cDNA Cloning and mRNA Expression of Androgen Receptor in Male Japanese Quail (\textit{Coturnix coturnix japonica})

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The biological activities of androgen are largely mediated via androgen receptor (AR). Although a partial sequence AR cDNA was revealed in canaries and zebra finch, AR cDNA has not been cloned in quail and chickens. To understand the physiological function of androgen and to apply it for other purposes, AR cDNA is necessary. Hence, this study was aimed to isolate the cDNA of AR and to reveal changes in mRNA expression using RT-PCR analysis in male reproductive organs of quail under different day-lengths and castration treatment. We found that a partial length of quail AR cDNA contains a 1385 bp sequence encoding 343 amino acid residues and had high homology to other vertebrates. Quail were raised under continuous light regimen from 4 to 6 weeks old (6LL) and they were divided into 3 groups under the following conditions up to 3 weeks old: (1) continuous light regimen group (24h light ; 9LL), (2) short days regimen group (8h light and 16h darkness ; 9SD) and (3) castration group in which testis were surgically removed at 6 weeks old and raised under continuous light regimen (9CAS). Although weekly changes in the cloacal gland protrusion area showed significantly progressive increase at 7–9 weeks old in 9LL group and a progressive decrease in 9SD group, there were no changes in AR mRNA levels in the gland. In contrast, in 9CAS group, although the cloacal gland protrusion area decreased after castration treatment, AR mRNA levels increased in the gland. On the other hand, AR mRNA levels in epididymis and vas deferens increased in 9LL group, but there were no differences in 9SD and 9CAS groups from 6LL. The testicular AR mRNA levels did not change after long or short days treatment. These results indicate there is tissue-specific regulation in AR mRNA expression in quail.

Key words: androgen receptor, castration, day-lengths, mRNA expression, quail

Introduction

Androgens play a central role in male reproduction of all vertebrates. In birds, a primary androgen, testosterone, not only promotes gonadal development and spermatogenesis but also stimulates the secondary sex characteristics of male. Among the latters in quail, cloacal gland protrusion area is well known as an external indicator of male reproductive condition. Long days treatment increases the size of testis and cloacal gland protrusion area, whereas short days treatment reduces them (Coil and Wetherbee, 1959; Tanaka et al., 1965; Follett and Farner, 1966; Sachs, 1967; Sachs, 1969; Siopes and Wilson, 1975). Furthermore, there is a high correlation between enlargement of cloacal gland protrusion area and plasma testosterone levels (Delville et al., 1985). It is known that testosterone administration to the castrated quail increases the cloacal gland protrusion area (Sachs, 1969) and it prevents cloacal gland growth from regression induced by a short days (Sachs, 1969; Massa et al.,
1980). These indicate that enlargement of cloacal gland protrusion area is androgen dependent. These biological activities are largely mediated via androgen receptor (AR) that binds DNA to regulate transcription of responsive gene in the targeting tissues.

AR cDNA was first cloned from rodents (Chang et al., 1988) and human (Chang et al., 1988; Lubahn et al., 1988). In birds, it was only partially cloned in canaries (Nastiuk and Clayton, 1994) and zebra finch (Perlman et al., 2003). Quail and chickens are excellent model species in birds to study reproductive functions and other basic and applied biology. For example, recently, the Organization for Economic Co-operation and Development (OECD) proposed the standardization of assay systems for evaluation of biological potencies of endocrine disrupters in birds and suggested the Japanese quail to be used as the model bird. We have established in vitro receptor assay to screen estrogenic activity of environmental chemicals by bacterial recombinant protein from quail estrogen receptor (ER) cDNA (Maekawa et al., 2004). Likely, to establish a method to evaluate endocrine disrupters with androgenic activity, AR cDNA is needed. After sequencing a partial length of quail AR cDNA, we conducted a study to reveal changes in AR mRNA expression of testis, cloacal gland, epididymis, vas deferens and kidney in relation to physiological changes in testis functions by manipulating environmental light conditions and castration.

**Materials and Methods**

**Experiment I: Polymerase chain reaction (PCR) cloning and sequencing of quail AR cDNA**

9 weeks old male Japanese quail were purchased from Chubu Kagaku Shizai Co., Ltd. (Nagoya, Japan). Total RNA was extracted from the testis according to the method described by Chomczynski (1993). Poly-(A+) RNA was extracted by Oligotex-dT30 (Takara Bio Inc., Otsu, Japan). The reverse-transcribed (RT) cDNA was synthesized using Superscript II (Invitrogen Japan K.K., Tokyo, Japan) with dT18 primer according to the manufacturer’s instructions. Based on the sequence of canary AR cDNA (Nastiuk and Clayton, 1994) primers were designed. The pairs of oligo-nucleotide (forward primer : 5’-GGGAGCTGCAAAGTGTCTCTGATGAAGGC-3’, reverse primer : 5’-GAGCATGAGGTGGTCTTCTCG-3’) were synthesized and subjected to PCR. To obtain the 3’-region of quail AR cDNA, the cDNA was synthesized using a SMART RACE cDNA Amplification Kit (BD Bioscience Clontech, Tokyo, Japan). For the 3’-region of quail AR cDNA cloning, the primer : 5’-CACGCCCAAGAGTTTCTCTGATGAAGGC-3’ were synthesized. All primers were purchased from Invitrogen Japan K.K. (Tokyo, Japan). All PCR products were cloned in pGEM-T EASY (Promega Co., Tokyo, Japan) and sequenced.

