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ABSTRACT—Although a number of immunohistochemical studies have been carried out on the differentiation of chicken gonadotropes during embryogenesis, the temporal and spatial properties of appearance of gonadotropes are not clear. In this study, we studied the appearance and morphological characteristics of gonadotropes in the embryonic and adult chicken anterior pituitary glands using RT-PCR, *in situ* hybridization and immunohistochemistry.

For this purpose, we raised specific antisera against chicken follicle-stimulating hormone β-subunit (cFSHβ) and chicken luteinizing hormone β-subunit (cLHβ) based on each putative amino acid sequence.

RT-PCR analysis revealed that cFSHβ mRNA was expressed from embryonic day 7 (E7). Chicken FSHβ mRNA-expressing (-ex) and -immunopositive (-ip) cells started to appear in the ventral part of the caudal lobe in the anterior pituitary gland at E8. Chicken LHβ-ip cells were also first observed there at E8, but cLH mRNA expression was confirmed from E4 by RT-PCR analysis. The distribution of these chicken gonadotropin-ex and -ip cells spread from the ventral part to dorsal part in the caudal lobe around E10 and subsequently expanded to the cephalic lobe from E12 to E20. These cells were morphologically classified into two types (round- and club-shaped cells). It was found that the density of gonadotropin-ip cells in the caudal lobe was always higher than that in the cephalic lobe throughout the period of development. To the best of our knowledge, this is the first report focusing on the differentiation of chicken gonadotropes by assessment of both protein and mRNA of chicken gonadotropin.

Key words: chicken, FSHβ, LHβ, pituitary gland, development

INTRODUCTION

The anterior lobe of the avian pituitary gland consists of well-defined cephalic and caudal lobes, and the hormone-producing cells in these lobes show unique distribution patterns; i.e., corticotropes, lactotropes and thyrotropes are mainly distributed in the cephalic lobe (Hansen *et al.*, 1977; Iturriza *et al.*, 1980; Mikami *et al.*, 1984; Thommes *et al.*, 1983), and somatotropes are found in the caudal lobe (Hansen *et al.,* 1977; Mikami *et al.,* 1984). On the other hand, gonadotropes are localized in both lobes (Ravona *et al.,* 1973; Mikami *et al.,* 1984).

It is well established that the mammalian pituitary gly-coprotein hormone consists of a common α-subunit (αGSU) and hormone-specific β-subunit (Pierce and Parsons, 1981), and these subunits are encoded by the different genes (Quérat, 1994). Since Stockell-Hartree and Cunningham (1969) successfully purified chicken follicle-stimulating hormone (cFSH) and luteinizing hormone (cLH), the avian pituitary gland is also thought to contain two kinds of gonadotropins. Thereafter, cDNA sequences of chicken αGSU (Foster *et al.*, 1992), cLH β-subunit (cLHβ; Noce et al., 1989) and cFSH β-subunit (cFSHβ; Shen *et al.*, 2002) were determined.

Owing to the effects of gonadtropins on cell proliferation in the gonads of chickens during embryogenesis (Pedernera *et al.*, 1999; Méndez-Herrera *et al.*, 1998), it is important to clarify the temporal properties of differentiation of gonadotropes during chicken pituitary gland development. Although several immunohistochemical studies have shown the ontogeny of chicken gonadotropes during embryogenesis,

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there is no agreement concerning the temporal and spatial appearance of gonadotropes (Mikami and Takahashi, 1987; Puebla-Osorio *et al.*, 2002). To clarify this, we immunized rabbits with synthetic peptides to make specific antisera for the cFSHβ and cLHβ, and we performed total analysis of both cFSHβ and cLHβ cells in the developing chicken pituitary gland using RT-PCR, *in situ* hybridization (ISH) and immunohistochemistry (IHC).

MATERIALS AND METHODS

Tissue preparation

Fertile eggs and adult female chickens (aged 26 months) of White Leghorn were purchased from a commercial dealer (Omiya Poultry Laboratory or Tanaka Broiler, Saitama, Japan). The fertile eggs were incubated at 38±1°C in a moisture incubator (P-800, SHOUWA, Saitama, Japan). The age of the embryo at the time of sacrificing (E) indicates the number of days that the egg had remained in the incubator. Embryos at various days to E20 and purchased adult chickens were sacrificed for RT-PCR, ISH, IHC, and Western blotting.

For RT-PCR analysis, the pituitary glands were excised immediately after sacrificing of embryos on various embryonic days or sacrificing adult chickens and were kept in a RNA extracting reagent (ISOGEN, Nippon Gene, Toyama, Japan) at –80°C until analysis. Tissues including the embryonic pituitary gland and adult pituitary were immediately removed and immersed in 4% paraformaldehyde in 1/15 M phosphate buffer (PB), pH 7.4, for 24 hr for ISH or in Bouin-Hollande fixative solution for 24 hr for IHC. For Western blotting analysis, adult pituitaries were homogenized in 10 mM phosphate-buffered saline (PBS), and the homogenate was centrifuged at 8,000 × g for 20 min at 4°C. The supernatant were kept at –80°C until analysis.

Cloning and sequence of chicken FSHβ cDNA

To identify the cFSHβ cDNA sequence, a search of a chicken expressed sequence Tag (EST) database based on the quail FSHβ cDNA sequence was made, and a chicken EST sequence similar to FSHβ cDNA (GenBank: BI392995) was found. This EST sequence was isolated from a pool of RNA of the chicken pituitary gland, hypothalamus and pineal gland, and it was found to have high homology (97.7%) to the quail FSHβ cDNA. To confirm the cFSH cDNA sequence, the following primers were designed to cover as much of the open reading frame of this EST sequence as possible: primer 1, 5'-CAGCACTCTACTAGAATACAGG-3'; and primer 2, 5'-CAAGCTGCCATATCAAGT-3' (Fig.1.) for RT-PCR.

