Developmental and Reproductive Regulation of NR5A Genes in Teleosts

Jonas von Hofsten
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ABSTRACT

In mammals sex chromosomes direct and initiate the development of male and female gonads and subsequently secondary sex characteristics. In most vertebrates each individual is predestined to either become male or female. The process by which this genetic decision is carried out takes place during the embryonic development and involves a wide range of genes. The fushi tarazu factor-1 (FTZ-F1) is a nuclear receptor and transcription factor, which in mammals has proven to be essential for gonad development and directs the differentiation of testicular Sertoli cells. A mammalian FTZ-F1 homologue subtype, steroidogenic factor-1 (SF-1), is a member of the nuclear receptor 5A1 (NR5A1) group and regulate several enzymes involved in steroid hormone synthesis. It also regulates the expression of the gonadotropin releasing hormone receptor GnRHR and the β-subunit of the luteinizing hormone (LH), indicating that it functions at all levels of the reproductive axis. Another mammalian FTZ-F1 subtype, NR5A2, is in contrast to SF-1, not linked to steroidogenesis or sex determination. Rather, NR5A2 is involved in cholesterol metabolism and bile acid synthesis in liver. Hormones and environmental factors such as temperature and pH can influence teleost development and reproductive traits, rendering them vulnerable to pollutants and climate changes. Very little is known about teleost FTZ-F1 expression, regulation and function. In this thesis, expression patterns of four zebrafish FTZ-F1 genes (ff1a, b, c and d) and two Arctic char genes (acFF1α and β) were studied during development, displaying complex embryonic expression patterns. Ff1a expression was in part congruent with expression of both mammalian NR5A1 and NR5A2 genes but also displayed novel expression domains. The complexity of the expression pattern of ff1a led to the conclusion that the gene may be involved in several developmental processes, including gonad development, which also was indicated by its transcriptional regulation via Sox9a. Two ff1a homologues were also cloned in Arctic char and were shown to be involved in the reproductive cycle, as the expression displayed seasonal cyclicity and preceded that of the down stream steroidogenic genes StAR and CYP11A. High levels were correlated to elevated plasma levels of 11-ketotestosterone (11KT) in males and 17β-estradiol (E2) in females respectively. Treatment with 11KT did not affect FTZ-F1 expression directly but was indicated to alter expression of CYP11A and 3β-hydroxysteroid dehydrogenase. E2 treatment was indicated to down-regulate the expression of testicular FTZ-F1, which may contribute to the feminising effect previously observed in E2 treated salmonids. Ff1d is a novel FTZ-F1 gene, expressed in pituitary and interrenal cells during development, suggesting steroidogenic functions. In adult testis and ovary ff1d was co-expressed with anti-Mullerian hormone (AMH), a gene connected to sex determination in mammals and previously not characterised in teleost fish. The co-expression between ff1d and AMH was found in Sertoli and granulosa cells, which is congruent with the co-expression of mammalian SF-1 and AMH. This suggests that ff1d and AMH may have similar functions in teleost sex differentiation and reproduction, as their mammalian homologues. In conclusion, this study present data that connects members of the teleost FTZ-F1 family to reproduction, cholesterol metabolism and sex determination and differentiation.
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ABBREVIATIONS

11KT 11-keto-testosterone
3βHSD 3β hydroxy steroid dehydrogenase
ACTH Adrenocorticotropic hormone
Ad4BP Adrenal 4-binding protein
AMH Anti Müllerian Hormone
BA Bile acid
CBF CAAT-binding factor
C/EBP CCAAT/Enhancer binding protein
CYP Cytochrome P450
Dax-1 dosage sensitive sex reversal x-chromosome
DBD DNA-binding domain
Dmrt1 Doublesex/mab3 related transcription factor 1
DRD Distal repressive domain
E2 17β-Estradiol
ELP Embryonal long term repeat-binding protein
FF1 Fushi Tarazu Factor 1
FSH Follicle stimulating hormone
FTF α-fetoprotein transcription factor
FTZ Fushi Tarazu
FTZ-F1 Fushi Tarazu Factor 1
GnRH Gonadotropin releasing hormone
LH Luteinizing hormone
LDB Ligand binding domain
LRH-1 Liver receptor homologue-1
N-CoR Nuclear receptor co-repressor
NR5A Nuclear receptor 5A
P450scc Cytochrome P450 side chain cleavage enzyme
PID Proximal interactive domain
PRD Proximal repressive domain
SF-1 Steroidogenic factor-1
SOX SRY-related HMG box-containing gene
StAR Steroidogenic acute regulatory protein
SRC-1 Steroid receptor co-activator-1
SRY Sex reversal region on the Y-chromosome
T Testosterone
USF Up-stream stimulatory factor
Wnt4a Wnt 4a, Wingless in Drosophila
WT1 Wilms tumour 1
INTRODUCTION

General introduction:

A considerable number of species have become prematurely extinct since the human population started to spread through the worldwide ecosystems 10 000 years ago. Although the natural environment is constantly changing, as humans began to alter their environment instead of adapting to it, the conditions of existence for many species have rapidly become distorted. Loss of genetic variation may arise from several factors, but is often due to decreased population sizes. However, it is not the actual number of individuals within a population that decides the genetic diversity within it. The genetically effective population size is of greater importance. An ideal population contains individuals which all have an equal probability of contributing genes to the next generation. Such populations are however unlikely to exist, but two of the cornerstones for achieving ideal populations are sex ratios of 1:1 and progeny evenly distributed among females. Hence, the genetic effective size may be substantially lowered if breeding sex ratios become uneven, or if the reproductive success among males or females becomes uneven.

Skewed sex ratios in fish have been observed all over the world during the most recent years and the reproductive success among wild fish has been under lively debate. Fish as a group are susceptible to environmental pollutants and hormonally active substances may cause altered sex ratios, decreased reproductive success and embryo mortality. However, few molecular studies have been conducted on fish and the mechanisms underlying the observed effects have not been elucidated.

Reduced reproductive success, including infertility, will therefore not just affect the number of offspring produced; the effective size may also be reduced leading to lowered genetic diversity within the entire species. Breeding and breeding systems are thereby obviously important features of conserving genetic diversity. The distribution of progeny among females also strongly affects the population’s genetic effective size. If a single female would account for a majority of the produced offspring, her genes would be over-represented in the next generation and increase the likelihood of inbreeding. This is also the case in large population fluctuations, where every population that crashes to a small size experiences a demographic bottleneck and loss of genetic variation. The magnitude of reduced genetic variability is dependent on the number of survivors through the bottleneck and the ensuing growth rate of the population. However, the importance of evenly distributed reproductive success and sex ratios within each species are obvious for its continuous existence and a requirement for the long-term preservation of biodiversity.

The strategy of keeping a mixed gene pool by combining DNA from the parental generation in the offspring have proven to be an outstanding method of surviving in a fluctuating environment. The mechanisms of sexual reproduction are diverse and the genetic backgrounds underlying the existence of sex are extremely varying between different groups of species. Sex determination is the process deciding what sex a developing embryo will generate. This is usually decided genetically, however it is a delicate process, which in many cases can be influenced by environmental factors. In mammals the foetuses develop in a sheltered uterine environment and gender is strictly regulated at the chromosome level, but this is hardly the case for the majority of vertebrates. The largest and most diverse group of vertebrates are the teleosts, which contains over 20 000 species. Within this group numerous sex determining and reproductive strategies have evolved. Different types of hermaphroditism are not unusual in fish and the appearance of a specific sex is not as well defined as in mammals. A handful of genes have been identified as controllers of sex determination in mammals. Just a few of these genes have so far been identified in fish, and
even fewer have clearly been documented as controllers of sex determination. One of the differences between mammalian and fish sex determination is that fish are much more sensitive to environmental influence. It is possible that steroidogenesis and hormonal regulation plays an important role in fish sex determination process. In this thesis I present data regarding expression of genes involved in steroid biosynthesis and putatively in sex determination in teleost fish, with emphasis on members of the *fushi tarazu* factor-1 gene family.

**History of the *fushi tarazu* factor-1:**

"*Fushi tarazu*" is Japanese for “not enough segments” and the Drosophila homeobox gene *fushi tarazu* (*ftz*) was first identified as an important factor for segmentation, as down-regulation of *ftz* lead to the development of fewer segments (Kuroiwa et al., 1984; Wakimoto et al., 1984). The *fushi tarazu* factor-1 (FTZ-F1) was a few years later identified as the regulator and transcription factor of *ftz* expression (Ueda et al., 1990; Lavorgna et al., 1991). FTZ-F1, or genes homologous to FTZ-F1, has since been isolated in several species in different phyla (Tugwood et al., 1991; Lala et al., 1992; Sun et al., 1994; Galarneau et al., 1996; Wong et al., 1996; Kudo and Sutou, 1997; Kawano et al., 1997; Liu et al., 1997; Watanabe et al., 1999; Chai and Chan, 2000; Higa et al., 2000). Also, different names have been designated to these homologues, such as Steroidogenic factor-1 (SF-1), adrenal-4-binding protein (Ad4BP), embryonal long terminal repeat-binding protein (ELP), \( \alpha \)-fetoprotein transcription factor (FTF) and liver receptor homologue-1 (LRH-1). However, the nuclear receptor committee recently announced a novel nomenclature system for all nuclear receptors where all the FTZ-F1 homologues constitute a phylogenetic clade of its own called NR5A (Nuclear receptors committee, 1999). Within the members of the NR5A group there are certain traits, which are conserved and links the group together.

