector into a bacterial strain was carried out by heat shock at 42°C or 37°C according to the manufacturers instructions, except 25µl aliquots of cells rather than 50µl aliqouts were used (JM109, Stratagene, UK; One Shot INFαF', Invitrogen Netherlands: DH5α, GibcoBRL Life Technologies™, Scotland). Cells were allowed to recover at 37°C for 1 hour by shaking at 225rpm in SOC (2% tryptone, 0.5% yeast extract, 10 mM Na Cl, 2.5mM K Cl, 10mM Mg SO_4, 20mM glucose). 50µl, 200µl or all of the cells were spread on L-agar containing 50µg/ml ampicillin, 5µg/ml X-gal and 1mM IPTG. Plates were inverted and incubated at 37°C overnight.

3.7. Size fractionation of nucleic acids by electrophoresis.

3.7.1. DNA.

DNA was size fractionated on a 1% agarose gel/1xTBE (0.8M Tris, 0.8M H_3BO_3, 0.002M EDTA) containing 0.2µg/ml ethidium bromide in a 1xTBE buffer containing 0.2µg/ml ethidium bromide. 24µl sample contained 4µl 6xTypeIII loading dye (0.25% bromophenol blue, 0.25% xylene cyanole FF in 30% glycerol). Elecrophoresis of DNA was routinely carried out at 95-100V/cm for 1 hour. DNA was visualised and photographed under U.V. light at a wavelength of 254nm. DNA gels were exposed to Polaroid black-and-white film type 55 (Sigma, UK) for 20-30 secs, on a Polaroid MP.4 Land Camera (Polaroid, Mass USA).

3.7.2. RNA .

5 different pooled male and pooled female RNA samples were used in Northern analysis, with 15 RNA gels being carried out. 5-10µg RNA in sample buffer (50% deionized formamide, 18% formaldehyde, 10% MOPS) and containing 0.3µg/ml ethidium bromide and 4µl 6xTypeIII loading dye was size fractionated on a 1% agarose/6.8% formaldehyde gel. The gel was electrophoresed at 80V for 2.5 - 4 hrs in 1x MOPS (0.2M 3[n-Morpholino]propane sulphonic acid, 0.05M Na OAc, 0.01M EDTA, pH7.0). RNA was visualised under U.V. light at a wavelength of 254nm, and exposed for 10 - 15 secs using Polaroid black-and-white film type 55 on a Polaroid MP.4 Land Camera.

3.8. Transfer of nucleic acid onto nylon membrane.

3.8.1. Southern analysis.

Genomic DNA (a gift from D. Morrice, Roslin Institute) and plasmid DNA size fractionated by electrophoresis in an agarose gel (without ethidium bromide) was stained for 30 mins in 1µg/ml ethidium bromide, 0.5xTBE. The gel was wrapped in Saran wrap (Dow Chemical Co.,) and U.V. irradiated at 60mJoules/cm^2 using a U.V. stratalinker 2400 (Stratagene Ltd, Cambridge UK). The gel was visualised and photographed for 5 sec exposure under U.V. light. The gel was soaked in 0.4N Na OH, 1.5M Na Cl. The DNA was transferred onto Hybond-N nylon membrane (Amersham International, UK). Transfer was carried out for 16 - 20 hrs. Once transfer was complete, the membrane was orientated and neutralised in 0.5M Tris, pH 7.0. DNA was fixed onto the nylon membrane by baking at 80°C for 2 hrs.

3.8.2. Northern analysis.

The gel containing size fractionated RNA was soaked in 5xSSC (3M Na Cl, 0.3M Na citrate). RNA was transferred onto Hybond-N nylon membrane (Amersham International). Transfer was allowed to take place for 20 - 24 hrs. Once transfer was complete, the membrane was baked at 80°C for 2 hrs.

3.8.3. Dot Blot.

DNA samples resuspended in 10xSSC were denatured and 50µl of each sample was drawn onto Hybond-N nylon membrane using a Hybri.Dot™ 96 well filtration manifold (GibcoBRL Life Technologies™, Scotland). The filter was washed in denaturing solution (1.5M Na Cl, 0.5M Na OH) and then neutralising solution (1.5M Na Cl, 0.5M Tris-HCl pH 7.2, 0.001M EDTA). DNA was fixed to the membrane by baking at 80°C for 2 hrs.

3.9. DNA extraction from agarose and polyacrylamide gels.

3.9.1. Agarose gel.

Extraction of DNA from agarose gels was carried out following the manufacturers instructions (Qiagen, Germany). DNA was eluted in 50µl distilled, deionized H_2O. Alternatively, the DNA fragment was excised from the agarose gel. The gel slice was loaded into a 1ml syringe barrel (Terumo Corporation, Belgium) and reduced to a fine slurry by ejection into a Spin-X tube, containing a 0.22µm cellulose acetate filter (Corning Costar Ltd, UK). The gel slurry was mixed with 200µl distilled dH_2O and centrifuged at 13 000rpm so that the DNA was expelled into the collection tube. DNA was concentrated by ethanol precipitation (Schwarz and Whitton, 1992).

3.9.2. Polyacrylamide gel.

DNA fragments excised from polyacrylamide gels were rehydrated in distilled dH_2O and boiled at 99°C for 15 mins. DNA was recovered by ethanol precipitation in the presence of a carrier, 2.5 µl of 20mg/ml glycogen. After centrifugation, DNA was resuspended in 10µl deionized, distilled H_2O.

3.10.1 Random prime labelling of DNA with ^{32}P.

25ng denatured DNA insert was added to a Rediprime labelling mix (Amersham International, Bucks UK) and incubated at 37°C in the presence of α-^{32}P

dCTP. The reaction was stopped by addition of 5µl 0.02M EDTA. The labelled probe was purified on a G50 Sephadex column (Pharmacia Biotech Inc., Herts UK). 1% of the probe was quantitated using a 1218 Rackbeta Liquid Scintillation Counter (LKB Instruments Ltd, Surrey UK). The probe was denatured at 95°C for 5 mins, cooled quickly on iced H_2O and pulse spun to collect the liquid at the bottom of the tube.

3.10.2. Probes used in hybridisation.

DNA fragments isolated by differential display analysis were used as probes in Northern analysis and were transcribed from T7, T3 or Sp6 in the vectors pCRII and pBK-CMV for wmISH. Sizes of inserts and sequence can be found in appendix 4.

Probes used in the analysis of candidate genes by Northern analysis were as follows:

1. WT-1: a 500bp fragment of the chick WT-1 gene.
2. AMH: a 1905bp fragment of the cAMH gene.
3. SF-1: a 500bp fragment of the cSF-1 gene.
4. Aromatase: a 351bp fragment of the cAromatase gene.
5. Sox-9: a 1200bp fragment of the cSOX-9 gene.

Probes used in the analysis of candidate genes by wmISH were as follows:

1. WT-1: the plasmid was restricted with NsiI and transcribed with T7 to give a 600bp riboprobe.
2. AMH: the plasmid was restricted with NcoI and transcribed with T7 to give a 459bp riboprobe.
3. SF-1: the plasmid containing a 1.3kb fragment of the ZFSF-1 gene was restricted with DdeI and transcribed with T3 to give a sense riboprobe of 570bp; and it was restristed with BglI and transcribed with T7 to give an anti-sense riboprobe of 530bp.

A schematic of these probes and from whom they were obtained are detailed in appendix 3.

3.11. Hybridization of ^{32}P labelled DNA probes to nylon membranes.

3.11.1. Southern analysis.

Southern blots were pre-hybridized at 55°C for 1 hr in 2xPIPES (1,4-piperazine-diethanesulfonic acid), 50% formamide, 0.5% SDS and 250µg/ml denatured, sonicated salmon sperm DNA. The denatured probe was added at a maximum concentration of 1.5 x 10^6 cpm (counts per min)/ml. Hybridization was carried out at 60°C overnight. Southern blots were washed to a final stringency of 0.2xSSC, 0.1% SDS at 65°C.

3.11.2. Northern analysis.

Northern blots were pre-hybridized for a minimum of 1 hr at 60°C in 0.25M Na PO_4, 7% SDS, 250µg/ml denatured salmon sperm DNA; or at 42°C in 2xPIPES, 50% formamide, 0.5% SDS, 250µg/ml denatured salmon sperm DNA. The denatured probe was added at a concentration of 2 million cpm/ml and hybridized at 60°C or 42°C overnight. Northern blots were washed to a final stringency of 10mM Na PO_4, 0.1% SDS at 65°C (Na PO_4 hybridization solution) or 0.1xSSC, 0.1% SDS at 65°C (PIPES hybridization solution).

3.11.3. Dot Blot analysis.

Dot blot membranes were pre-hybridized in 0.25M Na PO_4, 7% SDS, 250µg/ml denatured salmon sperm DNA at 60°C for 1 hr. The denatured probe was added at a concentration of 1.5 million cpm/ml. Hybridization was carried out at 60°C overnight. Dot blots were washed to a final stringency of 25mM Na PO_4, 1% SDS at 65°C.

3.12. Autoradiography.

Gels containing α-^{35}S dATP were exposed to Dupont Cronex film (Tech Photosystems,) or Kodak Biomax MR film (Sigma-Aldrich Techware, UK). Exposure time ranged from overnight to 3 days at room temperature.

Gels or nylon membranes containing α-^{32}P dCTP were exposed to Dupont Cronex film or Kodak Biomax MS film (Sigma-Aldrich Techware, UK). Exposure time was from 1hr at room temperature to 2 weeks at -70°C. All films were developed using a X-OGraph Compact x2 (supplied by H.A. West, Edinburgh).

3.13 Phosphorimager analysis.

Gels and blots containing ^{32}P were exposed to a phosphor screen from 1 hr to 2 weeks. The image was visualised using a Molecular Dynamics phosphorimager.

3.14. PCR for sexing embryos.

Approximately 50mg of tissue from the remainder of the chick embryo (after dissection of mesonephroi and gonads) was taken and incubated in 50µl digestion buffer (10mM Tris, 1mM EDTA, 1% SDS, pH8.0 containing 10mg/ml proteinase-K) at 45°C overnight. Samples were cooled to 4°C and centrifuged at 13 000rpm. The recovered supernatant was diluted to 1ml in 1xTE (10mM Tris-HCl, 1mM EDTA).

Diluted DNA (1µl) was used in a PCR reaction using W-chromosome specific primers (Clinton, 1994) and chick GAPDH primers (sequence obtained from D.Burt, Roslin Institute) or chick 18S ribosomal primers (Clinton *et al.*, unpublished data) as a control amplification (appendix 1). The W primer and GAPDH primer amplification was carried out as two separate reactions. The W and GAPDH reaction conditions were an initial denaturation at 94°C followed by 20 (W) or 30 (GAPDH) cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 5 min. The W and 18S ribosomal reactions were performed in a single tube under the following conditions; an initial denaturation step at 94°C for 1 min followed by 25 cycles of 96°C for 15 sec, 56°C for 15 sec, 72°C for 15 sec and a final extension step at 72°C for 5 min.

3.15. Differential display analysis.

Two different pooled male and pooled female RNA samples were used for differential display analysis. 5µg of total RNA extracted from pooled male and pooled female genital ridge tissue was used to prepare cDNA using a first strand cDNA synthesis kit (Pharmacia Biotech Inc., UK). Synthesis of cDNA utilised modified oligo dT_{12} MG or oligo dT_{12} MC or oligo dT_{12} MA primers (M = A, G or C). A fraction of the cDNA (0.4%) was used as a template in the differential display PCR reaction. Differential display analysis was performed as described by Liang and Pardee (1992). The modified oligo dT_{12}MN primer used to prepare cDNA, was used in combination with a 10mer of arbitrary sequence (see appendix 2 for primer sequences) in PCR reactions. Each reaction was performed in 20µl 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM K Cl, 0.1mg/ml gelatine, pH 8.3 containing 2mM dNTPs, 2.5µM modified oligo dT primer, 0.5µM primer of arbitrary sequence and 1 unit of Taq polymerase (Boehringer Mannheim, UK) in the presence of 10µCi α-^{35}SdATP. The reaction conditions for the differential display procedure were an initial denaturation step at 94°C for 30 sec followed by 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec for 40 cycles and a final extension step of 72°c for 5 min. PCR products (6µl) were separated on a 6% native polyacrylamide gel (Bauer *et al.*, 1993) using a standard sequencing apparatus (Stratagene Ltd, UK). Gels were dried using a Model 583 gel dryer (Bio-rad Laboratories Ltd, Herts UK).

3.16. Re-amplification.

DNA eluted from gel bands excised from the display gel were re-amplified under similar reaction conditions and using the original combination of primers as the display PCR; except that the primer and dNTP concentration was 2.5μM and 1mM respectively. A fraction of the re-amplified product (25%) was analyzed by agarose gel electrophoresis.

3.17. DNA sequencing.

3.17.1. Chain termination sequencing.

DNA was sequenced using the Sequenase™ Version 2.0 DNA sequencing kit (Amersham International, UK), using T7, T3 or M13 reverse primers in the presence of 5μCi α-^{35}S dATP.

3.17.2. Cycle sequencing.

DNA was sequenced using the fmol® DNA sequencing system (Promega Ltd, Southampton UK), using direct incorporation of radio-isotope. T7, T3 or M13reverse primers were used and each reaction was performed in the presence of 10μCi α-^{35}S dATP. Reactions were initially denatured at 95°C for 2 min, followed by 50 cycles of 95°C for 30 sec, 42°C for 30 sec, 70°C for 30 sec.

Sequencing reactions carried out by both methods were separated on a denaturing 5% Long Ranger polyacrylamide gel, on standard sequencing apparatus (Stratagene Ltd, UK) and a GenomyxLR™ Programmable DNA Sequencer (Beckman, UK).

3.18. Genomic library screening (chick).

The chick genomic library, cloned into the BamHI site of the lambda ZAP express™ vector (Stratagene Ltd, UK) was obtained from Dr. B. Earnshaw.

3.18.1. Preparation of host cells.

Glycerol stocks of cells were streaked onto L-agar containing 12.5μg/ml tetracycline and incubated at 37°C overnight. Plates were sealed with Parafilm M® (American National Can™) and stored at 4°C for up to 1 week. A single colony of XL1-Blue MRF' cells was grown overnight at 30°C in LB medium supplemented with

0.2% (w/v) maltose, 10mM $MgSO_4$. XLOLR cells were grown overnight at 30°C in NZY medium.

3.18.2. Titering library.

XL1-Blue MRF' cells grown overnight were diluted 1:100 and incubated at 37°C until they reached an OD_{600} of 1.0. Cells were diluted to an OD_{600} of 0.5. Serial dilutions of the phage were prepared in SM buffer (100mM Na Cl, 8mM Mg SO_4, 50mM Tris-H Cl pH 7.5, 0.01% (w/v) gelatin). Host cells were inoculated with diluted phage and plated out on NZY top agar. Plates were inverted and incubated overnight at 37°C. Plaques were counted and the concentration of plaque forming units (pfu)/ml calculated.

3.18.3. Primary screen.

Host cells inoculated with phage were plated out at 50 000 pfu/plate on NZY agar and incubated overnight at 37°C. Plates were chilled at 4°C for 2 hrs before performing duplicate plaque lifts using Hybond-N nylon membrane (Amersham International, UK) (Benton and Davis, 1977). Transfer of DNA was allowed to take place for 2 mins and 4 mins for the first and second lifts respectively. Membranes were marked for orientation at this stage. DNA was denatured in 1.5M Na Cl, 0.5M Na OH, neutralised in 1.5M Na Cl, 0.5M Tris-H Cl, pH 8.0, and rinsed in 0.02M Tris-H Cl pH 7.5, 2x SSC. DNA was fixed to nylon membranes by baking at 80°C for 2 hrs. Pre-hybridization was performed in 2xPIPES, 50% deionized formamide, 0.5% SDS, 100µg/ml denatured, sonicated salmon sperm DNA at 42°C for 2 hrs. Denatured probes were added to the hybridization solution at a concentration of $1x10^6$ cpm/ml. Hybridization was performed at 42°C overnight. Membranes were washed to a final stringency of 0.2xSSC, 0.1% SDS at 65°C. Filter hybridization was visualised by autoradiography.

3.18.4. Secondary screen.

Duplicate autoradiographs were orientated to line up putative positive clones with plaques on the plates. A Pasteur pipette (Fisher Scientific, UK) was used to core the plaques of interest. Cored plaques were stored in SM buffer containing a few drops of chloroform at 4°C. Each cored plaque was assumed to contain $1x10^7$ pfu/ml. Host cells were inoculated with diluted phage and plated out at a frequency of 50 - 1000 pfu/plate. Plates were incubated at 37°C overnight. Plaque lifts, pre-hybridization, hybridization and post-hybridization washes were performed as before

(see section 3.18.3). Isolated positive plaques were stored in SM buffer containing a few drops of chloroform.

3.18.5. Recovery of insert.

Excision rescue was carried out by combining XL1-Blue MRF' cells, at an OD_{600} of 1, with $>1x10^5$ phage particles and $>1x10^6$ pfu/ml ExAssist helper phage. XLOLR cells were inoculated with the excised phagemid and plated out on L-agar containing 50µg/ml kanamycin and incubated at 37°C overnight.

3.19. Whole mount *in situ* hybridization.

3.19.1. Collection and processing of chick material.

The whole mount *in situ* hybridization was based on the procedure by D. Wilkinson (1992) and its subsequent modification by D. Henrique and D. Ish-Horowicz (protocol supplied by C. Stern).

Chick mesonephroi and gonads were dissected out of embryos from d4.5, 5.5, 6.5, 7.5, 8.5 of incubation and d3.5 chick embryos were partially dissected removing the head and removing the overlying viscera to expose the urogenital ridge. Dissected tissue was fixed in fresh 4% (w/v) paraformaldehyde, 2mM EGTA pH 7.5 at 4°C overnight. Dissected tissue was subsequently dehydrated through 25%, 50%, 75% and 100% methanol washes and stored in 100% methanol for up to 4 months at -20°C.

Embryos from the same stage of development as dissected tissue were used to prepare embryo powder. Embryos were homogenized in PBS, washed in acetone and centrifuged at 10 000g. The pellet was ground into a powder on filter paper and air dried. Once dry, embryo powder was stored at 4°C.

3.19.2. *In vitro* transcription.

In vitro transcription of a sense and anti-sense probe using 1µg linearised plasmid containing insert was performed following the manufacturers instructions for the Digoxygenin (DIG) RNA labelling kit (Boehringer Mannheim, UK), using Sp6, T7 or T3 RNA polymerase. A fraction (1µl) of the transcribed product was electrophoresed on an agarose gel, transferred to nylon membrane (Hybond-N) and incubated in a 1:2500 dilution of the DIG antibody (Boehringer Mannheim, UK) at 4°C. The antibody was detected by a colour reaction (see section 3.20.4) to ensure the transcription reaction had worked. The remainder of the transcribed product was recovered by ethanol precipitation and diluted to 100ng/µl in hybridization solution

(50% formamide, 1.3xSSC pH 5.3, 5mM EDTA 50µg/ml yeast RNA, 0.002% Tween-20, 0.005% Chaps ((3-[3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), 100µg/ml Heparin (Falkirk Vet Drug Centre, Scotland) and stored at -20°C.

3.19.3. Preabsorption of antibody.

A certain amount (X) of embryo powder (X = 2x the number of ml of final antibody solution required) was weighed out and incubated in 1xTBST (1.4mM Na Cl, 0.03mM K Cl, 25M Tris-H Cl pH 7.5, 1% Tween-20) at 70°C. Embryo powder was washed in 1xTBST and incubated in blocking buffer (1xTBST, 5% heat inactivated sheep serum, 1mg/ml BSA) and the DIG antibody at a 1:250 dilution. The preabsorbed antibody was removed by centrifugation. The supernatant containing the antibody was diluted to a final concentration of 1:2500 and stored at 4°C.

3.19.4. *In situ* hybridization.

Dissected tissue was rehydrated through a 75%, 50% and 25% methanol washes and rinsed twice in PTW (Ca^{2+}, Mg^{2+} free PBS, 0.1% Tween-20). Tissue was incubated in 10µg/ml Proteinase-K (GibcoBRL Life Technologies™, UK) and post-fixed in 4% (w/v) paraformaldehyde, 0.1% gluteraldehyde. Tissue was pre-hybridized in hybridization solution at 62 - 66°C for a minimum of 4 hrs. The probe was added to fresh hybridization solution at a concentration of 1µg/ml and incubated at 62 - 66°C overnight. Tissue was washed in pre-hybridization solution, and 1xTBST:pre-hybridization solution (1:1) at the hybridization temperature and in 1xTBST at room temperature. Tissue samples were incubated in blocking buffer at room temperature. The DIG antibody, conjugated to alkaline phosphatase was added at a 1:2500 dilution and incubated overnight at 4°C. Post-antibody washes in 1xTBST were performed at room temperature for 24 hrs. Detection of alkaline phosphatase was performed by incubating in 1.5ml 1xNTMT containing 4.5µl 75mg/ml NBT and3.5µl 50mg/ml BCIP, protected from light at room temperature. The colour reaction was allowed to develop from 30 mins to 3 days.

Tissue was photographed using Kodak EPY64 film on a Leitz Wild Heerbrugg zoom microscope.

3.19.5. Embedding, sectioning and counterstaining of DIG whole mounts.

Tissue was rapidly dehydrated through 100% methanol, propan-2-ol and tetrahydronaphthalene. Tissue was incubated in wax at 60°C for 18 - 24 hrs. Tissue was embedded using a Reichert Jung tissue embedding centre. Sections were counterstained in 0.5% eosin in 25% ethanol and visualised and photographed using a Nikon Microphot-SA microscope and Kodak EPY64 film.

3.20. Histological staining of chick genital ridge tissue.

Between 3-5 mesonephroi and genital ridge tissue was used per day of incubation for histological staining. Mesonephroi and genital ridge tissue from d 4.5, 5.5, 6.5, 7.5 and 8.5 of incubation was collected and fixed in 4% (w/v) paraformaldehyde at room temperature overnight. Tissue was placed in 0.5M sucrose in PBS at room temperature overnight. Tissue samples were dehydrated through 0.85% Na Cl (saline), saline:ethanol (1:1), 70%, 85%, 95%, 100% ethanol and chloroform. Tissue was incubated in wax at 60°C for 3 hrs and embedded on a Reichert Jung tissue embedding centre and stored at 4°C. Tissue was sectioned on a microtome. The 6μm sections were placed on poly-L-lysine coated slides and dried at 37°C overnight. Sections were stained using a trichrome method. Briefly, sections were rehydrated through xylene, 100%, 85% and 75% ethanol and rinsed in water. Sections were stained in Weigert's haematoxylin for 15 mins and differentiated in acid/alcohol and Li CO_4. Sections were stained in picro-orange (0.25% Orange G in saturated picric acid in 80% ethanol) for 2 mins and acid fuchsin (1:1 volume of 0.5% Ponceau 2R in 1% acetic acid and 0.5% acid fuchsin in 1% acetic acid) for 10 mins. Sections were rinsed in 1% acetic acid and differentiated in red differentiator (10% phosphotungstic acid, 1% picric acid in 76% ethanol). Finally, sections were stained in aniline blue (2.5% aniline blue in 2.5% acetic acid) for 8 mins and differentiated in blue differentiator (10% phosphotungstic acid, 1% picric acid in 38% ethanol). Sections were dehydrated through 75%, 85% and 100% ethanol and xylene and then mounted using DPX mountant. Stained sections were visualised and photographed using a Nikon Microphot-SA microscope and Kodak EPY64 film.

3.21. Computing analysis.

Raw sequence data was converted to a Fasta format and compared to nucleotide and protein databases using the BLAST and Wisconsin analysis packages (Altschul *et al,* 1990; Genetics Computer Group, 1994). Sequence data was analyzed using GCG packages (Pearson and Lipman, 1988).

Chapter 4.

Results.

Morphology of chick gonadal development.

Gross morphological and histological analysis of chick genital ridge and mesonephros tissue from 4.5d to 8.5d (stage 25 to 35), was carried out in an attempt to better understand the morphological aspects of chick gonadal development and to identify the initial stages of morphological sex differentiation in the chick. These stages of development would encompass: the development of the indifferent gonad, the sex determination period and the initial stages of testis and ovary differentiation. It should be noted that not all eggs were incubated immediately after collection, thus an incubation time of 5.5d could result in a 6-12 hour developmental difference between embryos. Futhermore, not all embryos will develop simultaneously and thus reach the same stage at the same time throughout embryogenesis. Adult testis tissue was also histologically analyzed. However, a fully mature hen's ovary measures 40mm in diameter, and is considered too large to section onto slides (Rugh, 1977). In a trichrome stain, fibrin stains red, red blood cells stain orange, nuclei stain blue/black, collagen stains blue and connective tissue stains blue. After dissection of the genital ridge and mesonephric tissue, individual embryos were sexed using two W-PCR methods. For both W-PCR methods, presence of two bands of 415bp (W-repeat amplified fragment) and 720bp (GAPDH amplified fragment) (data not shown) or two bands of 415bp (W-repeat) and 256bp (18S amplified fragment) (fig 6) indicated the sample is female (ZW). If only one band is obtained corresponding to 720bp (GAPDH) or 256bp (18S), the sample is male (ZZ). This method of sexing was confirmed by a dot blot method. A GAPDH probe was used as a loading control. Female samples were identified by hybridising a W-repeat probe, to a membrane containing DNA from each embryo. Hybridization was visualised by autoradiography and those samples showing a black dot were identified as female (data not shown).

At 4.5d (stage 25 and 26) the urogenital ridge was observed with the indifferent gonads developing on the medial surface of the mesonephros (fig 7a, 7b). The gonads and mesonephroi are thin and elongated at this stage of development. At a higher magnification (x10), the gonad was observed to be continuous with the mesonephros at various places along its length and continuous with the mesentery (fig 7c). A germinal epithelium could also be observed on the outer edge of the gonads, exhibiting a compact cell structure. Below the germinal epithelium, a loose cell structure was observed. The indifferent gonad contained two basic cell types, the somatic cells and the PGCs (fig 7d). The PGCs were identified as being larger than the peritoneal cells with a large nucleus and clear cytoplasm (Dubois and Croisille, 1970). At this stage of development, the indifferent gonads were narrow and extended along the majority of the length of the mesonephros. No differences in morphology were observed between males and females.

Figure 6: W-PCR showing the amplification of an 18S ribosomal band of 256bp in all samples, and the amplification of a W-repeat band of 415bp only in female samples. F = female. M = male.

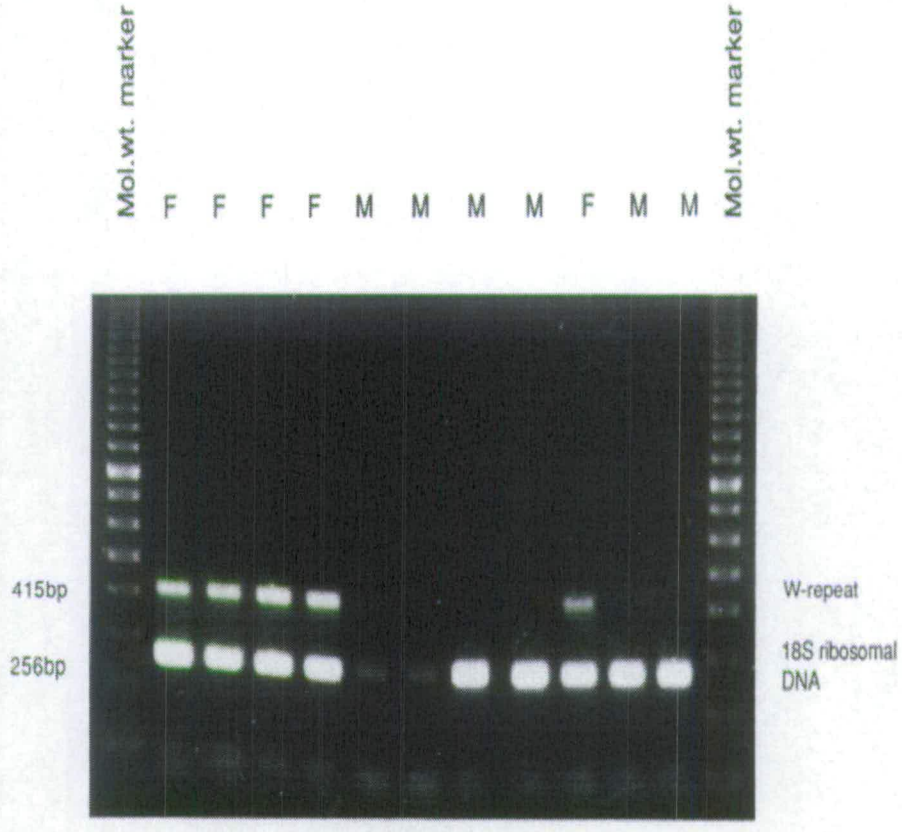
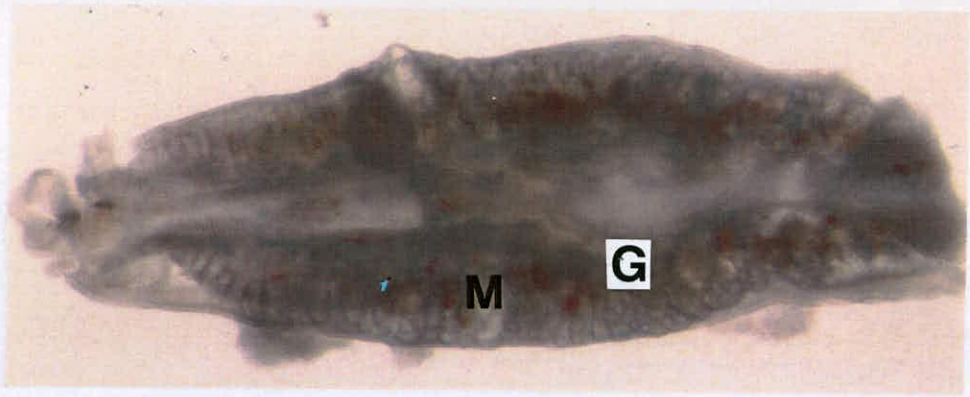
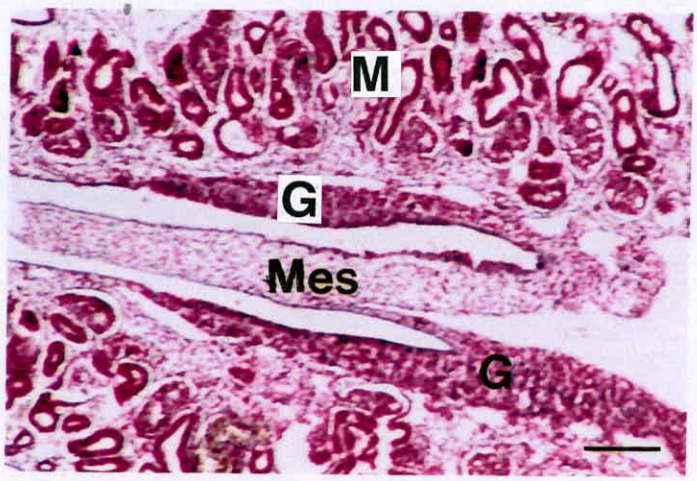

Mol.wt. marker
F F F F M M M M F M M
Mol.wt. marker
415bp
256bp
W-repeat
18S ribosomal DNA

Figure 7: Morphology of the indifferent gonad at day 4.5 of incubation (stage 25 and 26). (a) Gross morphology of the indifferent gonad, dissected from a chick embryo at day 4.5 of incubation. Actual length of the mesonephros is 4.3mm. (b) Sagittal section of the gonads developing on the medial surface of the mesonephros. Scale bar represents 105μm. c) Indifferent gonad is continuous with the mesonephros and mesentery. Scale bar represents 40μm. (d) High power magnification of the indifferent gonad showing the presence of somatic cell and primordial germ cell. Scale bar represents 10μm. Mesonephros (M), mesentery (Mes), gonad (G), germinal epithelium (GE), primordial germ cell (PGC). Staining was carried out on 3 separate genital ridge/mesonephric tissue.

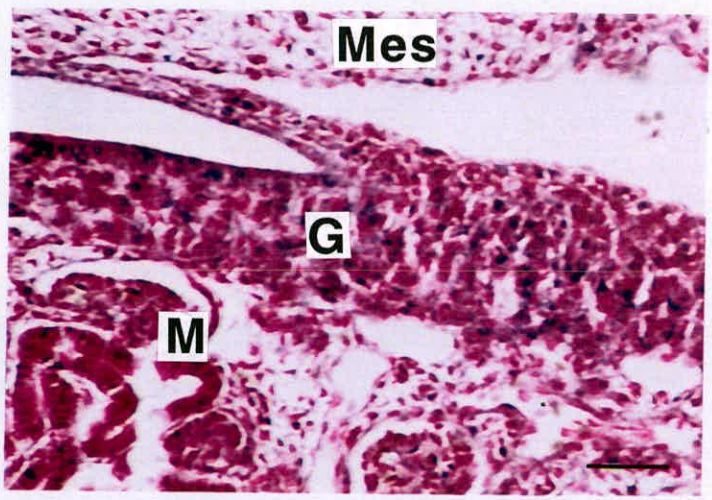
(a)



(b)



(c)



(d)

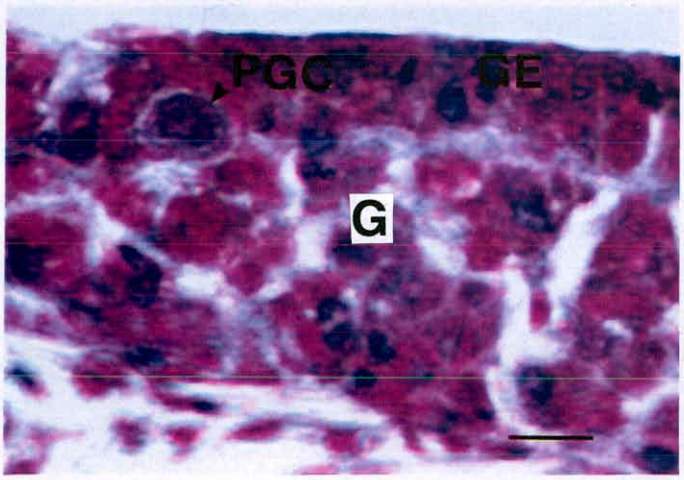
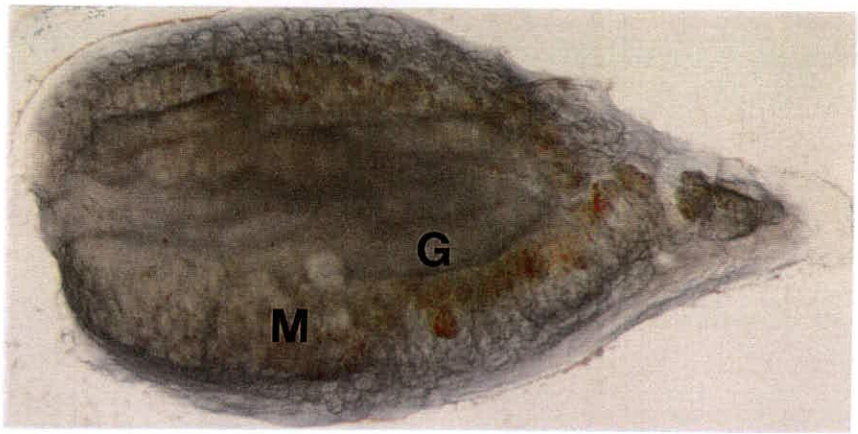
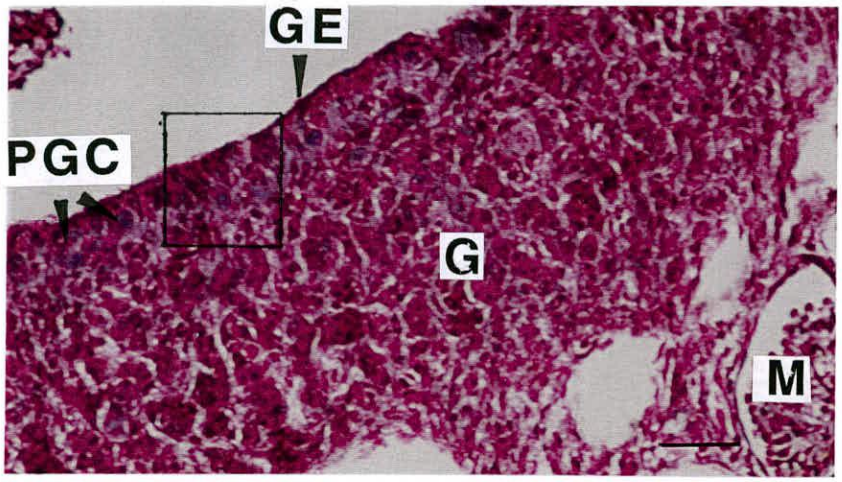


Figure 8: Morphology of the male gonads and mesonephros at day 5.5 of incubation (stage 28). (a) Gross morphology. Actual length of the mesonephros is 3.3mm. (b) Sagittal section of the left male gonad. Scale bar represents 40μm. c) High power magnification of a sagittal section of the male left gonad. Scale bar represents 10μm. (d) High magnification of a sagittal section of the left male gonad. Scale bar represents 10μm. Gonad (G), mesonephros (M), germinal epithelium (GE), primordial germ cell (PGC), primary sex cord (C). Box in 'b' represents the area that is seen in 'c' at a higher magnification. Staining was carried out on 4 separate genital ridge/mesonephric tissue.

(a)



(b)



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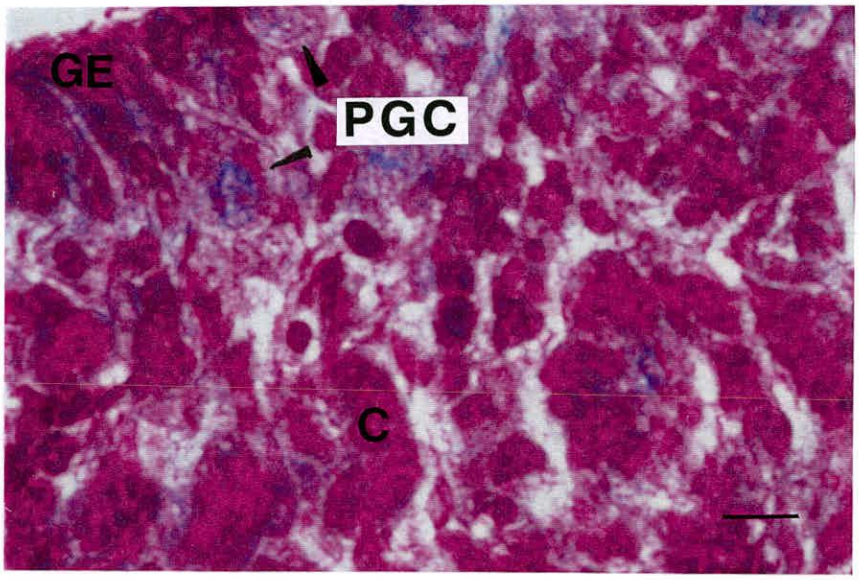


(d)

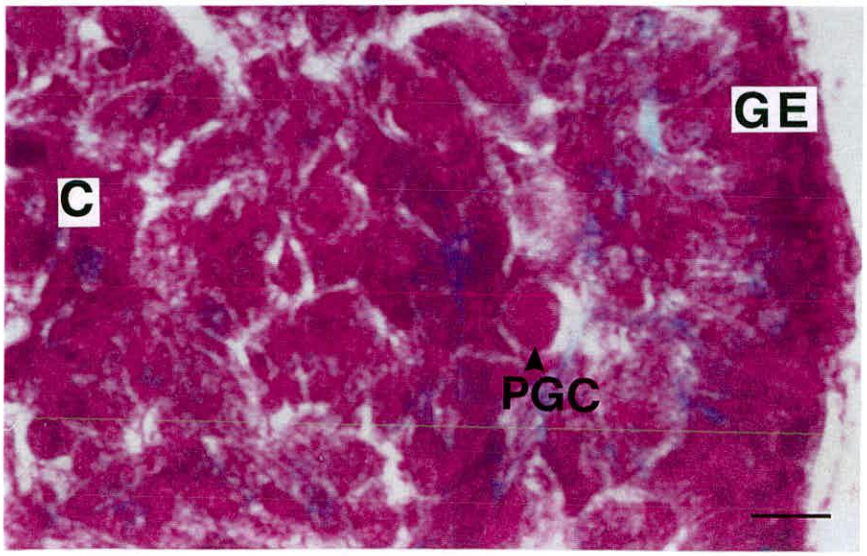
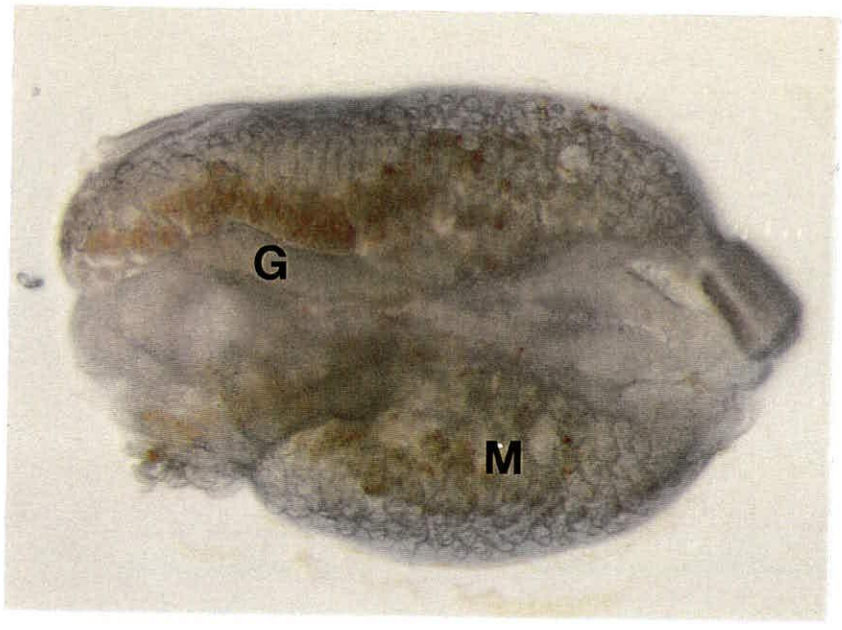
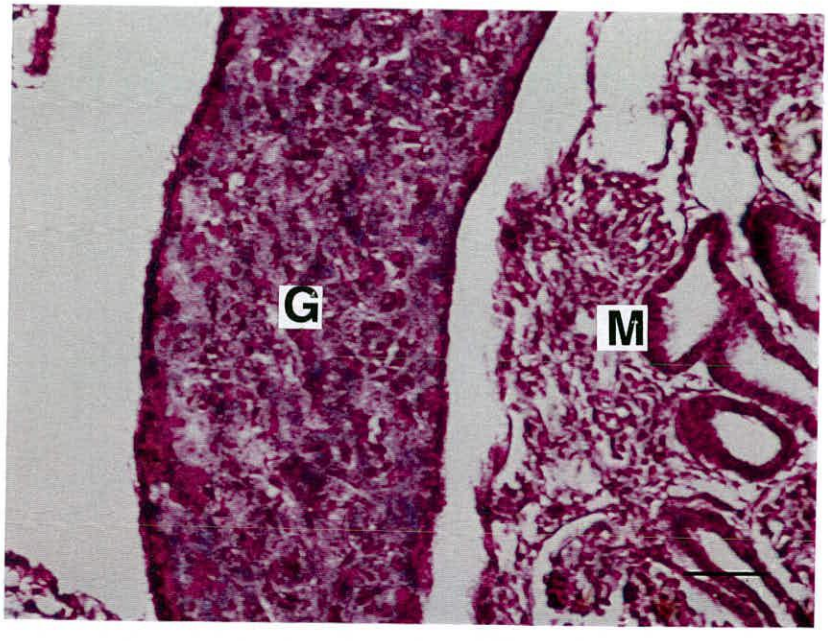


Figure 9: Morphology of the female gonads at day 5.5 of incubation (stage 28). (a) Gross morphology. Actual length of the meonephros is 3.5mm. (b) Sagittal section of the left female gonad. Scale bar represents 40μm. c) High power magnification of a sagittal section of the left female gonad. Scale bar represents 10μm. Primary sex cord (C), mesonephros (M), gonad (G), germinal epithelium (Ge), primordial germ cell (PGC). Staining was carried out on 3 separate genital ridge/mesonephric tissue.

(a)



(b)



(c)

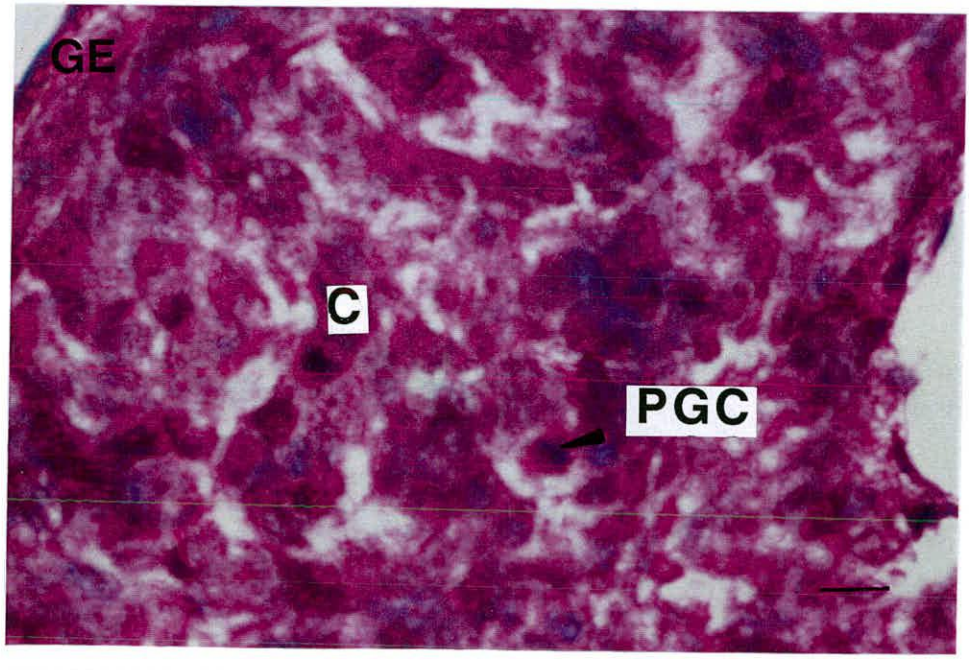
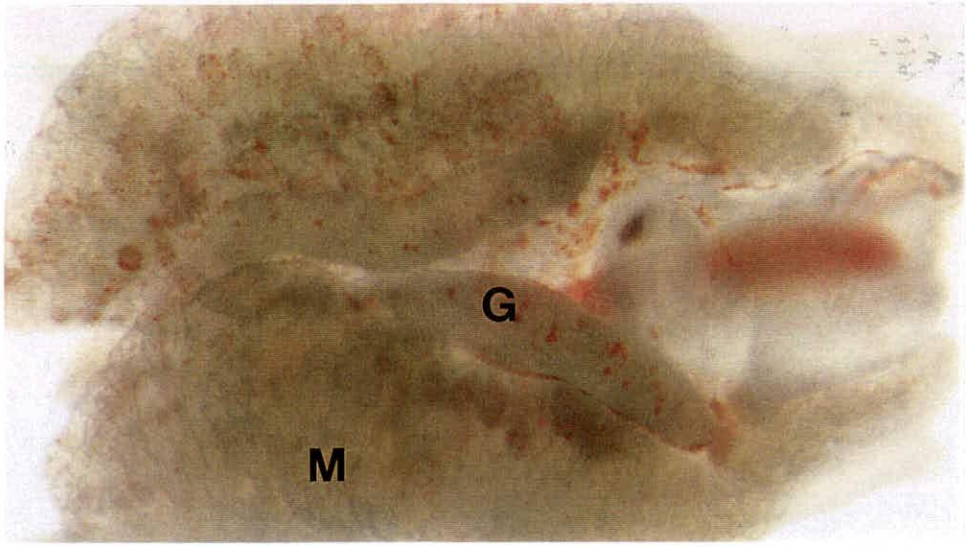
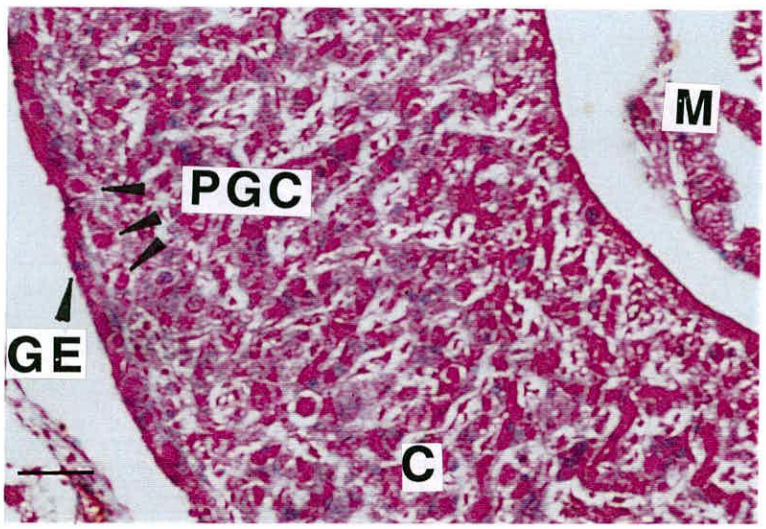


Figure 10: Morphology of the male gonads and mesonephros at day 6.5 of incubation (stage 30). (a) Gross morphology. Actual length of the mesonephros is 4mm. (b) Sagittal section of the male left gonad. Scale bar represents 40μm. (c and d) High power magnification of a sagittal section of the left male gonad. Scale bar represents 10μm. Gonad (G), mesonephros (M), primordial germ cell (PGC), primary sex cord (C), connective tissue (CT), germinal epithelium (GE). Staining was carried out on 3 separate genital ridge/mesonephric tissue.

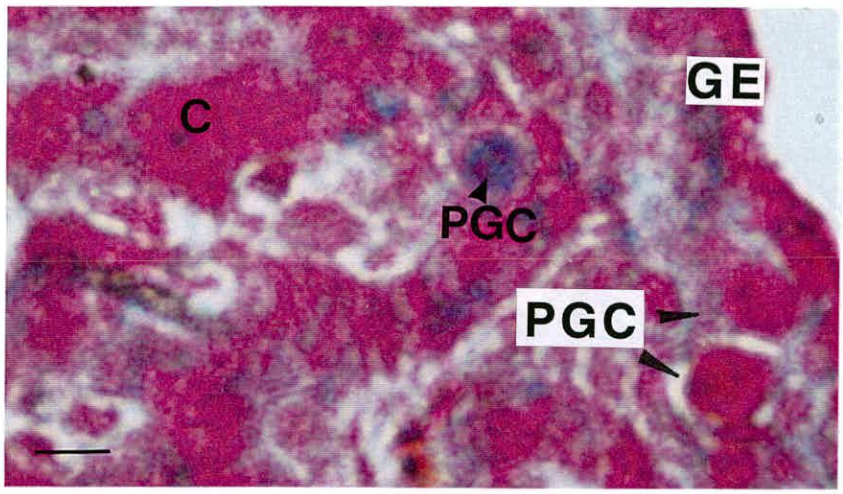
(a)



(b)



(c)



(d)

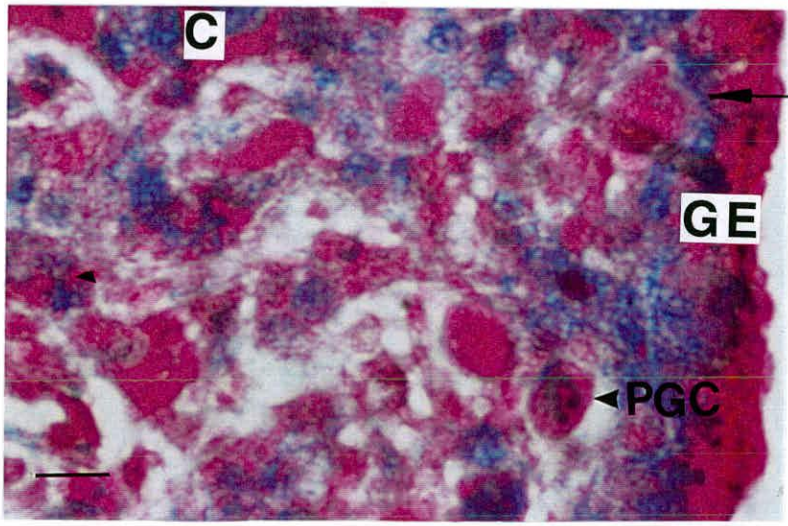
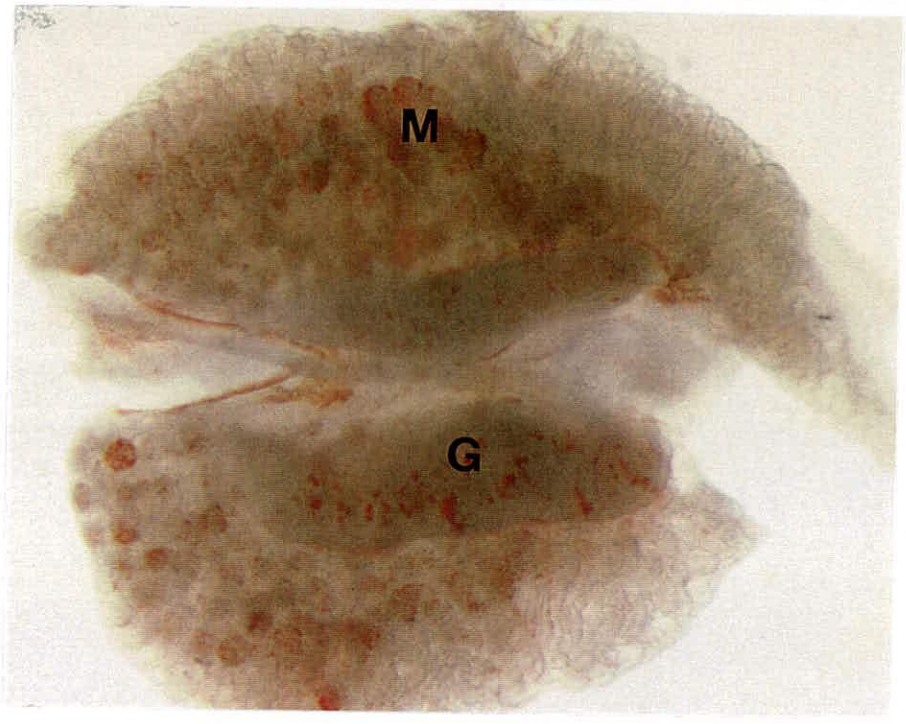
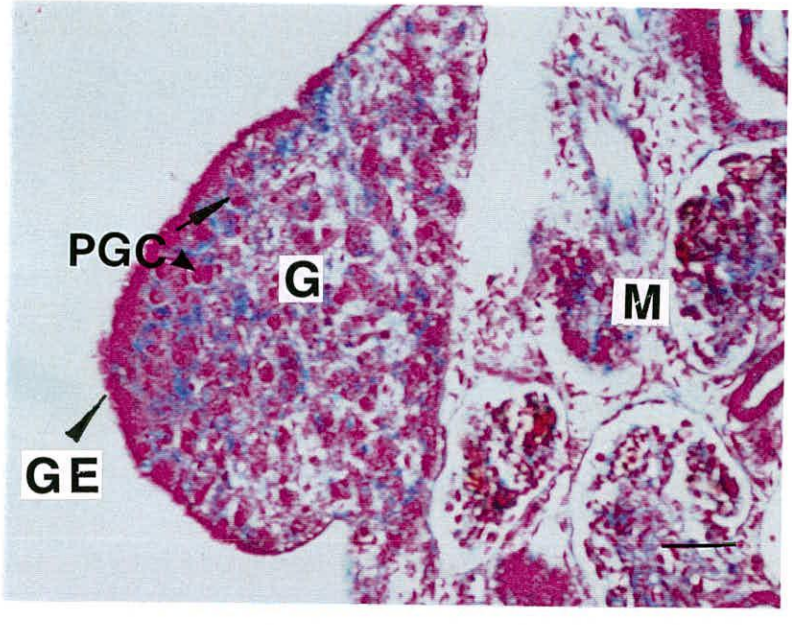


Figure 11: Morphology of the female gonads and mesonephros at day 6.5 of incubation (stage 30). (a) Gross morphology. Actual length of the mesonephros is 3.9mm. (b and c) Sagittal section of the female left gonad. Scale bar represents 40μm. Gonad (G), mesonephros (M), primordial germ cell (PGC), germinal epithelium (GE), primary sex cord (C). Staining was carried out on 5 separate genital ridge/mesonephric tissue.

