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Circadian Clock Gene Regulation of Steroidogenic Acute Regulatory Protein Gene Expression in Preovulatory Ovarian Follicles

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It is now known that circadian clocks are localized not only in the central pacemaker but also in peripheral organs. An example of a clock-dependent peripheral organ is the ovary of domestic poultry in which ovulation is induced by the positive feedback action of ovarian progesterone on the neuroendocrine system to generate a preovulatory release of LH during a daily 6–10 h "open period" of the ovulatory cycle. It has been assumed previously that the timing of ovulation in poultry is controlled solely by a clock-dependent mechanism within the neuroendocrine system. Here, we question this assumption by demonstrating the expression of the clock genes, *Per2* (Period 2) and *Per3*, *Clock*, and *Bmal1* (brain and muscle Arnt-like protein 1), in preovulatory follicles in laying quail. Diurnal changes in *Per2* and *Per3* expression were seen in the largest preovulatory follicle (F1) but not in smaller follicles. We next sought to identify clock-driven genes in preovulatory follicles focusing on those involved in the synthesis of progesterone. One such gene was identified, encoding steroidogenic acute regulatory protein (*StAR*), which showed 24-h changes in expression in the F1 follicle coinciding with those of *Per2*. Evidence that *StAR* gene expression is clock driven was obtained by showing that its 5′ flanking region contains E-box enhancers that bind to CLOCK/BMAL1 heterodimers to activate gene transcription. We also showed that LH administration increased the promoter activity of chicken *StAR*. We therefore suggest that the timing of ovulation in poultry involves an LH-responsive F1 follicular clock that is involved in the timing of the preovulatory release of progesterone. (*Endocrinology* 148: 3031–3038, 2007)

THE PERIOD OF the ovulation-oviposition cycle in domestic poultry is between 24 and 27 h, with ovulations and subsequent ovipositions occurring successively later on consecutive days, to form a sequence until a "pause day" occurs when no egg is laid (1). The next egg is then laid early in the day to start a new sequence of ovipositions. The timing of ovulation and subsequent oviposition is entrained by the daily lighting cycle, resulting in a daily 6–10 h, "open period" of the ovulatory cycle, which defines the period when coincidental preovulatory releases of LH and progesterone may be spontaneously initiated (2, 3). It was first suggested by Fraps (2, 4) and subsequently elaborated on by others (1, 3) that the open period of the ovulatory cycle is a consequence of a clock-driven diurnal rhythm of neuroendocrine responsiveness to the positive feedback action of progesterone from the preovulatory follicle on LH release (5). Although it has been shown that coincidental preovulatory peaks of plasma progesterone and LH are generated by the stimulatory effects of progesterone on GnRH (6) and LH release (7, 8), a diurnal rhythm in threshold response to the positive feedback action of progesterone on LH release has not been demonstrated.

The characteristic pattern of egg laying in sequences as observed in the chicken and quail (9, 10) is thought to be a consequence of asynchrony between the occurrence of the open period of the ovulatory cycle and a cycle of ovarian follicular growth and maturation, which is not considered to be clock controlled (1, 3). However, we reported the presence of clock gene expression in the ovary of the Japanese quail (11), which suggests that a circadian mechanism within the ovary might also play a role in the control of the avian ovulation-oviposition cycle. The ovulation-oviposition cycles of quail and domestic hens are essentially the same, except the quail cycle lags that of the domestic hen by approximately 8 h (9, 10). Consequently, most eggs are laid by quail in the afternoon and by domestic hens in the morning. In quail held on a 14 or 16 h photoperiod, oviposition is preceded by coincidental 8–10 h preovulatory surges of plasma LH and progesterone starting early in the photoperiod, with a peak 4–6 h before oviposition (12). As in the domestic hen, progesterone is synthesized predominantly in the granulosa layer largest (F1) preovulatory follicle (12, 13). Circadian rhythms are generated by a transcription-transla-

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Abbreviations: CT, Circadian time; F1, largest preovulatory follicle; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 16L:8D, 16-h light, 8-h dark cycle; LL, constant light; LSD, least significant difference; RACE, rapid amplification of cDNA ends; RNase, ribonuclease; StAR, steroidogenic acute regulatory protein; ZT, Zeitgeber time.

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tion-based oscillatory loop that involve clock genes, including *Per2* (Period 2) and *Per3*, *Clock*, and *Bmal1* (brain and muscle Arnt-like protein 1) (14–16). PER2 forms part of a complex of proteins that inhibits the transcriptional activator CLOCK/BMAL1 heterodimer that promotes the transcription of clock-controlled genes by binding to regulatory E-box (CACGTG) sequences. Clock genes in quail and chickens have high homologies with those in mammals, although no homolog of the mammalian *Per1* gene has been found in birds (11, 17).

