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Localization of Two Gonadotropin Receptors in the Salmon Gonad by In Vitro Ligand Autoradiography[1]

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ABSTRACT

Receptors for two salmon gonadotropins, GTH I and GTH II, were localized by use of in vitro ligand autoradiography of coho salmon gonads at various stages of sexual maturation. The results in both sexes revealed the presence of two types of GTH receptors: type I (GTH-RI), which interacts with both GTHs, and type II (GTH-RII), which interacts specifically with GTH II. GTH-RI was found at all stages of spermatogenesis examined and was localized on cells that were most likely Sertoli cells; however, it could not be determined whether GTH-RI was also localized on Leydig cells. In contrast, GTH-RII was found only in Leydig cells in the testis from a spermiating fish. In the vitellogenic ovary, GTH-RI was localized in the thecal layer and intensely on granulosa cells; in the preovulatory follicle, in contrast, GTH-RI was found in the thecal layer and in interstitial connective tissue, but not in the granulosa layer. Among all the stages of oogenesis examined, only granulosa cells of the preovulatory follicle exhibited GTH-RII. The appearance of GTH-RII coincides well with the increase in plasma levels of GTH II that occurs during final oocyte maturation and spermiation in coho salmon. The nature, distribution, and timing of appearance of these two receptors can explain, at least in part, the results of previous studies on steroidogenic activities of the two GTHs. The present study also suggests the functional homology of salmon GTH I and GTH II to mammalian FSH and LH, respectively, during gonadal development.

INTRODUCTION

In teleosts, two chemically different pituitary gonadotropins, GTH I and GTH II, were characterized first in salmonids [1–5] and later in a variety of non-salmonid species [6–10]. These two glycoproteins not only differ chemically, but also play distinct roles in controlling gonadal development. In salmonids, GTH I and GTH II are produced by separate pituitary cell types [11–13] and are differentially secreted during sexual maturation. GTH I levels in the blood increase during vitellogenesis or spermatogenesis and decline prior to final maturation; on the other hand, GTH II is low or nondetectable until the period just before ovulation or spermiation, when plasma GTH II levels increase [14–18]. In accordance with these findings, Tyler et al. [19] reported that GTH I, but not GTH II, significantly stimulated in vivo and in vitro ovarian uptake of vitellogenin in rainbow trout, *Oncorhynchus mykiss*. Furthermore, GTH II has higher in vitro steroidogenic potency than GTH I in matured fish gonad [8, 20–23], whereas the steroidogenic potencies of the two GTHs are similar in the gonads at earlier stages of gametogenesis [5, 7, 8, 10, 20–23]. Since hormonal effects must be preceded by the binding of the hormone to its specific receptor, it is natural to speculate that the functional differentiation of the two salmon GTHs might be accomplished through binding to at least two different types of receptors.

In mammals [24–29] and birds [30–34], FSH and LH have different receptors. However, the presence of separate FSH and LH receptors is not fully established in all classes of vertebrates. For instance, Yamanouchi and Ishii [35] proposed a cooperative binding mechanism by which FSH and LH may interact with a single type of receptor to produce different effects in the bullfrog (*Rana catesbeiana*) gonad. Before purified preparations of two chemically different fish GTHs were available, several studies reported the presence of a single type of GTH receptor in the fish gonad [36–39]. More recently, a two-receptor model was proposed for the postvitellogenic/preovulatory salmon ovary on the basis of binding studies using highly purified GTH I and GTH II [40]. The following model was proposed: 1) There are two types of GTH receptors, type I (GTH-RI) and type II (GTH-RII); 2) GTH-RI binds to both GTH, but with higher affinity for GTH I, whereas GTH-RII is highly specific for GTH II; 3) GTH-RI exists in both thecal and granulosa layers, whereas GTH-RII exists in only the granulosa layer.

Although the fundamental question of the presence of more than one type of GTH receptor in fish has been answered for at least one fish species, other important questions remain to be addressed. For example, 1) Does the salmon testis also have two types of GTH receptors? 2) Which testicular cell types have GTH receptors, and does the expression of the receptors change during spermatogenesis? 3) Is the two-receptor model applicable to all stages of oogenesis? The present study was conducted to address these questions through use of in vitro ligand autoradiography to locate GTH receptors.

MATERIALS AND METHODS

Hormone Preparations

Coho salmon GTH I and GTH II were purified according to Swanson et al. [5]. The GTH I preparation used in the present study contained both the stable (about 10%) and

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TABLE 1. Tissues and tissue sources used in this study.

Number	Fish	Gonadal maturity (stage)[a]	Body wt.	Gonadosomatic index
Testis				
#1	Juvenile, parr stage	contained only a small number of spermatogonia (I)	15 g	ND[b]
#2	Immature adult	contained only spermatogonia (I)	385 g	0.1
#3	Immature adult	contained spermatogonia and some spermatocytes (II)	400 g	0.2
#4	Adult	contained all stages of germ cells (IV) not spermiating	850 g	11
#5	Mature Adult	spermiating (V)	1200 g	10
Number	Fish	Gonadal maturity (stage)	Body wt.	Gonadosomatic index
Ovary				
#6	Juvenile, parr stage	pre-vitellogenic, perinucleolar oocytes (I)	17 g	ND
#7	Immature adult	vitellogenic (IV), maximal follicular diameter = 1.5 mm	345 g	1.1
#8	Immature adult	vitellogenic (IV), maximal follicular diameter = 2.0 mm	600 g	1.0
#9	Mature adult	post-vitellogenic, pre-ovulatory maximal follicular diameter = 6 mm	~2000 g	ND

[a]Determined according to the classification of Schultz [43].
[b]Not determined.

unstable (about 90%) forms. "Stable" refers to a form of GTH I that is not dissociated into subunits under acidic conditions in HPLC [1, 5]. In the present experimental conditions, neither of the forms of GTH I, nor GTH II, is dissociated into subunits. Both preparations are biologically active, and they show similar in vitro steroidogenic activities [5, 23]. A partially purified glycoprotein extract of mature coho salmon pituitaries (SG-G100; a mixture of GTH I and GTH II with less than 0.1% contamination by thyroid-stimulating hormone) was prepared by ethanol extraction and gel filtration chromatography [5].

Tissue Sampling

The fish used in the current study were coho salmon that were maintained in freshwater tanks under natural conditions of photoperiod and temperature at the National Marine Fisheries Service (Northwest Fisheries Science Center, Seattle, WA), with the exception of one mature adult female coho salmon that was obtained from Domsea Farms Inc. (Rochester, WA). Because spermiating male fish were not available at the time of the experiments, it was necessary to induce spermiation hormonally one month in advance of the normal spawning period. One adult male coho salmon was given an intramuscular implant containing 75 μg of LHRH (D-Ala^6,Pro^9-NET) in a copolymer of ethylene vinyl acetate [41]. We used the term "spermiating" to describe fish from which milt could be collected with gentle pressure to the abdomen. The gonadal tissues used in the present study are described in Table 1; in some cases the tissues used in the experiments are numbered as shown in Table 1. Developmental stages of spermatogenesis and oogenesis were determined according to procedures previously described [42–44]. Pieces of gonadal tissue excised from each fish were embedded in small molds made of aluminum foil with Tissue-Tek O.C.T. Compound (Miles Inc., Elkhart, IN) and were immediately frozen in a bath of dry ice and acetone. The frozen tissues were then stored at −64°C until sectioning. At the time of sampling, a small piece of gonadal tissue from each fish was also fixed in Bouin's fixative for routine histology to determine maturational stages of the gonad—except for the postvitellogenic ovary (tissue #9, Table 1). Follicles from the post-vitellogenic/preovulatory ovary were physically separated into theca-interstitial connective tissue layers and chorion-granulosa layers in ice-cold modified Hanks' balanced salt solution (HBSS: 106.9 mM NaCl, 5.4 mM KCl, 20 mM $MgCl_2$, 0.8 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 8 mM $NaHCO_3$, and 10 mM HEPES, pH 7.4) with fine-tipped forceps as reported by Kagawa et al. [45]. The separated theca-interstitial connective tissue layers were pooled and embedded as described above. The chorion-granulosa layers were rinsed in ice-cold HBSS to wash away egg yolk, pooled, and embedded similarly.

Radioiodination of Hormones

GTH I and GTH II were radioiodinated as reported previously [40, 46] with minor modifications. After the radiolabeled GTH was desalted, the peak fraction of the labeled hormone was subjected to gel filtration chromatography using a column of Sephadex G-75 superfine (1 × 51 cm; Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The column was equilibrated with HBSS containing 0.2% BSA (RIA grade, fraction V, Sigma Chemical Co., St. Louis, MO) and 0.05% NaN_3, and run at a flow rate of 8 ml/h. Fractions of 0.6 ml were collected. The specific binding of each fraction in the first peak of radioactivity was determined as previously described [39, 46]. Fractions with high specific binding (80–85% of total binding for GTH I and 51–80% of total

binding for GTH II) were used for subsequent experiments. Specific activities for radiolabeled hormones were determined by the self-displacement assay [47]; these ranged from 15 to 36 μCi/μg for GTH I (four iodinations) and from 12 to 105 μCi/μg for GTH II (five iodinations).

