Review

Endocrine regulation of gametogenesis in fish

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ABSTRACT The pituitary-gonadal axis plays an important role in regulating gametogenesis in vertebrates. In most cases, gonadotropins act through the biosynthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis. A series of studies in our laboratory using several species of teleost fishes as experimental animals has provided new information about the endocrine regulation of gametogenesis, including oocyte growth, oocyte maturation, spermatogenesis and sperm maturation. This article briefly reviews our findings on the identification of steroidal mediators involved in each process of gametogenesis, and the sites and mechanisms of action of the mediators. These observations collectively demonstrate the appropriateness of using teleost fishes as valid models for examining hormonal influences on gametogenesis. Such models could also have applications and validity for vertebrates in general.

KEY WORDS: *gametogenesis, gonadotropin, steroid hormones, fish*

Introduction

It is well established that in vertebrates, gonadotropins are the primary hormone to regulate gametogenesis. However, it appears that gonadotropins do not act directly, but work through the gonadal biosynthesis of steroid hormones which in turn mediate various stages of gametogenesis. Over the last several years, a series of studies in our laboratory using several fish species as experimental animals has provided new information about the hormonal regulation of gametogenesis. This article briefly reviews our results on the hormonal regulation of oocyte growth, oocyte maturation, spermatogenesis, and sperm maturation in fish (Fig.1).

Gonadotropin

Recent studies have indicated that fish pituitaries, like those of other vertebrates, secrete two kinds of gonadotropins. Two distinct carbohydrate gonadotropins, designated as GTH-I and GTH-II, have been isolated and characterized from pituitaries of spawning chum salmon, *Oncorhynchus keta* (Suzuki *et al.*, 1988a,b; Kawauchi *et al.*, 1989). Each of these gonadotropins consists of α and ß subunits, the ß subunits having amino acid sequence identities of 31%. GTH-I and GTH-II have also been purified in coho salmon, *Oncorhynchus kisutch*, (Swanson *et al.*, 1991) and they are chemically similar to the chum salmon gonadotropins. Both GTH-I and GTH-II are steroidogenic (Suzuki *et al.*, 1988c), and differ only in their relative potencies. Plasma levels of GTH-I show an extended increase during vitellogenesis/spermatogenesis, and a decline at the time of spawning. On the other hand, plasma levels of GTH-II are constantly low throughout vitellogenesis/spermatogenesis and increase dramatically at the time of spawning in the late autumn. Taken together, it is suggested that in addition to the established role of GTH-II during final gonadal maturation, GTH-I may play a role during vitellogenesis and spermatogenesis. In this review, the term gonadotropin is taken to refer to the glycoprotein-rich maturational gonadotropin or GTH-II.

Hormonal regulation of oocyte growth (vitellogenesis)

Oocytes of nonmammalian vertebrates grow while arrested in the first meiotic prophase. The principal events responsible for the enormous growth of fish oocytes occur predominantly during the phase of development termed vitellogenesis, and involve the sequestration and packaging of a hepatically derived plasma precursor, vitellogenin, into yolk protein. Estrogen, estradiol-17ß in most cases, produced by the ovary under the influence of gonadotropins, is introduced into the vascular system and stimulates the hepatic synthesis and secretion of vitellogenin (Fig. 2). Injection with pituitary extracts or various preparations of gonadotropins induces estradiol-17ß synthesis and subsequent vitellogenesis. Plasma levels of estradiol-17ß and vitellogenesis are positively correlated and increase in parallel. Exogenous estrogen can induce vitellogenin synthesis in the liver and its appearance in the plasma.

Vitellogenin is selectively taken up from the blood stream by developing oocytes (Wallace, 1985). Vitellogenin receptors were

Abbreviations used in this paper: 17α,20ß-DP, 17α,20ß-dihydroxy-4-pregen-3-one; HCG, human chorionic gonadotropin; GVBD, breakdown of germinal vesicle; MIH, maturation-inducing hormone; MPF, maturation promoting factor; 20ß-S, 17α,20ß, 21-trihydroxy-4-pregen-3-one.

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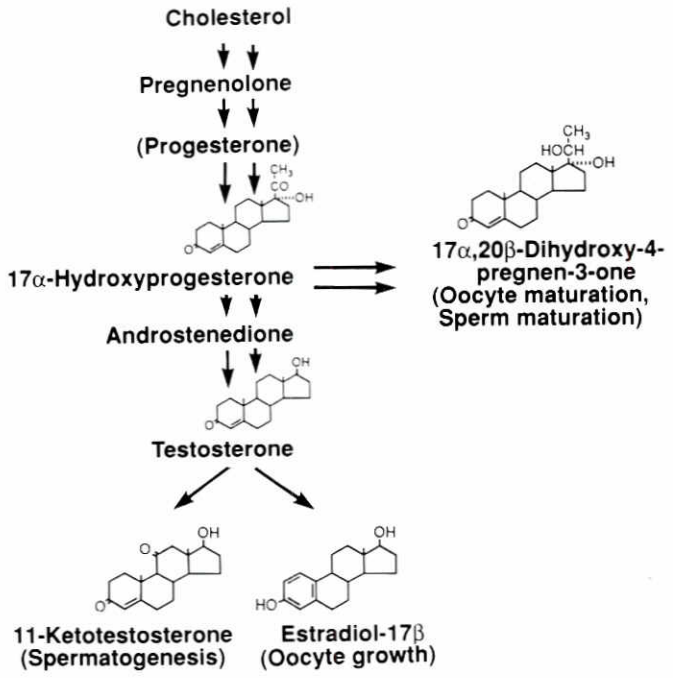

Fig. 1. Steroidogenic mediators of oocyte growth, oocyte maturation, spermatogenesis and sperm maturation in teleosts.

characterized in isolated oocyte membranes of coho salmon (Stifani *et al.*, 1990), tilapia, *Oreochromis niloticus* (Chan *et al.*, 1991) and rainbow trout, *Oncorhynchus mykiss* (Lancaster and Tyler, 1991; Le Menn and Nuñez Rodriguez, 1991). Saturation and Scatchard analyses reveal only a single class of binding site. In tilapia, the number and affinity of these bindings greatly increase from the previtellogenic to the vitellogenic stage, and remain unchanged until the preovulatory stage at which time the affinity of the bindings is also highest.

Several hormones, such as gonadotropins, thyroxine, triiodothyronine, insulin, and growth hormone, have been implicated in regulating oocyte growth, although the role of any of these hormones has yet to be determined. GTH-I, but not GTH-II, stimulates *in vitro* uptake of vitellogenin into ovarian follicles of intact vitellogenic rainbow trout, as well as *in vitro* incorporation of vitellogenin by partially denuded follicles (Tyler, 1991). Using follicle-free oocytes of rainbow trout, we have shown that insulin and thyroxine significantly increase vitellogenin uptake (Shibata *et al.*, 1993). In contrast, neither chum salmon GTH-I nor GTH-II is effective. Mature oocytes incorporate only 1/15 of vitellogenin. Insulin also has no effect on the mature oocytes. It is hypothesized from these findings that the previously observed stimulation of vitellogenin uptake into intact follicles or partially denuded oocytes of rainbow trout is most likely mediated by the actions of gonadotropins on the ovarian follicles.

Estradiol-17ß production

The teleost ovary consists of oogonia, oocytes, and surrounding follicle cells, supporting tissue or stroma, and vascular and nerve tissue. With the growth of the oocytes, follicle cells multiply and form a continuous follicular layer (granulosa cell layer). Simultaneously, the surrounding stromal connective tissue elements also become organized to form the distinct outer layer of the follicular envelope (the thecal layer). Therefore, vitellogenic oocytes, like those of other vertebrates, are composed of two major cell layers, an outer thecal cell layer and an inner granulosa cell layer which are separated from each other by a distinct basement membrane. The thecal layer contains fibroblasts, collagen fibers, and capillaries, and, in some species, special thecal cells (Nagahama, 1983). A simple dissection technique to separate the ovarian follicles of salmonids into two cell layers, the thecal and granulosa layers, has made it possible to elucidate the role of each layer and gonadotropins in the overall process of steroid production (Kagawa *et al.*, 1982). Our *in vitro* studies indicate that the thecal and granulosa cell layers cooperate in the production of steroidal mediators of oocyte growth and maturation (see below).