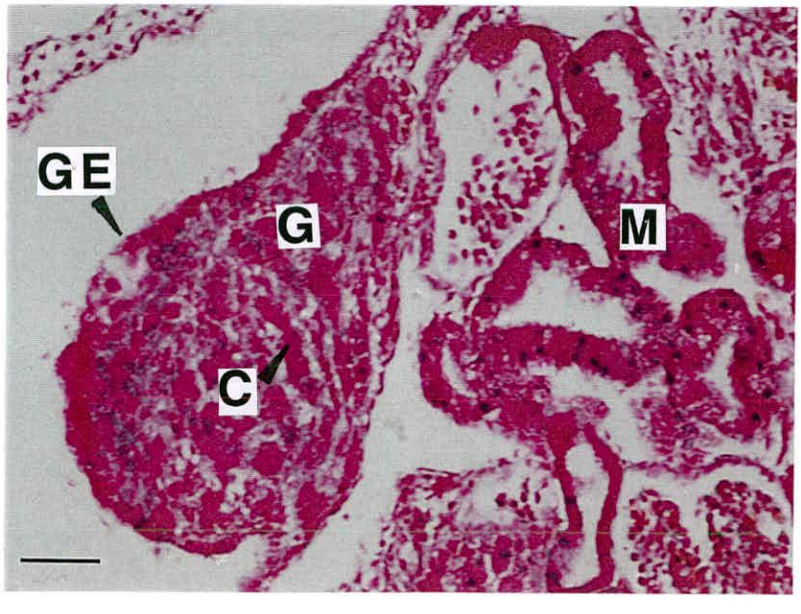
(a)



(b)



(c)

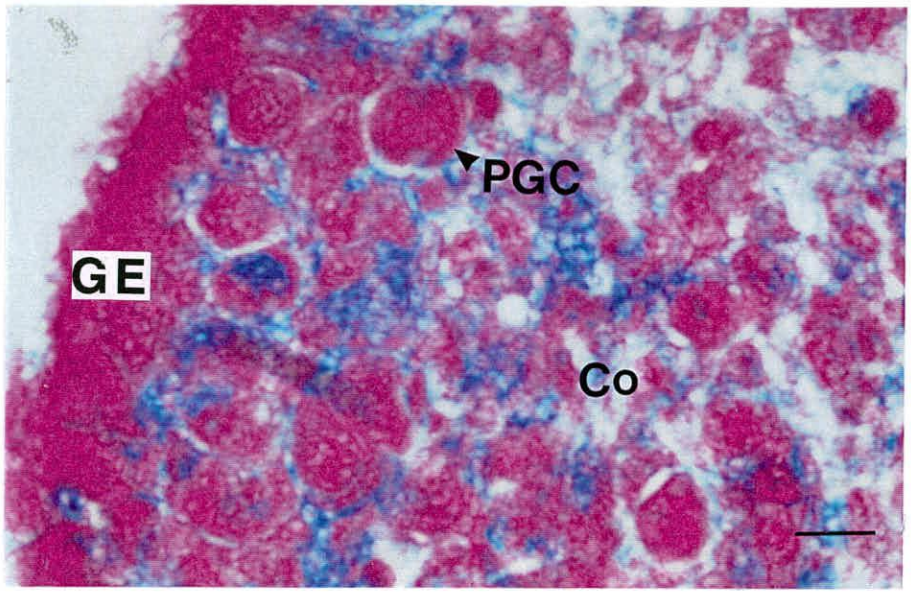


By 5.5d (stage 28), initial differences in the morphological development of the gonads was observed between male and female chick embryos, at the histological level. No differences were observed between male and females at the gross morphological level. The gonads and mesonephroi of both sexes were thicker and shorter compared with their morphology at 4.5d (fig 8a, 9a). In the male, a relatively thick germinal epithelium was observed with a connective tissue layer separating the germinal epithelium from the underlying medulla (fig 8b, c & d). Formation of cord structures was observed in the medulla of the male gonad. These tubules were located under the germinal epithelium layer towards the hilum of the gonad, and are the primary sex cords. PGCs were observed in the germinal epithelium and in the stroma of the medulla, near the edges of the tubules (fig 8c & d). In the female gonad at the same stage of development, cell structure appeared less organised and more homogeneous (fig 9b & c). The germinal epithelium was observed, but a connective tissue layer separating it from the medulla was not (fig 9b). A small number of cord-like structures were observed in the female gonad at a high magnification (x40) (fig 9c). It is, therefore, possible that by this stage of development, testicular cord formation has begun or the primary sex cords in the female have begun to partially degenerate. Degeneration of the primary sex cords in the female chick embryo does not occur in many instances, and instead the cords become distended (Romanoff, 1960).

At 6.5d (stage 30) at the gross morphological level, the mesonephroi and gonads had increased in width and length (fig 10a). The germinal epithelium had become much thinner in the male gonad (fig 10b & c). The testicular cord structure had begun to organise in the medulla of the gonad, and PGCs were observed inside or on the edge of these cords, in the stroma of the medulla, and in the germinal epithelium (fig 10b, c & d). The medulla had also increased in thickness, with more somatic cells observed between the cords. In the female at the gross morphological level, the right gonad was visibly smaller than the left gonad (fig 11a). The left gonad had increased significantly in size. It was thicker, but shorter than the male gonads at the same stage. Therefore, it was at this stage of development that gross morphological differences were first discernible between males and females. At the histological level, the germinal epithelium had increased significantly in thickness, with relatively compact cell structure (fig 11b). A connective tissue layer was observed in figure 11c, but due to the slight differences in the stages of the 6.5d female embryos, no connective tissue layer was observed in figure 11b. PGCs were clearly located in the cortex of the female gonad at this stage of development (fig 11b, 12a). In the medulla, the cord structures were still present but far less organised compared to the male (fig

Figure 12: Histological analysis of the female left gonad at day 6.5 of incubation (stage 30). (a) High power magnification of a sagittal section of the female left gonad. Scale bar represents 10μm. (b) Sagittal section of the female left gonad. Scale bar represents 40μm. Cortex (Co), gonad (G), primordial germ cell (PGC), primary sex cord (C), mesonephros (M), germinal epithelium (GE). Staining was carried out on 5 separate genital ridge/mesonephric tissue.

(a)



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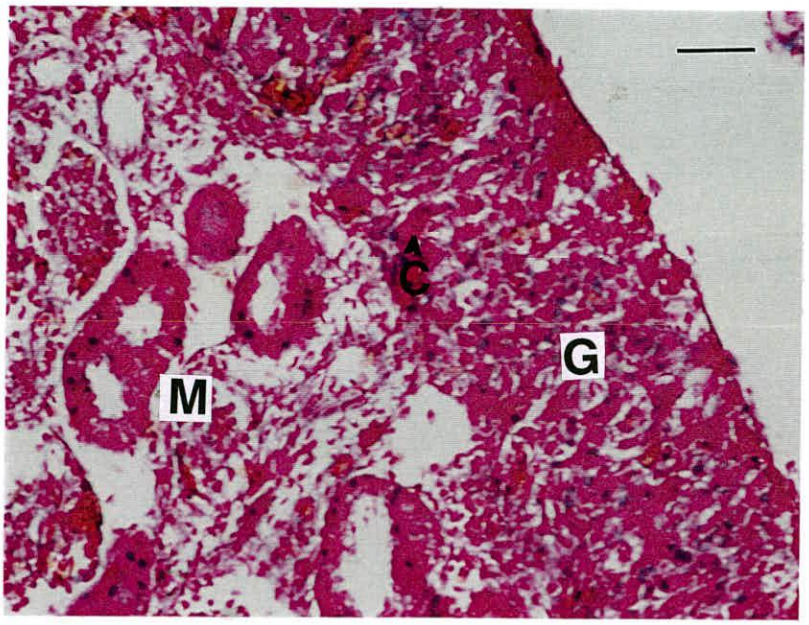
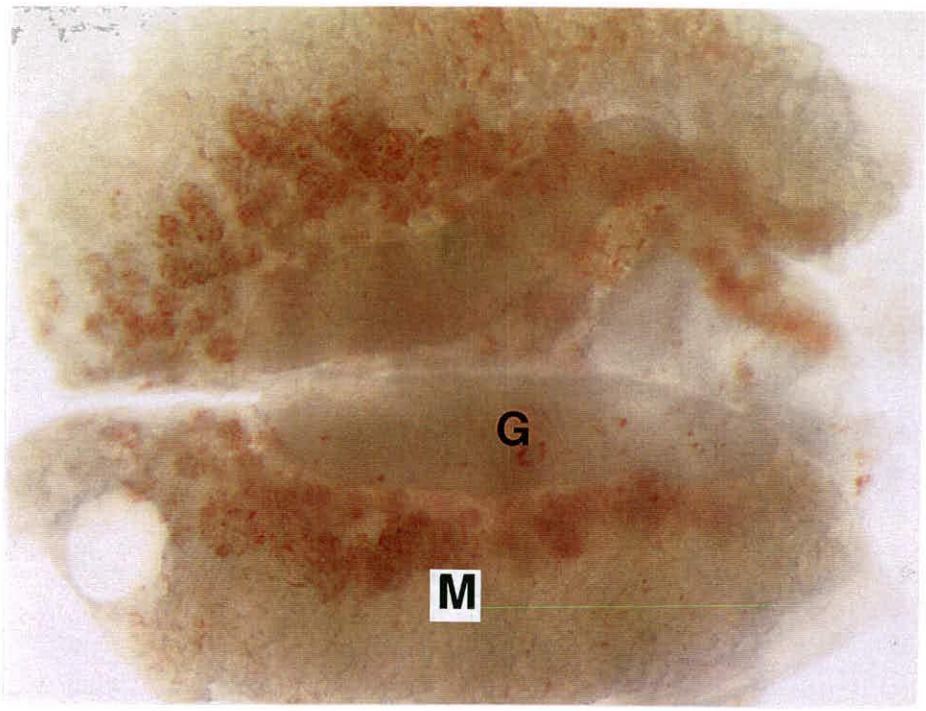


Figure 13: Morphology of the male gonads and mesonephros at day 7.5 of incubation (stage 32 and 33). (a) Gross morphology. Actual length of the mesonephros is 4.3mm). (b) Sagittal section of the left male gonad. Scale bar represents 40μm. c) High power magnification of a sagittal section of the left male gonad. Scale bar represents 10μm. Gonad (G), primordial germ cell (PGC), testicular cord/primary sex cord (C), mesonephros (M), mesentery (Mes). Staining was carried out on 3 separate genital ridge/mesonephric tissue.

(a)



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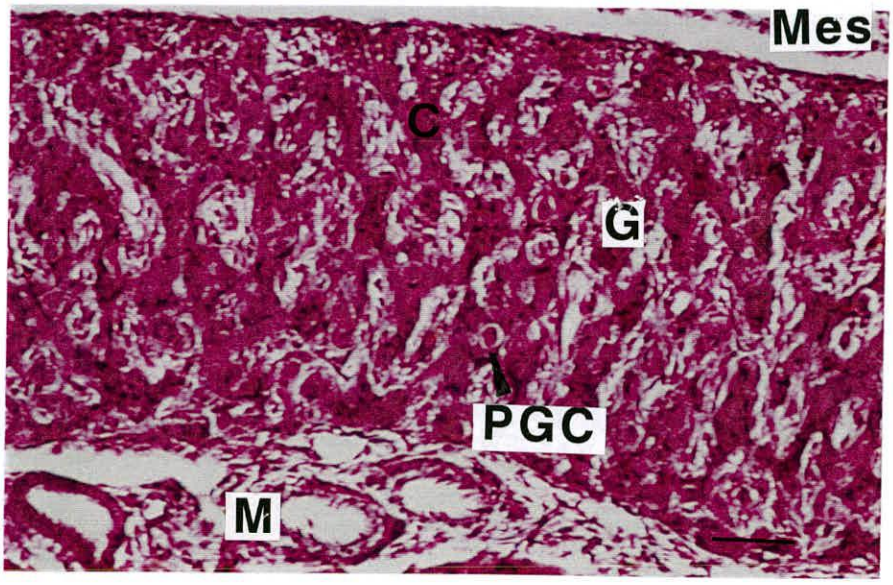

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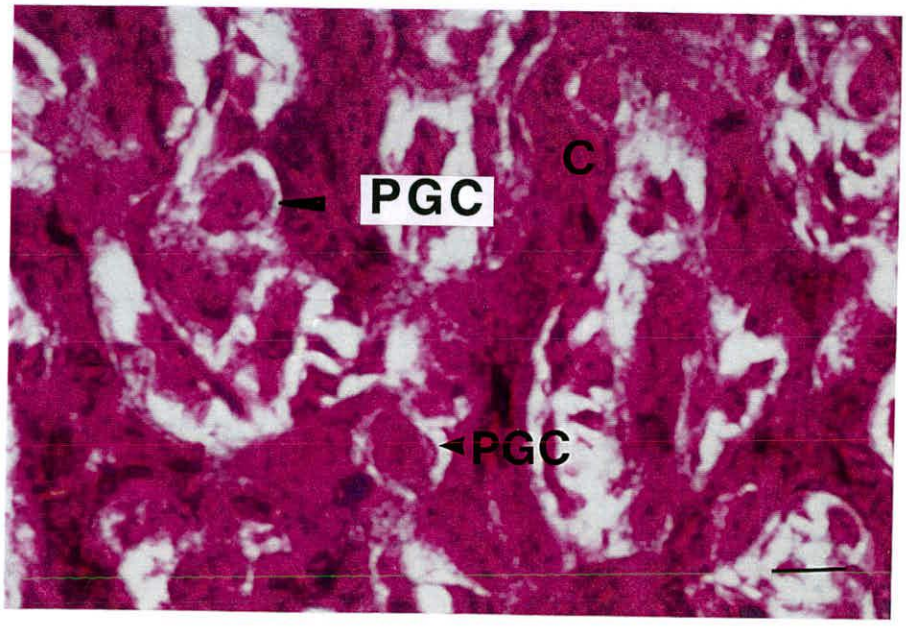
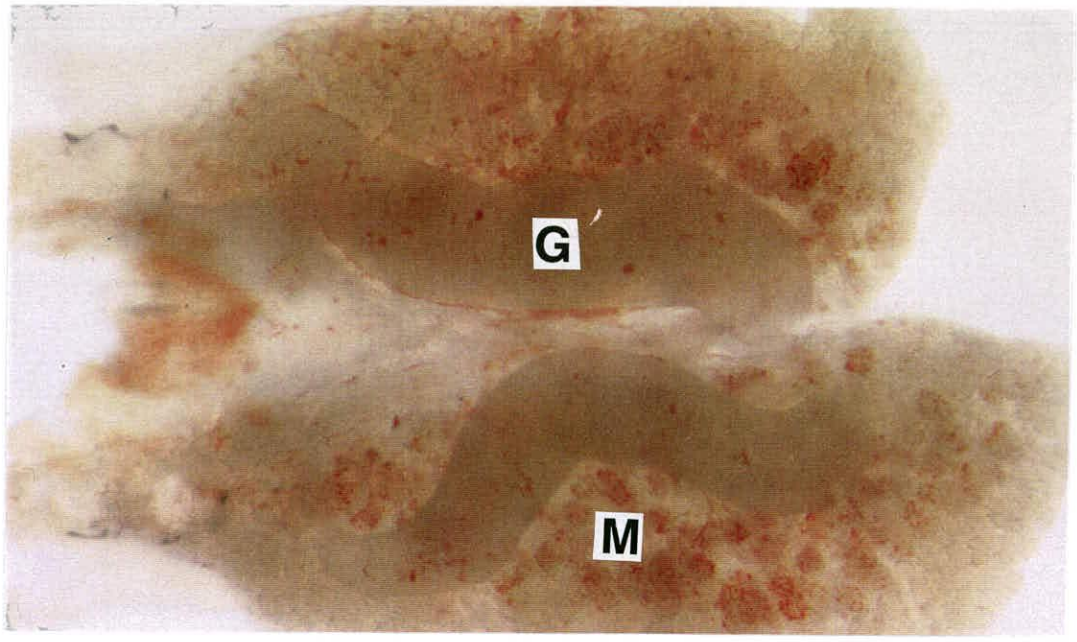
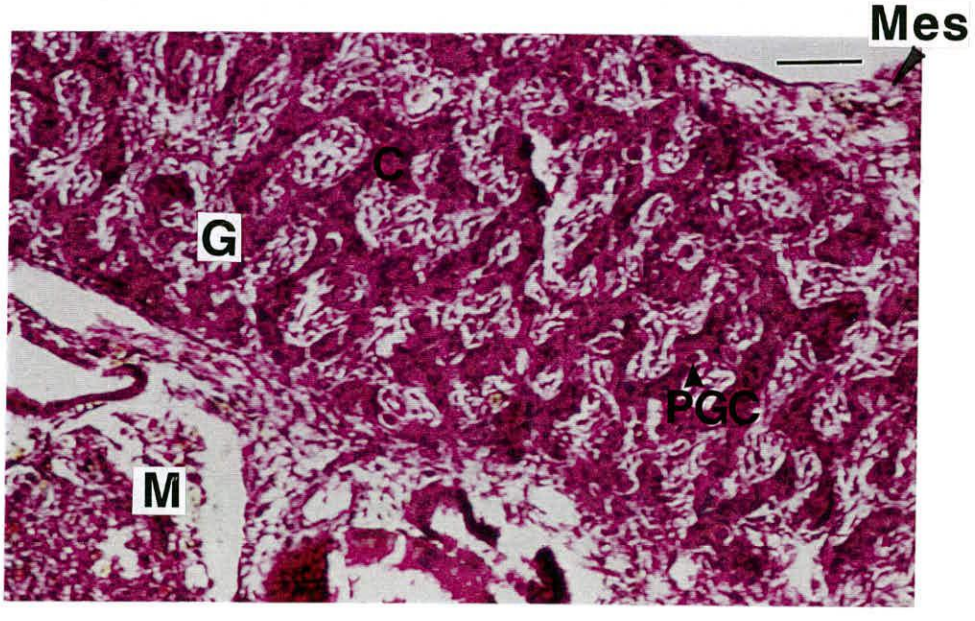


Figure 14: Morphology of the male gonads and mesonephros at day 8.5 of incubation (stage 34 and 35). (a) Gross morphology. Actual length of the mesonephros is 4.6mm. (b) Sagittal section of the male gonad. Scale bar represents 40μm. c) High power magnification of a sagittal section of the male gonad. Scale bar represents 10μm. Mesonephros (M), primordial germ cell (PGC), testicular cords/primary sex cords (C), gonad (G), mesentery (Mes). Staining was carried out on 3 separate genital ridge/mesonephric tissue.

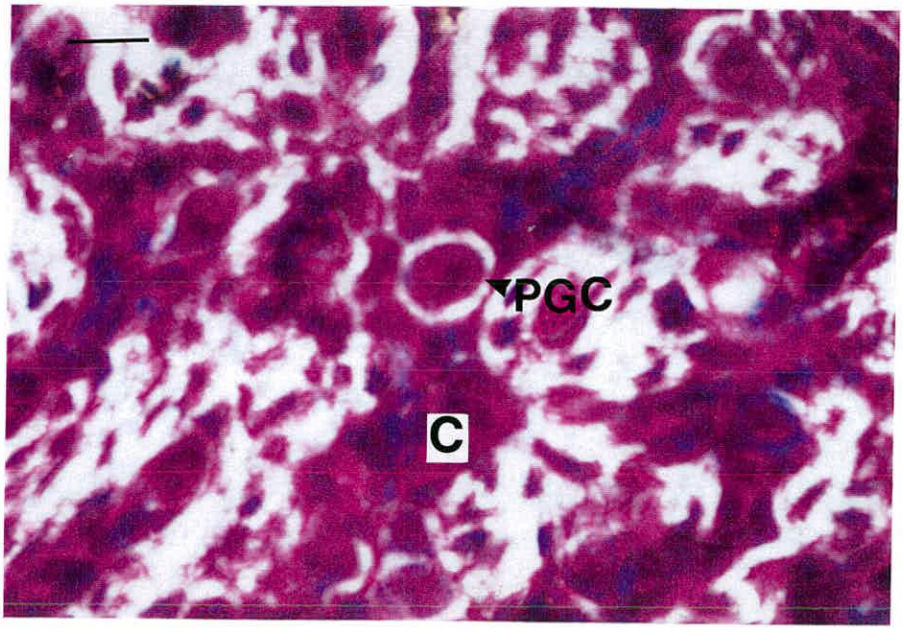
(a)



(b)



(c)



12b). In figure 11c, the cord structures had a distended appearance and fewer somatic cells were located between these cords compared with the male gonad.

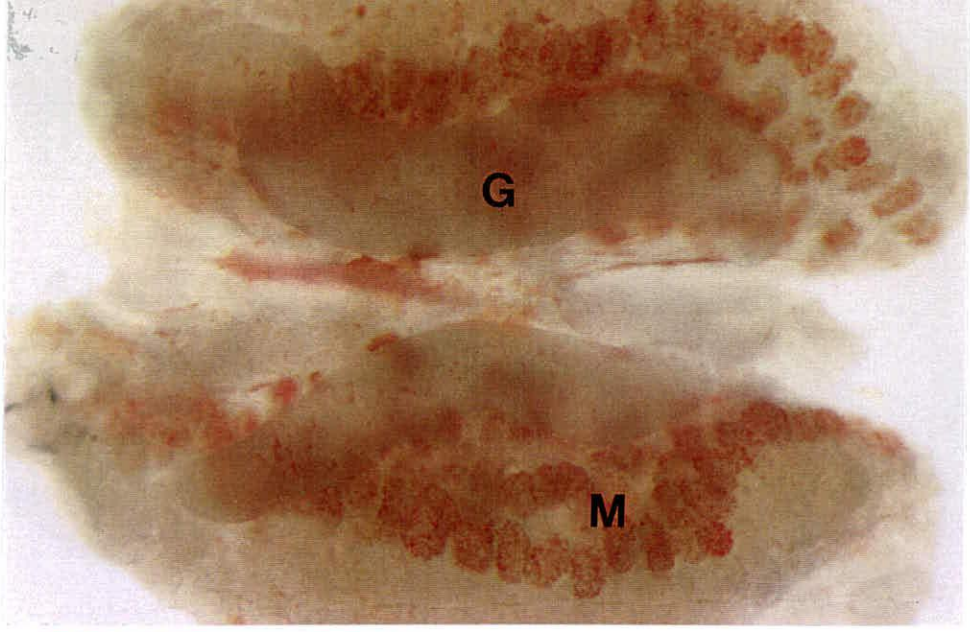
The gross morphology of the male gonad at 7.5d and 8.5d (stage 32 to 35) indicated an increase in size in both left and right gonads (fig 13a & b). At the histological level, a significantly more organised cord structure was observed in addition to an increase in the number of cords (fig 13b & c; 14b). The cord structure indicated an intricate connecting network and constituted a large majority of the developing testes. Due to the increase in cord number, the stromal cells between these cords had decreased compared with 6.5d. PGCs could now be clearly identified in the testicular cords (fig 13b; 14c).

At the gross morphological level in the female gonads at 7.5d and 8.5d, the difference in size between the left and the right ovary was clearly visible (fig 15a; 16a). The left ovary had increased in volume, whereas the right ovary remained a similar size to 6.5d. In the female gonad at the histological level, the germinal epithelium increased significantly in thickness in the left gonad (fig 15b & c; 16b & c). PGCs were observed in this thick germinal epithelium layer (fig 15c; 17a); and these are known to develop into the definitive germ cells (Lillie, 1908). In the cortex of the ovary, whorls of cells were identified, indicative of follicular formation (fig 17b & c). However, PGCs were also identified in the medulla region of the female gonad (fig 15c; 17a), which contained the partially degenerated primary sex cords (fig 15c). Lacunae were also identified in the medulla, and were especially apparent by 8.5d (fig 16b). These lacunae developed from the primary sex cords, in which a large lumen was surrounded by the cord cells. This cord distension caused the medulla region of the female left gonad to increase in size. The right gonad also developed lacunae (fig 16c). Figure 15d indicated the close association of the left gonad with the mesonephros and the left gonad with the mesentery, at this stage of development.

A histological analysis of the adult testis was also carried out. The cross section at low magnification (x4) indicated the majority of the adult testis consisted of many seminiferous tubules packed closely together (fig 18a). Located between these seminiferous tubules were Leydig cells, blood vessels and stroma. The seminiferous tubules were surrounded by a basement membrane and inside this basement membrane was a single cell thickness of Sertoli cells (fig 18b). The seminiferous tubules contained germ cells at various stages of spermatogenesis; with cells nearest the periphery in the earliest stages of spermatogenesis, the primary spermatocytes. Moving interiorly into the tubule, the cells progressively entered later stages of spermatogenesis; the secondary spermatocytes, spermatids and spermatozoa (fig 18c).

Figure 15: Morphology of the female gonads and mesonephros at day 7.5 of incubation (stage 32 and 33). (a) Gross morphology. Actual length of the mesonephros is 3.75mm. (b) High power magnification of a sagittal section of the female left gonad. Scale bar represents 10µm. © Sagittal section of the female left gonad. Scale bar represents 40µm. (d) Low power magnification of a sagittal section of the female left gonad. Scale bar represents 105µm. Gonad (G), germinal epithelium (GE), medulla (Me), mesentery (Mes), mesonephros (M), primordial germ cell (PGC), primary sex cord (C). Staining was carried out on 3 separate genital ridge/mesonephric tissue.

(a)



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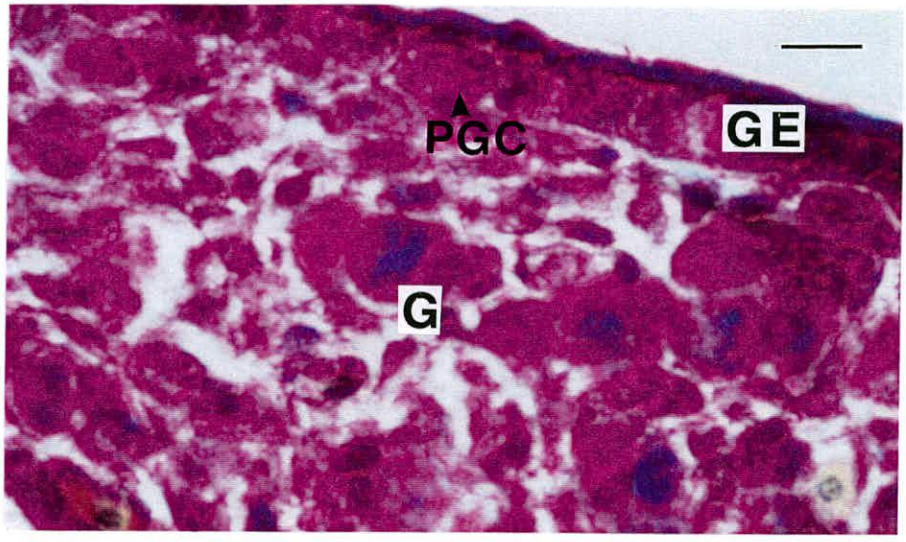

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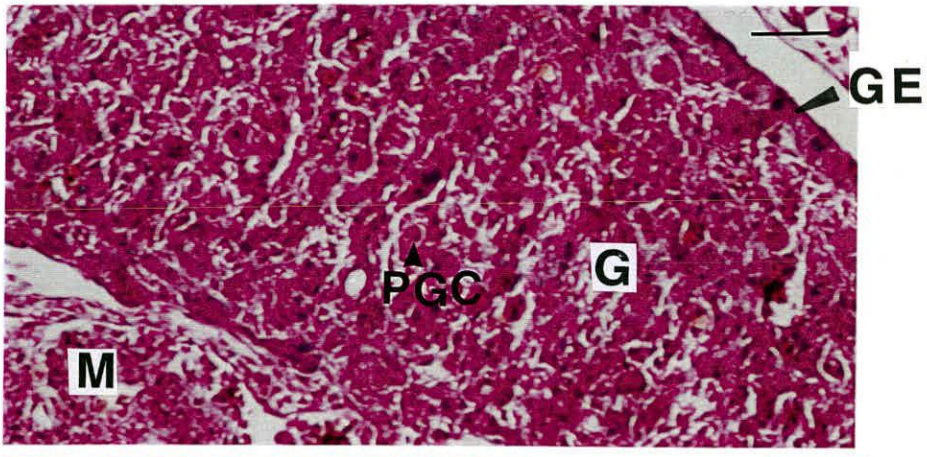


(d)

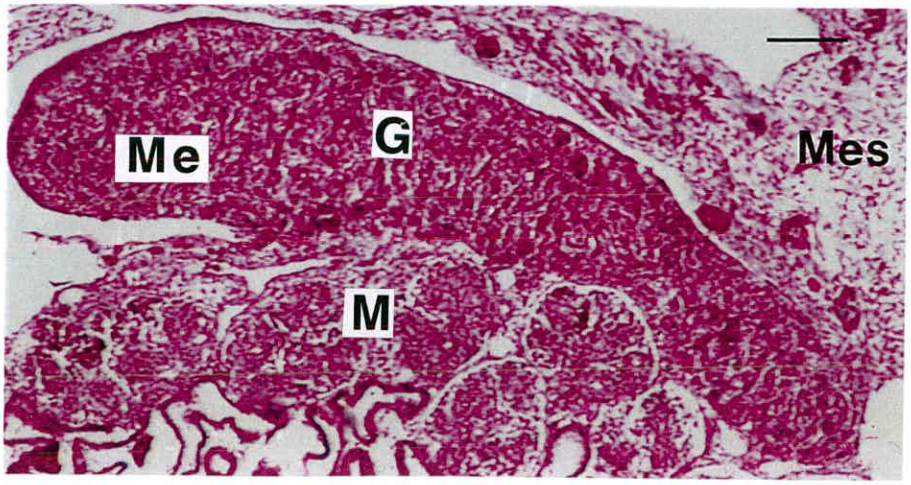
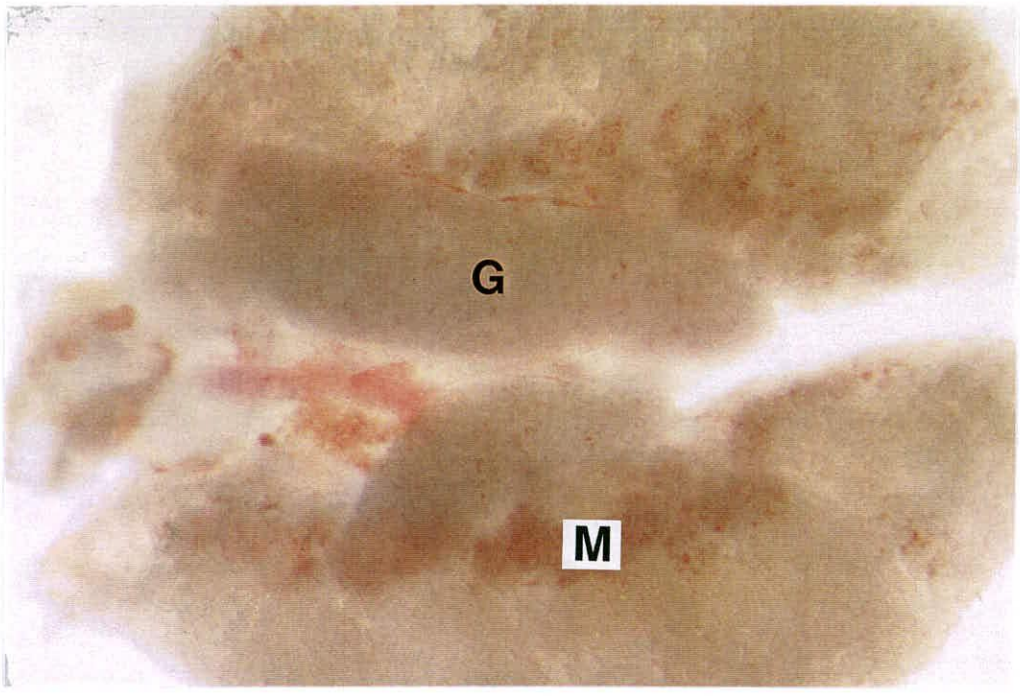


Figure 16: Morphology of the female gonads and mesonephros at day 8.5 of incubation (stage 34 and 35). (a) Gross morphology. Actual length of the mesonephros is 4.5mm. (b and c) Sagittal section of the female gonads. Scale bar represents 40μm. Gonad (G), lacunae (L), right gonad (RG), left gonad (LG), medulla (Me), cortex (Co). Staining was carried out on 3 separate genital ridge/mesonephric tissue.

(a)



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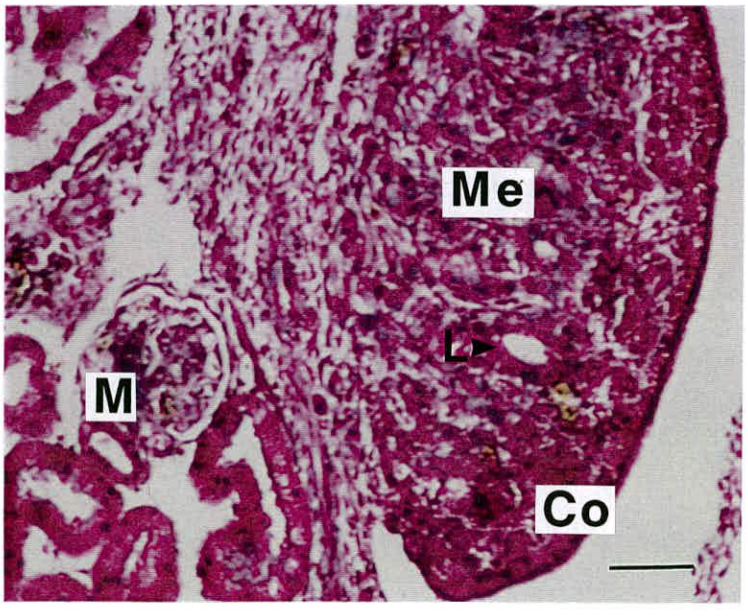

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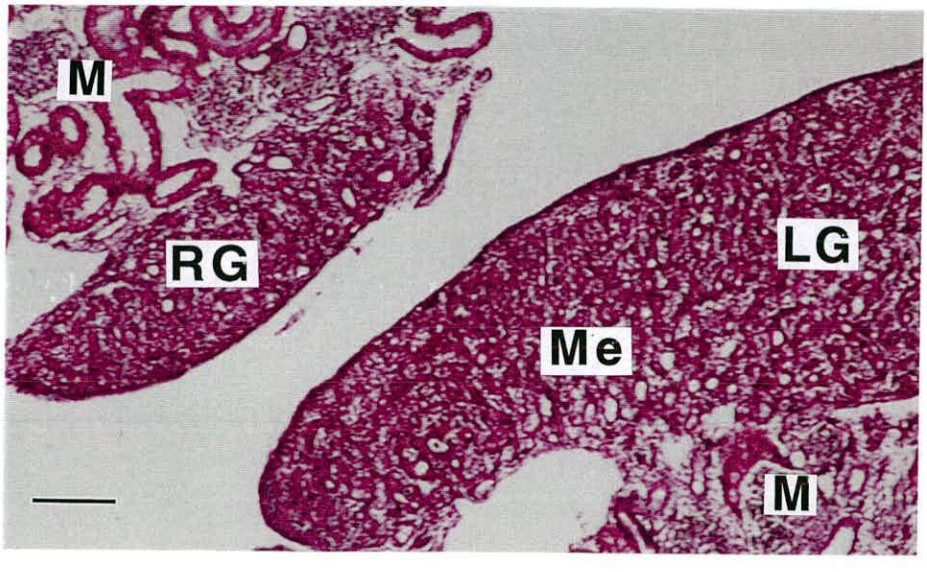
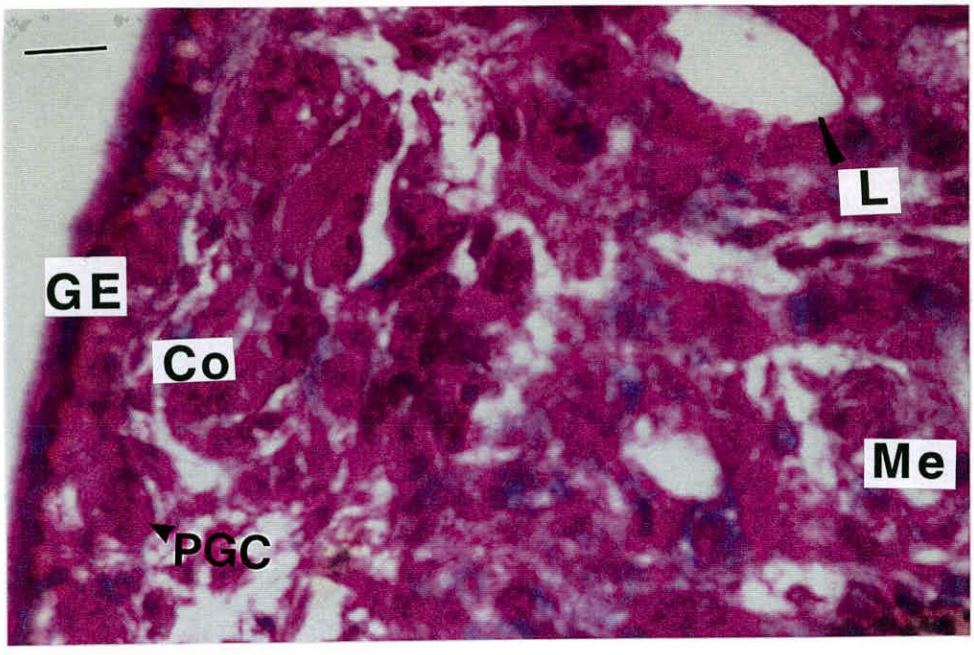


Figure 17: Histological analysis of the female left gonad at day 8.5 of incubation (stage 34 and 35). (a and c) High power magnification of a sagittal section of the female left gonad. Scale bar represents 10μm. (b) Sagittal section of the female left gonad. Scale bar represents 40μm. Cortex (Co), primordial germ cells (PGC), medulla (Me), lacunae (L), germinal epithelium (GE), whorls of cells in cortex (W), box in 'b' represents the area seen at higher magnification in 'c'. Staining was carried out on 3 separate genital ridge/mesonephric tissue.

(a)



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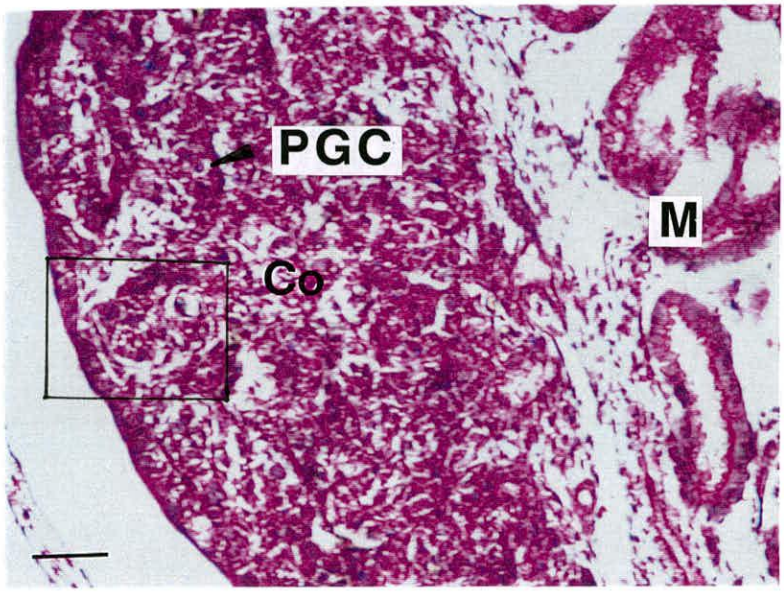


(c)

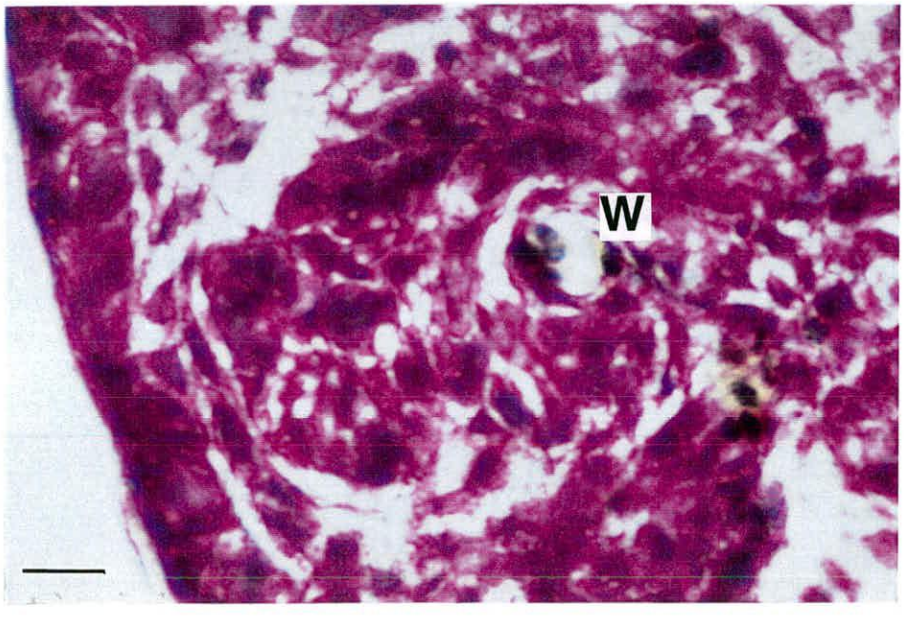
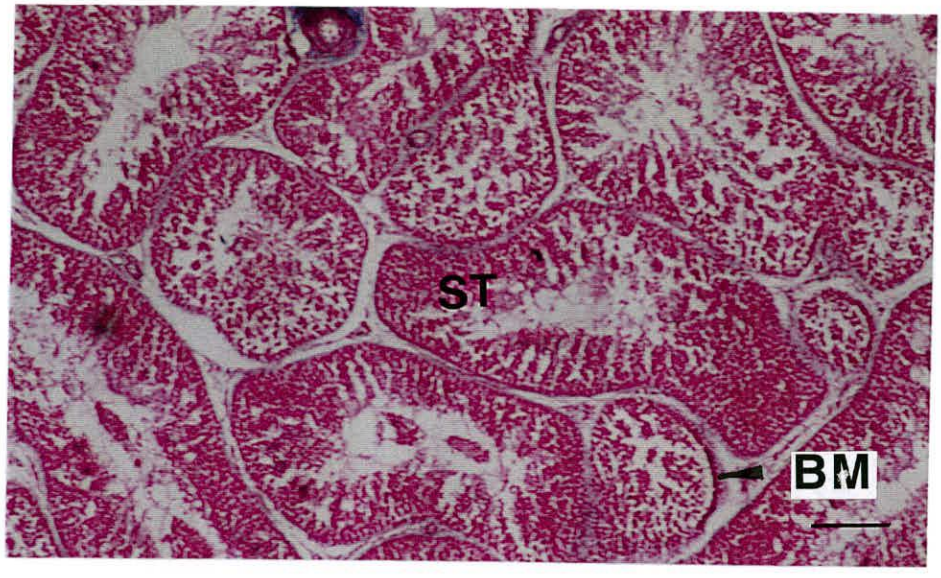


Figure 18: Histological analysis of the chicken adult testis. (a) Low magnification of a longitudinal section of the testis showing the close packing of the seminiferous tubules. Scale bar represents 105μm. (b) Longitudinal section of the testis, indicating the basement membrane and Sertoli cells. Scale bar represents 40μm. c) High power magnification of a logitudinal section of the testis. Scale bar represents 10μm. Basement membrane (BM), seminiferous tubules (ST), sertoli cells (SC), spermatozoa (S), interstitial cells (IC). Staining was carried out on 3 separate adult testes.

(a)



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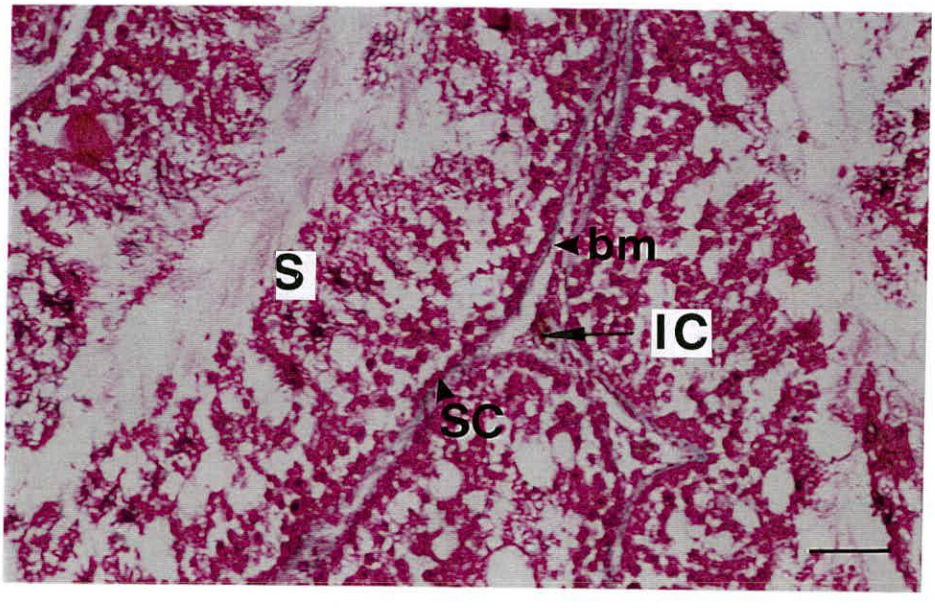

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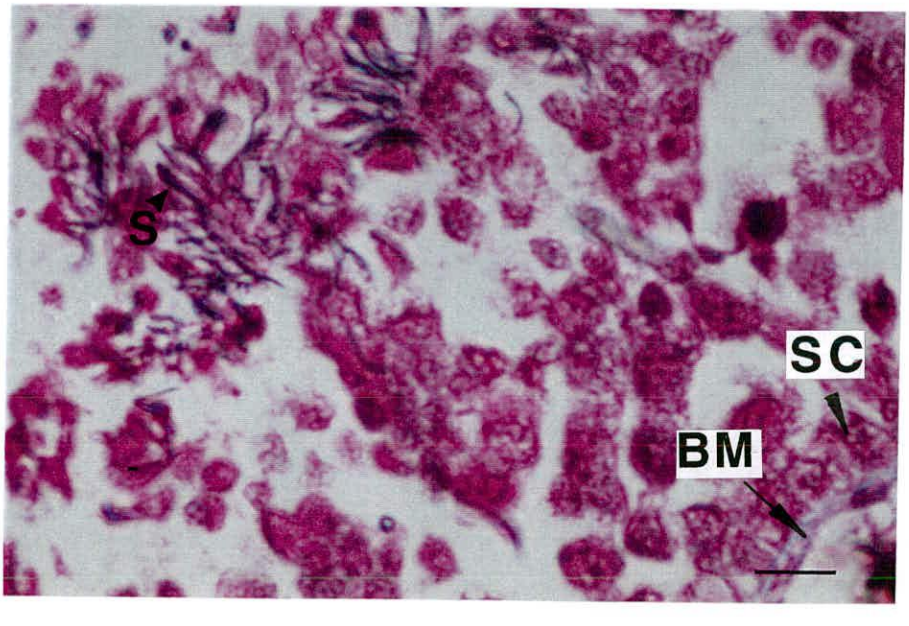


Chapter 5.

Discussion.

Morphological analysis of chick gonadal development.

Histological analysis of chick gonadal development provided a comparison between development in the male and female, and with mammalian gonadal development. At 4.5d no differences were observed, between the sexes, in the morphological development of the chick gonads. The gonads are considered to be bipotential or in the indifferent stage of development. The indifferent gonads were thin and elongated, and closely associated with the mesonephros and mesentery. However, a germinal epithelium was distinguishable from the underlying loose mesenchyme. As the genital ridge is reported to arise by the mid to end of 3d (Venzke, 1954a); by 4.5d the indifferent gonads will have begun developing. From the initial genital ridge development, the gonads are referred to as in the indifferent stage of development (Venzke, 1954a). PGCs were also observed in the germinal epithelium of the gonads at this stage of gonadal development as previously documented (Venzke, 1954a; Romanoff, 1960).

By 5.5d in the chick, slight differences in gonadal morphology between males and females were apparent. A distinct cord structure was visible in male gonads, however, in the females a disorganised tubule structure was visible. This observation indicated the unknown sex determination switch had occurred. Lillie (1908), Venzke (1954a) and Romanoff (1960) reported the development of the primary sex cords between 5d and 5.5d. It is possible that the results observed in this study indicate the cords had already formed and were either degenerating in the female or differentiating in the male, or both. It had previously been reported that testicular cord formation was apparent from 7.5d onwards (Venzke, 1954b). Histological analysis merely provides us with a "snapshot" of gonadal development at a particular stage. Therefore, due to the inability to determine the developmental state of the cords in the gonads, it is not possible to confirm from this study whether female or male differentiation begins first in chick gonadal development. However, with the obvious cord structure organising in the medulla of the male gonad and the formation of a connective tissue layer separating the germinal epithelium from the medulla compared to the female showing a more homogenous appearance, it is tempting to speculate that male differentiation occurs earlier than female differentiation.

The differences in gonadal structure between males and females at 5.5d suggests the sex determining event occurred earlier in gonadal development than previously reported (Venzke, 1954a; Romanoff, 1960). In comparison, morphological differences in the mouse were not apparent until 12.5 dpc (Capel, 1996). Chick gonadal development is analogous to mammalian gonadal development in that the indifferent period in the mouse begins at 10.5 dpc through to 12.5 dpc

(Capel and Lovell-Badge, 1993), and in the chick the indifferent period begins at 3.5d up to 5.5d, when we report morphological differences are first observed. The indifferent stage of development in both mice and birds is approximately two days. In the mouse, *Sry* is expressed during this indifferent period, between 10.5 dpc and 12.5 dpc, with a peak of expression at 11.5 dpc (Hacker *et al.,* 1995). If a similar genetic switch is utilised to determine sex in the chick, expression would occur between 3.5d and 5.5d.

After these initial differences were observed, the cords in the male gonad continued to proliferate so eventually a highly organised cord structure was visible, which constituted a large proportion of the gonad. This confirmed previously described work by Romanoff (1960) and Venzke (1954a and 1954b). These cords will ultimately form the seminiferous tubules.

There is also a cord structure in the medulla of the left ovary, but it is not as organised as the cord structure in the testis. The medullary cords in the female partially degenerate and often become distended, which is apparent by 8.5d. This corresponds to the observations of Romanoff (1960), although in this study the distension of the medullary cords is reported earlier in development than in previous reports. Cortical cords have also started to develop by 8.5d. This is 0.5 day of incubation earlier than reported by Swift (1915). Cortical cord proliferation is characterised by the grouping of germ cells by the inner edge of the germinal epithelium (Romanoff, 1960).

A second characteristic of male mammalian and male chick gonadal development is that the germinal epithelium becomes thinner compared to the germinal epithelium in the female. Comparing 4.5d and 5.5d, the difference in germinal epithelium thickness is negligible. By 6.5d to 8.5d, the germinal epithelium became considerably thinner. This confirmed the report by Venzke (1954a and 1954b).

