cDNA Cloning and mRNA Expression of Estrogen Receptor α in Japanese Quail

Kouhei Ichikawa, Ichiro Yamamoto, Akira Tsukada,
Noboru Saito and Kiyoshi Shimada

Laboratory of Animal Physiology, Graduate School of Bioagricultural Sciences,
Nagoya University, Nagoya, Aichi 464-8601, Japan

The biological actions of estrogen are mediated via estrogen receptors (ER). Although chicken ER cDNA for subtypes α and β have been identified, only the β form of ER cDNA was cloned in quail. Hence, in this study the cDNA of ER α subtype was cloned and levels of mRNA expression were studied in different tissues in quail. Quail ER α cDNA containing the sequence containing the open reading frame (1770 bp) encodes the predicted 589 amino acid residues and had high homology to chicken ER α (98.8%). Both RT-PCR and northern blot analyses indicate that relatively higher expression was observed in the liver, ovary, oviduct and testes. Relatively lower expression was detected in adrenal, cerebrum, hypothalamus, gizzard, heart, kidney and intestine by RT-PCR analysis. Conversely, low levels of expression of quail ER β mRNA was detected in all the examined tissues. The results indicate that ER α subtype plays a predominant role for estrogenic actions in reproduction in quail.

Key words : estrogen receptor α , mRNA, expression, quail, northern blot analysis, RT-PCR

Introduction

Estrogens cause diverse actions in vertebrates. In birds, notable actions are observed in reproduction such as gonadal development, the induction of the secondary sex characteristics of females, and synthesis of vitellogenin in the liver and albumen in the oviduct. These biological actions are mediated via estrogen receptor (ER). It is now recognized that ER is one of the transcription regulatory factors which, in response to the binding of estrogen, is believed to bind to specific DNA elements to activate transcription. In the chicken estrogens are produced in the female gonad as early as 3–4 days of incubation (Woods and Erton, 1978) and is involved in ovarian formation, differentiation of the oviduct (Palmiter and Mulvihill, 1978) and demasculinization of the brain (Balthazart *et al.* 1996). Around sexual maturity, more estrogens are synthesized primarily in the thecal cells of the ovarian follicles (Porter *et al.*, 1989 ;

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Correspondence : Kiyoshi Shimada, Ph.D.

Laboratory of Animal Physiology, Graduate School of Bioagricultural Sciences, Nagoya University,
Nagoya, Aichi 464-8601, Japan.

Tel & Fax : 052-789-4065 E-mail : kshimada@agr.nagoya-u.ac.jp

Kato *et al.*, 1995) which act to accelerate oviductal growth and stimulate the synthesis of yolk precursor material, vitellogenin in the liver (Elbrecht *et al.*, 1984). In the egg-laying period, estrogens also promote albumin production in the magnum segments of oviduct (Moen and Palmiter, 1980).

Although chicken ER cDNA for subtypes α and β have been identified (Krust *et al.*, 1986), only the β form of ER cDNA was cloned in quail (Foidart *et al.*, 1999). Since the ER α subtype is related to specific physiological actions in chickens and mammals, the cDNA clone is necessary for better understanding of molecular mode of estrogen actions in quail. Furthermore, recently, the Organization for Economic Co-operation and Development (OECD) expert consultation on endocrine disrupting testing in birds proposed the standardization of assay systems for evaluation of biological potencies of endocrine disrupters in birds and suggested the Japanese quail be used as the model bird. Furthermore, a new in vitro assay method using quail recombinant ER α is desired to monitor the effect of estrogenic environmental disrupters. For this purpose the cDNA cloning of the quail ER α is necessary. Herein, we report the cDNA cloning of the ER α and levels of mRNA expression in quail tissues.

Materials and Methods

Tissue Sampling and RNA extraction

Actively laying female Japanese quail were killed by decapitation and tissues were collected, frozen in liquid nitrogen and stored at -80°C until RNA extraction. The total RNA was extracted according to the method described by Chomczynski (1993). The amount of total RNA was estimated by spectrophotometry (Beckman Instruments Inc., Fullerton, CA).