A two-cell type model in the production of follicular estradiol-17ß has been proposed. In this model, the thecal cell layer, under the influence of gonadotropin, secretes the androgen substrate (probably testosterone) which diffuses into the granulosa cell layer where the aromatase is localized exclusively (Kagawa *et al.*, 1985; Nagahama, 1987b; Adachi *et al.*, 1990). Restricted distribution of testosterone and estradiol-17ß was confirmed immunohistochemically in vitellogenic ovarian follicles of rainbow trout (Schulz, 1986). We have found that the thecal cell layer from amago salmon *(Oncorhynchus rhodurus)* and the granulosa cell layer from rainbow trout could produce the same effect as has been reported using a combination of thecal and granulosa cell layers from the same species. The reciprocal use of amago salmon granulosa and rainbow trout thecal cell layers is also effective. This finding implies that there may be little species specificity of each of these cell layers among salmonids (Nagahama, 1987b). However, the two-cell type model described above does not seem to be valid for the *Fundulus heteroclitus* (killifish) and medaka, *Oryzias latipes*. In these species, in which special thecal cells are not evident in the thecal layer, the follicle cells (granulosa cells) are the major site of steroid synthesis and the production of estradiol-17ß does not require the involvement of two cell types (Onitake and Iwamatsu, 1986; Petrino *et al.*, 1989).

Gonadotropin acts on thecal cell layers to stimulate testosterone production through a receptor-mediated adenylate cyclase-cAMP system. More recent studies have demonstrated that other intracellular signalling molecules including calcium, calmodulin, protein kinase C, and arachidonic acid are involved in gonadotropin-induced testosterone production by intact preovulatory follicles of goldfish, *Carassius auratus* (Van Der Kraak, 1990, 1991; Van Der Kraak and Chang, 1990). It is interesting to note the presence of gonadotropin receptors in the granulosa cell of amago salmon vitellogenic follicles. It was found that in this species gonadotropins and several agents known to increase intracellular cAMP level failed to enhance aromatase activity in granulosa layers at all developmental stages. Nevertheless, aromatase activity in granulosa cell layers markedly increased during vitellogenesis. Thus, the mechanism of the activation of the amago salmon granulosa cell aromatase system is unknown at present.

We also investigated the mechanism of aromatase activation in early vitellogenic follicles of medaka, a species in which the sequence of events such as vitellogenesis, oocyte growth, meiotic maturation, and ovulation can be timed accurately (Sakai *et al.*, 1987). Using this model, we have demonstrated a progressive increase in aromatase activity, assessed indirectly by the conversion

of exogenous testosterone to estradiol-17ß, in vitellogenic follicles isolated between 28 and 20 h before spawning (Sakai *et al.*, 1988). In this species, aromatase activity is markedly enhanced by pregnant mare serum gonadotropin (PMSG) after 18 h of incubation of vitellogenic follicles isolated at 32 h before spawning. This action of PMSG is mimicked by forskolin and dbcAMP which are known to raise the cellular level of cAMP. This enhancing effect of PMSG, forskolin and dbcAMP on aromatization is completely inhibited by actinomycin D and cycloheximide. These results suggest that PMSG induces aromatase activity in medaka vitellogenic follicles via an adenylate cyclase-cAMP system. Furthermore this action of PMSG is dependent upon both transcriptional and translational processes (Nagahama *et al.*, 1991).

Oocyte maturation

A period of oocyte growth is followed by a process called oocyte maturation which occurs prior to ovulation and is a prerequisite for successful fertilization. Oocyte maturation is defined as the reinitiation and completion of the first meiotic division, subsequent progression to metaphase II. In some species such as goldfish, the first visible event of oocyte maturation is the migration of the germinal vesicle (GV) toward the animal pole and is often used as an indicator of the onset of oocyte maturation. The mechanisms of this process involve changes in cytoskeletal networks such as microtubule distribution (Lessman *et al.*, 1988; Jiang *et al.*, unpublished). Following the migration of the GV, several processes occur in the nucleus and cytoplasm of the oocyte, including the breakdown of the germinal vesicle (GVBD) (which indicates the end of prophase I), condensation of the chromosomes, formation of a spindle, and extrusion of the first polar body (which marks the completion of meiosis I) (Masui and Clarke, 1979; Goetz, 1983; Nagahama, 1987c). Studies from our laboratory and others have indicated that in teleosts, as in amphibians, oocyte maturation is regulated by three major mediators, namely, gonadotropin, maturation-inducing hormone (MIH), and maturation-promoting factor (MPF) (Nagahama, 1987c; Redding and Patino, 1993) (Fig. 3).

Primary mediator of oocyte maturation — gonadotropin

In vitro incubation studies have demonstrated that gonadotropin induces meiotic maturation in the follicle-enclosed postvitellogenic fish oocytes, but not in defolliculated oocytes (Goetz, 1983; Nagahama, 1987c). Cyanoketone, a specific inhibitor of 3ß-hydroxy-Δ^5-steroid dehydrogenase, completely abolished the maturational effects of gonadotropin (Young *et al.*, 1982; DeManno and Goetz, 1987). Taken together, these findings suggested that gonadotropins trigger maturation by stimulating the follicle cells to synthesize a Δ^4-steroid that acts directly on the oocyte to cause maturation.

Secondary mediator of oocyte maturation — Maturation-inducing hormone (MIH)

Numerous studies have been conducted to test the effects of various steroids on the induction of oocyte maturation *in vitro* (see reviews, Jalabert, 1976; Iwamatsu, 1978; Fostier *et al.*, 1983; Goetz, 1983; Nagahama *et al.*, 1983; Greeley *et al.*, 1986; Canario and Scott, 1988; Trant and Thomas, 1988). Among the C18, C19, and C21 steroids tested so far, the C21 steroids have always been reported to have more potent maturation-inducing activity than the other two groups of steroids. The MIH of amago salmon was purified and characterized from media in which immature but full-grown folliculated oocytes had been incubated with partially purified salmon gonadotropin (Nagahama and Adachi, 1985). Among 20 fractions separated by reversed phase high performance liquid chromatography, maturation-inducing activity was found only in one fraction which had a retention time coinciding exactly with 17α,20ß-dihydroxy-4-pregnen-3-one (17α,20ß-DP). The purity and final characterization of this active fraction were further confirmed by thin layer chromatography and mass spectrometry with authentic 17α,20ß-DP. Plasma levels of 17α,20ß-DP were found to be high only in mature and ovulated amago salmon females and were very low or nondetectable (less than 30 pg/ml) in females during the remaining period of their life (Young *et al.*, 1983a). It was also found that among the steroids tested, 17α,20ß-DP was the most effective inducer of GVBD in amago salmon postvitellogenic oocytes (Nagahama *et al.*, 1983). 17α,20ß-DP was thus identified as the naturally occurring MIH in amago salmon (Nagahama, 1987a). Further studies from our laboratory and others suggest that 17α,20ß-DP functions as the MIH common to several other teleost fishes including most species of salmonids (Jalabert, 1976; Goetz, 1983; Yamauchi *et al.*, 1984; Canario and Scott, 1988).