Characteristic to female mammalian and female chick gonadal development, is the proliferation of the left germinal epithelium (Kaufman, 1992). A thickening of the germinal epithelium was first observed, in the female chick gonad, at 6.5d and is obvious by 8.5d. This is slightly earlier than the stage in development of germinal epithelium thickening reported by Romanoff (1960) and Venzke (1954a). This suggested ovarian differentiation began after the differentiation of the testis, thus supporting the theory that female chick gonadal development occurred later than male chick gonadal development.

In this study, between 4.5d and 8.5d, PGCs were observed in the germinal epithelium (of the male gonad), the stroma of the medulla, the boundary between the stroma and the cords, and in the testicular cords. The number of PGCs located in the

cords increased with day of incubation in the chick. This study confirmed the observations reported by Lillie (1908), although accurate developmental stages were not recorded. Migration of these cells is possible because the testicular cords at this stage of development do not have a basement membrane (Lillie, 1908). This study contradicts the description of chick gonadal development by Romanoff (1960) which suggested that PGCs moved into the medulla with the testicular cords, after the cords had differentiated from the germinal epithelium. However, at 6d Venzke (1954a) reported that the majority of germ cells were located in the germinal epithelium, with some being present in the primary sex cords and the stroma. This agrees with the observations reported in this study.

PGCs in the female were also located in the germinal epithelium at 4.5d, 5.5d, 6.5d, 7.5d and 8.5d. Whorls of cells were observed at the later stages of development, indicative of follicular formation around the germ cells. PGCs were also visible in cords and stromal tissue of the medulla. These observations confirm previous reports by Romanoff (1960), Venzke (1954a) and Lillie (1908).

The histological analysis of the adult chick testis revealed a similarity in structure with the mammalian adult testis (Torrey, 1966). The seminiferous tubules constituted the majority of the testis, and were closely packed together. Contained within these seminiferous tubules were cells in various stages of spermatogenesis.

Overall, chick gonadal development exhibited similarity with mammalian gonadal development. The urogenital ridge arises from the intermediate mesoderm in both mammals and birds (Kaufman, 1992; Romanoff, 1960). Initial morphological differences between the sexes in both species is observed by the differentiation of the primary sex cords in the male. In the male, in both mammals and birds, the germinal epithelium is characteristically thin and the testicular cords proliferate to constitute the majority of the testes. In the female, the germinal epithelium proliferates rapidly and it is here that the secondary sex cords develop. Differences that do exist; such as the asymmetry in the chick female, where the right gonad degenerates and the complete degeneration of the primary sex cords in the mammalian female, does not mean the structure and function of the ovary and testis is significantly different between these species.

Chapter 6.

Results.

Expression studies of chick homologues to genes involved in mammalian gonadal development.

6.1. Northern analysis data.

In an attempt to compare the molecular control of gonadal development between mammals and avians, the expression of chick homologues to genes involved in mammalian gonadal development was examined by Northern analysis. This enabled a comparison between the expression profiles of the genes in mammals and avians.

6.1.1. Wilms' Tumour 1 (WT1).

A Northern blot containing male and female RNA from the genital ridge/mesonephros (which will be subsequently referred to as genital ridge), was used to study the expression of WT1 during early gonadal development. A transcript of approximately 3.1kb was identified in all samples examined on the Northern blot (fig 19A). This corresponded to the transcript detected by Kent *et al.*, (1995), which was described as 3kb in length.

Northern analysis showed WT-1 message was present in male and female samples, with a potentially sexually dimorphic expression pattern in males at 4.5d and 5.5d (fig 19A), however these expression levels might not be significantly higher. In both sexes, expression of the WT-1 transcript was low at 3.5d. Expression in female genital ridge tissue samples from 4.5d to 9.5d (stage 25 to 36) remained constant over this developmental period. Northern analysis using the WT-1 probe was repeated and consistent results obtained. However, the phosphorimager analysis (fig 19B) does not accurately replicate the expression profile observed by Northern analysis, because it accentuates the potential up-regulation of WT-1 in male samples at 4.5d and 5.5d.

Southern analysis of this clone resulted in no differences being detected between males and females, indicating the gene was not located on either of the sex chromosomes (data not shown).

6.1.2. Steroidogenic Factor-1 (SF-1).

Expression of SF-1 during early gonadal development, was analyzed by Northern analysis (fig 20A). After normalisation for loading, this transcript showed higher levels of expression in female samples at 4.5d (stage 25 and 26) (fig 20B). However, the level of SF-1 expression visualised by Northern analysis seemed lower in female genital ridge samples at 4.5d, compared to 5.5d and 6.5d in the same tissue type. The increase in female genital ridge tissue at 4.5d appears artificially high in phosphorimager analysis due to inconsistencies in loading.

By Northern analysis, expression of SF-1 was detected in male and female genital ridge samples throughout the developmental stages studied, with no obvious

up- or down-regulation of the gene (fig 20). This is inconsistent with phosphorimager analysis which showed down-regulation in female samples and up-regulation in male samples

The size of the SF-1 transcript detected by Northern analysis was approximately 1.85kb. This corresponds to the human SF-1 transcript which is 1895bp in length (de Santa Barbara *et al.*, 1996).

6.1.3. Anti-Müllerian hormone (AMH).

Analysis of AMH by Northern analysis exhibited a sexually dimorphic pattern of expression (fig 21A). After 20hr exposure, expression of the transcript was only detected in male genital ridge samples. Expression was first detected at 6.5d (stage 30), and peaked at 7.5d (stage 32 and 33) (fig 21B). Only low levels of expression were detected in the adult testis, equivalent to the expression levels detected at 6.5d in the male (fig 21).

A one week exposure resulted in low levels of expression being detected in the female samples (fig 21 A). Expression was first detected in the female genital ridge at 6.5d, and peaked at 7.5d. Highest levels of female AMH expression was detected in the adult ovary. In male genital ridge samples, at this longer exposure time, AMH was first detected at 5.5d (stage 28). The AMH transcript size was approximately 2.7kb which roughly corresponded to the published sequence of 2.8kb (Eusèbe *et al.*, 1996). Two other transcripts were also observed on the longer exposure. These transcripts were approximately 4.45kb and 6.35kb in size, which corresponded to two larger transcripts observed previously (4.5kb and 6.5kb) (Eusèbe *et al.*, 1996).

6.1.4. Aromatase.

Expression of aromatase was analyzed during male and female chick gonadal development by Northern analysis. A transcript of approximately 4.0kb in size was detected, which corresponded to the published size (McPhaul *et al.*, 1988).

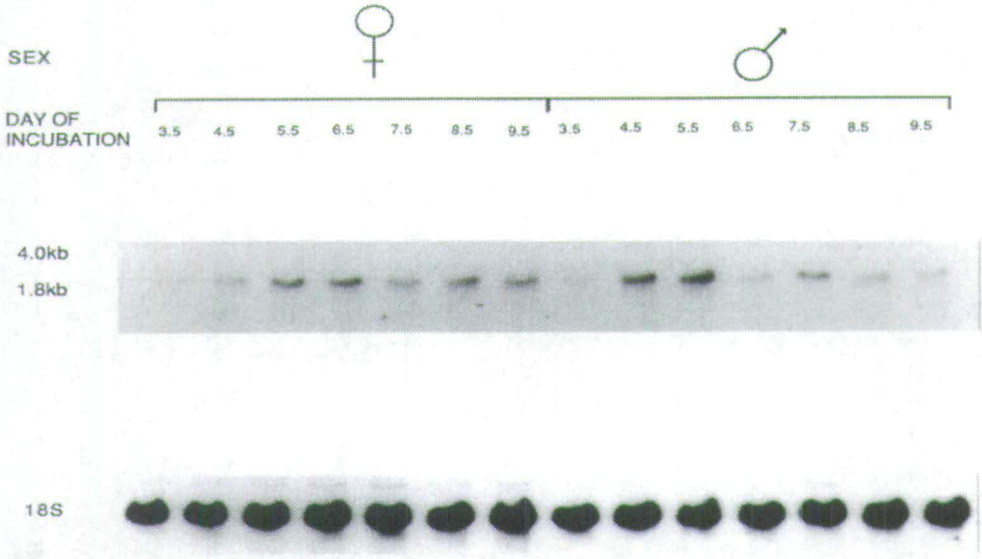
The aromatase transcript was never detected in male samples, at any developmental stage. In female samples, the transcript was first detected at 6.5d (stage 30), with higher expression levels at 8.5d (stage 34 and 35), and 9.5d (fig 22A & B).

The aromatase expression profile was also studied by Northern analysis, in various tissues from 4.5d to 8.5d (stage 25 to 35) (fig 23). Expression was only ever detected in the adult ovary. There was no expression of the aromatase transcript in the brain, where it had been detected in male and female reptiles (Jeyasuria and Place, 1997). The size of the two transcripts in the adult ovary were a major transcript of approximately 4.0kb and a minor transcript of 1.43kb.

Figure 19: (A) Northern analysis of cWT1 in the gonads of developing male and female chick embryos (top panel). Total RNA was isolated from pooled male and pooled femlae genital ridge/mesonephric tissue collected from day 3.5 to day 9.5 of incubation (stage 21 to 36), and 10μg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 60°C in 0.25M Na PO_4, 7% SDS. The blot was washed to a final stringency of 25mM Na PO_4, 1% SDS at 68°C and exposed to film for 2 days at -70°C. The loading of RNA in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). Northern analysis was carried out twice, using the same RNA pools each time, and consistent results were observed.

(B) Graph showing the expression of cWT1, analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female . M = male.

(A)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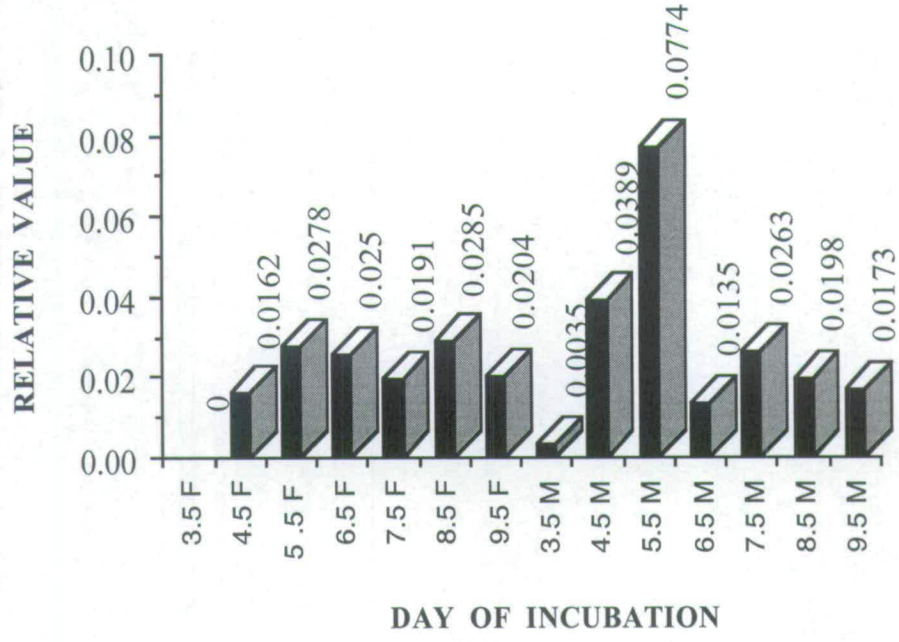
4.0kb
1.8kb
18S

(B)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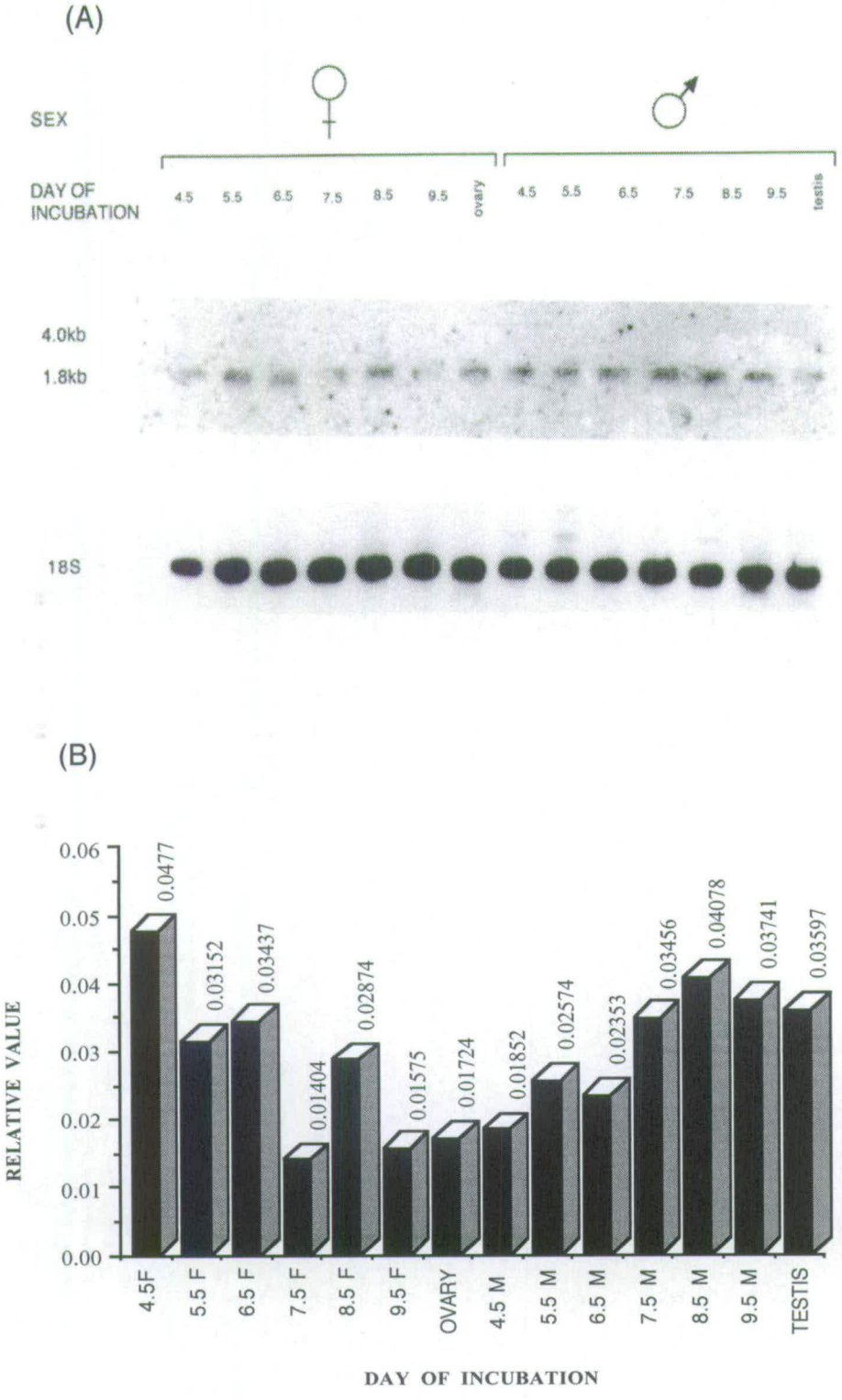
0.0285
0.0204
0.0035
0.0389
0.0774
0.0135
0.0263
0.0198
0.0173
3.5 F
4.5 F
5 .5 F
6.5 F
7.5 F
8.5 F
9.5 F
3.5 M
4.5 M
5.5 M
6.5 M
7.5 M
8.5 M
9.5 M
DAY OF INCUBATION

Figure 20: (A) Northern analysis of cSF-1 in the gonads of male and female developing chick embryos (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 4.5 to day 9.5 of incubation (stage 25 to 36) and from the adult ovary and testis. 5μg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 60°C in 0.25M Na PO_4, 7% SDS. The blot was washed to a final stringency of 15mM Na PO_4, 0.5% SDS at 65°C and exposed to film overnight at -70°C. The loading of RNA in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). A single observation was made by Northern analysis.

(B) Graph of the expression profile of cSF-1 in the genital ridge/mesonephric tissue of male and female chick embryos, analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.


(A)
SEX
DAY OF INCUBATION
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5.5
6.5
7.5
8.5
9.5
ovary
4.5
5.5
6.5
7.5
8.5
9.5
testis
4.0kb
1.8kb
18S
(B)
RELATIVE VALUE
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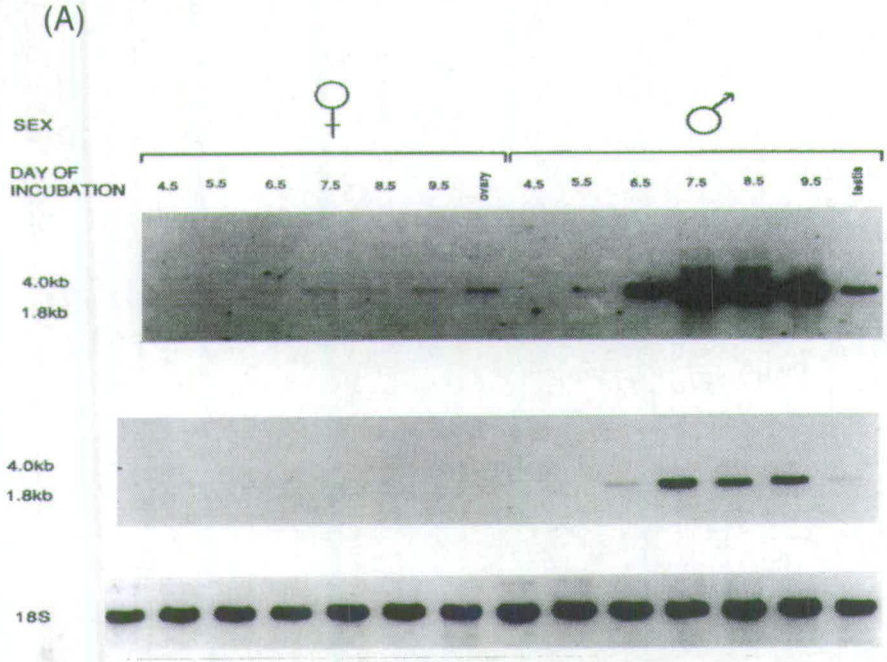
0.05
0.04
0.03
0.02
0.01
0.00
0.0477
0.03152
0.03437
0.01404
0.02874
0.01575
0.01724
0.01852
0.02574
0.02353
0.03456
0.04078
0.03741
0.03597
4.5F
5.5 F
6.5 F
7.5 F
8.5 F
9.5 F
OVARY
4.5 M
5.5 M
6.5 M
7.5 M
8.5 M
9.5 M
TESTIS
DAY OF INCUBATION

Figure 21: (A) Northern analysis of cAMH in the gonads of developing male and female chick embryos (top and middle panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 4.5 to day 9.5 of incubation (stage 25 to 36) and from adult ovary and testis. 5μg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 60°C in 0.25M Na PO_4, 7% SDS. A low stringency wash was carried out at 25mM Na PO_4, 1% SDS at 65°C and exposed to film overnight at -70°C (top panel). The Northern blot was washed to a final stringency of), 25mM Na PO_4, 1% SDS at 68°C and exposed to film for 3 days at -70°C (middle panel). The presence of approximately equal amounts of RNA in each lane was monitored by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). Northern analysis was carried out three times, on different RNA samples each time, with consistent results.

(B) Graph showing the expression profile of cAMH, analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.


(A)
SEX
♀
♂
DAY OF INCUBATION
4.5
5.5
6.5
7.5
8.5
9.5
ovary
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9.5
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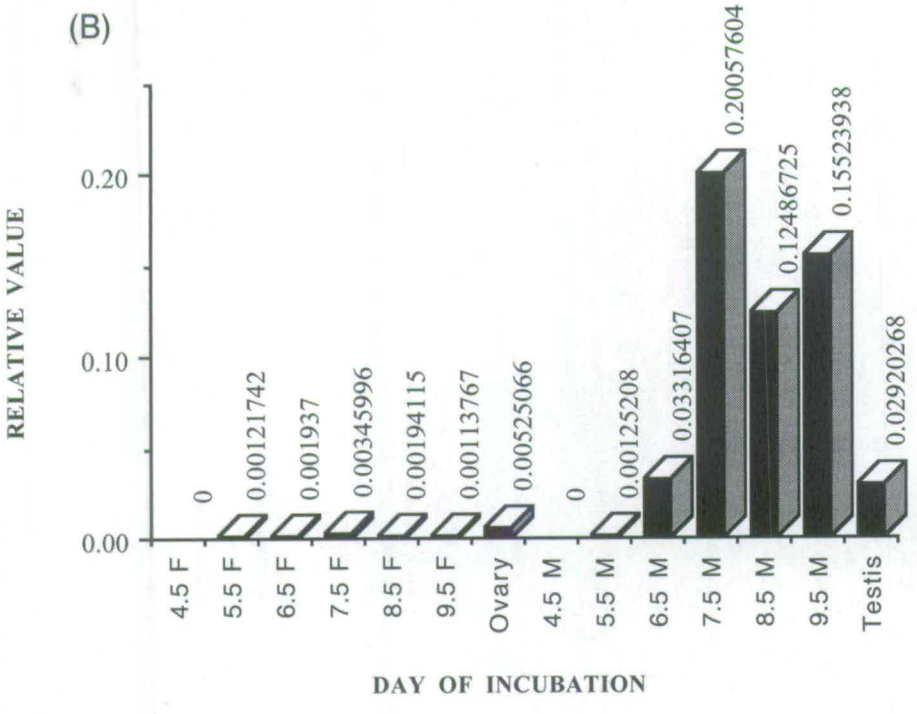
4.0kb
1.8kb
4.0kb
1.8kb
18S


(B)
RELATIVE VALUE
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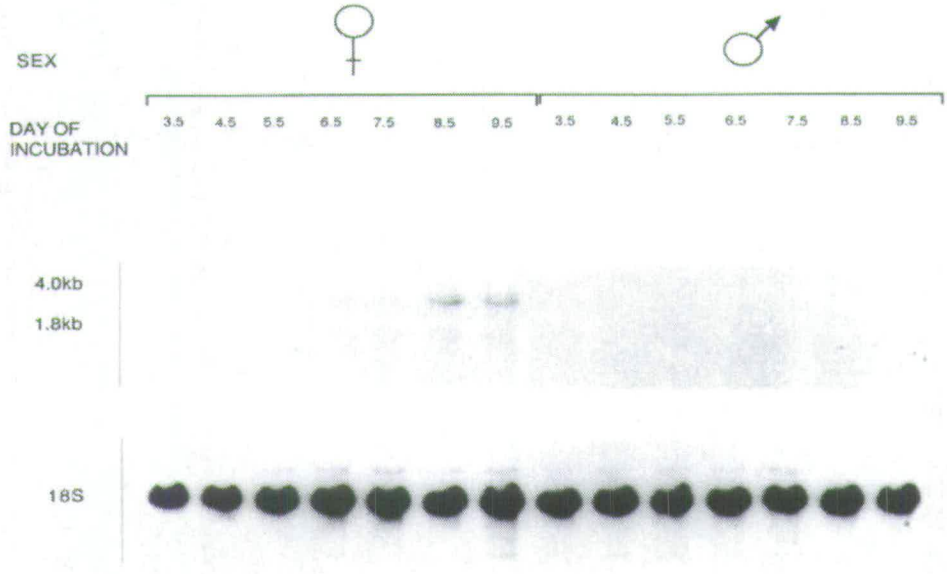
0.00525066
0
0.00125208
0.03316407
0.20057604
0.12486725
0.15523938
0.02920268
4.5 F
5.5 F
6.5 F
7.5 F
8.5 F
9.5 F
Ovary
4.5 M
5.5 M
6.5 M
7.5 M
8.5 M
9.5 M
Testis
DAY OF INCUBATION

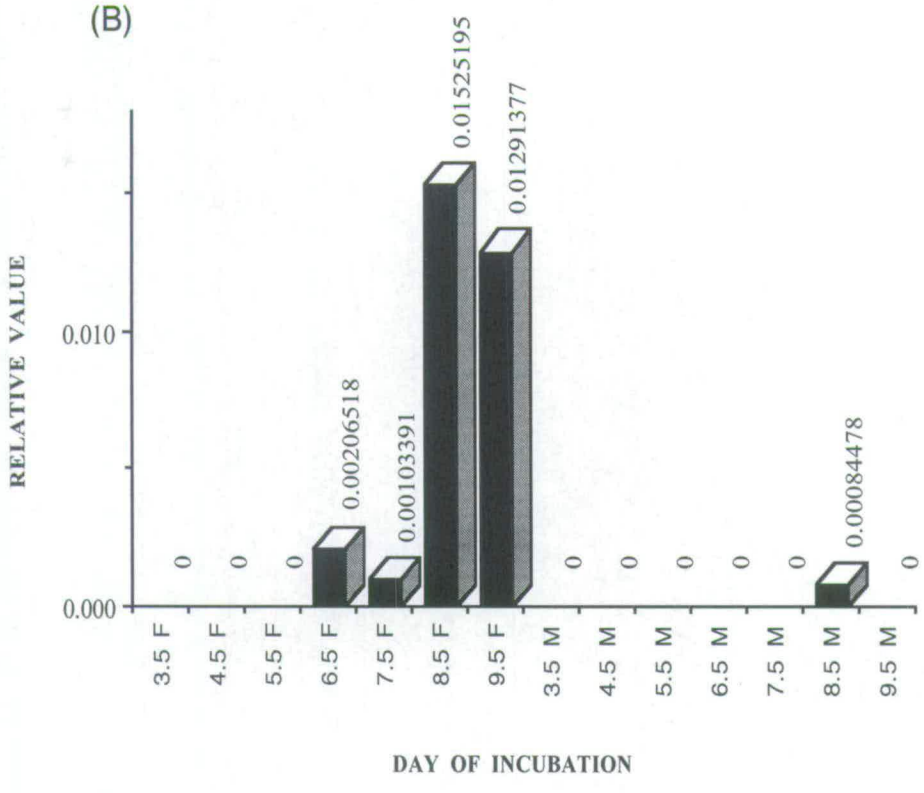
Figure 22: (A) Northern analysis of c-aromatase in the gonads of the developing male and female chick embryo (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 3.5 to day 9.5 of incubation (stage 21 to 36), and 10µg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 60°C in 0.25M Na PO_4, 7% SDS. The blot was washed to a final stringency of 25mM Na PO_4, 1% SDS at 65°C and exposed to film for 3 days at -70°C. The loading of the RNA in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). Northern analysis was carried out three times with consistent results.

(B) Graph of the expression profile of c-aromatase in the genital ridge/mesonephric tissue of male and female chick embryos, analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.

(A)


SEX
♀
♂
DAY OF INCUBATION
3.5 4.5 5.5 6.5 7.5 8.5 9.5
3.5 4.5 5.5 6.5 7.5 8.5 9.5
4.0kb
1.8kb
18S

(B)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0
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4.5 F
5.5 F
6.5 F
7.5 F
8.5 F
9.5 F
3.5 M
4.5 M
5.5 M
6.5 M
7.5 M
8.5 M
9.5 M
DAY OF INCUBATION

Figure 23: Northern analysis of c-aromatase expression in tissues in the developing chick embryo and in the adult ovary and testis (top panel). Total RNA was isolated from different chick embryonic tissues at day 4.5 to day 8.5 of incubation (stage 25 to 35) and from adult ovary and testis. 8μg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 60°C in 0.25M Na PO_4, 7% SDS. The blot was washed to a final stringency of 25mM Na PO_4, 1% SDS at 65°C and exposed to film for 2 days at -70°C. The RNA loading in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). Northern analysis was carried out twice, using different RNA sample pools, with consistent results.

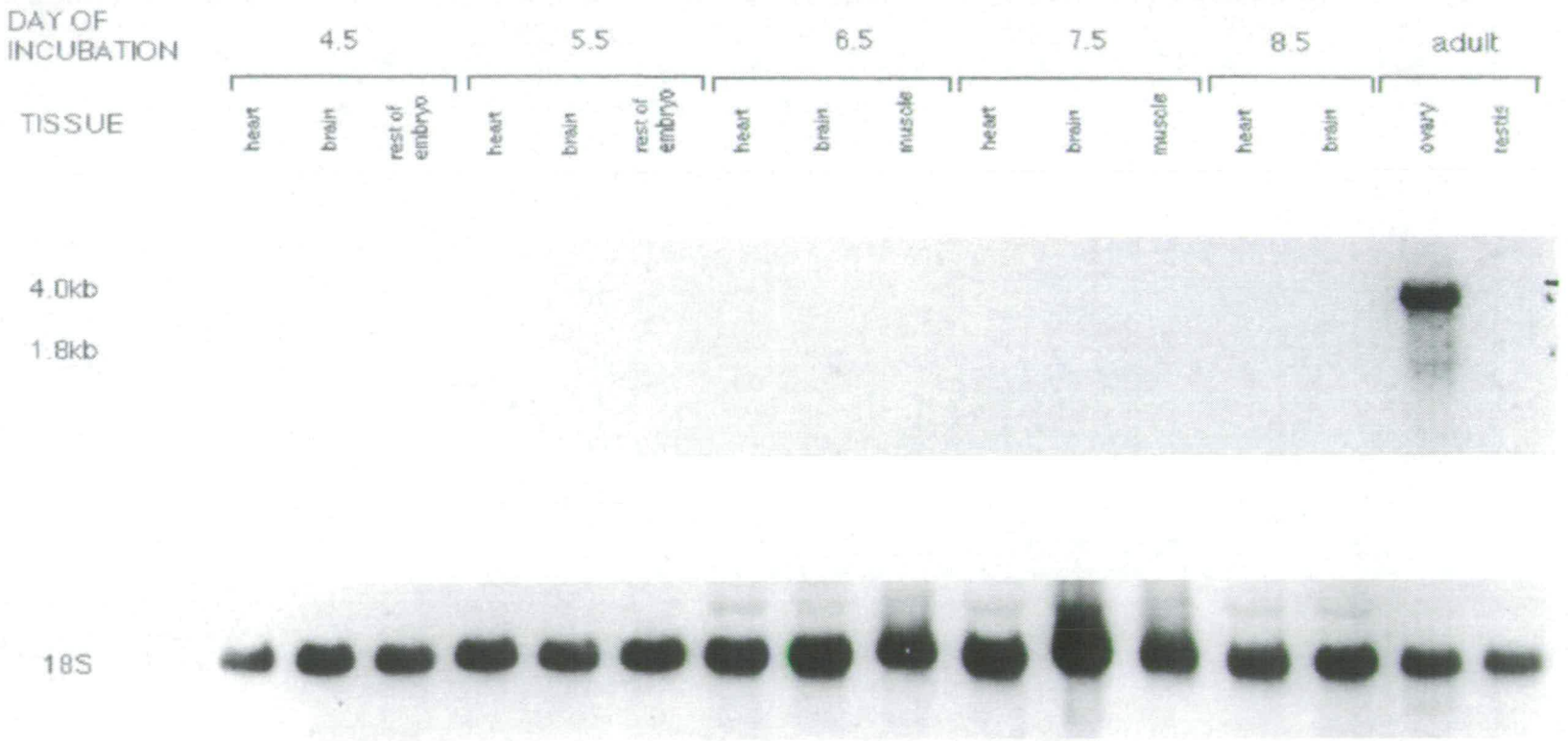
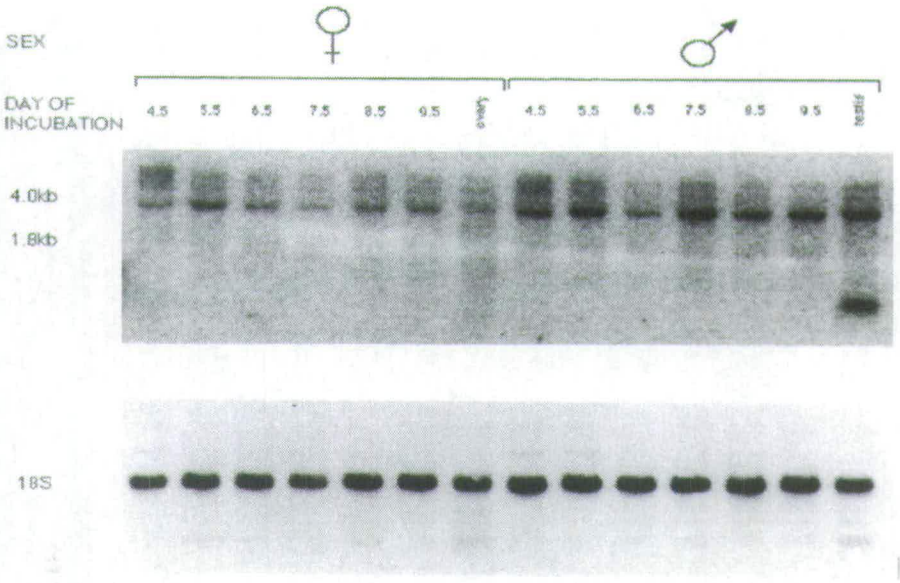

DAY OF INCUBATION
4.5
5.5
6.5
7.5
8.5
adult
TISSUE
heart
brain
rest of embryo
heart
brain
rest of embryo
heart
brain
muscle
heart
brain
muscle
heart
brain
ovary
testis
4.0kb
1.8kb
18S

Figure 24: (A) Northern analysis of cSox-9 in the gonads of male and female developing chick embryos (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 4.5 to day 9.5 of incubation (stage 25 to 36) and from the adult ovary and testis. 5µg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 42°C in 2x PIPES, 50% formamide, 0.5% SDS. The blot was washed to a final stringency of 0.2xSSC, 0.1% SDS at 65°C and exposed to film for two days. The loading of RNA in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). Northern analysis was carried out twice, using different RNA sample pools, with consistent results.

(B) Graph of the expression profile of cSox-9 in the genital ridge/mesonephric tissue of male and female chick embryos, analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.

(A)


SEX
DAY OF INCUBATION
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6.5
7.5
8.5
9.5
ovary
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5.5
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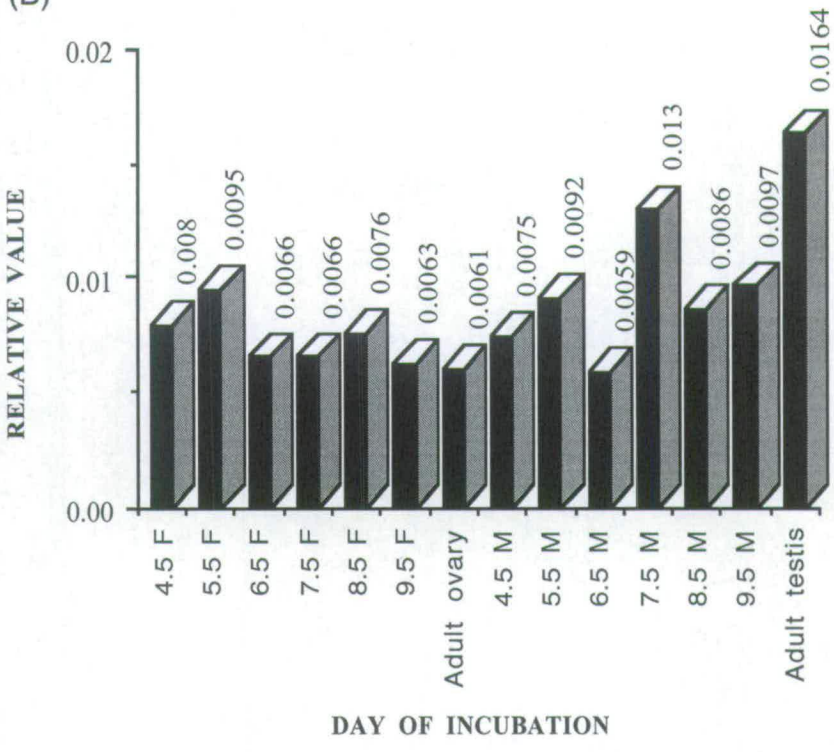
9.5
testis
4.0kb
1.8kb
18S

(B)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0.0075
0.0092
0.0059
0.013
0.0086
0.0097
0.0164
4.5 F
5.5 F
6.5 F
7.5 F
8.5 F
9.5 F
Adult ovary
4.5 M
5.5 M
6.5 M
7.5 M
8.5 M
9.5 M
Adult testis
DAY OF INCUBATION

6.1.5. Sox-9 (Sry-related HMG box proteins).

The expression profile of Sox-9 was analyzed by Northern analysis (fig24A). A transcript of approximately 3.9kb was detected in male and female samples in all developmental stages (fig 24A). This transcript size was more than twice the size of the published transcript, of 1640bp (Kamachi and Kondoh, 1998). However, it does correspond to the published transcript size for mouse (Wright *et al.*, 1995).

Expression levels on the Northern blot were similar between males and females at 4.5d to 9.5d (fig 24A). In the adult testis the expression levels of the 3.9kb transcript were significantly higher compared to the expression levels in the adult ovary. A second transcript was also detected, only in the adult testis. This transcript was approximately 1.36kb in length (fig 24A). Phosphorimager analysis confirmed the profile observed by Northern analysis, indicating no significant changes in expression levels between male and female genital ridge tissue samples at the embryonic stages studied (fig 24B).

6.2. Whole mount *in situ* hybridisation data.

In order to identify the tissue and cellular location of expression, whole mount *in situ* hybridisation (wmISH) was carried out on selected transcripts. Due to the low levels of expression of some transcripts, extended colour development times were frequently required. In some instances this lead to the formation of a precipitate which collected on the surface of the tissue. Unless stated, colour development times were the same in males and females.

6.2.1. WT1.

An anti-sense WT1 riboprobe of 600bp was used to carry out wmISH. No sense probe for WT1 was generated because the expression of WT1 had previously been reported (Kent *et al.*, 1995), and this study was carried out to confirm this expression. WT1 expression was studied at 4.5d and 5.5d (stage 25 to 28) (as this was the period of greatest expression by Northern), in order to identify those tissues expressing WT1 in the urogenital ridge.

In the female chick urogenital ridge, at 4.5d (stage 25 to 27), expression of WT1 was observed from the anterior to the posterior end of the urogenital ridge (fig 25a). WT1 expression was observed on the medial surface of the mesonephroi, encompassing the region where the indifferent gonads would develop. WT-1 expression was not confined to the gonads, but also in the mesonephros adjacent to the gonads. At 5.5d (stage 28), the area of WT1 expression had retracted anteriorly, corresponding to the shortening of the gonads (fig 25b).

In the male chick embryo, at 4.5d, WT1 expression was observed along the anterior two thirds of the urogenital ridge. This expression pattern was similar to that observed in the female, except that lower expression levels were detected in the male (fig 26a). This contrasted with the Northen analysis data, where expression levels of WT-1 appaered slightly higher in the male genital ridge tissue. At 5.5d, WT-1 expression was restricted to the medulla of the mesonephros and in the gonads (fig 26b,c & d). A longer colour development period (26hrs) resulted in expression being detected on the inner medial surface of the mesonephros, from the gonads to the posterior tip of the mesonephros (fig 26c & d). Expression was also observed along the edges of the Wolffian ducts (fig 26d).

6.2.2. SF-1.

An anti-sense SF-1 riboprobe of 530bp and a sense SF-1 riboprobe of 570bp were used to carry out wmISH. In partially dissected male and female chick embryos, at 3.5d (stage 21 and 22), expression of SF-1 was observed along the developing urogenital ridge on either side of the neural tube (fig 27a, 29a). At 5.5d (stage 28), very low levels of SF-1 expression were detected in the gonads in male embryos (fig 29b). In female gonads at this stage of development there appeared to be expression of SF-1, but levels were barely above background (fig 27b). In the mesonephric tissue, patches of colour development were observed indicating expression in clusters of cells.

Expression was observed in male and female gonads at 6.5d (stage 30) (fig 27c, 29c). There were no apparent differences in expression between the left and the right gonads, or in either sex. SF-1 expression, at 7.5d (stage 32 and 33), exhibited very low levels in male and female gonads (fig 28 & 29d). This does not agree with Northern analysis data, where expression levels remained fairly constant throughout 4.5d to 9.5d in both sexes. Expression was also observed in the mesonephric tissue, with differing patterns between the sexes; although it was likely that this was an artifact. A line of SF-1 expression in the mesonephros underneath the developing Müllerian ducts was also observed (fig 28b). Tissue probed with the sense SF-1 riboprobe was negative (fig 28c). Analysis by wmISH was carried out twice with consistent results.

In an attempt to elucidate which cells were expressing SF-1, genital ridge tissue at 6.5d, analyzed by wmISH, was sectioned and counterstained. Expression was observed in the inner surface lining the collecting tubules (fig 30a & b). Expression was also detected in the gonads in the male embryo, although it was

difficult to establish exactly which cells were expressing SF-1 (fig 30c). However, the more intense colour followed a pattern reminiscent of the seminiferous tubules.

6.2.3. AMH.

An anti-sense AMH transcript of 450bp was used to carry out wmISH. No sense probe was generated because analysis of chick AMH expression pattern had previously been reported (Euèsebe *et al.,* 1996), and this study was carried out to confirm this expression.

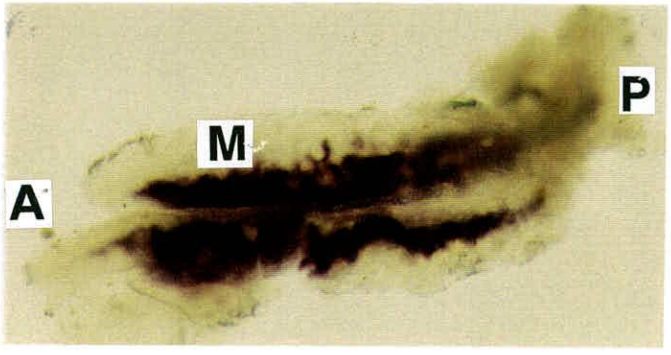
AMH expression was obvious in the gonadal portion of the male tissue sample at 7.5d and 8.5d (stage 32 to 35) (fig 31a,b & c). If the colour reaction was only allowed to develop for a short time period (45 min), a speckled appearance was observed in the male gonads, indicating not all cells in the gonad expressed AMH at high levels (fig 31a). A colour development longer time period, showed a more intense staining of the gonads (fig 31b). At 5.5d (stage 28), no colour development was observed in the gonads of the male chick embryo after 24 hrs of colour development (fig 31d).

AMH expression was also analyzed in chick female genital ridge tissue. No signal could be detected at 7.5d (stage 32 and 33) in female samples, after the same colour development time as the male sample (fig 32a). However, after 2.5 days of colour development, AMH expression was detected in the female gonads at 7.5d. Higher levels of expression were observed in the left ovary compared with the right ovary at this stage of development (fig 32b). AMH expression was also analyzed at 5.5d, 6.5d and 8.5d (stage 28 to 35) in the female genital ridge tissue, but no signal was detected in this tissue (fig 32c). Analysis by wmISH was carried out 4 times with consistent results.

In an attempt to more accurately elucidate in which cell type AMH was being expressed, chick genital ridge tissue from males and females at 7.5d were sectioned and counterstained (fig 33a,b & c). No expression was observed in female sections (fig 33a). In male sections, expression of AMH was located in the medulla of the gonad, corresponding to the location and pattern of the seminiferous tubules (fig 33b). At a higher magnification (x40), AMH expression was observed as a ring of cells. This indicated that as AMH expression had located to the seminiferous tubules, these ring of cells corresponded to the Sertoli cells of the seminiferous tubules. However, it must be noted that expression was not confined to cells in the cords.

Figure 25: Expression of WT1 in the female chick embryonic gonads and mesonephric tissue by wmISH. Hybridization was performed using an anti-sense DIG labelled cWT1 riboprobe at 65°C. (a) Day 4.5 (stage 25 and 26) female genital ridge. Colour development proceeded for 9½hr. actual length of the mesonephros is 3.4mm. (b) Day 5.5 (stage 28) female gonads and mesonephros. colour development proceeded for 9½hr. Actual length of the mesonephros is 3mm. Anterior (A), posterior (P), gonad (G), mesonephros (M). Analysis by wmISH was carried out twice with consistent results.

(a)



(b)

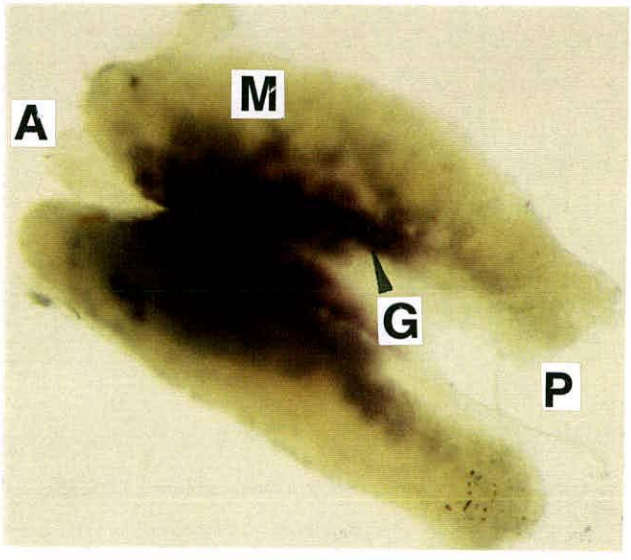
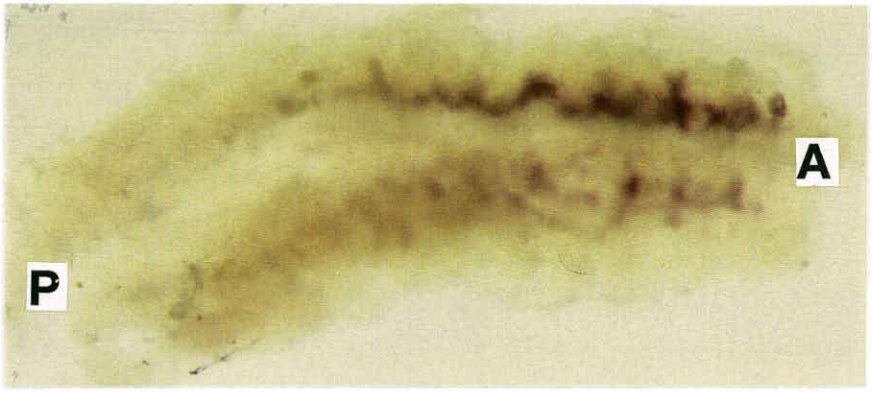
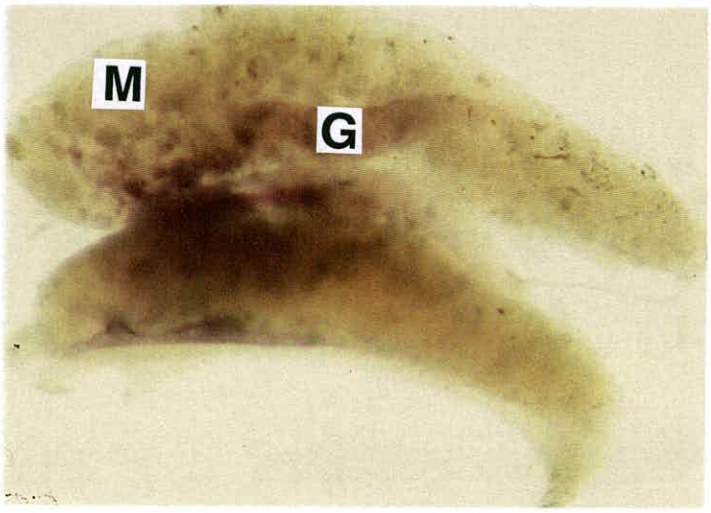


Figure 26: Expression of WT1 in the chick embryonic testes and mesonephric tissue by wmISH. Hybridization was performed using an anti-sense DIG labelled cWT1 riboprobe at 65°C. (a) Day 4.5 (stage 25 and 26) male urogenital ridge. Colour development proceeded for 9½ hr. Actual length of the mesonephros is 3.4mm. (b) Day 5.5 (stage 28) male gonad and mesonephros. Colour development proceede for 4½hr. Actual length of the mesonephros is 4mm. (c and d) Day 5.5 male gonads and mesonephros. Colour development proceeded for 24hr. Actual length of the mesonephroi are 3.4mm and 3.2mm respectively. Gonad (G), mesonephros (M), Wolffian duct (WD), anterior (A), posterior (P). Analysis by wmISH was carried out twice with consistent results.

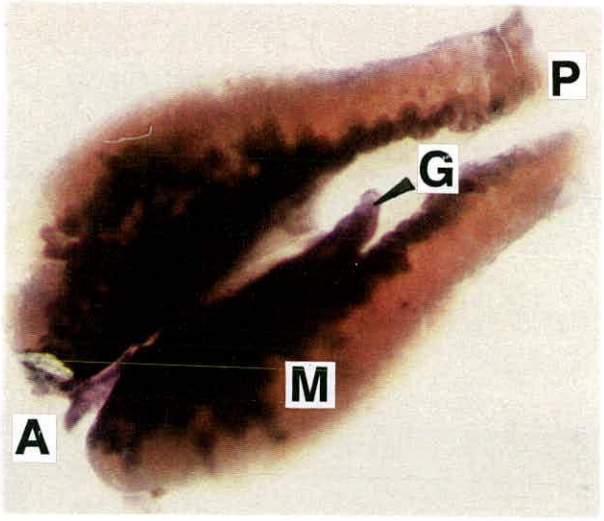
(a)



(b)



(c)



(d)

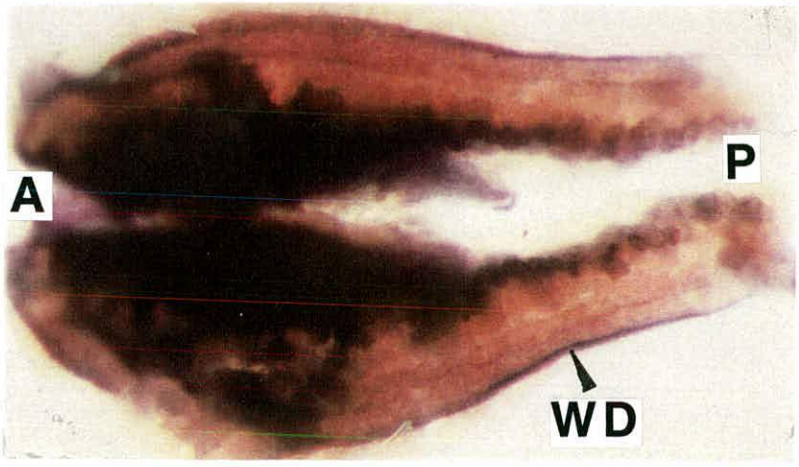
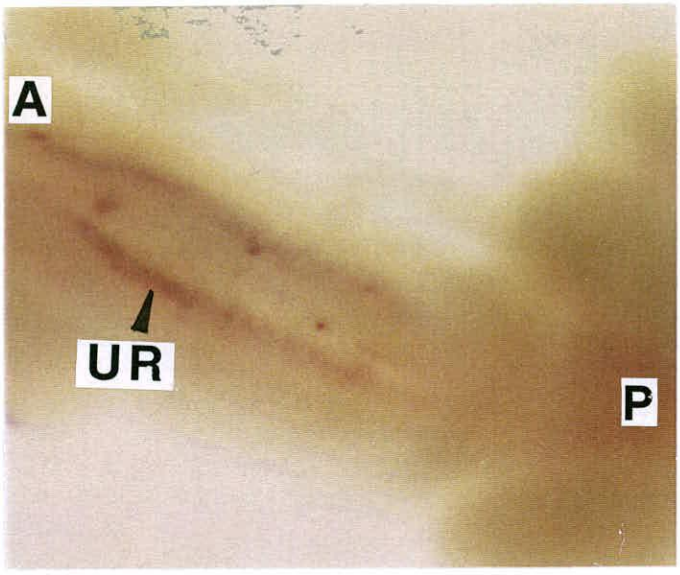
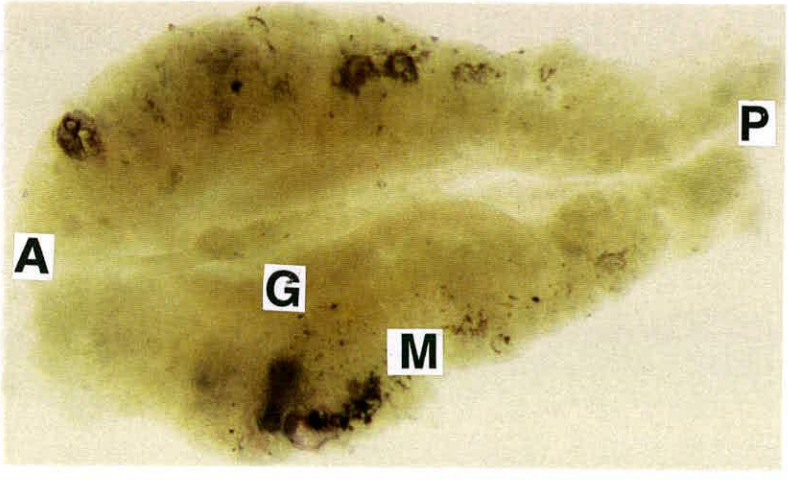


Figure 27: Expression of SF-1 in the female chick urogenital ridge and embryonic gonads and mesonephros by wmISH. Hybridization was performed using an anti-sense DIG labelled zfSF-1 riboprobe at 64°C. (a) Day 3.5 (stage 21 and 22) partially dissected female embryo, showing the expression of SF-1 in the urogenital ridge. Colour development proceeded for 20hr. Actual length of the embryo is 6mm. (b) Day 5.5 (stage 28) female gonads and mesonephros. Colour development proceeded for 20hr. Actual length of the mesonephros is 4mm. (c) Day 6.5 (stage 30) female gonads and mesonephros. Colour development proceeded for 20hr. Actual length of the mesonephros is 3.7mm. Anterior (A), posterior (P), urogenital ridge (UR), gonad (G), mesonephros (M). Analysis by wmISH was carried out twice with consistent results.