In the present study, we determined in combined granulosa and theca interna layers of preovulatory follicles of quail whether there is circadian rhythmicity in the expression of the clock genes *Per2*, *Per3*, *Bmal1*, and *Clock* and of potential clock-controlled genes involved in progesterone synthesis. Genes known to be involved in the progesterone synthesis selected for analysis encoded very-low-density lipoprotein receptor that mediates lipoprotein uptake in the avian ovary (18), sterol carrier protein-2 (SCP-2) that mediates intracellular cholesterol movement (19), and steroidogenic acute regulatory protein (StAR) required for cholesterol shuttling across the mitochondrial membrane (20). Additional genes of interest selected for analysis were AdRed (adrenodoxin reductase) and Adx (anaredoxin) (19) that mediate electron transport to mitochondrial cytochrome P450, cytochrome P450 side-chain cleavage enzyme that converts cholesterol to pregnenolone, and 3β-hydroxysteroid dehydrogenase (3β-HSD) that converts pregnenolone to progesterone (19). Finally, we further sought to demonstrate that possible clock-controlled genes we identified have E-box enhancers characteristic of clock-driven genes, binding to CLOCK/BMAL1 heterodimers to stimulate gene transcription. Because chicken and quail are Galliformes and nucleotide sequences between the two species are highly conserved, we used chicken sequences for promoter analysis to take advantage of the chicken genome sequences provided by the chicken genome project.

Materials and Methods

Animals

Nine-week-old laying Japanese quail (*Coturnix japonica*) were obtained from a local dealer and housed in light-tight boxes (55 × 210 × 62 cm) kept at 24 ± 1 C and exposed to a light cycle of 16-h light, 8-h dark cycle (16L:8D) or constant light (LL). The floors of the individual cages were sloped, and the time of egg laying was monitored using an interrupted-infrared beam system (Omron, Kyoto, Japan) installed in each cage and connected to a chronobiology analysis system (Stanford Software System, Santa Cruz, CA). Oviposition rhythmicity was monitored in all quail used in the present study. Because quail held on 16L:8D most often laid eggs at around 11 h after the onset of light corresponding to Zeitgeber time (ZT) 11 (Fig. 1A), the phase reference point for the ovulation-oviposition cycle in quail held on LL was taken as the time of oviposition and designated as circadian time (CT) 11. Under LL conditions, samples were collected at least 2 wk after transferred into LL. For the *StAR* gene promoter analyses, we used laying hens (*Gallus domesticus*) obtained from a local dealer. The birds were provided food and water *ad libitum*. The birds used in the present study were treated in accordance with the Animal Welfare Guidelines of Nagoya University.

In situ hybridization

Approximately 1 month after transfer to 16L:8D or LL, laying quail were killed at ZT or CT 1, 7, 13, and 19 by decapitation, and ovaries were immediately removed to avoid acute changes in gene expression. *In situ* hybridization was performed according to Yoshimura *et al.* (11). Antisense and sense 45-mer oligonucleotide probes were labeled with [^{33}P]dATP (PerkinElmer Life Sciences, Boston, MA) using terminal deoxyribonucleotidyl transferase (Invitrogen, Carlsbad, CA). For probe information, see supplemental Table 1 (published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Hybridization was performed overnight at 42 C. Two high-stringency posthybridization washes were performed at 55 C. The sections were air dried and apposed to Biomax-MR film (Eastman Kodak, Rochester, NY) for 2 wk. ^{14}C standards (American Radiolabeled Chemicals, St. Louis, MO) were included in each cassette, and relative OD was measured by using a computed image-analyzing system (MCID; Imaging Research, St. Catharines, Ontario, Canada) and converted into the radioactive value (nanocuries) using the ^{14}C standard measurements.

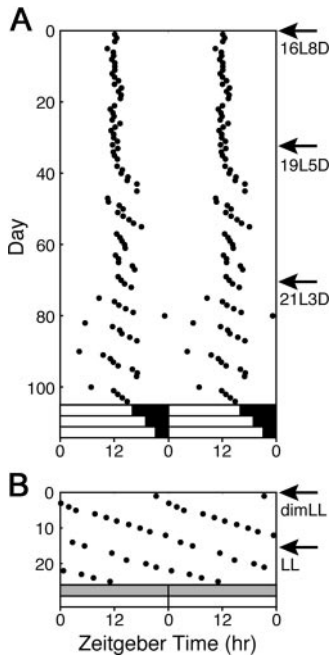

FIG. 1. Representative oviposition records of quail maintained under various light-dark [16-h light, 8-h dark (16L8D); 19-h light, 5-h dark (19L5D); and 21-h light, 3-h dark (21L3D)] (A) and LL (B) conditions. Dim LL, 10 lux; LL, 200 lux. The time of oviposition is indicated by a *black dot*. Records are double plotted.

RT-PCR analysis

Total RNA were extracted from granulosa cells and theca layers of F1 follicle at ZT 7 and ZT 19 by TRIzol (Invitrogen). RT was performed on total RNA (0.5 μg) preparation using ExScript RT reagent kit (TaKaRa, Shiga, Japan). PCR was performed at 95 C for 10 min and then run for 28 cycles (all genes except for *Per2* and *Per3*) or 30 cycles (*Per2* and *Per3*) at 95 C for 10 sec, 60 C for 1 min using AmpliTaq Gold (Applied Biosystems, Foster City, CA). For primer information, see supplemental Table 2 (published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). We confirmed the sequence of the PCR products.

cDNA cloning of the 5′ untranslated region of chicken StAR mRNA

A cDNA library was prepared from the chicken F1 follicle using the SMART RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA), and the 5′ region of *StAR* cDNA was synthesized by the rapid amplification of cDNA ends (RACE). This method used the SMART RACE-UPM primer and the antisense primer (5′-gccaccgtctccgtcttcca-3′), fol-

lowed by a nested PCR reaction using the SMART RACE-NUP primer and antisense primer (5′-agcttgctgagctcctggct-3′).