**Nuclear receptor 5A:**

Nuclear receptors are often transcription factors altering gene expression in response to hormone stimulation, which acts as a ligand to the receptor and activates it. This is achieved by conformation change in the receptor protein enabling it to bind DNA and initiate transcription, or repression, of its specific target genes. The binding of DNA is achieved by homo- or hetero dimerisation, or by monomer receptor binding, depending on the type of receptor. Some receptors are also able to bind DNA without ligand and are referred to as orphan receptors. The nuclear receptor 5A subfamily contains the *fushi tarazu* factor-1 genes, a group of orphan nuclear receptors.

The vertebrate FTZ-F1 homologues (NR5A) have been arranged in two groups (NR5A1 and NR5A2) based on sequence homology, function and expression patterns. NR5A1 contains genes closely connected to steroidogenesis. SF-1 and genes homologous to SF-1 are placed in the NR5A1 group and are, in mammals, expressed in steroiogenic tissues and are identified as transcriptional regulators of steroidogenesis (Ikeda et al., 1993, Ikeda et al., 1994) and have been linked to sex determination. The NR5A2 group contains genes coding for proteins linked to regulation of \( \alpha \)-fetoprotein (Galarneau et al., 1996), an estrogen-binding protein expressed in mammalian embryos and foetuses. In adults, NR5A2 homologues are highly expressed in liver and have been shown to be involved in cholesterol metabolism and production of bile acids (Nitta et al., 1999; del Castillo-Olives and Gil, 2000). Expression has also been observed in intestine, pancreas and chondroid tissue during development (Rausa et al., 1999). The mammalian NR5A2 genes are so far not connected to steroid synthesis or sex determination. However, studies of teleost FTZ-F1 genes have shown
that the mammalian classification system is not appropriate to apply on fish. So far, no teleost FTZ-F1 gene has been arranged in the NR5A1 group, containing the SF-1 homologues. Teleost FTZ-F1 genes have after sequence analyses been placed in the NR5A2 group, or in NR5A4 which only contains teleost gene homologues. NR5A3 does not contain any vertebrate homologues, but several insect and crustacean genes. A rainbow trout FTZ-F1 gene termed tFZR1 does not fit the description of NR5A2 or 4 and shows little similarity to any other FTZ-F1 gene. The function of the teleost NR5A genes and their putative involvement in sex determination and hormone synthesis are still to be determined.

FTZ-F1: Role in steroidogenesis:

Steroid synthesis in mammals:

Mammalian steroid biosynthesis occurs mainly in the adrenal cortex and the gonads. All steroids derive from the same precursor, cholesterol. Modifications are made by steroidogenic enzymes, converting cholesterol to different steroid hormones depending on which tissue localization of the biosynthesis and the modifying enzymes involved in the process. Three major pathways direct the synthesis of separate types of steroid hormones. Two of the pathways are located in the adrenal cortex, where the mineralocorticoid steroid aldosterone is the terminal product in zona glomerulosa and the glucocorticoid cortisol in zona fasciculata/reticularis (Fig. 1). The third pathway is active in Leydig cells and in theca and granulosa cells, resulting in the production of the sex hormones testosterone (T) and estradiol (E2). Sertoli cells in the testis do not produce androgens, but can convert androgens into estrogens and are also able to directly synthesise estrogens (Ganong, 1997). Steroids are produced in the appropriate cell type in response to signals from the anterior pituitary gland. These signals consist of the tropic hormones, adrenocorticotropic hormone (ACTH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The tropic hormones transduce their signals through the cAMP second messenger pathway influencing the rate of steroid biosynthesis. Cytochrome P450 (CYP) enzymes and hydroxysteroid dehydrogenases (HSDs) catalyse the modifications. These enzymes carry out the conversion of the steroids by catalysing redox reactions including hydroxylation by adding a hydroxyl group and dehydrogenation by inserting a keto group to the carbon backbone of the steroid ring. Many of the enzymes involved are able to catalyse the same reaction on several different substrates.
Figure 1. Steroidogenic pathways in mammalian adrenal cortex and gonad. A single precursor protein, cholesterol, is modified in the cell by various enzymes in the cytosol and mitochondria. The initial step of steroid synthesis is the transportation of cholesterol to the inner membrane of the mitochondria. This is accomplished by a cholesterol transporter protein, the Steroidogenic Acute Regulatory protein (StAR) (Clark et al, 1994) that delivers cholesterol to the side chain cleavage enzyme, a product of the CYP11A gene, which converts cholesterol into pregnenolone by cleaving the side chain of cholesterol through hydroxylation. Pregnenolone can subsequently be transformed into 17-OH-Pregnenolone by CYP17 in the glucocorticoid pathway, or progesterone by 3βHSD in the mineralocorticoid pathway. Enzymes transcriptionally regulated by SF-1 are indicated by *.

Steroidogenic Factor-1 in steroid biosynthesis:

The regulation of mammalian steroidogenesis is carried out by enzymes, which are controlled by transcription factors. One of these transcription factors, SF-1, is involved in the regulation of numerous steroidogenic enzymes (reviewed by Hammer and Ingraham, 1999). Promoter studies of several of these genes, for example StAR (Caron et al., 1997; Sandhoff et al., 1998), CYP11A (Clemens et al., 1994; Liu and Simpson, 1999; Hu et al., 2001) and 3βHSD (Leers-Sucheta et al., 1997) have revealed binding sites for the SF-1 protein and both in vivo and in vitro experiment supports this observation. Secondly, SF-1 has been shown to be a regulator of the β-subunit of luteinizing hormone (LH) (Halvorson et al., 1996; Liu et al., 1997), which is a tropic hormone secreted from the pituitary. SF-1 has also been identified as a regulator of the gonadotropin releasing hormone (GnRH) receptor (Duval et al., 1997), confirming that SF-1 acts on all levels of the reproductive axis. Expression of SF-1 also has an important function in the pituitary, although the development of the pituitary is not affected by an SF-1 knockout. However, specific knock down of SF-1 in the pituitary by the cre-lox method affected gonad growth and function, probably due to reduced gonadotropin release (Zhao et al., 2001). In addition, SF-1 is also essential for gonad development.

The StAR protein is an essential component in the acute regulatory phase of steroidogenesis and functions by mediating the transfer of cholesterol from the outer to the inner mitochondrial membrane where steroidogenesis is initiated. A mutation that inactivates
the StAR gene (a disease named congenital lipoid adrenal hyperplasia) results in severe effects on gonadal and adrenal steroidogenesis and a substantial accumulation of cholesterol in the cytoplasm (Lin et al., 1995; Tee et al., 1995). Mouse StAR knock out models require corticosteroid replacement to survive and display effects on adrenal and gonad development (Caron et al., 1997 b; Hasegawa et al., 2000). These mice accumulated large amounts of lipid deposits, confirming StARs involvement in cholesterol metabolism. The regulation of StAR gene expression is mediated by tropic hormonal up-regulation of cAMP and is accomplished by SF-1 and a CCAAT/Enhancer Binding Protein (C/EBP) (Reinhart et al., 1999). Repression of StAR transcription may be mediated by Dax-1 (dosage sensitive sex reversal X-chromosome) (Zazopoulos et al., 1997), which is a nuclear receptor protein often opposing the effect of SF-1. In addition Dax-1 inhibits expression of CYP11A and 3βHSD (Lalli et al., 1998) by interacting directly with SF-1 resulting in inhibition of SF-1 mediated transactivation (Ito et al., 1997 a).

CYP11A is included in the super family of CYP enzymes, which display diverse cellular functions from detoxification functions in liver to steroid hydroxylation and modification in gonads. The product of the CYP11A gene, the side chain cleavage enzyme, is a crucial component of the production of bioactive steroids. In the initial reaction the six-carbon side chain is cleaved off by a reaction catalysed by CYP11A. This step occurs in the mitochondria and involves two hydroxylations, both requiring molecular oxygen and a pair of electrons. NADPH donates the electrons to CYP11A via adrenodoxin reductase (reviewed in Greenspan and Strewler, 1997). In mammals ACTH is the major stimulatory factor of CYP11A expression in adrenals and gonadotropins in gonads (Hu et al., 2001 a). Regardless of stimulatory hormone, the intracellular messenger is cAMP (Hu et al., 2001 a; Hu et al., 2001 b). A few regulatory elements in the CYP11A promoter have been identified, including cAMP responsive sequences and SF-1 binding sites (Hu et al., 2001 a; Hu et al., 2001 b).

3βHSD is an enzyme essential for all classes of steroid biosynthesis, including glucocorticoids, mineralocorticoids and sex steroids. It acts by catalysing the dehydrogenation and isomerisation of the 3β-hydroxysteroids to keto-steroids, for example pregnenolone, 17α-hydroxy pregnenolone and dehydroepiandrosterone to progesterone, 17α-hydroxyprogesterone and androstenedione respectively (Fig. 1). Promoter studies of the 3βHSD gene revealed SF-1 response elements, which were shown to activate transcription in vitro (Leers-Sucheta et al., 1997).

CYP19, also called aromatase is another gene transcriptionally regulated by SF-1. Aromatase is an enzyme that converts T into E2 and is primarily expressed in the ovarian granulosa cells, but also in testicular Leydig cells and is necessary for both ovarian and testicular functions.

In summary, the steroidogenic biosynthesis is based on modifications of the precursor molecule cholesterol. The transporter protein STAR delivers cholesterol to the inner membrane of the mitochondria where CYP11A initiate the synthesis pathway. The steroids may subsequently be transformed into glucocorticoids, initially by CYP17, or into mineralocorticoids by 3βHSD. In gonads, steroids can also be modified into androgens or estrogens.