(a)



(b)



(c)

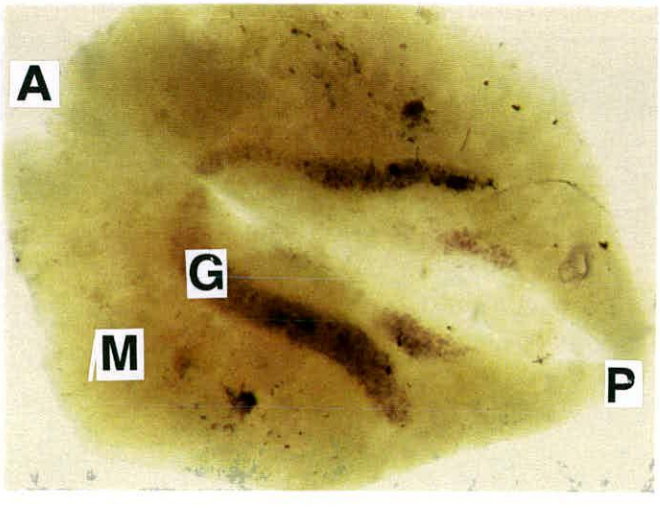
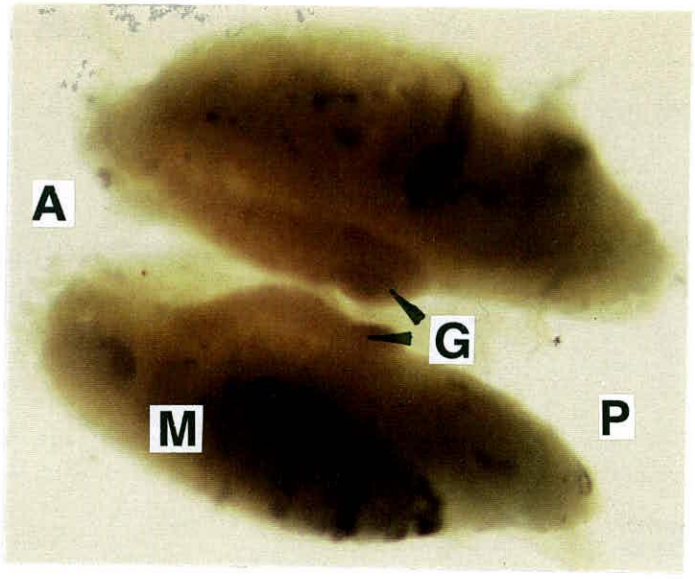
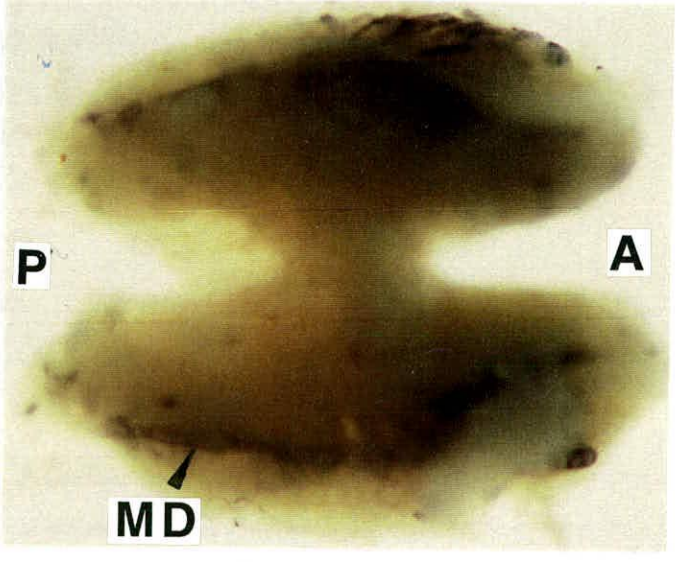


Figure 28: Expression of SF-1 in the chick embryonic ovaries and mesonepric tissue by wmISH. Hybridization was carried out using a sense and anti-sense DIG labelled zfSF-1 riboprobe at 64°C. (a) Front view of day 7.5 (stage 32 and 33) female gonads and mesonephros. Colour development proceeded for 20hr. Actual length of the mesonephros is 4mm. (b) Reverse view of day 7.5 (stage 32 and 33) female mesonephros. Colour development proceeded for 20hr. Actual size of the mesonephros is 4mm. (c) Day 5.5 (stage 28) female gonads and mesonephros using the sense probe. Colour development proceeded for 20hr. Actual length of the mesonephros is 4mm. Anterior (A), posterior (P), gonad (G), mesonephros (M), Müllerian ducts (MD). Analysis by wmISH was carried out twice with consistent results.

(a)



(b)



(c)

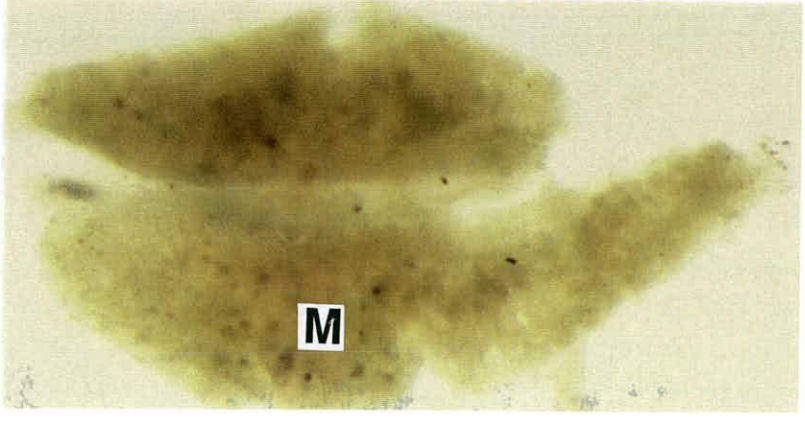
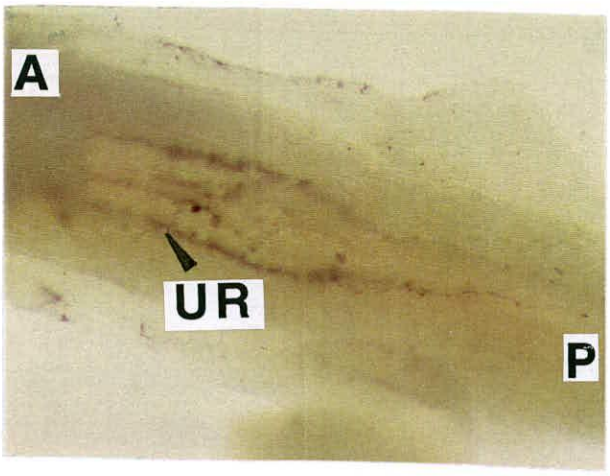
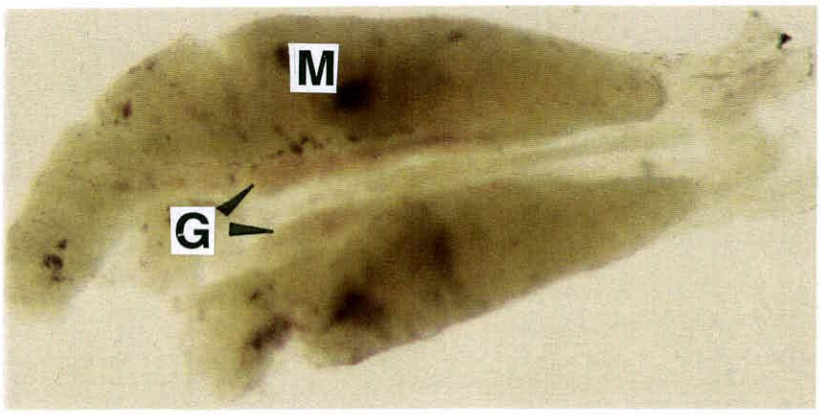


Figure 29: Expression of SF-1 in the male chick urogenital ridge and embryonic gonads and mesonephros by wmISH. Hybridization was performed using a sense and anti-sense DIG labelled zfSF-1 riboprobe at 64°C. (a) Day 3.5 (stage 21 and 22) partially dissected male embryo, showing expression in the urogenital ridge. Colour development proceeded for 20hr. Actual length of the embryo is 6.2mm. (b) Day 5.5 (stage 28) male gonads and mesonephros. Colour development proceeded for 20hr. Actual length of the mesonephros is 4mm. (c) Day 6.5 (stage 30) . Colour development proceeded for 11hr. Actual length of the mesonephros is 4mm. (d) Day 7.5 9stage 32 and 33). Colour development proceeded for 11hr. Actual length of the mesonephros is 4.5mm. Anterior (A), posterior (P), urogenital ridge (UR), gonad (G), mesonephros (M). Analysis by wmISH was carried out twice with consistent results.

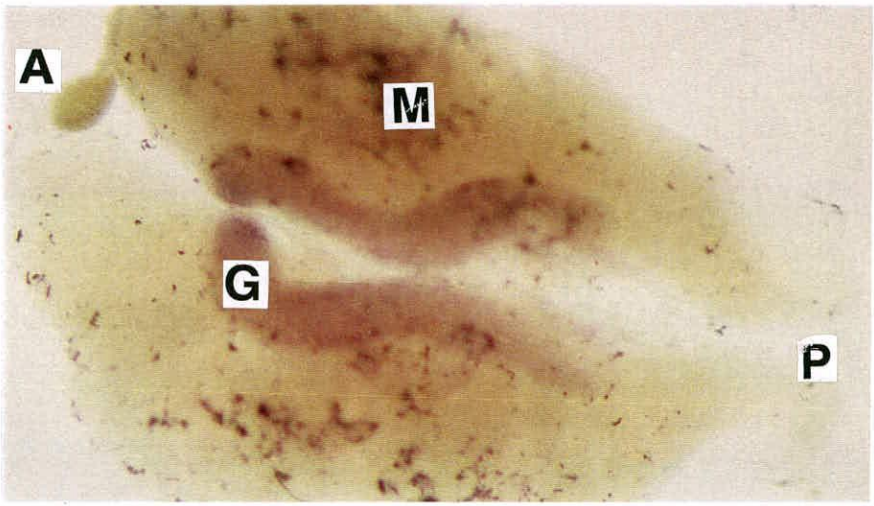
(a)



(b)



(c)



(d)

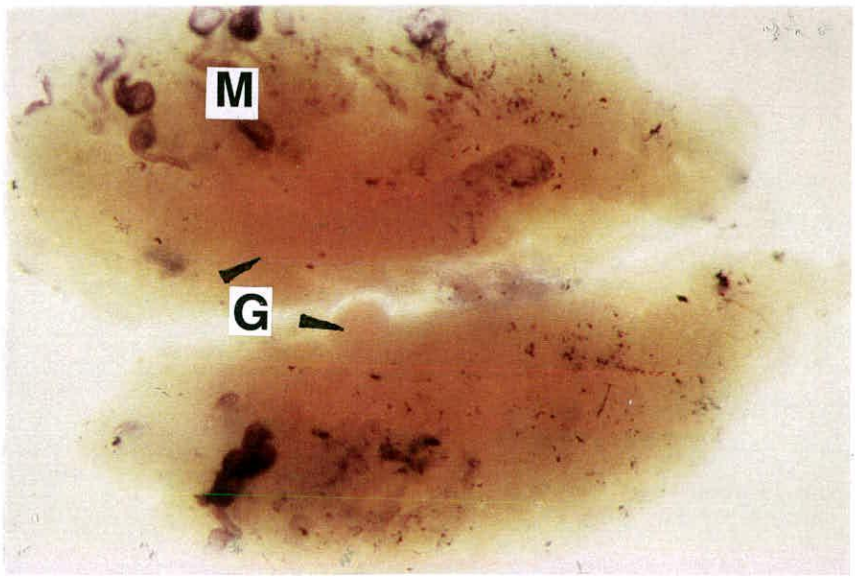
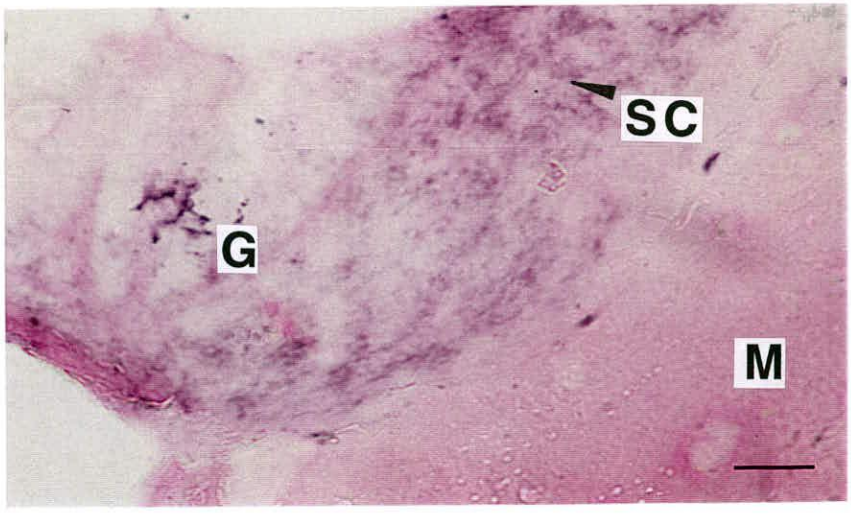
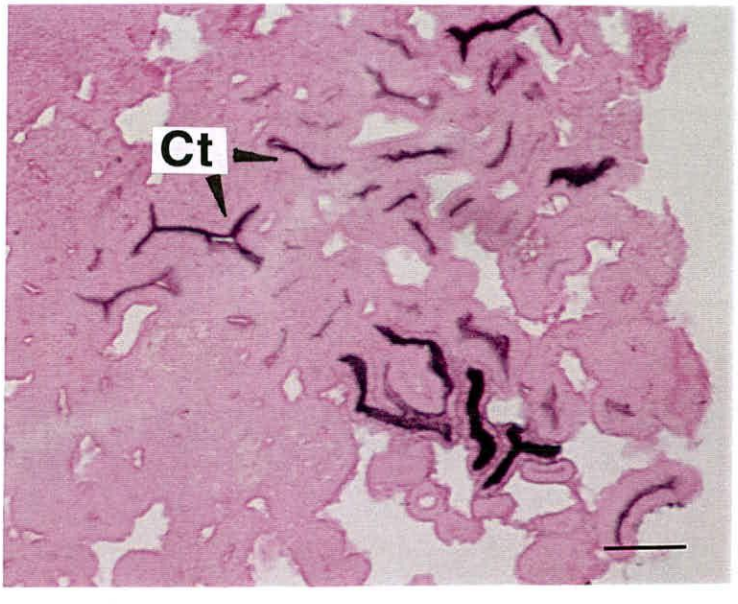


Figure 30: Expression of SF-1 in the male chick gonad and mesonephros wmISH, which has been sectioned and counterstained. (a) Day 7.5 (stage 32 and 33) male gonad. Scale bar represents 40μm. (b) Day 7.5 male mesonephros. Scale bar represents 40μm. (c) Day 7.5 male mesonephros. Scale bar represents 10μm. Gonad (G), mesonephros (M), collecting tubule (Ct), seminiferous cords (SC).

(a)



(b)



(c)

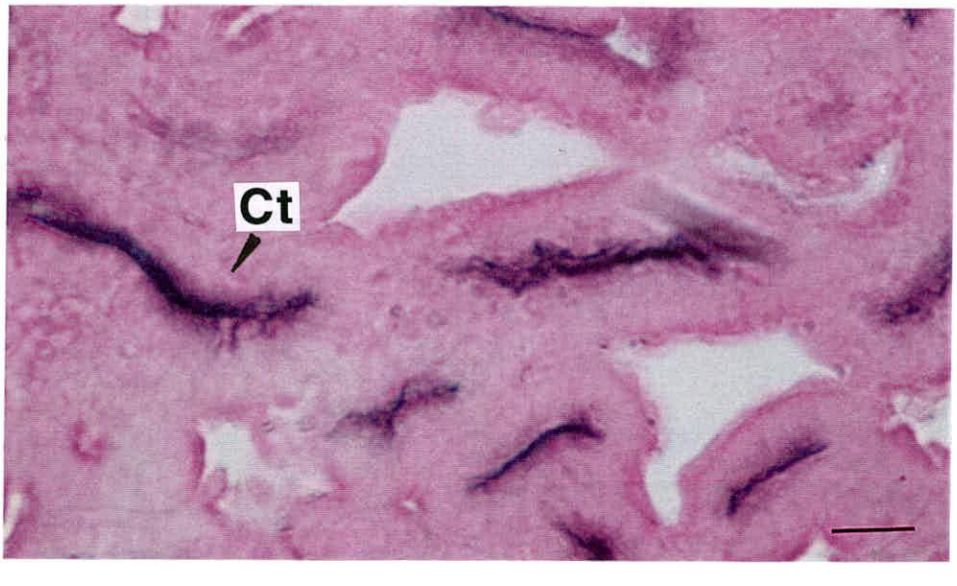
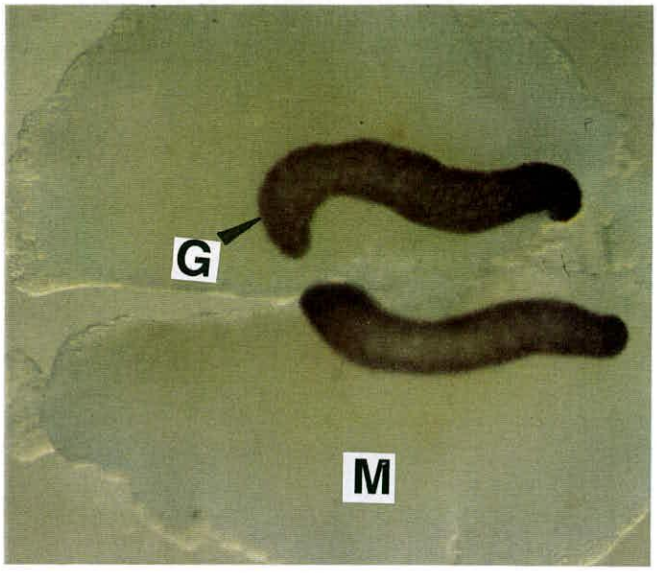
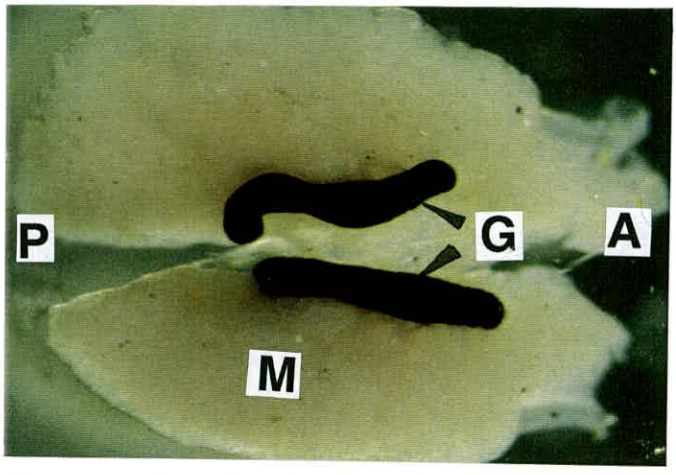


Figure 31: Expression of AMH in the chick embryonic testis by wmISH. Hybridization was performed with an anti-sense DIG labelled cAMH riboprobe at 66°C. (a) A day 7.5 of incubation (stage 32 and 33) testes and mesonephroi, showing the "speckled" appearance of the gonads after a colour development of 45mins. Actual length of the mesonephros is 3.75mm. (b) Day 7.5 testes and mesonephroi, showing a more intense staining of the gonads. Colour development proceeded for 15hr. Actual length of the mesonephros is 3.8mm. (c) Day 8.5 (stage 34 and 35) testes and mesonephroi, indicating the intense staining in the gonads. Colour development proceeded for 15hr. Actual length of the mesonephros is 4.5mm. (d) Day 5.5 testes and mesonephroi which are negative. Colour development proceeded for 24hr. Actual length of the mesonephros is 3.2mm. Gonad (G), mesonephros (M), anterior (A), posterior (P). Analysis by wmISH was carried out 5 times with consistent results.

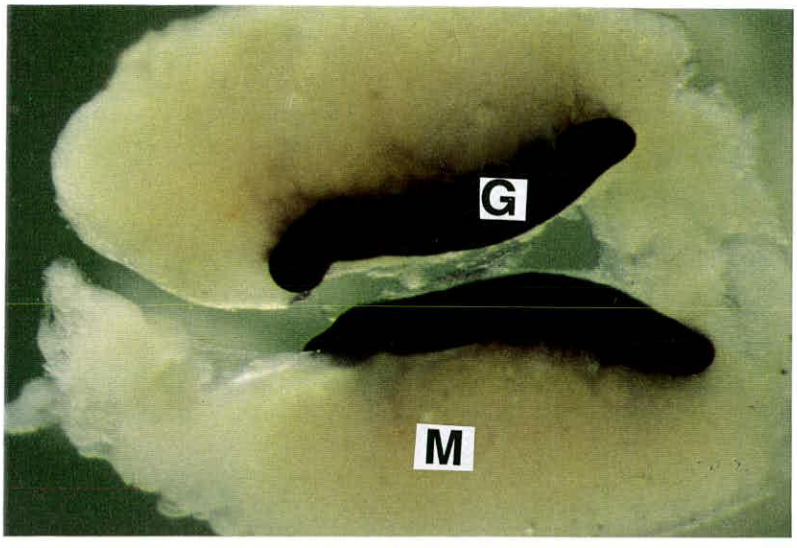
(a)



(b)



(c)



(d)

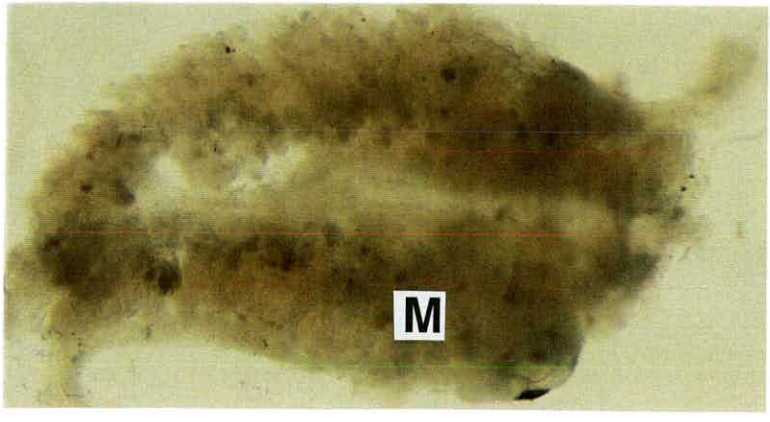
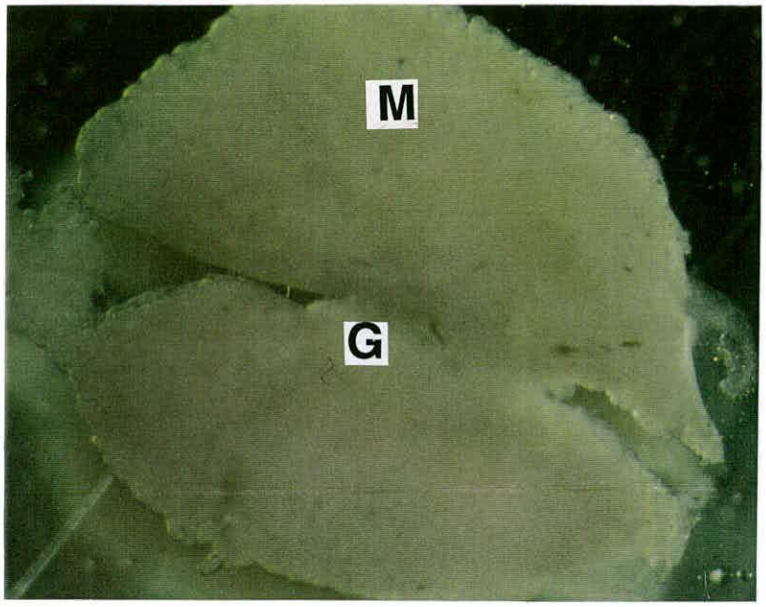
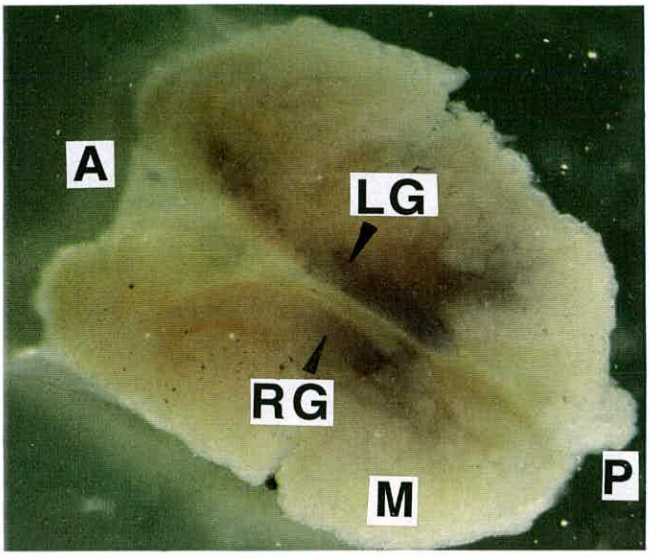


Figure 32: Expression of AMH in the chick embryonic ovaries by wmISH. Hybridization was performed with an anti-sense DIG labelled cAMH riboprobe at 66°C. (a) Day 7.5 (stage 32 and 33) ovaries and mesonephroi, which show no colour development after 5hr. Actual size of the mesonephros is 3.8mm. (b) Day 7.5 ovaries and mesonephroi, showing colour development in the gonads after 2½ days. Actual length of the mesonephros is 3mm. (c) Day 6.5 (stage 30) ovaries and mesonephros, showing no colour development after 3 days. Actual length of the mesonephros is 3.2mm. Anterior (A), posterior (P), left gonad (LG), right gonad (RG), mesonephros (M). Analysis by wmISH was carried out 5 times with consistent results.

(a)



(b)



(c)

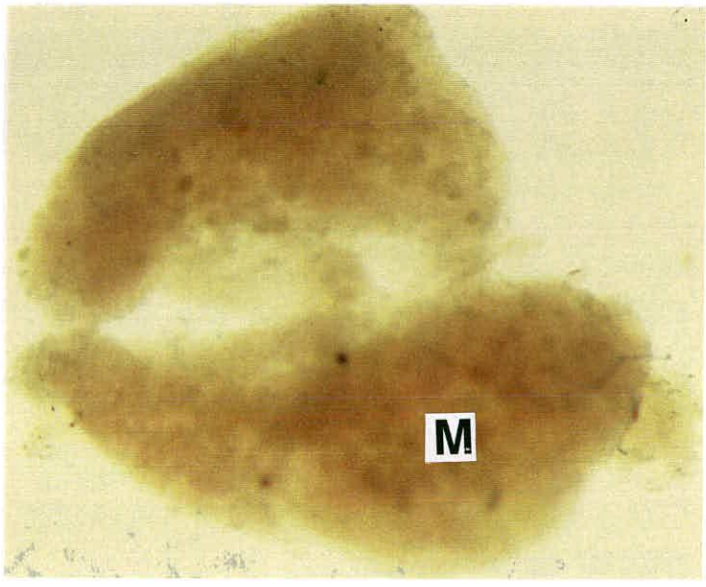
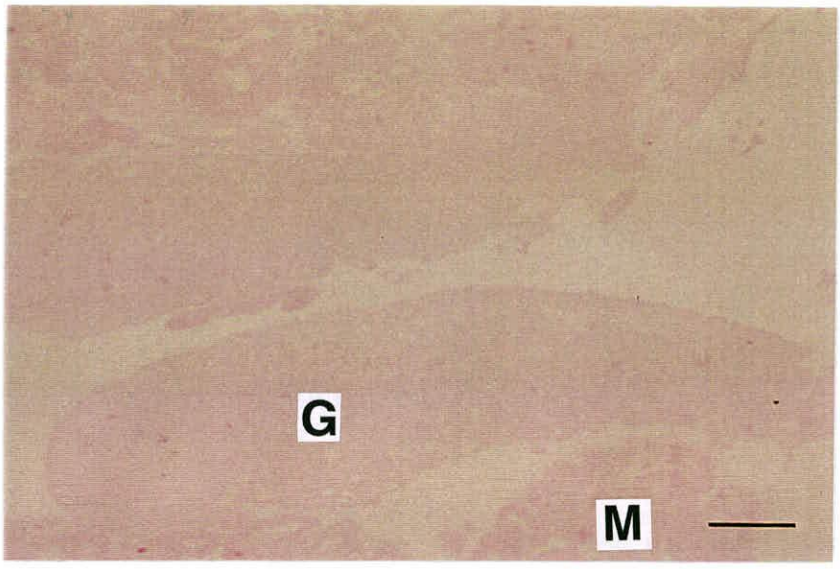
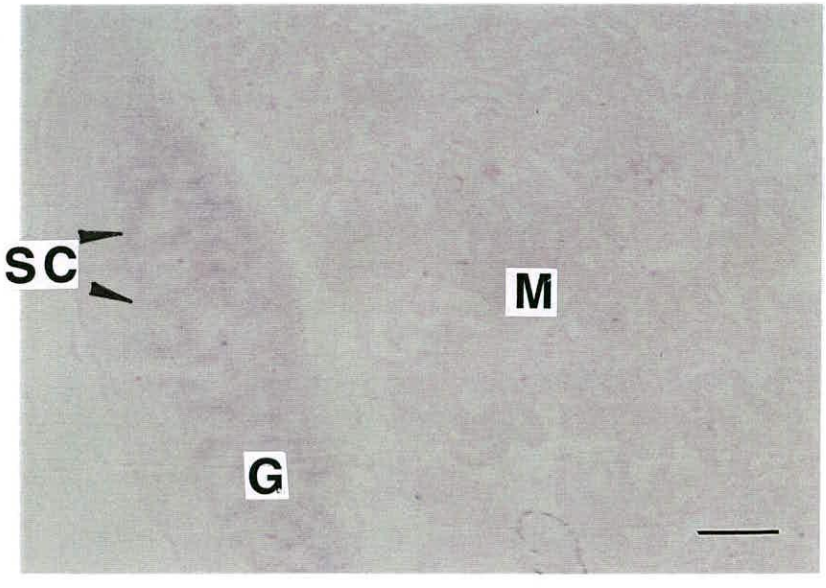


Figure 33: Expression of AMH in male and female chick gonads at day 7.5 of incubation by wmISH , which have been sectioned and counterstained. (a) Female left gonad, showing no expression could be detected. Scale bar represents 40μm. (b) Male gonad , showing expression of AMH is confined to the medulla and locates to the testicular cords. Scale bar represent 40μm. (c) High power magnification of the male gonad showing expression of AMH in cells in the medulla. Scale bar represents 10μm. Mesonephros (M), gonad (G), seminiferous cords (SC), medulla (Me).

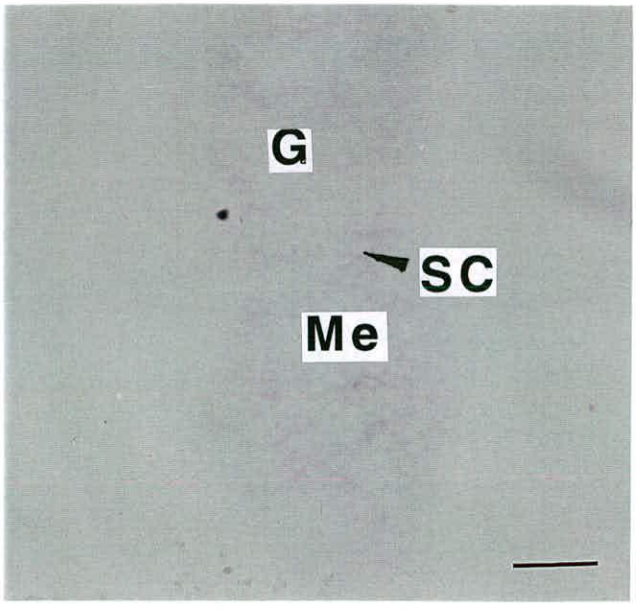
(a)



(b)



(c)



Chapter 7.

Discussion.

Expression studies of chick homologues to genes involved in mammalian gonadal development.

This study was carried out to compare whether gene regulation of mammalian gonadal development was conserved in the chick, and thus followed a similar genetic pathway. In addition, as a number of the genes expressed during mammalian gonadal development are a consequence of the sex determination switch, a systematic study of these genes might refine the timing of sex determination in the chick. One gene potentially involved in female mammalian gonadal development and not included in this study is Dax-1 (Burris *et al.,* 1996). Using primers based on mammalian sequences, attempts to amplify a chick homologue of Dax-1 were unsuccessful. Reports have suggested that conservation of this gene between different species is not very high (Zanaria *et al.,* 1994) and, therefore, it was considered that a mammalian Dax-1 probe would be unlikely to detect a chick transcript.

The major transcript size of AMH was approximately 2.7kb and two larger transcripts were approximately 4.45kb and 6.35kb, which corresponded with the size of the transcripts previously reported (Eusèbe *et al.,* 1996). AMH was first detected in male genital ridge tissue at 5.5d and peaked at 7.5d. This does not correspond to expression studies carried out by Eusèbe *et al.* (1996) where the expression levels peaked at 10d. However, in the study reported here, expression in male and female gonads at 10d was not analyzed and in the Eusébe *et al.* (1996) study expression was not analysed prior to 8d. The profile observed here agrees with the ELISA assay analysis (Teng, 1987), which reported highest levels of AMH expression between 6d and 8d. Our study reports highest levels of expression between 7.5d and 9.5d. Slight differences in the developmental days of expression between these two reports might be due to differences in the timing of egg incubation. When analyzed by wmISH, AMH transcripts were detected in the male gonads. This expression mainly corresponded to the location of the testicular cords and confirmed the *in situ* analysis by Eusèbe *et al.* (1996).

In the female gonads, expression was detected from 6.5d to 9.5d, but at much lower levels than in the male gonads (fig 34). Expression in the adult ovary was higher than in any of the embryological stages studied, but lower compared to the adult testis. Eusèbe *et al.* (1996) reported the expression levels of AMH being approximately 14 times higher in the male gonads compared to the female gonads. However, in this study, the expression levels of AMH is 57 times higher in male gonads compared to female gonads at 7.5d.

Low levels of expression were detected by wmISH, in the female gonads at 7.5d. This correlates with the study by Teng (1987), where low AMH expression levels were detected in both left and right ovaries between 6d and 12d. However, work carried out by Eusèbe *et al.* (1996) indicated expression was never detected in

the right ovary and only detected in the left ovary at 17d. Both in this study and in the study carried out by Eusèbe *et al.* (1996) higher AMH expression levels were observed in the left ovary compared to the right ovary. By 7.5d, the right gonad is reported to be regressing and non-functional (Romanoff, 1960). However, results from this study indicate the right gonad is producing AMH at this stage of development and thus is functional. Due to AMH being expressed in the female chick, AMH is therefore not a marker of specifically Sertoli cell differentiation; as suggested for mammalian gonads (Münsterberg and Lovell-Badge, 1991). In males, expression of AMH may be confined to the Sertoli cells but that not all Sertoli cells have organised into testicular cords by 7.5d.

In mammalian male gonads AMH is first detected at 11.5 dpc and peaks at 13 dpc (Hacker *et al.*, 1995; Münsterberg and Lovell-Badge, 1991) (fig 35). Expression at 11.5 dpc coincides with a peak of SRY expression in the male gonads (Hacker *et al.*, 1995). If AMH expression in the chick follows the same pattern as AMH expression in the mammal and there is conservation of gonadal development, then the sex determining gene in the chick would be expected to be expressed before 5.5d. In fact, to follow the same pattern of gonadal development as in the mammal, the sex determining gene in the chick would be expressed in the male gonads between 3.5d and 5.5d. If previous reports on the timing of sex determination are correct (Romanoff, 1960, Venzke, 1954a) then initial expression of AMH at 5.5d would be prior to the postulated sex determination event.

It has previously been reported that female gonadal differentiation precedes male gonadal development in the chick (McCarrey and Abbott, 1979). The initial expression of AMH in the male chick is at 5.5d and in the female at 6.5d. These results disagree with the report by McCarrey and Abbott (1979) and instead suggest that male gonadal differentiation occurs prior to female gonadal development.

Expression of WT1 was detected in male and female genital ridge tissue from 3.5d to 9.5d. A potentially sexually dimorphic pattern of WT1 expression was observed in chick gonads at 4.5d and 5.5d, with slightly higher levels of expression in the male gonads (fig 34). Analysis by wmISH detected expression in male and female gonads and mesonephric tissue immediately adjacent to the gonads, but it was difficult to identify exactly which cell types were expressing WT1. However, wmISH analysis (where expression levels appeared lower in the male) did not correlate with Northern analysis data. Further analysis to quantitate the expression levels of the WT-1 transcript at 4.5d and 5.5d, in different pools of male and female genital ridge RNA, must be carried out to resolve this discrepancy. The expression in the mesonephros observed in this study correlates with the study by Kent *et al.* (1995), which reported

expression of WT1 in the glomeruli of the chick mesonephros at 4.5d to 6.5d. Expression was also observed on the outer edge of the Wolffian duct in males at 5.5d. Kent *et al.* (1995) have previously reported expression of WT1 in the chick at 2.5d, where the urogenital ridge develops.

In the mouse, WT1 was first detected in the intermediate mesoderm at 9 dpc (Armstrong *et al.*, 1995) (fig 35), and in the urogenital ridge at 9.5 dpc; analogous to WT1 expression in the initial stages of urogenital ridge development in the chick. Expression of WT1 was observed in the gonads from the earliest stages of gonadal development through to the adult (Armstrong *et al.*, 1995). While expression in the mesonephros was detected only from 9.5 dpc to 12 dpc, after which point the mesonephros regresses. Expression was located to the glomerular structures in the mouse mesonephros (Armstrong *et al.*, 1995), which correlates with the expression of WT1 in the chick mesonephros (Kent *et al.*, 1995; this study).

The present study indicates that expression of WT1 in the chick has a similar profile to that in the mammal. However, one potential difference we observed between mammals and chicks is the slightly higher levels of expression in the male chick genital ridge tissue at 4.5d and 5.5d. If we assume the sex determination period in the chick is prior to 5.5d, then the higher levels of expression at this stage might suggest an involvement for WT1 in the sex determining mechanism in the chick. Southern analysis of DNA from males and females revealed that WT1 was not located on either of the sex chromosomes in the chick, suggesting that if WT1 has a role in sex determination, it is not a primary role analogous to *SRY*.

Targeted knockout of the mouse *wt1* gene resulted in gonadal and metanephric development arresting at a very early stage (Kreidberg *et al.*, 1993). This suggested WT1 had an important role in the development and maintenance of the urogenital ridge. This theory is supported in the chick as expression of WT1 was detected in both sexes from the earliest stage of urogenital ridge development through to the initial stages of ovary and testis differentiation. WT1 has been proposed to be involved in the mesenchymal cell to epithelial cell transition (Armstrong *et al.*, 1992; Rauscher, 1993). It is possible, therefore, that WT1 is required for the differentiation of the mesenchymal cells into epithelial cells of the future seminiferous cords.

Expression of aromatase was first detected in the female genital ridge at 6.5d, and peaked at 8.5d. No expression was detected by Northern analysis in the male genital ridge from 3.5d to 9.5d (fig 34), agreeing with previous studies (Smith *et al.*, 1997; Yoshida *et al.*, 1996). This suggested that male gonads could not synthesise oestrogens as they do not express aromatase. Detection of aromatase in both the left and the right female gonad at 9.5d and 7.5d was reported by Smith *et al.* (1996) and

Andrews *et al.* (1997) respectively, and indicates oestrogens are produced in both female gonads even though the right gonad has begun to regress by this stage.

Analysis of aromatase in reptiles (with a temperature dependent sex determination mechanism) has shown expression in the female gonads, female brain and the male brain (Jeyasuria and Place, 1997), from which a brain-gonadal axis was proposed to be involved in sex determination. Aromatase expression was not detected in other chick embryonic tissues, suggesting a brain-gonadal axis was not involved in chick sex determination. The detection of aromatase transcripts in embryonic and adult ovary but not in embryonic or adult testis indicates oestrogens are required for female but not male sexual development.

Expression of aromatase in the female genital ridge at 6.5d coincides with oestradiol production which is first detected by radioimmunoassay at 6.5d in female chick embryos (McBride *et al.,* unpublished observations). This conflicts with earlier studies carried out by Woods and Erton (1978), in which oestrogens were initially detected at 3.5d in the indifferent gonads, using an immunofluoresence procedure. However, three different studies using three different procedures have not detected aromatase expression prior to 6.5d (this study; Yoshida *et al.,* 1996; Smith *et al.,* 1997). As aromatase is the rate limiting step for oestrogen production, this suggests the study carried out by Woods and Erton (1978) was not sufficiently accurate and a more sensitive method (radioimmunoassay) has not detected oestrogens before 6.5d in female embryos (McBride *et al.,* unpublished observations).

Oestrogen receptor expression in the chick has also been studied (Smith *et al.,* 1996; Andrews *et al.,* 1997). Differences in oestrogen receptor expression or oestrogen concentration between males and females was considered to be a mechanism by which chick sex determination could be regulated (Andrews *et al.,* 1997; Woods and Erton, 1978). However, the initial detection of aromatase and oestrogens in female gonads at 6.5d suggests oestrogen production is a consequence of chick sex determination. Nevertheless, the reported differences in aromatase and oestrogen receptor expression between male and female do implicate oestrogens in an important role in chick female gonadal development.

Expression of SF-1 was detected in male and female genital ridge from 4.5d to 9.5d, and exhibited a fairly constant pattern of expression (fig 34). This did not correlate with phosphorimager analysis, which showed down-regulation in female samples and up-regulation in male samples. This may be due to inconsistencies in loading, the difficulties in relating expression of the probe of interest to that of 18S, or the mechanics of quantitating signal intensity on a background that is not uniform. Expression levels observed by wmISH, where SF-1 levels appear to be down-

regulated by 7.5d in the gonads of both sexes, were also inconsistent with Northern analysis data. Also, Northern analysis data and wmISH data obtained on the chick does not correspond with data reported for the mouse after the sex determination period (Parker and Schimmer, 1997). However, by wmISH, SF-1 expression was detected in the urogenital ridge in 3.5d male and female embryos, corresponding with early SF-1 expression in the urogenital ridge in the mouse (Ikeda *et al.*, 1994).

Expression of SF-1 was observed in the mesonephros with a different pattern in males and females. A closer analysis of the expression pattern in the mesonephros indicated SF-1 expression was detected in the inner surface of the cells lining the collecting tubules. However, not all tubules expressed this transcript, and thus could be an artifact due to trapping of the transcript or antibody in the tissue.

One role for SF-1 in the developing gonads is thought to involve the regulation of steroid biosynthesis (Parker and Schimmer, 1997). It is also proposed that it positively regulates AMH (Shen *et al.*, 1994). As AMH is expressed in the male and female chick, it would be expected that SF-1 would be detected in the gonads of both sexes. Relatively high levels of SF-1 expression in male and female chick gonads at 6.5d supports this hypothesis. However, there does not appear to be a direct correlation between SF-1 expression and AMH expression at other developmental days. It is likely that other factors are involved in the activation of AMH. One possible factor is the unknown sex determining switch in chicks, as *SRY* has been proposed as a potential regulator of SF-1 in mammals (Parker and Schimmer, 1997).

Another potential role for SF-1 is the up-regulation of aromatase (Lynch *et al.*, 1993). In female chicks, estrogens are required for female development, therefore, SF-1 and aromatase would be expressed in the gonads during chick embryological development. In this study, expression of SF-1 was observed in the female chick gonads, but expression levels remained the same in contrast with aromatase expression, which increased rapidly from 6.5d to 8.5d. Thus it is unlikely that just SF-1 positively regulates aromatase expression in the female chick and that additional factors are involved.

Finally, another putative target or regulator of SF-1 is WT1 (Parker and Schimmer, 1997). Both of these genes are expressed in the chick and mouse urogenital ridge prior to sex determination (Kent *et al.*, 1995; this study). If *wt1* or SF-1 knockout mice are generated, both have arrested development of or lack of gonads (Kreidberg *et al.*, 1993; Luo *et al.*, 1994; Luo *et al.*, 1995). Both WT1 and SF-1 are thought to have an important role in the development of the urogenital ridge after it has arisen, and might interact with one another. However, WT1 knockout mice expressed normal levels of SF-1 and vice versa (Parker and Schimmer, 1997). The

expression of these two genes did not appear to colocalise in the chick mesonephros, and thus are unlikely to regulate one another.

The expression of *Sox-9* exhibited a relatively constant level in the female and male genital ridge from 4.5d to 9.5d (fig 34). The constant levels of expression of *Sox-9* in the chick male disagrees with studies carried out by da Silva *et al.* (1996) and Kent *et al.* (1996). The higher level of expression in the chick adult testis compared to the adult ovary, and the detection only in the testis of a 1.36kb transcript suggested *Sox-9* had an important role in gonadal development in the adult, in addition to a role in male and female chick gonadal development.

The expression profile of *Sox-9* in the chick does not correspond to the expression profile observed in the mouse gonads, where expression is initially detected in the urogenital ridges of both males and females. Expression levels increase in the mouse male gonads but decrease to undetectable levels in the mouse female gonads (da Silva *et al.,* 1996; Kent *et al.,* 1996) (fig 35).

Sox-9 is reported to be highly conserved between mammals and birds, indicating a conserved function. Studies on the structure of the protein indicated it might function as a transcription factor (Foster, 1996; Kent *et al.,* 1996; Goodfellow and Lovell-Badge, 1993). Two of its proposed targets are SF-1 and AMH, due to the up-regulation of these two genes during male gonadal development in the mammal (Kent *et al.,* 1996). The expression profiles of SF-1 AMH and *Sox-9* in the male chick would support this theory. *Sox-9* is proposed to enforce the male sex determining pathway, by activating genes in this pathway. Alternatively, *Sox-9* could act to inhibit genes in the female determining pathway, thus leading to male gonadal development. However, in the chick, our results suggest the *Sox-9* must also have a role in female gonadal development. Therefore, although the expression profile of *Sox-9* has been well characterised in mammals, the actual function of this gene during gonadal development is unknown.

Overall AMH, SF-1, aromatase, WT1 and *Sox-9* are all expressed during chick gonadal development and that AMH, WT-1 and aromatase exhibit similar expression profiles compared to mammals; indicating a conserved gonadal differentiation pathway between these classes (fig 34 & 35). Although SF-1 and *Sox-9* in the chick, do not exactly follow the expression profile observed in the mammal, both are initially expressed at similar stages of gonadal development in both classes. If the regulation pathway and function of these genes are conserved, then the timing of expression of these genes indicates the sex determining switch in chicks occurs earlier in development than previously reported.

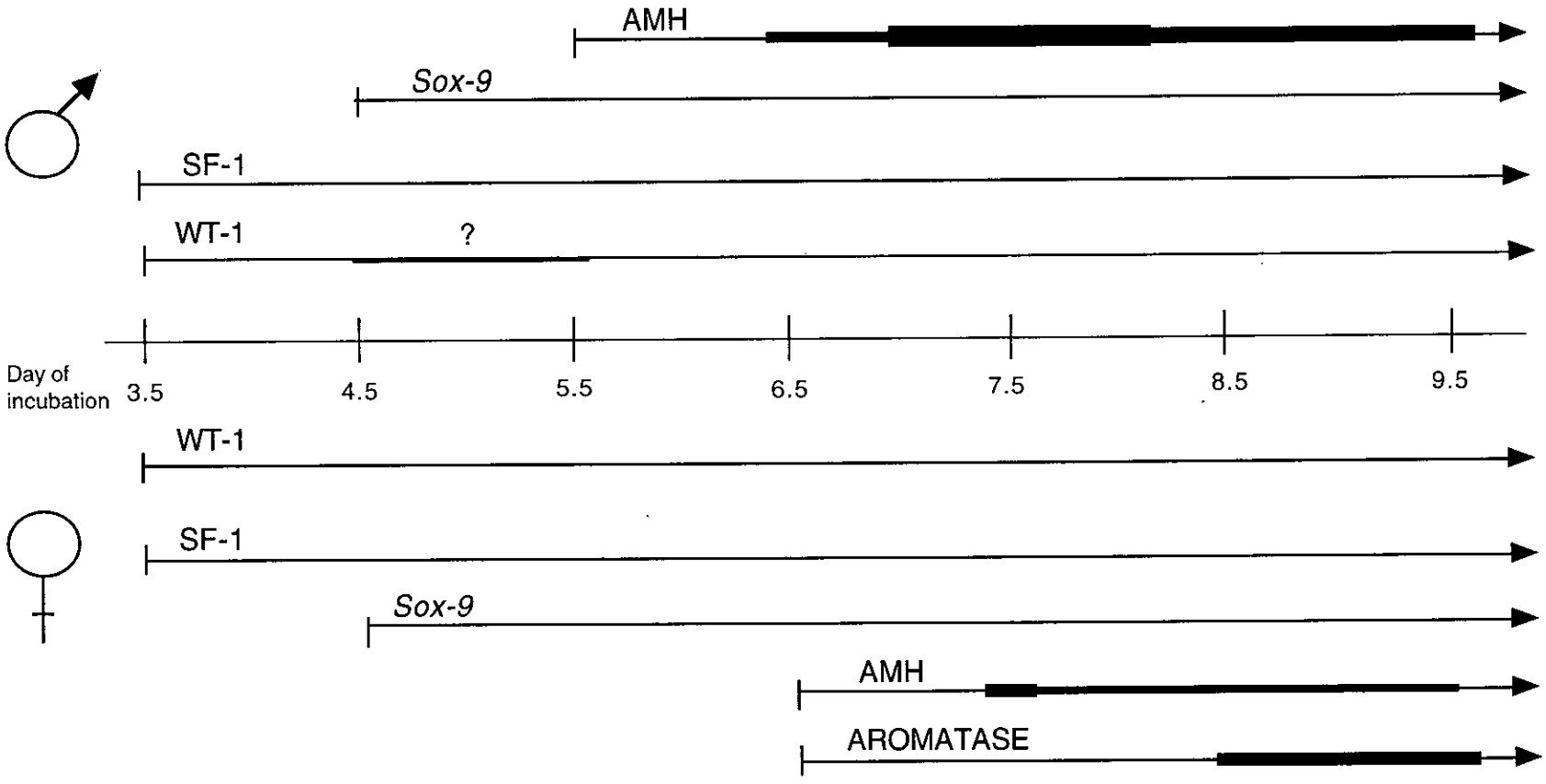


Figure 34: Schematic representation of gene expression during the early stages of chick gonadal development, based on the expression profiles observed by Northern analysis. Thicker black lines represent differences in increases in expression levels. ? represents a potential up-regulation of the transcript.

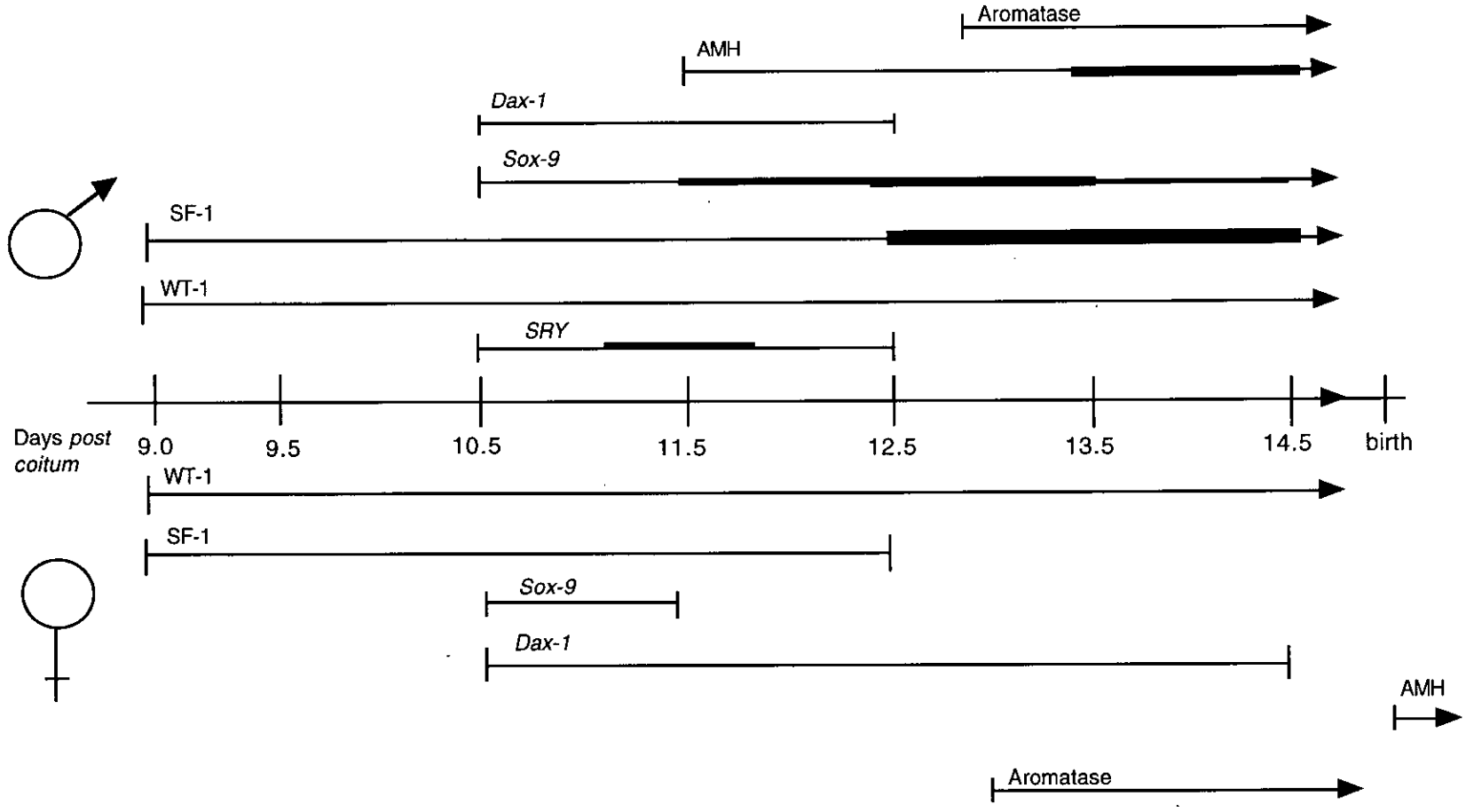


Figure 35: Schematic representation of gene expression during the early stages of mammalian (mouse) gonadal development. Thicker black lines represent increases in expression levels.