Total RNA was extracted from the adult pituitary gland using ISOGEN according to the manufacturer's instructions. Trace contamination of DNA was removed by DNase digestion. cDNA was synthesized from 4.5 µg total RNA using a Ready-To-Go T-primed First-strand Kit (Amersham Pharmacia, Biotech, Uppsala, Sweden). PCR amplifications were carried out with AmpliTaq Gold polymerase (Roche Molecular Systems Inc., NJ, USA). Initial template denaturation was programmed for 10 min at 95°C. The cycle profile was programmed as follows: 1 min at 94°C (denaturation), 1 min at 56°C (annealing and extension). Forty cycles of the profile were run, and the final extension step was increased to 10 min at 60°C.

PCR fragments were ligated in pGEM-T Easy vector Systems (Promega, Madison, WI, USA), and the nucleotide sequence was determined by using an ALFexpress DNA sequencer (Amersham

Fig. 1. Nucleotide and deduced amino acid sequences of the chicken FSHβ. The underlined nucleotide primers (primer 1 and primer 2) were used for RT-PCR. The nucleotide sequence obtained for chicken FSHβ covered the coding region. The white letters on the black box indicate our synthesized peptide sequence of cFSHβ. The asterisk shows a stop codon.

Pharmacia Biotech, Buckinghamshire, England).

Peptide synthesis

A custom peptide with the sequence CSFSHNGSNQ, corresponding to the C-terminal 10 amino acid residues of the predicted β-subunit of chicken FSH (shown by white letters on black in Fig. 1.), was obtained from a commercial source (Sawady Technology, Inc., Tokyo, Japan). As well as FSH peptides, a custom peptide with the sequence CGAPGGFGGE, corresponding to the C-terminal 10 amino acid residues of the β-subunit of chicken LH, was obtained. These peptide sequences were chosen by prediction of antigenicity using commercial software (MacVector ver .4.5; Eastman Kodak Co., Rochester, NY, USA).

Production of antiserum for chicken FSHβ and antiserum for chicken LHβ

The synthetic peptides of cFSHβ and cLHβ described above were coupled to a carrier protein, keyhole limpet hemocyanin (Sigma Chemical Co., St. Louis, MO, USA), at a peptide/protein ratio of 500:1 (mol: mol) with 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's instructions. Female Japanese White rabbits were immunized with the peptide-protein conjugated with an adjuvant (FREUND'S COMPLETE ADJUVANT, ICN Pharmaceuticals, Inc, Ohio, USA) intradermally into multiple sites on the back every week, and then whole blood from each rabbit was collected after 2 months, and anti-cFSHβ (cF31F) and -cLHβ (cL32F) antisera were obtained.

Western blotting

The protein extracts of the pituitary gland (30 μg) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) based on the method of Laemmli as previously described (Ogawa et al., 1997). Briefly, the discontinuous slab gels consisted of 4% stacking gel and 12.5% running gel. The protein sample was dissolved by boiling for 1 min in a mixture of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.025% bromophenol blue in 62.5 mM Tris-HCl, pH 6.8. The proteins in the gel were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (PVDF Protein Sequencing Membrane, Bio-Rad Laboratories) for 50 min at 120 mA in a semi-dry transfer apparatus (Model RB-310, Biocraft Co., Japan). The transfered membrane was cut to two sheets, one of the membrane was stained with 0.1% Amido Black 10B (Bio-Rad Laboratories). For immunoblotting, the other membrane was blocked with 15% skim milk (Yukijirushi, Inc., Tokyo, Japan) in PBS overnight and then cut into each lane. After washing with PBS, the each membrane were incubated overnight with anti-cFSHβ and -cLHβ antisera (diluted 1:16,000 or 1:200,000 with 10 mM PBS containing 1% normal horse serum and 0.4% Triton X-100 (TNBS), respectively) or pre-immune rabbit serum (diluted 1:16,000). After incubation, the membranes were washed extensively with PBS and then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., CA, USA) diluted 1:300 in TNBS for 2 hr. After washing with PBS, the membranes were incubated with avidin-biotinylated HRP-complex (ABC; Vector Laboratories, Inc., CA, USA) for 30 min, washed with PBS, and then placed inside a plastic sheet. Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Inc. Boston, MA, USA) was added. Finally, the membranes were exposed X-ray film (Eastman Kodak Company, Rochester, NY, USA). All procedures were performed at room temperature.

Developmental analysis of chicken FSHβ mRNA expression by *in situ* hybridization

The fixed tissue blocks were dehydrated with an ascending ethanol series, immersed in xylene, and then embedded in PARAPLAST (Oxford Labware, MO, USA), and sagittal sections of 7 μm in thickness were made. *In situ* hybridization was performed basically according to the previously reported procedure (Wada et al., 2003). Briefly, the sections was hybridized by Digoxigenin (DIG)-labeled anti-sense and sense cRNA probes (1 ng/μl), synthesized from the above-mentioned 441 bp cFSHβ fragment using a labeling kit (DIG RNA Labeling Kit, Roche Diagnostics GmbH, Mannheim, Germany), for 16 hr at 42°C in a humidity chamber. After washing the probes, the sections were incubated at 4°C overnight with a chicken pituitary powder-absorbed alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics GmbH) diluted 1:1000 in 100 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 0.01% tween 20. After washing, visible signals were detected with a chromogen solution (337 μg/ml 4-Nitro blue tetrazolium chloride (NBT), 175 μg/ml 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) in 100 mM Tris-HCl buffer (pH 9.5) containing 100 mM NaCl and 50 mM MgCl$_2$).