**Cholesterol synthesis and metabolism:**

The supply of cholesterol is necessary for proper steroid biosynthesis. Both NR5A1 and 2 are directly involved in cholesterol metabolism, not only by increasing the consuming of cholesterol in steroid synthesis, but also by de novo production and receptor mediated cellular endocytosis via the High Density Lipoprotein (HDL) and the Low Density Lipoprotein (LDL).
NR5A1 regulates the expression of the 3-OH-3-methylglutaryl-CoA (HMG-CoA) gene, which is essential for the endogenous synthesis of cholesterol (Mascaro et al. 2000). NR5A1 also synergise with the sterol regulatory element binding protein 1a (SREBP1a) in inducing the HDL gene expression, leading to increased endocytosis of cholesterol (Lopez and McLean, 1999).

NR5A2 acts as a cofactor to the liver nuclear receptor LXR in regulating the cholesterol catalyzing enzymes 7α-hydroxylase (CYP7A) and 12α-hydroxylase, which leads to an increased production of bile acids (BA) in the liver (Nitta et al., 1999; del Castillo-Olives and Gil, 2000). As a response to increased bile acid levels the nuclear receptor FXR is up-regulated together with the short heterodimer partner (SHP), which repress LXR and NR5A2 and subsequently leads to a down regulation of BA (reviewed in Fayard et al., 2004). LXR has also been shown to directly activate NR5A2 expression by binding to a direct repeat 4 (DR4) cis-element in its promoter region (Paré et al., 2004). Thus both NR5A1 and NR5A2 genes are essential for proper cholesterol synthesis, distribution and metabolism.

Steroid synthesis in teleosts:
Steroidogenesis in teleosts is regulated in a similar manner as in mammals. However, one difference between mammals and fish is that fish lack adrenals. The teleost kidney is located in the body cavity along the vertebra and the anterior part (closest to the head) contains interrenal cells which are functional homologues to the corresponding cells in the mammalian adrenal. The teleost interrenal consists of two functionally different cell types with separate developmental origin, adrenocortical steroidogenic cells and medullary chromaffin cells, which secrete different products (Hanke and Kloas, 1995). The chromaffin cells are of neuro-ectodermal origin, and are the equivalent to mammalian medullary adrenergic cells and secrete epinephrine and nor-epinephrine. The steroidogenic cells derive from mesodermal precursors and secrete steroid hormones, such as cortisol.

In many fish species 11-keto-testosterone (11KT) is the most active androgen. It can be synthesised through three separate pathways from androstenedione involving the enzymes CYP11β, 11βHSD and 17βHSD (Fig. 2).

![Figure 2](Modified from Baroiller et al. 1997.)

**Figure 2.** Three possible transformation pathways of androstenedione into 11KT in teleost fish.
Testosterone is present at comparable levels in both male and female salmonids, whereas 11KT expression is higher in males and more efficient in stimulating male secondary sex characteristics, reproductive behaviour and spermatogenesis (Miura et al., 1991; Borg, 1994; Yaron, 1995). The regulation of steroidogenic enzymes in this pathway has so far not been studied.

**FTZ-F1: Role in sex determination:**

**Mammalian sex determination:**

“Sex determination can be defined as the proliferation, migration, and differentiation of supporting cells to become Sertoli cells, committing the fate of the gonad to the testis pathway” (Harley et al., 2003). The first developmental difference between a male and a female embryo is the differentiation of Sertoli cells in the genital ridge. As a consequence of the presence of Sertoli cells a testis will form. Sex characteristics can be divided into primary, e.g. type of gonad and secondary, which refers to hormone dependent traits such as mammary gland development or facial hair growth in humans. The primary sex characteristics are in mammals dependent on the mammalian chromosomal XY sex determination system. An individual with a Y sex chromosome will develop testes regardless of number of X chromosomes. The system is based on the presence of a testis-determining factor (TDF) on the Y chromosome, a region known as the Sex reversal region on the Y chromosome (SRY) (Sinclair et al., 1990; Berta et al., 1990).

SRY acts as a transcription factor, it contains a High Mobility Group (HMG)-Box, which functions as a DNA-binding and DNA-bending domain. A number of recognition sites have been found for SRY in vitro, but the in vivo target for SRY during testis differentiation has still not been identified.

The use of SRY as TDF is however limited to mammals and is not a universal sex determining system in the animal kingdom.

**Formation of the bipotential gonad:**

During embryogenesis the uro-genital ridge is initially formed from the intermedial mesoderm. This is a paired structure that gives rise to kidneys, adrenals and gonads. In order for the uro-genital ridge to differentiate into these separate organs, the expression of certain genes is needed in addition to SRY (Fig. 3).

Kidneys will not differentiate without the presence of the Wilms Tumour 1 (WT1) (Kriedberg et al., 1993) and SF-1 is essential for the development of the steroidogenic components of the uro-genital ridge as SF-1 knockout mice are unable to form either adrenals or gonads (Sadovsky et al., 1995).

In the presence of SF-1 a bipotential gonad will initially be formed from the uro-genital ridge (Fig. 3). Before the differentiation of specific gonads occurs, two layers are formed in the genital ridge where the outer epithelial layer proliferates into the loose connective mesenchyme above to form the sex cords. The sex cords contain germ cells and pre-Sertoli cells surrounded by peritubular myolid cells, and an interstitial region containing steroidogenic cell precursors.
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Figure 3. Gonad differentiation. Genes involved (boxed in black) are placed at its functional level during differentiation.

Differentiation of sex specific gonads:

Testis differentiation:
In the presence of the Y chromosome the SRY will induce the differentiation of a testis by directing the development of sex cords cells into Sertoli cells and subsequently the cells in the intermedial mesenchyme differentiate into Leydig cells. After the initial induction of testis differentiation, a cascade of genes is triggered, all essential for a successful testis formation. Among these genes are Sox9 (SRY-related HMG box-containing gene 9) (Wagner et al., 1994; Foster et al., 1994), SF-1 (Sadovsky et al., 1995), Dmrt1 (Raymond et al., 1998) and GATA4.

Sox9 is a member of the SRY-like HMG-box (Sox) gene family of transcription factors. Sox9 is important for cartilage formation and the development of gonads (Foster et al., 1994; Wagner et al., 1994). During development Sox9 is expressed in mesenchymal cells and directs their differentiation into condrocytes by regulating the expression of type II collagen (Ng et al., 1997; Lefebvre et al., 1997). Lack of Sox9 causes campomelic dysplasia (CD), which leads to skeletal defects, such as poorly developed limbs and jawbones (Foster et al., 1994; Wagner et al., 1994). CD also cause gonadal and, in particular, testis dysgenesis and feminisation, where XY-individuals display a female phenotype to a variable extent (Meyer et al., 1997). Sox9 is together with SF-1, WT1 and GATA4 positive transcriptional regulators of human Anti-Mullerian Hormone (AMH) (Giuili et al., 1997; De Santa Barbara et al., 1998; Arango et al., 1999; Tremblay and Viger, 2001) and thereby linked to the male sex-determining pathway. During gonadal development Sox9 is expressed at low levels in the bi-potential gonad, and up-regulated in Sertoli cells as the initiation of testis differentiation commences (Kent et al., 1996) possibly due to SRY expression. During the same period Sox9 is down regulated in ovary. The transcriptional regulation of Sox9 is however not elucidated.
SF-1 has been associated with sex determination and is an important factor for the development of XY-male sexual features. During mammalian development SF-1 is expressed in the uro-genital ridge of both sexes until sex specific features occur, when it subsequently diminishes in females but is continuously expressed in males (Ikeda et al., 1994). During embryogenesis SF-1 is crucial for the development of steroidogenic tissues and organs. SF-1 null-mice lack adrenal and gonadal tissue, both in genetically male and female individuals (Sadovsky et al., 1995). Expression analyses of several genes downstream of SF-1, such as CYP11A and StAR, indicate massive down regulation and the mice eventually die due to lack of steroid production.

SF-1 is together with GATA4 and WT1 responsible for the expression of the Anti Müllerian Hormone (AMH) (Giuili et al., 1997), a dimeric glycoprotein belonging to the TGFβ super family of growth factors, which initiate the regression of the Müllerian ducts. The Müllerian ducts and the Wolffian ducts are embryonic tissues present in both genetically male and female individuals before sex differentiation. The Müllerian ducts will in XX individuals and in the absence of AMH develop into parts of the female sex organ (oviducts and part of uterus) and the Wolffian ducts will regress. In genetical males AMH will initiate the regression of the Müllerian ducts, which paves the way for the Wolffian ducts to continue differentiation into vas deferens and parts of the male sex organ.

The only gene that so far has been identified to be involved in sex determination in several phyla is the *Drosophila* doublesex (dsx) and *Caenorhabditis elegans* mab-3 (Shen et al., 1988), which contain a conserved zinc-finger like DNA binding motif called the DM-domain. One DM-domain containing gene (Dmrt1) has been isolated in a variety of vertebrates (Raymond et al., 1998; Raymond et al., 1999; Guan et al., 1999) and has been implied to be important in testis development on the basis of embryonic expression patterns. Dmrt1 has also been identified in fish and is implicated to have a similar role during gonad development as implied for mammals.

**Ovary differentiation:**

In the ovary pathway the sex cords will degenerate at the onset of sex differentiation, but new cords are formed from the epithelial layer of the genital ridge. These will penetrate the mesenchyme and surround the germ cells with layers of granulosa and theca cells and form follicles. Since the presence of SRY will induce testis differentiation and the absence of SRY leads to differentiation of an ovary, the mechanism of developing a female gonad is sometimes referred to as the default way. However, there are genes suggested to be of importance for ovary differentiation as well.