Characterization of Binding Sites on Tissue Sections

To quantify binding sites on cryostat-cut, slide-mounted tissue sections, the following procedure was used unless otherwise indicated. Frozen tissue blocks were trimmed into a columnar shape so that the area of each section would be the same. Serial sections (25–100 sections at a time) were cut at 10 μm (0.5–0.8 cm^2) on a cryostat microtome, thaw-mounted on gelatin-coated glass slides, and dried with cool air by means of a hair dryer for 10–20 sec for each section. A square was drawn around the tissue section on each glass slide with a PAP Pen (Daido Sangyo Co., Tokyo, Japan) so that radioactive incubation medium would not flow off the slide. The slides were stored at −64°C until use. For the analyses, many serial sections with homogeneous quality and rather large area were needed. Hence, quantitative estimates were usually made with mature testes (tissues #4 and #5, Table 1).

For the binding experiments, slides were arranged horizontally on a stainless steel mesh in a moist chamber and preincubated with 150 μl of HBSS per section for 20 min at 0–5°C. Subsequently, the preincubation medium was aspirated and each section was incubated with 70 μl of HBSS containing 0.2% BSA and 33 000 dpm of labeled hormone at 4–5°C for 20 h. Nonspecific binding was estimated in the presence of 1–1.25 μg of SG-G100 for ^{125}I-GTH I binding and 0.5 μg of purified unlabeled GTH II for ^{125}I-GTH II binding, since preliminary experiments indicated that even 10 μg of SG-G100 was sometimes less potent than 0.16 μg of purified GTH II to inhibit binding of ^{125}I-GTH II. After the incubation, the medium was aspirated and sections were washed three times (5 min each) with 150 μl of ice-cold HBSS per section. Subsequently, the slides were allowed to dry at room temperature. When necessary, the wet weight of tissue sections was calculated as follows. First, the shape of a section was traced on graph paper; then the enclosed area was cut out and weighed. The area of the section was calculated by an equation previously determined. The wet weight of the section was calculated according to the preliminary analysis of the area and the weight of sections of a defined thickness. Subsequently, each section was wiped off with two small pieces of filter paper (immersed in 99% ethanol) until no tissue remnants remained on the slide. The two pieces of filter paper containing the sections were put in a polystyrene test tube and the radioactivity was measured with a Micromedic γ-counter (efficiency = 61%).

Binding of Labeled Hormones to Tissue Sections

The time course of labeled hormone association with its binding sites on tissue sections was determined by varying incubation times up to 48 h. Saturation of binding sites on tissue sections was tested by varying the concentration of labeled hormone. For the saturation experiments, concentrations of unlabeled hormones were also changed so that the ratio of labeled and unlabeled hormone concentrations would be constant. Sections of testicular tissue in stages IV (^{125}I-GTH I binding; tissue #4, Table 1) and V (^{125}I-GTH II binding; tissue #5, Table 1) of spermatogenesis were used. To examine competitive inhibition of radioligand binding by unlabeled hormones, sections were incubated with radiolabeled hormones in the presence of either unlabeled GTH I or unlabeled GTH II at a concentration 0, 40, or 400 times greater than that of labeled hormone in molar ratio. Sections of testicular tissue from a spermiating male (tissue #5, Table 1) were used for the binding inhibition studies.

Effects of Prefixation

To test the effect of prefixation of tissue sections on the specific binding, sections were incubated with HBSS containing 0.05% paraformaldehyde for 20 min at 0–4°C and then washed three times (5 min each) with ice-cold HBSS before being incubated with radioligands. A preliminary study indicated that this prefixation could slightly enhance the quality of tissue morphology and did not affect the distribution pattern of silver grains in the resultant autoradiography. For binding of ^{125}I-GTH I, sections of testicular tissue in stage IV of spermatogenesis (tissue #4, Table 1) were used. For binding of ^{125}I-GTH II, the chorion-granulosa layers of the postvitellogenic/preovulatory ovary (tissue #9, Table 1) were used, since GTH-RII had been previously shown to be localized in this tissue [40]. To detect specific binding of ^{125}I-GTH II to the GTH-RII, binding inhibition was conducted with either unlabeled GTH I or unlabeled GTH II. The rationale for detection of the two types of receptors is discussed below.

Statistical Analyses

Student's *t*-test or Cochran-Cox test was used to compare means of different experimental groups.

Autoradiography

For ligand autoradiography, sections were freshly prepared on the day of incubation, since storage of cryostat-cut, slide-mounted tissue sections causes deterioration of tissue morphology [48]. Several sections were cut from each tissue, prefixed, incubated, and washed as described above. After the slides were dried, the sections were fixed with paraformaldehyde vapor for 2 h at 80°C. Then the slides were vented overnight and coated with photographic emulsion (NTB2; Eastman Kodak, Rochester, NY) at 40–45°C by dipping in liquid emulsion diluted with the same volume of distilled water. After the emulsion was dried, slides were exposed for 14–90 days at 4°C. Subsequently, slides were developed with D-19 developer (Kodak) for 4 min at 17°C,

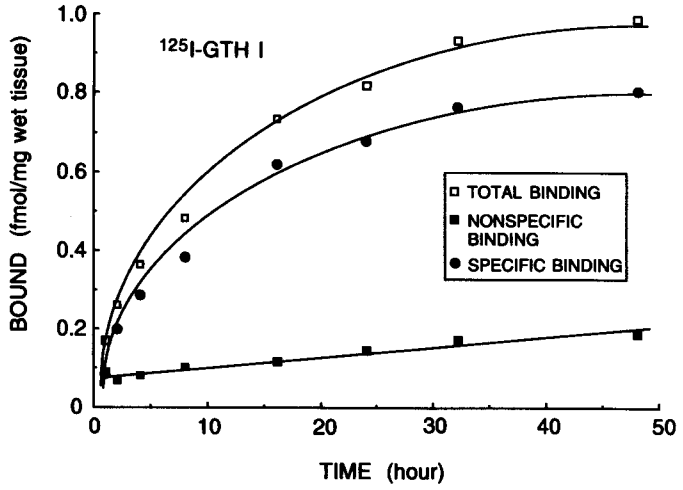


FIG. 1. Time-course association for ^{125}I-GTH I with sections of the testis in stage IV of spermatogenesis (tissue #4, Table 1). Data shown are from one of three experiments yielding similar results.

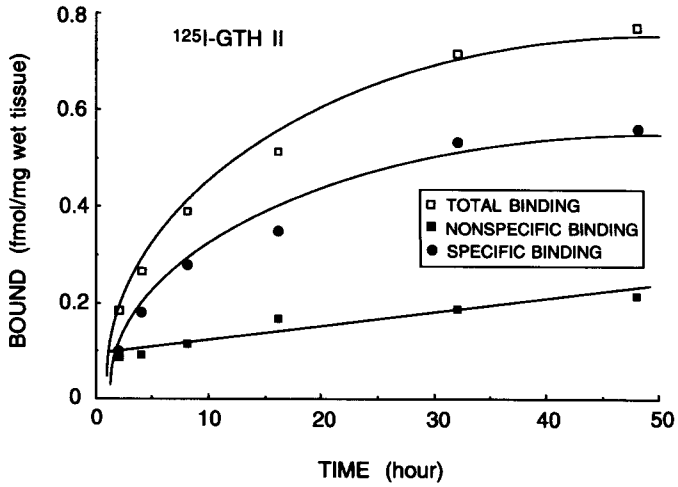


FIG. 2. Time-course association for ^{125}I-GTH II with sections of testis from a spermiating fish (stage V of spermatogenesis; tissue #5, Table 1). Data shown are from one of three experiments yielding similar results.

fixed, and washed with running tap water. Sections were then routinely stained with hematoxylin-eosin, cleared, and mounted. A preliminary experiment revealed that this wet emulsion technique was as effective for demonstrating salmon GTH receptor binding as the coverslip method of Young and Kuhar [49].

Detection of the Type I Receptor (GTH-RI)

Because Yan et al. [40] reported that GTH I binds specifically to GTH-RI, but not to GTH-RII, ^{125}I-GTH I was used as the ligand to detect the GTH-RI. A pair of slides with serial sections was used. One slide was incubated with 33 000–60 000 dpm of labeled GTH I to measure "total" binding. The other slide was incubated with the same amount of the radioligand in the presence of 1–1.25 μg of SG-G100 to estimate nonspecific binding. SG-G100 was used since it contains both GTH I and GTH II and since it had been previously shown that both GTH I and GTH II could displace binding of labeled GTH I to the GTH-RI [40].

