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Expression of Protamine mRNA in Relation to Spermatogenic Activity in Japanese Quail

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This study was conducted to elucidate relationship between testicular protamine mRNA expression and spermatogenic activity in the quail as the following experiments. (1) Quail were raised under continuous light condition after hatching and body weights, testis weights, spermatogenic activity and protamine mRNA levels were determined weekly between 3 and 8 weeks. (2) The other group of quail raised under continuous light condition up to 6 weeks after hatching were kept in either continuous light or reduced light-length (8-hour light, 16-hour darkness) conditions for 3 weeks. At 6 and 9 weeks old, the same parameters were measured as in the foregoing experiment. Spermatogenic activity was evaluated by Bartholomew's classification after histological examination of the testis and mRNA expression of testis protamine was analyzed by Northern blot. Between 3 and 5 weeks old, body weights, testis weights, and cloacal gland protrusion areas significantly increased and thereafter they stayed at plateau levels. Spermatogenic activity markedly increased from stage II to VI between 3 and 5 weeks old and stayed at stage VI up to 8 weeks old. Spermatids and immature sperm were firstly observed at 4 weeks old. Protamine mRNA levels were also first detected at 4 weeks old and increased at 5 weeks old. A marked expression were maintained after 6 weeks old. In contrast, short daylengths treatment after 6 weeks old significantly reduced testis weights, cloacal gland protrusion areas, spermatogenic activity, and protamine mRNA levels at 9 weeks old ($P < 0.05$) when compared to those raised under continuous light condition. Spermatogenic activity dropped from the full activity of stage VI to elongated spermatid (IV) or spermatocyte (II-III) stage after the treatment. Protamine mRNA levels also significantly decreased in response to short daylengths. These results suggest that protamine mRNA expression is highly related to spermatogenic activity in the quail.

Key words : Protamine mRNA, spermatogenesis, quail, short day treatment, testis

Introduction

In the course of spermiogenesis, the last process of spermatogenesis, spermatids remarkably changes in shape, from round cells to spermatozoa with species-specific head and filiform tail. At this period, nucleoproteins are replaced from histones to

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protamines. According to a model of DNA conformation in late spermatid in mammals, protamines by their highly basic characteristics of arginine-rich amino acid sequence and cysteine potentiate them to package DNA into a very condensed doughnut loop (Balhorn, 1982 ; Ward, 1993). In relation to histone-protamine replacement, stringent timetable of post-meiotic exclusive protamine gene expression has been extensively studied in mice (Balhorn *et al.,* 1984 ; Hecht and Penshow, 1987 ; Mali *et al.,* 1989).

In birds, unlike in mammals, information about protamine gene is still meager. Most of the knowledge has been derived from studies on chicken and quail. Although protamine in chicken (Oliva and Dixon, 1989) and quail (Oliva *et al.,* 1989) have no cysteine residue, the proposed mammalian DNA conformation may be applied in birds since other conserved motifs and arginine clusters are present as reviewed by Oliva and Dixon (1991 a). *In situ* hybridization study (Oliva and Dixon, 1991 b) and Northern blot analysis (Oliva *et al.,* 1988) demonstrated that, like in mammals, the protamine mRNA was transcribed at post-meiotic stages of spermatogenesis in the chicken. In quail, Oliva *et al.* (1989) found 400-base protamine transcripts by Northern analysis, but they have not shown a relationship between protamine expression and spermatogenic activity under more physiologically different conditions. Therefore, the aim of this study was to reveal changes of protamine mRNA levels of testes in relation to the changes in spermatogenic activity during the growing stage and those in response to short daylengths treatment that manipulates spermatogenic activity in quail.

Materials and Methods

Animals

Sex-linked, fertile eggs were obtained by crossing black female ($Z^{B}W$) with brown male ($Z^{b}Z^{b}$), which were purchased from Tokai Yuki (Toyohashi, Japan) and kept in pairing under 14 h light (lights on, 6 : 00) and 10 h darkness (lights off, 20 : 00) regimen (14L10D). The eggs were incubated at 37.5℃ in a commercial incubator using standard conditions. Male chicks were provided with water and food *ad libitum.* From 37℃ at hatching time, temperature were gradually decreased by 3℃ for the first week and 4℃ each weekly until reached the ambient temperature of 22℃ at 4 weeks old.