More recently, 17α,20ß,21-trihydroxy-4-pregnen-3-one (20ß-S) has been identified as a naturally occurring MIH of the Atlantic croaker, a marine perciform fish (Trant *et al.*, 1986; Trant and Thomas, 1989a). In this species, 20ß-S was found to be one of the most potent steroids to induce GVBD *in vitro* (Trant and Thomas, 1988). Exposure of maturing ovarian follicles to HCG *in vitro* greatly increased their production of 20ß-S, with a concomitant increase in the rate of oocyte maturation *in vitro*. Furthermore, *in vitro* production and plasma levels of 17α,20ß-DP during the period of oocyte maturation were very low in this species (Trant and Thomas, 1989b). These results strongly support the hypothesis that 20ß-S

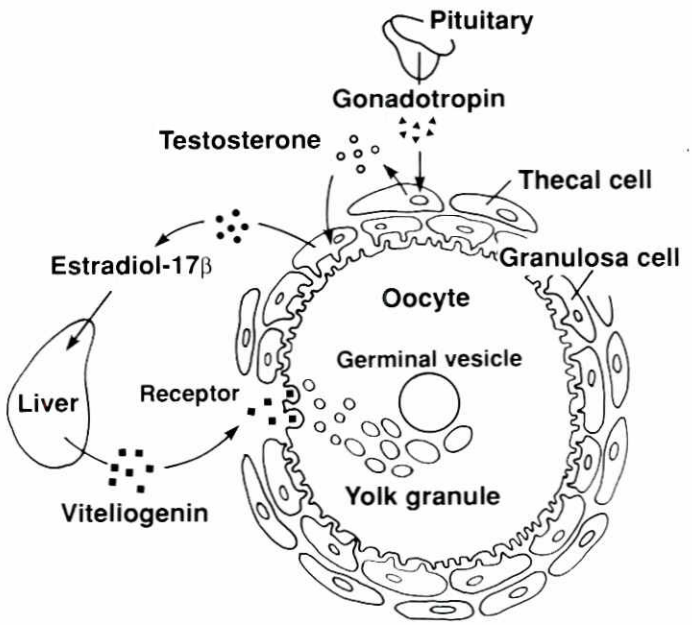

Fig. 2. Hormonal regulation of oocyte growth (vitellogenesis) in teleosts.

is the natural, major MIH in Atlantic croaker. The involvement of 20ß-S in the induction of oocyte maturation is also suggested in a closely related species, spotted seatrout, *Cynoscion nebolusus* (Thomas and Trant, 1989). However, 20ß-S does not appear to be involved in the induction of oocyte maturation in salmonids, since there is no evidence for the presence of large amounts of this steroid in the blood of female salmonids undergoing maturation or ovulation (Scott and Canario, 1987).

17α,20ß-DP production

Using incubation techniques of isolated salmonid follicular preparations similar to those used for the studies on follicular estradiol-17ß production, a two-cell type model has been proposed, for the first time in any vertebrate, for the follicular production of MIH. In this model, the thecal cell layer produces 17α-hydroxyprogesterone that traverses the basal lamina and is converted to 17α,20ß-DP by the granulosa cell layer where gonadotropin acts to enhance the activity of 20ß-hydroxysteroid dehydrogenase (20ß-HSD), the key enzyme involved in the conversion of 17α-hydroxyprogesterone to 17α,20ß-DP (Young *et al.*, 1986; Nagahama, 1987a,b).

The first step of the stimulating effect of gonadotropin in thecal cell layers is receptor-mediated activation of adenylate cyclase and formation of cAMP, the major site of action probably occurring at the steroidogenic step between cholesterol and pregnenolone involving cholesterol side-chain cleavage enzyme systems (Kanamori and Nagahama, 1988a,b).

The action of gonadotropin on 20ß-HSD enhancement in granulosa cells is mimicked by forskolin, by dbcAMP, but not dbcGMP, and by two phosphodiesterase inhibitors (Nagahama *et al.*, 1985a; Kanamori and Nagahama, 1988b). Furthermore, gonadotropin and forskolin caused a rapid accumulation of cAMP with maximum levels at 30-60 min. These findings are consistent with the view that cAMP is the second messenger of gonadotropin action in granulosa cells.

In vitro experiments utilizing both protein synthesis inhibitors (cycloheximide and puromycin) and RNA synthesis inhibitors (actinomycin D, cordycepin and α-amanitin) have revealed that the gonadotropin and cAMP induction of 20ß-HSD activity in granulosa cell layers is dependent on the activation of new RNA and protein synthesis. Our time course studies further suggest that *de novo* synthesis of 20ß-HSD *in vitro* in response to gonadotropin and dbcAMP occurs, and consists of gene transcriptional events within the first 6 h of exposure to gonadotropin and dbcAMP and transcriptional events 6-9 h after the exposure to gonadotropin and cAMP (Nagahama *et al.*, 1985b). Taken together, these results suggest that gonadotropin causes the *de novo* synthesis of 20ß-HSD in the amago salmon granulosa cell through a mechanism dependent on RNA synthesis.

Steroidogenic shift occurring in follicle cells immediately prior to oocyte maturation

The capacity of intact follicles to produce estradiol-17ß in response to gonadotropin stimulation increases during oocyte growth, but rapidly decreases in association with the ability of the oocyte to mature in response to gonadotropin (Kagawa *et al.*, 1983). Testosterone production by the thecal layer preparations obtained each month during oocyte growth and maturation has revealed that the capacity of the thecal layer to produce testosterone in response to gonadotropin gradually increases during the course of oocyte growth and peaks during the postvitellogenic period; this capacity of thecal cell layers is maintained by the period of oocyte maturation and ovulation (Kanamori *et al.*, 1988). Aromatase activity in granulosa cell layers increases during vitellogenesis and decreases rapidly in association with the ability of the oocyte to mature in response to gonadotropin (Young *et al.*, 1983b; Kanamori *et al.*, 1988). This decrease in aromatase activity appears to be coincident with the decreased ability of intact follicles to produce estradiol-17ß in response to gonadotropin. Since testosterone production in thecal cell layers do not decline during this time, the reduced production of estradiol-17ß by postvitellogenic follicles is due, in part, to decreased aromatase activity in granulosa cell layers.

Immediately prior to oocyte maturation, intact ovarian follicles of salmonid fish acquire an increased ability to produce 17α,20ß-DP in response to gonadotropin. Thecal cell layers do not develop the ability to produce 17α-hydroxyprogesterone in response to gonadotropin until immediately prior to oocyte maturation. Although granulosa cell layers first acquire the ability to convert exogenous 17α-hydroxyprogesterone to 17α,20ß-DP in response to gonadotropin about 2 months prior to oocyte maturation, an increase in endogenous 20ß-HSD activity in granulosa cells occurs during oocyte maturation. Thus, a decrease in C17-20 lyase and/or 17ß-hydroxysteroid dehydrogenase activity in thecal cells and an increase in 20ß-HSD in granulosa cells appear to be the major factors responsible for the rapid increase in 17α,20ß-DP production by intact follicles during oocyte maturation.

Since the steroidogenic shift from estradiol-17ß to 17α,20ß-DP occurs in association with increasing concentrations of gonadotropins, it is important to investigate the molecular events by which the shift is regulated by gonadotropins. The first step toward elucidation of this problem is the identification and characterization of cDNA clones encoding steroidogenic enzymes responsible for estradiol-17ß and 17α,20ß-DP biosynthesis. We have recently isolated and cloned full-length cDNA inserts complementary to the mRNAs encoding rainbow trout cholesterol side-chain cleavage cytochrome (P-450scc) (Takahashi *et al.*, 1993), 3ß-hydroxysteroid dehydrogenase (3ß-HSD) (Sakai *et al.*, 1993), 17α-hydroxylase/C17-20 lyase cytochrome (P-450c17) (Sakai *et al.*, 1992), and aromatase cytochrome (P-450arom) (Tanaka *et al.*, 1992b). The cDNA inserts were confirmed to encode each steroidogenic enzyme by introducing it into nonsteroidogenic COS-1 monkey kidney tumor cells. Unfortunately, unlike most of the ovarian steroidogenic enzymes, 20ß-HSD cDNA has not been cloned in any animal species. Therefore, as a first step, we isolated a cDNA encoding pig testis 20ß-HSD, since purified preparations of 20ß-HSD is available only in pig (Nakajin *et al.*, 1988). Using synthetic oligonucleotides deduced from the partially determined amino acid sequences, we have, for the first time, isolated and cloned cDNA encoding 20ß-HSD from a pig testis cDNA library (Tanaka *et al.*, 1992a). The cDNA contains an open reading frame predicted to encode 289 amino acid residues. Surprisingly it has 85% amino acid homology with human carbonyl reductase. We are now using this cDNA clone to isolate cDNAs encoding rainbow trout 20ß-HSD from a rainbow trout ovarian cDNA library.