Immunohistochemistry

After fixation, the tissue blocks were decalcified using the Plank-Rychlo method. The tissue blocks were saturated in a mixture of 7% aluminum chloride, 1 N hydrochloric acid and 1.3 N formic acid for 24 hr and then neutralized with 5% sodium sulfate for 5 hr. After neutralization, the tissue blocks were dehydrated with an ascending ethanol series, methyl benzoate and benzene, then embedded in PARAPLAST. Immunohistochemical staining was performed basically according to the previously reported procedure (Sakata et al., 2002). Briefly, sections (sagittal sections of 6 μm in thickness) were treated with 0.5% sodium metaperiodate for 15 min. After blocking with TNBS, they were incubated overnight with anti-cFSHβ antiserum diluted 1:16,000, anti-cLHβ antiserum diluted 1:10,000 or pre-immune rabbit serum. The sections were washed extensively with PBS and then incubated with biotinylated goat anti-rabbit IgG diluted 1:300 with TNBS for 2 hr. After washing with PBS, the sections were incubated with avidin-biotinylated HRP-complex for 30 min and then reacted with 0.02% 3,3'-diaminobenzidine-tetrachloride and 0.006% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6).

To examine the specificity of immunoreactivity, an absorption test was performed as follows. The antisera used in this study were incubated overnight at room temperature with 1 μg/ml of each synthetic peptide, and mixtures were centrifuged at $10,000 \times g$ for 20 min at 4°C. The supernatant was used as the primary antiserum for the absorption test. In addition, for double staining with FSH-ex cells, fluorescent immunohistochemistry was performed using goat anti-rabbit IgG conjugated with fluorescein-5-isothiocyanate (ICN Pharmaceuticals, Inc., Aurora, Ohio, USA)

Morphometric analysis of chicken FSHβ- and LHβ-producing cells

The densities of cFSHβ- and cLHβ-ip cells in the chicken pituitary gland were estimated. After taking digital photographs under a light microscope (BX60, OLYMPUS, Tokyo, Japan) with a digital camera (COOLPIX950, Nikon, Japan), the numbers of cFSHβ- and cLHβ-ip cells in the cephalic and caudal lobes were counted, and the area of each lobe was measured using a computerized image analysis program, Scion Image (Scion Corporation, USA). The density of cFSHβ- or cLHβ-ip cells was calculated as the number of immunopositive cells per unit area (mm^2). All of the data are expressed as means ± SEM.