Experiment 1 : Weekly changes in protamine mRNA levels of growing quail

Thirty quail were subjected to continuous light regimen from hatching to the tissue collection time. Body weights were measured by digital balance and width and length of cloacal gland were measured using a ruler as described by Sachs (1969) to get cloacal gland protrusion area (CPA) value. Five birds were weekly sacrificed from 3 to 8 weeks by decapitation. The left testis was weighed and cut into 3 parts. The middle portion was fixed with Bouin's solution, the remaining portions were snap-frozen in liquid nitrogen and stored at −80℃ until the day of RNA extraction.

Experiment 2 : Changes in protamine mRNA levels under short daylengths

Fifteen quail were raised under continuous light regimen (LL) for the first 6 weeks after hatching. Five birds of the first group (6LL) were sacrificed at 6 weeks old and the remaining birds were divided into 2 groups, kept either under continuous light (9

LL) or short daylengths regimen of 8 h L : 16 h D (9SD) for 3 weeks and all birds were sacrificed. The measurement of parameters and tissue collection were same as the experiment 1.

Histological assessment

The fixed tissues were trimmed, placed into a tissue-processing cassette (Histosette I, Simport Ltd., Canada), and rinsed several times by 70% ethanol. Dehydration steps were applied using increasing ethanol series, immersed in methyl benzoate, clarified with xylene and embedded in paraplast$^+$ (Oxford labware, USA). The continuous cross sections of 5μm thickness were mounted to the slides, stained with haematoxylin eosin, and observed under light microscope. Spermatogenic activity was put into one of stages proposed by Bartholomew (1949), namely stage I-resting spermatogonia only ; stage II-spermatogonia dividing, but only a few spermatocytes present ; stage III-many spermatocytes ; stage IV-spermatocytes with spermatids ; stage V-spermatids with a few sperm ; and stage VI-full spermatogenic activity with many sperm.

Probe preparation

The total RNA was extracted from adult quail testis using TRIZOL (Gibco-BRL, Life Technologies, Rockville, MD) based on the method described by Chomczynski (1993). Total RNA (5μg) of testis was denatured at 65℃ for 10 minutes with oligo dT primers and reverse-transcribed with 100 units of SuperScript II (Gibco-BRL, Life Technologies, Rockville, MD) in a 10-μl mixture. Based on database of quail protamine (Oliva *et al.,* 1989), primers were designed as follows, sense primer 5′-ACG CCG CCG CTA CGG GAG GT-3′ and antisense primer 5′-CCC GGT GCC ACG GCA GTT TAT-3′. First strand cDNA were synthesized and subjected to 30 cycles of PCR amplification using Taq polymerase (Takara, Tokyo, Japan) with total volume of 10μl. The cDNA was denatured at 97℃ for 1 minute, 5 cycles of 97℃ for 5 sec and 72℃ for 30 sec, 5 cycles of 97℃ for 5 sec, 65℃ for 10 sec and 72℃ for 30 sec, 30 cycles of 97℃ for 5 sec, 55℃ for 10 sec and 72℃ for 30 sec, and finally 72℃ for 10 minutes. Using the same methods, cDNA for S17 gene was synthesized using following primers, sense primer 5′-GGC GCG GGT GAT CAT CGA GAA-3′ and antisense primer 5′-GAG AGC GCC TCG CGG CGT TT-3′. The PCR products were then separated on 1× TAE agarose gel, and the DNA fragment of interest was cloned into pGEM-T Easy (Promega, Madison, WI). DNA sequencing was performed on plasmids using dye terminator chemistry on an applied chain-termination method (Sanger *et al.,* 1977).