The mRNA levels of P-450scc, 3ß-HSD, and P-450c17 are barely detected in the follicles during the mid-vitellogenic stage, and abundant in follicles during the postvitellogenic stage and oocyte maturation (Takahashi *et al.*, 1993; Sakai *et al.*, 1992,

1993). The P-450arom RNA transcripts are present in the ovary during active vitellogenesis, but are absent in the ovary in the stage of oocyte maturation or in the ovary containing postovulatory follicles (Tanaka *et al.*, 1992b). These results are consistent with the rapid decrease in aromatase activity in the granulosa cell layers during the postovulatory period (Young *et al.*, 1983b). It is thus concluded that the ability of the granulosa cells to produce estradiol-17ß is regulated by the amount of the RNA transcripts present (Tanaka *et al.*, 1992b). Our preliminary results indicate that forskolin-induced 17α,20ß-DP production is accompanied by a dramatic decrease in P-450arom mRNA levels by granulosa cells isolated from postvitellogenic follicles. A 2- to 3-fold increase in P-450scc and 3ß-HSD mRNAs and a slight decrease in P-450c17 mRNA are also observed during the forskolin-induced 17α,20ß-DP production (Tanaka and Nagahama, unpublished). Our preliminary Northern hybridization analysis using the pig 20ß-HSD cDNA as a probe has revealed that 20ß-HSD mRNA transcripts are low in granulosa cells prior to oocyte maturation, but markedly increased during natural and gonadotropin-induced oocyte maturation.

Site of MIH action

Previous *in vitro* studies in fish have shown that the mechanism of action involved in the steroid-stimulation of oocyte maturation has special characteristics not typical of the classic steroid mechanism of action (Dettlaff and Skoblina, 1969; Jalabert, 1976; DeManno and Goetz, 1987). We also found that 17α,20ß-DP is ineffective in inducing oocyte maturation when microinjected into fully grown immature oocytes of goldfish, but was effective when applied externally. Taken together, these *in vitro* results suggest that the site of MIH action in inducing meiotic maturation in fish oocytes is at or near the oocyte surface.

More direct evidence for the existence of MIH receptors in oocyte plasma membranes has been obtained by binding studies using labelled MIH. We have identified and characterized specific binding of [^{3}H]17α,20ß-DP to plasma membranes prepared from defolliculated oocytes of rainbow trout (Yoshikuni *et al.*, 1993). Scatchard analysis reveals two different binding sites: a high affinity binding site with a Kd of 18 nM and a Bmax of 0.2 pmoles/mg protein and a low affinity binding site with a Kd of 0.5 μM and a Bmax of 1 pmole/mg protein. Maneckjee *et al.* (1989) also reported 17α,20ß-DP binding activity in the zona radiata-oocyte membrane complex of rainbow trout and brook trout, *Salvelinus fontinalis*. Recently, we also described a specific 17α,20ß-DP receptor for 17α,20ß-DP in the oocyte cortices of the Japanese flounder, *Paralichthys olibaceus* (Yoshikuni *et al.*, unpublished). This 17α,20ß-DP binding increases and reaches an equilibrium within 1 h. Scatchard analysis reveals a single binding site with a Kd of 63 nM and a Bmax of 25 fmoles/cortex. The 17α,20ß-DP receptor is present in oocyte cortices from both postvitellogenic oocytes (500-600 μm in diameter) and ovulated eggs, but not in those from either mid-vitellogenic oocytes (425-500 μm) or early-vitellogenic oocytes (<425 μm).

The binding of 20ß-S to plasma membranes from the ovaries of spotted seatrout was also characterized (Patino and Thomas, 1990a). Scatchard analyses suggest a single class of high affinity (Kd, 10^{-10} M), low-capacity (10^{-13}-10^{-12} mol/g ovary) binding sites for 20-S. A specific binding site for 20ß-S exists in plasma membranes from spotted seatrout ovaries (Patino and Thomas, 1990b). The concentrations of 20ß-S receptors in ovarian plasma membranes are three times higher in seatrout undergoing oocyte maturation than in vitellogenic females. This increase in receptor concentrations in fish collected during their natural spawning cycle is similar to that previously observed in seatrout undergoing final maturation following LHRH injections (Thomas and Patino, 1991). These observations suggest that changes in the concentration of MIH receptors in the ovaries are of physiological importance during natural oocyte maturation. Similarly, a specific progesterone (a proposed MIH of amphibian oocytes) receptor has been demonstrated on the surface of *Xenopus* oocytes (Sadler and Maller, 1982). Taken together, these observations emphasize that the action of maturation-inducing steroid on the oocyte is rather novel in the light of conventional concept that steroids act through cytosolic or nuclear receptors. Clearly, further work is necessary to fully characterize steroid receptors on oocyte membranes.

Acquirement of oocytes to mature in response to hormonal stimulation

It has been reported that intrafollicular oocytes of several teleosts develop the ability to undergo GVBD in response to hormonal stimulation immediately prior to the final oocyte maturation stage. For example, medaka follicles acquire the ability to undergo maturation in response to gonadotropin 28 h before the expected time of spawning (Sakai *et al.*, 1987). In oocytes of kisu *(Sillago japonica,* Kobayashi *et al.*, 1988), dragonet *(Repomucenus beniteguri,* Zhu *et al.*, 1989) and Atlantic croaker (Patino and Thomas, 1990b,c), there is a stage in which they can undergo oocyte maturation *in vitro* in response to gonadotropin stimulation but not to MIH. However, these oocytes become sensitive to MIH if exposed to gonadotropin *in vitro*. These results suggest that gonadotropin is responsible for the induction of maturational competence of oocytes. Furthermore, HCG-induced maturational competence was inhibited by cycloheximide and actinomycin D (Patino and Thomas, 1990c). Thomas and Patino (1991) examined the relationship between 20ß-S receptor concentrations and the development of maturational competence using an ovarian incubation system. Treatment of spotted seatrout ovarian follicles with gonadotropin causes a two-fold increase in 20ß-S receptor concentrations and, concomitantly, full-grown oocytes acquire the ability to mature in response to 20ß-S. In contrast, 20ß-S treatment does not induce an increase in receptor concentrations or the development of oocyte maturational competence. Similarly, *in vitro* treatment of Japanese flounder follicles with HCG also causes a three-fold increase in 17α,20ß-DP receptor concentrations. Moreover, this HCG-induced elevation in receptor concentrations is accompanied by the appearance of maturational competence in the follicle-enclosed oocytes (Yoshikuni *et al.*, unpublished). Taken together, these results suggest that a gonadotropin-induced increase in MIH receptor concentrations is responsible for the development of oocyte maturational competence in seatrout and the Japanese flounder, and is not mediated by increases in the production of the MIH.