The Dax-1 gene, located on the X chromosome, has been placed in focus. Dax-1 has been isolated in several mammals and deletions of the gene results in female to male sex reversals. SF-1 and Dax-1 have also been shown to interact and SF-1 dependent transcription activity and SF-1 can be down regulated by Dax-1 (Lalli et al., 1998). Dax-1 lacks the, among nuclear receptors, conserved DNA-binding domain but is considered to be able to dimerise with other proteins (SF-1) and has been suggested to bind DNA through a hairpin structure (Zazopoulos et al., 1997). The gene is co-expressed with SF-1 in multiple cell lineages (Ikeda et al., 1996) and in steroidogenic tissues at early embryonic development, but disappears in the testis at the time of SRY expression while it remains normal in the ovary (Swain et al., 1996; Swain et al., 1998). Interestingly, a recently cloned amphibian Dax-1 was suggested to be of importance for testicular development (Sugita et al., 2001).

Wnt4a (Wint-4a; wingless in *Drosophila*) is another gene suggested to be involved in ovary development. It is expressed in the uro-genital ridge and the metanephric kidney (reviewed by Sariola and Sainio, 1998) and later in ovaries while it is down regulated in the testis. It is essential for development of the Müllerian ducts in female and as a suppressor of the male testis and is suggested to function up-stream of Dax-1 (Jordan et al., 2001).
**Teleost sex determination:**

As in mammals, sex determination in many fish species has a chromosomal background. Several species, including most salmonids, have heterogametic males and homeogametic females, similar to the mammalian XY/XX-system. Other fish have homeogametic males and heterogametic females, including *Gambusia*, which also is the case for birds (ZZ/ZW). In some species, as the platyfish *Xiphophorus*, both types of heterogametic sex exist. A different type of sex determination is also present where the determination process is decided more strictly by environmental factors such as the temperature surrounding the developing embryo (Fig. 4). Hermaphroditism is a common feature of several fish species. In many cases studies have shown that species with genetical sex determination can be directed to produce genetically sex-reversed offspring. Either by treating the fish with hormones, which can induce sex reversal in synchronous hermaphroditic fish (Tang et al., 1974; Yeung et al., 1993) and masculinisation/feminisation in gonochoristic species, or by incubating embryos in certain temperatures or pH (reviewed in Baroiller et al., 1999). The proportion of males usually increases with temperature whereas lower temperatures favour females. In the case of pH more individual differences are observed. The fact that fish sex determination and differentiation is sensitive to hormone treatment, temperature and pH puts them in a defenceless position when exposed to environmental pollutants and climate changes, since reproductive traits in both developing and adult fish may be affected. Sex determination has been poorly studied in fish and genetic mechanisms behind sex determination even less. The developmental mechanisms by which the gonads are formed have been thoroughly studied in mammals and several genes involved have been identified. Only a few of these genes have been identified in fish (Sox9, Dmrt1 and FTZ-F1), but it has not been fully elucidated if the functions of these genes are conserved between mammals and teleosts.

**Diagram:**

![Diagram showing factors influencing sex determination and differentiation in teleosts.](image)

**Figure 4.** Factors influencing sex determination and differentiation in teleosts. The chromosomal sex is considered to be a default sex-determining pathway, but surrounding conditions may influence this. Normal factors are temperature, hormones and social factors. Environmental pollution is not considered to be normal, but may influence the sex-determining pathway.

**Formation and differentiation of gonads:**

There is a close anatomical relationship between the development of the genital ridge and excretory system during early ontogeny of all vertebrates, including fish. A mesodermal layer
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ventral to the somites differentiate into structures involved in excretion and reproduction. There are however species differences in how closely connected these structures are in regard of sharing ducts for secretion. The teleost gonads are in general similar to those in mammals. The testis contains Sertoli and Leydig cells in addition to germ cells while the ovary consists of thecal cells and granulosa cells surrounding the ovum. In both teleosts and mammals the interstitial cells (Leydig and theca) and Sertoli cells and granulosa cells are of the same origin. An important difference is that the mammalian gonads are terminally developed into either a testis or an ovary, while fish gonads often retain the ability to change, making them hermaphroditic. Immature teleost gonads can be directed to develop into testes or ovaries, regardless of chromosomal background, after hormone treatments (Kobayashi et al., 2003; Barollier et al., 1999). Far to few fish species have been studied with regard to gonad development to be able to develop a general model of how this occurs.

The zebrafish has become a useful vertebrate model system and is probably the most studied fish in developmental biology. The zebrafish diploid genome consists of 50 chromosomes. The existence of specific sex chromosomes has not been verified by the use of synaptonemal complex studies but rather indicate that no sex chromosomes exist in zebrafish (Traut and Winking, 2001; Wallace and Wallace, 2003). However, there may still be smaller genomic differences that account for or direct the development toward two separate sexes. By studying patterns of inheritance zebrafish have been suggested to have XY-like chromosomal background (Uchida et al., 2002). The zebrafish is sexually mature after approximately three months, but separate sexes can be detected after 21-23 days post fertilisation (dpf) (Uchida et al., 2002). Before the sexual differentiation occurs all zebrafish develop ovary-like gonads, regardless of chromosomal background. Ovarian development is the default pathway, which is initiated after 10 dpf and goes on until 20 dpf. At 21 dpf until approximately 30 dpf testis development is initiated in genetic males simultaneously with ovarian apoptosis.

The gonad development in zebrafish begins during embryogenesis. Using Vasa as a marker gene, germ cells can be detected in the area ventral to the third to fifth somite at the six-somite stage (Yoon et al., 1997). While germ cells can be detected earlier, they are not properly positioned until around the 6 somite stage. So far no studies have been made regarding markers for gonadal steroidogenic precursor cells, rendering it difficult to know exactly where in the embryo these cells are located. But, it is likely that these cells derive from an area close to where Vasa is detected at the six-somite stage. The zebrafish WT1 is also initially detected in an area corresponding to that of Vasa but is later expressed in the pronephric ducts (Drummond et al., 1998), making this region probable for the development of the rest of the zebrafish gonadal cells.

Candidate genes in teleost sex determination:

Even though sex determination in teleost fish usually has genetic a background, they lack an equivalent to the testis-determining factor SRY found in mammals. However, several HMG-box containing genes, Sox-genes, have been identified in fish (Takamatsu et al., 1997; Kanda et al., 1998; Wang et al., 2003). In zebrafish, two Sox9 genes, termed Sox9a and Sox9b, have been identified. Both contain the HMG-box and are able to bind the AACAAAG recognition site in a similar manner as murine Sox9 (Chiang et al., 2001). The expression patterns of Sox9a and b are however dissimilar in adult zebrafish. Sox9a displays a broad expression pattern and has been found in brain, kidney, muscle, testis and pectoral fin, whereas Sox9b only has been found in ovary. During embryogenesis Sox9a and b are both expressed in cells involved in craniofacial development and in the brain (Chiang et al., 2001). In addition Sox9a has been shown to be essential for chondrogenic development (Yan et al., 2002) and Sox9b has been implicated to be involved in neural crest development (Li et al., 2002). Whether
Sox9a and/or b are involved in sex determination or differentiation has so far not been studied. The fact that no testis-determining factor similar to SRY has been found in fish is not a unique phenomenon. This is also the case for many other lower vertebrates. Genes containing a DM-domain (Dmrt1) have however been identified in fish. Here, different Dmrt1 homologues have been shown to be involved in gonad development (Guan et al., 2000; Marchand et al., 2000) and somitogenesis (Meng et al., 1999).

In the teleost Japanese medaka the DM-domain gene DMY has been mapped to the Y-chromosome and has been shown to be essential for testis differentiation (Matsuda et al., 2002), but was later discarded as a universal teleost sex-determining gene due to the fact that it was shown to be a species specific sex-determining strategy (Volff et al., 2003).

AMH may not be excluded as a factor involved in the process. Although fish lack Müllerian ducts, other AMH functions may be important for gonad development. In mammals AMH is, in addition to Müllerian degeneration, involved in regulation of steroidogenesis in the gonad. AMH inhibits the expression of aromatase in developing gonads (di Clemente et al., 1992). It also negatively modulates the differentiation and function of Leydig cells (Racine et al., 1998) by down regulating several enzymes involved in the steroidogenic pathway. Ovarian cell growth is inhibited by AMH in vitro (Ha et al., 2000). No study has been published on teleost AMH so far, but an AMH related gene, designated eel spermatogenesis related substances 21 (eSRS21), have been studied in the Japanese eel (Miura et al., 2002). eSRS21 was mainly expressed in Sertoli cells and was shown to down regulate 11KT induced spermatogenesis, which suggests that eSRS21 and genes related to AMH have functions important for reproduction as well as sex determination and differentiation in fish.

Homologues of FTZ-F1 have been identified in several teleosts (Liu et al., 1997; Ito et al., 1997 b; Watanabe et al., 1999; Chai and Chan, 2000; Higa et al., 2000), but whether these genes have a sex determining or hormone-regulating function has not been clarified. It is likely that more genes will be found due to the variety of sex determining mechanisms in teleosts.

