

Expression of Estrogen Receptor α mRNA in Theca and Granulosa Layers of the Ovary in Relation to Follicular Growth in Quail*

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Changes in expression of estrogen receptor alpha (ER α) mRNA were studied in special reference to follicular growth of the ovarian follicles in laying quail. Levels of mRNA were determined by RT-PCR in the ovarian stroma, each class of the ovarian follicles, oviductal parts and in the liver. Low levels of ER α mRNA were detected in stroma, the small white follicles, large white follicles and small yellow follicles and in the theca layer of the three largest preovulatory follicles. Although the level in the granulosa layer of the F3 was also low, the level significantly increased in F2 and F1. Relatively higher levels were found in the liver and oviduct, and that in the magnum was the highest among all tissues examined. The level in the F1 granulosa layer was comparable to that in the liver which actively synthesises egg yolk proteins for the sake of estrogen and ER. The results of the present study demonstrate that (1) ER α mRNA is present in each compartment of the reproductive tissue in quail, (2) the marked expression of ER α mRNA in the granulosa layer of the largest follicle may indicate the involvement of estrogens in the biosynthesis of inhibin/activin, progesterone and yolk perivitelline layer protein, (3) very high expression of ER α in the oviductal tissues may be related to the role of estrogens in cell proliferation and protein synthesis in the oviduct.

Key words: ER α , reproductive system, RT-PCR, quail.

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The synthesis of estrogens begins in the chicken gonads during the 3rd-4th day of incubation (WOODS & ERTON 1978) and progressively increases at sexual maturity (WILIAMS & HARVEY 1986; SECHMAN *et al.* 2000). In adult birds, estrogens are produced mainly by the thecal cells of the ovarian follicles (HUANG *et al.* 1979; BAHR *et al.* 1983; ETCHES & DUKE 1984; KATO *et al.* 1995). Small follicles and ovarian stroma with numerous cortical follicles produce most of the ovarian estrogens (SENIOR & FURR 1975; ARMSTRONG 1984).

Estrogens control reproductive functions in vertebrates through a variety of processes such as gonadal differentiation and development, reproductive behaviour, control of GnRH secretion, cell proliferation in the ovary and the oviduct. Moreover, in oviparous species estrogens induce the syn-

thesis of a group of egg yolk proteins including very-low-density apolipoprotein II, vitellogenin in the liver and proteins in the oviduct (SAKIMURA *et al.* 2001; ICHIKAWA *et al.* 2003a).

The biological, multiple effects of estrogens are mediated *via* two forms of estrogen receptors, alpha (ER α) and beta (ER β) (KRUST *et al.* 1986; SHUGHRUE *et al.* 1997; LAKAYE *et al.* 1998; GRIFFIN *et al.* 1998; BERNARD *et al.* 1999; FOIDART *et al.* 1999) which are ligand-inducible transcription factors and members of a nuclear receptor superfamily (EVANS 1988; BEATO 1989). Structurally, these receptors contain a DNA binding domain, nuclear localisation signals, a ligand binding domain, and several transcription activation domains (GRONEMEYER 1991). Transcriptional regulation of estrogen target gene expression

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is the mechanism by which estrogens induce biological activity.

In birds, ER cDNA of subtype α and β has been identified in chicken (KRUST *et al.* 1987) and quail (FOIDART *et al.* 1999; ICHIKAWA *et al.* 2003b). Moreover, two variants of chicken ER α protein (cER α 66 and cER α 61) have been demonstrated (GRIFFIN *et al.* 1998, 1999) and its distribution in the neuroendocrine system (GRIFFIN *et al.* 2001) and the liver, ovary and oviduct has been shown (GRIFFIN *et al.* 1999). Furthermore, the previous study demonstrated that the ER β mRNA is widely expressed at low levels throughout tissues in adult quail, but ER α is expressed mainly in the reproductive organs (ICHIKAWA *et al.* 2003b). However, there has been no study on changes in ER α mRNA expression in the hierarchical follicles of the ovary. Accordingly, the present study was conducted to reveal the changes in expression of ER α mRNA in the ovarian and oviductal tissues in special reference to follicular growth of the ovarian follicles in laying quail.