Northern blot analysis of quail protamine mRNA

Total RNA (10μg) was denatured with formaldehyde at 65℃ and electrophoresed with 1×MOPS in 1.5% (w/v) agarose gel containing 2.2 M formaldehyde for 2 and half hours at 50 V. After ethidium bromide staining, the bands of 28S and 18S ribosomal RNA (rRNA) were observed to check the integrity of RNA. After washing by DEPC-treated water, the RNA was then blotted onto a Hybond N$^+$ membrane (Amersham Inc., U.K.) using capillary action with 10×SSC (1×SSC=0.15 M sodium chloride, 0.05 M sodium citrate pH 7.0) for 16 hours. The membrane was air-dried and cross-linked using a UV cross-linker (Stratagene, La Jolla, CA), prehybridized at 42℃ for 1 hour, and probed with ^{32}P-labelled-quail protamine cDNA prepared using a

BcaBest labeling kit (Takara, Tokyo, Japan). The hybridization was performed at 42℃ for 18 hours in a solution mixture of 50% formamids, 5×Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinilpyrrolidone, 0.1% BSA), 0.1% SDS, 5×SSPE (750 mM NaCl, 5 mM EDTA, 50 mM NaH_2PO_4 $2H_2O$) and sonicated salmon sperm DNA (200 μg/m*l*). To remove non specific radioactive probe, the membrane was washed at 65℃, 2 times of 15 minutes with 2×SSC, 0.1% SDS, and once with 1×SSC, 0.1% SDS. Finally, membrane was exposed to the Image Plate (Fuji Photo Film Co., Ltd. Japan) for 24 hours and the radioactive signals were quantified using BAS-2000 Bio-Imaging Analyzer. All membranes were rehybridized by S17 ribosomal protein mRNA as internal control.

Statistical analysis

Data were presented as means ± S.E.M. Statistical significance of the differences between groups were examined by one-way ANOVA and Tukey-Kramer test or Student's *t*-test (Zar, 1999).

Results

Experiment 1 : Weekly changes in protamine mRNA levels of growing quail

Weekly changes in body weights, testis weights, and cloacal gland protrusion areas between 3 and 8 weeks old were shown in Fig. 1. The average body weight at 3 weeks was about 64 g and it increased significantly to 88 g at 4 weeks and 99 g at 5 weeks ($P < 0.05$). Thereafter, it remained unchanged up to 8 weeks. At 3 weeks, the average weight of the left testis was about 72 mg. It significantly increased weekly up to 5 weeks ($P < 0.001$). The average weights were 452 mg and 1,414 mg at 4 weeks and 5 weeks, respectively, but stayed at a plateau after 5 weeks. Average protrusion area was only 32 mm^2 at 3 weeks but it increased to 101 mm^2 at 4 weeks and 177 mm^2 at 5 weeks. No marked changes were found thereafter.

The histology results were shown in Fig. 2. At 3 weeks, the spermatogenic activity of all birds was at stage II and most developed tubuli at this age contained spermatogonia and primary spermatocytes (Fig. 2A, G). The border between tubuli was obvious and the size of tubuli was relatively small but the intertitial area was relatively broad. Neither sperm nor spermatids were observed. At 4 weeks, a few sperm have already appeared in some lumen of tubuli seminiferi of all birds (Fig. 2B, H). Hence the spermatogenic activity was classified at stage V. Comparing to those of 3 weeks, the tubuli became larger. The height of the epithelial seminiferi and the number of primary spermatocytes increased. Sertoli cells were dispersed among spermatogenic cells. Round spermatids and sperm were observed. This spermatogenic activity was more distinct at 5 weeks when all birds were at stage VI, showing full spermatogenic activity with more sperm in the lumen and the adluminal areas (Fig. 2C, I). Round spermatids and primary spermatocytes were more abundant. Between 6 and 8 weeks, the spermatogenic activities stayed at stage VI and higher densities of sperm in the lumen and the adluminal areas were observed (Fig. 2D-F, J-L).

Northern blot analysis of protamine transcript at about 400 bases were shown in Fig. 3 A and changes of protamine mRNA levels in testes were shown in Fig. 3 B, as