Ribonuclease (RNase) protection assay

The 304-bp fragment of the 5′ end of the *StAR* gene containing the first exon was amplified by PCR using the sense 5′-cacgtatctgggcagcagca-3′ and antisense 5′-ggtcacgttgcgcaggtgtt-3′ primers derived from chicken *StAR* genomic DNA sequence subcloned into the pGEM-T Easy vector (Promega Japan, Tokyo, Japan). The antisense RNA probe was synthesized using T7 polymerase and [α-^{32}P]CTP. The RNA probe (5×10^4 cpm/assay) was hybridized with 20 μg of the total RNA obtained from the F1 follicle at 50 C for 16 h in 30 μl of the solution containing 40 mM 1,4-piperazinediethanesulfonic acid (pH 6.5), 0.4 M NaCl, 1 mM EDTA, and 80% formamide. The samples were digested with RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) at 37 C for 30 min and then incubated with proteinase K (50 μg/ml) at 37 C for 15 min. The RNA fragment protected from RNase digestion was separated by electrophoresis in a 6% polyacrylamide-7 M urea gel and detected by autoradiography. *Msp*I-digests of the pUC19 plasmid DNA were used as the size marker.

Granulosa cell culture

Granulosa layers from the F1 follicles of laying chickens were collected as described previously (21) and dispersed in 500 U/ml collagenase. The granulosa cells were plated out in 24-well polystyrene culture plates at a density of approximately 2.5×10^5 cells per well in 1 ml of M199-HEPES supplemented with 10% fetal calf serum; the plates were incubated at 41 C in an atmosphere containing 5% CO_2/95% O_2.

Construction of StAR promoters and E-box mutations

Chicken genomic DNA was extracted from liver by using the DNeasy Tissue kit (Qiagen, Hilden, Germany). The chicken *StAR* 5′ flanking region was amplified by the inverse PCR method (22) using sense 5′-atctcctaccaacacctgcgcaacgtg-3′ and antisense 5′-ccgagataagggccatcacttacggca-3′ primers derived from a chicken *StAR* mRNA sequence (GenBank accession no. AF220436). A 2011-bp chicken *StAR* promoter-luciferase vector and a 1500-bp *Per2* promoter-luciferase vector were constructed by PCR using sense 5′-ctagctagcagcagcagcagcagcttt-3′ and antisense 5′-cccaagcttgcctcagccccggcgctcggc-3′ primers, and sense 5′-ctagctagcaacctcacgcacgcagcatgt-3′ and antisense 5′-cccaagcttggccgcggctacgtgacgcag-3′ primers containing respective *Nhe*I and *Hin*dIII restriction site sequences (underlined), derived from the Ensembl database (http://www.ensembl.org). The amplified DNA fragments were digested with *Nhe*I-*Hin*dIII and were subcloned into the pGL3-Basic Vector (Promega), which had been digested with *Nhe*I-*Hin*dIII. A mutated E-box (GGACCT) was created by PCR-based site-directed mutagenesis (23).

Transfection and luciferase reporter gene assay

Five microliters of the Effectene transfection reagent (Qiagen) containing 0.2 μg DNA and 1.6 μl enhancer were added to a 24-well dish. Chicken *StAR* promoter-luciferase or *Per2* promoter-luciferase construct was cotransfected with either chicken *Bmal1* (10 ng) and *Clock* (50 ng) expression vectors or with an empty vector, pcDNA3.1 (Invitrogen). Cells were also cotransfected with the *Renilla* luciferase, to act as an internal control for transfection efficiency. The cells were harvested 24 h after transfection, and transcriptional activity was determined using a luciferase reporter assay. In addition, 24 h after transfection, the cells were cultured for 3 h in the absence or presence of 100 ng/ml ovine LH (Sigma, Kanagawa, Japan). The luciferase assay was performed using a dual-luciferase assay system (Promega) with Lumat LB950 (Berthold, Tokyo, Japan) according to the protocols of the manufacturer. Firefly relative luciferase unit measurements were normalized to *Renilla* relative luciferase units.