Tertiary mediator of oocyte maturation — Maturation-promoting factor (MPF)

In a previous study, Dettlaff *et al.* (1977) reported MPF-like activity in the sturgeon, *Acipenser stellatus,* oocytes. However, this activity was not detected in the presence of cycloheximide, a protein synthesis inhibitor, so that pretranslational factors upstream of true MPF activity were not excluded. To determine whether MPF

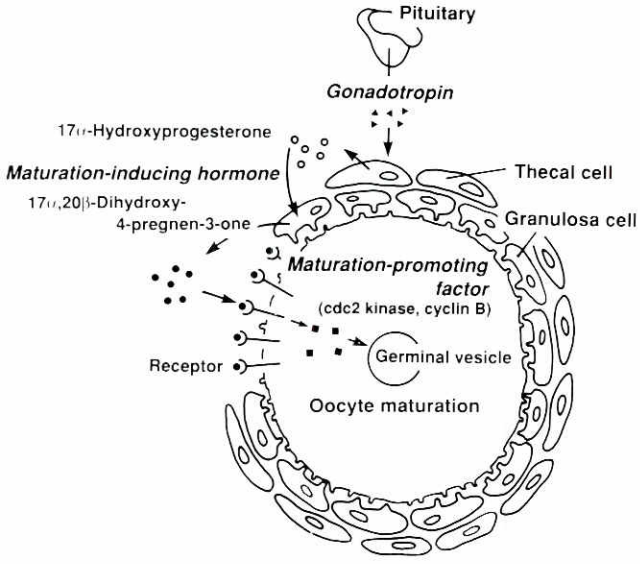

Fig. 3. Hormonal regulation of oocyte maturation in teleosts.

activity is present in the mature fish oocyte, we extracted MPF activity from human chorionic gonadotropin (HCG)-injected mature goldfish oocytes and naturally spawned carp oocytes (Nagahama and Yamashita, 1988). These mature oocytes were crushed by ultracentrifugation at 100,000xg for 1 h in a buffer containing phosphatase inhibitors and EGTA. After centrifugation, the five major layers obtained, from centripetal to centrifugal pole, were: supernatant, green layer, yolk, chorion, and cortical alveoli. The green layer was enriched with cellular organelles such as mitochondria and endoplasmic reticulum. MPF activity of each layer was determined using the *Xenopus* oocyte microinjection assay. MPF activity was localized in the supernatant and the green layer; microinjection of the green layer caused cell lysis. MPF activity was also extracted from oocytes matured *in vitro* by 17α,20ß-DP, but not from immature oocytes or activated oocytes. MPF activity extracted from fish was also effective when injected into immature fish oocytes under conditions of inhibited protein synthesis. The injected oocytes matured much faster than oocytes induced to mature by incubation with 17α,20ß-DP: $GVBD_{50}$ was around 2.5 h in MPF-induced maturation, but about 6 h when induced by 17α,20ß-DP. GVBD usually occurred at the center of the MPF-injected oocytes, because the movement of the GV to the animal pole did not take place. GV migration always occurred in the oocytes matured naturally, *in vivo* by HCG, or *in vitro* by 17α,20ß-DP. Thus, post-translational MPF activity is present in mature goldfish and carp oocytes.

MPF from mature oocytes of goldfish induced GVBD when injected into *Xenopus* oocytes and *vice versa*. It was also shown previously that goldfish MPF induced maturation of immature oocytes of the starfish, *Asterina pectinifera* (Kishimoto, 1988). These findings are consistent with the notion that MPF is similar among vertebrates and invertebrates (Kishimoto, 1988).

MPF purification and characterization

In spite of efforts in several laboratories, progress in MPF purification had been very slow until very recently. The breakthrough in purification was achieved by Lohka *et al.* (1988), who purified *Xenopus* MPF approximately 3,000-fold; this MPF fraction contained two predominant proteins, with relative molecular mass of 34 and 45 kDa, respectively. The former protein has been identified as the *Xenopus* homolog of a fission yeast protein encoded by a gene ($cdc2^+$), which is required for the G2/M transition in mitotic cell cycle (Gautier *et al.*, 1988). The product of the $cdc2^+$ gene is a 34 kDa serine/threonine protein kinase, designated $p34^{cdc2}$ or cdc2 kinase. Another component of the *Xenopus* MPF, the 45 kDa protein, has been identified as a *Xenopus* B-type cyclin (Gautier *et al.*, 1990). Similar MPF has also been purified in starfish (Labbe *et al.*, 1989a,b).

We recently purified and characterized MPF (Yamashita *et al.*, 1992a) and histone H1 kinase (Yamashita *et al.*, 1992b) from 100,000xg supernatant of crushed, naturally ovulated, unfertilized eggs of carp using four chromatographic steps including Q-Sepharose Fast-Flow, $p13^{suc1}$-affinity Sepharose, Mono S, and Superose 12. MPF activity of various fractions during purification was determined by the *Xenopus* oocyte microinjection assay. Analyses by SDS-polyacrylamide gel (PAGE) showed that the most active fraction after Mono S contained four proteins, with molecular masses of 33, 34, 46, and 48 kDa. When the active fractions after Mono S were applied to Superose 12, MPF eluted as a single peak with an apparent molecular weight of about 100 kDa. SDS-PAGE of the active fraction after Superose 12 revealed the same four proteins as those obtained after Mono S, indicating that these proteins form complex(es) of about 100 kDa in their native form. The final preparation was purified over 1,000-fold with a recovery of about 1%.

It is suggested from the comparison of the components of purified carp MPF with those of *Xenopus* MPF that among the four proteins found in carp MPF, the 34-kDa protein, and the 46- and 48-kDa proteins correspond to cdc2 kinase and cyclin B of *Xenopus* MPF, respectively. To fully characterize these four proteins, we isolated cDNA clones encoding fish homologs of MPF- related proteins, including cdc2 kinase (Kajiura *et al.*, 1993), cdk2 kinase (Hirai *et al.*, 1992b), cyclin A (Katsu *et al.*, unpublished), and cyclin B (Hirai *et al.*, 1992a) from cDNA libraries of goldfish oocytes. Monoclonal antibodies against the C-terminal sequence of goldfish cdc2 kinase (Kajiura *et al.*, 1993), a unique stretch of 16 amino acids (EGVPSTAIREISLLKE, termed the PSTAIR sequence) that is perfectly conserved in cdc2 kinase and its homologs from yeast to man (Yamashita *et al.*, 1991), the C-terminal sequence of goldfish cdk2 kinase (Hirai *et al.*, 1992b), and *E. coli*-produced full-length goldfish cyclin A (Katsu *et al.*, unpublished) and B (Hirai *et al.*, 1992a) were raised.

These monoclonal antibodies were used to characterize the purified carp MPF which contains 33-, 34-, 46- and 48-kDa proteins. Both the 33- and 34-kDa proteins are recognized by the anti-PSTAIR antibody, indicating that they are the cyclin-dependent kinases. The anti-cdc2 C-terminal antibody reacts with the 34-kDa, but not the 33-kDa protein. The latter is recognized with the anti-cdk2 C-terminal antibody (Hirai *et al.*, 1992b). The 46- and 48-kDa proteins are recognized by the anti-full length cyclin B antibody, but not by an anti-cyclin A antibody (Yamashita *et al.*, 1992a; Katsu *et al.*, unpublished). It is concluded from these findings that carp MPF is a complex of cdc2 kinase (34-kDa) and cyclin B (46- and 48-kDa).