Material and Methods

Animals

A commercial strain of laying Japanese quail (Tokaiyuki Company, Toyohashi, Japan; n=3), 12 weeks old, were caged individually under a photoperiod of 14 h light/10 h darkness (light on at 05:00) with free access to food and water. They regularly laid eggs in sequences of more than 5 eggs. The time of oviposition was recorded for each quail as previously reported (HRABIA *et al.* 2003a, b). The birds were killed by decapitation 0.5 h after ovulation, and the following tissues were collected: liver, ovarian stroma with follicles <1 mm, small and large white follicles (SWF, 1-2 mm diameter, and LWF, 2-4 mm diameter), yellow follicles (small yellow follicles as SYF, 4-6 mm diameter; 3 largest preovulatory follicles: F3, F2 and F1, the third, second and first largest, respectively) and oviductal parts (infundibulum, magnum, isthmus and shell gland). From preovulatory follicles the granulosa and theca layers were separated according to the procedure of GILBERT *et al.* (1977). Tissues were quickly frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction and RT-PCR

The total RNA was extracted using TRIzolTM reagent (Invitrogen, Carlsbad, CA, USA) as described previously (SAKIMURA *et al.* 2001). Two and a half μg of total RNA of each tissue was dena-

tured at 70°C for 10 min with 250 ng Oligo (dT)₁₂₋₁₈ primers (Invitrogen) and reverse-transcribed with 0.5 μl of PowerScriptTM Reverse Transcriptase (CLONTECH Laboratories, Palo Alto, CA, USA) in a 10 μl mixture. One microliter of each RT product was used for PCR amplification. For ER α the sequences of primers were 5'-GTG CCT TAA GTC CAT CAT CCT-3' (1522-1542) and 5'-GCG TCC AGC ATC TCC AGT AAG-3' (1801-1821) (KRUST *et al.*, 1986). The primers for the S17 ribosomal protein (internal control) were 5'-GGC GCG GGT GAT CAT CGA GAA-3' (73-93) and 5'-GAG AGC GCC TCG CGG CGT TT-3' (423-442) (GeneBank, AY215074). For PCR, a mixture containing buffer (20 mM Tris-HCl, pH 8.0; 100 mM KCl; 2 mM MgCl₂), 0.2 mM each dNTP, 0.5 μM sense and antisense primers for ER α and S17, and 0.25 unit of Taq DNA polymerase (Takara Bio inc., Shiga, Japan) in a total volume of 10 μl was used. After the initial denaturation for 2 min at 94°C , the amplification conditions for ER α were as follow: 25 cycles ($97^{\circ}\text{C}/10$ sec; $55^{\circ}\text{C}/30$ sec; $72^{\circ}\text{C}/1$ min), in the case of ribosomal protein S17: 25 cycles ($96^{\circ}\text{C}/10$ sec; $53^{\circ}\text{C}/1$ min; $72^{\circ}\text{C}/1$ min). Amplifications were completed with an additional extension at 72°C for 2 min. All PCR reactions were carried out on a Perkin-Elmer 9700 thermocycler. PCR products were electrophoresed on 1.5% agarose gel in 0.5x TBE buffer and bands were visualised by ethidium bromide staining. The bands were analysed using NIH Image software and the level of ER α gene expression in tissues was measured in respect to ribosomal protein S17 mRNA expression.

Statistical analysis

For statistical evaluation of the results a one-way ANOVA followed by Duncan's multiple range test was used. The significance of differences was considered at the level of $P < 0.05$. Values are expressed as the mean \pm SEM from 5 determinations. Analysis of data was done using the computer program SigmaStat 2,03 (SPSS Science Software Gm, Germany).

Results

RT-PCR analysis was adapted to detect ER α gene expression in the quail reproductive system. The presence of ER α mRNA was found in the liver (used as a positive control), as well as all other ovarian and oviductal tissues examined (Fig. 1). As an amplification product there was a single band with the expected size of 300 bp for ER α cDNA and 370 bp for ribosomal protein S17 cDNA, respectively (Fig. 1).