Polymerase chain reaction (PCR) cloning of quail ER α cDNA

Total RNA ($5\mu\text{g}$) of the liver was denatured at 65°C for 10 min with oligo dT (17) primers and reverse-transcribed with 200 units of SuperScript II (Invitrogen Life Technologies, Carlsbad, CA) in a $20\text{-}\mu\text{l}$ mixture. Based on the sequences of chicken ER α (Krust *et al.*, 1986) primers were designed. The pairs of oligonucleotide primers (sense primer S1 : $5'\text{-CTGCT GAGCC TCAGA ATAGG T-3'}$, S2 : $5'\text{-GTGCC TTAAG TCCAT CATCC T-3'}$, antisense primer A3 : $5'\text{-GCGTC CAGCA TCTCC AGTAA G-3'}$, A4 : $5'\text{-GGTGG CTAAT GTCAT TGGAG T-3'}$) were synthesized and was subjected to PCR. The reverse-transcribed (RT) product was subjected to 35 cycles of PCR amplification using Taq polymerase (Takara, Kyoto) in a total volume of $50\mu\text{l}$. The cDNAs were denatured at 97°C for 1 min, followed by 35 cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 1 min, and finally 10-min extension at 72°C . The PCR products were separated on an agarose gel, and a DNA fragment of the desired size was subcloned into pGEM-T (Promega Corp., Madison, WI). DNA sequencing was performed on plasmids using dye terminator chemistry on an applied chain-termination method (Sanger *et al.*, 1977).

Northern blot and RT-PCR analyses for ER α and ER β mRNA expression

Twenty μg of total RNA were denatured with formaldehyde at 65°C and electrophoresed in 1% (w/v) agarose gel containing 2.2 M formaldehyde. The RNA was

then blotted onto a Hybond N+ membrane (Amersham Biosciences Corp., NJ) by capillary action with 10x SSC (1x SSC=0.15 M sodium chloride, 0.05 M sodium citrate, pH 7.0). Following blotting, air-dried membranes were cross-linked using a UV cross-linker (Stratagene, La Jolla, CA). The membranes were prehybridized and probed with ³²P-labeled cDNAs of ER α , ER β or GAPDH, in turn. The hybridization was carried under conventional conditions and was washed to remove nonspecific radioactivity twice for 15 min at 65°C in 1x SSC-0.1% SDS and twice for 15 min at 65°C in 0.1x SSC-0.1% SDS. Membranes were exposed to x-ray film with intensifying screens (Amersham Biosciences Corp., NJ) at -80°C for 1 day.

For PCR amplification 2 μ l of each RT reaction was used. For ER α and ER β mRNA, the sequences of the upstream and downstream primers used were listed in Table 1. PCR reactions were carried out in 25 μ l PCR buffer (20 mM Tris HCl, 100 mM KCl, 2 mM MgCl₂, pH 8.0) contains 0.2 mM dNTPs, 0.5 μ M primers and 0.3125 unit Ex Taq DNA polymerase (Takara, Kyoto). After the initial denaturation for 2 min at 94°C, the amplification profile consisted of 10 sec of denaturation at 97°C, 30 sec of annealing at 55°C, and 1 min of extension at 72°C for 25 cycles. Amplification was 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 run on a 1.5% agarose gel in 1 X TAE buffer, and bands were visualized by ethidium bromide staining.

Results

Figure 1 shows coding sequences of the quail ER α . The quail ER cDNA contains the sequence of open-reading frame of 1770 bp and encodes the 589 predicted amino acid residues. According to the chicken ER α , the quail ER α amino acid residues were divided into 6 regions (A-F). A-F regions consist of 37, 136, 83, 39, 251 and 43 amino acid residues, respectively (Fig. 1). Comparison of the quail ER α amino acid residues to those of human, mouse and frog are shown in Figure 2 and as shown in Table 2, overall identity of their amino acid residues was 98.8% in the proteins of quail and chicken ER α . Two regions of high homology were found in C (DBD, 100% amino acid identity) and E regions (LBD, 99.6%). In contrast, overall identity of the amino acid residues was low (50.9%) between quail ER α and ER β .

Table 1. Position and sequence of synthetic oligonucleotide primers used for assay of ER α and ER β mRNA expression

Gene	Location	Sequence (5'→3')	Reference and accession number
ER α	1522-1542	GTG CCT TAA GTC CAT CAT CCT	Krust <i>et al.</i> , 1986 X03805
	1801-1821	GCG TCC AGC ATC TCC AGT AAG	
ER β	670-689	CCT GGC TCA AAA AGA GAT GC	Lakaye <i>et al.</i> , 1998 AF045149
	953-972	GCC CAT GCA ATC TTC TGA AT	
GAPDH	608-627	GTG GAG AGA TGA CAG AGG TG	Dugaiczky <i>et al.</i> , 1983
	937-956	AAC AAG CTT GAC GAA ATG GT

Fig. 2. Alignment of ER α proteins among quail (a), chicken (c), human (h), mouse (m) and frog (f). The amino acids alignment of ER α was divided into six regions (A-F) as described in Fig. 1. Numbers in each side represent the position of the amino acid residues in each sequence. Identical amino acids in 3-4 sequences are shadowed and in all 5 sequences are darkened.