**FTZ and FTZ-F1 in non vertebrates:**

Homologues to the Drosophila FTZ-F1 have been isolated in a wide variety of species both in vertebrates and in more primitive organisms, such as the silk worm (Bombyx mori) (Ueda and Hirose, 1990) and the tobacco hornworm (Manduca sexta) (Weller et al., 2001). The function of these homologues is not conserved between different phyla and perhaps not even within separate phyla. The function of Drosophila and Bombyx mori FTZ-F1 is to control segmentation during embryogenesis by regulating transcription of the homeobox gene fushi tarazu. (Ueda et al., 1990; Sun et al., 1994). Recently the gene product of fushi tarazu was identified as an LXXLL motif-dependent co-activator for orphan receptor FTZ-F1 (Suzuki et al., 2001), indicating that FTZ-F1 carry the function during segmentation rather than FTZ. The FTZ gene has not been found in any vertebrate and the function of FTZ-F1 homologues are mainly linked to functions in the reproductive axis or cholesterol metabolism. Recently FTZ-F1 in C. Elegans was shown to be involved in regulating a gene (lin-3), which is crucial for vulval development (Hwang and Sternberg, 2004), indicating additional functions for non-vertebrate FTZ-F1.
Regulatory mechanisms of FTZ-F1:

Studies of the regulation of FTZ-F1 homologues is so far limited and restricted to mammals. In the promoters of FTZ-F1 genes, there is usually an E-box (CACGTG) for bHLH protein USF (Upstream Stimulatory factor) and CAAT-box (CCAATTGG) for CBF (CAAT-binding factor) (Harris et al., 1998; Daggert et al., 2000). There is also an auto-regulatory loop in the first intron (Ninomiya et al., 1995). In the mouse NR5A2 promoter a Direct Repeat 4 (DR4) response element for the Liver X receptor (LXR) receptor was shown to be important for activation of the gene (Paré et al., 2004). This response element (GGTTTAnnnnAGGTCA) was also shown to be conserved in other species, including zebrafish, and may be essential for the NR5A2 expression in response to altered cellular levels of cholesterol in the liver.

FTZ-F1 is regarded to be an orphan nuclear receptor and no ligand has yet been presented, although 25-hydroxycholesterol has been implied as such (Bertherat et al., 1998). However, the results are controversial and recent studies suggest that both NR5A1 and 2 are active as monomeric orphan receptors (Sablin et al., 2003). The crystal structure of the NR5A2 ligand-binding domain was shown to possess a large empty hydrophobic pocket that may harbour a ligand, but was also shown to work fine without one as no effect on activational function was observed after making alterations in the pocket. The NR5A general protein structure and conserved regions have been mapped. As members of the nuclear receptor family the SF-1 (NR5A1) protein contains a DNA-binding domain (DBD), a hinge region and a ligand-binding domain (LDB) (fig 4) (Mangelsdorf et al., 1995). The DBD contains two Zinc-finger regions and a FTZ-F1 box, recognizing the FTZ-F1 consensus site 5’-PyCAAGGPyCPu-3’ (Ueda et al., 1992). The LDB is the region where the protein interacts with other proteins. It contains a proximal interactive domain (PID), which interacts with co-factors, such as the steroid receptor co-activator 1 (SRC-1), to form complexes involved in the trans-activation machinery (Crawford et al., 1997; Ito et al., 1998). The AF-2 domain is essential for DNA binding and trans-activation. There are also proximal- and distal repressive domains (PRD and DRD) which interacts with co-repressors, such as Dax-1 and the nuclear receptor co-repressor (N-CoR), to block trans-activation complex formation (Crawford et al., 1998).

FTZ-F1 in teleosts:

FTZ-F1 homologues have been identified in a number of teleost species (Liu et al., 1997; Ito et al., 1998; Watanabe et al., 1999; Chai and Chan, 2000; Higa et al., 2000). Several teleosts have multiple variants of FTZ-F1 genes. The roles and functions of these genes are not completely elucidated, but the studies conducted so far all indicate an involvement in the reproductive axis or in steroidogenesis.

**FTZ-F1 in zebrafish:**

Zebrafish is the most extensively studied teleost in that aspect and four FTZ-F1 genes have been identified so far (ff1a, b, c and d). The arrangement of FTZ-F1 genes into the nuclear receptor 5A subgroups is a suitable system for genes in higher vertebrates, as no indistinguishable genes have been described so far in these animals. However, teleosts and particularly zebrafish are different in more than one way compared to higher vertebrates. Firstly, zebrafish have four different FTZ-F1 genes described, whereas mammals and higher vertebrates only possess two. The zebrafish genes are also not as easily arranged within the NR5A subgroups compared with their mammalian homologues (Fig. 5). Zebrafish ff1a and Arctic char ff1 aligns well within the NR5A2 subgroup, but their expression patterns and
suggested functions do not fit the description of the mammalian NR5A2 genes. Secondly, the zebrafish ff1c does not align well with any of the described subgroups, which further raises the question of how appropriate the subdivisions really are to teleost FTZ-F1. Ff1d aligns in the NR5A4 subgroup and is most similar to ff1b.

Figure 5. Comparative sequence analysis of vertebrate FTZ-F1 proteins. The radial tree was constructed using Tree View (Version 1.6.2) (Page, 1996) following alignment of the protein sequences by the Clustal W algorithm (Version 1.7). Modified in adobe Photoshop. Branch lengths are proportional to evolutionary distances. GenBank Accession Numbers: Salvelinus alpinus FF1α (acFF1α) (AF468978); Mus musculus LRH-1 (mLRH-1) (P45448); Rattus norvegicus SF-1 (rSF-1) (P50569); Mus musculus SF-1 (mSF-1) (P33242); Rana rugosa FTZ-F1 (rrFTZ-F1) (BAA94077); Danio rerio FF1A (zFF1A) (AAC60274); Danio rerio FF1b (zFF1b) (AF198086); Danio rerio FF1c (zFF1c) (AAK19303); Danio rerio FF1d (zFF1d) (AAO59489); Rattus norvegicus FTF (rFTF) (AAC52645); Oryzias latipes FTZ-F1 (mdFTZ-F1) (BAA32394); Oncorhynchus mykiss FTZ-F1 (rtFTZ-F1) (BAB11689); Gallus gallus SF-1 (cSF-1) (BAA22839); Gallus gallus FTF (cFTF) (BAA22838); Drosophila Melanogaster (dmFTZ-F1a) (NP_524143).

**Ff1α:**

The first FTZ-F1 gene described in zebrafish was ff1a (Liu et al., 1997). Since no other zebrafish ff1 genes were known at that time the gene was named zff1, but has later been referred to as ff1a. It was shown to possess two splicing variants, designated ff1a-A and ff1a-B, and ff1a-A was shown to transcriptionally activate a gonadotropin promoter in synergy with ER, whereas ff1a-B acted as an inhibitor of ff1a-A due to its lack of trans-activator domain AF-2. The expression of zebrafish ff1a was later shown to be driven by two separate gene promoters, giving rise to a total of four separate gene transcripts, ff1a-IA, ff1a-IB, ff1a-
IIA and ff1aIIIB (Fig. 6) (Lin et al., 2000). The gene consists of eight exons. There is a splice site in exon seven, dividing it into exon 7a and 7b, where exon 7b is kept in the truncated transcripts (ff1a-IB and ff1a-IIIB). In ff1a-IA and ff1a-IIA exon 7b is spliced of and exon eight is used in its place. This leads to a differing LDB between the truncated and not truncated ff1a proteins.

![Figure 6](image).

(A) The genomic structure of ff1a with exons 1 to 8. Exons 1 and 2 are transcribed alternatively by dual promoter usage to form separate 5'UTRs and modulator domains. A splicing site is also present in exon 7, where exons 7b and 8 are alternatively used. The transcripts containing exon 7b (ff1a-IB and –IIB) codes for truncated protein. (B) General structure of the FTZ-F1 protein with an A/B modulator region, a C region containing the DNA-binding domain (DBD) with two Zinc-fingers and an FTZ-box. The D region contains a hinge region and the E region contains a ligand-binding domain (LBD) with an AF-2 domain essential for trans-activation.

The ff1a protein shares the general structure of other nuclear receptors. It contains a DNA binding domain (DBD) with two Zn- fingers and the FTZ-F1 box for DNA interaction and recognition, a hinge domain that connects to the ligand-binding domain (LBD), which contains the I-box for protein-protein interaction and the activator function-2 (AF-2) domain for transcriptional activation (Figs. 6b and 8).

The functional difference between ff1a protein consisting of exon one, transcribed via promoter I, and protein transcribed from promoter II has so far not been studied. However, they differ slightly in their tissue distribution, where promoter one derived transcripts are lacking in brain and heart (Lin et al., 2000). Since the two ff1a promoters contain several putative response elements (Fig. 7), not shared between them, it leads to the conclusion that the two promoters are used in regulation of ff1a in a tissue specific manner.
and during different developmental stages rather than rendering them separate functions. The use of two separately regulated NR5A promoters have also been found in mouse (Ikeda et al., 1993) and in rat (Nomura et al., 1995).

Figure 7. The ffla gene is transcriptionally regulated via two separate promoters. Promoter sequence is illustrated in black, cis-elements are boxed, transcribed sequence in light gray and the first atg and translated sequence is highlighted.