The earliest detectable expression of both SF-1 and WT1 is prior to the sex determination event in mammals (fig 35), and here detection of SF-1 and WT1 expression was prior to detectable morphological changes in the chick gonads (5.5d). In the mouse, *Sox-9* is expressed from 10.5 dpc, prior to sex determination, and here *Sox-9* expression was first detected at 4.5d, prior to morphological differences.

In the mouse, AMH was first detected in the male gonads at 11.5 dpc, at the peak of *Sry* expression (Hacker *et al.*, 1995). This expression was a consequence of the sex determining switch resulting in the gonads developing along the male pathway. If it is assumed that AMH in the chick is also expressed as a consequence of a sex determining gene, then initial expression of AMH in the male chick gonads at 5.5d suggested that sex determination occurred prior to this developmental stage in the chick. Finally, aromatase expression in the female chick gonads was detected approximately one day after morphological changes were first apparent between male and female chick gonads and one day after AMH expression in the male. This corresponds with the detection of significant levels of oestrogen in the female chick gonads from 6.5d (McBride *et al.*, unpublished observations). In the mouse low levels of aromatase expression are detectable from 13 dpc in both the ovary and the testis (Greco and Payne, 1994). This corresponds to approximately 1.5 days after AMH is first expressed in the testis and approximately 1 day after morphological changes are first observed in the mouse gonads. Therefore, the results of this study indicate sex determination in the chick occurs prior to 5.5d. This theory is supported by slight morphological differences being observed, at the histological level, from 5.5d.

Chapter 8.

Results.

Identification of novel transcripts by differential display.

8.1. Identification of novel transcripts by differential display.

Differential display analysis was performed on pooled RNA from male genital ridge tissue and pooled female genital ridge tissue. Embryos from 3.5d to 9.5d (stage 21 to 36) were used in the analysis. PCR products generated from the display analysis were separated on a native 6% acrylamide gel and visualised by autoradiography (fig 36, 37, 38, 39 & 40).

A total of 24 primer combinations were carried out. It was calculated that each primer combination displayed approximately 80 bands representing different transcripts. Therefore, the total primer combinations used in this study resulted in approximately 1920 different transcripts being examined. This represents only 9.6% to 19.2% of the postulated 10 000 to 20 000 different transcripts normally observed in a chick cell (Alberts *et al.,* 1994). While a proportion of the primer combinations produced identical banding patterns in the RNA samples tested, the majority of primer combinations did exhibit differences in banding patterns.

One of the aims of this study was to assess the application of differential display for isolating differentially expressed genes in complex developmental systems. Therefore, the expression profiles isolated in this study did not just focus on differences in gene expression between males and females. Expression differences that exhibited changes over a developmental time period in both males and females were also isolated.

A total of twelve different bands were excised from the display gel and the DNA eluted for further analysis. DNA was re-amplified in a PCR reaction utilising the same primer combination as in the original display reaction (fig 41). In some cases, more than the expected one band was re-amplified, suggesting either contamination or more than one sequence was present in the DNA isolated from the display band. Each PCR product was sub-cloned and a minimum of six sequences were obtained for each band of DNA isolated. Sequences were analyzed using a multiple sequence alignment programme from the GCG package and consensus sequences are presented in appendix 4. The consensus sequence for each clone was compared to sequences in nucleotide and protein databases using BLAST and FASTA analysis packages (Altschul *et al.,* 1991; Genetics Computer Group, 1994). Information concerning the clones isolated and their expression profiles on the display gels is summarised in table 2.

Clone 771 had 96% homology to human RhoA at the amino acid level (Yeramian *et al.,* 1987) and exhibited stage-specific expression (fig 36). RhoA is a GTP-binding protein and a member of the Ras superfamily (Vojtek and Cooper,

Figure 36: Time course differential display analysis comparing a subset of male and female genital ridge/mesonephric transcripts from days 3.5, 4.5, 5.5, 7.5 and 8.5 of incubation. Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue and 5µg used to prepare cDNA by reverse transcription. A fraction (0.4%) of the cDNA was used as template in each display reaction. Differential display products were separated on a 6% native acrylamide gel. MG = oligo dT_{12}MG primer used in the reverse transcription and display analysis. R5, R12 and Sox = the random 10mers used in the display analysis. Bands excised from the display gel are indicated by a clone number at the side of the figure.

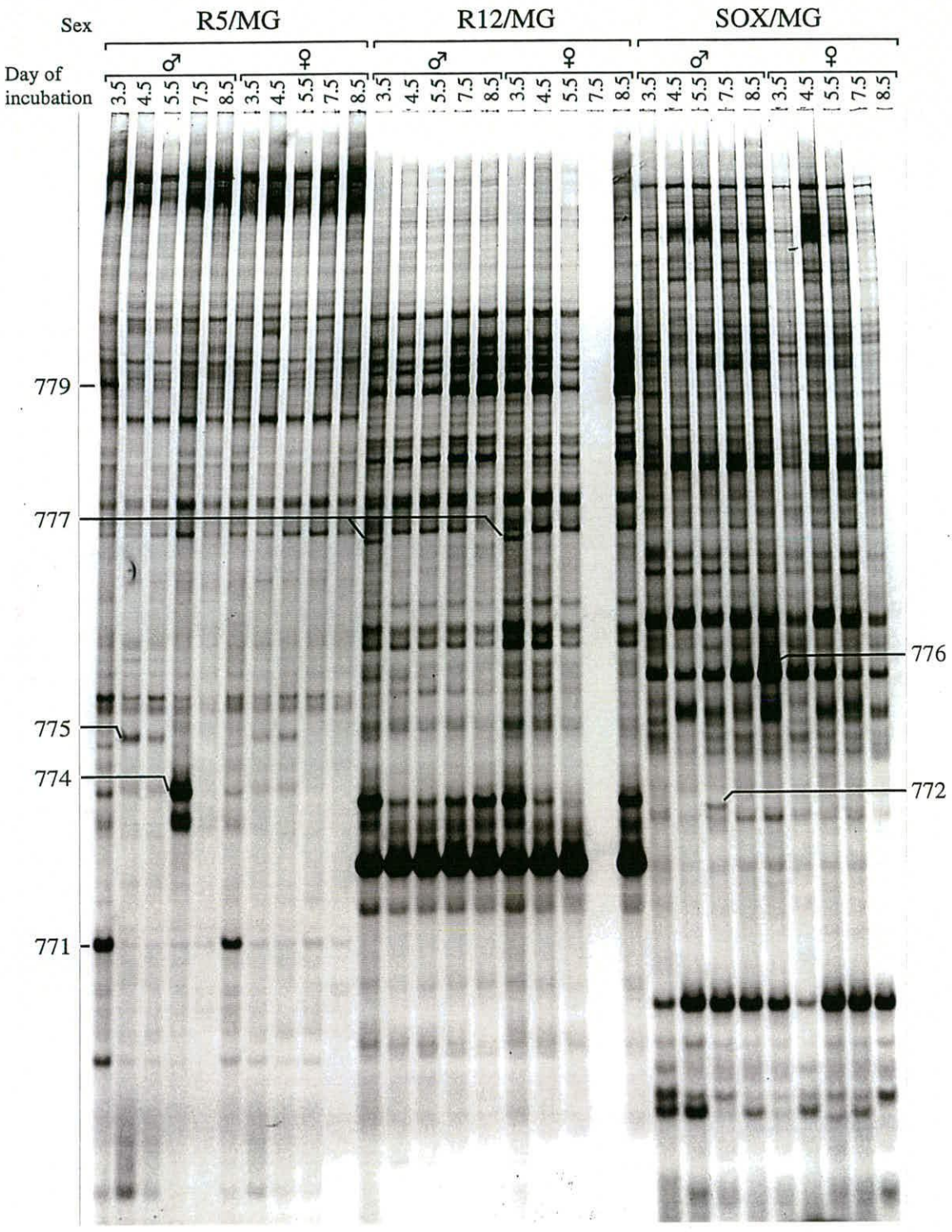

Sex
R5/MG
R12/MG
SOX/MG
♂
♀
Day of incubation
3.5
4.5
5.5
7.5
8.5
779
777
776
775
774
772
771

Figure 37: Time course differential display analysis comparing a subset of male and female genital ridge/mesonephric transcripts from day 5.5 to day 9.5 of incubation. Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue and 5µg used to prepare cDNA by reverse transcription. A fraction (0.4%) of the cDNA was used as a template in each display reaction. Differential display products were separated on a 6% native acrylamide gel and visualized by autoradiography. MC = oligo dT_{12}MC primer used in the reverse transcription and display reaction. Sox, R5 and R11 = random 10mers used in the display analysis. Bands excised from the display gel are indicated by clone numbers at the side of the figure.

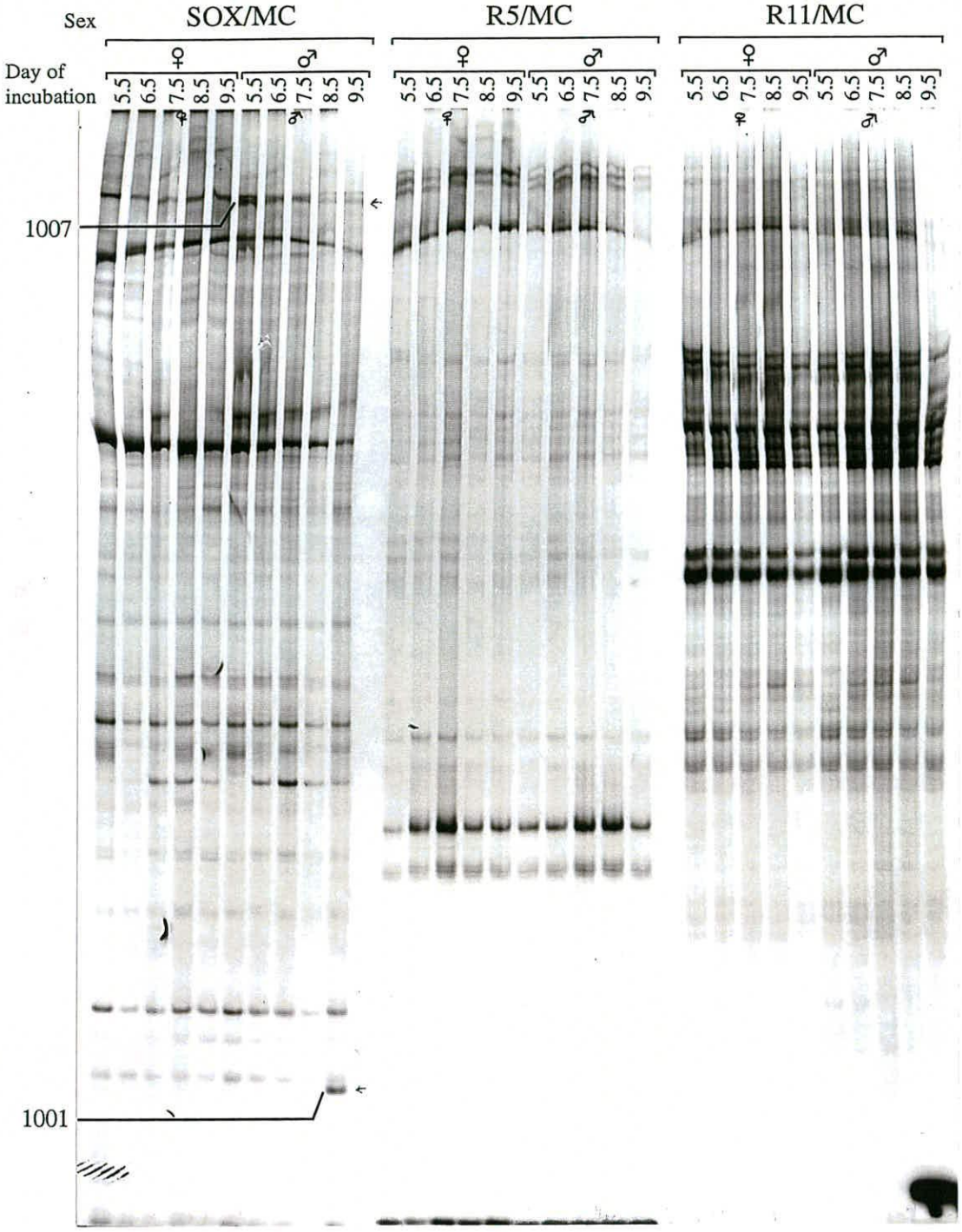

Sex
SOX/MC
R5/MC
R11/MC
♀
♂
Day of incubation
5.5
6.5
7.5
8.5
9.5
1007
1001

Figure 38: Time course differential display analysis comparing a subset of male and female genital ridge/mesonephric transcripts from day 5.5 to day 9.5 of incubation. Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue and 5µg used to prepare cDNA by reverse transcription. A fraction (0.4%) of the cDNA was used in each display reaction. Differential display products were separated on a 6% native acrylamide gel and visualized by autoradiography. MG = oligo dT_{12}MG primer used in the reverse transcription reaction and display analysis. R8, R14 and R19 = random 10mers used in the display analysis. Bands excised from the display gel are indicated by clone numbers at the side of the figure.

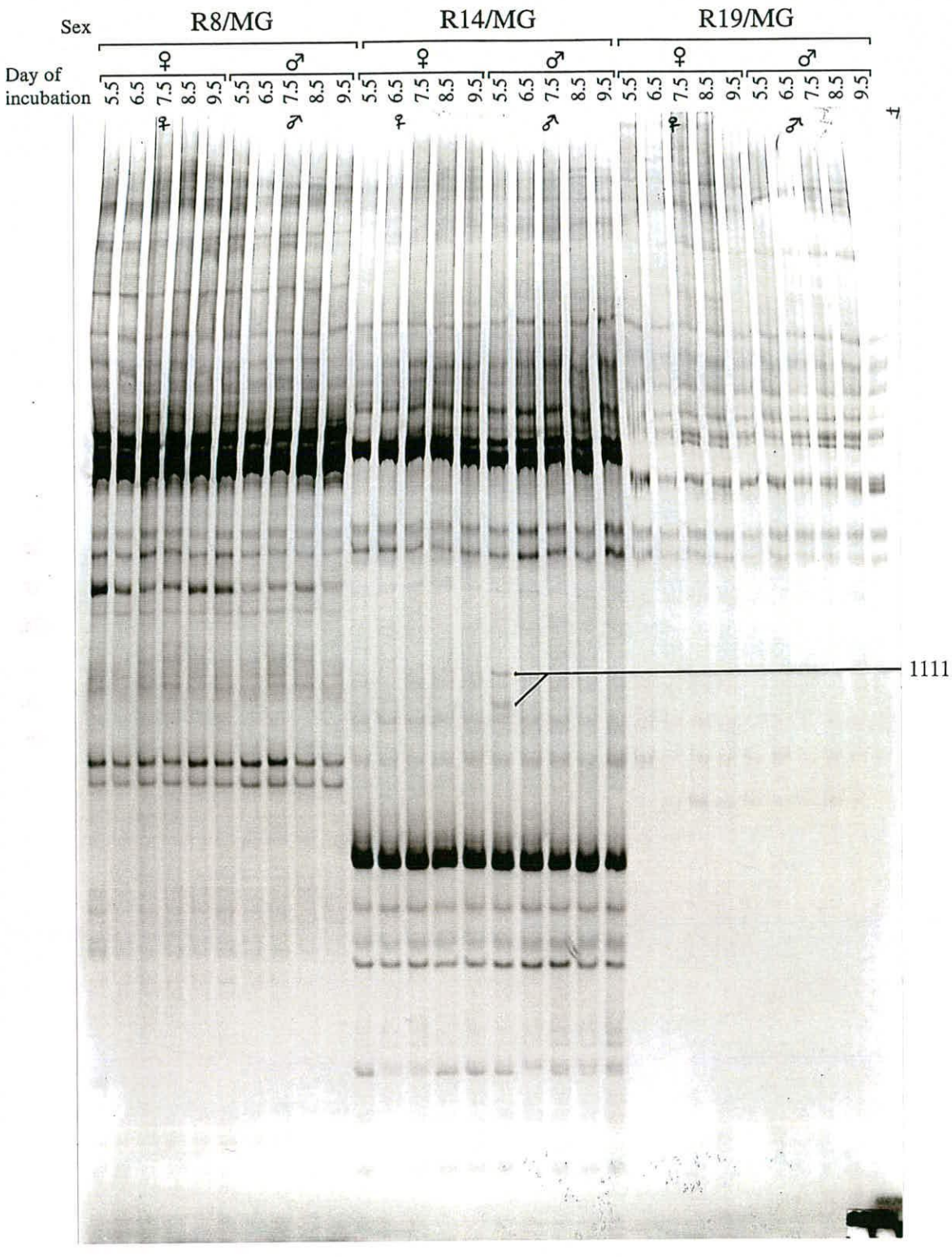

Sex
R8/MG
R14/MG
R19/MG
♀
♂
Day of incubation
5.5
6.5
7.5
8.5
9.5
1111

Figure 39: Time course differential display analysis comparing a subset of male and female genital ridge/mesonephric transcripts from day 5.5 to day 9.5 of incubation. Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue and 5μg used to prepare cDNA by reverse transcription. A fraction (0.4%) of the cDNA was used in each display reaction. Differential display products were separated on a 6% native acrylamide gel and visualized by autoradiography. MG = oligo dT_{12}MG primer used in the reverse transcription reaction and display analysis. R8, R14, R19 = random 10mers used in the display analysis. Bands excised from the display gel are identified by clone numbers at the side of the figure.

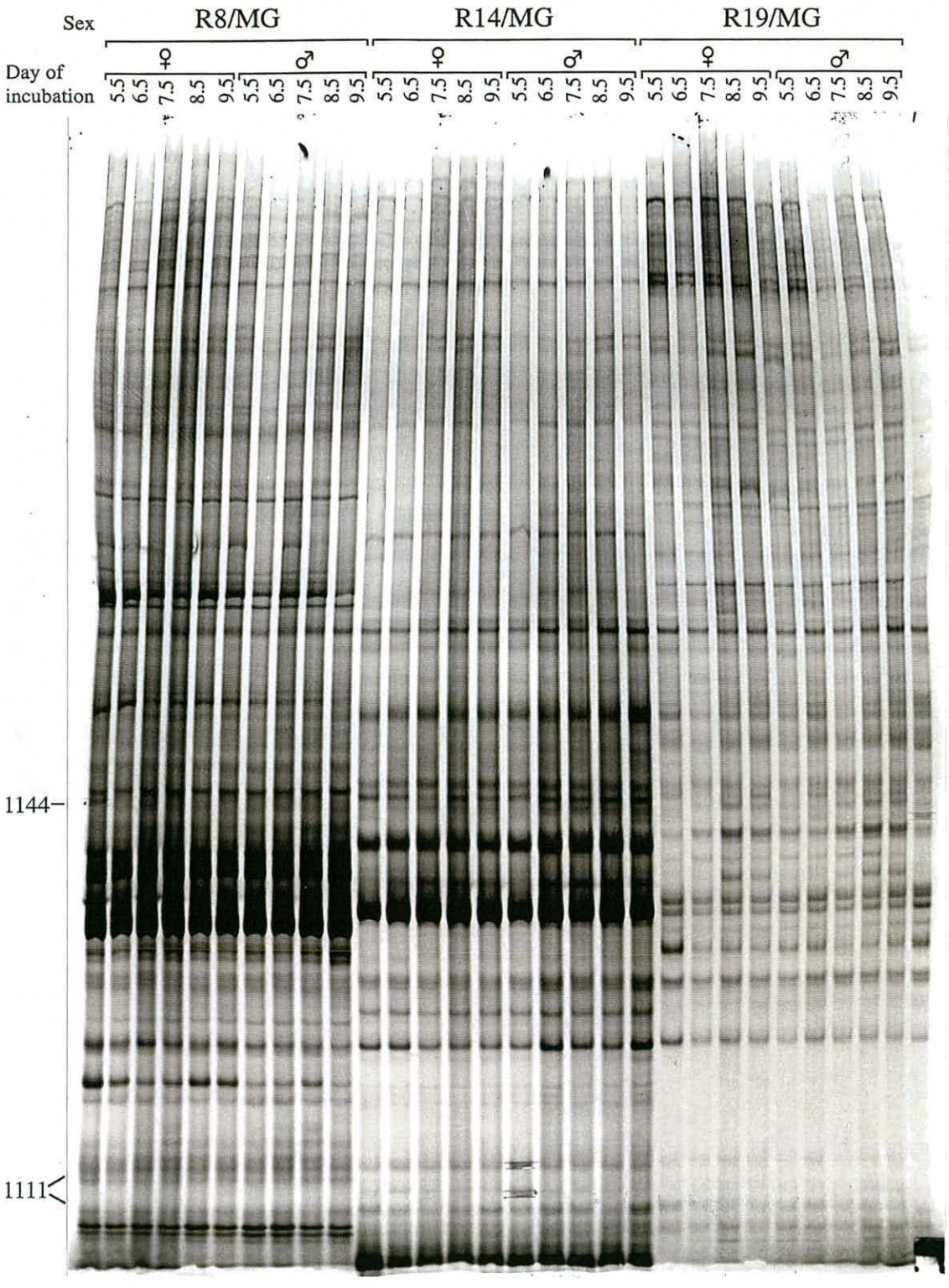

Sex
R8/MG
R14/MG
R19/MG
♀
♂
Day of incubation
5.5
6.5
7.5
8.5
9.5
1144
1111

Figure 40: Time course differential display analysis comparing a subset of male and female genital ridge/mesonephric transcripts from day 5.5 to day 9.5 of incubation. Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue and 5µg was used to prepare cDNA by reverse transcription. A fraction (0.4%) of cDNA was used in each display reaction. Differential display products were separated on a 6% native acrylamide gel and visualized by autoradiography. MC = oligo $dT_{12}MC$ primer used in the reverse transcription and display analysis. R2, R13 and R16 = random 10mers primers used in the display analysis. The band excised from the display gel was identified by the clone number at the side of the figure.

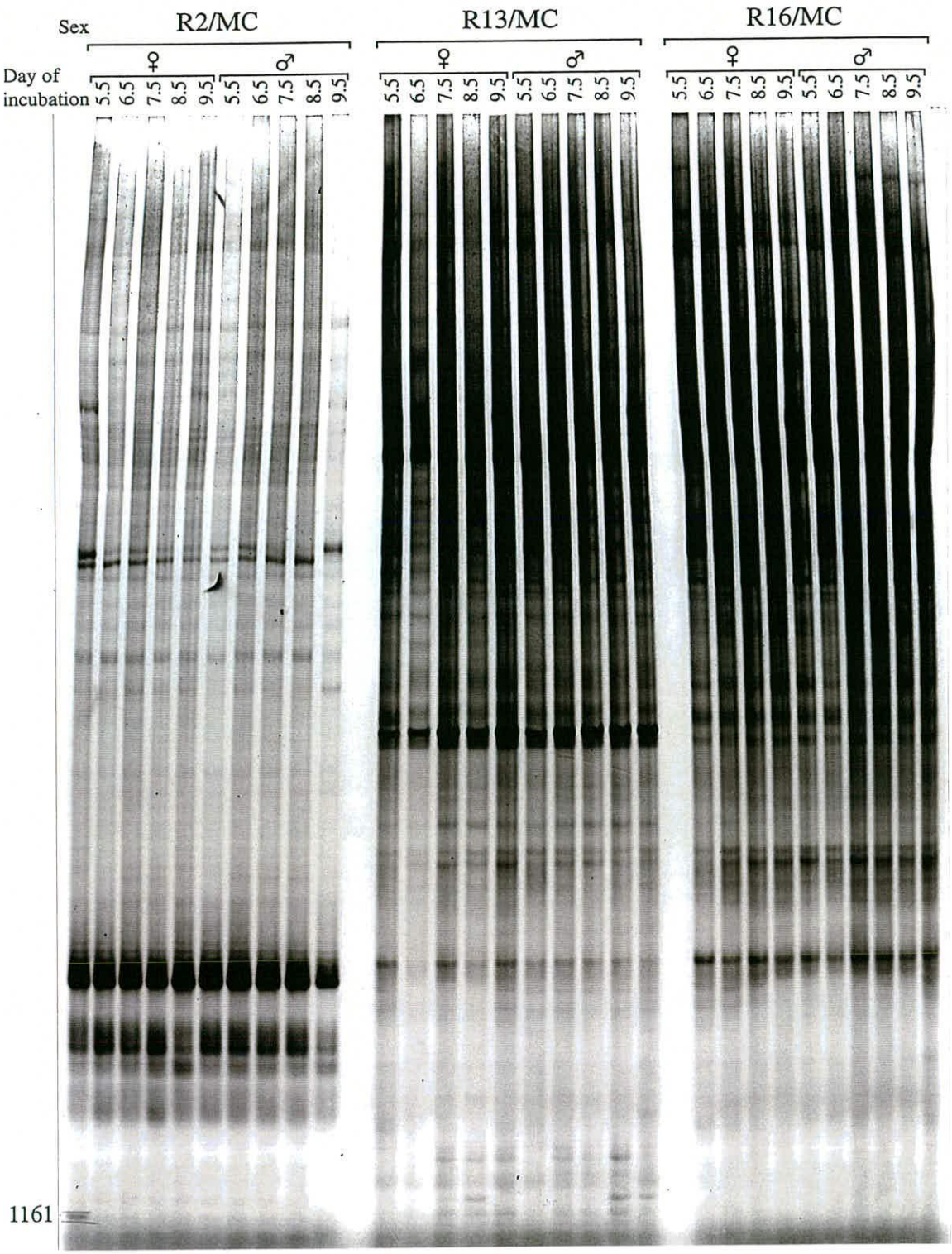
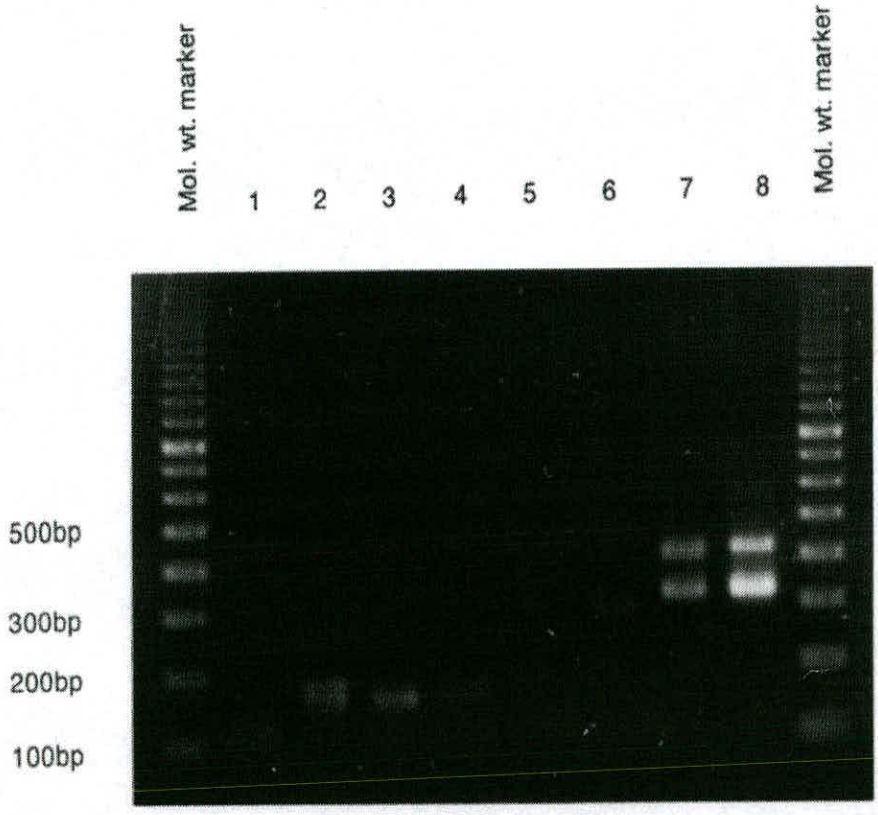

Sex
R2/MC
R13/MC
R16/MC
♀
♂
Day of incubation
5.5
6.5
7.5
8.5
9.5
1161

Figure 41: An example of PCR products of a reamplification reaction, carried out on DNA recovered from bands excised from the differential display gel. The majority of reamplification products exhibited one band, but in some reactions more than one band was observed. Sample numbers 1 to 8 indicates reamplified DNA from different bands excised from the display gel.


Mol. wt. marker
1
2
3
4
5
6
7
8
Mol. wt. marker
500bp
300bp
200bp
100bp

Clone	Size	Identity	Display pattern
771	106bp	96% to human RhoA at the amino acid level	Expression detected in all days with highest levels of expression in 3.5d males and females
772	132bp	no homology to sequences in the databases	Expression only detected in day 5.5 males
774	140bp	99% to chick mitochondrial genome at the nucleotide level	Higher expression in 7.5d males, but present in other samples of both sexes
775	156bp	100% to chick rho-globin	Expression detected in both sexes but highest expression levels in 4.5d males
776	160bp	no homology to sequences in the databases	Expression detected in all samples with highest expression levels in 8.5d males
777	216bp	no homology to sequences in the databases	Expressed only in 3.5d male and female samples
779	299bp	no homology to sequences in the databases	Expression detected only in 3.5d males
1001	174bp	97% to chick mitochondrial genome at the nucleotide level	Expression detected in all samples with higher expression levels in 9.5d males
1007	289bp	73% to human mef-2A at the nucleotide level	Highest expression levels in day 5.5 males. No expression detected in females
1111	176bp	92% to rat TBP-1 and rat Sata at the nucleotide level	Expression detected in all samples with highest expression levels in 5.5d males
1144	252bp	84% to rat TCP-1 at the nucleotide level	Highest expression levels detected in 5.5d female samples
1161	71bp	no homology to sequences in the databases	Expression detected in all days with highest expression levels in 5.5d females

Table 2: A summary of the sequence and expression profile data obtained on the differential display candidates.

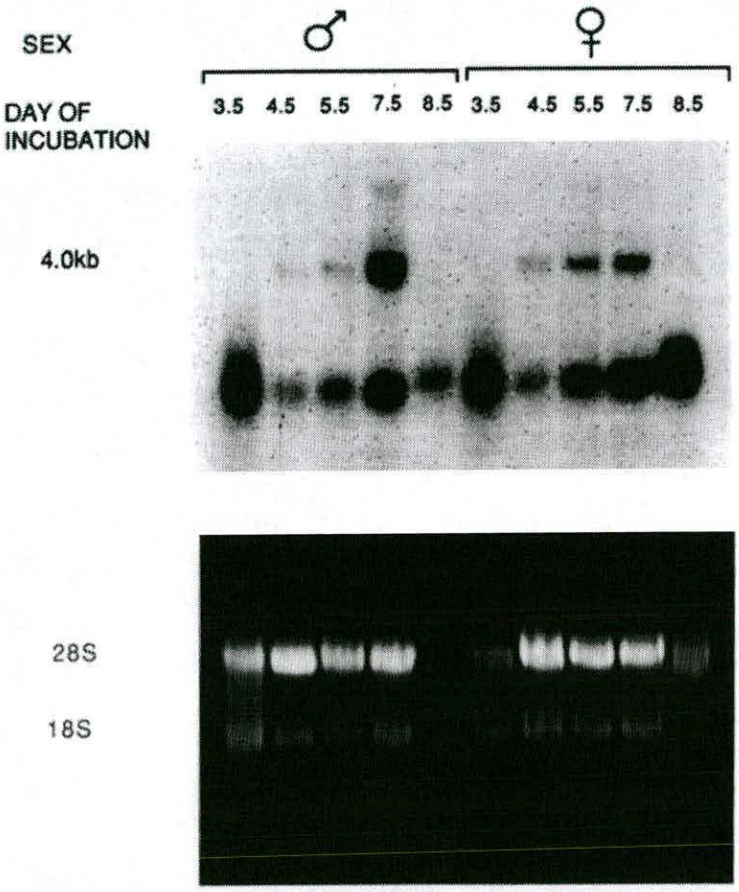
1995). Clone 772 had a sex-specific expression profile on the display gel, with expression only being detected in male genital ridge samples at 5.5d. Clone 774 exhibited sexually dimorphic expression with higher levels in the male sample at 7.5d (fig 36). This clone had 99% homology at the nucleotide level to a portion of the chick mitochondrial genome, covering tRNAs; alanine, asparagine and cysteine (Desjardins and Morais, 1990). Clone 775 gave 100% homology at the nucleotide level to the chick rho-globin (ρ–globin) gene (Roninson and Ingram, 1981). The expression pattern on the display gel showed a transcript with highest levels of expression in males and females at 4.5d and 5.5d (fig 36). Clones 776, 777 and 779 exhibited no sequence homology to any of the sequences present in the nucleotide and protein databases. Clone 776 showed highest levels of expression at 8.5d in the male genital ridge sample (fig 36). Clone 777 exhibited a stage-specific profile, and clone 779 a sex-specific profile (fig 36). Clone 1001 gave 97% homology at the nucleotide level to the chick 16S rRNA gene (Morais, 1990), and showed highest expression levels in male samples at 9.5d. Clone 1007 had 73% homology at the nucleotide level to human *mef2A* (Suzuki *et al.*, 1996), and exhibited a sex-specific profile (fig 37). Clone 1111 exhibited higher expression levels in male samples at 5.5d (fig 38). This clone gave 92% homology at the nucleotide level to rat TBP-1 (tat binding protein 1) (Makino, 1996) and 92% homology to rat spermatogenic cell/sperm-associated Tat binding protein homologue (Sata) (Rivkin *et al.*, 1996). Clone 1144 exhibited higher expression levels in female samples at 5.5d (fig 39). Clone 1144 gave 84% homology at the nucleotide level to rat *Tcp-1* (t complex polypeptide 1) (Morita *et al.*, 1991), a subunit of a chaperone that assists in protein folding (Kubota *et al.*, 1995). Clone 1161 exhibited highest expression levels in female samples at 5.5d (fig 40), but gave no homology to sequences present in the databases.

8.2. Isolation and characterisation of a clone exhibiting differential expression between males and females.

8.2.1. Clone 774.

In figure 42, the ethidium bromide gel exhibited relatively equal loading of RNA at day 4.5d, 5.5d and 7.5d, for male and female samples. However, the amount of RNA at 3.5d was not equal between males and females or other timepoints and so 3.5d RNA samples were disregarded in this analysis. Two transcripts, approximately 3.8kb and 1.1kb, were detected. The larger transcript exhibited the same expression profile as observed on the display gel, with higher levels of expression in male samples at 7.5d (stage 32 and 33) (fig 42).

Figure 42: Northern analysis of display clone 774 expression in the gonads of developing male and female chick embryos (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at days 3.5, 4.5, 5.5, 7.5 and 8.5 of incubation (stage 21 to 35). 5µg of each sample was electrophoresed on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 60°C in 0.25M Na PO_4, 7% SDS. Northern blots were washed to a final stringency of 25mM Na PO_4, 1% SDS at 65°C and exposed to film for 2 days at -70°C. The presence of approximately equal amounts of RNA in each lane was monitored by staining the gel with ethidium bromide and visualizing under U.V. light (bottom panel). A single observation by Northern analysis was made.


SEX
♂
♀
DAY OF INCUBATION
3.5 4.5 5.5 7.5 8.5 3.5 4.5 5.5 7.5 8.5
4.0kb
28S
18S

Clone 774 was also used to probe a Northern tissue blot (data not shown) and revealed a number of hybridising transcripts, which indicated 774 was not gonadal specific. A transcript of approximately 2.5kb was detected in various embryonic tissue samples but not in the adult ovary or testis. Another transcript, of approximately 1.1kb, showed strong expression in adult testis with weak expression in the ovary. Two other transcripts, of approximately 0.48kb and 7.6kb were also detected (data not shown).

An anti-sense 774 of 244bp and a sense probe of 274bp were used to perform wmISH. Expression was studied at 7.5d in male and female chick genital ridge and mesonephros tissue. Expression of 774 was observed in the male gonads at this stage of development (fig 43a), although expression was not observed at the anterior and posterior tips. There was also patchy expression in the medulla region of the mesonephros. In the female, expression was observed in the medulla of the mesonephros (fig 43b) . Expression was not obvious in the gonads, which appeared as transparent tubes overlying expression in the mesonephros. The sense probe exhibited no colour development over the same time period.

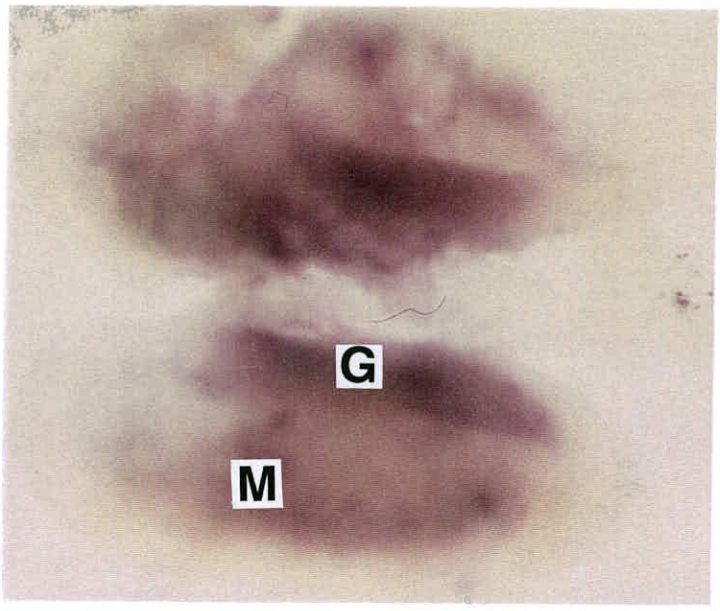
8.2.2. Library screen data.

The clone chosen for further study was 774. Although this clone had 99% homology to the chick mitochondrial genome, there was clearly a higher level of expression in male genital ridge samples at 7.5d compared with female samples at the same stage. In addition, the transcript size of clone 774, observed by Northern analysis, did not correspond to the expected sizes of the mitochondrial genes which it matched.

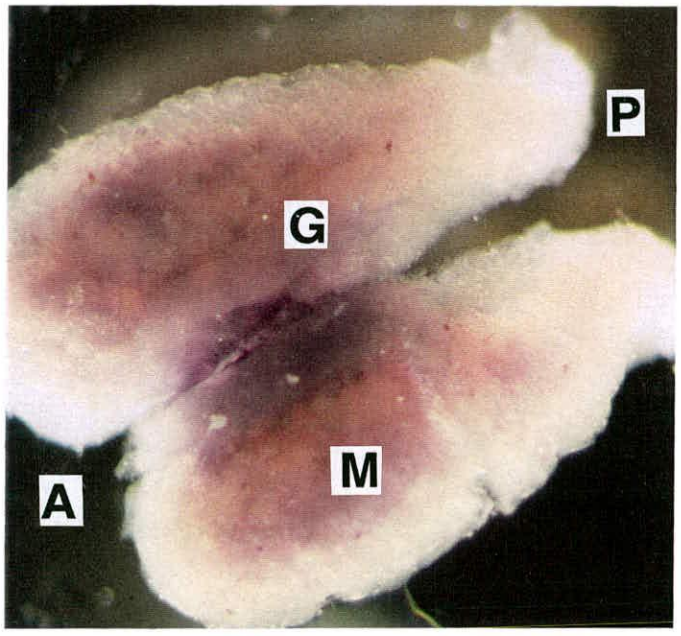
The entire genomic library was represented by plating out approximately 50 000 p.f.u/plate on 20 large NZY plates, so that a total of $1x10^6$ plagues were screened. The primary screen resulted in 118 positives being identified and cored. Of these, 20 were chosen at random and plated out for a secondary screen, at approximately 400 p.f.u./plate for each plaque plug. From these 20 plates, 138 positives were identified. At least one positive from each plate was chosen, so that a total of 21 positives were cored and the DNA insert and phagemid vector excised from the lambda vector. A tertiary screen was not required as the plating frequency of plaque forming units, allowed a single, well isolated plaque to be cored for each positive identified. In order to confirm which positive plaques picked contained the correct insert; each clone was studied by Southern analysis (fig 44). Of the 21 positives picked, 13 were positive for the display clone 774 by Southern analysis.

Figure 43: Expression of display clone 774 in the embryonic chick gonads ar mesonephric tissue at day 7.5 of incubation (stage 32 and 33) b wmISH. Hybridization was performed using a sense and anti-sens DIG labelled 774 riboprobe at 66°C. Colour development proceeded fo 3 days. (a) Male gonads and mesonephros. Actual length of th mesonephros is 3.3mm. (b) Female gonads and mesonephros. Actu length of the mesonephros is 3mm. (c) Female gonads hybridized wi the sense probe. Actual length of the mesonephros is 4.3mm. Anteri (A), posterior (P), left gonad (LG), right gonad (RG), mesonephro (M). Analysis by wmISH was carried out twice with consistent results

(a)



(b)



(c)

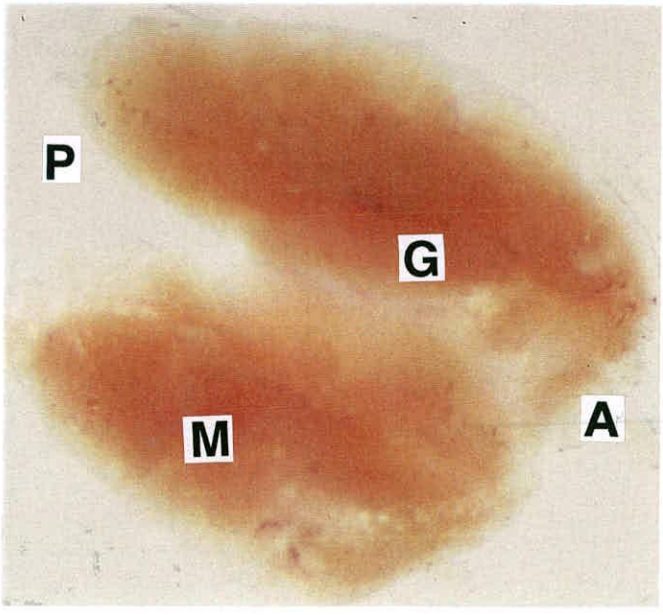


Figure 44: Southern analysis of clones isolated from a genomic library screen, using clone 774 as a probe. The top panel indicates the ethidium bromide gel of each clone, digested with EcoRI and separated on a 1% agarose gel. The bottom panel indicates clones which contain a homologous sequence to the 774 probe, and are identified by the binding of radioactivity to the relevant band on the Southern membrane. Southern hybridization was carried out at 42°C in 2x PIPES, 50% formamide, 0.5% SDS. The blot was washed to a final stringency of 0.2xSSC, 0.1% SDS at 68°C and was exposed to film for four hours. No. 1 to 21 indicates different clones analyzed.

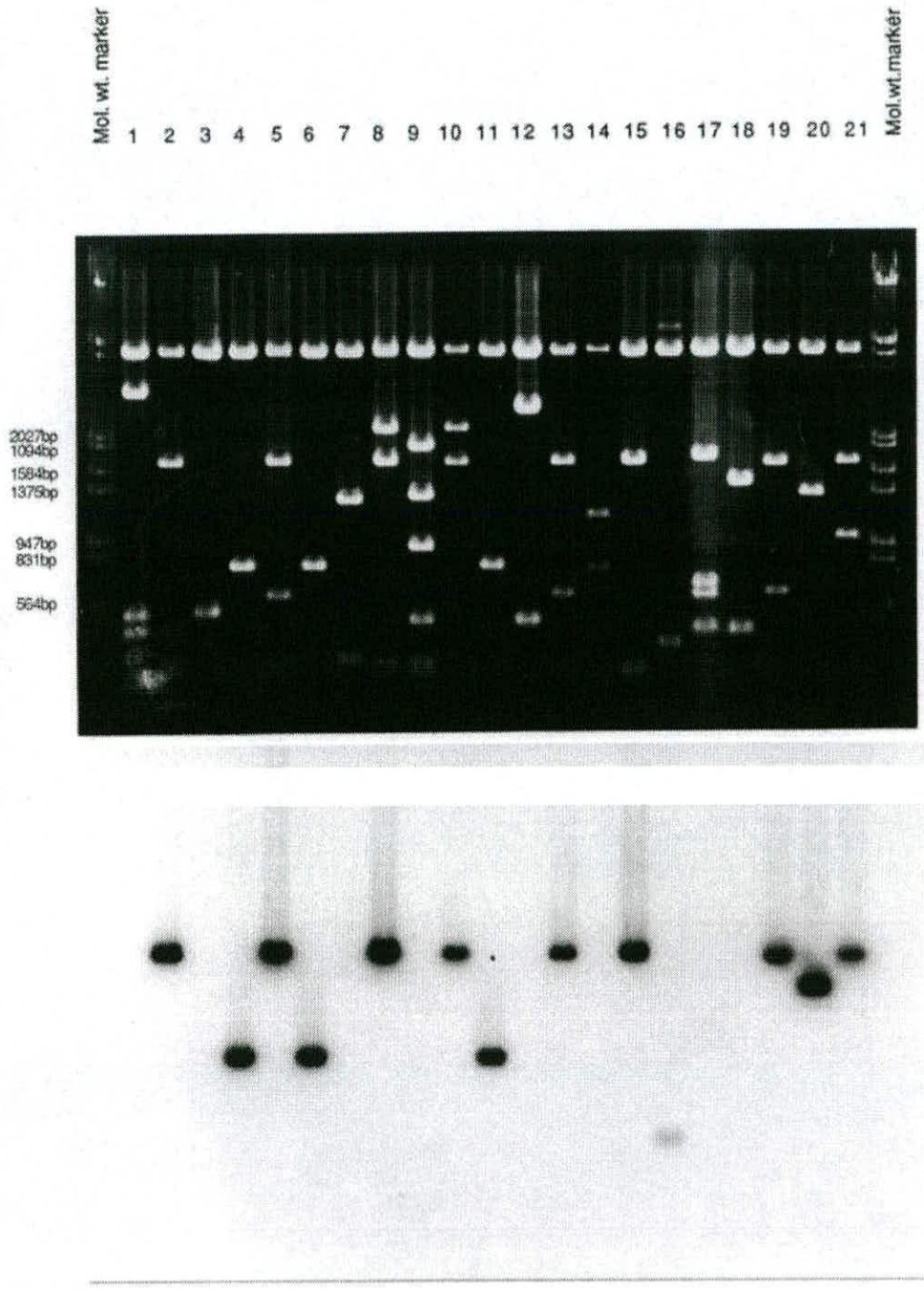
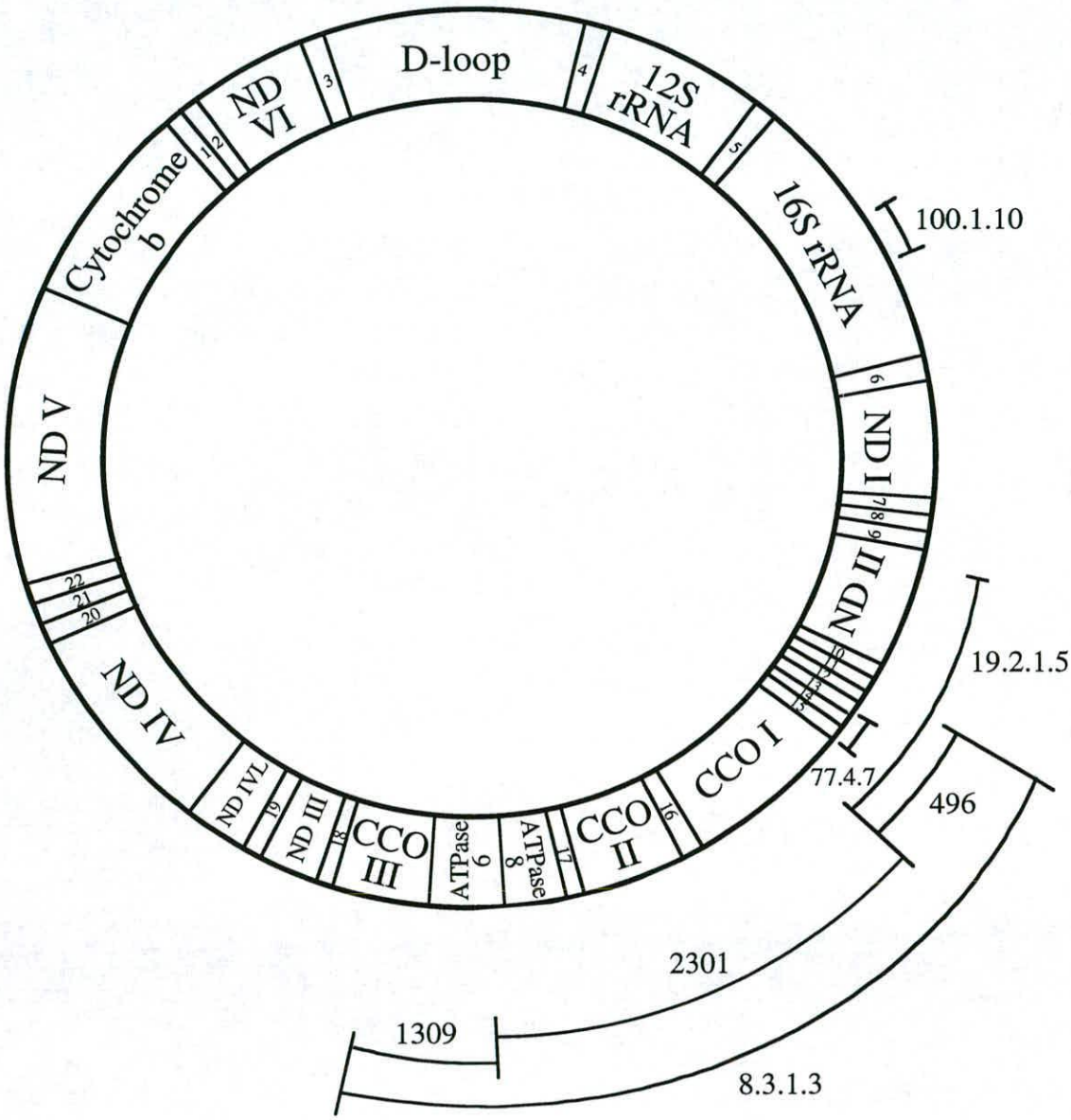

Mol. wt. marker
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Mol.wt.marker
2027bp
1094bp
1584bp
1375bp
947bp
831bp
564bp

Figure 45: Schematic representation of the chick mitochondrial genome. The chick mitochondrial genome is 16775bp in length. No.s 1 to 22 represent the positions of the tRNA genes. CCO = cytochrome c oxidase. ND = NADH dehydrogenase.


D-loop
12S rRNA
16S rRNA
ND I
ND II
CCO I
CCO II
ATPase 8
ATPase 6
CCO III
ND III
ND IV
ND V
Cytochrome b
ND VI
100.1.10
19.2.1.5
77.4.7
496
2301
1309
8.3.1.3

All 13 positive clones were sequenced, and analyzed using a multiple sequence alignment programme from the GCG package. A consensus sequence for each clone was compared to the sequences in the databases using the BLAST and FASTA analysis packages (Altschul *et al.,* 1990; Genetics Computer group, 1994). All clones showed homology to the chick mitochondrial genome. The extent of the fragment isolated was 5078bp, and was contained in two overlapping clones (19.2.1.5 and 8.3.1.3.) (fig 45). Clone 19.2.1.5 was 1322bp in length and encoded the region from the 3' end of the cytochrome c oxidase I (CCOI) gene through to the 3' end of the NADH dehydrogenase II gene. Clone 8.3.1.3 was 4.1kb in length. It exhibited homology from the midpoint of the cytochrome c oxidase III (CCO III) gene through to the gene encoding the alanine tRNA. The position of the original display clone (774) is shown on figure 45.

8.2.3. Northern analysis data on genomic clones.

In an attempt to identify approximately which part of the isolated chick mitochondrial genome exhibited the same differentially expressed pattern as the display clone, Northern analysis was carried out. The full length of the insert 19.2.1.5 was utilised in the Northern analysis procedure. Clone 8.3.1.3 was cut with restriction enzyme into three fragments of: 496bp, 2301bp and 1309bp (fig 45). Clone 8.3.1.3 (496) encoded the region from the 3' end of the cytochrome c oxidase I (CCO I) gene to the alanine tRNA gene. Clone 8.3.1.3 (2301) encoded the region from the 3' end of the ATPase 6 gene to the 3' end of the CCO I gene. Finally, clone 8.3.1.3 (1309) encoded the region from the middle of the CCO III gene to the 3' end of the ATPase 6 gene.

Clones 8.3.1.3 (496), 8.3.1.3 (1309) and 8.3.1.3 (2301) did not show the same differentially expressed pattern as observed with the display clone (fig 46, 47 & 48).

Northern analysis using clone 19.2.1.5. detected two major transcripts of approximately 1.5kb and 0.82kb and three minor transcripts of approximately 5.1kb, 3.3kb and 2.8kb. Transcript 3.3kb exhibited an expression profile and transcript size similar, but not identical to, that observed with the display clone (774). After normalisation for loading, female expression levels were seen to be relatively constant for this transcript (fig 49), from 5.5d to 9.5d and in the adult ovary. In the male, expression of the 3.3kb transcript was relatively constant except for the peak of expression at 7.5d. This peak of expression corresponded to the peak of expression observed with the display clone (774), although expression levels were much lower by Northern analysis using the 19.2.1.5 clone. Another difference between 19.2.1.5 and

Figure 46: Northern analysis of clone 8.3.1.3 (496) in the gonads of the developing male and female chick embryo (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 4.5 to day 9.5 of incubation (stage 25 to 36), and 10μg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 60°C in 0.25M Na PO_4, 7% SDS. The blot was washed to a final stringency of 25mM Na PO_4, 1% SDS at 65°C and exposed to film for 2 days at -70°C. The loading of the RNA in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). A single observation by Northern analysis was carried out.