Results

Twenty-four-hour changes in expression of clock genes in the F1 follicle in the quail ovary

Under 16L:8D, the quail laid very long sequences, and consequently most eggs were laid at the same time in the late afternoon (Fig. 1A). Quail held on LL laid eggs at about 27 h intervals but at any time of day (Fig. 1B). Expression of clock genes was observed in both granulosa and theca layers of the quail F1 ovarian follicle (Fig. 2). The expression sites of clock genes were similar in smaller F2–F4 yellow yolky follicles and in small white yolky follicles. No hybridization signal was observed in the granulosa and thecal layers using control sense probes (Fig. 2). In quail exposed to 16L:8D, the expression of *Per2* and *Per3* changed over 24 h, with the highest values at ZT 7 for *Per2* and at ZT 1 for *Per3* in the F1 follicle, respectively (one-way ANOVA, $P < 0.05$; Fisher's *post hoc* test, $P < 0.05$) (Figs. 2 and 3). In smaller follicles, the expression of these genes was not significantly different (ANOVA, $P > 0.05$) over a 24-h period but appeared to become rhythmic in the largest follicle (Fig. 3). Although no statistically significant difference was detected in *Clock* and *Bmal1*, their expression tended to show 24 h changes in F1 follicle. Similar observations were made on ovarian follicles taken from quail exposed to LL (Fig. 4), and peak time of *Per2* expression under LL (CT 7) was consistent with LD cycles (ZT 7).

Identification of clock-controlled genes in the progesterone production pathway

Of the seven genes investigated known to be involved in progesterone synthesis (see introductory section), only the expression of *StAR* and *3β-HSD* genes changed over 24 h in ovarian follicles. These changes were seen in the F1 follicle but not in the F2, F3, or small white follicles (Fig. 5). Con-

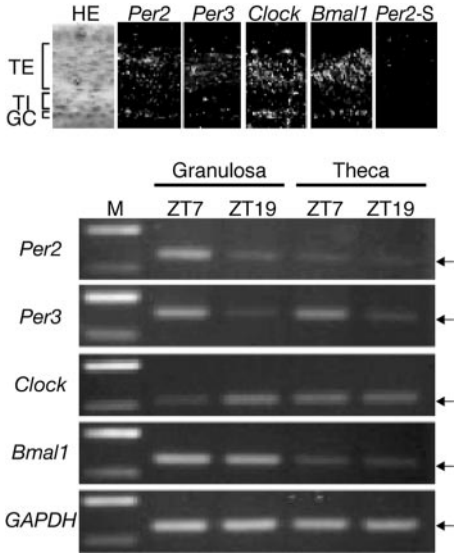

FIG. 2. Localization of *Per2*, *Per3*, *Clock*, and *Bmal1* mRNA in the quail ovary. *Top row* includes representative dark-field photomicrographs showing the expression of clock genes and sense control of *Per2* (*Per2*-S) and the light-field photomicrograph counterstained by hematoxylin and eosin. *Bottom rows* are the results of RT-PCR analyses of mRNA extracted from granulosa layers and theca layers of quail F1 follicle at ZT 7 and ZT 19. The position of size markers is 147 and 110 bp (lane M). *Arrows* indicate positions of PCR products. PCR products were electrophoresed in 4.0% agarose gel and stained with ethidium bromide. GC, Granulosa layer; TI, theca interna; TE, theca externa. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

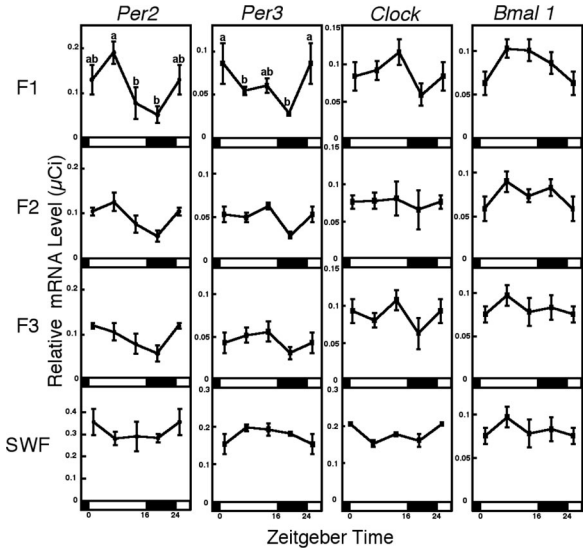

FIG. 3. Expression patterns of the clock genes in various quail follicles under 16L:8D. Each value represents the mean ± SEM (n = 3–7). Samples were obtained at ZT 1, ZT 7, ZT 13, and ZT 19. Data at ZT 1 and ZT 25 are double plotted. Significant differences are indicated by *different letters* [*Per2*: ANOVA, $F_{(3,12)} = 4.999$, $P < 0.05$, Fisher's least significant difference (LSD) *post hoc* test, $P < 0.05$; *Per3*: one-way ANOVA, $F_{(3,12)} = 4.416$, $P < 0.05$, Fisher's LSD *post hoc* test, $P < 0.05$]. SWF, Small white follicles.