Two of the putative response elements in promoter I indicate an involvement in somitogenesis. MyoD and Snail are both transcription factors shown to be involved in somite development (Weinberg et al., 1996; Thisse et al., 1993), indicating that promoter 1 may drive the ffla expression during somitogenesis. Promoter II contains a HMG-Box response element 24-31 bp up stream of the transcription start. A response element identical to this has been shown to bind Sox9a in vitro (Chiang et al., 2000) and Sox9 is hence a putative regulator of gonadal expression of ffla.
**Ff1b:**

Zebrafish ff1b and medaka FTZ-F1 are the only members of the NR5A4 subgroup. The ff1b gene was initially assigned to functions regarding pancreatic development due to its co-expression with pancreas duodenum homeobox-1 (pdx-1) and proinsulin (Chai and Chan, 2000). However, more recent publications suggest that ff1b is more likely an important factor for steroidogenic cell development and that ff1b is required for the differentiation of the interrenal organ (Chai et al., 2003; Liu et al., 2003). The expression of ff1b precedes that of CYP11A and 3βHSD in the embryonic interrenal cells and ff1b morpholino knock down experiments abolishes the expression of these two genes (Chai et al., 2003). The placing of ff1b in the NR subgroup NR5A4 has, due to these results, been questioned and suggestions have been made that NR5A1 is a more appropriate group for ff1b. Additional studies are however needed to link ff1b to the NR5A1 group.

The downstream transcriptional activation function of ff1b is modulated by protein-protein interactions with homeodomain protein Prox1 (Liu et al., 2003). Two domains are needed for the interaction, the I-box and the AF-2 domain, both situated in the LBD (Fig. 8). Binding to Prox1 leads to a repression of downstream trans-activation. There is also a co-localisation of ff1b and Prox1 expression in the developing interrenal. Due to the conserved I-boxes and AF-2 domains, both ff1a and ff1c are probably able to interact with Prox1, although less efficiently than ff1b.

**Ff1c and ff1d:**

There is little information available regarding ff1c and ff1d functions, regulation or expression patterns. Except for the weak interaction between Prox1 and ff1c presented in Liu et al. (2003), only sequences have been published on GenBank, ff1c (AF327373) and ff1d (AY212920). Both ff1c and ff1d are similar to ff1a and b in their DNA-binding domains where the FTZ-F1 box is situated and in the ligand binding domains, but are less conserved in their hinge regions (Fig. 8). All zebrafish ff1 have highly conserved AF-2 domains and I-Boxes in their LBD.
Figure 8. Protein alignment of zebrafish fli1a, b, c and d. fli1a is represented by the protein generated by transcription from exon 2. FTZ-F1 box is underlined inside the indicated area of DBD. The interactive I-box and AF-2 cores are underlined in the LBD. GenBank sequences for fli1a (NM_131463), fli1b (NM_131794), fli1c (AF327373) and fli1d (AY212920)
**Other teleosts:**

Ftz-F1 genes have been identified in several fish species, although zebrafish is by far the most studied. Numerous salmonids have also been shown to possess FTZ-F1 related genes, such as csFF1-I and II in the chum salmon (*Oncorhynchus keta*) and ssFF1-I and II in the sockeye salmon (*Oncorhynchus nerka*) (Higa et al., 2000), tFZR1 (*Oncorhynchus mykiss*) (Ito et al., 1998) and tFTZ-F1 in the rainbow trout (GenBank no: BAB11689). Other fish FTZ-F1 genes are the medaka (*Oryzias latipes*) FTZ-F1 (Watanabe et al., 1999), and ff1a and ff1b in black porgy (*Acanthopagrus schlegelii*) (GenBank no: AY491378 and AY491379).

A clear link between reproductive maturation and elevated pituitary levels of both csFF1 and ssFF1 is established in fish migrating to spawn (Higa et al., 2000). An increase in FSH was related to up regulation of GnRH, but could not be directly induced by GnRH implants. This indicates other or additional regulatory mechanisms than a direct interaction with GnRH (Higa et al., 2000). The relationship between GnRH and mammalian SF-1 is complex and there is evidence that supports that SF-1 is transcriptionally regulated by GnRH (Haisenleder et al., 1996) and there are studies that show SF-1 dependent regulation of the GnRH receptor (Duval et al., 1997; Reinhart et al., 1997). Also, in the teleost spotted sea bass (*Morone saxatilis*) the GnRH gene promoter contain a FTZ-F1 binding site, suggesting that FTZ-F1 is involved in GnRH transcriptional regulation (Chow et al., 1998).

The medaka FTZ-F1 is co expressed with CYP19 in the ovary and binds to the aromatase promoter, suggesting a potential role of mdFTZ-F1 in the transcriptional regulation of CYP19 in ovarian follicles (Watanabe et al., 1999).

Few studies have presented a general function of these genes and it is likely that their functions vary between species. However, they are in all cases involved in steroidogenesis and reproduction, making this a common feature the teleost FTZ-F1 genes. Regarding embryonic expression and developmental function of teleost FTZ-F1 the information is even more limited and is at the present time not studied in any other species than the zebrafish, except for paper III in this thesis, where the embryonic expression of the salmonid Arctic char was examined.
AIM

The aim of this thesis was to study teleost FTZ-F1 homologues and to investigate expression patterns and putative actions of FTZ-F1 during embryogenesis, in adult fish and during the reproductive cycle. Initially, the emphasis was set on establishing the wild type expression patterns of teleost FTZ-F1 genes in order to link their expression to tissues involved in reproduction and steroidogenesis. Specific functions and regulatory mechanisms were thereafter studied both up-stream and down-stream of the FTZ-F1 genes. In papers I and III the work was focused on embryonic and adult expression patterns. In paper II the work was aimed at studying FTZ-F1 in hormonal influence and reproductive maturation. Paper IV focused at determining up-stream regulation of ff1a. Paper V was aimed at characterizing the novel FTZ-F1 gene ff1d and the zebrafish homologue to the AMH gene.
RESULTS AND DISCUSSION

Paper I

We isolated two zebrafish FTZ-F1 cDNA homologues (ff1a) and ff1b) to elucidate whether they were developmentally regulated and possibly linked to sex determination. Ff1a is in this paper named zFF1 due to the fact that when this paper was published the gene was known as zFF1 and has thereafter been renamed zff1a. The clones were used to generate cDNA and RNA probes in order to study mRNA expression *in situ* during several stages of zebrafish development.

To our knowledge at the time of writing the manuscript the zFF1 (ff1a) gene gave rise to two mRNAs (zFF1A and zFF1B), by alternative splicing in exon 7 (Lin et al., 2000). As described above more recent studies have shown that the gene gives rise to four transcripts and the zFF1A transcript studied in (I) is the combined transcripts of ff1a IA and IIA, and zFF1B is ff1a IB and IIB (See fig. 6). The zFF1A and zFF1B transcripts codes for amino acid sequences identical in the DBD and hinge regions. They differ in the LDB where zFF1B is truncated and lacks the AF-2 region needed for protein trans-activation (Liu et al., 1997). The mRNAs have separate UTRs, which made it possible to study them separately. The other homologue, ff1b, is a product of another gene, which was confirmed by southern analyses (I). To study the developmental regulation of zFF1A/B and ff1b we used a semi-quantitative RT-PCR method to compare expression levels during four developmental stages between somitogenesis and hatch (Fig. 9).

![Figure 9. Developmental stages of zebrafish (Modified from Kimmel et al 1995).](image)

The developmental stages were chosen from early somitogenesis (10 somites) until hatch (long pec). The Prim 5 stage occurs just after completed somitogenesis and Prim 16 is in the
middle of the organogenesis process. Using 18s rRNA as control, the relative expression levels of each transcript was determined. zfF1A and B had a similar pattern with peaks at the prim 5 and long pec stages (I). The highest levels were reached at the long pec stage. ff1b showed more constant expression during the examined stages. However, the highest levels were again found at the long pec stage indicating that both genes are up regulated at hatch (I). The diverging relative levels of expression of zfF1 and ff1b transcripts indicated separate regulatory processes and possibly separate functions during development. To study the spatial expression during development in situ hybridisations were conducted at the earlier described stages, plus two additional stages, 14 somites and 22 somites. The additional stages were used to give a more complete picture of expression patterns after the onset of somitogenesis. Digoxigenin-labelled RNA probes were generated to map the expression of two FTZ-F1 genes and the specificities were confirmed by generating different hybridisation patterns on a genomic southern blot. The southern blot also confirmed that the transcripts were derived from separate gene loci (I). A study by Chai and Chan (2000) described the developmental expression of ff1b, which was congruent with our own observations. Expression of ff1b mRNA was observed in the diencephalon, in the area corresponding to the pituitary and hypothalamus and in the area of the developing pancreas (fig 10).

Figure 10. Zebrafish embryo at long pec after whole mount in situ hybridisation with a DIG-labelled anti-sense ff1b RNA probe visualised by NBT/BCIP. (A) Head view. (B) Dorsal view. p: pituitary/hypothalamus, pa: pancreas

Three probes at separate locations in the RNA sequences monitored expression of zfF1. Sense and anti-sense probes recognising the splicing variants A and B were generated to hybridise with the 3'UTRs, specific for each splice variant. A third probe was generated to recognise both variants indifferently (I). This probe gave the strongest signal and was used to illustrate the zfF1 expression. A complex spatial and temporal expression pattern was observed. The signals were first detected in the area of the somites and ventral to the somites, indicating an involvement of zfF1 during somitogenesis. The expression in the somites was consistent during the subsequent somite formation process and disappeared when the formation was completed (I). So far, no mammalian version of FTZ-F1 has displayed any expression or function during somitogenesis, suggesting a different or additional role of this FTZ-F1 version. Expression of FTZ-F1 has later been observed during somitogenesis in frog (Kawano et al., 2001), indicating conservation for FTZ-F1 in this developmental mechanism in lower vertebrates. The signal ventral to the somite expression corresponded to that of the uro-genital ridge and later the pronephric duct (Westerfield et al., 1997). This is an area previously identified expressing SF-1 and genes in the NR5A1 group (Ikeda et al., 1994), linking zfF1 to NR5A1. During the late phase of somite development, expression of zfF1 was detected in the pituitary area (I). This signal was found in Prim 5, Prim 16 and Long Pec. At the prim 16 stage a signal in the developing liver appeared (I). Mammalian SF-1 and genes in the NR5A1 group have so far not been observed in the liver of either developing or adult individuals. NR5A2 genes are however abundantly expressed in liver (Galarneau et al., 1996), suggesting that zfF1 would be linked to NR5A2. In addition to these areas, zfF1 was expressed in the first pharyngeal arch, giving rise to the mandibular arch. This is also a tissue
observed to developmentally express NR5A2 (Rausa et al., 1999). zFF1A gave the same expression pattern, although weaker whereas zFF1B was detected in the pituitary and liver only.