Developmental analysis of chicken FSHβ and LHβ mRNA expression by RT-PCR

For RT-PCR, total RNA was extracted from the embryonic or adult pituitary gland, and DNase treatment and reverse transcription were performed as described above. The following primers were used for cLHβ and chicken β-actin: cLHβ sense primer, 5'-GTATG-GCTGTGACCACCACGG-3'; cLHβ antisense primer, 5'-CTGCAC-GGTGCAGTCGGAG-3', chicken β-actin sense primer, 5'-TGGCA-
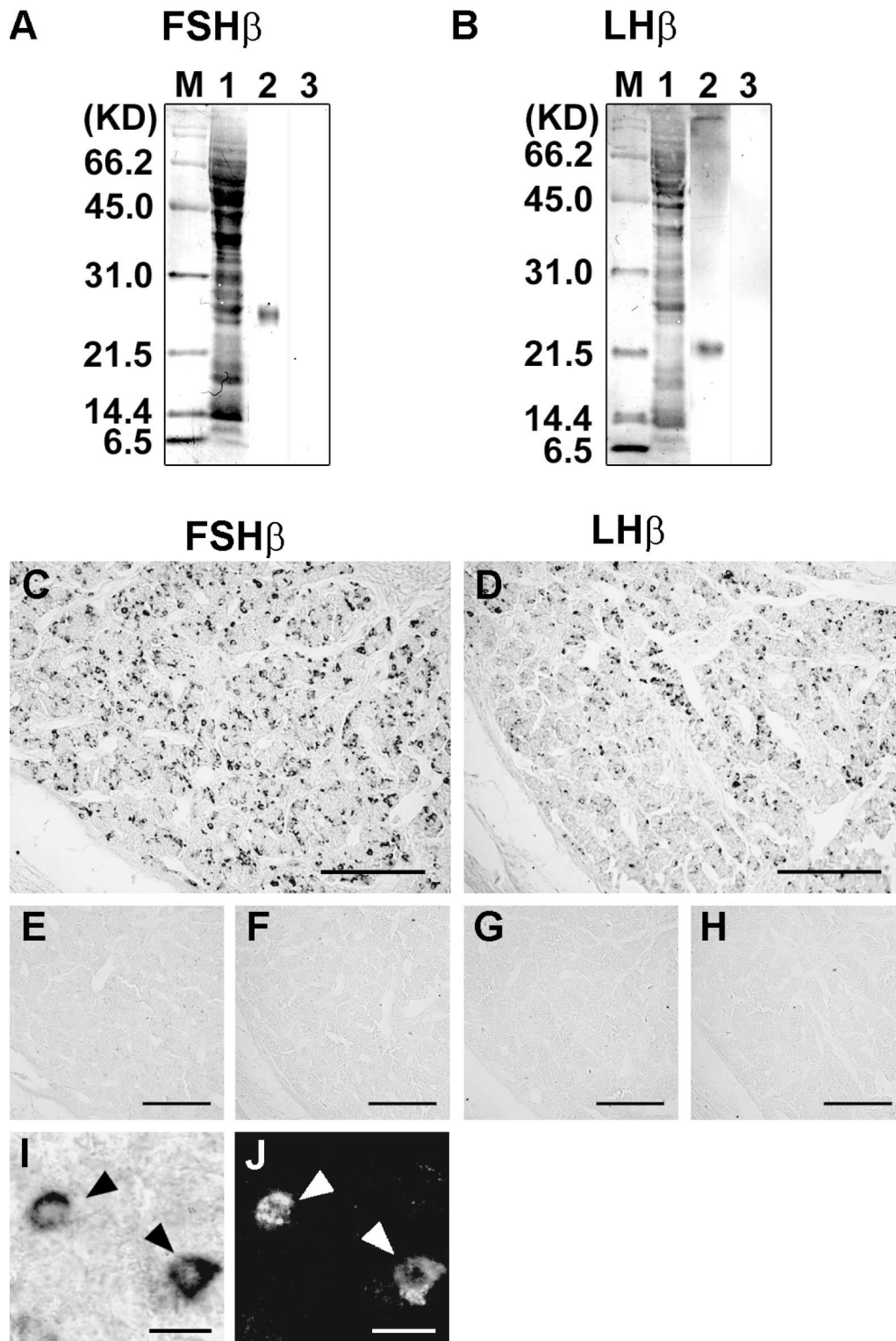
Fig. 2. Analysis of specificity for rabbit antisera against chicken FSHβ and LHβ. A and B show the results of analysis by western blotting, and C-J show the results of histological analysis. Proteins extracted from whole adult chicken pituitaries were separated by SDS-PAGE. Lane M (molecular weight marker) and lane 1 (extracted protein) were stained with amide black. Lane 2 was immunostained with anti-cFSHβ (A) or -cLHβ (B) serum, and lane 3 was the result of immunostaining with pre-immune rabbit serum. Immunoreactivities for cFSHβ (C) and cLHβ (D) were observed at E20 in the chicken anterior pituitary gland. Anti-cFSHβ and -cLHβ antisera absorbed with each synthetic cFSHβ and cLHβ peptide (1 