MPF activation

It has been reported that in immature oocytes of *Xenopus* and starfish, cdc2 kinase forms a complex with cyclin B as pre-MPF. In

contrast, immature goldfish oocytes contain the 35-kDa inactive cdc2 kinase but no cyclin B, and mature oocytes contain both the 35-kDa inactive and the 34-kDa active cdc2 kinases and cyclin B (Hirai *et al.*, 1992a; Katsu *et al.*, 1993). The appearance of the 34-kDa active cdc2 kinase coincides with the appearance of cyclin B just before GVBD. Anti-cyclin B immunoblots of the $p13^{suc1}$ precipitates and anti-cdc2 kinase immunoblots of anti-cyclin B immunoprecipitates show that the binding of cdc2 kinase and cyclin B coincides with the appearance of cyclin B and the 34-kDa active cdc2 kinase. The cyclin B which appears during oocyte maturation is labeled with ^{35}S-methionine (Hirai *et al.*, 1992a), demonstrating *de novo* synthesis during oocyte maturation. On the other hand, anti-cyclin B immunoprecipitates from mature oocyte extracts sometimes contain the 35-kDa inactive cdc2 kinase (Hirai *et al.*, 1992a), and the 35-kDa cdc2 kinase, as well as the 34-kDa form, can bind to cyclin B in a cell-free system (Yamashita *et al.*, unpublished). Therefore, it is most likely that the 35-kDa inactive cdc2 kinase binds to *de novo* synthesized cyclin B at first, then is rapidly converted into the 34-kDa active form (Fig. 4).

The preceding results indicate that the appearance of cyclin B is required and is sufficient for inducing oocyte maturation in goldfish. To confirm that the appearance of cyclin B is sufficient for inducing oocyte maturation, *E. coli*-produced full length goldfish cyclin B protein was injected into immature oocytes. Even under conditions of inhibited protein synthesis, injected cyclin B induces oocyte maturation within 1 hr after injection in a dose-dependent manner. Introduction of goldfish cyclin B into immature goldfish oocyte extracts also induced cdc2 kinase activation, concurrent with the shift in apparent molecular weight of cdc2 kinase from 35- to 34-kDa, as found in oocytes matured with 17α,20ß-DP. Phosphoamino acid analysis shows that threonine (Thr) phosphorylation of the 34-kDa cdc2 kinase and serine phosphorylation of cyclin B are associated with the activation. The same phosphorylation is found in oocytes matured by 17α,20ß-DP. Cyclin B-induced cdc2 kinase activation is not observed when threonine phosphorylation of cdc2 kinase and serine phosphorylation of cyclin B are inhibited by protein kinase inhibitors, although the binding of the 35-kDa cdc2 kinase to cyclin B occurs even in the presence of the inhibitors. In contrast, cdc2 kinase is activated by mutant cyclins that undergo no serine phosphorylation during the activation. The site of threonine phosphorylation on cdc2 kinase was mapped to residue Thr161. These findings indicate that the Thrl61 phosphorylation of cdc2 kinase, but not serine phosphorylation of cyclin B, is required for cdc2 kinase (MPF) activation in goldfish oocytes.

Recently we have isolated a cDNA clone encoding a goldfish homolog of $p40^{MO15}$, the catalytic subunit of a protein kinase which has been shown to activate cdc2 kinase through phosphorylation of Thrl61, from a goldfish oocyte cDNA library (Onoe *et al.*, 1993). Northern and Western blot analyses reveal that both $p40^{MO15}$ mRNA and protein are already present in goldfish immature oocytes and do not appear to exhibit any measurable changes during hormonally induced maturation (Onoe *et al.*, 1993; Onoe, unpublished). Thus, it is suggested that $p40^{MO15}$ may itself be regulated by subunit association and/or protein phosphorylations. The substrate preference of $p40^{MO15}$ also needs to be determined.

In *Xenopus* oocytes, dephosphorylation of threonine (Thr14) and tyrosine (Tyr15) is required for cdc2 kinase activation (Maller, 1991). However, it is doubtful that tyrosine (Tyr15) dephosphorylation of cdc2 kinase is a prerequisite for its activation during goldfish oocyte maturation, since the activation is not inhibited by vanadate, a protein phosphatase inhibitor commonly used for inhibiting cdc25 activity that dephosphorylates tyrosine (Tyr15) of cdc2 kinase, thereby inhibiting its activation. Furthermore, anti-phosphotyrosine antibodies do not cross-react with the 35-kDa cdc2 kinase, as well as the 34-kDa form, which binds to cyclin B. Also, an antibody which recognized the dephosphorylated form of Tyr15 cross-react with both the 34- and 35-kDa cdc2 kinase. Therefore, the 35-kDa cdc2 kinase found in goldfish immature oocytes may be already dephosphorylated on Thr14 and Tyr15 and the threonine phosphorylation (Thr161) may only be required for the activation. Taken together, it is strongly suggested that 17α,20ß-DP induces oocytes to synthesize cyclin B, which in turn activates preexisting 35-kDa cdc2 kinase through its threonine (Thr161) phosphorylation, producing the 34-kDa active cdc2 kinase. These mechanisms of MPF activation in fish apparently differ from those in *Xenopus* and starfish, in which cyclin B is present in immature oocytes and forms a complex with cdc2 kinase (pre-MPF).

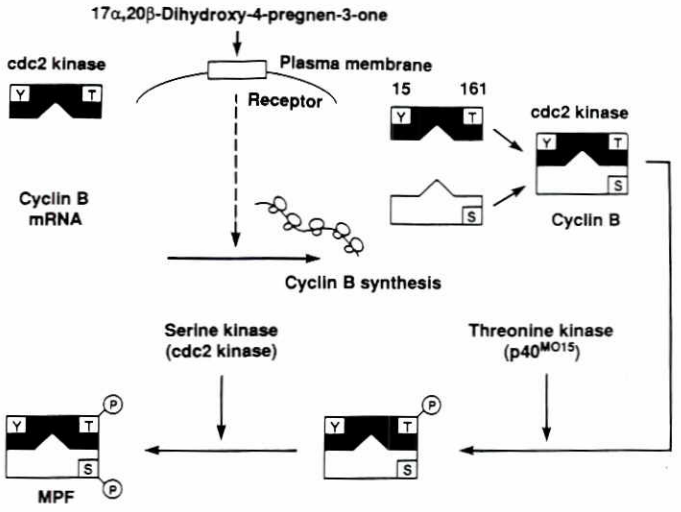

Fig. 4. Mechanisms of MPF activation during 17α,20ß-DP-induced meiotic maturation in goldfish oocytes.

Structure of teleost testis

The testis of teleosts, as that of other vertebrates, is composed of interstitial and lobular (tubular) compartments. The interstitium between lobules consists of interstitial cells (Leydig cells), fibroblasts, and blood and lymph vessels. Various enzymes involved in steroid hormone synthesis have been demonstrated by histochemistry in the Leydig cells of a number of teleosts. These cells possess ultrastructural features commonly found in steroid-producing cells such as agranular endoplasmic reticulum and mitochondria with tubular cristae (Nagahama, 1983). The lobular (tubular) component of the teleost testis contains two cell types: germ cells and distinct somatic cells lining the periphery of the lobule, Sertoli cells. It is still uncertain whether Sertoli cells can produce steroids. Ultrastructural evidence generally does not indicate steroid synthetic capacity for these cells. However, certain species do seem to have some of the appropriate histochemical and ultrastructural features for steroidogenesis (Nagahama, 1983).

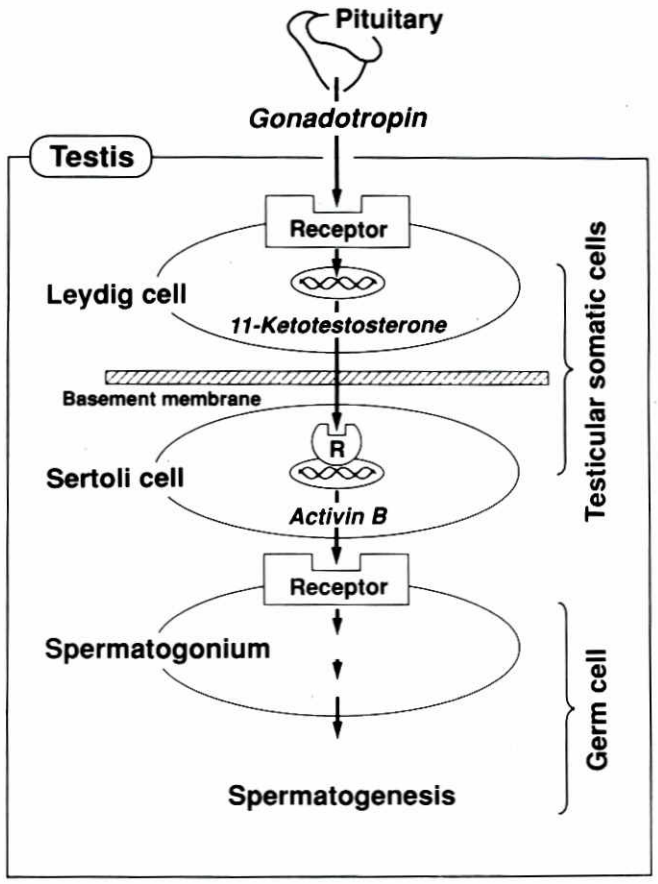

Fig. 5. Hormonal regulation of spermatogenesis in the eel testis.