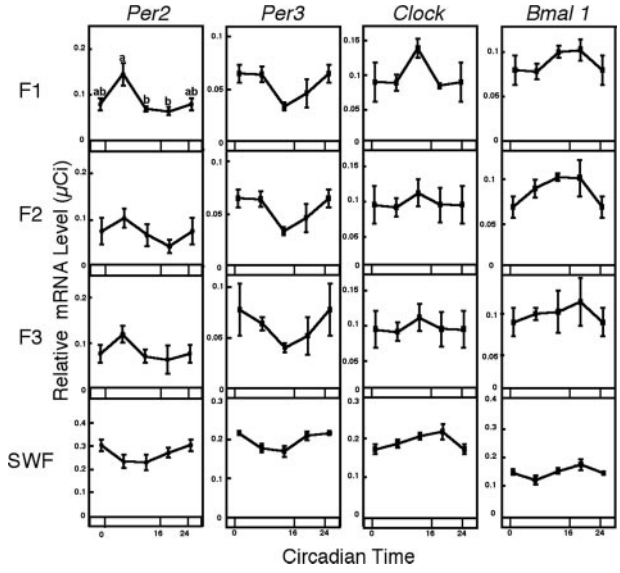

FIG. 4. Expression patterns of the clock genes in various quail follicles under LL conditions. Each value represents the mean ± SEM (n = 3–7). Samples were obtained at CT 1, CT 7, CT 13, and CT19. Data at CT 1 and CT 25 are double plotted. Significant differences are indicated by *different letters* (*Per2*: ANOVA, $F_{(3,11)} = 3.717$, $P < 0.05$, Fisher's LSD *post hoc* test, $P < 0.05$). SWF, Small white follicles.

sistent with previous reports (24–26), expression of these genes was observed predominantly in the granulosa layers in which all of the clock genes were expressed (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The temporal expression profile of the *StAR* gene in the F1 follicle was similar to that of *Per2*, whereas that of the *3β-HSD* gene was inversely related to the expression of the *StAR* and *Per2* genes.

Determination of the transcription initiation site for the chicken StAR *gene*

The 5′ flanking region of the chicken *StAR* gene was isolated, and the transcription initiation site, identified using 5′ RACE, was further confirmed by RNase protection assay with an RNA probe obtained from a genomic fragment (GenBank accession no. AB258391) (Fig. 6A). The size of the protected fragment, 115 bp, was consistent with that obtained using 5′ RACE (Fig. 6A). A transcription initiation site was observed 53 bp upstream of the ATG translational start codon (Fig. 6A). No signal was detected using *in situ* hybridization and oligonucleotide probes designed to target upstream sequences (nucleotides −84 to −40 and −69 to −25) of the *StAR* gene, which is consistent with RACE and the RNase protection assays. The DNA sequence upstream from the transcription initiation site contained a TATA-like element (TTTAA) and other putative transcription factor-binding sites such as SF-1 and YY-1 (Fig. 6). A comparison of this promoter sequence with that for mammalian species (27) demonstrated high conservation (Fig. 6B). This sequence was therefore considered to be validated for functional characterization of the *StAR* 5′ flanking region.

Functional characterization of the StAR 5′ flanking region

Three CACGTG E-box enhancers that are putative binding sites for the CLOCK/BMAL1 heterodimers were found in the 5′ flanking region of the *StAR* gene, at positions −1640 to −1635, −1320 to −1315, and −250 to −245, upstream from the transcription initiation site (Fig. 7). In addition, a TGACGTCA cAMP response element-binding site was observed at the −556 to −549 position.

To obtain evidence that the CLOCK/BMAL1 heterodimer is involved in the regulation of *StAR* gene transcription, we conducted a luciferase assay in cultured granulosa cells using *Clock* and *Bmal1* expression vectors cotransfected with wild-type and mutant-type *StAR* promoter constructs (Fig. 7). A *StAR* promoter construct carrying mutations in all of the E-boxes was completely unresponsive to CLOCK/BMAL1, whereas promoter constructs with wild-type E-boxes showed additive activity (Fig. 7). These results suggested that *StAR* transcription is under the control of clock proteins. Because *StAR* levels are known to be increased by LH stimulation in granulosa cell (24, 25), we also examined the effect of LH on the transcriptional activity of the *StAR* gene with or without CLOCK/BMAL1. The transcriptional activity of *StAR* was increased by both CLOCK/BMAL1 and LH in an additive manner (Fig. 8A). Although the LH stimulus alone did not increase *Per2* transcriptional activity, it increased the transcriptional activity of the *Per2* gene in the presence of CLOCK/BMAL1 (Fig. 8B).

Discussion

In the present study, we examined the temporal expression of clock genes and of possible clock-driven genes involved in progesterone synthesis in ovarian follicles during the ovulation-oviposition cycle of the Japanese quail. We found a robust change over 24 h in *Per2* and *Per3* gene