Detection of the Type II Receptor (GTH-RII)

Yan et al. [40] reported that GTH-RII binds GTH II with high affinity, but does not bind GTH I. Therefore, it was necessary to use ^{125}I-GTH II as the ligand for detection of the GTH-RII. However, GTH II can also bind to the GTH-RI, although with lower affinity than GTH I [40]. As a result, the GTH-RII cannot be distinguished from the GTH-RI by conventional methods using labeled GTH II. To overcome this problem and to detect GTH-RII, the following method was applied. Three slides with serial sections were prepared. The first slide was incubated with 33 000–40 000 dpm of ^{125}I-GTH II to estimate the total binding. On this slide, the radioligand would presumably bind to both types of GTH receptors. The second slide was incubated with the same amount of the labeled hormone in the presence of 0.31–0.67 μg (105–228 nM) of unlabeled GTH I. On this slide, the binding of ^{125}I-GTH II to GTH-RI would be inhibited by an excess amount of unlabeled GTH I (which has a higher affinity for GTH-RI and cannot inhibit binding of GTH II to GTH-RII) and thus only the binding of ^{125}I-GTH II with the GTH-RII would remain and could be visualized. The third slide was incubated with the same amount of ^{125}I-GTH II in the presence of 0.31–0.67 μg (114–245 nM) of unlabeled GTH II, which would inhibit binding of labeled GTH II to both types of receptors to yield an estimate of nonspecific binding.

RESULTS

Binding of Labeled Hormones to Tissue Sections

Association curves for ^{125}I-GTH I and ^{125}I-GTH II with the sections of testes in stages IV and V of spermatogenesis are shown in Figures 1 and 2, respectively. Binding of the labeled hormones with slide-mounted sections was dependent on the incubation time. The specific binding of both radioligands approached equilibrium at 48 h after the start of incubation, while nonspecific binding increased linearly throughout the incubations. The specific binding of ^{125}I-GTH I to sections of the testis was saturable, whereas nonspecific binding increased linearly as the radioligand concentration increased (Fig. 3). In contrast, clear saturation of specific binding was not observed for ^{125}I-GTH II (Fig. 4a). In four out of five repeated experiments, both specific and nonspecific binding increased linearly as ^{125}I-GTH II concentrations increased, although saturation of specific binding was observed in one experiment (Fig. 4b). In the binding inhibition experiments, unlabeled GTH I and unlabeled GTH II at high concentrations inhibited the binding of ^{125}I-GTH I to almost the same degree. However, at low concentrations, GTH I was clearly more potent than GTH II ($p <$

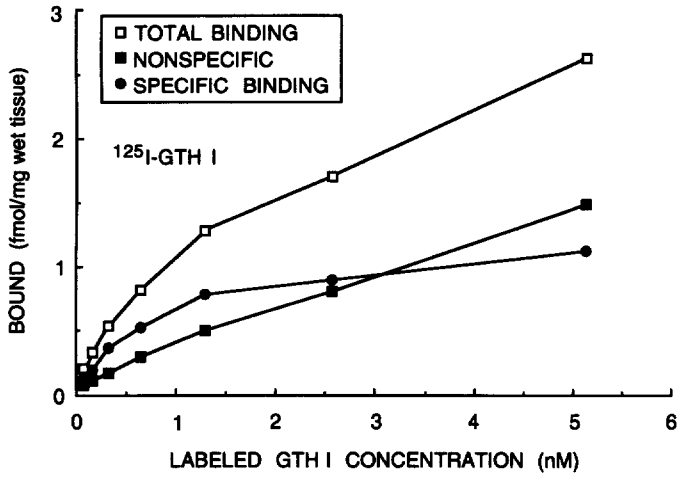


FIG. 3. Saturation analysis for ^{125}I-GTH I binding to sections of the testis in stage IV of spermatogenesis (tissue #4, Table 1). Data shown are from one of three experiments yielding similar results.

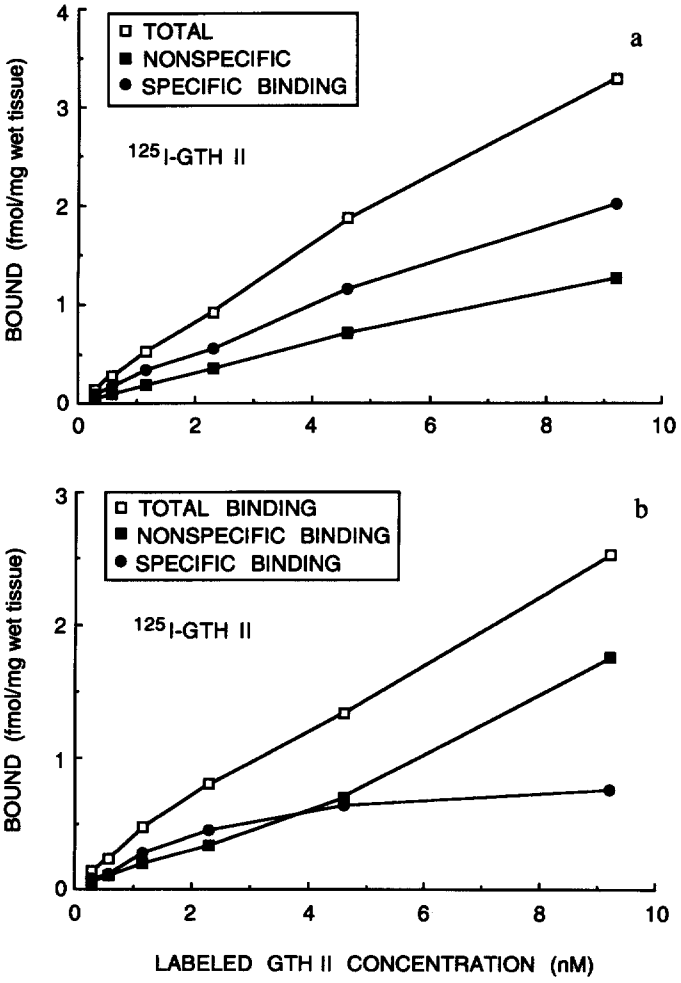


FIG. 4. Saturation analysis for ^{125}I-GTH II binding to sections of the testis from a spermiating fish (stage V of spermatogenesis; tissue #5, Table 1). Data shown are from two of five repeated experiments. Results of the other three experiments were similar to those shown in Figure 4a.

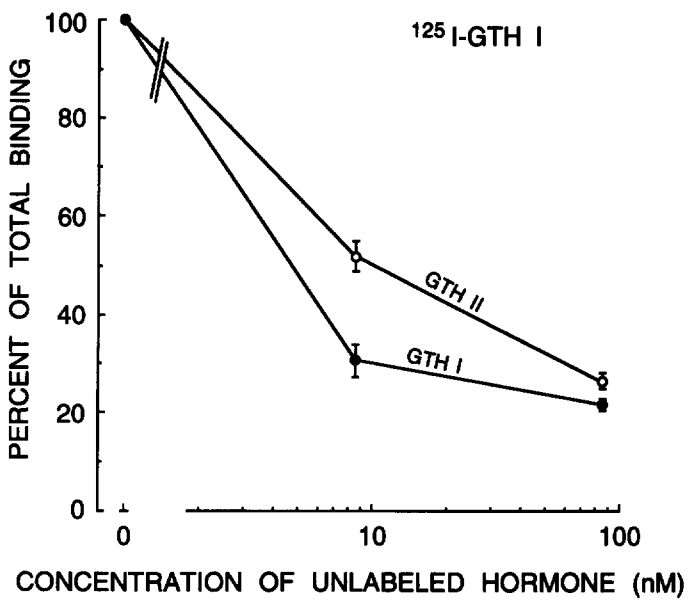


FIG. 5. Competitive inhibition by unlabeled GTH I and GTH II of ^{125}I-GTH I (210 pM) binding to sections of the testis from a spermiating fish (tissue #5, Table 1). Data are expressed as mean ± SE (n = 4).

0.01) in displacing ^{125}I-GTH I binding (Fig. 5). On the other hand, the two unlabeled GTHs inhibited binding of ^{125}I-GTH II to the same degree at the low concentrations. Nevertheless, GTH I was significantly less potent ($p < 0.001$) than GTH II in displacing ^{125}I-GTH II binding at high concentrations (Fig. 6).

Effects of Prefixation

No significant difference was observed between mean values of specific binding of prefixed and control (untreated) tissue sections (Figs. 7 and 8), suggesting that the prefixation procedure did not adversely affect specific binding of the radiolabeled hormones. Thus, the prefixation was applied to subsequent incubations for autoradiography.