To further characterise the two zebrafish homologues a protein sequence alignment was analysed in PAUP, to possibly clarify the origin of the genes. A bootstrap analysis confirmed that the zFF1 sequence was homologous to the NR5A2 genes and ff1b to NR5A4 (I), which has no mammalian homologue. The majority of our data suggested zFF1 to be a NR5A2 and if this would be the case, less interesting for sex determination and hormone regulation studies. However, so far has no teleost FTZ-F1 homologue aligned with the NR5A1 group and none have displayed a consistent expression pattern. Functional studies have identified zFF1 as a regulator of the LHβ receptor subunit (Liu et al., 1997), linking zFF1 to the NR5A1 group. The expression detected in the pituitary during the later developmental stages is congruent with this documented function.

Our conclusions were that the FTZ-F1 homologues, zFF1 and ff1b, were transcribed from separate loci. They were developmentally expressed in an individual manner, but both up-regulated at hatch. Both genes had a complex temporal and spatial expression pattern, while only zFF1 was expressed in the area of the developing gonads. The sequence analysis linked zFF1 to the NR5A2 genes, but the expression pattern was not consistent with other genes in this category, displaying expression in somites and uro-genital ridge, suggesting that zFF1 and other teleost FTZ-F1 genes may be different than mammalian FTZ-F1 genes with functions in common with both NR5A1 and NR5A2.

**Paper II**

As we confirmed that zFF1 was developmentally expressed and putatively involved in the formation of gonadal tissue we chose to further study this FTZ-F1 subtype in adult fish to investigate possible involvement in reproductive maturation and steroid synthesis. Since zebrafish are tropical fish without obvious reproductive cycles, we chose to study the salmonid Arctic char (Salvelinus alpinus). The aim was to monitor expression of the Arctic char version of zFF1, StAR and CYP11A during the reproductive maturation and after hormone treatment to determine seasonal alterations and possibly find links between expression patterns. Since none of the previously mentioned genes had been identified in Arctic char, we initiated the study by isolating partial cDNA of StAR, CYP11A and two homologues of FTZ-F1 closely related to the zebrafish zFF1. We also isolated the Arctic char version of 3βHSD.

The two Arctic char sequences (designated acFF1α and acFF1β) were closely related to the other teleost FTZ-F1 sequences (See fig 5), zFF1 and trout FTZ-F1 indicating that they are derived from the same ancestral gene.

Co-expression of acFF1, StAR and CYP11A mRNA was observed in the gonads and head kidneys (II), leaving a possible regulatory mechanism open. CYP11A expression was restricted to these tissues. StAR expression was also observed in liver, where no documented function has been established. Possibly, StAR is involved in cholesterol trafficking in liver. Using a FF1 antibody, putative post-transcriptional regulation was observed for acFF1, which had fewer tissues expressing the protein than the mRNA (II). Posttranscriptional regulation has previously been suggested for the amphibian rrFTZ-F1, where a steady-state level of mRNA was detectable in the testis through the whole year cycle, but the protein was only detected at spawning and pre-hibernation (Takase et al., 2001). acFF1 mRNA and protein expression in testis and ovary correlated during reproductive maturation (II). A sharp peak of acFF1 mRNA preceded the onset of protein expression. Different regulatory mechanisms of acFF1 mRNA expression in ovary and testis were
However observed in the hormone treatment study, where E2 inhibited acFF1 expression in testis, but did not affect ovarian levels (II). This is a novel finding in teleosts that may have important effects on reproductive success after exposure to environmental estrogenic pollutants. In this study 11KT was observed to down-regulate CYP11A and 3βHSD in the Arctic char head kidney and up-regulate CYP11A and 3βHSD in testis (II). No clear effect was seen on acFF1 expression and the down- and up-regulation of CYP11A and 3βHSD was not indicated to be dependent on decreased or increased acFF1 transcription. Previous studies have reported decreased cortisol levels in the head kidney after 11KT treatments (Young et al., 1996; Pottinger et al., 1996) and a decrease of CYP11A and 3βHSD transcription may account for such a decrease. The mechanism by which this decrease occurs has not yet been elucidated, but a decrease in acFF1 is a strong candidate and acFF1 cannot yet be ruled out until the existence of post-transcriptional regulation has been determined.

The process of hormone synthesis is complex and feedback loops to the hypothalamus acts as a main regulator by triggering LH and FSH secretion from the pituitary. The main biological activity of LH is to regulate Leydig-cell steroid production. While piscine FSH also appears to have steroidogenic activity, specific roles have not been described yet in the testis. However, different regulatory mechanisms between testis and ovary are evident. A link between 11KT and FTZ-F1 levels have previously been observed in salmonid fish (Higa et al., 2001) and was also observed in the present study. Female E2 plasma levels were also correlated to high acFF1 levels (II), indicating a link between E2 and acFF1. This link was not verified by the hormone treatment study. But, expression levels are correlated to 11KT and E2 during the reproductive maturation and hormones are able to modulate expression of acFF1 and putative downstream genes.

The fact that acFF1 is involved in reproduction is well supported by the results in this study, but in what way and in what specific mechanisms acFF1 functions during reproduction is less evident. Since the tissue specific expression of acFF1, StAR and CYP11A was overlapping and followed a similar profile during the reproductive cycle it is likely that they are associated. The hormone treatment study is in a way in conflict with these results as CYP11A was down regulated by hormones independently to acFF1, which argues for another regulatory pathway for CYP11A than via acFF1. But, hormones create systemic effects when they are introduced in the fish. Many regulatory pathways may be affected, which can lead to down- or up-regulation of a battery of genes in steroid production. The transcription of steroidogenic genes can be dependent of several transcription factors and co-factors and the systemic effect achieved by injecting hormones may have altered the balance in a way that the acFF1 effect is overlooked. Based on the results in this paper and the findings previously seen in other species, my conclusion is that acFF1 is an important factor in Arctic char reproduction, likely as a transcriptional regulator steroidogenic genes such as StAR, CYP11A and 3βHSD.

**Paper III**

To confirm that acFF1 was the Arctic char equivalent to zebrafish ff1a we examined its developmental expression pattern. A whole mount in situ hybridisation on Arctic char embryos, using antisense DIG-labelled acFF1 RNA, displayed a similar expression pattern as observed for zFF1 in paper I and verified the putative involvement in gonad development for these FTZ-F1 genes. The signal seen for ff1a (or zFF1 as it is called in paper I) in somites was not observed in the Arctic char. Neither was the signal in the pituitary. The Arctic char development was however not as thoroughly examined and only two developmental stages were studied by in situ hybridisation (stages 21 and 25) (II).
Since the expression domains of zFF1 were temporally regulated during embryogenesis, it is not unlikely that expression in somites and pituitary exist in the Arctic char even though we were unable to detect them at these particular stages. However, the precise function of these genes during development remains to be determined. The staging of embryos were made using the combined information from Balon (1980), on the development of *Salvelinus* embryos based on water temperature and days of development, and from Vernier (1966) depicted staging series on *Oncorhynchus mykiss* (Fig. 11). The relative expression levels of acFF1 mRNA and protein were monitored through these developmental stages and were all found to up regulated towards the time of hatching, which occur after stage 29.

**Figure 11.** Selected stages of salmonid embryonic development. The stages illustrated are labelled as they are in the original reference (Vernier, 1966).

The conclusion drawn from this study is that acFF1 is developmentally expressed and regulated. AcFF1 does undoubtedly belong to the NR5A2 subgroup, which is strongly connected to cholesterol metabolism in mammals. The connection to cholesterol metabolism was also made by the expression pattern in liver and intestine. A correlation to zFF1 (ff1a) was found as both were expressed in the uro-genital region during embryogenesis indicating a function in gonad development. In adult fish acFF1 was also expressed in steroidogenic and non-steroidogenic tissues matching both the expression patterns of NR5A1 and NR5A2 genes. The developmental function of acFF1 needs to be studied further, but based on tissue distribution and the correlation between acFF1 and ff1a, the results indicate an involvement in steroidogenic- and cholesterol metabolising organ development.

**Paper IV**

The dual promoter region of zebrafish ff1a contains several putative binding sites for transcription factors. The emphasis in this study was to examine the dual promoter and to learn more about its regulation. By linking the promoter region to luciferase and EGFP vectors the activity of different parts of the promoter was studied, both *in vitro* and *in vivo*.
The full length EGFP-1 linked ff1a dual promoter was microinjected in 1-cell stage embryos in order to study the expression in vivo. The results indicated that the injected promoter construct was active in transient transgenic fish in the same tissues as previously observed by in situ hybridisation (Fig. 12, also see paper I). Ff1a promoter driven EGFP expression was also detected in early gonadal tissue (Fig 12G), which argue for ff1a involvement in gonad differentiation.