**Experiment II: Effects of long and short days and castration on mRNA expression of AR**

Animals

4 weeks old male Japanese quail were purchased from Chubu Kagaku shizai Co., Ltd. (Nagoya, Japan). All birds were kept at constant condition of room temperature at 24±1°C with continuous light regimen (LL) until 6 weeks old. They were provided with food and water ad libitum. Then, 5 birds of the first group (6LL) were decapitated at 6 weeks old and tissues such as left testis, cloacal gland, vas deferens, epididymis and kidney were collected. The 21 remaining birds were divided into 3 groups under the following the conditions up to 9 weeks old : (1) continuous light regimen (9LL) ; (2) short days regimen (9SD) : a photoperiod of 8 h L : 16 h D ; (3) castration (9CAS) : birds were castrated at 6 weeks old by surgery under continuous light regimen. Body weight and cloacal gland protrusion area were measured according to the method described by Sachs (1969) every week until 9 weeks old, when they were decapitated and the tissues were collected and immediately frozen with liquid nitrogen. All tissues were stored at −80°C until the extraction of total RNA. The castrated birds were inspected for completeness of castration at the tissue sampling.

**RT-PCR analysis for AR mRNA expression**

Total RNA was isolated from each tissue mentioned above. Total RNA was treated with DNase to avoid contamination of genomic DNA. The amount of RNA was estimated by spectrophotometry at 260 nm. RT was performed with 0.5 μg total RNA and oligo-(dT)12–18 primers using PowerScript™ Reverse Transcriptase (BD Bioscience Clontech, Tokyo, Japan) according to the manufacturer’s protocol. The total volume was 10 μl. Following RT, 0.5 μl reverse transcription solution was used for PCR amplification. Separate reactions were carried out for each cDNA with rTaq™ DNA polymerase
(Takara Bio Inc., Otsu, Japan) according to the manufacture’s instructions. After the initial denaturation for 1 min at 94°C, for S17 and AR, the amplification followed by 35 to 40 cycles of 94°C for 15 sec, 55°C for 20 sec, 72°C for 30 sec on each tissue sample. A linear range of amplification was determined by first carrying out PCR reactions at the different number of cycles (between 30 and 50 cycles). And to confirm the accuracy of the semi-quantitative RT-PCR method, we measured the protamine mRNA levels in the testis by RT-PCR as protamine mRNA levels in the testis correlated with the development of testis described by Chairun Nisa et al. (2003). For protamine, the amplification followed by cycles of 31°C for 3 sec, 72°C for 1 sec, 100°C for 0.5 sec and 72°C for 30 sec. All PCR-amplified fragments were run on 1.5% agarose gels stained with ethidium bromide. Gels were photographed using the Printgraph™ imaging system (ATTO Co., Ltd., Toyko, Japan). A semi-quantitative measurement of specific mRNA expression was obtained using the ImageJ 1.29x (NIH), which quantifies the density of bands on the gel. For AR, S17 and protamine mRNA, the sequences of the forward and reverse primers used were listed in Table 1.

Statistics

All data were analyzed using one-way analysis of variance followed by Newman-Keuls multiple comparison test. Significance was achieved when P < 0.05.

Results

Cloning of AR

Figure 1 shows coding sequence of the quail AR (accession number, AB188828). The partial cDNA contains 1385 bp sequence and encodes the 343 pre-
dicted amino acid residues. Full length AR cDNA contains total amino acid residues of 919 and 902 in human (Lubah et al., 1988) and rat (Chang et al., 1988), respectively. According to vertebrates AR, the quail amino acid residues consisted of partial DNA-binding and hormone-binding domains and hinge. Assuming the same number of DNA-binding domain as those of human and rat (68 amino acid residues), the DNA-binding domain of quail AR (48 amino acid residues) shows 70.6% of full length. The hormone-binding domain of quail AR includes 253 amino acid residues which is the same as those of human and rat (Chang et al., 1988). Comparison of the quail AR amino acid residues to those of other vertebrates are shown in Fig. 2. The overall identity of their amino acid residues was 94.9, 89.2, 88.9, 82.7 and 64.1% in canary, human, mouse, frog and eel protein, respectively.

**Body Weight and Cloacal Gland Protrusion Area**

Figure 3a shows the weekly changes in body weights between 6 and 9 weeks. The body weights remained relatively stable in the LL and CAS groups between 6 and 9 weeks. However, in the SD group, body weights at 9 weeks old increased from those at 6, 7 and 8 weeks old. They were higher than in other groups (P < 0.05).