Autoradiography

Specific binding of ^{125}I-GTH I, which was considered to be binding to GTH-RI, was found in all of the tissues used in our experiments. On the other hand, specific binding of ^{125}I-GTH II to GTH-RII (binding that could be inhibited by unlabeled GTH II, but not by GTH I) was detected only in the spermiating testis and in the postvitellogenic/preovulatory ovary.

Testis

The external appearance of the testes of the juvenile coho salmon used in the present study was very immature; in their gross morphological appearance the testes were resembled a pair of thin strands of tissue. The testes contained only a small number of spermatogonia and no sper-

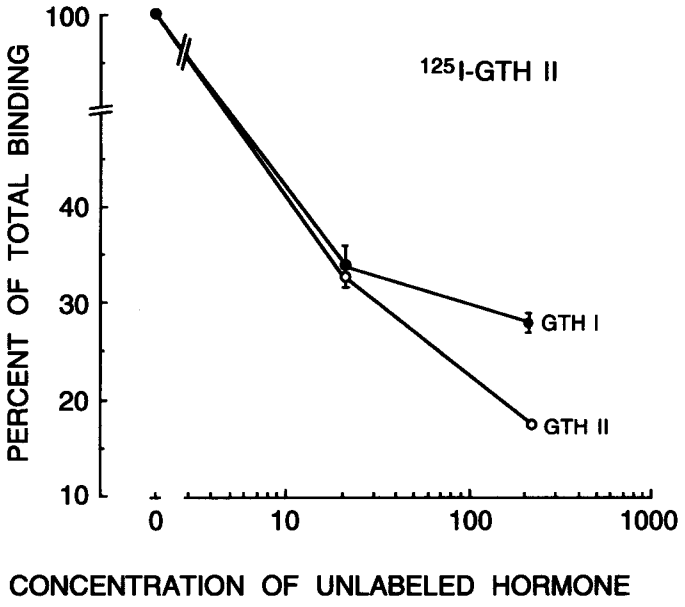


FIG. 6. Competitive inhibition by unlabeled GTH I and GTH II of ^{125}I-GTH II (510 pM) binding to sections of the testis from a spermiating fish (tissue #5, Table 1). Data are expressed as mean ± SE (n = 4).

matocytes, and were judged to be in stage I of spermatogenesis according to the studies by Grier [42] and Schulz [43]. Specific ^{125}I-GTH I binding sites (GTH-RI) were clearly observed in this immature tissue (Fig. 9). Most of the binding sites were located on somatic cells that were near the spermatogonia. However, the spermatogonia were almost devoid of binding sites (Fig. 9c). Some binding sites were also found in the connective tissue layer.

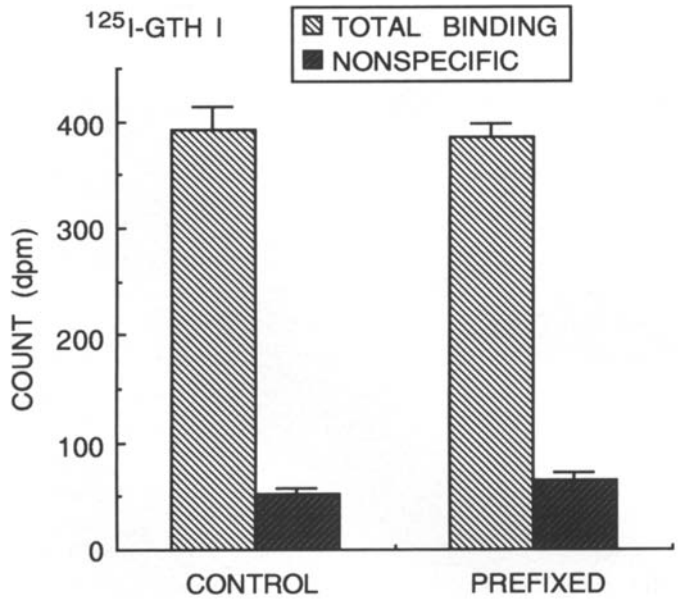


FIG. 7. Effect of prefixation with 0.05% paraformaldehyde on ^{125}I-GTH I binding to sections of the testis in stage IV of spermatogenesis (tissue #4, Table 1). Data are expressed as mean ± SE (n = 5).

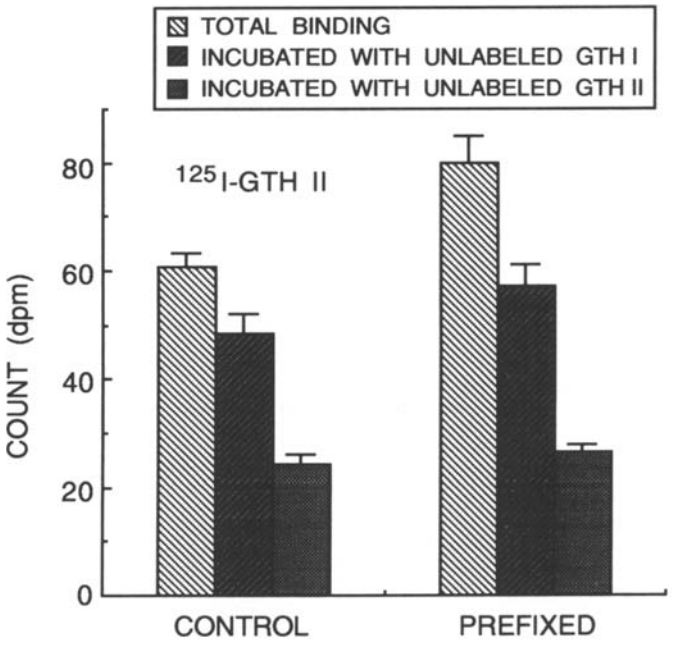


FIG. 8. Effect of prefixation with 0.05% paraformaldehyde on ^{125}I-GTH II binding to sections of separated chorion-granulosa layers from the preovulatory follicle (tissue #9, Table 1). Data are expressed as mean ± SE (n = 4).

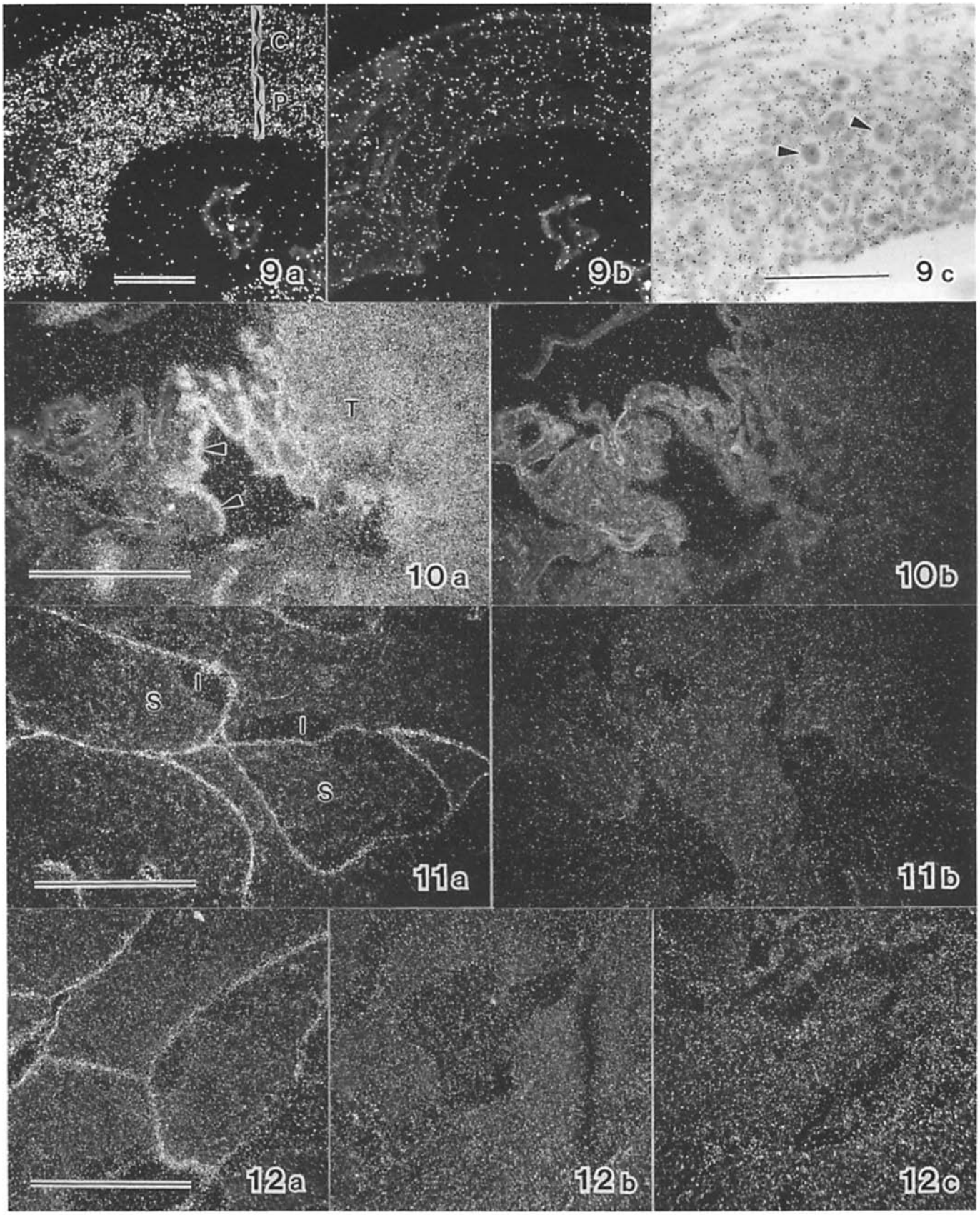
PLATE I. FIGS. 9–12.