μg/ml) showed complete disappearance of immunoreactivity (E, F). The use of each pre-immune serum also showed no immunoreactivity (G, H). In the double-staining, cFSHβ immunoreactivities were observed in cFSHβ-ex cells (I, ISH; J, IHC). Bars: 100 μm (C-H), 10 μm (I, J).
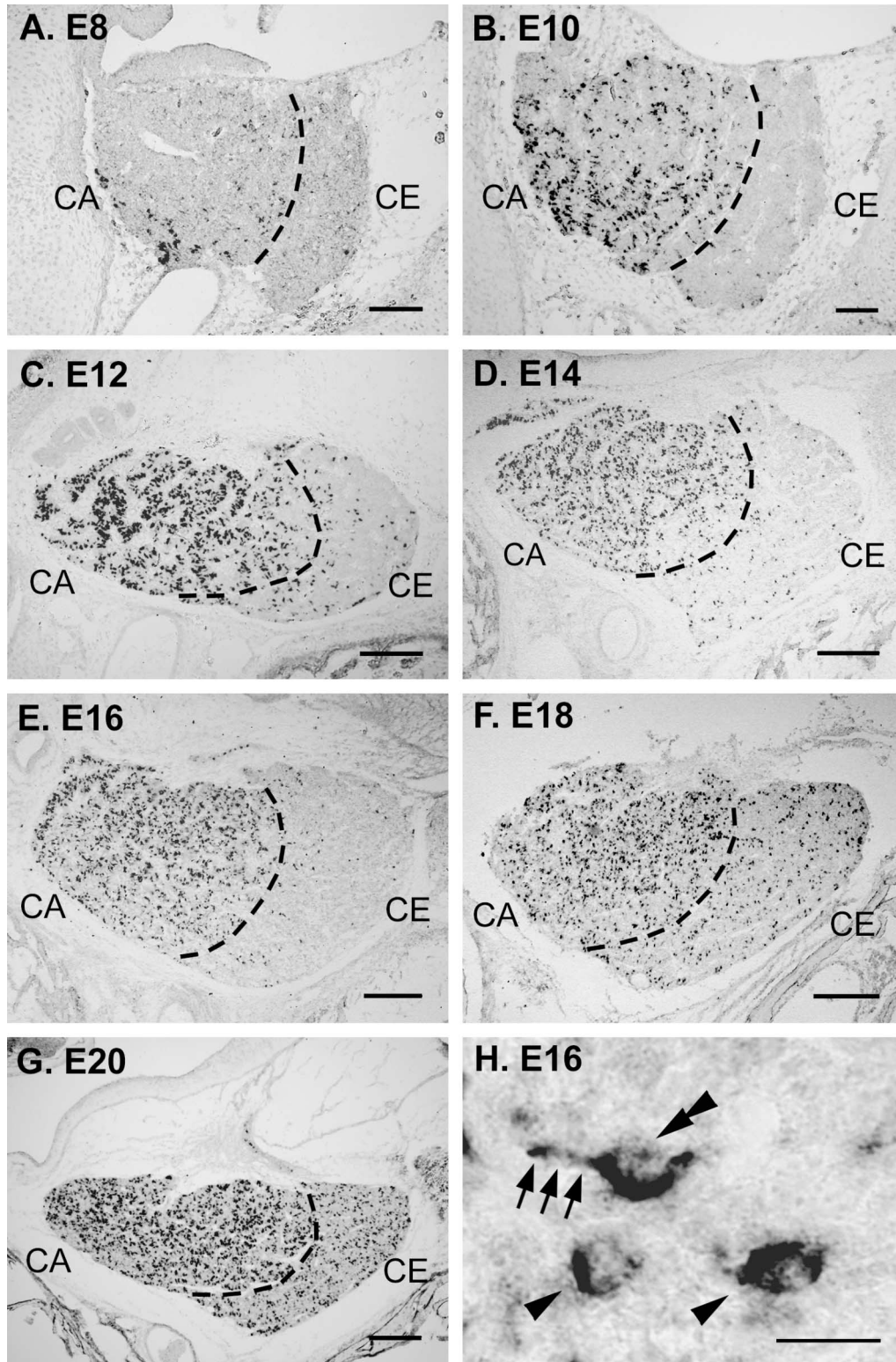
Fig. 3. Microphotographs of FSHβ-ex cells detected by ISH in the chicken pituitary gland during development. The chicken pituitary gland at E8 (A), E10 (B), E12 (C), E14 (D), E16 (E) E18 (F), E20 (G), and higher magnification at E16 (H). Chicken FSHβ-ex cells first appeared at E8 (A), and the number of cFSHβ-ex cells increased markedly in the caudal lobe (B). Chicken FSHβ-ex cells spread in a scattered pattern from the caudal lobe to cephalic lobe with progress of development (C-G). Two types of cFSHβ-ex cells, round-shaped cells (arrowheads) and club-shaped cells (double arrowhead) that have long cytoplasmic processes (arrows), were observed throughout the period of embryonic development (H). CE, cephalic lobe of the chicken anterior pituitary gland; CA, caudal lobe; dashed line, boundary of the CA and CE. Bars: 100 μm (A, B), 200 μm (C-G) and 10 μm (H).