**Figure 12.** The EGFP-1 linked ff1a full length wild type promoter is active in tissues consisting with in situ detected ff1a expression. (A, C, E and F) EGFP expression. (B, D and F) in situ hybridisation of ff1a expression. (A) 28 somites, (B) Prim 5, (C) Prim 16, (D-F) Long Pec, (G) 5 dpf. Pd: pronephric ducts, s: somites, m: mandibular arch, l: liver, g: gonads.

Using a series of deletion mutants the ff1a promoter was examined in a zebrafish liver cell line (ZFL). An HMG box/sox9 cis-element was identified in a region of 32 bp prior to transcription initiation, which was sufficient to drive the luciferase expression at high levels (IV). When the AACAAAG cis-element was point mutated the levels of luciferase expression decreased indicating that the response element was important for the regulation of the gene. When the part of the promoter containing the HMG box/sox9 response element was removed, the luciferase levels decreased (IV). The decrease was most significant when the promoter sequence between −30 and +10 was removed from the full length dual promoter. This decrease was even more significant than the decrease resulting from the promoter deleted in its 5’ end to +10, indicating that there may be a repressor present acting up-stream of the HMG box/sox9 response element. The promoter region between the HMG box/sox9 response element and transcription initiation may also harbour additional cis-element important for positive regulation. This is indicated by the moderate decrement in luciferase levels after deleting the HMG box/sox9 element.

Sox9a was found to be expressed in ZFL cells, suggesting that Sox9a may be the transcription factor that binds and regulates the promoter. To investigate if Sox9a was able to bind the cis-element we expressed Sox9a using a T7 promoter linked in vitro expression vector system and used the protein as template in an electrophoretic mobility shift assay (EMSA). Point mutations in the HMG-Box/Sox9 response element resulted in diminished capability to compete for binding. But, using the wild type sequence as a competitor the labelled DNA/ Sox9a protein complex was decreased indicating that Sox9a bound specifically to the wild type sequence (IV).

There are numerous Sox-proteins and theoretically many may bind to the AACAAAG sequence in the zebrafish ff1a promoter. Based on the function of Sox9 in mammals and the connection between Sox9 and SF-1, the zebrafish Sox9 homologue Sox9a was the natural candidate for the in vitro studies. The EMSA showed that Sox9a bind to the
HMG box/sox9 cis-element sequence. Down regulation of Sox9a by siRNA also resulted in decreased luciferase levels (IV). This suggests that Sox9a is a transcriptional regulator of ff1a. Since a similar transcriptional activation has been shown in mammals, this study indicates that the Sox9/FTZ-F1 interplay may be a conserved vertebrate mechanism. While in vivo studies are needed to establish if the function of this interplay has any influence on reproduction or development, a putative mechanism could be that Sox9a acts as an up-stream transcriptional regulator of ff1a during zebrafish gonad development in a way similar to the mammalian system.

**PaperV**

The mammalian sex determination process involves SF-1 and AMH, both crucial for the development of a male phenotype. In this paper we present two novel zebrafish genes ff1d and AMH, homologous to their mammalian counterparts and both previously not studied in fish. Ff1d was found to align within the NR5A4 subgroup, close to the NR5A1 clade, which makes ff1d the zebrafish gene closest associated to the SF-1 genes by sequence similarity. The NR5A4 gene, ff1b, has previously been suggested to be the zebrafish SF-1 functional homologue based on its expression and steroidogenic regulatory functions during interrenal development (Hsu et al., 2003). During development, ff1d was detected in the pituitary and in the developing interrenal, in a way similar to ff1b (V). The expression in the pituitary persisted through out embryogenesis and at 30-dpf ff1d was expressed both in hypothalamus and the steroidogenic anterior pituitary. Its expression pattern was identical with ff1b in adult tissue in an RT-PCR analysis and was found in brain, gonads and weakly in liver (V). The strongest expression detected by RT-PCR was found in testis, where in situ hybridised sections detected ff1d mainly in Leydig and Sertoli cells (V). The partial zebrafish AMH mRNA was cloned and the translated product aligned with AMH of human, chick and eel (V). The function of teleost AMH has not been studied previously, but in this study we found that the expression pattern of zebrafish AMH corresponded to mammalian AMH. The expression was restricted to Sertoli cells in testis and granulosa cells in ovarian follicles (V).

The specific functions of ff1d and AMH needs to be determined further, but since the expression patterns of the two genes are identical to mammalian SF-1 and AMH it is likely that they also share functional features. As fish in general lack a sex-determining gene like SRY, ff1d and AMH are candidates for directing zebrafish sex determination and differentiation.

In mammals, AMH is responsible for the degeneration of Mullerian ducts during male reproductive development. These ducts will in the lack of AMH develop into parts of the female reproductive system, such as oviducts and part of the uterus. Since the teleost female reproductive system differs significantly from the mammalian system and no Mullerian ducts exist in fish, such functions are redundant. AMH may still be important for teleost gonad development. Zebrafish develop as females with early ovarian-like gonads that around 20dpf differentiate into testicular tissue in male fish. The time of male development occur at the same time as AMH expression is upregulated (V), suggesting a putative role of zebrafish AMH in masculinization. In mammals AMH is also involved in the regulation of steroidogenesis in the gonad. A teleost AMH related gene, designated eel spermatogenesis related substances 21 (eSRS21), has been studied in the Japanese eel and was found to be regulated by 11KT. As steroid levels affect fish sex determination and differentiation during development, this indicates that eSRS21 and genes related to AMH may have functions important both for reproduction as well as sex determination and differentiation in fish.
CONCLUDING REMARKS

Reproductive success is fundamental for the survival and evolution of teleosts. The large group of species have evolved diverse reproductive strategies. The division into separate sexes is however a common feature although fertilisation and rearing of offspring may differ. Based on the results in this study, I find that the teleost NR5A genes are to the highest degree involved in both reproductive success and sexual differentiation. Papers I and IV presents data that links zebrafish ff1a to numerous developmental features, including gonad development. This by detection of ff1a mRNA in the uro-genital region in early development, by detection of GFP linked to the ff1a promoter in developing gonads in zebrafish larvae and by finding a functional regulatory mechanism by Sox9a in the ff1a promoter. These data taken together give a clear picture of ff1a as a transcription factor important for gonad development.

Arctic char ff1α and β also belong to the NR5A2 genes and are in many ways similar to zebrafish ff1a. During embryonic development they have a similar expression pattern, indicating conserved functions. During reproduction acff1 is up regulated, which indicates a function in the reproductive maturation process. Testicular levels of acff1 were down regulated after E2 treatment. This fact may have serious consequences to the reproductive success in a population as many environmental pollutants, such as PCBs have estrogenic effects. This could lead to a disturbance in the maturation process if fish, males in particular, are exposed to estrogenic compounds during the maturation process. As teleost NR5A2 genes also seem to be important for gonad development and possibly sex determination, exposure to estrogenic compounds also could cause altered sex ratios and in the long run it could lead to weaker populations and extinction.

The zebrafish NR5A family contain a total four genes (ff1a, b, c and d). Ff1b and c are not extensively studied in this thesis. However, ff1b and ff1d (a novel zebrafish gene) are developmentally expressed in steroidogenic tissues and are both closer related to mammalian SF-1 than ff1a or ff1c. The cellular distribution of ff1d indicates in involvement in reproduction as it is highly expressed in both Leydig and Sertoli cells in the testis and in the theca and granulosa cells in the ovary. The gonadal expression also overlaps the expression of AMH, which was found mostly in Sertoli cells and the follicular layer in the ovary. AMH is previously not described in fish and the function is therefore not known. However, in mammals AMH is a crucial sex-determining factor, transcriptionally regulated by SF-1. In this study the overlapping expression patterns indicate that this may be a conserved mechanism in vertebrates. In addition, AMH and related genes in other species are involved in gonadal steroidogenesis. As fish sex determination and differentiation is dependent on certain steroid levels during development this indicates that AMH may have functions important for sex determination and differentiation in fish.

Teleost reproductive success and sex determination are both depending on a hormonal balancing act, which make them vulnerable to environmental changes. A battery of candidate genes has been identified as important for maintaining the system. The teleost NR5A genes are, based on the results and literature cited in this study, important factors involved in multiple levels of this system.
• Teleost FTZ-F1 genes are diverse and located to at least four groups coding for FTZ-F1 proteins. The genes cannot be grouped together with equivalent mammalian FTZ-F1 homologues after comparing sequences and expression patterns.
• The developmentally regulated expression of teleost ff1a is restricted to previously observed NR5A1, NR5A2 and NR5A3 domains, indicating developmental functions spanning over the borders of the mammalian subdivision of FTZ-F1 genes.
• Developmental expression of ff1a and acFF1 in the uro-genital area is indicated to be conserved between mammals and teleosts and correlates with the documented function in sex determination and gonad development in mammals.
• The ff1a dual promoter is positively regulated by Sox9a, which also implies an involvement in gonad development.
• mRNA and protein expression of acFF1a does not fully correlate, suggesting a potential post-transcriptional regulation.
• The acFF1 protein is up regulated during early reproductive maturation suggesting a role in hormonal regulation.
• Different regulatory mechanisms of acFF1 mRNA expression in ovary and testis were observed in the hormone treatment study, where E2 inhibited acFF1 expression in testis, but did not affect ovarian levels. This is a novel finding in teleosts that may have important effects on reproductive success after exposure to environmental estrogenic pollutants.
• Ff1d is a novel zebrafish FTZ-F1, which is developmentally expressed in pituitary and highly expressed in adult testis.
• AMH exist in fish and is expressed in Sertoli and granulosa cells, similar to mammals, and may be involved in sex determination in fish, possibly together with ff1d.
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