FIG. 9. Photomicrographs of the autoradiography for two adjacent sections of the testis in stage I of spermatogenesis (tissue #1, Table 1), incubated with 140 pM ^{125}I-GTH I (36 μCi/μg) and exposed for 63 days. a) Darkfield photomicrograph of total binding. Specific binding sites are observed not only in the testicular parenchyma (P; the layer that contains spermatogonia), but also in the connective tissue layer (C). The bar indicates 100 μm. ×150. b) Darkfield photomicrograph of nonspecific binding; sections incubated in the presence of 1.0 μg/70 μl of SG-G100. ×150. c) Lightfield photomicrograph of the same specimen shown in Figure 9a at a higher magnification. Arrowheads indicate spermatogonia. Note that spermatogonia are almost devoid of binding sites. The bar indicates 50 μm. ×460.

FIG. 10. Darkfield photomicrographs of the autoradiography for two adjacent sections of the testis in stage I of spermatogenesis from an immature adult salmon (tissue #2, Table 1), incubated with 140 pM ^{125}I-GTH I (36 μCi/μg) and exposed for 63 days. The bar indicates 400 μm. a) Total binding: specific binding sites are found in the testis (T) and on the associated connective tissue (arrowheads). ×75. b) Nonspecific binding: sections incubated in the presence of 1.0 μg/70 μl of SG-G100. ×75.

FIG. 11. Darkfield photomicrographs of autoradiography for the testis in stage IV of spermatogenesis (tissue #4, Table 1), incubated with 600 pM ^{125}I-GTH I (8 μCi/μg) and exposed for 90 days. The bar indicates 400 μm. a) Total binding: specific binding sites form a reticular pattern surrounding testicular cysts. I, immature germ cells; S, spermatozoa. ×75. b) Nonspecific binding: sample incubated in the presence of 1.25 μg/150 μl of SG-G100. ×75.

FIG. 12. Darkfield photomicrographs of autoradiography for the testis in stage IV of spermatogenesis (tissue #4, Table 1), incubated with 470 pM ^{125}I-GTH II (16 μCi/μg) and exposed for 35 days. The bar indicates 400 μm. a) Total binding: specific binding sites form a pattern similar to that shown in Figure 11a. ×75. b) Tissue incubated in the presence of 220 nM unlabeled GTH I; specific binding is completely inhibited. ×75. c) Nonspecific binding: tissue incubated in the presence of 230 nM unlabeled GTH II. ×75.



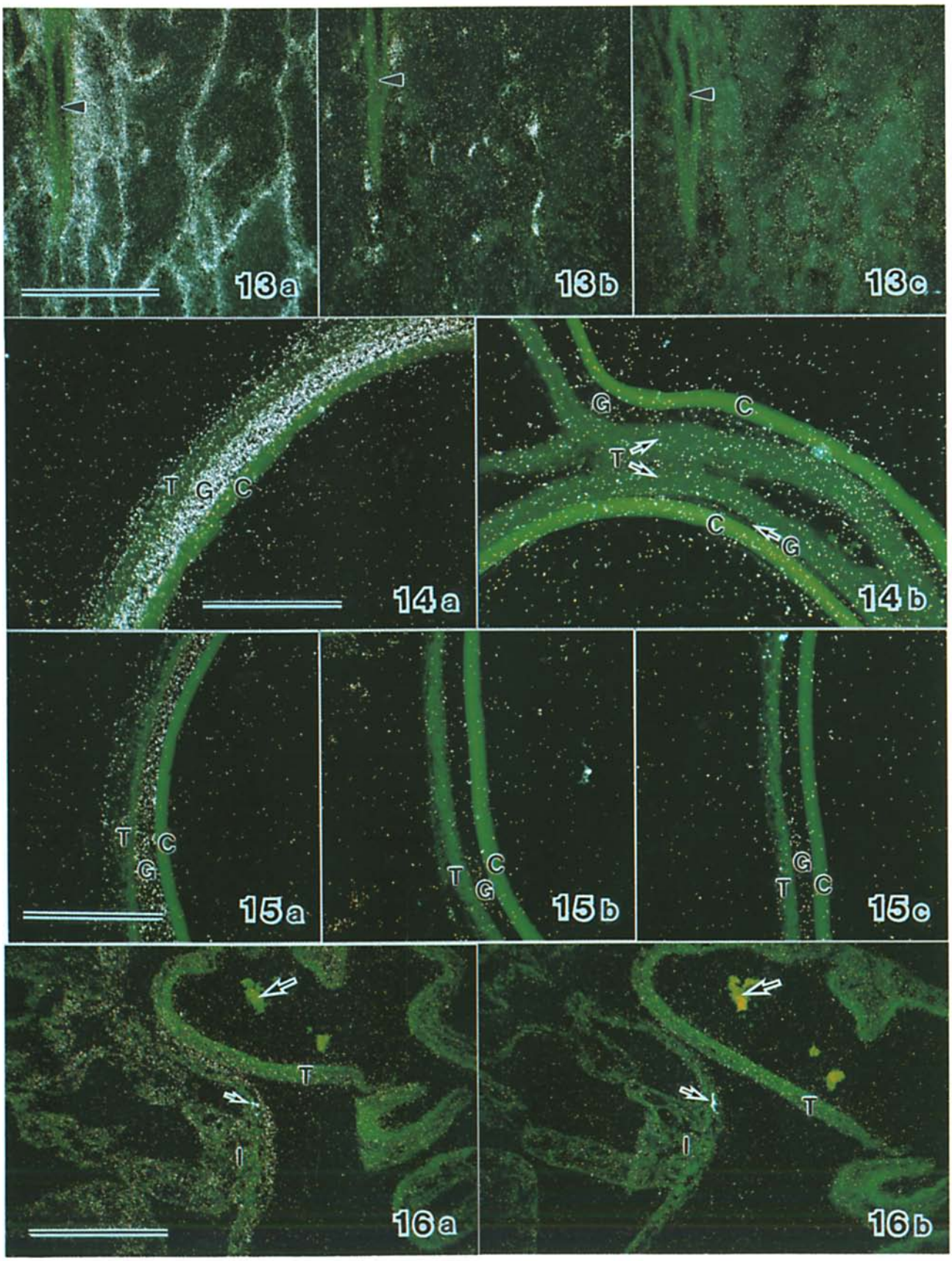


Figure 10 shows ^{125}I-GTH I binding to sections of a testis from an immature adult (tissue #2, Table 1). In the testes of the two immature adult coho salmon that were sampled (tissues #2 and #3, Table 1), specific ^{125}I-GTH I binding sites (GTH-RI) were homogeneously distributed. Histological observation of the paraffin sections revealed that one sample (tissue #2) had large numbers of spermatogonia and no spermatocytes; this sample was considered to be in stage I of spermatogenesis. The sample from the second immature adult (tissue #3) contained large numbers of spermatogonia and some spermatocytes; therefore, this testis was considered to be in stage II of spermatogenesis. Specific binding was also found on testis-associated connective tissue (Fig. 10a). This connective tissue may be the tissue that forms either the deferent duct or the mesorchium. Specific binding sites for ^{125}I-GTH II were also observed in these testes (tissues #2 and #3). However, the binding could be inhibited with unlabeled GTH I as well as unlabeled GTH II.

In the maturing adult coho salmon male (tissue #4, Table 1), the testis contained primary and secondary spermatocytes as well as spermatozoa and was judged to be in stage IV of spermatogenesis. The specific binding sites for ^{125}I-GTH I (GTH-RI) on sections of this testis were located on the border of testicular cysts or lobules, forming a reticular pattern (Fig. 11). Specific binding of ^{125}I-GTH II was also observed at this stage and formed a similar reticular pattern on the tissue sections. This specific binding was inhibited with unlabeled GTH I as well as unlabeled GTH II (Fig. 12). No specific binding was seen on germ cells.