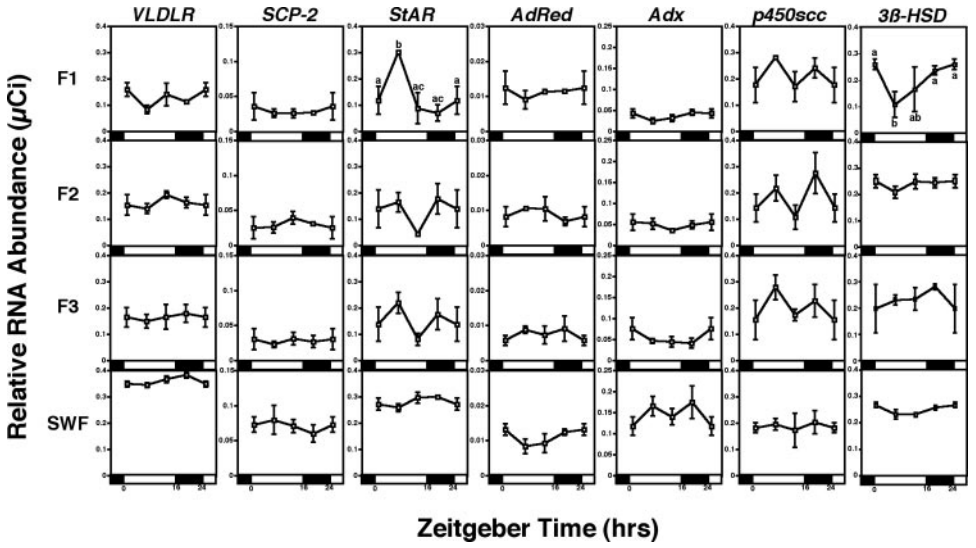

FIG. 5. Search for the clock-controlled gene in progesterone production in 16L: 8D. Each value represents the mean ± SEM (n = 3–7). Samples were obtained at ZT 1, ZT 7, ZT 13, and ZT 19. Data at ZT 1 and ZT 25 are double plotted. Significant differences are indicated by *different letters* (*StAR*: ANOVA, $F_{(3,8)} = 6.177$, $P < 0.05$, Fisher's LSD *post hoc* test, $P < 0.05$; *3β-HSD*: ANOVA, $F_{(3,12)} = 5.236$, $P < 0.05$, Fisher's LSD *post hoc* test, $P < 0.05$). SWF, Small white follicles.

expression in the mature F1 follicle but not in less mature follicles that was directly correlated with *StAR* gene expression and inversely with *3β-HSD* gene expression. Although expression of *Clock* and *Bmal1* also appeared to change over 24 h in the F1 follicle, no statistically significant difference was observed, possibly because of lower-amplitude changes in expression relative to the *Per* genes. There was no evidence of a change in expression over 24 h of five other genes involved in progesterone synthesis. The expression of *Per2* and *StAR* was highest at ZT 7, indicating that it coincides with the initiation with the preovulatory peaks of LH and progesterone (12). Conversely, *3β-HSD* expression was lowest at this time. Although the temporal expression of the *StAR* gene in the F1 follicle is consistent with involvement in synthesizing progesterone to generate the preovulatory surge, it is not consistent with the associated depression in *3β-HSD* gene expression. However, increased progesterone accumulation in the quail F1 granulosa cells before ovulation (12) may in part reflect a shift in the balance of progesterone synthesis and metabolism in favor of a decrease in metabolism. In support of this view, in the quail, the activity of a key enzyme involved in progesterone metabolism, 17α-hy-