Steroid production during spermatogenesis

In a number of teleost species, as in other teleost species, plasma levels of testosterone and 11-ketotestosterone are high during the later stages of spermatogenesis and rapidly decline after the onset of spermiation (Fostier *et al.*, 1983; Ueda *et al.*, 1983a). *In vitro* incubation studies on 11-ketotestosterone production by testicular fragments at different stages of development have revealed that the capacity of the fragments to produce this steroid in response to gonadotropin is high during the later stages of spermatogenesis and rapidly declines after the onset of spermiation (Sakai *et al.*, 1989). These results suggest that androgens are involved in spermatogenesis in amago salmon.

Hormonal regulation of spermatogenesis

Spermatogenesis in teleost fish takes place within testicular cysts formed by Sertoli cells. Within each cyst germ cell development occurs synchronously (Grier, 1981; Billard *et al.*, 1982; Nagahama, 1983; Callard, 1991). In the cultivated male Japanese eel, *Anguilla japonica*, the only germ cells present in the testis are the type A and early type B spermatogonia which are primitive spermatogonia that have not begun to proliferate. Thus, studies using these animals may provide considerable advantages over the use of highly complex spermatogenic systems in higher vertebrates. A single injection of HCG to these males induced spermatogenesis within 18 days (Miura *et al.*, 1991a). Within 1 day after the HCG injection, Leydig cells and Sertoli cells are markedly activated. Subsequently (3 days after HCG injection), spermatogonia begin proliferation, and type B spermatogonia appear. Following approximately 10 mitotic divisions, spermatogonia begin meiosis. Spermatocytes with synaptonemal complex first appear in testes 12 days after the injection. Spermatids and spermatozoa are observed after 18 days.

A serum-free, chemically defined organ culture system for eel testes has been developed, and used to investigate the effect of HCG and various steroid hormones on the induction of spermatogenesis *in vitro*. Cultures of testicular fragments in media containing HCG induced the entire process of spermatogenesis from premitotic spermatogonia to spermatozoa within 24 days (Miura *et al.*, 1991c). This HCG-induced spermatogenesis was accompanied by a marked activation of Sertoli cells and Leydig cells, occurring prior to the beginning of spermatogonial proliferation. Leydig cell activation was characterized by ultrastructural features of active steroid production. Furthermore, the addition of HCG to culture media containing testicular fragments induced a marked, rapid increase in 11-ketotestosterone production. These results indicate that gonadotropin triggers spermatogenesis in the Japanese eel, and further suggest that this effect of gonadotropin is mediated through the actions of testicular somatic cells, probably through the production of 11-ketotestosterone by Leydig cells. This suggestion was confirmed by organ culture experiments demonstrating that 11-ketotestosterone can induce all stages of spermatogenesis including spermatogonia proliferation, meiotic division and spermiogenesis *in vitro* within 21 days (Miura *et al.*, 1991d) (Fig. 5). 11-ketotestosterone also causes a marked cytological activation of Sertoli cells, but not Leydig cells, suggesting that the action of 11-ketotestosterone on spermatogenesis is mediated through the action of Sertoli cells. This is the first animal system in which the entire process of spermatogenesis has been induced by hormonal manipulation *in vitro*.

Activin B: a potential mediator of gonadotropin-induced spermatogenesis

As described above, morphological changes in germ cells, Leydig cells, and Sertoli cells are first visible one day after HCG treatment. We have used subtractive hybridization to identify genes that are expressed differentially in eel testes in the first 24 h after HCG treatment *in vivo*, which ultimately induces spermatogenesis. One up-regulated cDNA was isolated from subtractive cDNA libraries derived from mRNA extracted from control testes and testes one day after a single injection of HCG. From its deduced amino acid sequence, this clone was identified as coding for the activin B subunit. The nucleotide sequence of eel activin B cDNA is 3.3 kb. A methionine codon at nucleotide 1572 initiates a long open reading frame specifying a protein of 395 amino acids. Using Northern blot analysis and *in situ* hybridization techniques, we examined sequential changes in transcripts of testicular activin B during HCG-induced spermatogenesis. No transcripts for activin B were found in testes prior to HCG injection. In contrast, 3.3 kb mRNA transcripts for activin B were prominent in testes one day after the injection.

The transcript concentration began to decrease three days after the injection, followed by a further sharp drop by nine days. The HCG-dependent activin B mRNA expression in the testes was

confirmed by *in situ* hybridization using a digoxigenin-labeled RNA probe; the signal was restricted to Sertoli cells in testes treated with HCG for one to three days.

Taken together, these findings suggest the following sequence of the hormonal induction of spermatogenesis in the eel. Gonadotropin stimulates the Leydig cells to produce 11-ketotestosterone, which, in turn, activates the Sertoli cells to produce activin B. Activin B then acts on spermatogonia to induce mitosis leading to the formation of spermatocytes.

Steroid production during the final testicular maturation

17α,20ß-DP has been reported to be high in the plasma or serum of several species of salmonids during spermiation (Scott and Baynes, 1982; Ueda *et al.*, 1983a,b, 1984). Furthermore, the capacity of testicular fragments to produce 17α,20ß-DP is low during spermatogenesis and increases sharply at the time of spermiation (Ueda *et al*, 1983b; Sakai *et al.*, 1989). Thus, a distinct shift in the steroidogenic pathway from androgens to 17α,20ß-DP appears to occur in the testis of amago salmon around the onset of spermiation (Sakai *et al.*, unpublished). These results suggest that 17α,20ß-DP plays a role in the process of final testicular or sperm maturation in some teleosts.

Using three different testicular preparations (intact testicular fragments, sperm-free testicular fragments and sperm preparations), we examined the roles of testicular somatic and germ cell elements in the gonadotropin-induced testicular production of 17α,20ß-DP (Ueda *et al.*, 1984). Intact testicular fragments produced a large amount of 17α,20ß-DP in response to gonadotropin or 17α-hydroxyprogesterone. Gonadotropin did not stimulate 17α,20ß-DP production by either sperm-free testicular fragments or sperm preparations, but markedly stimulated 17α-hydroxyprogesterone production by sperm-free testicular fragments. Neither sperm-free testicular fragments nor sperm preparations alone were capable of producing 17α,20ß-DP in response to gonadotropin. 17α-Hydroxyprogesterone markedly stimulated 17α,20ß-DP production by sperm preparations, but not by sperm-free testicular preparations. Finally, 17α,20ß-DP was identified as the only major metabolite when sperm preparations were incubated with [^{14}C]17α-hydroxyprogesterone.

These results suggest that the site of 17α,20ß-DP production is sperm, but its production depends on the provision of a precursor steroid (probably 17α-hydroxyprogesterone) which is produced by testicular somatic cells under the influence of gonadotropin. The results also suggest that the distribution of 20ß-HSD in the testis of spermiating rainbow trout is limited to only to sperm. More recently, Asahina *et al.* (1990) reported that isolated sperm of the common carp contain 20α-HSD. In contrast, Loir (1988) reported that in spermiating rainbow trout the various testicular cell preparations mostly devoid of sperm secrete 17α,20ß-DP regardless of gonadotropin stimulation.