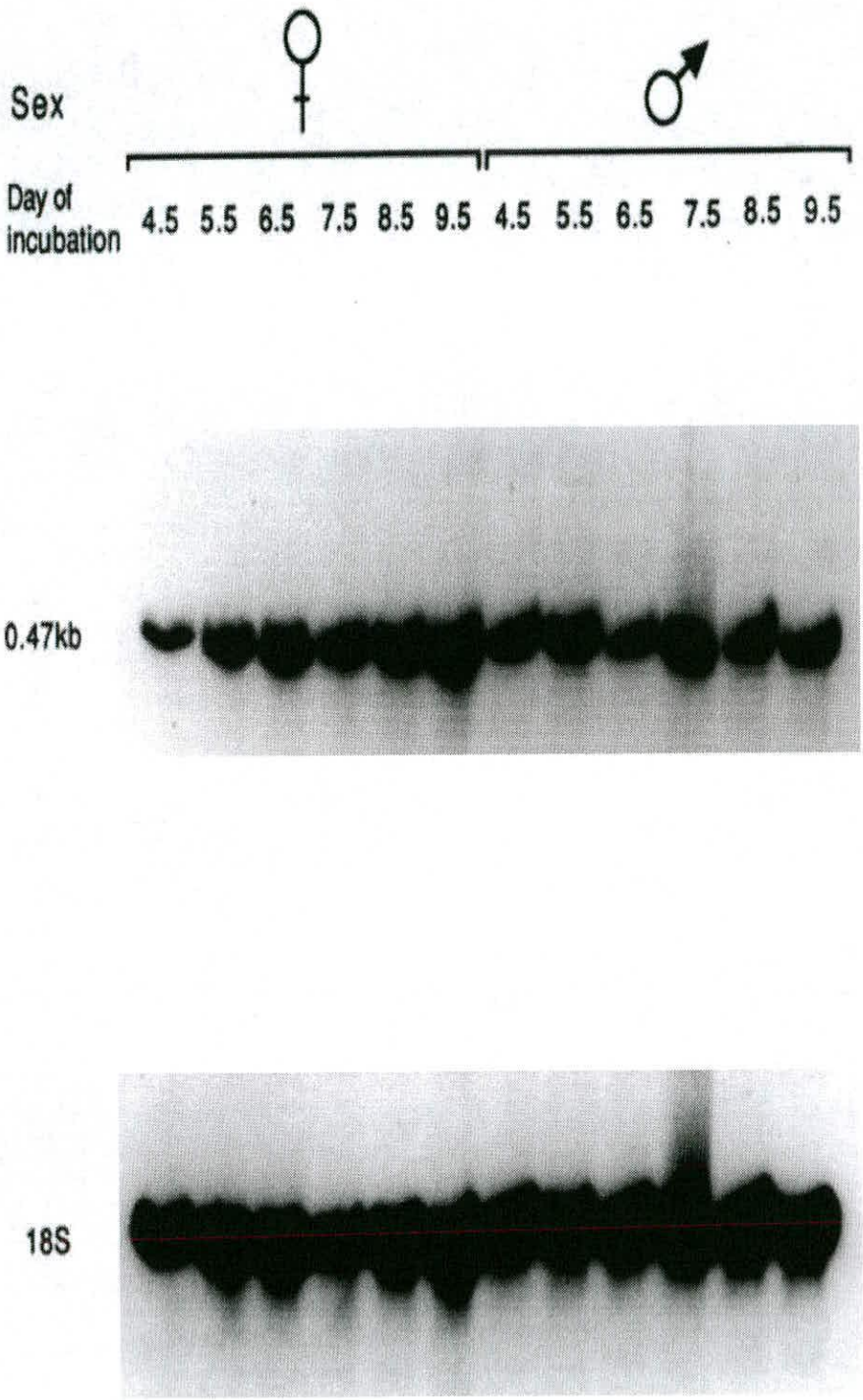

Sex
♀
♂
Day of incubation
4.5 5.5 6.5 7.5 8.5 9.5 4.5 5.5 6.5 7.5 8.5 9.5
0.47kb
18S

Figure 47: Northern analysis of clone 8.3.1.3 (1309) in the gonads of the developing male and female chick embryo (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 4.5 to day 9.5 of incubation (stage 25 to 36), and 10μg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 60°C in 0.25M Na PO_4, 7% SDS. The blot was washed to a final stringency of 10mM Na PO_4, 1% SDS at 65°C and exposed to film overnight at -70°C. The loading of the RNA in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). A single observation by Northern analysis was made.

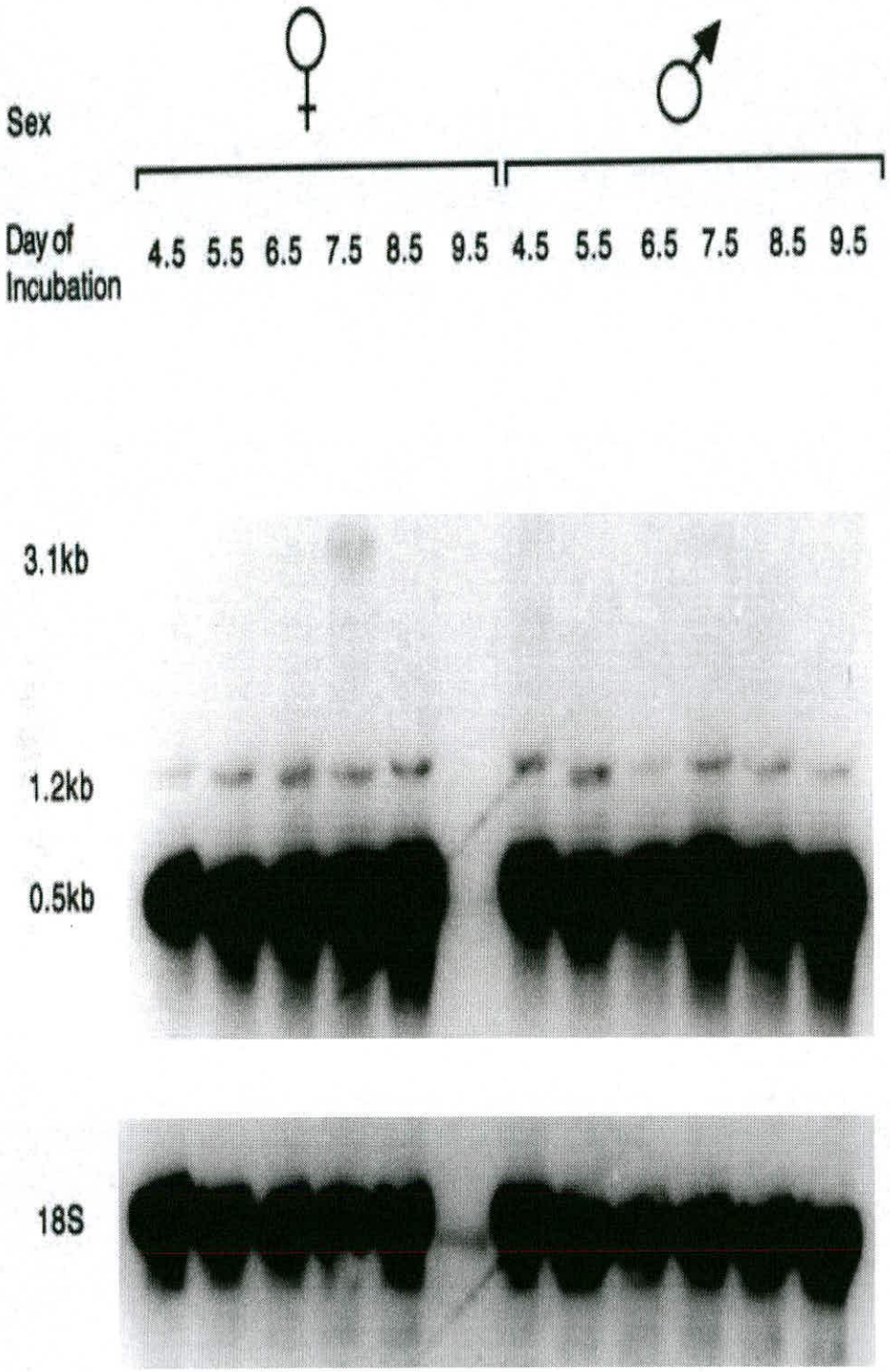

Sex
♀
♂
Day of Incubation
4.5 5.5 6.5 7.5 8.5 9.5 4.5 5.5 6.5 7.5 8.5 9.5
3.1kb
1.2kb
0.5kb
18S

Figure 48: Northern analysis of clone 8.3.1.3 (2301) in the gonads of the developing male and female chick embryo (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 4.5 to day 9.5 of incubation (stage 25 to 36) and adult ovary and testis. 10μg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 60°C in 0.25M Na PO_4, 7% SDS. The blot was washed to a final stringency of 25mM Na PO_4, 1% SDS at 68°C and exposed to film for 2 days at -70°C. The loading of the RNA in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). A single observation by Northern analysis was made.

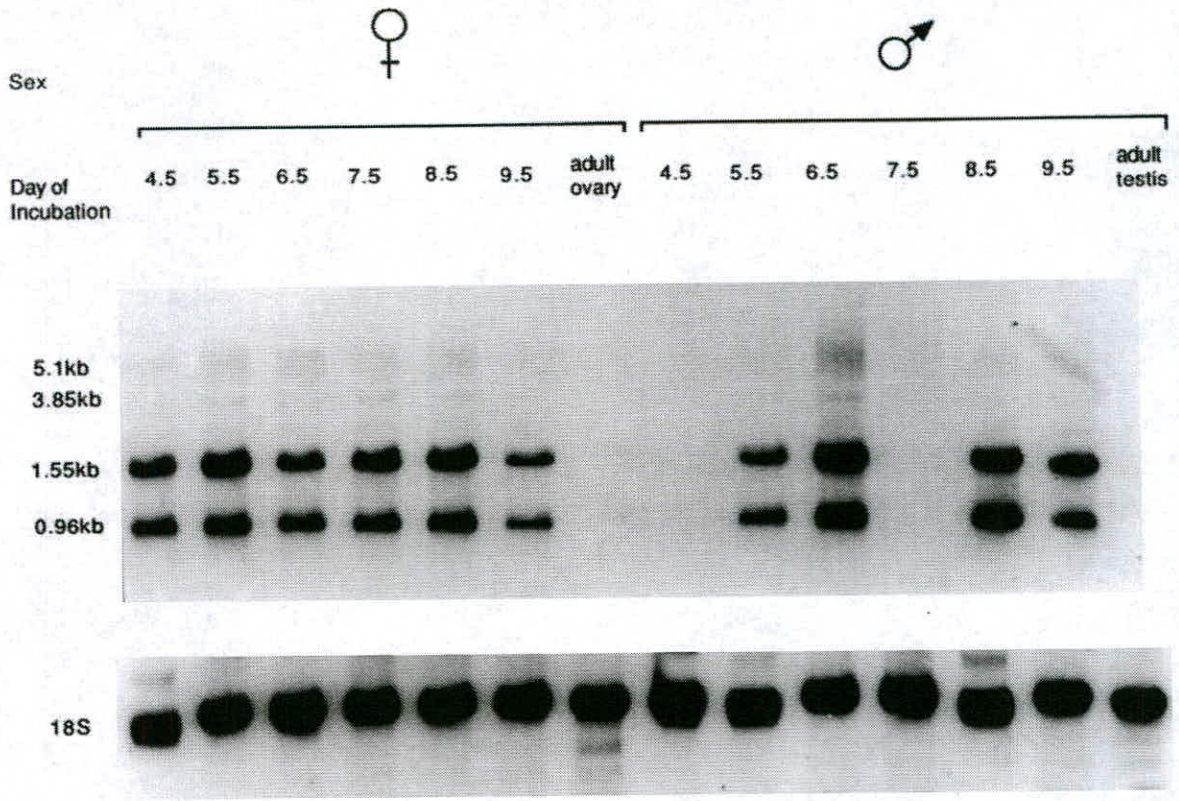
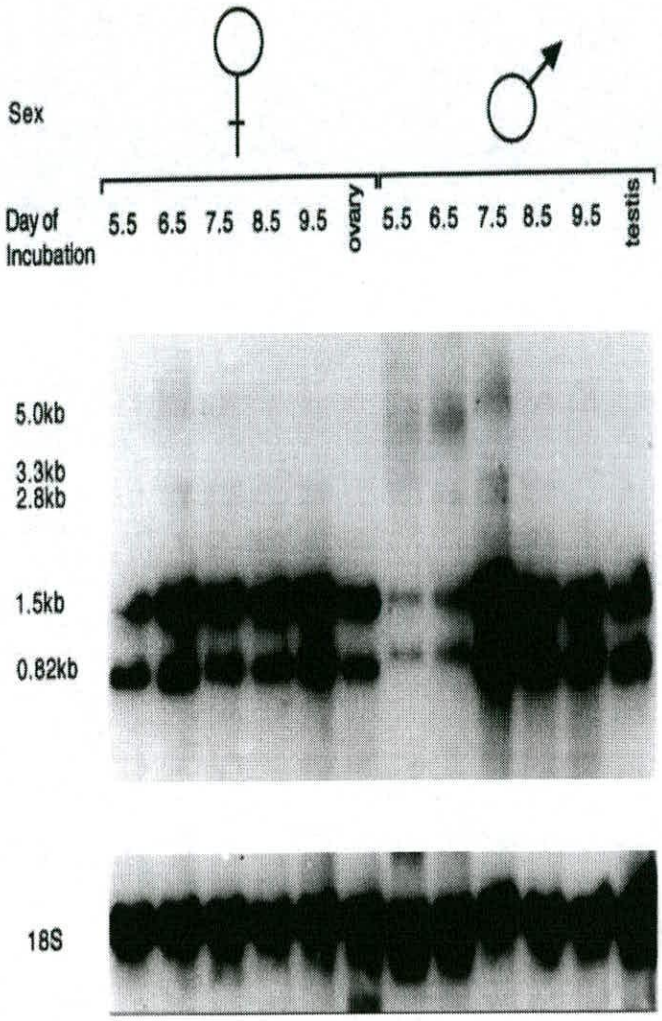

Sex
♀
♂
Day of Incubation
4.5
5.5
6.5
7.5
8.5
9.5
adult ovary
4.5
5.5
6.5
7.5
8.5
9.5
adult testis
5.1kb
3.85kb
1.55kb
0.96kb
18S

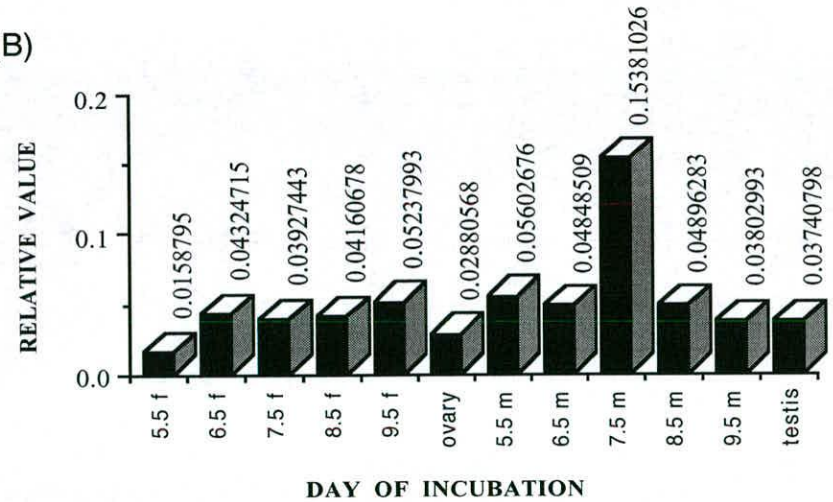
Figure 49: (A) Northern analysis of clone 19.2.1.5 in the gonads of the developing male and female chick embryo (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 5.5 to day 9.5 of incubation (stage 28 to 36) and adult ovary and testis. 5µg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridisation was carried out at 60°C in 0.25M Na PO_4, 7% SDS. The blot was washed to a final stringency of 10mM Na PO_4, 1% SDS at 65°C and exposed to film for 3 days at -70°C. The loading of the RNA in each lane was standardised by hybridising with a fragment of the chick 18S ribosomal gene (bottom panel). A single observation by Northern analysis was made.

(B) Graph of the expression profile of the 3.3kb transcript of clone 19.2.1.5 in the genital ridge/mesonephric tissue of male and female chick embryos, analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.

(A)


Sex
Day of Incubation
5.5 6.5 7.5 8.5 9.5
ovary
5.5 6.5 7.5 8.5 9.5
testis
5.0kb
3.3kb
2.8kb
1.5kb
0.82kb
18S

(B)


RELATIVE VALUE
0.2
0.1
0.0
0.0158795
0.04324715
0.03927443
0.04160678
0.05237993
0.02880568
0.05602676
0.04848509
0.15381026
0.04896283
0.03802993
0.03740798
5.5 f
6.5 f
7.5 f
8.5 f
9.5 f
ovary
5.5 m
6.5 m
7.5 m
8.5 m
9.5 m
testis
DAY OF INCUBATION

the display clone (774) is the number of transcripts detected. However, the fact that clone 19.2.1.5 covers the area encoded by the display clone; its approximate size is within 300bp of the display clone and its expression peaks at day 7.5 of incubation suggests this part of the chick mitochondrial genome is comparable to the differential expression observed with the display clone 774.

8.3. Confirmation of differential expression of display clones.

The twelve clones isolated from the display gel and their expression profile by Northern analysis in comparison to their expression on the display gel is summarised in table 3. In an attempt to localise the expression of each display clone to a tissue type, wmISH was performed.

8.3.1. Clone 771.

By Northern analysis, 771 exhibited elevated levels of expression at 3.5d (stage 21 and 22) in both male and female samples (fig 50A). However, the band obtained was diffuse and gave a transcript size of approximately 0.9kb to 1.2kb. Two other transcript sizes were also detected. These were approximately 1.8kb and 1.5kb and were identified in 4.5d, 5.5d and 7.5d (stage 25 to 33) in male and female samples. These transcript sizes correspond to previously published sizes for RhoA (Moscow *et al.*, 1994). A third transcript for RhoA has also been reported, however the size reported is over 1.2kb (Moscow *et al.*, 1994). Expression of 771 was higher in female samples than males when compared at the same developmental day, with the exception of 3.5d. After normalisation, no differences were observed between male and female samples for the same developmental stages (fig 50B). Expression levels decreased after 3.5d and then increased in both sexes at 7.5d. The expression pattern for 771 detected by Northern analysis replicates exactly the expression profile observed on the display gel.

The expression profile was also studied in different tissues at different developmental stages in the chick embryo (data not shown). Two main transcripts, of approximately 1.8kb and 1.5kb, were observed in all tissues studied. Minor transcripts of 0.85kb and 3.5kb were also detected in various tissues.

An anti-sense 771 probe of 254bp and a sense probe of 224bp were used to perform wmISH. Partially dissected male and female chick embryos at 3.5d showed fairly widespread expression of 771 throughout the developing embryo (data not shown).

Expression of clone 771 was also analyzed in male and female genital ridge and mesonephric tissue, at 7.5d (fig 51a & b). In the male, expression of clone 771

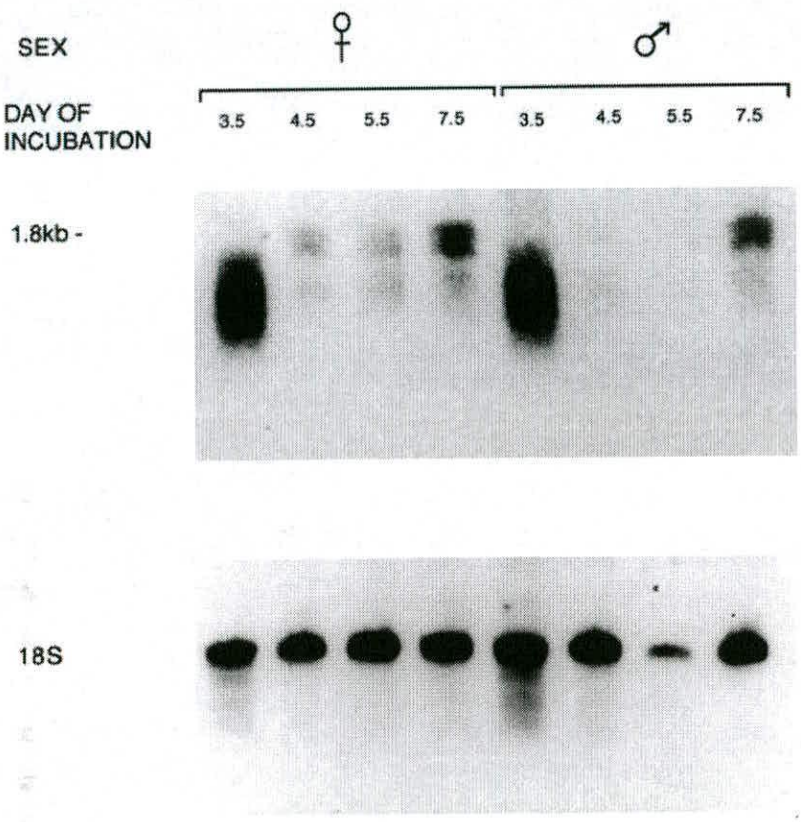
Clone	Display Data	Northern Data
771	Highest levels of expression, d3.5 males and females	Highest expression of smallest transcript on d3.5 in males and females. Two larger transcripts present, higher in females.
772	Expression only detected in d5.5 males.	Increase in expression at d5.5 in males and females, followed by a decrease in expression.
774	Higher expression in d7.5 males.	Higher expression levels in males at d7.5.
775	Expression levels peak at d4.5 in males and females.	Expression levels peak at d4.5 in males and females.
776	Higher expression levels in males at day 8.5 of incubation.	No transcript detected by this method.
777	Expression in males and females only at day 3.5 of incubation.	No transcript detected.
779	Expression only in males at day 3.5 of incubation.	No transcript detected.
1001	Higher expression in males at d9.5 of incubation	Increasing levels of expression, which peaks at day 9.5 of incubation in both sexes.
1007	Highest expression in males at d5.5 of incubation. No expression in females.	Peak of expression in males and females at d5.5 of incubation.
1111	Higher levels of expression in males at d5.5 of incubation.	Higher levels of expression in females compared to males, at all developmental stages analysed.
1144	Higher expression levels in females at d5.5 of incubation.	Higher levels of expression in males and females at d5.5, but expression detected at other developmental stages.
1161	Higher expression levels at d5.5 of incubation.	No transcript detected.

Table 3: A summary of the expression profile of the display clones on the display gel and by Northern analysis.

Figure 50: (A) Northern analysis of display clone 771 in the gonads of developing male and female chick embryos (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue at days 3.5, 4.5, 5.5 and 7.5 of incubation, and 5µg of each sample electrophoresed on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 42°C in 2xPIPES, 50% formamide and 0.5% SDS. Northern blots were washed to a final stringency of 0.5xSSC, 0.1% SDS at 65°C and exposed to film overnight at -70°C. The presence of approximately equal amounts of RNA in each lane was monitored by hybridizing with a fragment for the chick 18S ribosomal gene (bottom panel). A single observation was made by Northern analysis.

(B) Graphical representation of the expression profile of display clone 771 analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a Molecular Dynamics phosphorimager and related to a loading control. F = female. M = male.

(A)


SEX
♀
♂
DAY OF INCUBATION
3.5
4.5
5.5
7.5
3.5
4.5
5.5
7.5
1.8kb -
18S

(B)

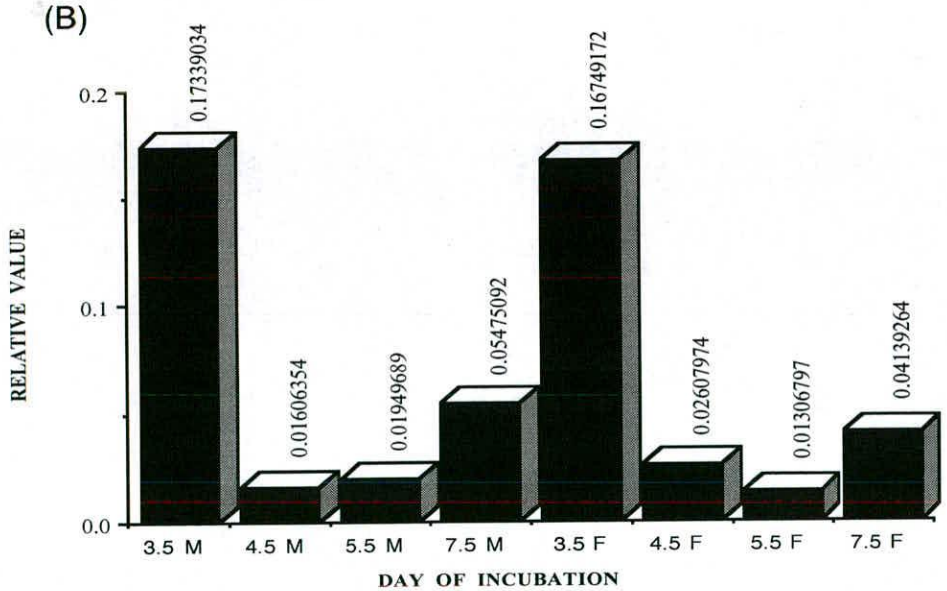
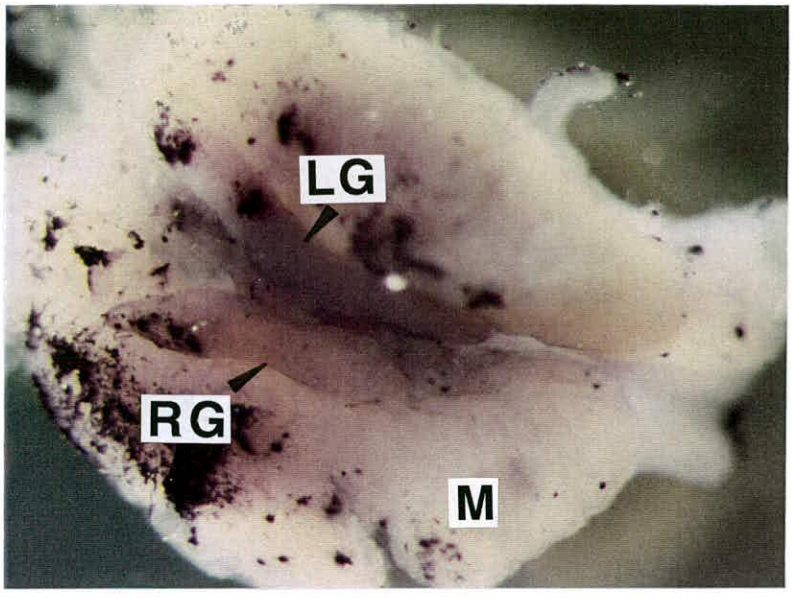
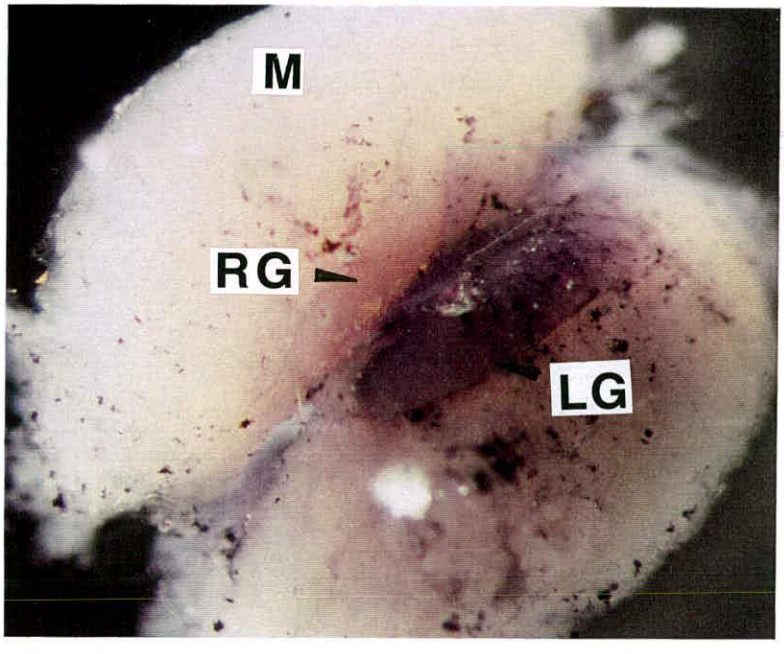

0.17339034
0.01606354
0.01949689
0.05475092
0.16749172
0.02607974
0.01306797
0.04139264
0.2
0.1
0.0
RELATIVE VALUE
3.5 M
4.5 M
5.5 M
7.5 M
3.5 F
4.5 F
5.5 F
7.5 F
DAY OF INCUBATION

Figure 51: Expression of display clone 771 in the embryonic chick gonad an meonephric tissue at day 7.5 (stage 32 and 33) by wmISH Hybridization was performed using a sense and anti-sense DIG labelle 771 riboprobe at 63°C. Colour development proceeded for 15hr. (a Male gonads and mesonephros. Actual length of the mesonephros 3.9mm. (b) Female gonads and mesonephros. Actual length of th mesonephros is 3.6mm. (c) Male gonads and mesonephros hybridize with a sense probe. Actual length of the mesonephros is 4mm. Anteric (A), posterior (P), left gonad (LG), right gonad (RG), mesonephro (M). Analysis by wmISH was carried out twice with consistent results

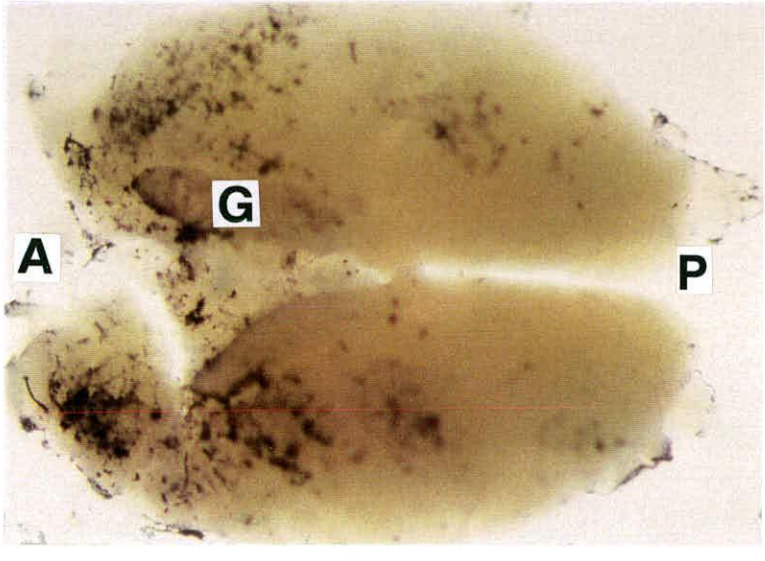
(a)



(b)



(c)



was detected in both gonads. Expression could also be detected in the mid-part of the mesonephros. In the female, expression of clone 771 was detected in the gonads. However, expression levels were significantly higher in the female left gonad compared to the right gonad. If the sense 771 probe was used, no specific expression was observed in the tissue (fig 51c).

8.3.2. Clone 772.

Northern analysis carried out on clone 772 showed two transcripts of approximately 1.6kb and 3.7kb, after a low stringency wash and a two day exposure (fig 52A). The 1.6kb transcript exhibited a gradual increase in levels of expression in both male and female samples over 4.5d to 9.5d (stage 25 to 36). After a high stringency wash and a one week exposure, only the 3.7kb transcript was detectable. The expression profile of this transcript was similar in both males and females. Expression levels peaked at 5.5d and then gradually decreased to 9.5d. After normalisation, the female samples showed a peak of expression at 5.5d followed by the gradual decrease in expression (fig 52B). The results of the male samples were less clear (fig 52B). The very low levels of expression at 4.5d followed by the rapid increase a day later were evident. There was another peak of expression at 7.5d, much greater than the expression levels observed in the female at the same stage, although phosphorimager data accentuates the level of expression of 77.2 in the male at this stage. Levels of expression in the remaining developmental stages were equivalent to those in the female at the same stages. Overall, both sexes followed the pattern of a rapid increase of expression levels at 5.5d, followed by a decrease in these levels. This pattern of expression did not correspond to that observed on the display gel, where expression was only detected in male samples at 5.5d.

The expression profile of clone 772 was also analyzed on a Northern blot containing different embryonic tissues from different days. A transcript of approximately 3.75kb was detected in various embryonic tissues and in the adult ovary and testis, with higher levels of expression in the adult ovary compared to the adult testis (data not shown). In addition, a larger transcript of approximately 4.2kb was detected in various tissues at 5.5d and 6.5d.

An anti-sense 772 probe of 269bp and a sense probe of 239bp were used to perform wmISH. Expression of clone 772 was studied in male and female chick genital ridge and mesonephros tissue at 4.5d to 6.5d. At all three days of incubation, high levels of expression, of clone 772, were observed in the male and female genital ridge and mesonephric tissue, which does not correlate with the low levels of

Figure 52: (A) Northern analysis of display clone 772 in the gonads of developing male and female chick embryos (top and middle panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 4.5 to day 9.5 of incubation (stage 25 to 36), and 5µg of each sample was electrophoresed on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 42°C in 2xPIPES, 50% formamide, 0.5% SDS. Northern blots were washed in 0.5xSSC, 0.1% SDS at 60°C (low stringency) and exposed to film overnight at -70°C (top panel). Northern blots were washed to a final stringency of 0.2xSSC, 0.1% SDS at 68°C and exposed to film for 1 week at -70°C (middle panel). The RNA loading in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). Northern analysis was carried out twice, on different RNA samples, with consistent results.

(B) Graphical representation of the expression profile of display clone 772 analyzed by Northern analysis. The amount of isotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.

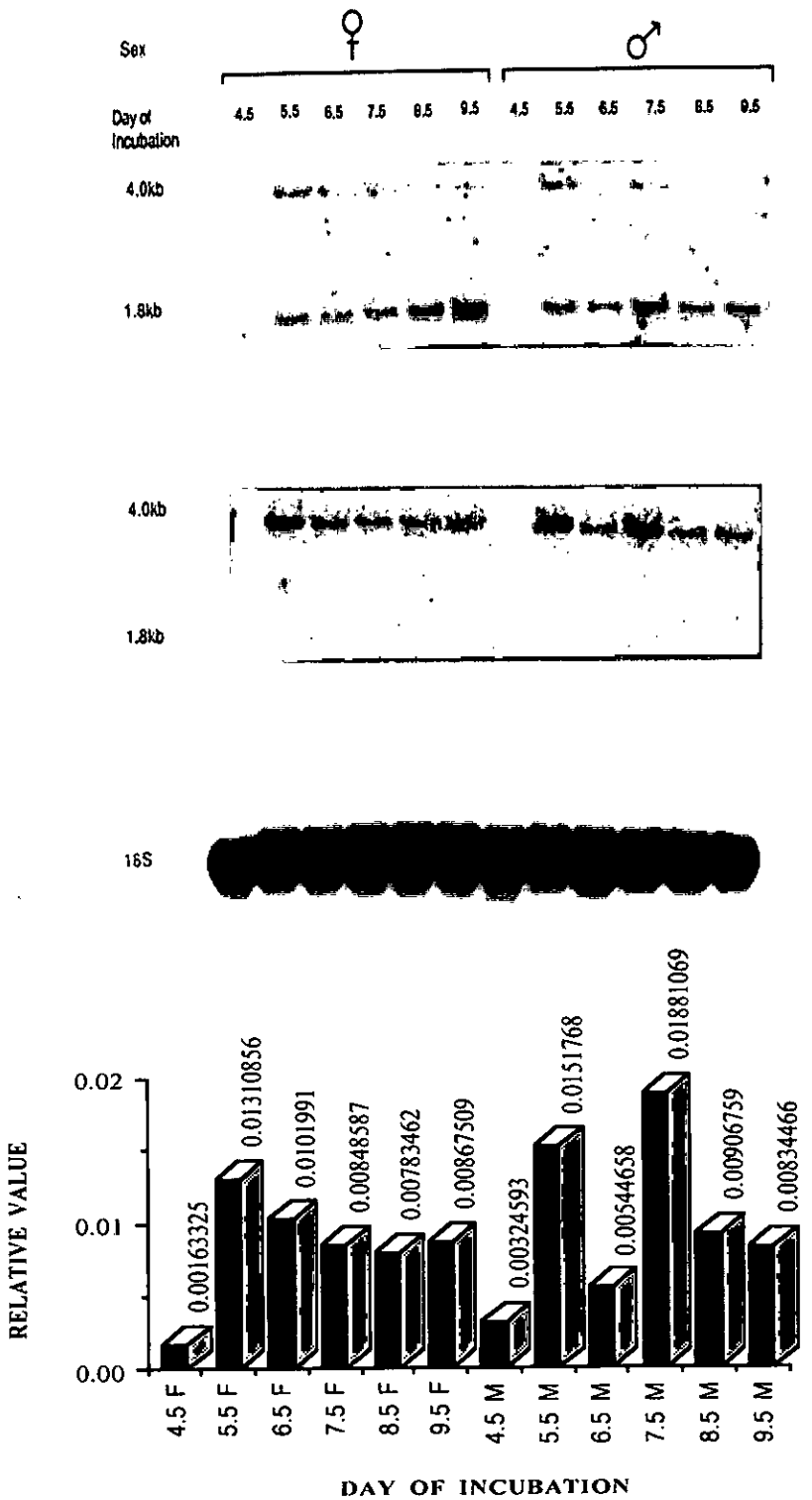
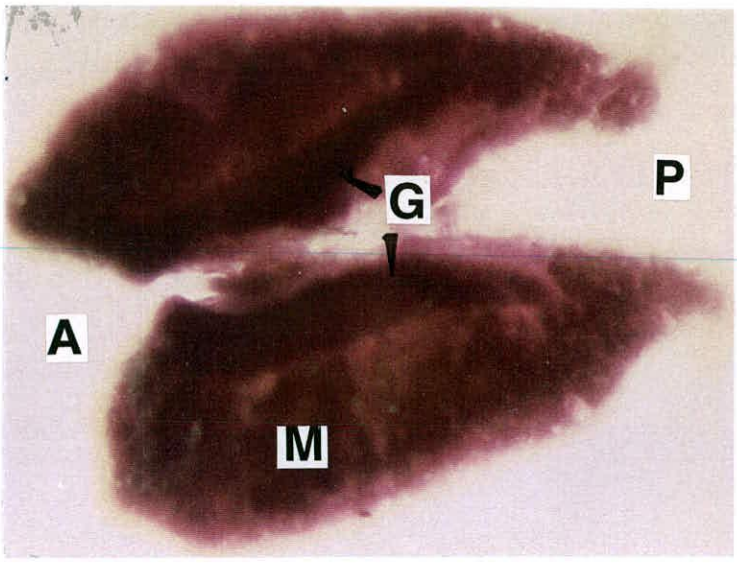
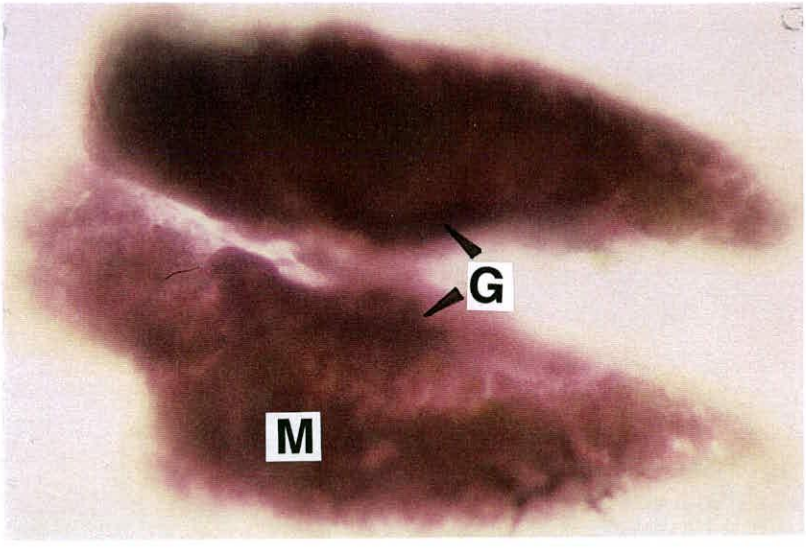

Sex
♀
♂
Day of Incubation
4.5 5.5 6.5 7.5 8.5 9.5 4.5 5.5 6.5 7.5 8.5 9.5
4.0kb
1.8kb
4.0kb
1.8kb
16S
RELATIVE VALUE
0.02
0.01
0.00
0.00163325
0.01310856
0.0101991
0.00848587
0.00783462
0.00867509
0.00324593
0.0151768
0.00544638
0.01881069
0.00906759
0.00834466
4.5 F
5.5 F
6.5 F
7.5 F
8.5 F
9.5 F
4.5 M
5.5 M
6.5 M
7.5 M
8.5 M
9.5 M
DAY OF INCUBATION

Figure 53: Expression of display clone 772 in the chick embryonic gonads an mesonephric tissue by wmISH. Hybridization was performed using sense and anti-sense DIG labelled 772 riboprobe at 66°C. (a) Day 6. (stage 30) male gonads and mesonephros. Colour developme proceeded for 1½hr. Actual length of the mesonephros is 4mm. (b Day 6.5 female gonad and meonephros. Colour development proceede for 3½hr. Actual length of the mesonephros is 3.8mm. (c) Day 6. female gonads and mesonephros hybridized with the sense probe Colour development proceeded for 3½hr. Actual length of th mesonephros is 3.6mm. Anterior (A), posterior (P), gonad (G) mesonephros (M). Analysis by wmISH was carried out twice wit consistent results.

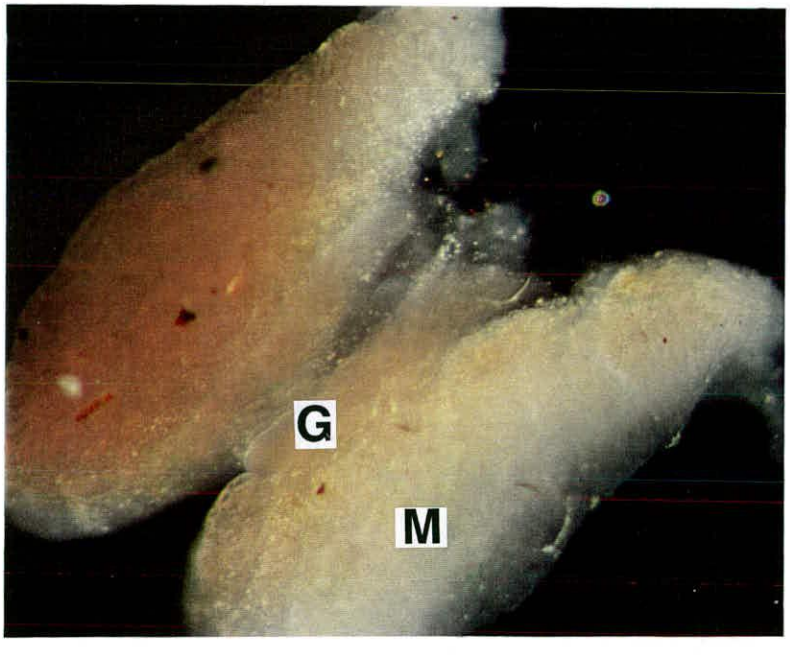
(a)



(b)



(c)



expression detected in male and female genital ridge tissue at 4.5d by Northern analysis (fig 53a & b). No specific expression was observed with the sense probe.

8.3.3. Clone 775.

A low stringency wash resulted in many different size transcripts being detected on the Northern blot (fig 54), due to binding to related transcripts. After a high stringency wash (10mM Na PO_4, 0.1%SDS at 70°C), only a single transcript could be detected (fig 54). The size of this transcript was approximately 0.6kb, corresponding to the published size of 546bp for rho-globin (Roninson and Ingram, 1981). The expression showed the highest levels of expression in male and female genital ridge tissue was at 4.5d (stage 25 and 26), matching the expression profile on the differential display gel. Expression levels decreased after this developmental day so that only very low levels could be detected at 7.5d.

8.3.4. Clone 776.

When clone 776 was used to probe a Northern blot, containing genital ridge tissue from different days, no transcript could be detected, precluding a comparison of the patterns obtained by Northern analysis and differential display. However, a transcript, approximately 4.0kb, was detected on a Northern tissue blot (data not shown), with expression in the brain at 5.5d, kidney tissue at 6.5d and adult testis.

8.3.5. Clone 777.

By Northern analysis, probes derived from clone 777 failed to detect expression in either the genital ridge or other embryonic tissue, between 3.5d and 9.5d. No expression could be detected by wmISH.

8.3.6. Clone 779.

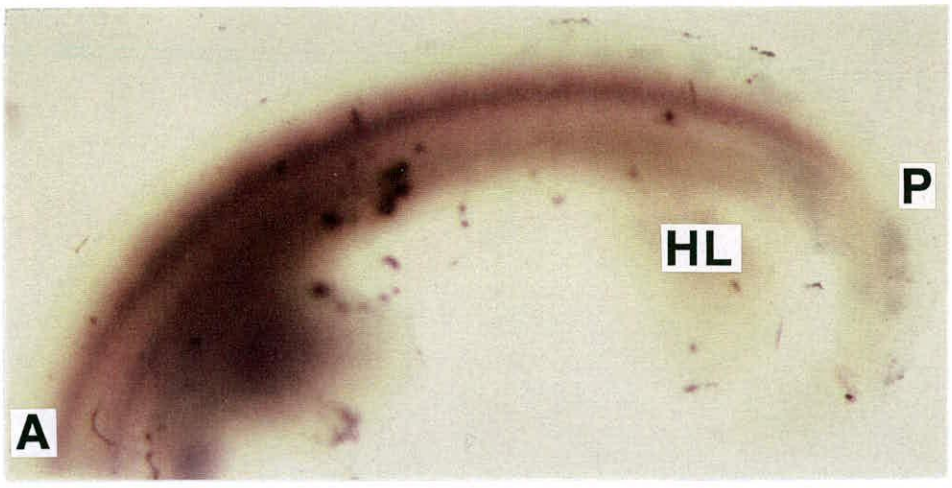
By Northern analysis, probes derived form clone 779 failed to detect expression in the genital ridge, between 3.5d and 9.5d.

This clone was also used to probe a Northern blot containing various embryonic tissues (data not shown). A transcript, approximately 9.2kb, was detected in the brain at 7.5d (stage 32 and 33). A second transcript of approximately 3.45kb was also detected in brain samples at 4.5d to 8.5d, and in the adult ovary and testis, with slightly higher levels of expression in the testis (data not shown).

An anti-sense 779 transcript of 434bp and a sense transcript of 404bp were used to perform wmISH. In partially dissected male embryos at 3.5d, expression was detected along the main body of the embryo (fig 55a & b), with expression in the

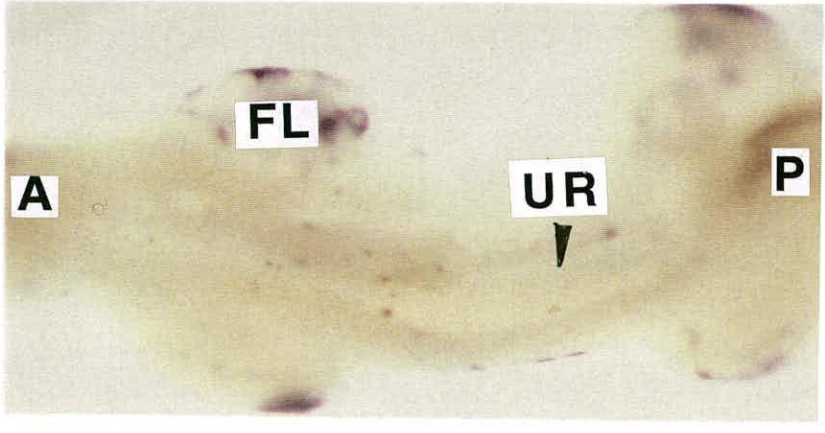
Figure 54: Northern analysis of display clone 775 in the gonads of developing male and female chick embryos. Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at days 3.5, 4.5, 5.5, 7.5 and 8.5 of incubation (stage 21 to 35), and 5µg of each sample was electrophoresed on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 65°C in 0.25M Na PO_4, 7% SDS.The top panel shows the Northern blot washed in 25mM Na PO_4, 1% SDS at 55°C and exposed to film overnight at -70°C. The middle panel shows blots washed to a final stringency of 10mM Na PO_4, 0.1% SDS at 70°C and exposed to film for 2 days at -70°C. The presence of approximately equal amounts of RNA in each lane was monitored by staining the gel with ethidium bromide and visualizing under U.V. light (bottom panel). A single observation by Northern analysis was made.

(a)

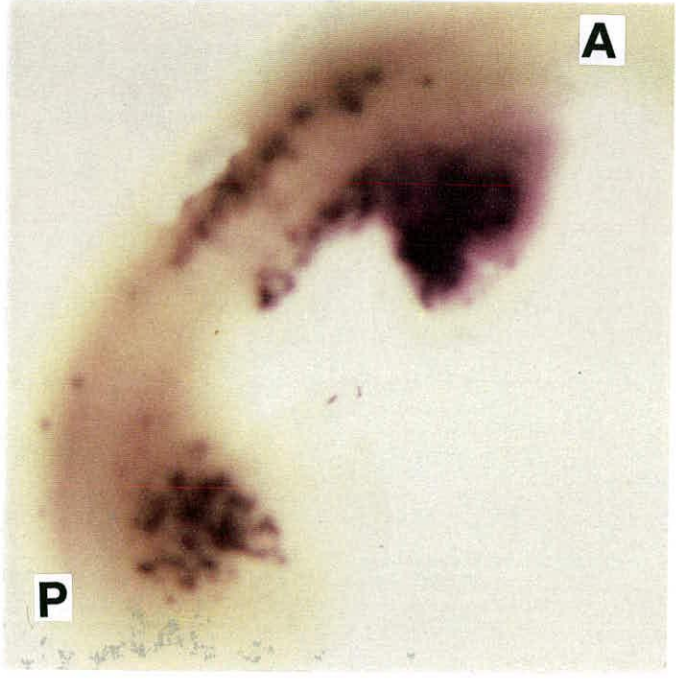


(b)

(c)



(d)



neural tube and the mesoderm. Expression was also detected in the proximal region of the fore and hind limb bud (fig 55a). In female partially dissected embryos, at 3.5d, expression levels were much lower compared with those detected in the male (fig 55d), with a more restrictive pattern of expression. However, this figure is of poor quality and the length of time required (3 days) to detect a signal suggests the result observed is an artifact. No specific expression was observed with the sense probe. Also, no expression was detected in the genital ridge from males or females at 4.5d to 6.5d (data not shown).

8.3.7. Clone 1001.

Northern analysis carried out on clone 1001 revealed one transcript of approximate size 2.3kb (fig 56A) detectable in every developmental day studied. Expression gradually increased from 4.5d to peak at 9.5d, in both male and female genital ridge. After normalisation, the overall trend was a gradual increase in the expression levels of this transcript in male samples over the initial developmental stages (fig 56B). While the expression pattern exhibited relatively constant levels in female samples from 5.5d to 9.5d, which does not accurately represent the increase in expression levels in female samples observed by Northern analysis.

The expression profile of clone 1001 was examined in different embryonic tissues (data not shown). Three transcripts of approximate size: 2.3kb, 4.65kb and 9.1kb were detected. The 2.3kb transcript showed the strongest expression and was detected in all tissues at all developmental stages studied.

8.3.8. Clone 1007.

Northern analysis, with clone 1007, revealed expression at all stages of gonadal development studied in both males and females, with very low levels at 4.5d (fig 57A). After normalisation for loading, expression levels were seen to peak at 7.5d for males and thereafter decrease, while in females expression levels were relatively constant from 5.5d to 9.5d (fig 57B). No significant differences were observed between expression levels in adult ovary and adult testis. The transcript size of approximately 4.7kb, corresponds well to the documented size of human mef-2A (Suzuki *et al.*, 1996). The expression profile was also studied in other tissues during certain stages in embryonic development (data not shown). A band, approximately 4.7kb, was detected at 5.5d in the rest of the embryo (minus: heart, brain, genital ridge and mesonephros). A faint band was also detected in the heart at 8.5d.

A 1007 anti-sense probe of 416bp and a sense probe of 446bp were used to perform wmISH. Expression was detected in the gonads and the mesonephros of

Figure 33: Expression of AMH in male and female chick gonads at day 7.5 of incubation by wmISH , which have been sectioned and counterstained. (a) Female left gonad, showing no expression could be detected. Scale bar represents 40μm. (b) Male gonad , showing expression of AMH is confined to the medulla and locates to the testicular cords. Scale bar represent 40μm. (c) High power magnification of the male gonad showing expression of AMH in cells in the medulla. Scale bar represents 10μm. Mesonephros (M), gonad (G), seminiferous cords (SC), medulla (Me).