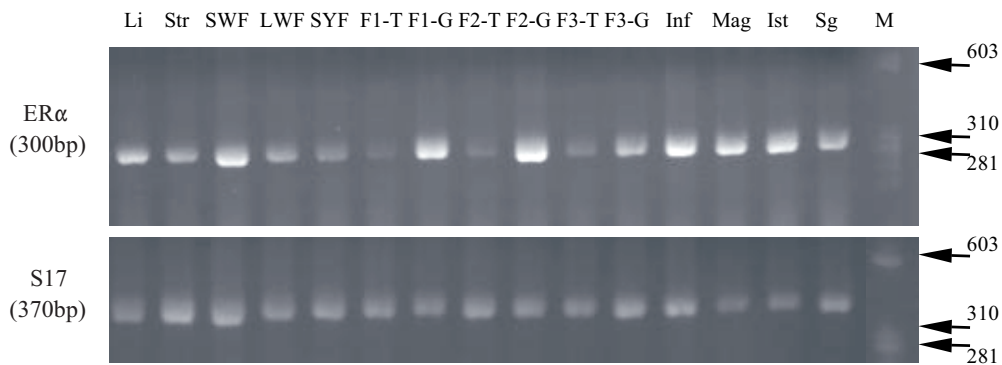


Fig. 1. RT-PCR analysis of estrogen receptor alpha (ER α) gene expression in the liver and the reproductive tissues of laying quail. M – molecular marker; Li – liver (positive control); Str – stroma; SWF – small white follicles; LWF – large white follicles; SYF – small yellow follicles; F3, F2, F1 – large preovulatory follicles (T – theca; G – granulosa); Inf – infundibulum; Mag – magnum; Ist – isthmus; Sg – shell gland.

The relative levels of ER α mRNA expression are shown in Fig. 2. Low levels of ER α mRNA were detected in the ovarian stroma, small white follicles, large white follicles and small yellow follicles and in the theca layer of the three largest preovulatory follicles. Although the ER α mRNA level in the granulosa layer of the F3 follicle was also low, the level significantly increased in F2 and F1. Relatively higher levels were found in the liver and oviduct, and that in the magnum was the highest among all tissues examined. The level in

the F1 granulosa layer was comparable to that in the liver, which actively synthesizes egg yolk proteins for the sake of estrogen and ER.

Discussion

To our knowledge, this is the first report demonstrating the unique distribution of ER α mRNA in the ovary and the oviduct in laying quail. Very high expression of ER α mRNA was found in the