Figure 3b shows the weekly changes in cloacal gland protrusion area between 6 and 9 weeks old. In LL group, the cloacal gland protrusion area significantly increased at 7 weeks old and high levels were sustained until 9 weeks old. In SD group, the area decreased significantly at 8 and 9 weeks old as compared to 6 weeks old. In CAS group, the area rapidly decreased at 7 weeks old and the low levels were maintained at 8 and 9 weeks old. The areas in LL group were significantly larger at the corresponding weeks of 7 to 9 than those in the other groups (P < 0.05).

**mRNA Expression of Protamine and AR**

Figure 4 shows changes in protamine and AR mRNA levels in testis of the quail under different day-lengths. Protamine mRNA level significantly increased 3 weeks after long days treatment, while the expression level decreased 3 weeks after short days treatment. On the other hand, testicular AR mRNA levels did not change after long or short days treatment (P < 0.05).

Figure 5 shows changes in AR mRNA levels in reproductive organs and kidney under different conditions. AR mRNA levels in the cloacal gland did not change 3 weeks after long or short days treatment, but increased after castration. AR mRNA levels in epididymis and vas deferens increased after long days treatment, but those in 9SD and 9CAS groups did not change from 6LL. In kidney AR mRNA levels in 9LL group were significantly higher.
This study revealed nucleotide sequence of the partial length of quail AR cDNA encoding the 343 predicted amino acid residues. The full length of human and rat includes the 919 and 902 amino acid residues, respectively. The encoded protein has a part of DNA-binding domain (70.6% of predicted full length) and full length of hormone binding domain. The predicted amino acid residues had a high homology to other vertebrates. Particularly, the homology of DNA binding domain is identical to all of the mammalian AR homologs (Gaspar et al., 1990). The DNA-binding domain of AR is required for binding to specific DNA element of target gene. The hormone-binding domain is also well conserved in amino acid residues among other vertebrates. The first step for biological action of androgen must be the binding of androgen to this region of AR. These high conservations of the DNA- and hormone-binding domains indicate the structural and functional constraints on these regions are quite strong throughout the species.

This study also clearly demonstrated AR mRNA expression in reproductive tissues (i.e. testis, epididymis, vas deferens and cloacal gland) of quail. The epididymis and vas deferens are androgen dependent organ whose structure and functions such as acquisition of sperm maturation and attainment of fertilizability of spermatozoa (Munro, 1938; Fujihara and Nishiyama, 1976; Lofts and Massa,
and cloacal gland is a unique organ with a large amount of foam whose production is the androgen dependent and which is transferred to the female during copulation, enhancing male fertilization success (Coil and Wetherbee, 1959). In addition, AR mRNAs were observed in kidney of quail in this study as seen in the rat (Lubahn et al., 1988). Although little is known about the function of androgens in the kidney of quail, androgens stimulate generation of two organic anion transporters, OAT1 and OAT3, which are p-aminohippurate (PAH) transporters in rat (Ljubojevic et al., 2004).

This study confirmed our previous study using Northern blot analysis that protamine mRNA expression in testis is up-regulated by long days, whereas it is down-regulated by short days (Chairun Nisa et al., 2003). It is known that protamine expression is regulated by androgen through AR binding in rat testis, since lowered protamine expression by administration of cyproterone acetate (CPA), an androgen receptor blocker (Aleem et al., 2005). In contrast, testicular AR mRNA expression was not affected by long and short days. Such an unrelated phenomenon is also observed in the cloacal gland. AR mRNA expression in the gland were not affected by long and short days, although cloacal gland protrusion area increased or decreased, respectively. On the other hand, although castration caused regression of cloacal gland protrusion area, AR mRNA expressions markedly increased. The results indicate no correlation between cloacal gland protrusion area and AR mRNA expression. These reason for this discrepancy cannot be explained fully, but high correlation between concentration of testosterone in plasma and cloacal gland protrusion area under any circumstances such as shot days or castration treatments suggests that testosterone concentrations in plasma may account in large part for

![Graphs showing changes in AR mRNA levels](image-url)

**Fig. 5.** Changes of AR mRNA levels in the cloacal gland (a), epididymis (b), vas deferens (c) and kidney (d) at 6 weeks old (6LL), and in LL group (9LL), SD group (9SD) and CAS group (9CAS) at 9 weeks old. Results are represented at the means±SEM (n=5-7) relative values to the means of 6 weeks old. Means with different letters are significantly different (P<0.05).
degree of development of the cloacal gland. In another word, nuclear AR may not be a limiting factor for the cloacal gland protrusion area.

In spite of no response of AR mRNA expressions in cloacal gland and testis to the lighting manipulations, AR mRNA expressions in both epididymis and vas deferens were up-regulated by long days and the increase was in parallel with both cloacal gland protrusion area and testis weight. In these tissues, the activity such as sperm maturation and fertilizability of spermatozoa may be largely mediated by functional AR.

References


Gaspar ML, Meo T and Toshi M. Structure and size distribution of the androgen receptor mRNA in wild-type and Tfm/y mutant mice. Molecular Endocrinology, 4, 1600–1610. 1990.


Sachs BD. Photoperiodic control of reproductive behavior and physiology of the male Japanese quail (Coturnix coturnix japonica). Hormones and Behavior, 1, 7–24. 1969.