Primarily the same reticular pattern of distribution for specific ^{125}I-GTH I binding sites (GTH-RI) was found on testis sections from the spermiating male coho salmon (stage V of spermatogenesis; tissue #5, Table 1) as in the testis in stage IV of spermatogenesis. The width of the distribution area of the specific binding in the stage V testis was enlarged compared to that found in stage IV. Figure 13 shows photomicrographs of the autoradiography for ^{125}I-GTH II binding to the spermiating testis. A reticular pattern of distribution of the binding sites for ^{125}I-GTH II, basically the

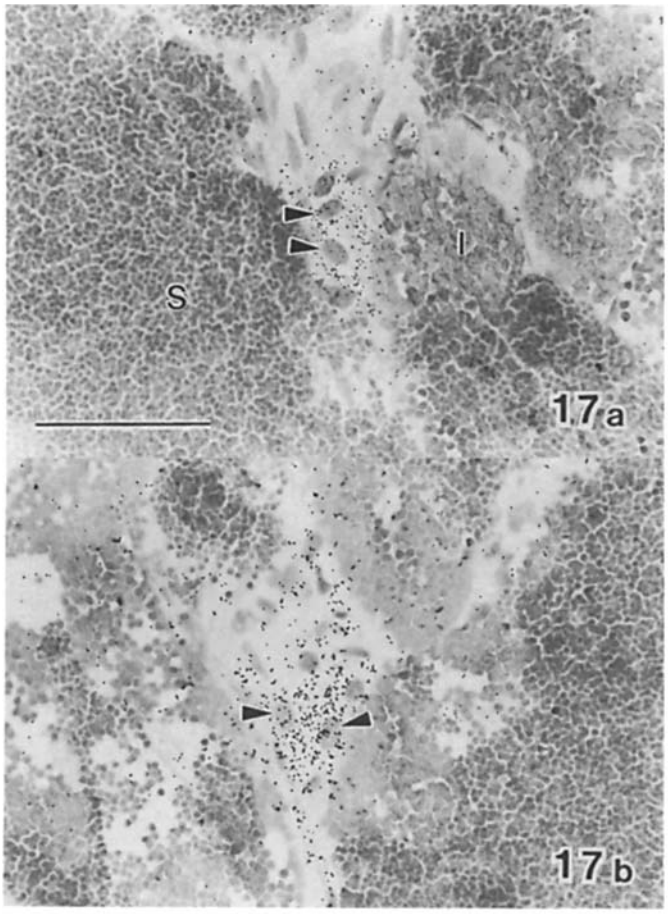


FIG. 17. Brightfield photomicrographs of autoradiography for testis from spermiating fish (stage V of spermatogenesis; tissue #5, Table 1), incubated with 460 pM ^{125}I-GTH II (12 μCi/μg) in the presence of 170 nM unlabeled GTH I to detect GTH-RII. The figures show two examples of the area where GTH-RII receptors are detected. In both cases, specific binding to GTH-RII is concentrated on some interstitial cells with large elliptical nuclei (arrowheads). The bar indicates 50 μm. a) Exposure for 23 days. Some nonspecific binding sites were also found on spermatozoa (S) and immature germ cells (I) but are not seen in this picture because of the difference in the focal planes. ×460. b) Exposure for 31 days. ×460.

PLATE II. FIGS. 13–16.

FIG. 13. Darkfield photomicrographs of autoradiography for three adjacent sections of the testis from a spermiating fish (stage V of spermatogenesis; tissue #5, Table 1), incubated with 460 pM ^{125}I-GTH II (12 μCi/μg) and exposed for 31 days. The bar indicates 400 μm. Arrowheads indicate the same blood vessel in the three photomicrographs. Connective tissues can be seen by green fluorescence of eosin. a) Total binding: specific binding sites form a pattern similar to that shown in Figures 11a and 12a. ×63. b) Tissue incubated in the presence of 170 nM unlabeled GTH I. Small patches of specific binding remain, indicating the presence of GTH-RII. ×63. c) Nonspecific binding: sample incubated in the presence of 180 nM unlabeled GTH II. Specific binding that forms the reticular pattern and the small patches is inhibited. ×63.

FIG. 14. Darkfield photomicrographs of autoradiography for the ovary in stage IV of vitellogenesis (tissue #8, Table 1), incubated with 140 pM ^{125}I-GTH I (36 μCi/μg) and exposed for 34 days. The bar indicates 200 μm. T, thecal layer; G, granulosa cells; C, chorion. a) Total binding: most of the specific binding sites are localized on the granulosa cells; some are also found on the theca layer. ×125. b) Nonspecific binding: sample incubated in the presence of 1.0 μg/70 μl of SG-G100. Two adjacent follicle walls are seen. ×125.

FIG. 15. Darkfield photomicrographs of autoradiography for the ovary in stage IV of vitellogenesis (tissue #8, Table 1), incubated with 150 pM ^{125}I-GTH II (42 μCi/μg) and exposed for 50 days. The bar indicates 200 μm. T, thecal layer; G, granulosa cells; C, chorion. a) Total binding: for the most part the same binding pattern is observed as in Figure 14a. ×125. b) Tissue incubated in the presence of 238 nM unlabeled GTH I. No specific binding remains on this slide. c) Nonspecific binding: tissue incubated in the presence of 255 nM unlabeled GTH II. ×125.

FIG. 16. Darkfield photomicrographs of autoradiography for two adjacent sections of the postvitellogenic/preovulatory salmon ovary (separated theca-interstitial tissue layers of tissue #9, Table 1) incubated with 140 pM ^{125}I-GTH I (36 μCi/μg) and exposed for 63 days. Large arrows and small arrows indicate remnants of the chorion and a melanophore, respectively. T, thecal layer; I, interstitial connective tissue. The bar indicates 400 μm. a) Total binding: most of the specific binding sites are observed on the interstitial connective tissue. ×63. b) Nonspecific binding: sample incubated in the presence of 1.0 μg/70 μl of SG-G100. ×63.

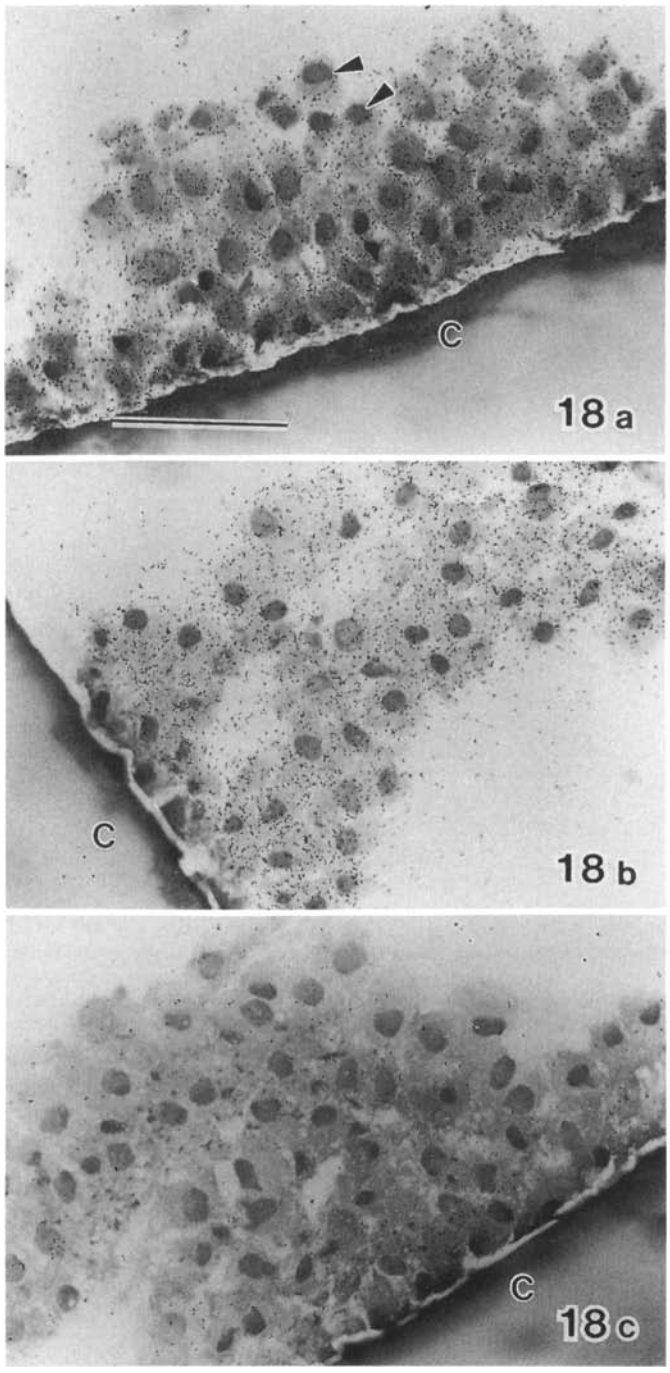


FIG. 18. Brightfield photomicrographs of autoradiography for the postvitellogenic/preovulatory salmon ovary (separated chorion-granulosa layers of tissue #9, Table 1), incubated with 150 pM ^{125}I-GTH II (42 μCi/μg) and exposed for 31 days. The bar indicates 50 μm. a) Total binding: specific binding sites are intensely located on the granulosa cells (arrowheads). ×460. b) Incubation in the presence of 231 nM unlabeled GTH I. Many specific binding sites, which cannot be displaced by unlabeled GTH I, remain, indicating the presence of GTH-RII. ×460. c) Nonspecific binding: incubation in the presence of 260 nM unlabeled GTH II. Specific binding to granulosa cells is inhibited. ×460.