CCACACTTTCTACAATGAG-3'; chicken β-actin antisense primer, 5'- TGTCATCTTCTCTCTGTTGGCTTTG -3'. PCR amplifications were carried out with AmpliTaq Gold polymerase. Initial template denaturation was programmed for 10 min at 95°C. The cycle profile was programmed as follows: 1 min at 94°C (denaturation), 1 min at 56°C for FSHβ and at 64°C for LHβ (annealing and extension). Forty cycles of the profile were run, and the final extension step was increased to 10 min at 60°C.

1. Chicken FSHβ-immunopositive cells
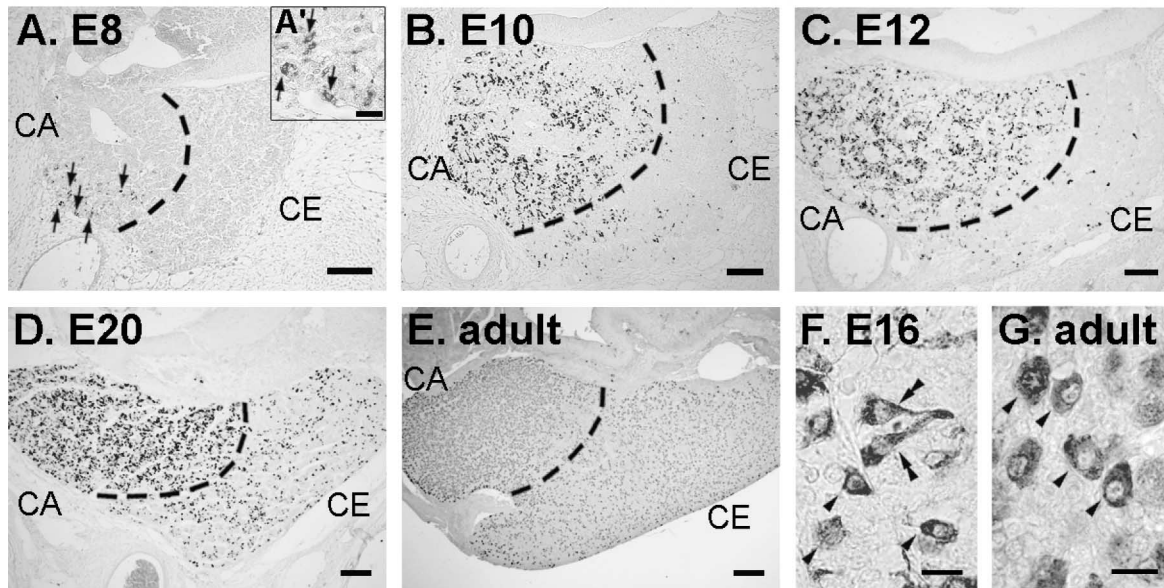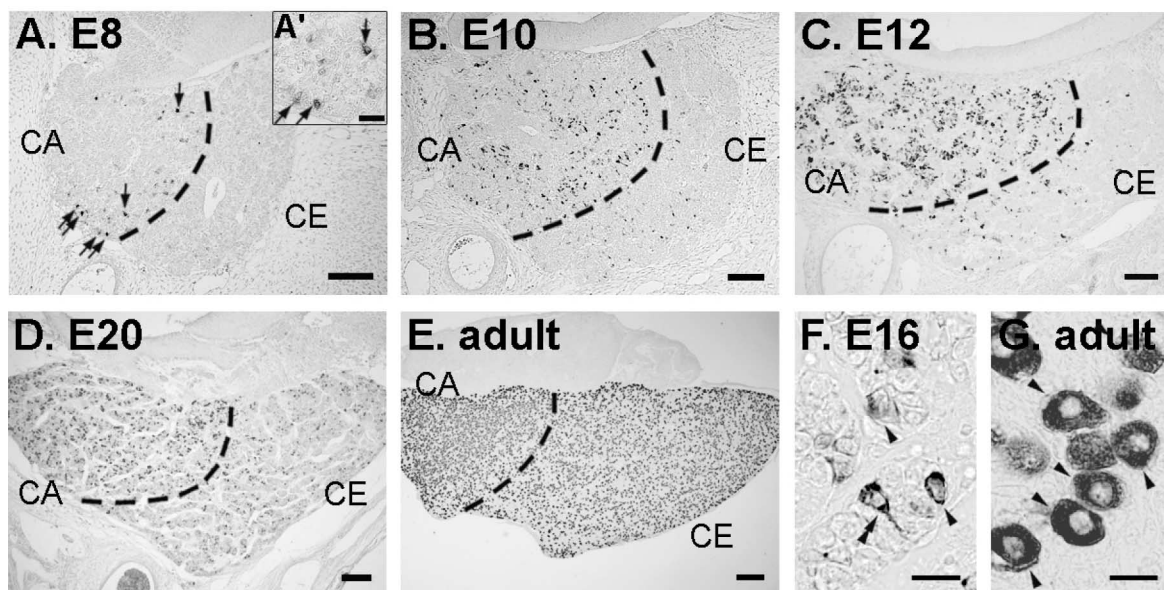

2. Chicken LHβ-immunopositive cells

Fig. 4. Microphotographs of chicken FSHβ-ip cells (1A -G) and LHβ-ip cells (2A-G) detected by IHC in the pituitary gland during development. Immunopositive cells were found at E8 (1A, 2A), E10 (1B, 2B), E12 (1C, 2C), E20 (1D, 2D) and adulthood (1E, 2E). Higher magnification microphotographs at E16 (1F, 2F) and adulthood (1G, 2G) are also shown. 1 A' and 2A' are higher magnifications of 1A and 2A, respectively. These cells first appeared at E8 (1A, 2A). As the numbers of these cells increased, the cells spread from the caudal lobe to cephalic lobe with the progress of development (1B-E, 2B-E). These cells included both round-shaped cells (arrowheads) and club-shaped cells (double arrowheads) in the embryonic pituitary gland (1F, 2F), but few club-shaped cells were found in the adult pituitary gland (1G, 2G). CE, cephalic lobe of the chicken anterior pituitary gland; CA, caudal lobe; dashed line, boundary of the CA and CE. Bars: 100 μm (1A-D, 2A-D), 200 μm (1E, 2E), 10 μm (1A', F, G; 2A', F, G).

RESULTS

Cloning and sequencing of chicken FSH β cDNA

PCR products of the expected size of 441 bp for cFSHβ were amplified from the chicken pituitary gland cDNA using primer 1 and primer 2 (Fig. 1.), which were designed on the basis of the chicken EST sequence obtained from the database. The sequence of the obtained PCR products was identical to the EST sequence and to the previously reported cFSHβ cDNA sequence (Shen *et al.*, 2002). This sequence exhibited high homology (97.7%) to the quail FSHβ cDNA.

Specificity for newly raised antisera

Both antisera were confirmed by western blot analysis to recognize each single molecule in the extracts from pituitary glands, and single bands corresponded to the predicted sizes for cFSHβ and cLHβ, respectively (Fig. 2. A, B-lane 2). Immunoreactivities of FSHβ and LHβ completely disappeared when synthetic peptide-absorbed antiserum was used (Fig. 2. E, F). Moreover, cFSHβ-ip cells corresponded well to the cFSHβ-ex cells (Fig. 2. I, J).

Distribution of chicken FSHβ mRNA-expressing cells

No cFSHβ mRNA-expressing cells (cFSHβ-ex cells) were found until E7 (data not shown), and a few cFSHβ-ex cells first appeared in the ventral part of the caudal lobe in the anterior pituitary gland at E8 (Fig. 3. A). The number of cFSHβ-ex cells increased markedly in the ventral part of the caudal lobe, and the cells had spread from the ventral part to dorsal part in the caudal lobe by E10 (Fig. 3. B) and then extended in a scattered pattern from the caudal to cephalic lobes (Fig. 3. C–G). At E20, many cFSHβ-ex cells were observed in the cephalic lobe, though, the density of these cells was much higher in the caudal lobe than in the cephalic lobe (Fig. 3. G). Chicken FSHβ-ex cells can be classified into two types: round-shaped cells (Fig. 3. H, arrowheads) and club-shaped cells (Fig. 3. H, double arrowhead). Many cells of both types were observed at all embryonic stages.

Distribution of chicken FSHβ-and LHβ-immunopositive cells

Chicken FSHβ-immunopositive cells (cFSHβ-ip cells) were first observed in the ventral portion of the caudal lobe in the anterior pituitary gland at E8 (Fig. 4. 1A), and the number of these cells had markedly increased at E10 (Fig. 4. 1B). On the other hand, a gradual increase in the number of cFSHβ-ip cells in the cephalic lobe was observed from E12 to E20 (Fig. 4. 1C, 1D, Fig. 5. A). In addition, the density of cFSHβ-ip cells was higher in the caudal lobe than in the cephalic lobe both at E20 and at the adult stage (Fig. 4. 1D, 1E, Fig. 5. A). Chicken FSHβ-ip cells were also classified into two types, i.e., round-shaped cells (Fig. 4. 1F, arrowheads) and club-shaped cells (Fig. 4. 1F, double arrowheads), but, few club-shaped cells were found in the adult pituitary gland (Fig. 4. 1G).