Figure 3 shows northern blotting analyses of ER α and ER β in quail tissues. The 3.6-kb transcript of ER α was detected. Relatively higher expression was observed in the liver, ovary (white follicle) and oviduct (magnum and shell gland), whereas no expression in ER β was detected by northern blot in any tissues examined. RT-PCR analysis also showed higher expression of ER α in liver, adrenal, ovary (white follicle), oviduct (magnum and shell gland) in the adult female and liver, heart and testis in the adult male (Fig. 4). On the other hand, weak signals were detected in the cerebrum, hypothalamus, gizzard, adrenal, hypothalamus, gizzard and intestine. RT-PCR analysis for ER β mRNA indicated low levels of expression in all the examined tissues of the adult female and male but no signal was detected in adrenal (Fig. 4).

Discussion

This study revealed nucleotide base sequences of the full-length of quail ER α encoding the 589 predicted amino acid residues. The encoded protein has several unique characteristics common to other steroid receptors. They consist of 6 regions (A-F) in which the C region contains 9 cysteines and forms the unique Zinc finger motif responsible for DNA binding as reported previously in the chicken (Krust *et al.*, 1986). Of the 6 regions, this region is highly conserved in various vertebrate species (Table 2)

Table 2. Comparison of quail ER α amino acid residues to those of chicken, human, mouse and frog and those of ER β of quail and chicken. Identity is shown in %

Gene	ORF	DBD (C)	LBD (E)
chicken ER α	98.8	100	99.6
human ER α	78.4	100	93.6
mouse ER α	77.3	100	93.6
frog ER α	76.3	98.6	85.3
quail ER β	50.9	81.7	58.4
chicken ER β	50.9	81.7	57.8

ORF, open reading frame ; DBD, DNA binding domain ; LBD, ligand binding domain.

Fig. 3. Northern blot analyses of ER α and ER β in quail tissues. Autoradiograph exposure was 1 day. Cbr, cerebrum ; Cbl, cerebellum ; Hyp, hypothalamus ; Giz, gizzard ; Hrt, heart ; Spl, spleen ; Liv, liver ; Kid, kidney ; Adr, adrenal ; Wf, white follicle ; Mag, magnum ; She, shell gland ; Int, intestine ; Tes, testis.

Fig. 4. RT-PCR analyses of ER α and ER β in quail tissues. Abbreviation : same as in Fig. 3. M, molecular marker (*Msp* I digested pUC19).

and is required for binding to specific cis element of the target gene, called as estrogen response element (ERE) (Martinez and Wahli, 1991). In chickens and quail target genes such as vitellogenin II (VTGII), very low density apolipoprotein II (apoVLDLII) and ovalbumin genes contain the specific cis element in the promoter region (Martinez and Wahli, 1991). Our previous study demonstrated a remarkable induction of VTGII mRNA expression in the liver of chicken embryos (Sakimura *et al.*, 2001) and of VTGII and apoVLDLII mRNA expression in the liver of developing embryos of quail after injection of estrogen (Ichikawa *et al.*, 2003). The other crucial region of the open reading frame of the quail ER α is the E region known as the ligand binding domain which is also well conserved in amino acid residues among steroid receptors (Martinez and Wahli, 1991). The first step for biological effect of estrogen must be the binding of estrogen to this region of ER α , which in turn, leads to modification of conformation of ER α protein, so as to facilitate the binding of the ER α complex to the target gene.

High expression of ER α mRNA in the reproductive organs (i.e. ovary, testes and oviduct) as well as the liver that is necessary for biosynthesis of yolk protein may indicate that this receptor plays a predominant role in reproduction. In contrast, the physiological significance of low levels of ER α mRNA expression in non-reproductive

organs is not clear. Furthermore, as reported by Foidart *et al.* (1999) and confirmed in this study extremely low levels of expression of ER β mRNA occurs in many tissues of quail.

An *in vitro* method to screen estrogenic activity of environmental chemicals is available which utilizes the expression vector (pGEX-4T-1, Amersham Biosciences Corp., NJ) for GST fusion proteins. For this assay the E region (LBD) of quail ER α cDNA is essential. Our cDNA of the E region of quail ER α can be applied for the *in vitro* assay system. Thus, establishment of an enzymeimmuno-competitive binding assay of estrogenic activity is now in progress.

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