same as that for ^{125}I-GTH I binding, was observed in the section showing total binding (Fig. 13a). Much of this binding was inhibited when the section was incubated in the presence of excess unlabeled GTH I. However, some specific binding remained, forming small patches on the section (Fig. 13b). Figure 17 shows two brightfield photomicrographs of these small patches of the GTH II-specific binding sites at a higher magnification. The binding sites were always located on a single type of interstitial somatic cells that possessed large elliptical nuclei. These cells were observed only in the stage V or spermiating testis. The binding of ^{125}I-GTH II to these cells was inhibited only with unlabeled GTH II (Fig. 13c), and therefore the binding sites were identified as GTH-RII. We could not determine whether these cells had GTH-RI or not.

Ovary

Stages of oogenesis were determined according to methods previously reported [43–44]. Yolk material was washed away during preincubation. In the previtellogenic or perinucleolar stage (tissue #6, Table 1), specific binding of ^{125}I-GTH I was detected in the sections of the ovary; however, we could not identify the cell types on which binding was localized because of the poor preservation of tissue morphology.

The ovarian follicles of the immature adult female (tissues #7 and #8, Table 1) were at an early stage of vitellogenesis as revealed by light microscopy of paraffin sections. In these early vitellogenic ovaries, most of the specific binding sites for ^{125}I-GTH I were located predominantly on the granulosa cells of the ovarian follicles (Fig. 14). Some specific binding sites were also found in the thecal layer. For ^{125}I-GTH II binding to these tissues, a similar pattern was observed, although the binding was inhibited by unlabeled GTH I and GTH II, indicating that these binding sites were GTH-RI (Fig. 15).

In the postvitellogenic/preovulatory ovary (tissue #9, Table 1), specific binding of ^{125}I-GTH I was not detected on the granulosa cells. Most of the ^{125}I-GTH I binding sites (GTH-RI) were localized in the interstitial connective tissue and in thecal layers (Fig. 16a). On the other hand, specific binding sites for ^{125}I-GTH II were intensely localized on granulosa cells (Fig. 18). Incubation with an excess amount of unlabeled GTH I could not inhibit binding of ^{125}I-GTH II to these sites (Fig. 18b). Binding of ^{125}I-GTH II to the granulosa cells at this stage was inhibited only by unlabeled GTH II (Fig. 18c); hence, these binding sites were identified as GTH-RII. In the interstitial connective tissue and in the thecal layer, binding of ^{125}I-GTH II could be inhibited by unlabeled GTH I.

In the present study, specific GTH binding sites could not be discerned in the plasma membrane of oocytes.

DISCUSSION

Receptor Characterization

The results of characterization of receptors on tissue sections suggest that on the whole, gonadal receptors for salmon GTHs retain their characteristics even on cryostat-cut, slide-mounted tissue sections. The two types of GTH receptors described by Yan et al. [40] were found in sections of both the ovary and testis of coho salmon. The radioligand association analyses (Figs. 1 and 2) showed saturation of the displaceable (specific) binding of both GTHs at the end of the incubations. In saturation analysis for ^{125}I-GTH I (Fig. 3), specific binding approached equilibrium as the radioligand concentration increased, suggesting a limited number of binding sites. On the other hand, clear saturation for specific binding of ^{125}I-GTH II was observed in only one of five repeated experiments (Fig. 4b). In the other four experiments, the displaceable ^{125}I-GTH II binding increased rather linearly as the radioligand concentration increased. The simplest explanation for this discrepancy is that in the four experiments in which saturation was not observed, the radioligand concentration was too low relative to the binding capacity of the tissue section to saturate all of the binding sites. This may not have been the case, however, because saturation was observed in the third of five repeated experiments and in the middle portion of the ninety successive sections used in the study. It is difficult to envision that the capacity of the specific binding was small only for the sections used in the third experiment unless there were regional variations in the density of receptors or cell types within the testis. So far, we have no clear explanation for these results, and further characterization may be needed for the GTH II binding sites.

Results for competitive inhibition of radioligand binding to sections of the testis from the spermiating male salmon by GTH I and GTH II (Figs. 5 and 6) are comparable to those observed in similar experiments using conventional membrane preparations from postvitellogenic salmon ovarian follicles [40]. The two unlabeled GTHs inhibited binding of ^{125}I-GTH I to almost the same degree at high concentrations; but at low concentrations, the extent of the inhibition was greater ($p < 0.01$) for unlabeled GTH I (Fig. 5). These data suggest that both GTHs interacted with a single type of receptor that has higher affinity for GTH I. On the other hand, the presence of two different binding sites for GTHs in the testis from the spermiating fish was suggested when ^{125}I-GTH II was utilized (Fig. 6). At high concentrations, GTH II was clearly more potent than GTH I in inhibiting ^{125}I-GTH II binding. This is readily explained by the two-receptor model originally proposed by Yan and colleagues [40]; unlabeled GTH I inhibited virtually all the ^{125}I-GTH II binding to the GTH-RI, whereas the ^{125}I-GTH II binding to GTH-RII (which is highly specific for GTH II) could be inhibited only by GTH II and not by GTH I. Therefore, provided that the concentration of the intact hormones (400 times higher than the radioligand in molar ratio) was high enough to attain equilibrium, the 10% difference in the inhibition of ^{125}I-GTH II binding by unlabeled hormones at high concentrations theoretically reflects the amount of ^{125}I-GTH II binding to GTH-RII, while the remaining specific binding (70% of the total binding) represents the amount of binding to GTH-RI.

Autoradiography

The results of the present in vitro ligand autoradiography study revealed not only tissue-specific distribution of GTH receptors, but also a stage-specific appearance of GTH-RII. The GTH-RI was detected in all tissues examined, but the GTH-RII was detected only in the gonads from fish that were close to spawning.

Testis

The present results suggest that specific binding sites are localized on somatic cells but that germ cells do not have any GTH receptors.

In stages IV and V of spermatogenesis, binding to GTH-RI was observed on the border of testicular cysts or lobules and formed a reticular pattern (Figs. 11a, 12a, and 13a). The only somatic cells that show such a distribution pattern in the salmon testis are Sertoli cells and fibroblasts surrounding testicular lobules [42, 50]. It is unlikely that fibroblasts possess GTH receptors; therefore, we concluded it to be likely that GTH-RI is localized on Sertoli cells in the salmon testis. In the testis from the spermiating fish, the width of the reticular pattern of distribution of GTH-RI was enlarged (data not shown). Since Sertoli cells are hypertrophied at the time of spermiation [51], this supports our conclusion that GTH-RI is localized on Sertoli cells.

Specific GTH I binding sites were also detected in the connective tissue layer of the testis of a juvenile coho salmon (Fig. 9) and on testis-associated connective tissue from an immature adult (Fig. 10). The presence of Sertoli cells in these tissues has not been reported; rather, Sertoli cells are situated within the basal lamina surrounding testicular lobules [42, 50]. One possible explanation for our observation is that Leydig cells are distributed in these tissues and that Leydig cells also possess the GTH-RI. However, this awaits further investigation.

GTH-RII was found only in the testis from the spermiating adult fish. At this stage of spermatogenesis, two different types of binding sites for the two GTHs could be visualized by autoradiography (Fig. 13). This is consistent with the results for the competitive inhibition of radioligand binding to the same tissue (Figs. 6). Figure 13b clearly shows that the difference in the extent of inhibition of ^{125}I-GTH II binding by unlabeled GTHs, at the high concentrations shown in Figure 6, is attributable to the binding of these small patches of binding sites (GTH-RII). The cells containing GTH-RII were localized in the interlobular space;

they had large nuclei and formed clusters of a few to several cells (Fig. 17). On the basis of these morphological features, these cells were identified as Leydig cells.