Chicken LHβ-immunopositive cells (cLHβ-ip cells) were first observed mainly in the ventral part of the caudal lobe and sparsely in the cephalic lobe of the anterior pituitary gland at E8 (Fig. 4. 2A, Fig. 5. B), and the number of these cells had markedly increased in the caudal lobe at E10 (Fig. 4. 2B). On the other hand, the number of cLHβ-ip cells in the cephalic lobe gradually increased from E12 to E20 (Fig. 4.
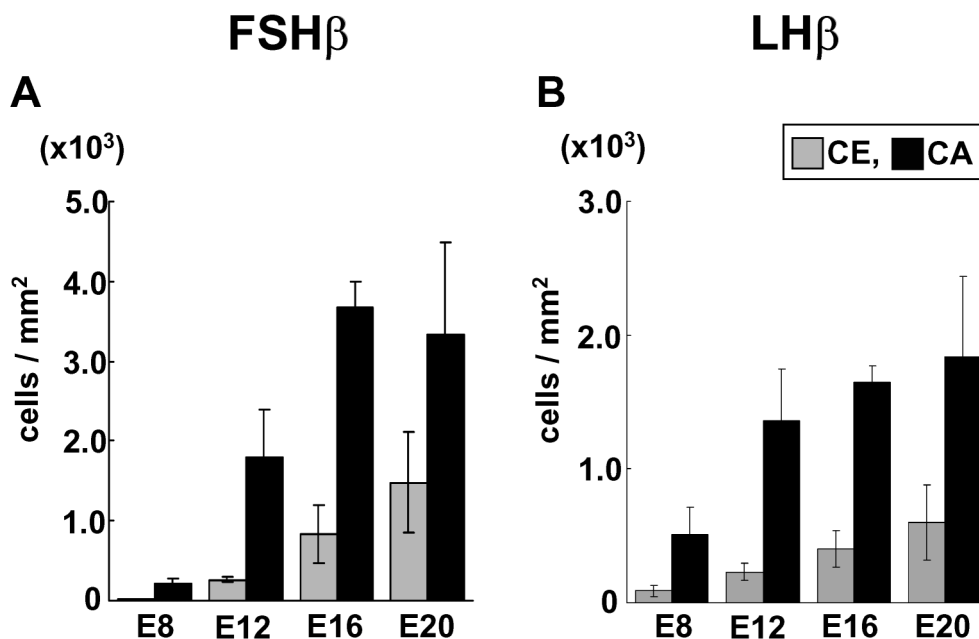

Fig. 5. Bar graphs showing the densities of chicken FSHβ- and LHβ-ip cells (cells/mm^2) in the pituitary gland during development. (A) Chicken FSHβ-ip cells. (B) Chicken LHβ-ip cells. The numbers of cFSHβ- and cLHβ-ip cells increased from E8 to E16 in the caudal lobe (CA) and increased gradually from E12 to E20 in the cephalic lobe (CE), and more cFSHβ- and cLHβ-ip cells were found in the caudal lobe than in the cephalic lobe. n=3/stage. The column and vertical bar indicate the mean and standard error of the group.
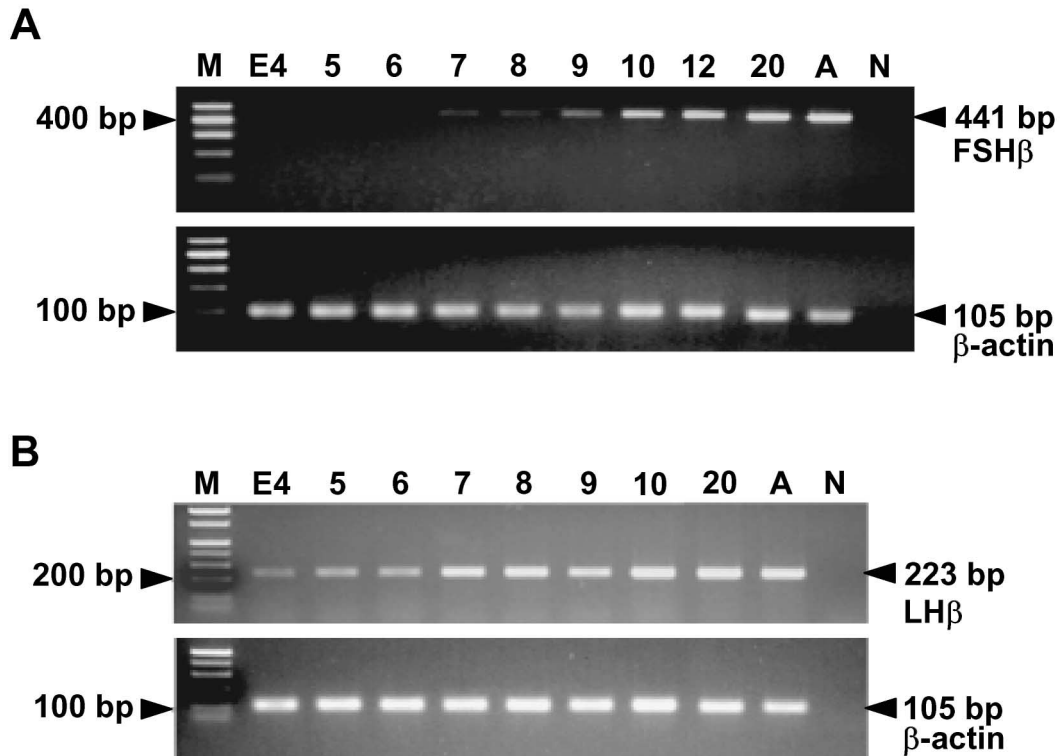
A

B

Fig. 6. Expression of chicken FSHβ and LHβ mRNA in the pituitary gland determined by RT-PCR analysis during development. Agarose gel (2%) electrophoresis of RT-PCR products showed 441 bp fragments for chicken FSHβ (A), 223 bp fragments for chicken LHβ (B) and 105 bp fragments for chicken β-actin. FSHβ mRNA in the chicken pituitary gland was first detected at embryonic day 7 (E7), and then the expression continued until the adult stage. On the other hand, LHβ mRNA in the chicken pituitary gland was detected from E4. β-actin was used as an internal control. M, 100 bp DNA molecular marker. Numbers are embryonic days. A, adult; N, negative control.