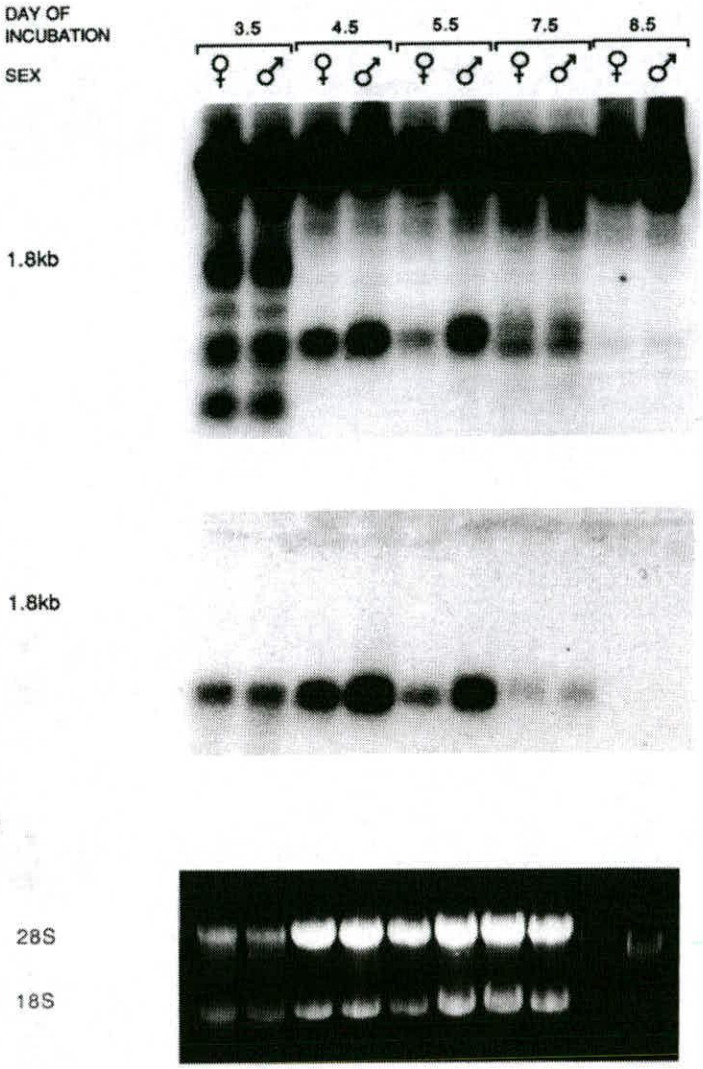
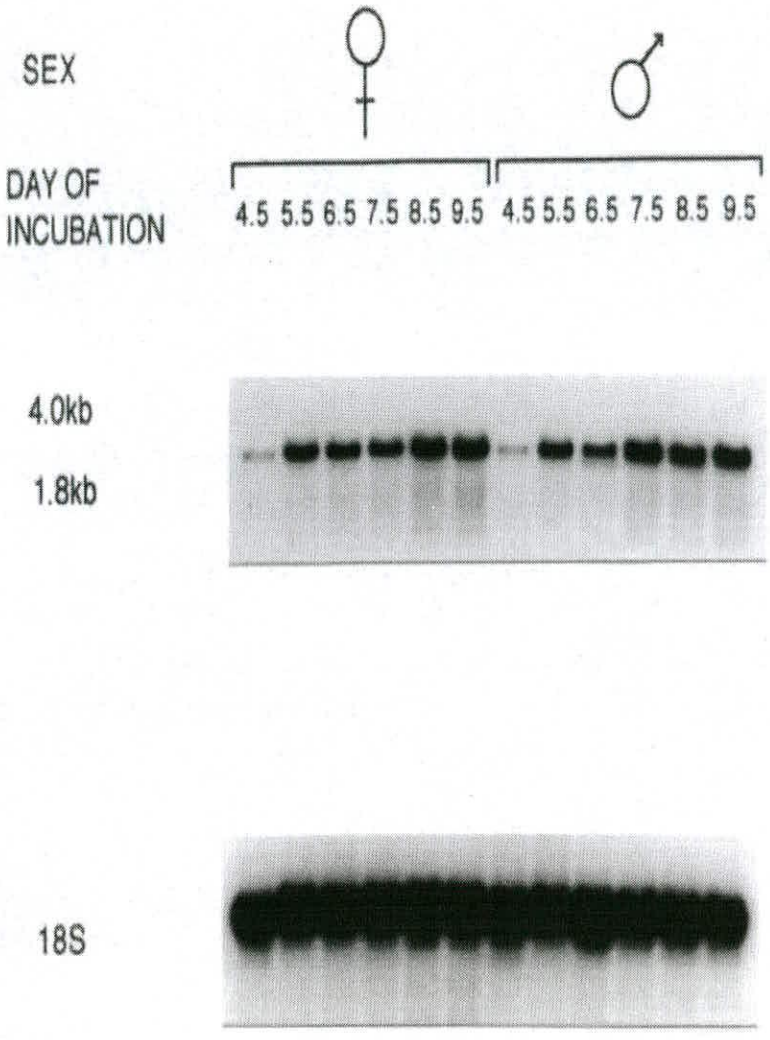

DAY OF INCUBATION
3.5
4.5
5.5
7.5
8.5
SEX
♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂
1.8kb
1.8kb
28S
18S

Figure 55: Expression of display clone 779 in male and female chick embryos day 3.5 of incubation (stage 21 and 22) by wmISH. Hybridization w performed using a sense and anti-sense DIG labelled 779 riboprobe 65°C. Colour development proceeded for 3 days. (a) Lateral view of partially dissected male embryo. Actual length of the embryo (minus t head) is 5mm. (b) Partially dissected male embrto. Actual length of t embryo (minus the head) is 5.2mm. (c) Partially dissected fema embryo hybridized with the sense probe. Actual length of the embr (minus the head) is 5.5mm. (d) Lateral view of a partially dissect female embryo. Actual length of the embryo (minus the head) is 5m (a), (b) and (d) were hybridized with the anti-sense probe. Anterior (A posterior (P), urogenital ridge (UR), hind limb (HL), forelimb (FL). single observation by wmISH was made.

Figure 56: (A) Northern analysis of display clone 1001 in the gonads of developing male and female chick embryos (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at days 4.5 to 9.5 of incubation (stage 25 to 36), and 5µg of each sample was electrophoresed on a 1% agarose/formaldehyde gel. Northern hybridization was carreid out at 42°C in 2xPIPES, 50% formamide, 0.5% SDS. Blots were washed to a final stringency of 0.2xSSC, 0.1% SDS at 65°C and exposed overnight to film at -70°C. The RNA loading in each lane was standardized by probing with a fragment of the chick 18S ribosomal gene (bottom panel). Northern analysis was repeated twice, on different RNA samples, with consistent results.

(B) Graph showing the expression profile of display clone 1001, analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.

(A)


SEX
♀
♂
DAY OF INCUBATION
4.5 5.5 6.5 7.5 8.5 9.5 4.5 5.5 6.5 7.5 8.5 9.5
4.0kb
1.8kb
18S

(B)

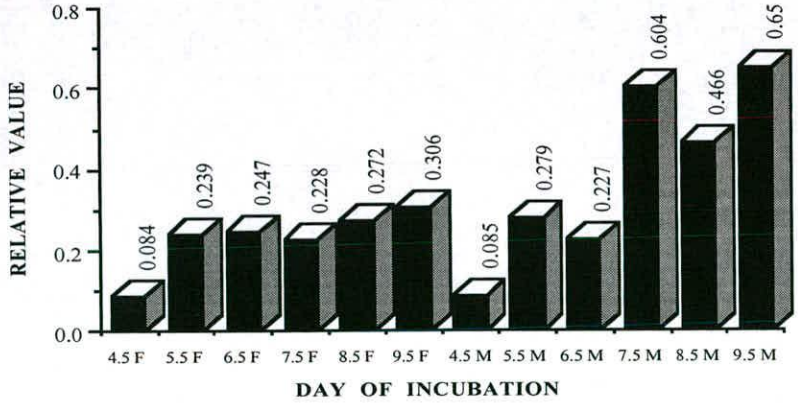
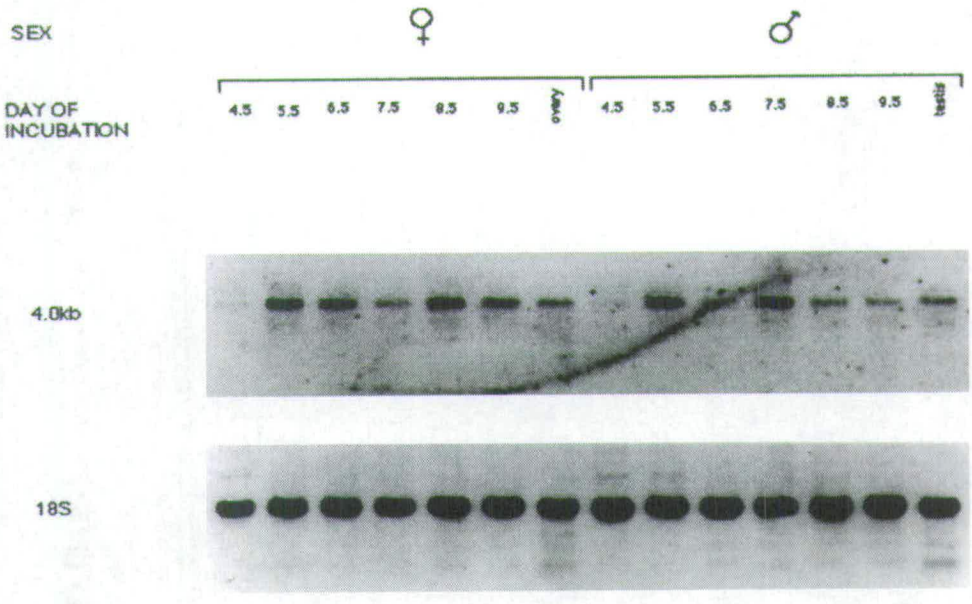

RELATIVE VALUE
0.0
0.2
0.4
0.6
0.8
0.084
0.239
0.247
0.228
0.272
0.306
0.085
0.279
0.227
0.604
0.466
0.65
4.5 F 5.5 F 6.5 F 7.5 F 8.5 F 9.5 F 4.5 M 5.5 M 6.5 M 7.5 M 8.5 M 9.5 M
DAY OF INCUBATION

Figure 57: (A) Northern analysis of display clone 1007 expression in the gonads of developing male and female chick embryos (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at day 4.5 to day 9.5 of incubation (stage 25 to 36), and 5µg of each sample was electrophoresed on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 42°C in 2xPIPES, 50% formamide, 0.5% SDS. The blot was washed to a final stringency of 0.2xSSC, 0.1% SDS at 60°C and exposed to film for 2 days at -70°C. The RNA loading in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). A single observation by Northern analysis was made.

(B) Graph showing the expression profile of display clone 1007 analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.

(A)


SEX
♀
♂
DAY OF INCUBATION
4.5
5.5
6.5
7.5
8.5
9.5
ovary
4.5
5.5
6.5
7.5
8.5
9.5
testis
4.0kb
18S

(B)

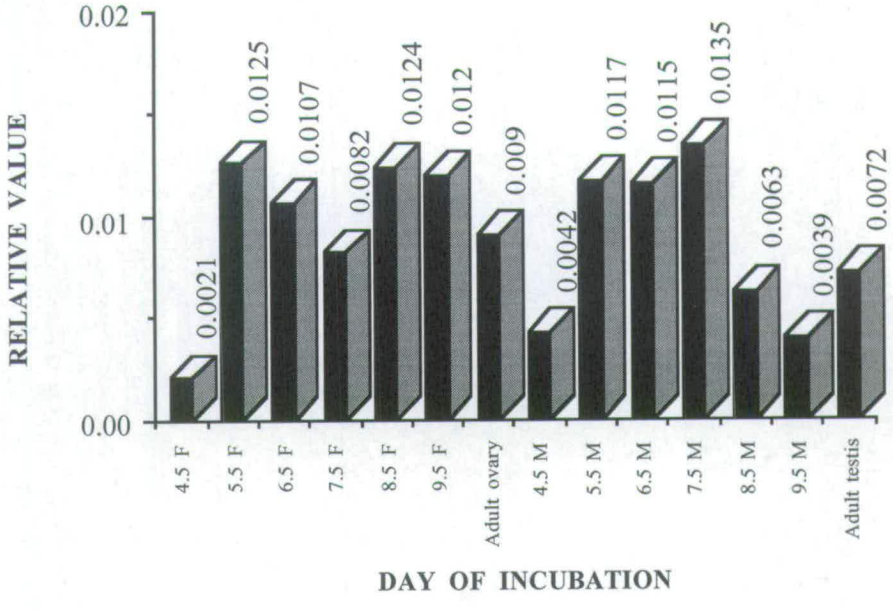
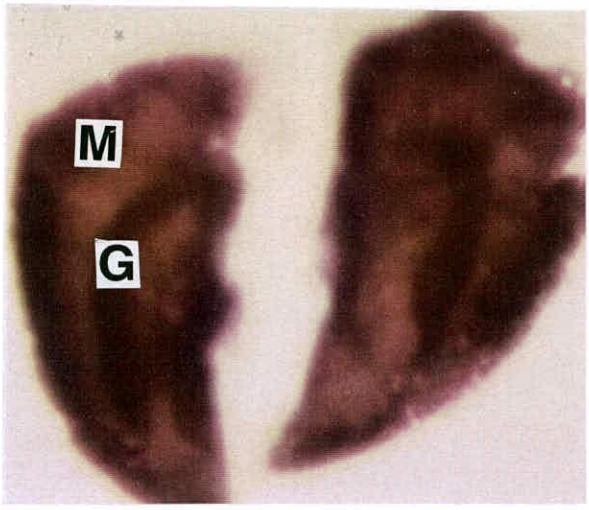
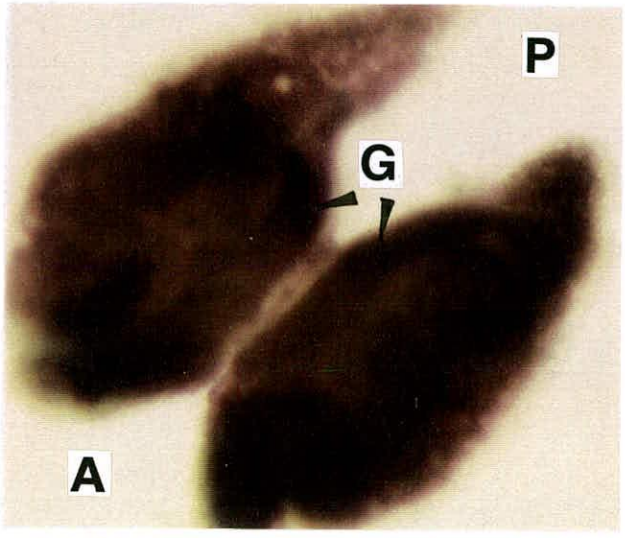

RELATIVE VALUE
0.02
0.01
0.00
0.0021
0.0125
0.0107
0.0082
0.0124
0.012
0.009
0.0042
0.0117
0.0115
0.0135
0.0063
0.0039
0.0072
4.5 F
5.5 F
6.5 F
7.5 F
8.5 F
9.5 F
Adult ovary
4.5 M
5.5 M
6.5 M
7.5 M
8.5 M
9.5 M
Adult testis
DAY OF INCUBATION

Figure 58: Expression of dispaly clone 1007 in the chick embryonic gonads an mesonephric tissue at day 6.5 of incubation (stage 30) by wmISH Hybridization was performed using a sense and anti-sense DIG labelle 1007 riboprobe at 66°C. Colour development proceeded for 1½hr. (a Male gonads and mesonephros. Actual length of the mesonephros i 3.8mm. (b) Female gonads and mesonephros. Actual length of th mesonephros is 3.3mm. (c) Male gonads and mesonephros hybridize with the sense probe. Actual length of the mesonephros is 4mm. (d Female gonads and mesonephros hybridized with the sense probe Actual length of the mesonephros is 3.4mm. Anterior (A), posterio (P), gonad (G), mesonephros (M). A single observation by wmISH was made.

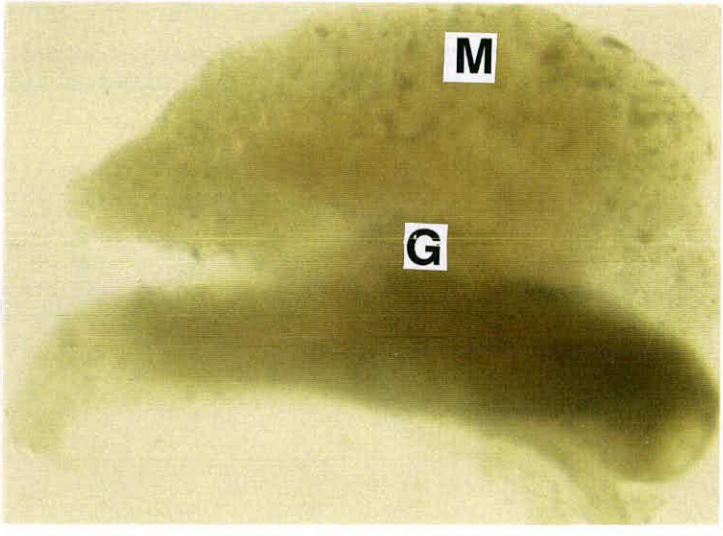
(a)



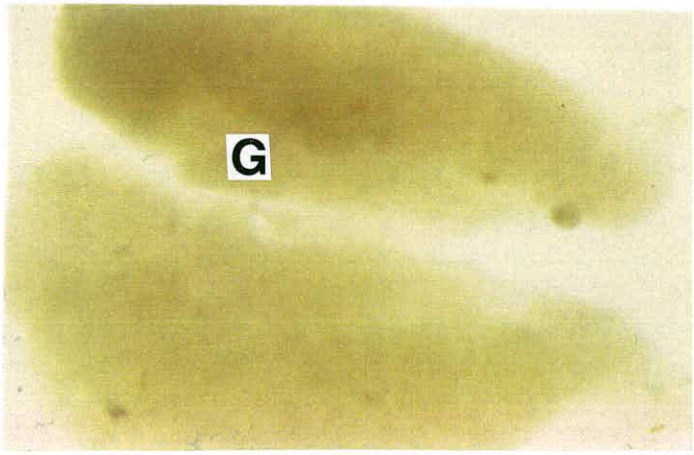
(b)



(c)



(d)



both sexes at 6.5d (fig 58a & b). In the male, expression levels were highest in the gonads and the cortex of the mesonephros. In the female, 1007 expression levels were higher in the posterior of both gonads (fig 58b). No expression was observed in the female or male genital ridge or mesonephros when using the sense probe (fig 58d).

8.3.9. Clone 1111.

Northern analysis, using clone 1111 as a probe, detected a transcript of approximately 1.7kb which is greater than the reported size of rat TBP-1 cDNA (1459bp) (Makino, 1996) and the cDNA size of rat *Sata* cDNA (1627bp) (Rivkin *et al.*, 1996). A transcript was detected in every male and female sample on the Northern blot; and in the adult ovary and adult testis. After normalisation and on the Northern blot, the female samples were seen to have higher levels of expression at all developmental stages compared with the male samples (fig 59A & B). This indicates a sexually dimorphic expression pattern for this transcript, at all developmental stages studied. This expression pattern, however, does not match the result obtained on the display gel.

The expression of this clone was also analyzed in various embryonic tissues and showed a similar sized transcript (data not shown). Expression was detected in all tissues studied at 5.5d. Expression levels at 6.5d and 7.5d were much lower, with possibly highest levels in the kidney at 7.5d. A transcript was detected in the majority of tissues analyzed at 8.5d.

8.3.10. Clone 1144.

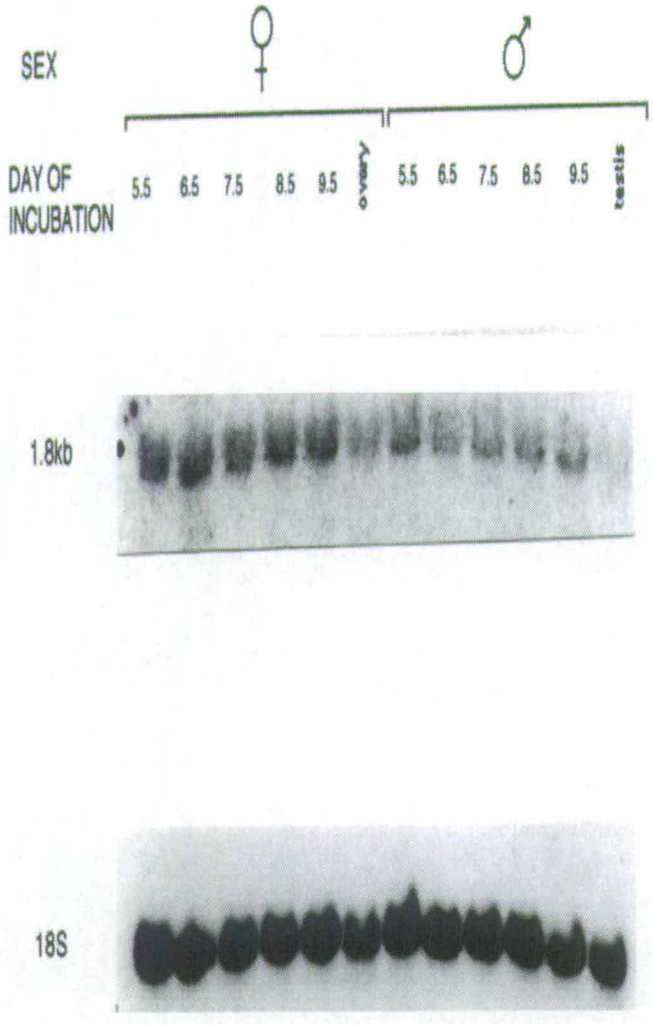
A major transcript of approximately 1.9kb and a minor transcript of 3.9kb were detected by Northern analysis. The smaller transcript corresponds to the size of the reported rat cDNA for TCP-1 (1831bp) (Morita *et al.*, 1991). The major transcript was detected at 4.5d to 9.5d on the Northern blot (fig 60A). After normalisation, expression of the smaller transcript was seen to peak at 5.5d for both males and females (fig 60B). In female and male genital ridge samples, transcript expression levels then showed a gradual decrease to 9.5d. Although this decrease in expression levels does not appear significant by Northern analysis.

Analysis of clone 1144 on a Northern tissue blot revealed the same two transcripts were present (data not shown). Highest levels of expression of the major transcript were observed at 5.5d and in the adult testis. A lower level of expression was seen at 6.5d, which increased at 7.5d and 8.5d in brain, heart, kidney and lung. Analysis of expression levels in the adult ovary and testis showed much higher levels

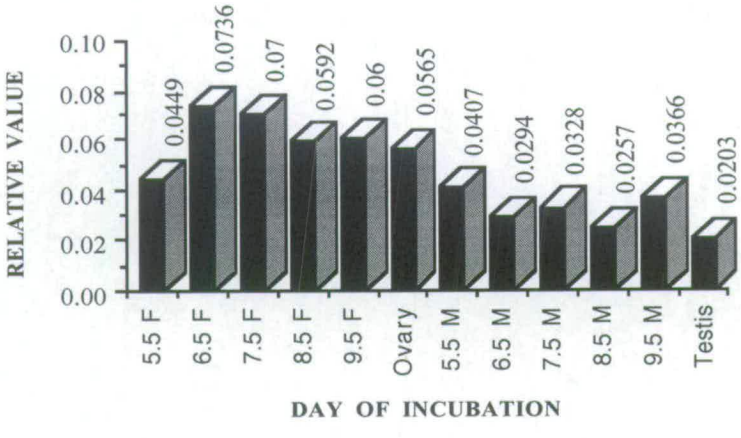
Figure 59: (A) Northern analysis of display clone 1111 in the gonads of developing male and female chick embryos (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected from day 5.5 to day 9.5 of incubation (stage 28 to 36) and from adult ovary and testis. 5μg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 42°C in 2xPIPES, 50% formamide, 0.5% SDS. The blot was washed to a final stringency of 0.2xSSC, 0.1% SDS at 65°C and exposed to film for 2 days at -70°C. The RNA loading in each lane was standardized by hybridizing with a fragment of a chick 18S ribosomal gene (bottom panel). A single observation by Northern analysis was made.

(B) Graph showing the expression profile of display clone 1111, analyzed by Northern analysis. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.

(A)


SEX
♀
♂
DAY OF INCUBATION
5.5
6.5
7.5
8.5
9.5
ovary
5.5
6.5
7.5
8.5
9.5
testis
1.8kb
18S

(B)


RELATIVE VALUE
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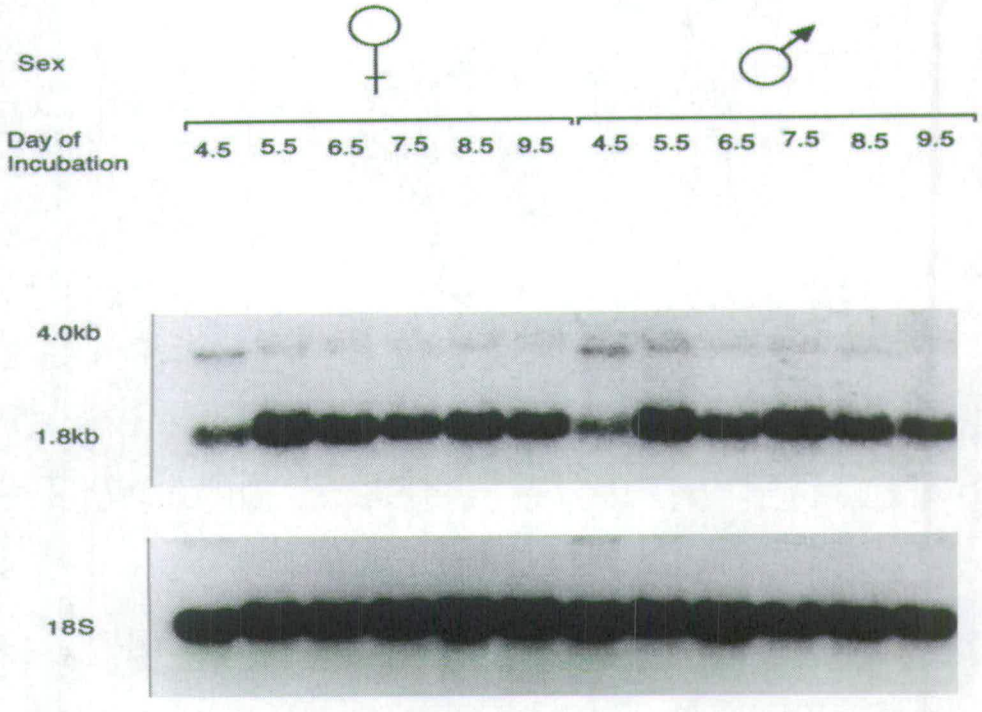
0.04
0.02
0.00
0.0449
0.0736
0.07
0.0592
0.06
0.0565
0.0407
0.0294
0.0328
0.0257
0.0366
0.0203
5.5 F
6.5 F
7.5 F
8.5 F
9.5 F
Ovary
5.5 M
6.5 M
7.5 M
8.5 M
9.5 M
Testis
DAY OF INCUBATION

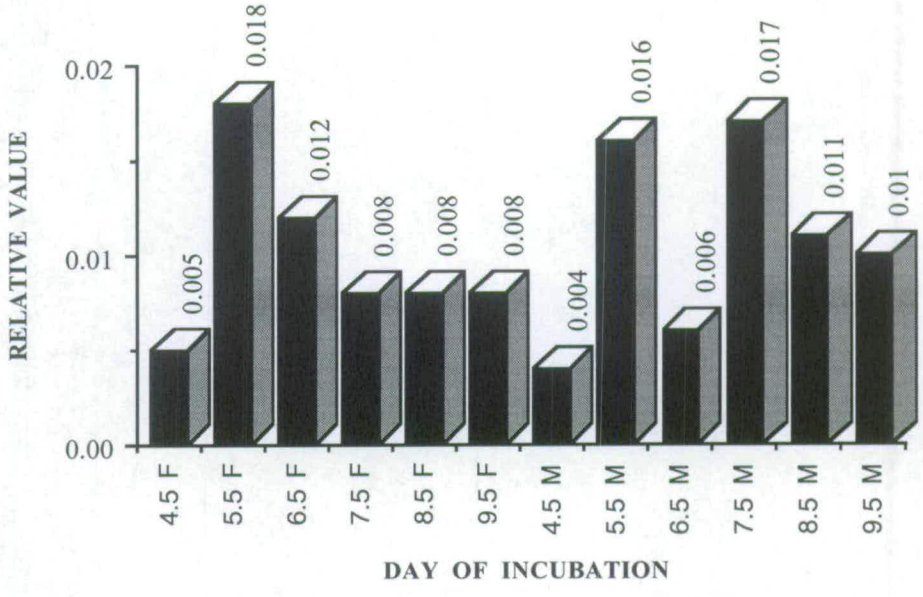
Figure 60: (A) Northern analysis of display clone 1144 in the gonads of developing male and female chick embryos (top panel). Total RNA was isolated from pooled male and pooled female genital ridge/mesonephric tissue collected at days 4.5 to 9.5 of incubation (stage 25 to 36), and 5µg of each sample was separated on a 1% agarose/formaldehyde gel. Northern hybridization was carried out at 42°C in 2x PIPES, 50% formamide, 0.5% SDS. The blot was washed to a final stringency of 0.2xSSC, 0.1% SDS at 65°C and exposed to film for 2 days at -70°C. The RNA loading in each lane was standardized by hybridizing with a fragment of the chick 18S ribosomal gene (bottom panel). Northern analysis was carried out twice, on the same RNA samples, with consistent results.

(B) Graph showing the expression profile of display clone 1144, analyzed by Northern. The amount of radioisotope bound in each lane was quantitated using a phosphorimager and related to a loading control. F = female. M = male.

(A)


Sex
Day of Incubation
4.5 5.5 6.5 7.5 8.5 9.5 4.5 5.5 6.5 7.5 8.5 9.5
4.0kb
1.8kb
18S

(B)


RELATIVE VALUE
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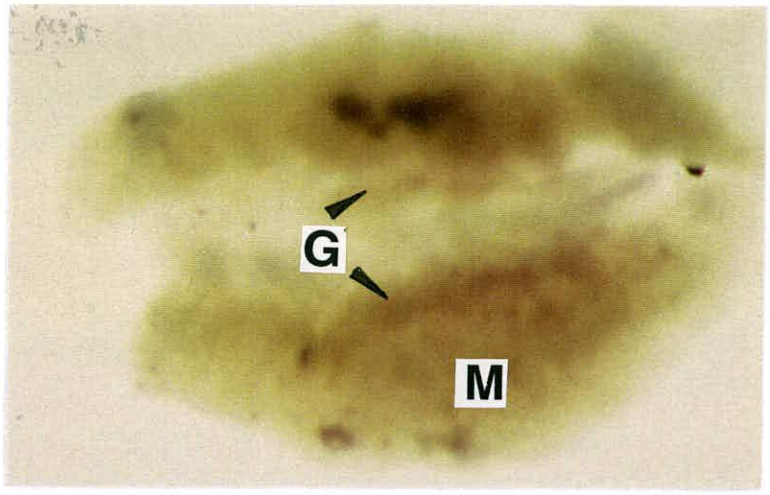
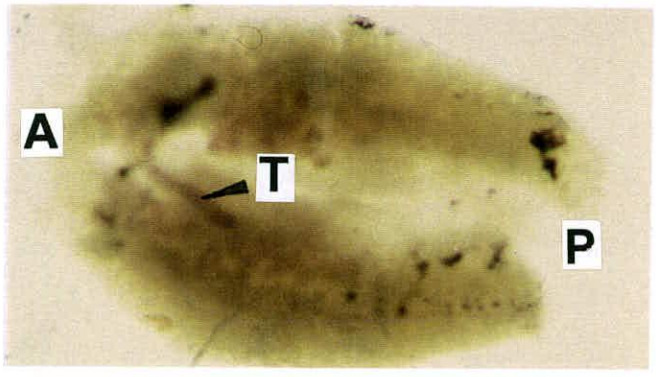
0.012
0.008
0.008
0.008
0.004
0.016
0.006
0.017
0.011
0.01
4.5 F
5.5 F
6.5 F
7.5 F
8.5 F
9.5 F
4.5 M
5.5 M
6.5 M
7.5 M
8.5 M
9.5 M
DAY OF INCUBATION

Figure 61: Expression of display clone 1144 in the female chick embryonic gonad and mesonephric tissue at day 5.5 and day 6.5 of incubation (stage 28 t 30) by wmISH. Hybridization was performed using an anti sense DI labelled 1144 riboprobe at 66°C. Colour development proceeded fo 20hr. (a) Front view of Day 5.5 female gonadal and mesonephri tissue. Actual length of the mesonephros is 3.5mm. (b) View of th reverse side of day 5.5 female mesonephris tissue. Actual length of th mesonephros is 3.5mm. (c) Front view of day 6.5 female gonadal an mesonephric tissue. Actual length of the mesonephros is 4mm. (d View of the reverse side of day 6.5 female mesonephris tissue. Actua length of the mesonephros is 4mm. Anterior (A), posterior (P), gona (G), mesonephros (M), proposed tubule structure (T). Analysis b wmISH was carried out twice with consistent results.

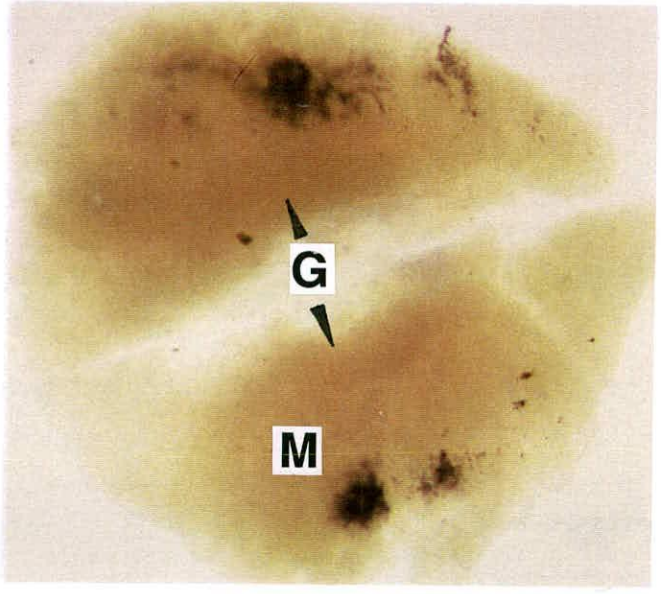
(a)



(b)



(c)



(d)

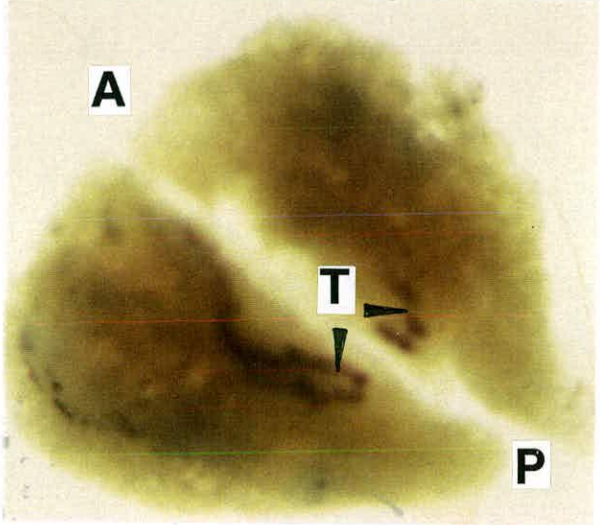
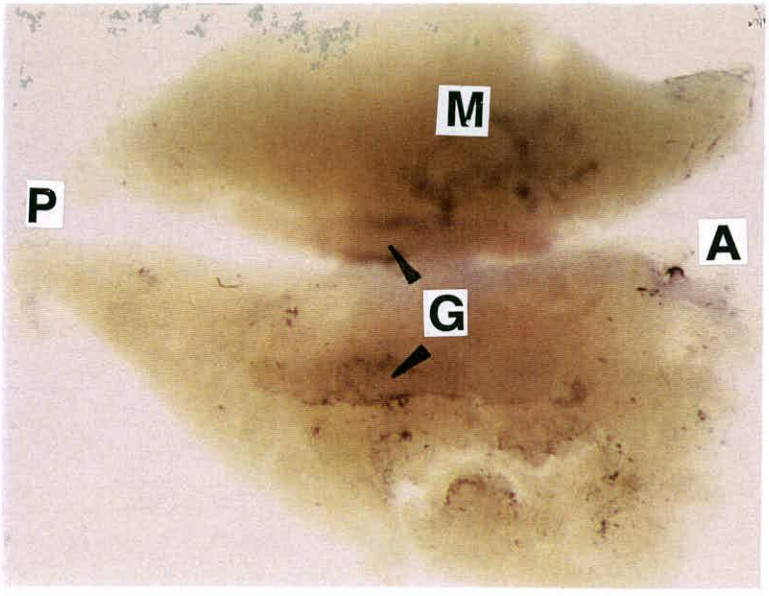
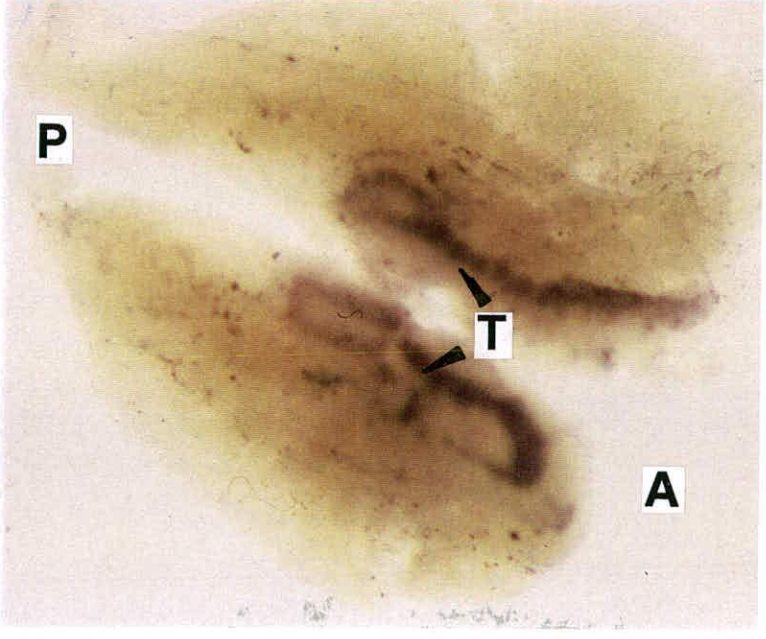


Figure 62: Expression of display clone 1144 in the male chick gonadal an mesonephric tissue at day 6.5 of incubation (stage 30) by wmISH Hybridization was performed using an anti-sense DIG labelled 114 riboprobe at 66°C. Colour development proceeded for 20hr. (a) Fron view of the male gonads and mesonephric tissue. Actual length of th mesonephros is 4mm. (b) View of the reverse side of the mal mesonephric tissue. Actual length of the mesonephros is 4mm Anterior (A), posterior (P), gonad (G), mesonephros (M), propose tubule structure (T). Analysis by wmISH was carried out twice wit consistent results.

(a)



(b)



of expression were present in the testis. Lower levels of expression of the minor transcript were detected in all tissues except adult ovary and testis.

To perform wmISH, an anti-sense 1144 probe of 386bp and a sense probe of 356bp were used. No specific signal was detected in any genital ridge and mesonephros tissue from male and female embryos, analyzed using a sense probe.

At 5.5d, expression of clone 1144 was detected in the male gonads and the anterior two thirds of the mesonephric tissue (data not shown). In the female, expression was detected in both gonads. A dorsal view of both male and female mesonephric tissue showed low levels of expression in a tubule structure in the anterior of the mesonephros (fig 61a & b).

At 6.5d, 1144 expression levels were low in the male gonads and the medulla of the mesonephros (fig 62a). On the dorsal side of the male mesonephros, expression of 1144 was detected in a tubule structure, beginning at the anterior end of the mesonephros and extending two thirds posteriorly before looping back (fig 62b). In the female, no expression was detected in the gonads or the mesonephros (fig 61c). The dorsal side of the female mesonephros also exhibited expression of 1144 in a tubule structure, but at lower levels and not as extensively as in the male (fig 61d). It was impossible to confirm whether expression could not be detected as a result of the tubule not developing or being lost or damaged during the dissection procedure.

8.3.11. Clone 1161.

By Northern analysis, probes derived from clone 1161 failed to detect expression in either the genital ridge or other embryonic tissue, between 4.5d and 9.5d.

Chapter 9.

Discussion.

Isolation and characterisation of one clone that exhibits differential expression.

Transcript 774 was chosen for further study due to the interesting transient sexually dimorphic expression pattern observed on the display gel, which was replicated exactly by Northern analysis and wmISH, and indicated a possible role in the initial stages of testicular development. Higher levels of expression of the larger transcript (3.8kb) was observed in the male genital ridge/mesonephric tissue at 7.5d. By whole mount *in situ* hybridization, expression was detected in the male gonads and the medulla of the mesonephros at 7.5d. In the female, at the same stage of development, expression was detected only in the medulla of the mesonephros. No transcript of a similar size was detected in other embryonic tissues from 5.5d to 8.5d.

Surprisingly, 774 exhibited 99% homology at the nucleotide level to a portion of the chick mitochondrial genome. However, the size of the sexually dimorphic transcript did not correspond to any of the known transcript sizes reported for the chick mitochondrial genome (Desjardins and Morais, 1990). It has been documented that transcription of the mammalian mitochondrial genome results in a polygenic transcript, which releases mRNAs and rRNAs after cleavage and folding of tRNAs (Clayton, 1991; Wallace, 1992; Ragnini and Frontali, 1994). Unlike mammalian mitochondrial transcription, initiation of both strands in the chick mitochondria begins from a bi-directional promoter (L'Abbé *et al.,* 1991). However, the proposed generation of a polygenic transcript is still likely to occur in birds. Therefore, it is possible that the 3.8kb transcript is made up of a number of chick mitochondrial transcripts that have not yet been completely processed.

Prior to the display analysis it was decided that one transcript isolated by differential display would be analysed further. To cover all possibilities in terms of developmental day and sex, preparations for the isolation of a full length cDNA would require the construction of approximately 12 cDNA libraries prior to the display analysis. The construction of this many cDNA libraries was impractical, therefore, it was proposed that a genomic library be screened to isolate a longer transcript of any selected clone.

Two clones were isolated that extended both 3' and 5' from the original 774 transcript. These two clones overlapped and covered an area of 5078bp. Homology of these clones to regions of the chick mitochondrial genome are shown in the schematic figure 45. The 8.3.1.3 transcript was 4.1kb in length, and was digested into three fragments of: 496bp, 2301bp and 1309bp. The expression profile of transcripts encoded by these three fragments and the 19.2.1.5 clone (1322bp) were analyzed by Northern analysis.

Transcript 8.3.1.3 (496) did not exhibit the same expression profile as the 774 transcript. This result was unexpected because clone 8.3.1.3 (496bp) included

homology to the original 774 probe. Transcript 8.3.1.3 (1309bp) detected three transcripts by Northern analysis, but none displayed an expression profile similar to that observed with clone 774. A probe derived from 8.3.1.3 (2301) detected two minor and two major transcripts, but none showed the same expression pattern as detected on the display gel.

One transcript detected by 19.2.1.5 of 3.3kb, exhibited an expression profile similar to that observed with transcript 774. The transcript was of a similar size to the transcript size detected using clone 774. This suggested that a transcript containing sequences corresponding to: cytochrome c oxidase I (CCO I), NADH dehydrogenase II (ND II) and various tRNAs were up-regulated in male genital ridge/mesonephric tissue at 7.5d. Differential expression of cytochrome c oxidase subunits has been reported in different mouse tissues (Kim and Song, 1996), and between the sexes in rat adrenal cortex (El-Migdadi *et al.,* 1995) indicating that differential expression of mitochondrial transcripts and tissue- and organ-specific regulation of ATP metabolism can occur. Alternatively, the increase of these transcripts could be due to an increase in mitochondrial number in this tissue, and not necessarily an up-regulation of transcription of existing mitochondria.

Oxidative phosphorylation (OXPHOS) consists of five enzyme complexes, made up from mitochondrial and nuclear genes. ND II is a member of complex I and CCO I and CCO II are members of complex IV, both complexes are involved in the electron transport or respiratory chain (Wallace, 1992; Alberts *et al.,* 1994). The potential up-regulation of these transcripts at 7.5d in male genital ridge samples indicates a higher requirement for ATP production at this point in testis differentiation and growth compared with ovarian differentiation. It has previously been reported that expression levels of both nuclear and mitochondrial OXPHOS genes can change during development, cell growth and neoplastic transformation (Torroni *et al.,* 1990; Kurauchi *et al.,* 1995).

An increased requirement of energy in the testis might result in a mechanism that co-ordinates the expression of mitochondrial- and nuclear-encoded respiratory enzyme genes. One possible means to achieve this could be via steroid and thyroid hormones (Sekeris, 1990; Demonacos *et al.,* 1996; Lucas and Granner, 1992). Hormone response elements (HREs), which mediate hormonal regulation of gene transcription, have been identified in nuclear-encoded mitochondrial genes (Lucas and Granner, 1992). Partial homology to glucocorticoid and estrogen response elements (GREs and EREs) have been identified in the mouse and human mitochondrial genome, within the D-loop and in structural genes (Sekeris, 1990; Demonacos *et al.,* 1996). It has been previously shown that imperfect palindromic HREs are able to

confer hormone inducibility on a gene, even though it may weaken the affinity of the hormone receptor for the HRE (Berry *et al.*, 1989). Studies carried out by Demonacos *et al.* (1996) suggest that steroid and thyroid hormones have a primary action on regulating mitochondrial gene transcription. This is based on: 1) the localisation of HREs to various regions of the human, mouse and bovine mitochondrial genome; 2) the detection of glucocorticoid receptor (GR) translocation into the mitochondria after injection of rat liver with dexamethasone; 3) the *in vitro* binding of GRs to the putative mitochondrial GREs, within CCO I and CCO III genes, and 4) the activation by glucocorticoids of a CAT reporter gene linked to the thymidine kinase promoter containing partially homologous GREs identified in the mitochondrial genome (Demonacos *et al.*, 1996). The identification of regulatory regions in nuclear and mitochondrial oxidative phosphorylation genes and these regulatory elements being recognised by the same protein receptors, would support this theory (Suzuki *et al.*, 1991).

It is possible, therefore, that different hormones regulate different mitochondrial genes via HREs. This would mean differential regulation of OXPHOS subunits, and this regulation could be specific to certain tissues depending upon hormone secretion. Therefore, mitochondrial genes in the male developing chick gonad could be responding to different hormone levels compared to the female, resulting in an up-regulation of certain transcripts. Also, as mitochondrial initiation occurs within the D-loop, HREs identified in structural genes could act as enhancers for transcription or the termination of mitochondrial transcription could be hormonally regulated (Demonacos *et al.*, 1996).

Clone 19.2.1.5 hybridised to five transcripts. This is more than was detected by Northern analysis using the clone 774 as a probe. These additional transcripts are most likely partially processed polygenic transcripts from the chick mitochondrial genome and are detected because of the longer length probes, which have homology to more than one chick mitochondrial gene. It is interesting to note that although mitochondrial genes are proposed to give rise to polygenic transcripts, this study has shown that a probe (encompassing a small portion of the chick mitochondrial genome) can detect different transcripts with differing expression profiles. This would not be expected if the polygenic transcript encompassed the entire or the majority of the genome. However, Lonergan and Gray (1996) have reported detection of an open reading frame (ORF) encoding CCO I and CCO II, which gives rise to a single transcript. The mature CCO I and CCO II proteins are then proposed to be translated from this single transcript.

Alternatively, identification of novel transcripts in the ATPase 8, ATPase 6 and CCO III region of the human mitochondrial genome, which do not correspond to the existing mitochondrial transcripts (Nardelli *et al.*, 1994), suggests that some of the transcripts observed in this study by Northern analysis might not be the previously documented genes of the chick mitochondrial genome. Identification of novel transcripts from human and rat mitochondria has also been reported by Sbisà *et al.* (1992) and Koga *et al.* (1993). The presence of an alternative transcript is possible because clone 774 exhibited expression in both testes, but not in the ovaries at 7.5d. And it would be expected that both ovaries and testes require ATP during their development and differentiation.

A final possibility is that the display clone 774 isolated a mitochondrially-derived sequence that has become inserted into genomic DNA. This has previously been reported by Hirano *et al.* (1997), Wallace *et al.* (1997) and Davis II and Parker Jr (1998). Therefore, the sexually dimorphic difference detected on the display and by Northern analysis might not represent mitochondrial transcripts, but a nuclear transcript containing a portion of a mitochondrial sequence. The attempt to isolate a full-length genomic clone resulted in the incorrect isolation of additional mitochondrial genes, due to the high proportion of mitochondrial sequences in this genomic library. This theory is supported by 19.2.1.5 not exactly replicating the expression profile of clone 774 , as observed by Northern analysis.

Chapter 10.

Discussion.

Expression profiles of transcripts isolated by differential display from he developing chick genital ridge.

This study was carried out with two main aims: 1) an attempt to isolate novel transcripts involved in chick gonadal development using the technique of differential display and 2) to assess the suitability of using differential display in the isolation of developmentally regulated genes from such a complex developmental system. Clones were isolated for transcripts that displayed developmental regulation, and transcripts which displayed sexually dimorphic or sex specific expression. Of the twelve clones isolated: four failed to detect expression by Northern analysis or wmISH and so differential expression could not be confirmed. Four of the display clones replicated the expression pattern observed on the display gel, by Northern analysis or wmISH. The remaining four clones exhibited differential expression by Northern analysis, but did not confirm the differential expression detected on the display gel.

No confirmation of differential expression.

For four of the clones (776, 777, 779, 1161), no transcripts could be detected in the genital ridge tissue by Northern analysis or by wmISH. This suggests that these transcripts are of low abundance and are unable to be detected by either of these two techniques. The display profile of transcript 776 in the male genital ridge/mesonephric tissue showed an increase in expression levels as developmental time increased. This contrasted with expression detected in female samples, where expression levels decreased as development proceeded. The expression of this transcript was not gonadal specific and was detected in various other tissues. Differences in expression of this transcript suggest gene regulation differences in male gonadal tissue compared with female gonadal tissue and other embryonic tissues.

The display expression profile of transcript 777 was only detected in the genital ridge tissue at 3.5d in males and females. This transcript did not exhibit homology to any sequences present in the databases. Therefore, studies carried out at present indicate this transcript is transiently expressed in males and females and has a potential role in indifferent gonad or mesonephric development.

Transcript 779 did not exhibit homology to sequences present in the databases. Expression was detected in male genital ridge sample at 3.5d on the display gel. A transcript was also detected in the brain at 4.5d to 8.5d and in the adult ovary and testis. By wmISH at 3.5d, expression was detected at significantly higher levels and with a less restricted pattern of expression in the male compared to the female. However, the result observed is probably an artifact due to the time length required for colour development (3 days). As the identity of this transcript is unknown, it is difficult to theorise as to the function of this transcript in chick gonadal development. By 3.5d, the urogenital ridge will begin to arise. Transient expression of this transcript with higher expression levels in male embryos prior to morphological

differences suggests a potential role in chick gonadal development and possibly the sex determination mechanism. However, Southern analysis indicated transcript 779 was not located on either of the sex chromosomes, which argued against a role as the primary sex determining switch. However, from the display analysis, it suggests the 779 transcript has an important male-biased function, due to the higher levels of expression at 3.5d and in the adult testis. It is interesting to note that at this early stage of development differences in transcript expression are already apparent between males and females.

Expression levels of the display transcript 1161 were highest in female genital ridge samples at 5.5d. The 1161 transcript was only 75bp in length, possibly too short to make a probe that can be detected by Northern analysis or wmISH. Also, the short length of the isolated transcript resulted in no significant match to any of the sequences present in the databases. As this expression profile can not be confirmed, it can only be speculated that this transcript is expressed as a consequence of the sex determination event and may have a role in ovarian differentiation.

Transcripts that confirm differential expression.

Four of the transcripts isolated by differential display replicated the display pattern by Northern analysis and/or wmISH. One of these transcripts (775) had 100% homology at the nucleotide level to chick rho-globin (Roninson and Ingram, 1982), an embryonic form of β-globin. The expression pattern observed by Northern analysis corresponds to the well documented developmentally regulated profile of this gene (Bruns and Ingram, 1973; Lois and Martinson, 1989; Minie *et al.*, 1992; Roninson *et al.*, 1982). Rho-globin is expressed in the primitive erythrocytes, produced in the chick embryo between 3d and 6d. By 6d the definitive erythrocytes begin to be produced, expressing the adult form of β-globin but not the embryonic rho-globin. The expression levels of rho-globin, in our analysis, are coincident with the maximal production of primitive erythrocytes, which is followed by the progressive replacement by definitive erythrocytes. Expression of rho-globin after 6d is due to some primitive erythrocytes remaining in the foetal circulation. The isolation of a well documented transcript which exhibits differential expression during chick embryogenesis demonstrates the effectiveness of differential display in the isolation of differentially regulated transcripts from a complex developmental system. This effectiveness is supported by the isolation of other transcripts, some known and some novel transcripts, by differential display which have confirmed their differential expression profile by Northern analysis or wmISH. Other transcripts with an expression profile confirming the differential display profile were: 771, 774 and 1001.

Transcript 771 exhibited 96% homology to human RhoA at the amino acid level (Yeramin *et al.*, 1987). Expression of this transcript by Northern analysis replicated exactly the expression profile observed on the display gel. By Northern analysis three different transcripts were detected. Two of the transcripts corresponded to previously published transcripts sizes for RhoA (Moscow *et al.*, 1994). These different transcripts exist due to the presence of multiple polyadenylation signals in the 3' untranslated region of RhoA. A third transcript size has been reported of 1.2kb (Moscow *et al.*, 1994) and the transcript size in genital ridge samples corresponds to approximately 0.9 kb to 1.2kb. The different isoforms of RhoA, detected by Northern analysis, could have different functions at different stages of gonadal development. Expression of 771 is not gonadal specific as transcripts were detected in various other embryonic tissues.

By wmISH, 771 was detected in the developing intermediate mesoderm at 3.5d. At later stages of development, expression was detected in the testes and ovaries, with slightly lower levels of expression being detected in the right ovary and right testis, indicating a potential role for RhoA in the asymmetric growth and differentiation of the female chick left ovary and the male left testis. At this stage of development in the chick, the testes and left ovary will be entering a period of rapid growth and differentiation, involving cell shape changes and migration. However, in the chick right ovary development does not occur and the structure has begun to regress by this stage; which corresponds to the lower levels of RhoA expression. Also, in the male chick, the left testis is always slightly larger than the right, indicating the asymmetry in chick gonadal development. It is possible there is a down-regulation of RhoA in the right gonad or an up-regulation in the left gonad, because expression levels on both the left and right side of the 3.5d embryo were similar.