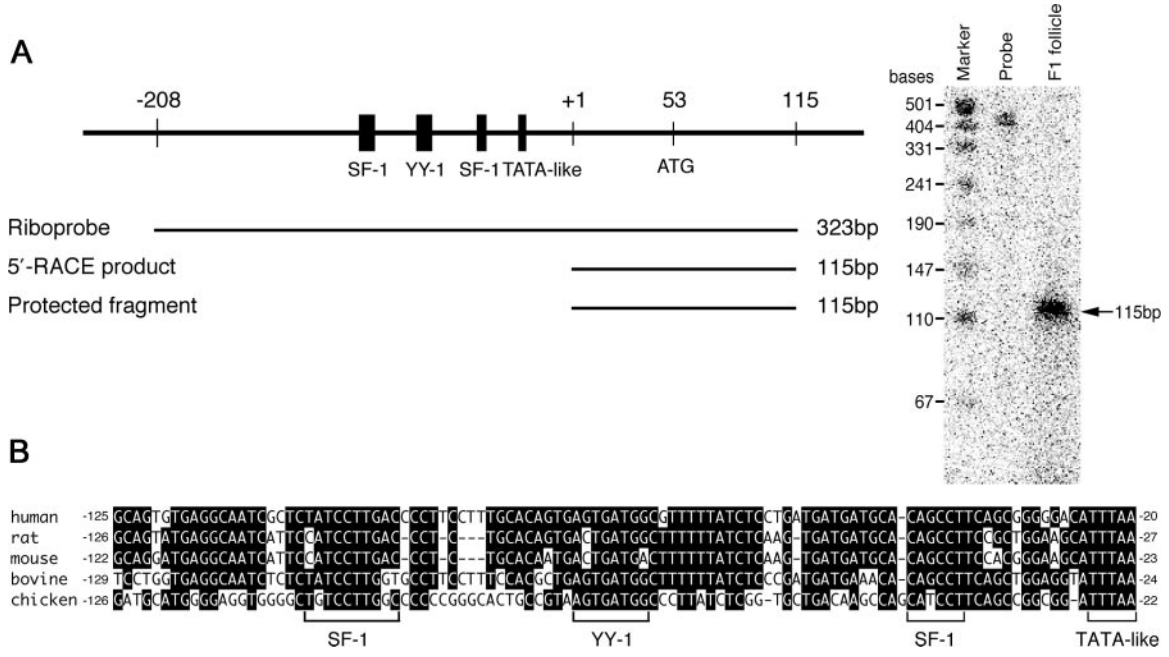

FIG. 6. Sequence analysis of the 5′ flanking region of the *StAR* gene. A, Analysis of the transcriptional initiation site of the *StAR* gene by 5′ RACE and an RNase protection assay. The RNA probe synthesized from the genomic region containing the first exon, 5′ RACE product, and the protected fragment are shown. *Msp*I digests of pUC19 plasmid DNA were used as the size marker. The position of the size marker is shown in the *left margin* of the autoradiogram. The location of the putative SF-1 and YY-1 binding sites, and TATA-like element are indicated by boxes. The transcription initiation site is designated as +1 and is shown by an *arrow*. The translational start codon is indicated by ATG. B, Alignment of the 5′ flanking sequence of the chicken *StAR* gene with sequences of the human, rat, mouse, and bovine *StAR* gene promoters in the region immediately adjacent to the transcription initiation site. Conserved residues are indicated by *black boxes*.

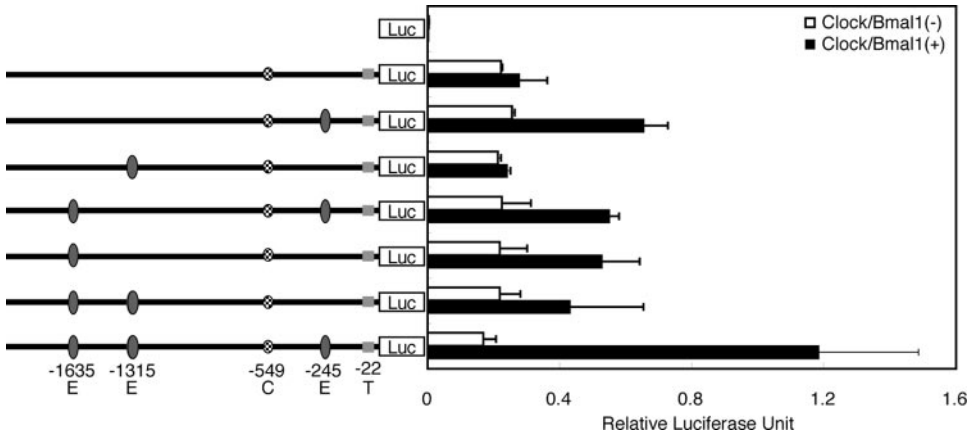

FIG. 7. Functional assessment of the E-boxes in the 5′ flanking region of the *StAR* gene. The transcription activation of a luciferase (Luc) reporter containing a 2011-bp fragment of the 5′ flanking region of the chicken *StAR* gene that included the endogenous promoter and either the CACGTG E-box or a mutated E-box (GGACCT) was assessed by cotransfection with *Clock* (50 ng) and *Bmal1* (10 ng) expression vectors or empty vector in cultured granulosa cells. The luciferase activity obtained from the reporter was normalized to the positive control *Renilla* luciferase reporter. Each value represents the mean ± SEM of three replicates for a single assay. The results shown are representative of three independent experiments. E, E-box; C, cAMP-responsive element; T, TATA-like element.

droxylase, which is present in the thecal layer of preovulatory follicles, drops to very low values from about 12 h before ovulation in the F1 follicle (28). If a factor controlling the increase in progesterone in the preovulatory follicle is a decrease in its metabolism within the follicle, the maintenance of a high level of expression of *3β-HSD* may not be the key factor sustaining the preovulatory surge of progesterone. It is therefore suggested that increased *StAR* expression in the F1 follicle may play a key role in initiating the preovulatory release of progesterone.

Previous observations on the chicken *StAR* gene show that it is evolutionarily conserved (29) and that its expression in F1 granulosa cells is up-regulated by gonadotropins, in part via cAMP signaling (24), and is necessary but not sufficient for the full potentiation of the preovulatory surge of progesterone (25). To establish whether chicken *StAR* gene expression is controlled by CLOCK/BMAL1 and therefore likely to be clock driven, we first characterized the 5′ regulatory region and identified the transcriptional initiation site (Fig. 6). In a previous study, Bauer *et al.* (29) identified a longer chicken *StAR* 5′ cDNA sequence (GenBank accession no. AF220436) than we found using 5′ RACE (GenBank accession no. AB258391). We could not confirm their results using 5′ RACE, RNase protection, or *in situ* hybridization and found no evidence for transcriptional activity of a luciferase construct using their 5′ sequence (data not shown). In the chicken *StAR* 5′ promoter, we identified a 125-bp sequence upstream from the transcription initiation site that was highly homologous with 5′ sequences in several mammalian *StAR* genes. This contained putative transcription factor binding sites for SF-1 and YY-1 and TATA-like elements in similar positions to those observed in the mammalian sequences (27). The 5′ chicken *StAR* sequence we obtained therefore appeared likely to be of functional significance in transcriptional regulation of the chicken *StAR* gene. The identification of three E-box sites farther upstream suggested putative binding sites for CLOCK/BMAL1.