2C, 2D, Fig. 5. B). In addition, the density of cLHβ-ip cells was higher in the caudal lobe than in the cephalic lobe both at E20 and at the adult stage (Fig. 4. 2D, 2E, Fig. 5. B). Chicken LHβ-ip cells were also classified into two types, round-shaped cells (Fig. 4. 2F, arrowheads) and club-shaped cells (Fig. 4. 2F, double arrowhead), but few club-shaped cells were found in the adult (Fig. 4. 2G).

Chicken FSHβ and LHβ mRNA expression during pituitary development

Specificities of the PCR products, 411 bp fragments for cFSHβ and 223 bp fragments for cLHβ (Fig. 6. A, B), were confirmed by cDNA sequencing. By RT-PCR analysis, cFSHβ mRNA and cLHβ mRNA in the chicken pituitary gland were first detected at E7 and E4, respectively.

DISCUSSION

Although cDNA sequences of avian LHβ (for chicken LHβ: Noce *et al.*, 1989; quail LHβ: Ando and Ishii, 1994; turkey LHβ: You *et al.*, 1995) and glycoprotein hormone α-subunit (αGSU) (for turkey αGSU: Foster, 1991; chicken αGSU: Foster *et al.*, 1992; quail αGSU: Ando and Ishii, 1994) have been determined in many species, the avian FSHβ cDNA sequence had been cloned only in the quail (Kikuchi *et al.*, 1998). In the present study, we determined the open reading

frame of the cFSHβ cDNA precursor molecule (accession number: AB077362), and this enabled us to detect the expression of cFSHβ mRNA by RT-PCR and ISH. During our study, Shen *et al.* (2002) reported the cloning of the full-length cDNA encoding the FSHβ precursor molecule from the chicken pituitary gland, and our determined cFSHβ cDNA sequence is completely consistent with the sequence that they reported.

In this study, we raised antisera against the β-subunits of cFSH and cLH based on putative amino acid sequences. Each antiserum recognized a single molecule in the extracts from pituitaries, and immunoreactivities observed by IHC completely disappeared in absorption tests. Moreover, immunoreactivities for cFSHβ-antiserum were found in the same cells that express FSHβ mRNA. These results clearly indicate that our newly raised cFSHβ- and cLHβ-antisera specifically recognize each β-subunit of chicken gonadotropins.

There are some discrepancies in the reported results of immunohistochemical studies on differentiation of chicken gonadotropes. For example, Mikami and Takahashi (1987) demonstrated that cLH-ip cells first appeared at E4 and that cFSH-ip cells appeared at E8, whereas Puebla-Osorio *et al.* (2002) reported that cLH-ip cells were first observed at E9 and that cFSH-ip cells were observed at E13. Moreover, the reported distributions and positions of appearance of gonad-

otropes are different. The former group reported that cLH-ip cells first appeared in the epithelium of the posterior process of Rathke's pouch, the site of the prospective cephalic lobe of the pituitary gland, and that cFSH-ip cells first appeared in the caudal lobe of the anterior pituitary gland, and then both cLH-ip and cFSH-ip cells become distributed throughout both lobes, whereas the latter group reported that cLH and cFSH-ip cells first appeared in both lobes. These discrepancies may be due to different antibodies or methods used in these studies. In the present study, using IHC, we clearly demonstrated that chicken gonadotropes appeared in the ventral region of the caudal lobe at E8, spread to the dorsal region of the caudal lobe by E10, and then expanded gradually toward the cephalic lobe of the chicken anterior pituitary gland from E12 to E20. Moreover, morphometric analysis demonstrated that, even after the cells had expanded throughout both lobes of the pituitary gland, the densities of LHβ- and FSHβ-producing gonadotropes were higher in the caudal than in the cephalic lobe. To our knowledge, this is the first detailed report on the distribution of chicken gonadotropes, focused on the differences in densities in the cephalic and caudal lobes.

Our RT-PCR and ISH analyses revealed that cFSHβ-mRNA started to be expressed at E7 and E8, respectively, and these results are almost in agreement with the results obtained from IHC during embryogenesis, indicating that the differentiation of chicken gonadotropes expressing FSHβ begins around E7. On the other hand, although cLHβ-mRNA expression was confirmed from E4 by our RT-PCR analysis, cLHβ-ip gonadotropes were not observed until E7. These results suggest that the cLHβ mRNA expressed at a low level at E4, and then a detectable amount of cLHβ protein started to be expressed at E8.

As well as cFSHβ, we also tried to detect cLHβ-ex cells using in situ hybridization, but we were not able to obtain good staining. The GC-rich structure of cLHβ mRNA may be one of the causes of this failure.

In mice, αGSU-ex cells first appeared at E11.5 in the anterior region of Rathkes pouch (Burrows *et al.*, 1996), and then gonadotropes differentiated at E16.5 in the anterior pituitary gland (Burrows *et al.*, 1999), indicating that expression of αGSU-ex cells precedes that of gonadotropes. Moreover, transgene ablation of αGSU cells terminated the expression of gonadotropes in the mouse pituitary gland, suggesting that αGSU cells are precursor cells of gonadotropes (Burrows *et al.*, 1996). On the other hand, in the chicken, Kameda *et al.* (2000) demonstrated that αGSU-ex cells appeared in the epithelium of the posterior process of Rathke's pouch at E3.5. We also found that αGSU-ex cells appeared in the prospective cephalic lobe at E4 and then spread into the ventral region of caudal lobe at E7 (manuscript in preparation). Interestingly, as shown in this study, chicken gonadotropes appeared at E8 in the ventral region of the caudal lobe, where αGSU-ex cells were found just one day before. These results strongly suggest that chicken gonadotropes also differentiated from αGSU cells.

In conclusion, our total analysis of FSHβ and LHβ cells during chicken embryogenesis has revealed the precise temporal and spatial properties of differentiation of gonadotropes. These results may be useful for future studies on differentiation of gonadotropes in the chicken pituitary gland.

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