The appearance of GTH-RII in the Leydig cells in stage V of spermatogenesis coincides with elevated plasma GTH II levels [17] and enhanced production of the maturation-inducing steroid 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) by the testis in response to GTH II [21–23] or to a preparation of chum salmon gonadotropin (SGA; a preparation that probably contains GTH II) [52–55]. The appearance of GTH-RII in Leydig cells of the spermiating testis suggests that GTH II plays an important role in the final maturation of sperm and spermiation. In salmon, it has been demonstrated that SGA stimulates sperm maturation via the production of 17,20βP [52–53]. It has also been demonstrated in rainbow trout that 17α-hydroxy-progesterone (17α-P) can be converted to 17,20βP by sperm [54]. However, this process is not stimulated by GTH. In teleosts, Leydig cells are known to have 3β-hydroxysteroid dehydrogenase activity [50]. Thus, GTH II may stimulate 17,20βP production via stimulation of 17α-P production, which might occur in Leydig cells. The observed changes in types and distribution of GTH receptors in the testis may help to explain the changes in steroidogenic potencies of GTH I and GTH II that have been reported in the male coho salmon during sexual maturation [21–23]. GTH II had significantly higher potency than GTH I in stimulating in vitro production of 17,20βP and 11-ketotestosterone (11-KT) by testicular fragments from fish that were spermiating or were near spermiation. In contrast, in earlier stages of spermatogenesis, GTH I and GTH II were almost equipotent in stimulating production of 11-KT and 17,20βP. It is likely that the similar activities of GTH I and GTH II observed during the early phases of spermatogenesis are due to the presence of only GTH-RI (which binds both GTH); on the other hand, the enhanced steroidogenic potency of GTH II during the late stages of spermatogenesis is probably due to the appearance of GTH-RII. These findings emphasize the importance of the interaction of GTH II with the highly specific GTH-RII at the time when the testis is producing mature sperm.

As discussed above, it appears that Sertoli cells are the target for GTH I and that Leydig cells are the target for GTH II, at least in the spermiating testis. If it is assumed that salmon GTH I and GTH II are homologous to tetrapod FSH and LH, respectively, there is apparent homology between salmon and mammals in terms of the GTH-target tissue relationship. For both animals, Sertoli cells are the target for FSH and Leydig cells are the target for LH. However, it is important to emphasize that Leydig cells may also contain GTH-RI. We could not determine whether or not Leydig cells in the stage V testis also contained GTH-RI. We were also unable to identify Leydig cells in the testis except in the tissue from the spermiating fish, although it is well established that these cells are present in the salmonid testis during spermatogenesis [42, 50].

Ovary

In the ovaries from vitellogenic fish, GTH-RI was intensely localized on the granulosa cells and to a lesser extent in the thecal layer (Figs. 14 and 15), whereas no GTH-RII was detected. The thecal layer is principally connective tissue, but it contains steroid-producing cells, designated special thecal cells [50, 56]. Thus, it is most likely that the GTH-RI found in the thecal layer is localized on the special thecal cells. Studies using purified GTH I and GTH II have shown that the two GTH equally stimulated in vitro estradiol-17β (E) production by whole follicles from vitellogenic salmon [20] and previtellogenic juvenile salmon [15]. The similar steroidogenic potencies of the two GTHs can also be explained (as in the case of the male) by the presence of GTH-RI, which interacts with both GTHs, and the absence of GTH-RII, which is very specific for GTH II.

In mammals, FSH stimulates aromatase activity in the granulosa cells, thereby enhancing E production [59–60]. In salmon, as in mammals, cells in the thecal layer produce testosterone (T), which is converted to E by aromatase in granulosa layers during vitellogenesis [45, 50, 56–58]. In other studies, a GTH preparation (designated SGA) did not stimulate conversion of T to E by isolated granulosa layers; this suggests that aromatase activity is not regulated by GTH in salmon, although production of T by thecal layers is stimulated by the same preparation [45, 56, 58]. However, the high amount of GTH-RI found in the present study in the granulosa cells still strongly suggests that the granulosa layer is an important target for GTH I in the vitellogenic ovary. Thus in future studies, the effects of GTH I on granulosa cells during vitellogenesis should be re-examined.

In the postvitellogenic/preovulatory salmon ovary, GTH-RI was detected in the thecal layer and in the interstitial connective tissue. As Figure 16 shows, the GTH-RI appears to be more concentrated in the interstitial connective tissue than in the thecal layer. However, this may be an artifact caused by the shrinkage of interstitial tissue at the time of separation of the tissue from the follicles. In contrast with the results for vitellogenic follicles, GTH-RI was not detected on the granulosa cells of the postvitellogenic follicles. Instead, the GTH-RII was concentrated on granulosa cells (Fig. 18). This indicates that there is a shift of GTH receptor types in the granulosa cell from GTH-RI to GTH-RII. The lack of GTH-RI in granulosa cells of the preovulatory follicle contrasts with observations in previous binding studies indicating the presence of both GTH-RI and GTH-RII in granulosa cells at this stage [40]. The apparent conflict in the data may be due to low sensitivity of the present autoradiography compared to quantitative methods for detection of the receptors, particularly when receptors are not sufficiently concentrated. A quantitative experiment in the present study also showed that unlabeled GTH I par-

tially inhibited ^{125}I-GTH II binding to the separated chorion-granulosa layer of the preovulatory ovary (Fig. 8), suggesting the presence of GTH-RI.

In any case, the present data demonstrate clearly that the dominant receptor type on granulosa cells shifts from GTH-RI to GTH-RII during the process of follicular maturation in salmon. The precise timing of this shift was not ascertained, because ovaries in very late stages of vitellogenesis were not examined. However, the appearance of GTH-RII may coincide with the shift in steroid production from E to the maturation-inducing steroid, 17,20βP, since GTH II has much higher potency than GTH I in stimulating conversion of 17α-P to 17,20,βP by isolated granulosa layers from preovulatory follicles [20]. These data suggest that GTH-RII is coupled to the 17,20βP production system. If this is the case, an interesting homology in the hormonal control of follicular development is found between salmon and mammals.

In mammals, granulosa cells of immature follicles have numerous FSH receptors and no LH receptors. However, before ovulation, the LH receptor appears and markedly increases in number, and granulosa cells acquire the capability of producing progesterone in response to gonadotropin stimulation [61]. Thus, assuming that GTH I is salmon FSH and that GTH II is salmon LH, granulosa cells of both animals at first have only FSH receptors; then, prior to ovulation, the LH receptor appears, and this enables granulosa cells to produce progestins (progesterone for mammals and 17,20βP for salmon). One major difference between salmon and mammals in the GTH receptor distribution was suggested in the present study: we could not find the GTH-RII (LH receptor) in the theca or in interfollicular connective tissue of either the vitellogenic or the postvitellogenic ovary. A finding inconsistent with our observation, reported by Suzuki et al. [20], was that GTH II was slightly more potent than GTH I in stimulating the production of 17α-P in the isolated thecal layers of amago salmon. Their finding may suggest the presence of a small amount of GTH-RII in the theca at the time of ovulation.

In summary, for both male and female salmon, the present study suggests that a receptor highly specific for GTH II (designated GTH-RII) appears only in the gonads of fish that are close to spawning (postvitellogenic/preovulatory ovaries and spermiating testes), while a receptor that binds both GTH I and GTH II (designated GTH-RI) is present in gonads at all stages of gametogenesis examined. The presence of a GTH receptor that binds both GTH (GTH-RI) can explain, at least in part, previous findings of no significant difference in steroidogenic activities of salmon GTH I and GTH II in previtellogenic ovaries in the perinucleolar stage [15], in vitellogenic ovarian follicles [20], and in testes in stage I [15] or stage IV [21–23] of spermatogenesis. It should also be noted that plasma GTH II levels are very low or nondetectable until the final stages of gametogenesis [17–18] and that consequently the GTH-RI virtually does not interact with GTH II during vitellogenesis in females or prior to spermiation in males under normal physiological conditions. Hence, GTH I seems to be virtually the only GTH that controls the early stages of gonadal development in salmon. In mammals, however, LH stimulates T production by the ovarian theca or by testicular Leydig cells. Thus, in immature salmon, GTH I apparently plays roles homologous not only to those of FSH, but also to those of LH. In fully matured salmon, both types of GTH receptors are present and both GTHs are present in the plasma; therefore, at this stage, GTH-RI probably interacts with GTH II as well as GTH I in vivo whereas GTH-RII interacts with only GTH II.

To further verify this two-receptor model for GTH I and GTH II in salmon, and to fully clarify the ontogeny of GTH-receptor expression as well as the mechanisms involved in regulating GTH-receptor expression in specific cell types during gametogenesis, a molecular approach will be necessary and is being implemented in our current investigations.

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