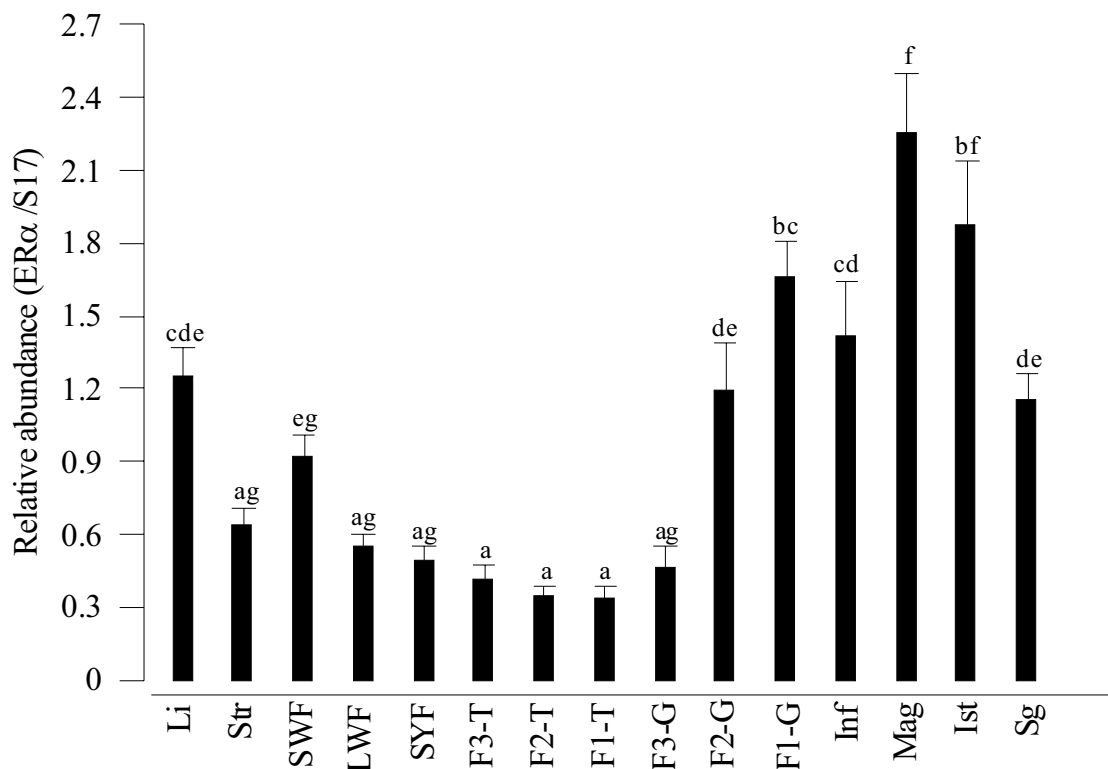


Fig. 2. Comparison of estrogen receptor α mRNA abundance in liver and reproductive tissues of laying quail measured by RT-PCR. Abbreviations are the same as those in the legend to Fig. 1. Each value represents the mean \pm SEM from 5 determinations that were measured as relative density of RT-PCR products compared to ribosomal protein S17. Means with different letters are significantly different from each other ($P < 0.05$).

oviduct, high in the liver and granulosa layer of F2 and F1 follicles and relatively low in other tissues of the ovary.

It is well known that the oviduct is a target tissue for estrogens in which they are key hormones for development and differentiation of the oviduct in female birds. Proliferation of the luminal epithelium and differentiation of ciliated and glandular cells are estrogen controlled processes (BERG *et al.* 2001, and references therein). Moreover, synthesis of the major egg white proteins is modulated by estrogens (PALMITER 1972, and references therein; GAUB *et al.* 1990). Hence the highest level of ER α mRNA expression found in this study is in the magnum, the segments of the oviduct where the synthesis of egg white protein is at the highest level. When compared to the magnum, the function of the isthmus for protein synthesis is not known. However, very high expression of ER α observed in the isthmus is worth attention. This result may be associated with the involvement of estrogens in the production of proteins for the formation of keratinous eggshell membranes.

A more important finding of the present study was the observation of very high expression of ER α mRNA in the granulosa layer of the largest preovulatory follicle and that this expression increased during development from the F3 to F1 follicle. One of the functions of estrogens in the granulosa layer, mediated by ER α , may be involvement in the transcriptional regulation of inhibin/activin gene expression. Inhibin and activin belong to the transforming growth factor- β superfamily (KINGSLEY 1994) and a previous study of CHEN & JOHNSON (1996) revealed that the granulosa layer of the large preovulatory follicles is the primary site for the expression of inhibin/activin α - and β _A-subunit mRNAs among a variety of chicken tissues examined. Other functions of estrogens in granulosa cells may pertain to steroidogenesis. KAMIYOSHI *et al.* (1992) showed that culture of hen granulosa cells with estradiol-17 β caused a significant increase in progesterone production in response to LH. Next SASANAMI & MORI (1999) demonstrated that LH-stimulated progesterone production in cultured granulosa cells of Japanese quail is promoted by estradiol-17 β . Recently, a marked increase in mRNA and protein levels of GP42 specific to the granulosa layer of the preovulatory follicle has been reported by TAKEUCHI *et al.* (1999). GP42 is a major protein constituent for the perivitelline layer in the chicken egg-envelope. The upper regulatory region of this gene has not been annotated. However, whether marked ER mRNA expression in the granulosa layer is reflected in the regulation of GP42 expression warrants further studies.

In conclusion, the results of the present study demonstrate that (1) ER α mRNA is present in each compartment of reproductive tissues in laying quail, (2) the marked expression of ER α mRNA in the granulosa layer of the largest follicle may indicate ER α mediated involvement of estrogens in the biosynthesis of inhibin/activin, progesterone and yolk perivitelline layer protein, (3) very high expression of ER α in the oviductal tissues may be related to the role of estrogens in cell proliferation and protein synthesis in the oviduct.

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