RhoA is a member of the Ras superfamily of GTP-binding proteins (Ridley, 1997), which are able to transduce signals to effector proteins and regulate cell growth and differentiation under the influence of growth factors (Vojtek and Cooper, 1995). It is, therefore, possible that RhoA has a role in chick urogenital ridge and gonadal development involving regulation of transcription via growth factors.

Members of the Rho family have been implicated in the regulation of the actin cytoskeleton and actin polymerisation (Vojtek and Cooper, 1995; Tanaka and Takai, 1998; Ridley, 1997; Symons, 1996; Hotchin and Hall, 1995; Hall, 1998; Van Aelst and D'Souza-Schorey, 1997). In particular RhoA is proposed to be involved in the regulation of actin stress fibres and focal adhesion complexes (Hotchin and Hall, 1995; Hall, 1998). Regulation of the actin cytoskeleton is essential for cell motility, migration, polarisation and differentiation and may possibly indirectly affect gene

transcription (Hotchin and Hall, 1995; Symons, 1996; Barrett *et al.*, 1997; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998).

Embryonic development requires changes in cell polarity, cell shape, cell migration and gene expression. Therefore, it is pertinent to consider the occurrence of co-ordinated changes in gene transcription and actin cytoskeleton. An example of this is cell polarity in the *Drosophila* eye, controlled by the *Frizzled (Fz)* receptor (Strutt *et al.*, 1997). RhoA is proposed to act downstream of the *Fz* receptor and mediate tissue polarity changes necessary for correct ommatidia arrangement in the eye (Strutt *et al.*, 1997). A second example of co-ordinated gene expression, cell polarity changes and actin cytoskeleton changes in *Drosophila* and yeast via RhoA have also been documented (Barrett *et al.*, 1997; Drgonová *et al.*, 1996; Qadota *et al.*, 1996). In the mammalian testis, cell morphological changes in co-ordination with changes in gene expression are suggested to occur during the formation of the testicular cords. In this instance, morphological and structural changes in the testis and the formation of the basement membrane around the testicular cords could affect or be affected by gene expression (Capel, 1996). If RhoA is involved in cell shape changes, it would be logical for RhoA to be expressed in the developing gonads due to: 1) differences in cell migration and cell morphology that probably occur as general development and differentiation take place and 2) differences in cell shape due to different morphology and function of the tissue between male and female gonads.

Transcript 1001 exhibited 97% homology at the nucleotide level to the 16S transcript of the chick mitochondrial genome. The 16S rRNA subunit complexes with the 12S subunit to form a mitochondrial ribosome required for translation of mitochondrial genes involved in oxidative phosphorylation (OXPHOS) (Wallace, 1992; Alberts *et al.*, 1994). Expression of this transcript by Northern analysis reproduced the expression profile observed on the display gel in the male, where expression levels exhibited a general increase to 9.5d in male genital ridge tissue, but was also detected at other developmental days in both sexes. However, in the female by Northern analysis, there was also a general increase in 1001 expression levels which was not observed on the display gel. Expression of 1001 was also detected in other embryonic tissues, with increasing expression levels coincident with increasing developmental age. This indicates the embryo in general requires more of the 16S rRNA subunit transcript as it develops, possibly due to an increase in energy requirement which is generated by the oxidative phosphorylation pathway. As differentiation proceeds, and structural changes and cell morphogenesis continue in the embryonic gonads, an increase in energy requirement is a reasonable assumption.

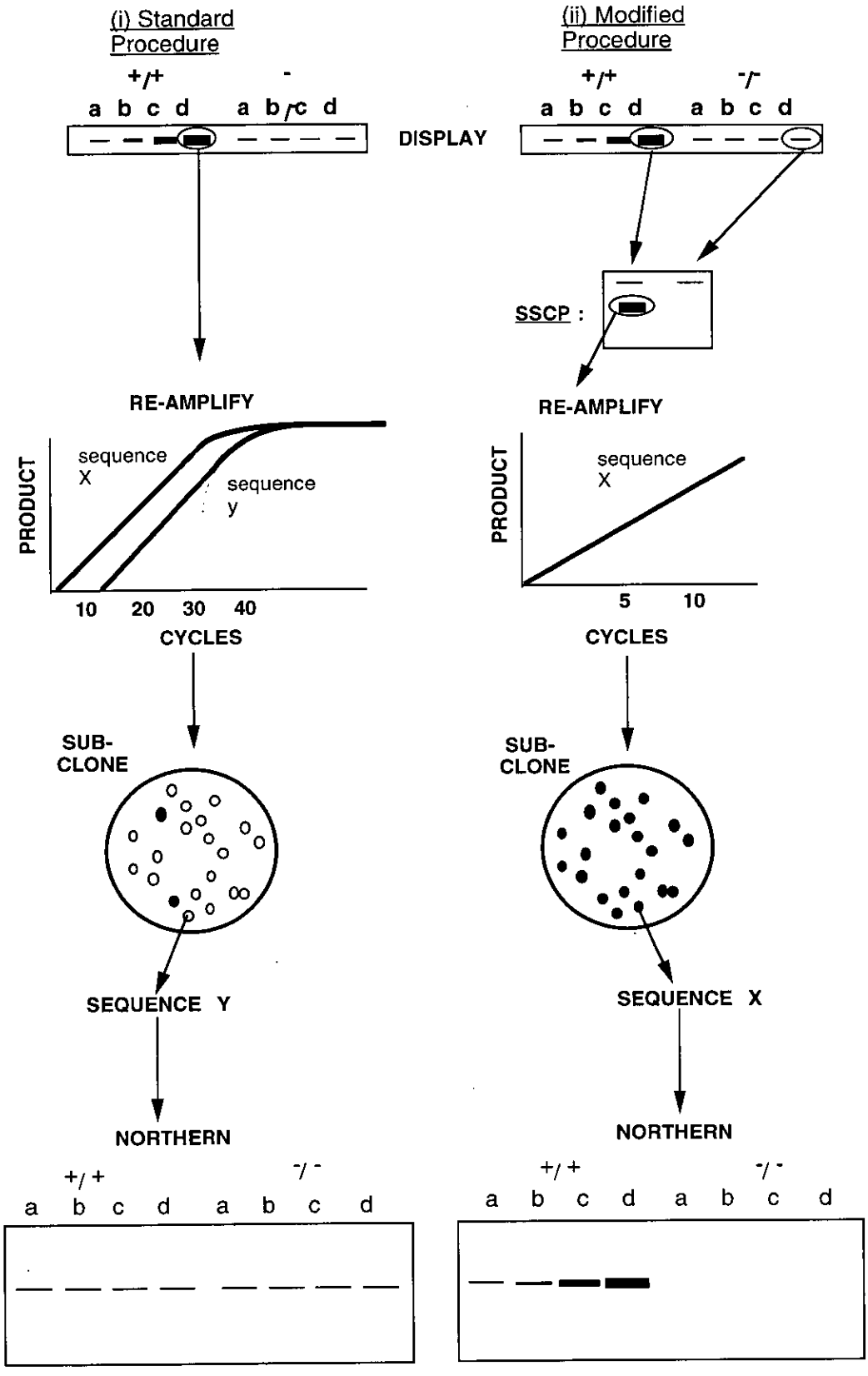


Figure 63: Schematic representation for the rationale for introducing the SSCP step into the differential display procedure. Reamplification using 40 cycles may result in equivalent concentrations of the band of interest and the co-migrating sequence. If cloning efficiency is better for the co-migrating sequence, it may indicate that this is the only sequence present, but confirmation of differential expression will not be observed. If SSCP is introduced into the procedure, identification of the correct sequence can be made and thus confirm differential expression by Northern analysis (or other methods). Figure from Miele *et al*. 1998.

Transcripts that do not confirm differential expression.

Four of the transcripts isolated and which exhibited differential expression on the display gel, did not exhibit the same expression pattern by Northern analysis. These transcripts were: 772, 1007, 1111, 1144. There are a number of reasons as to why Northern or whole mount *in* situ analysis did not confirm the differential expression pattern observed on the display gel. Firstly, there could be differences created in the RNA extraction procedures. This could lead to a false positive being generated, where differences in expression levels are observed that are not real. Secondly, a co-migrating transcript might have been isolated from the display gel. The reamplification procedure carried out using our differential display method has 40 cycles. Therefore, any DNA excised from the display gel will be amplified to the same extent as the band of interest. The wrong transcript will be analyzed to confirm differential expression if the cloning efficiency of the co-migrating transcript is higher than the cloning efficiency of the band of interest, so that the wrong transcript is over-represented in the sub-clone population (fig 63). Thirdly, DNA contamination in the reamplification PCR procedure could result in the wrong candidate being cloned, in the same way as described above. In fact, the reamplification procedure carried out in this study did not always result in one band being observed, indicating more than one sequence was present.

The actual RNA fingerprint obtained by DDRT-PCR was probably an accurate representation of gene expression in the developing chick gonads. Unfortunately, the subsequent analysis of these clones of interest was hampered by the difficulties encountered in the isolation procedure. These isolation difficulties resulted in a number of "wrong" clones being analysed, even though the expression profile of these "wrong" clones was potentially interesting with regard to chick gonadal development.

The main problem with the differential display procedure occurs with the co-migratory sequences which can be reamplified with the band of interest, and thus produce multiple products of similar or identical size. Recently the technique of single-stranded conformation polymorphism (SSCP) has been modified to aid in the isolation of the band of interest from a display gel (Mathieu-Daudé *et al.*, 1996; Miele *et al.*, 1998). SSCP was first developed to distinguish between point mutations in molecules that were otherwise identical (Hayashi , 1991). It was based on the fact that single stranded DNAs are able to fold into a relatively stable secondary structure which affects their mobility on polyacrylamide gel. The secondary structure they form depends upon the sequence of the molecule (Mathieu-Daudé *et al.*, 1996). Therefore, if the reamplification products contain multiple sequences of the same length, they can be separated by SSCP. Miele *et al.*, (1998) have also introduced a 5 cycle

reamplification step after the SSCP procedure, to minimalise the DNA contamination in the reamplification step and to maintain the relative levels of different cDNA species. These improvements to the differential display technique should enable the isolation and analysis of future bands of interest to be less time consuming and less labour intensive.

Transcript 772 was isolated from a display gel where expression was limited to the male genital ridge at 5.5d. This transcript did not exhibit homology to any sequences in the databases. By Northern analysis, the 772 transcript was approximately 3.7kb in length, and it showed a dramatic increase in expression after 4.5d in both male and female samples. The 772 transcript is not gonadal-specific because it was detected in other embryonic tissues. By wmISH, the 772 transcript was detected in the gonads and the mesonephric tissue of both sexes from 4.5d to 6.5d. This did not correlate with the display or Northern analysis expression profiles

Transcript 1007 exhibited highest expression levels at 5.5d in male genital ridge on the display gel, but could also be detected in male samples at the other developmental days studied. Expression was never detected in female samples over the same developmental period by differential display analysis. However, by Northern analysis expression of 1007 was detected in both male and female samples at all stages analysed. By wmISH, expression of 1007 was located in the gonads and mesonephros of both sexes. Transcript 1007 exhibited 73% homology at the nucleotide level to human *mef-2A* (Suzuki *et al.,* 1996). MEF-2A is a member of the myocyte enhancer factor 2 (MEF2) family of transcription factors, which have been shown to play a role in the activation of muscle-specific and growth-factor regulated gene transcription (Olson *et al.,* 1995; Pollock and Triesman, 1991; Shore and Sharrocks, 1995). MEF-2A transcript is reported to be widely expressed, but were most abundant in myotubes, skeletal muscle, heart and brain (Pollock and Triesman, 1991; Yu *et al.,* 1992). This does not correlate with the expression pattern observed in chick embryonic tissues from this study. In contrast, high levels of expression of the transcript were detected in the embryonic and adult ovary and testis tissue.

Yu *et al.* (1992) reported the MEF-2A protein to be restricted to mainly skeletal muscle, smooth muscle, cardiac muscle and the brain. However, Dodou *et al.* (1995) reported expression of the MEF-2A protein in non-muscle and muscle cell lines. This suggests other functions for MEF-2A not specific to muscle cell lineages. This is bourne out in these studies by the up-regulation of 1007 at 5.5d in male and female chick genital ridge/mesonephric tissue.

The actual role of MEF-2A in the gonadal and mesonephric tissue is unknown. However, MEF-2A and MEF-2D have been reported to be up-regulated during the

differentiation of a P19 cell line (which differentiates into endoderm or mesoderm lineages) (Hidaka *et al.,* 1995). It is possible that the initial up-regulation of 1007 at 5.5d in male and female genital ridge tissue could be due to the beginning of differentiation in the gonads. Expression studies by Northern analysis indicates high levels of this transcript are required transiently in the male genital ridge up to 7.5d, after which expression levels decrease. In the female, expression levels remain relatively constant from 5.5d to 9.5d, indicating differential regulation of this transcript during male and female chick gonadal development. Although it is unknown if these differences in expression are significant.

Transcript 1111 exhibited 92% homology at the nucleotide level to rat TBP-1 (Makino, 1996) and rat *Sata* (Rivkin *et al.,* 1996). TBP-1 is an ATPase and a subunit of the 26S proteasome, which is involved in ubiquitin-conjugated protein degradation (Tanaka, 1995; Gottesman *et al.,* 1997). The proteasome consists of a central cylindrical region made up of proteases. At each end of this region are different regulatory subunits (approximately 10 polypeptides, including ATPases) which regulate and specify proteolysis (Alberts *et al.,* 1994; Gottesman *et al.,* 1997). *Sata* is 99% homologous at the amino acid level to human TBP-1.

Transcript 1111 expression profile by display analysis was highest at 5.5d in males. However, by Northern analysis, expression levels were clearly higher in female samples at all embryonic developmental stages studied, and was higher in the adult ovary compared to the adult testis. Expression was also detected in other embryonic tissues, indicating 1111 is not gonadal specific.

From the Northern analysis studies, higher levels of expression of TBP-1 are required in the developing chick ovary compared with the developing testis. It has been proposed that different regulatory subunits of the 26S proteasome are required for selection and initial interactions with the protein substrate that is to be degraded (Gottesman *et al.,* 1997), thus indicating combinations of different regulatory subunits specify for different protein substrates (Gottesman *et al.,* 1997). Tanaka (1995) reported that ATPase subunits are developmentally regulated during programmed cell death in abdominal intersegmental muscle of *Manducta sexta.* Therefore, it is possible that a higher turnover of certain proteins is required during ovarian differentiation compared with testicular differentiation. For example, proteasomes are proposed to regulate the cell cycle via the degradation of mitotic cyclins (Hilt *et al.,* 1993). A human TBP-1 related gene (*Sata* or rtTBP-1) has been isolated from a rat pachytene spermatocyte cDNA library (Rivkin *et al.,* 1997). It was shown to be associated with elongating spermatids, spermatocytes and spermatids in the epithelial region of the

seminiferous tubules, indicating a role in spermatogenesis. However, a role for this gene in the ovary has not been documented.

Transcript 1144 exhibited 84% homology at the nucleotide level to rat TCP-1 (Morita *et al.*, 1991), a subunit of a chaperone, CCT (chaperone containing TCP-1) (Kubota *et al.*, 1995). Chaperones are involved in regulating protein folding pathways, thus preventing misfolding or promoting refolding (Ellis, 1990; Kubota *et al.*, 1995). Transcript 1144 expression profile by differential display analysis exhibited higher levels of expression at 5.5d in female genital ridge, although a transcript could be detected in males and females at other developmental stages. By Northern analysis, two transcripts were detected. The smaller transcript corresponded to the published length of the rat TCP-1 transcript (Morita *et al.*, 1991) and exhibited a dramatic increase in expression levels at 5.5d in both sexes. Expression levels remained fairly high through to 9.5d in both sexes, even though phosphorimager data suggested the transcript was down-regulated. Studies of mouse *Tcp-1* expression has indicated it is up-regulated in the testis, post-implantation embryos, early- to mid-gestation embryos and certain cell lines, where cells are rapidly proliferating (Willison *et al.*, 1986; Kubota *et al.*, 1992). Another proposed function for chaperones is that they complex with steroid receptors via their hormone-binding domains (Pratt, 1993) and promote binding of the steroid to its receptor (Caplan, 1997).

The larger transcript (3.9kb) could be due to the utilisation of different polyadenylation sites, or it might encode a TCP-1 related gene. Additional and larger transcript sizes have been reported for the mouse *Tcp*-1 mRNA (Kubota *et al.*, 1992). Transcript 1144 was not gonadal specific.

As TCP-1 is a subunit of a chaperone, it is possible that the increase in expression at 5.5d in male and female genital ridge is due to an increase in new protein translation required for differentiation of the ovary and testis. By wmISH expression of the 1144 transcript was detected in the gonads and mesonephros of male and female chick embryos. Although expression levels were fairly low by wmISH in comparison to Northern analysis data. On the dorsal side of the mesonephros, the transcript was detected in two tubule structures. These tubule structures are unknown and have not been described previously, but are in the correct location to be nephric tubules.

Expression of transcript 1144 was also analyzed in the adult gonads, with higher levels being detected in the testis. It is possible that the adult testis is a more dynamic structure than the adult ovary, due to spermatogenesis and the continued meiotic division. Therefore, production and turnover of proteins might be greater in the testis at this stage.

TCP-1 is located in the t-complex on chromosome 17 in the mouse (Willison *et al.*, 1986; Kubota *et al.*, 1992). The t-complex is a naturally occurring variation in wild type mouse populations and persists in the mouse genome due to 1) four inversions relative to the wild type chromosome, which suppress recombination and 2) due to transmission ratio distortion (TRD) (Willison *et al.*, 1990). TCP-1 has been implicated in TRD. In this instance, t-complex sperm from a heterozygous mouse preferentially fertilise an egg (Willison *et al.*, 1990). Other genes involved in spermatogenesis are also located in or near the t-complex, such as *Dazla* (Elliott and Cooke, 1997). Analysis of expression of TCP-1 in the mouse testis has identified transcript in the pachytene primary spermatocytes and in the haploid spermatid (Willison *et al.*, 1990), supporting a role for TCP-1 function in the chick testis other than assisting in protein folding.

Overall, the approach to isolate differentially expressed genes from a complex developmental system such as the developing gonads in the chick, by differential display was rational. A number of transcripts exhibiting differential expression on the display gel were isolated and the expression profile successfully confirmed by Northern analysis or wmISH. The fact that different developmental timepoints could be visualised at the same time, enabled a comparison of sexually dimorphic and developmentally regulated genes, and made this comparison easier.

The importance of carrying out longer runs of the display PCR products was indicated by the fact that it was able to resolve higher molecular weight PCR products. This is important for providing more accurate matches to sequence databases and generating more reliable probes with higher specific activity, to confirm differential expression by Northern analysis or other methods. Secondly, a longer run enabled bands of similar size, that migrated closely together on a display gel, to be resolved; thus reducing the number of false positives generated. A new development is the use of a GenomyxLR™ Programmable DNA Sequencer (Beckman, UK), enabling a significantly longer run of display PCR reactions, facilitating the isolation of higher molecular weight PCR products.

Chapter 11.

General Discussion and Future Research.

The overall objective of these studies was to investigate the molecular regulation of chick gonadal development and relate it to the morphological differentiation of the gonads. These studies would be allow an assessment of the level of conservation of early gonadal development between mammals and chicks.

Firstly, the morphology of the developing chick gonads was studied, from the indifferent gonad stage through to the beginning of ovarian and testicular differentiation. This study has shown that slight differences between the male and female chick gonad are first apparent, at the histological level, at 5.5d. This is 2.5 days earlier in development than the sex determination event has been previously documented (Romanoff, 1960). This observation is supported by the detection of AMH in the male gonads by 5.5d, assuming AMH is expressed as a result of the sex determining switch as is the case with mammals. Therefore, by 5.5d in the chick, the fate of the gonad has been decided. This means that future gene isolation and gene expression studies can be more easily categorised for a role in indifferent gonad development, the sex determination process or in ovarian or testicular differentiation.

These histological studies have also shown that, morphologically, gonadal development is essentially similar between mammals and chicks. Expression studies carried out on homologues to genes involved in mammalian gonadal development, indicated similar expression pattern of these transcripts between mammals and chicks. The failure to isolate a SRY homologue in birds (Griffiths, 1991; Tiersch *et al.,* 1991; McBride *et al.,* 1997) implies a conserved gonadal differentiation pathway exists between mammals and birds, differing primarily in the sex determining switch.

Histological analysis of the chick embryonic gonads indicated characteristic signs of testicular differentiation were evident prior to those characteristics of ovarian differentiation. The testicular characteristics include cord formation in the medulla and the thinning of the germinal epithelium. This evidence is further supported by the initial detection of AMH at 5.5d in male gonads compared to 6.5d in female gonads. AMH is proposed to be the first marker of Sertoli cell differentiation in mammals (Münsterberg and Lovell-Badge, 1991). This is not the case in chicks as AMH is expressed in both male and female gonads. However, with differences in expression levels and the initial stage of detection, it acts as a marker for gonadal differentiation in chicks.

A difference between mammals and birds is the expression of AMH in the female chick during embryonic development. This is necessary for the regression of the right Müllerian duct, resulting in the asymmetry observed in the female chick reproductive tract. AMH was expressed at 7.5d in the left and the right ovaries, but with lower levels in the right ovary. Unfortunately, histological analysis of the right

ovary, in this study, was inconclusive. Therefore, we infer from previously documented work (Romanoff, 1960) that although the right ovary never develops to the same extent as the left ovary, it is still functional at the early stages of gonadal development.

Although examination of the histology of the gonads and of the gene expression of homologues to mammalian genes involved in gonadal development indicated that overall a similar gonadal development pathway between mammals and chicks existed; the gene expression data revealed some differences. Most notably was the constant levels of expression of SF-1 and *Sox-9* from 3.5d to 9.5d in the genital ridge tissue of both sexes. Also there were slightly higher levels of expression of WT1 in the male chick genital ridge tissue at 4.5d and 5.5d, prior to and during the first signs of morphological differentiation in the chick gonads. WT1 is not located on either of the sex chromosomes, which 1) argued against a role as the sex determining gene and 2) suggests the higher levels of WT1 expression are not due to lack of dosage compensation of the Z chromosomes. Therefore, there is a potential, additional role for WT1 in male chick gonadal development if this difference in expression is significant. It would be prudent to further investigate the expression pattern of WT1 in the chick gonads, by repeating Northern analysis on different pools of male and female genital ridge RNA to confirm this difference; and by *in situ* hybridization in an attempt to identify the cell types expressing this gene and to investigate if any differences in transcript localisation exist between male and female chick gonads. In addition, further characterisation of the SF-1 cellular localisation in the gonads and mesonephros would contribute to a greater understanding of its role during chick gonadal development and elucidate if WT1 and SF-1 colocalise in the gonads or mesonephros. Finally, protein analysis of these homologues would indicate that these genes have a role and are functional in the tissue types in which their transcripts have been detected.

The application of differential display to identify transcripts involved in chick gonadal development was logical and feasible. The problems encountered with this procedure during this study, and by many others, have largely been overcome with the utilisation of SSCP, which aids in the separation of multiple sequences of the same length generated during the differential display technique. Secondly, the use of a GenomyxLR™ Programmable DNA Sequencer (Beckman, UK), has enabled significantly larger display PCR products to be separated. This results in longer sequence data for comparison with the databases and more reliable probes for confirming differential expression.

However, even without these recent refinements to the technique, this study showed that differentially expressed transcripts could be successfully isolated by differential display from the genital ridge/mesonephric tissue of male and female chick embryos. This strengthened the rationale for utilising differential display for isolation of novel transcripts from complex developmental systems. A significant number of these clones were confirmed as differentially expressed by Northern analysis or whole mount *in situ* hybridization. Due to the close association of the genital ridge to the mesonephros at the early stages of chick gonadal development, it was necessary to include a small portion of mesonephric tissue in with the gonadal tissue at each day analysed. Therefore, it was possible that the display analysis was isolating transcripts only from the mesonephric tissue, due to the empirical amount of mesonephric tissue included. Analysis by whole mount *in situ* hybridization showed that many of the transcripts isolated were expressed in the developing chick gonads, therefore, the display analysis was not isolating transcripts solely from the mesonephric tissue.

Only a small proportion of the potential transcripts expressed in the developing chick gonads have been analysed in this study. Therefore, it is important to continue the differential display analysis with new primer combinations, while incorporating the new refinements into the procedure. This will hopefully enable isolation of other genes with an important role in gonadal development. The application of other gene isolation techniques, such as Representational Difference Analysis (RDA) (Lisitsyn *et al.*, 1993) would also be a method for identifying differentially expressed transcripts and further increase our understanding of the gene regulation that occurs in this complex developmental system.

Another possibility would be to repeat the primer combinations for those clones that exhibited differences on the display gel, but did not show the same expression pattern by Northern analysis or whole mount *in situ* hybridization. In this instance, incorporation of the new refinements to the procedure should result in the isolation of the correct clone from the display gel, which can then be analysed for a role in chick gonadal development. Also, those clones that could not be confirmed as being differentially expressed, due to low expression levels, could be analysed by more sensitive detection methods such as ribonuclese protection assay (RPA) or RT-PCR.

Of the detectable transcripts, 50% replicated the expression profile observed on the display gel. Of these, clone 771 exhibited homology to RhoA, a GTP binding protein. The expression of this clone in the male and female urogenital ridge and in the developing testes and left ovary suggests a possible role in the general development of the gonads. From our data, it seems likely that different isoforms of this protein are

expressed at different levels at different stages during gonadal development. A future possibility would be to isolate transcripts for these different forms and identify differences between these developmentally regulated isoforms. As RhoA appears to signal via a number of different pathways, it would be of interest to establish which other genes in these pathways alter their expression during chick gonadal development.

Another clone that exhibited differential expression by display and Northern analysis was clone 774. The isolation of a genomic clone did not sufficiently elucidate the portion of the chick mitochondrial genome that was up-regulated in the male genital ridge at 7.5d. Also, the identification of mitochondrially-derived sequences in the genome (Hirano *et al.*, 1997; Wallace *et al.*, 1997; Davis II and Parker Jr., 1998) indicate that the mitochondrial transcripts might not be the transcripts of interest that are up-regulated in chick male gonads at 7.5d. Isolation of a full-length cDNA clone might result in the identification of the 3.8kb transcript up-regulated in males at 7.5d. Regardless of the outcome of its identity, the transcript is differentially expressed as a consequence of the sex determination process and obviously has an important role in chick testicular development.

While clone 1144 did not exhibit the same expression profile by Northern as observed on the display gel, it did exhibit an up-regulation of the transcript after 4.5d in both sexes and the transcript was detected in the gonads of male and female embryos. The fact that clone 1144 exhibited homology to TCP-1, which is located in the t-complex involved in fertility, raises the possibility that this clone may have a role in gametogenesis in the chick. Detection of this transcript in other tissues suggests it has a more diverse role in embryonic development. Localisation of the transcript to particular cell types within the gonads and mesonephros might help to elucidate its role in these tissues.

In summary these results have indicated a certain degree of conservation of morphology and gene regulation between the mammalian and chick gonadal development pathway. Figure 64 is a schematic representation of the potential genetic regulation of chick gonadal development by chick homologues to transcripts involved in mammalian gonadal development and by a number of the differential display candidates. The role of these transcripts in chick gonadal development is merely speculative being based on their localisation to the embryonic gonads (in addition to other tissues) and their expression profiles on the display gel. These studies also suggest that sex determination occurs at an earlier time in development than previously reported. This information will facilitate the future assignment of the role of novel transcripts in chick gonadal development. While the isolation of the sex determination

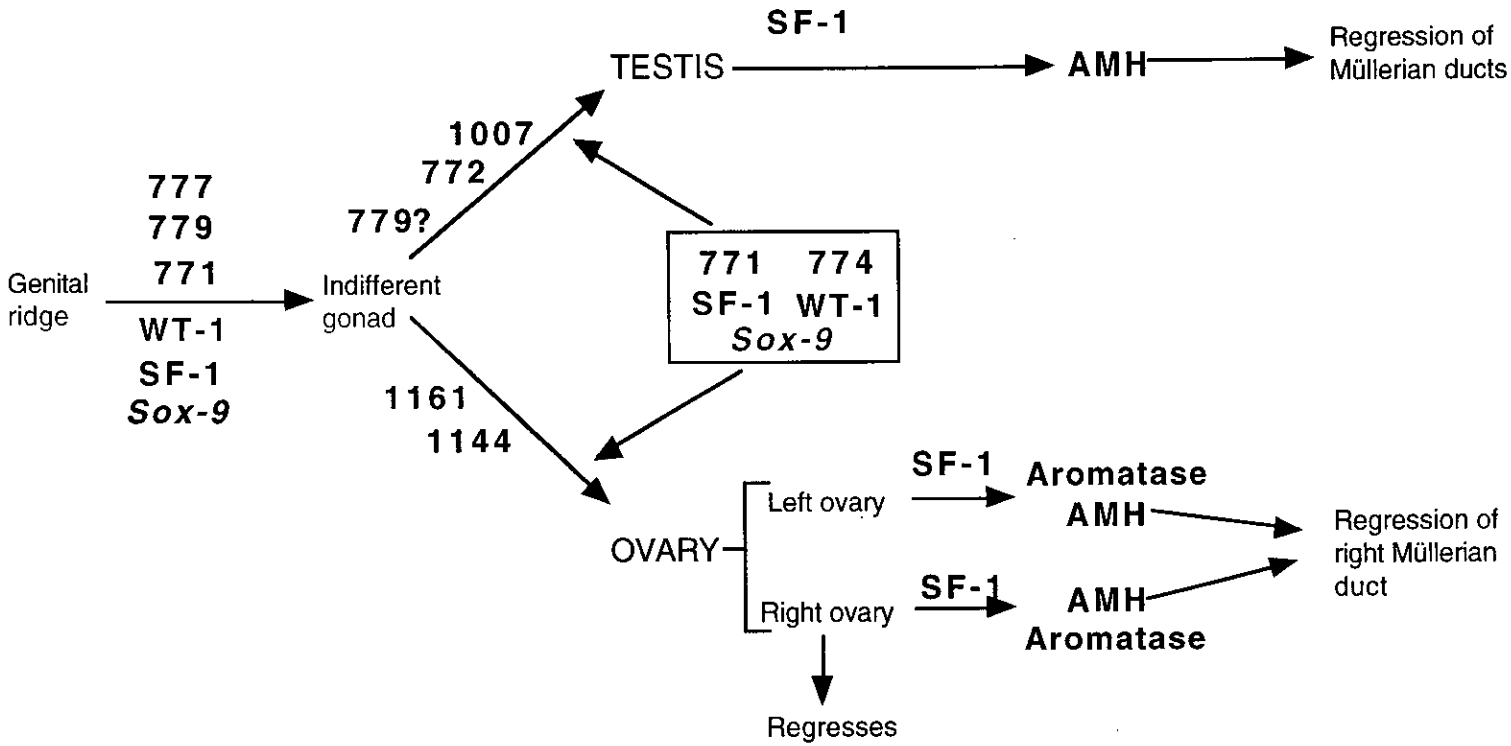


Figure 64: Diagram representing the potential gene expression during chick gonadal development. Chick homologues to genes involved in mammalian gonadal development and display candidates have been given a putative position in the developmental pathway. Some of the clones isolated by differential display analysis have been given a putative position based on their expression profile on the display gel. Due to the timing of the sex determining switch being unknown, gene regulation during ovarian or testis differentiation is considered as being day 5.5 of incubation or later (due to slight sex differences being apparent by this stage).

gene in chicks is of prime importance, the uncertainty regarding the sex determination mechanism, plus the paucity of genetic resources makes it unlikely that this gene will be identified by the approach used to identify *SRY* in mammals. Therefore, the isolation of novel genes by alternative methods such as differential display is a possible means to the identification of the chick sex determination gene, and should provide valuable information on the regulation of the chick gonadal development process as a whole. These studies have provided an insight into a small proportion of gene expression in the chick gonads and have shown that the differential display technique is a useful tool to isolate developmentally regulated genes, with both a low and high abundance. Extension of these studies to further characterise transcripts already isolated and to isolate additional transcripts will provide additional information on gonadal development in chicks and possibly other species.

APPENDICES.

APPENDIX 1.

Sequences for the primers used in the W-PCR procedure.

1. W primers:

From W-chromosome specific repetitive DNA (XhoI family).

W-5 5' - CCC AAA TAT AAC ACG CTT CAC T - 3' (127-148)

W-3 5' - GAA ATG AAT TAT TTT CTG GCG AC - 3' (542-520)

2. GAPDH primers:

GAPDH 5' primer 5' - TGT GAC TTC AAT GGT GAC A - 3'
(exon 10: 3862-3880)

GAPDH 3' primer 5' - CAG ATC AGT TTC TAT CAG C - 3'
(exon 11: 4563-4547)

3. 18S primers:

R18S1 5'- AGC TCT TTC CTC GAT TCC GTG - 3' (1267 - 1287)

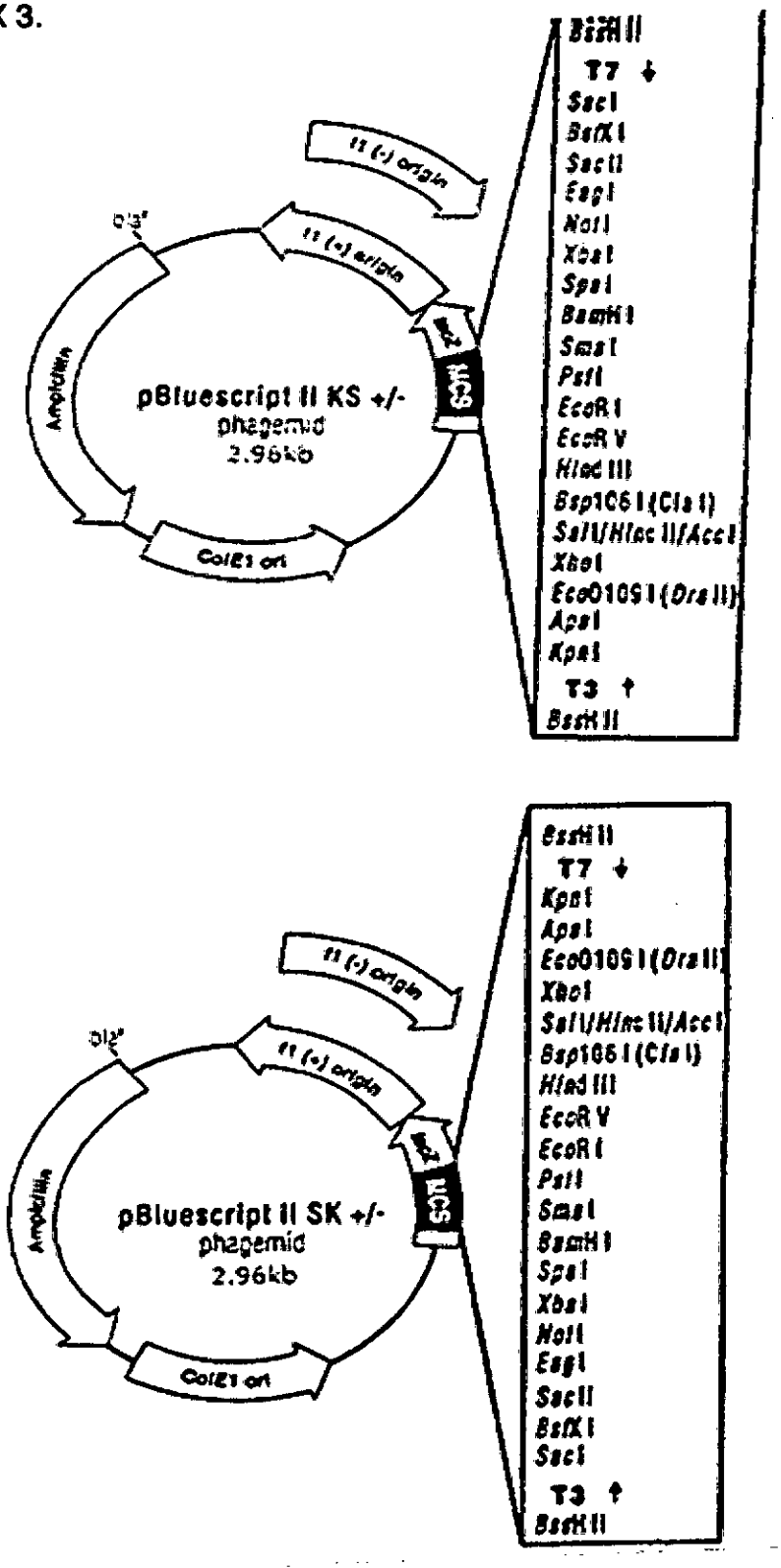
R18S2 5' - GGG TAG ACA CAA GCT GAG CC - 3' (1522 - 1503)

APPENDIX 2.

Sequences for the random 10mer primers used in the differential display RT-PCR.

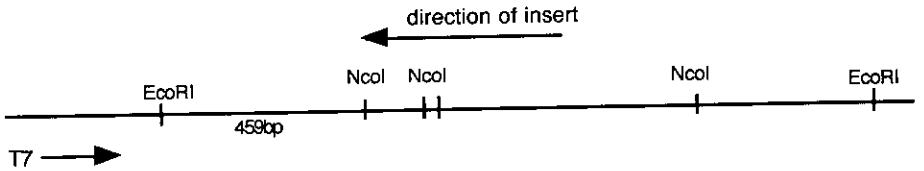
R1	5' - GGA ACT CCG T - 3'
R2	5' - GGC AAG TCA C - 3'
R3	5' - CCT CCG TAA G - 3'
R5	5' - CGG ACC CCG G - 3'
R7	5'- TAC AAC GAG G - 3'
R8	5' - TGG ATT GGT C - 3'
R9	5' - TGG TAA AGG G - 3'
R10	5' - TCG GTC ATA G - 3'
R11	5' - TAC CTA AGC G - 3'
R12	5' - CTG CTT GAT G - 3'
R13	5' - GAT CTG ACA C - 3'
R14	5' - GAT CGC ATT G - 3'
R15	5' - GAT CTG ACT G - 3'
R16	5' - GAT CTA AGG C - 3'
R18	5' - GGA ACC AAT C - 3'
R19	5' - CTT TCT ACC C - 3'
MAX2	5' - CAC AGT TTG C - 3'
MAX3	5' - CCA CAG AGT A - 3'
SOX	5' - GCG ACC CAT G - 3'
TK2	5' - CTT GAT TGC C - 3'

APPENDIX 3.

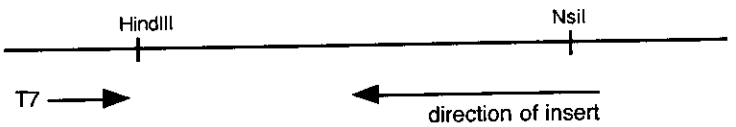


Schematic diagram of the two vectors used to clone the chick homologues to genes involved in mamamalian gonadal development. Diagram taken from Stratagene catalogue.

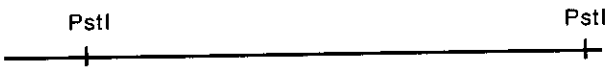
1. cAMH: cloned into EcoRI site of pBS KSII (+). 1905bp insert (not to scale) (Eusébe *et al.*, 1996).



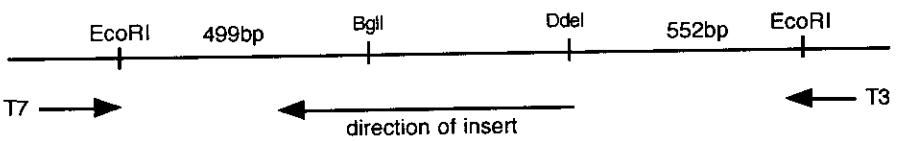
2. cWT-1 cloned into HindIII and NsiI sites of pBS SK II(+), 500bp insert (not to scale).



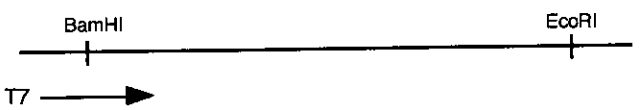
3. cSF-1 cloned into PstI site of pBS SKII (-). 500bp insert (not to scale).



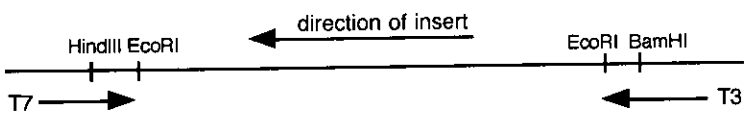
4. ZFSF-1 cloned into EcoRI site of pBS SKII(-). 1.3kb insert (not to scale).



5. cAromatase cloned into BamHI and EcoRI sites in pBS KS II (+). 351bp insert (not to scale).



6. c*Sox-9* clones into EcoRI site of pBS M13(-). 1200bp insert (not to scale).

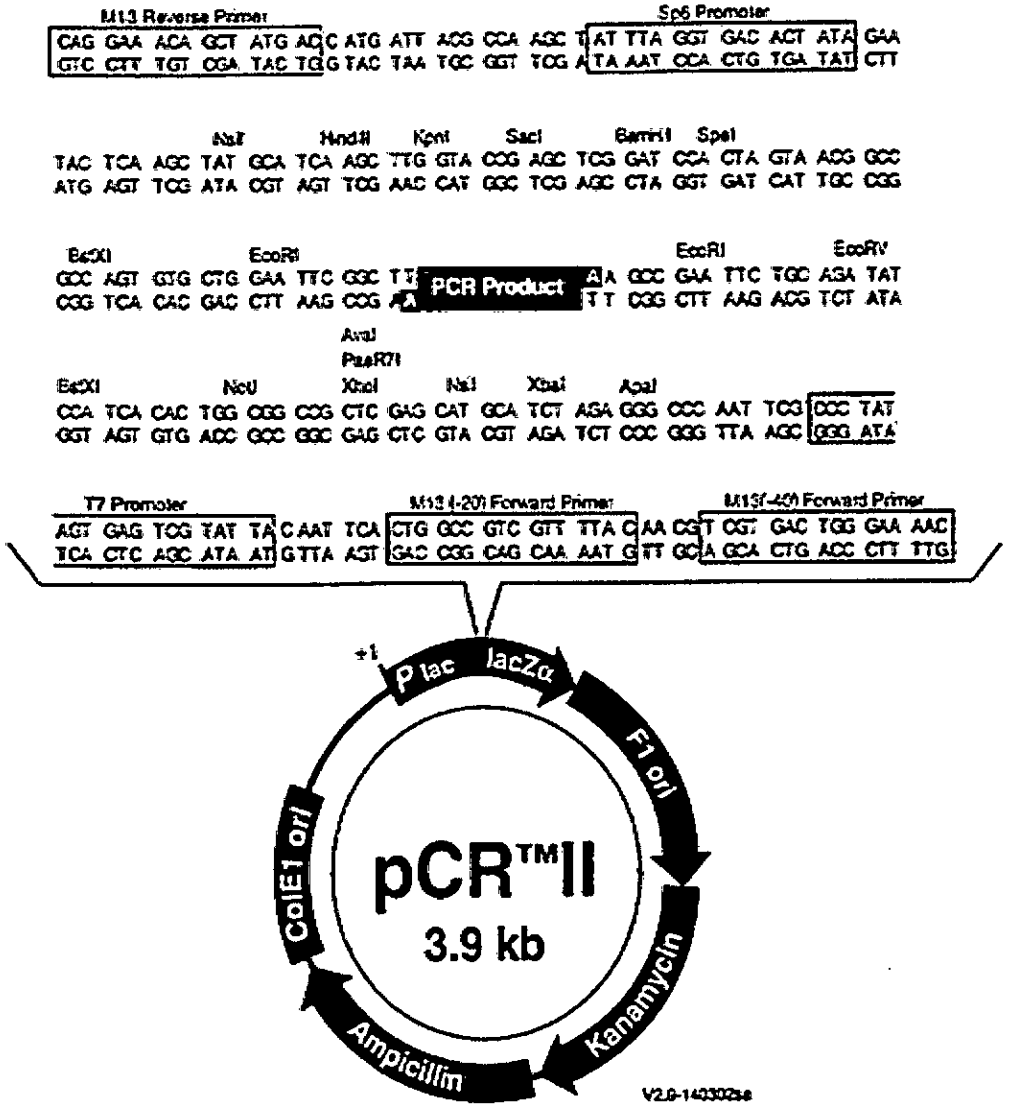


Available clones were chick homologues of AMH (gift from D. Eusèbe, INSERM Montrouge France), WT-1 (gift from V. van Heyningen, Human Genetics Unit Edinburgh), Sox-9 (gift from P. Sharpe, UMDS Guy's Hospital London) and aromatase (gift from D. Armstrong, Roslin Institute Edinburgh). There were chick and zebrafinch homologues to mouse SF-1(zebrafinch homologue was a gift from A. Arnold, UCLA).

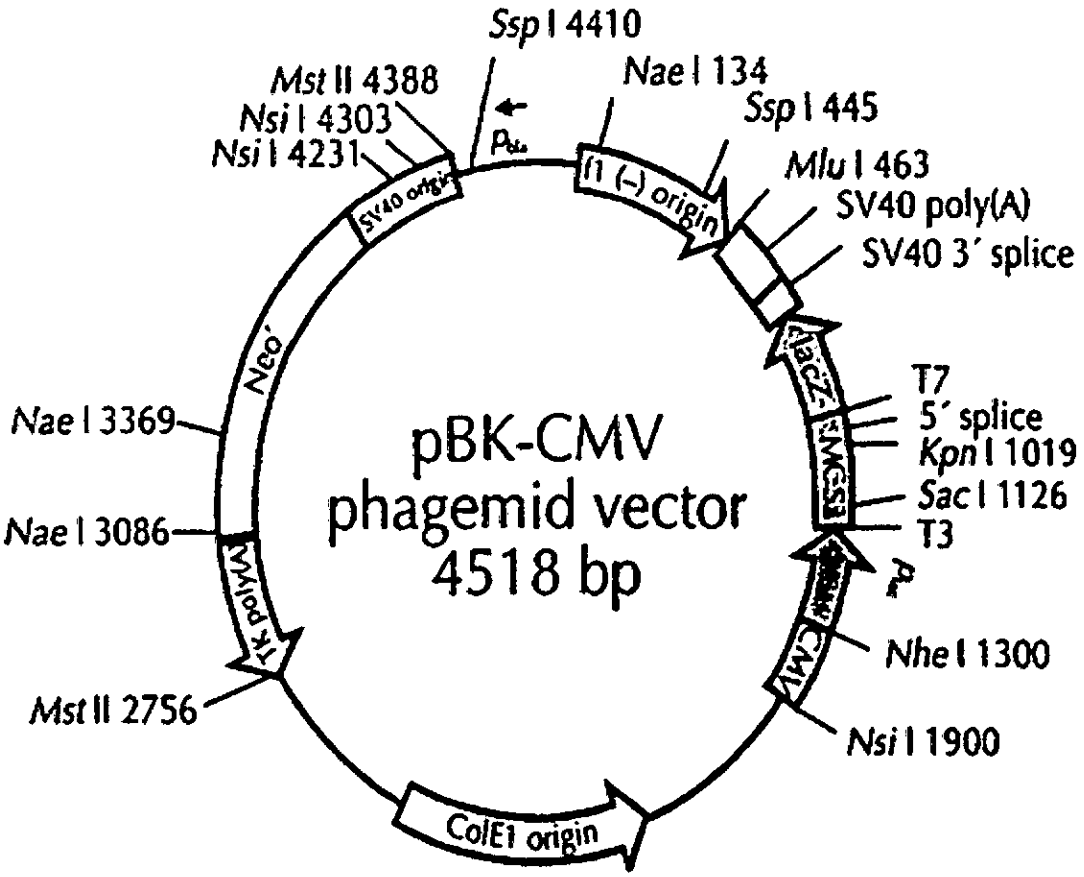
APPENDIX 4.

pCR™II Vector

of pCR™II The map of the linearized vector, pCR™II, is shown below. The sequence of the multiple cloning site is shown with a PCR product inserted by TA Cloning®.



Schematic diagram of the vector used to clone the sequences obtained by differential display. Diagram taken from the Invitrogen catalogue.



Schematic diagram of the vector used to clone the genomic clones (8313 and 19215). Diagram taken from the Stratagene catalogue. For further details concerning the MCS etc. see the Stratagene catalogue.

A list of the sequences obtained by differential display analysis.

Clone 771:
CGG ACC CCG GAA GTG AAG CAT TTC TGT CCC ACC GTG CCT ATC ATC TTG GTA GGA AAC AAG AAG GAC CTG AGG AAT GAC GAG CAC ACA AGA CGA GAG CTG GCC AAA A

Clone 772:
GCG ACC CAT GGC AAC ACA GAG AAA TCC CAG CTT TCC CTG GAG AAG ACA TTC AGA ATG TAC ATA ATG ATG TGT TGA TGA TGA TTC TCT ATA CAC TGA CAT TAC TGT GAC AGA CGT CCC TTT CCC CCC CCC AAA

Clone 774:
TTT CGG ATG GCT GAG TGT TGA AGC GTT AGG CTG TAG TCC TTT TTA CAG AGG TTC AAT TCC TCT TCT TAT CGA CTC TGT AGT GAA GTT CAT AAT GAG TTG CAA ACT CGT TGA TGT ACA CTA AAG TGT GCC GGG GTC GAA GC

Clone 775:
TTT TCG GGC AAT GTG CTT TTT TAT TGA CTT TTA CAC TGT GTC CTG CTC TGG GAG CTC AGT GGT ACT TGT AGG CCA GGG CAT GGG CCA CCA CGC TGA CCA GCT TCT GCC AGA CAG CCT GGC AGG TCG TCT ACG AAT GAT TTA GCA CCG GGG TCC GAA

Clone 776:
GGC ATT AAG ATA ACT GTT GTC ACT GTT TGA ACT GCA CTG TTC TTT GTT GAC TTC TCA AGT TCT TGA ACA ATG TTG TTT AGG TTA CTG TGA AAT GAA CTG TAT GGC TTG TTT GCA GTA TTT TTA ATG CAT AAT AAA TGT TTG GAA TAT TCT TTG CGA AAA A

Clone 777:
CTG CTT GAT GTG CCA GAA TAG TTT GGT GCT TGA TAA AGA AGT GAG ATC CTT TAA AAC ACT GTA ATG TTT CTG TCT TTT GAA GGA CCT ATT AGG AGA AAA CTC GAT TTG TGC ATT GTA TCC AGA AAT ACT CCC CAG GCA GAG CAC GTT TAG TTC TTT GTA AAT GTT GAA GCC CAC AGA CAG TGT TTG TGC TAA CGG GAA TCC TGA GTT CTG CCA AAA

Clone 779:
TTT GGG GAG ATA CAG CTG GAA TGG CTT TAT GCA TTC ACA GGC TGC AAA TCA GCT CTG TAT AGT AAT CAT TCC TTG AAT CAT CTA ATG CCT GAA GAT GAT CTG CTT CCC CCC CAC CTT ATG TGT GTT ACT TGT GTA CAG GAG AGA GCT GTT CCC ATG TGC TCT GCA GTA AGC AGC AAA GGA GGA TGT TGA TGT GTA CCA TGG CAG CTT GCT CTG GTT GTT TTG TCT GCC CCG TGC TGA TGG AAG CTA GCA GCC TGC TCT GGT AAT GGG AAG GAT GAG GAG GAA TCC TGT AAT TTG CGA AA

Clone 1001:
ACC CAT GTA CAT ACA GCC GCC AGT ATA CTG TGA GCG TAT TTT TTC TTA TGT ATT GTC GAG AAT GAA TTT TAT GGA AGG GTT TAT TTT TTT GGG GTT TGG GAA ACA GTC GGG TCT TTG GTT TGC CGA GTT CCT TCT GTA GGT TTT AAT CAT CCT GTG GGC GCT CAT GGG TCG CAA

Clone 1007:
CCC ATG CCA GGT GGG GTA ATA TAA AGG TCA TTT TAT GTA CAG TAT TGC TTT CTT CTT CCT GCT TTT CTA CTG CTC TGC TGC TAC AAA GCA TTA TTT CTT AAC TAC AAG TAA CCA TAC AGC TTT AAT AAA ACA CAC AGG GAC GTT ACT ACA GGC GAG CCC TCT TGG TTT CTG CAA TCT AGT GTG GTA GGA TTA TAA TAC GGG ACA ATG TTT CTT TGC AAA ACA GGT TAT ATT ATT AAA ACA ACA AGT CCA GGT AAG TGC CAG ACT AGA TCA ATA CCT ATA GGA TTG TTC T

Clone 1111:
GCA TTG CGC AGG GGA GCC ACG GAA CTC ACT CAT GAG GAC TAC ATG GAG GGC ATC CTG GAG GTT CAG GCC AAG AAG AAA GCC AAC CTA CAA TAC TAT GCC TAG GGG ACA CCT CTA GTC TGT CCG CTG GTC TCA GGG CTA AAG TTG ATA ATA AAA GGT GTT TTC TGT TCT TCG CCA AA

Clone 1144:
GGA TTG GTC TCG ACT TGG TCA ACG GGA AGC CTC GTG ATA ATA AGC AAG CCG GTG TCT TTG AAC CAA CCA TGG TCA AAA CTA AGA GCC TGA AGT TTG CAA CAG AAG CTG CAA TTA CTA TTC TTC GAA TTG ATG AAT CTT ATC AAA CTG CAC CCT GAA CCT AAA GAG GAT AGA GGG TGC TAT GAA GAT TGA CGG TTC ACT CTG GAG CAC TTG AAG AAT AAT TAG GCT TTA TTT ATG TTT ATC TGA CTA CAC TTC

Clone 1161:

TTT CCC AGA CAG TAA GGC CAA ATG GTA TTT GGC ACA CAT TCA TTT TAG AGT ATA GGG TGA CTT GCC AAG CC

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