Spermiation

Although fish spermatozoa in the testis have already completed two meiotic divisions, they may not be fertile. For example, salmonid spermatozoa in the testis are immotile, and acquire their motility during passage through the sperm duct (Morisawa and Morisawa, 1986). Furthermore, spermiation, which is prerequisite for successful fertilization, generally occurs in teleosts immediately before or during spawning period.

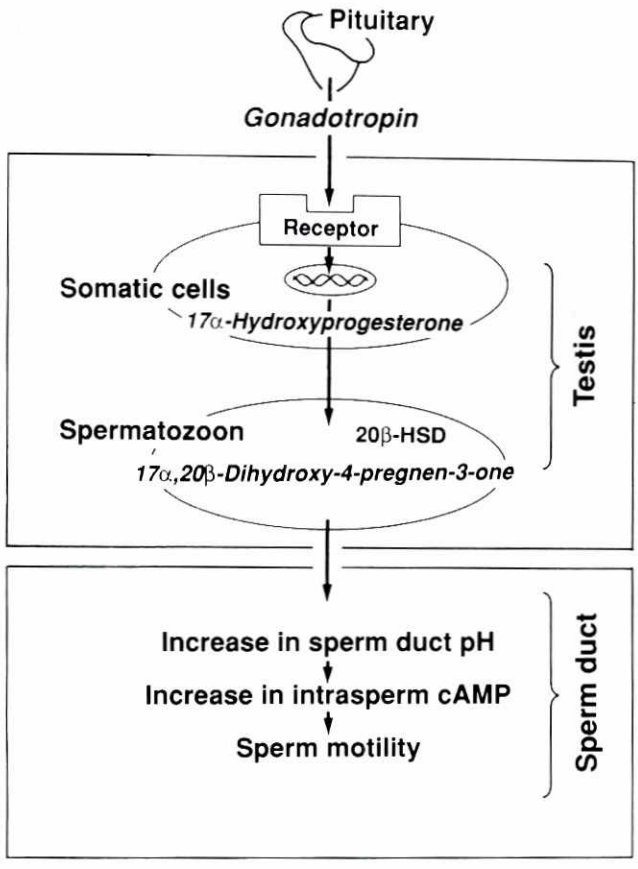

Fig. 6. Hormonal regulation of acquirement of sperm motility in salmonids.

The results described above suggest the possible involvement of 17α,20ß-DP in the process of spermiation. This is strongly supported by our studies demonstrating that a single injection of gonadotropin to non-spermiating amago salmon induced precocious spermiation 1-2 months prior to the normal spermiation period, concomitant with a marked increase in plasma levels of 17α,20ß-DP. Similarly, two successive injections of 17α,20ß-DP caused precocious spermiation, but the response to 17α,20ß-DP was of lesser magnitude than to gonadotropin. Neither testosterone nor 11-ketotestosterone was effective (Ueda *et al.*, 1985). Considered together, these results provide evidence to suggest that 17α,20ß-DP is a testicular steroidal mediator of gonadotropin-induced spermiation in salmonids. A similar suggestion has also been made for goldfish (Kobayashi *et al.*, 1986).

Sperm motility

In salmonid fishes such as masu salmon, *Oncorhynchus masou*, sperm in the testis and sperm duct are not motile. If sperm duct sperm are diluted with fresh water, then for the first time, they gain motility. If testis sperm are diluted with fresh water, however, they will not become motile. Thus, two separate processes are involved in the induction of sperm motility in salmonids. One is the acquisition of motile ability when shifting from the testis to sperm duct, and the other is the initiation of motility when diluted with fresh water. Early

in the breeding season, however, sperm from the sperm duct do not gain motility even when they are diluted with fresh water. This characteristic, namely the period during which sperm become motile, makes the masu salmon a good system for studying the endocrinological mechanisms involved in the acquisition of sperm motility.

The effects of two successive daily intramuscular injections of three steroid hormones on the acquisition of sperm motility in masu salmon during the early breeding season were examined (Miura *et al.,* 1991b). Injections of 17α,20ß-DP significantly raised the percentage of motile sperm and the duration of sperm motility. In contrast, the other two steroids showed no significant effect on sperm motility. Similar stimulatory effects of 17α,20ß-DP on sperm motility were observed in the Japanese eel (Miura *et al.,* 1991e). These results suggest that 17α,20ß-DP is involved in the acquisition of sperm motility in masu salmon and eel.

We then examined the effects of 17α,20ß-DP on the development of sperm motility *in vitro.* In this case, sperm duct immotile spermatozoa did not develop motility when they were incubated in artificial seminal plasma at pH 7.4 containing various doses of 17α,20ß-DP, suggesting that 17α,20ß-DP has no direct action on the development of sperm motility. The next experiment was designed to determine the effects of hormone injections on the pH of seminal plasma in the sperm duct, since we found that the pH seminal plasma of male masu salmon during the early breeding season, when spermatozoa in the sperm duct are still immotile, was significantly lower (pH 7.4-7.5) than that of the sperm duct of males during the active breeding season (pH 8.5). Both SGA and 17α,20ß-DP significantly increased the pH of the sperm duct from 7.4 to 8.0. In contrast, these injections did not increase the pH of the testis. Neither 11-ketotestosterone nor testosterone injections were effective in raising the pH of the testis or sperm duct. Collectively, these results suggest that the action of 17α,20ß-DP on the acquisition of sperm motility is mediated through an increase in pH of the sperm duct.

In the next experiment, the effect of pH on the acquisition of sperm motility was investigated. When immotile spermatozoa collected from the testis were incubated in artificial seminal plasma of varying pH, these spermatozoa acquired the capacity for motility in artificial seminal plasma of pH higher than 7.8. Furthermore, spermatozoa incubated in the pH range 8.0-9.0, which is the same pH range at which the acquisition of sperm motility occurred, had significantly elevated levels of cAMP. Immotile testis spermatozoa also acquired motility when incubated with dbcAMP, but not with dbcGMP (Miura *et al.,* unpublished).

Taken together, the findings described above led us to conclude the following sequence for the acquisition of sperm motility in masu salmon; gonadotropin stimulates the testis to induce the production of 17α,20ß-DP, which acts to increase sperm duct pH, this in turn increases cAMP in sperm allowing the acquisition of sperm motility (Fig. 6).

Conclusion

This article presents a current picture of the hormonal regulation of gametogenesis in teleosts. Steroidal mediators of oocyte growth, oocyte maturation, spermatogenesis, and sperm maturation have been identified in several teleost species, particularly in salmonids. In both females and males, the steroidogenic shifts that occur immediately prior to oocyte and sperm maturation seem to be prerequisite steps for growing germ cells to enter the final stage of maturation. These switches require a complex and integrated network of gene regulation involving cell-specificity, hormonal regulation, and developmental patterning. Our current efforts center on the cloning and sequencing of the genes encoding several steroidogenic enzymes responsible for estradiol-17ß, 11-ketotestosterone and 17α,20ß-DP biosynthesis. We will then investigate how gonadotropin and other factors act on gonadal somatic cells to turn on and turn off the expression of these specific genes at specific times during various stages of gametogenesis. Other non-steroidal mediators which regulate fish gametogenesis have also been determined. These include MPF (a mediator of 17α,20ß-DP action on oocyte maturation) and activin B (a mediator of 11-ketotestosterone action on spermatogenesis). These findings on teleosts may be one of the best documented examples of the role of steroid hormones in the regulation of gametogenesis in vertebrates, and will certainly provide the basis for a study at the molecular levels of hormonal regulation of germ cell development and maturation.

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