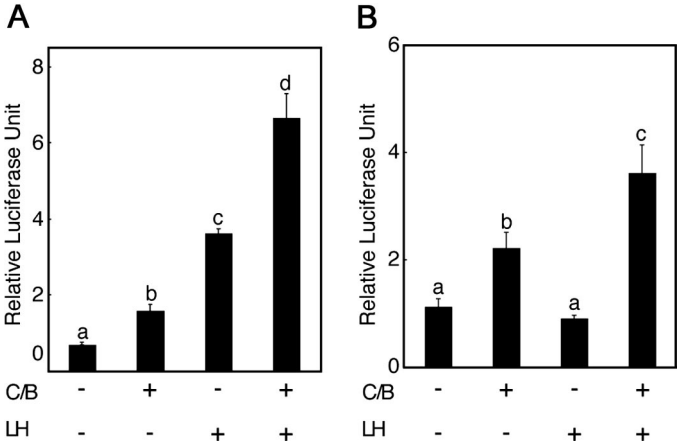

FIG. 8. LH-induced transcription activity of *StAR* (A) and *Per2* (B) in cultured granulosa cells. Cells were cotransfected with (+) or without (−) *Clock* and *Bmal1* (C/B) expression vectors. After 24 h of transfection, the cells were incubated for 3 h with or without ovine LH (100 ng/ml). The luciferase activity obtained from the reporter was normalized to the positive control *Renilla* luciferase reporter. Each value represents the mean ± SEM of three replicates for a single assay. The results shown are representative of three independent experiments. Significant differences are indicated by *different letters* (a–d) [panel A: ANOVA, $F_{(3,8)} = 67.596$, $P < 0.01$, Fisher's LSD test, $P < 0.05$; panel B: ANOVA, $F_{(3,8)} = 14.073$, $P < 0.05$, Fisher's LSD test, $P < 0.05$].

We demonstrated that these E-box sequences are likely to mediate clock-driven regulation of *StAR* gene expression using a luciferase reporter gene assay in which the reporter gene constructs were cotransfected with *Clock* and *Bmal1* expression vectors. When the native chicken *StAR* 5′ region was used in the reporter construct, reporter gene expression was stimulated in the presence of *Clock* and *Bmal1* expression vectors, but, when E-box mutations were created in the *StAR* 5′ promoter, reporter gene expression was attenuated. It is therefore concluded that the chicken *StAR* gene has the functional capacity for clock-driven expression. Using the same reporter system, chicken *Per2* gene expression was similarly demonstrated to be clock driven as expected because CLOCK/BMAL1 regulation of *Per* gene expression is fun-

damental to the molecular mechanism of the biological clock. To our knowledge, this is the first demonstration of clock gene regulation of *StAR* gene expression in any species. It is of interest that circadian expression of clock genes have been reported recently in the rat ovary (30, 31). We found two E-boxes and one E′-box (CACGTT) within the previously reported 2.2-kb upstream sequence of mouse *StAR* gene (32). The evidence suggests the possibility of the existence of similar circadian clock gene regulation of *StAR* gene expression in mammals.

The increase in *Per2* and *StAR* gene expression in the F1 preovulatory follicle at ZT 7 is associated with preovulatory surge of LH. The increased expression of both genes could therefore be LH dependent. Using our luciferase reporter assay, we demonstrated that this was partially true for *StAR* but not for *Per2* gene expression. However, LH had an additive effect on expression of both genes in the presence of CLOCK/BMAL1. The functional significance of clock-driven/LH-dependent *StAR* gene expression in the F1 follicle is therefore likely to be related to the timing of its maturation and ovulation. This view is reinforced by the absence of similar pronounced daily changes in *Per2,3* and *StAR* gene expression in less mature preovulatory follicles.

Our demonstration that *StAR* gene expression in the F1 follicle is likely to be clock driven suggests a novel alternate to the Fraps hypothesis (see introductory section) to explain the mechanism underlying the timing of ovulation in the chicken/quail ovulatory cycle. It is possible that a circadian clock controlling the timing of ovulation is in the ovary. A clock-driven increase in *StAR* gene expression in the F1 follicle could be responsible for timing an increase in plasma progesterone sufficient to initiate the preovulatory surge of LH. The observation that LH acts additively with CLOCK/BMAL1 to enhance *StAR* gene expression suggests that the LH may serve to amplify this increase in progesterone and thereby accelerate the development of a preovulatory surge. It is of note that LH also stimulated increased *Per2* gene expression in the presence of CLOCK/BMAL1, suggesting that this also is part of a mechanism to accelerate the development of a preovulatory LH surge. Our hypothesis suggests additional work to prove that clock gene expression in the F1 follicle drives a circadian rhythm of StAR production in the granulosa layer causally related to the preovulatory release of progesterone required for ovulation.

In conclusion, this paper reports the first demonstration in the ovary that *StAR* gene expression is potentially clock driven. It remains to be established whether this observation can be extended mammals or nonavian species. This observation is of particular relevance to species in which the timing of ovulation is controlled by a circadian rhythm, including poultry. It is widely accepted that the circadian control of the ovulation in poultry is controlled solely by reproductive neuroendocrine system. In contrast, our findings suggest that the circadian control of steroidogenesis within the preovulatory follicle may be involved in the circadian control of the timing of ovulation.

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