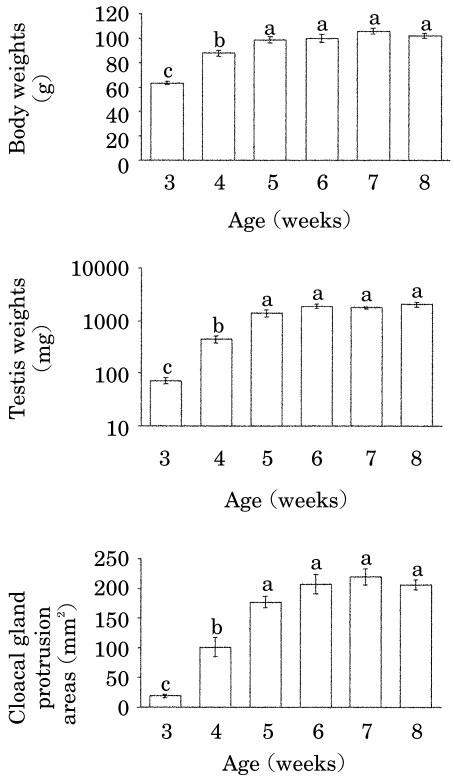

Fig. 1. Changes of body weights, testis weights, and cloacal gland protrusion areas of growing quail. Results were represented as means±SEM (n=5). Note log transformation for testis weights. Means with different letters are significantly different (body weights at $P<0.05$, testis weights at $P<0.001$ and cloacal gland protrusion areas at $P<0.01$).

relative values to the mean value of 6 weeks. Protamine mRNA levels was undetectable at 3 weeks but was detected at 4 weeks and gradually increased to remarkable levels at 6 weeks and thereafter.

Experiment 2 : Changes in protamine mRNA levels under short daylengths

The changes of testis weights and protrusion areas of cloacal gland under different daylengths were shown in Fig. 4. Data were presented as relative values to those at 6 weeks old. Average testis weight of 9LL group (2,082 mg) was significantly different ($P<0.05$) to that of 9SD group (67.6 mg). The difference between the average cloacal gland protrusion area of 9LL group (240.35 mm^2) and 9SD group (84.5 mm^2) were also significant ($P<0.05$).

Histology of testes of quail under different daylengths was depicted in Fig. 5. Full spermatogenic activity (stage VI) was found in all birds at 6 weeks old (Fig. 5A, 6LL). Primary spermatocytes and round spermatids were observed with abundant immature sperm. Full spermatogenic activity was maintained after 3 weeks with continuous illumination (Fig. 5B, 9LL). In contrast, 3 weeks after short daylengths (Fig. 5C, 9

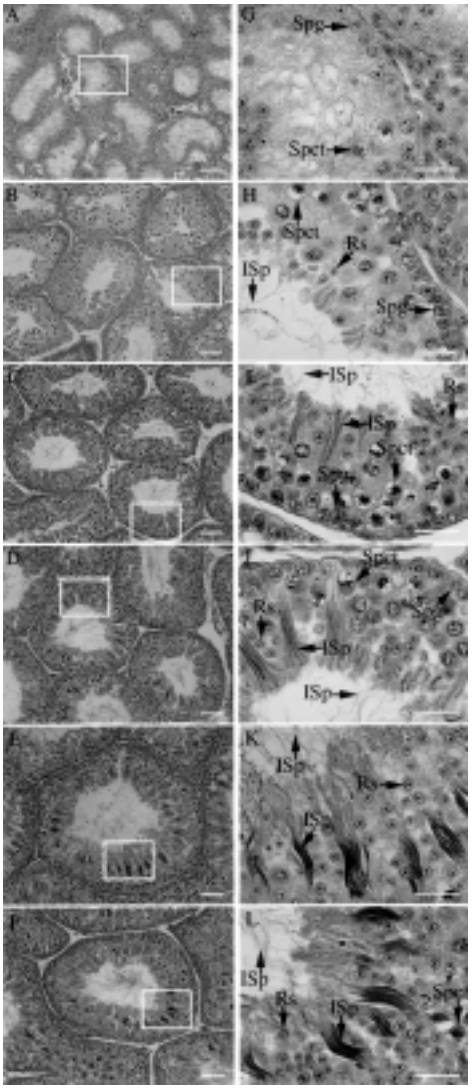

Fig. 2. Weekly histological changes of testes of growing quail of 3 to 8 weeks old. The marked areas in A-F were magnified orderly in G-L sections. Arrows show spermatogonium (Spg), primary spermatocyte (Spct), round spermatid (Rs), and immature sperm (ISp). Scale bars=50μm (A-F) and 20μm (G-L).

SD), their spermatogenic activity dropped from mature stage VI to immature stage. Several round spermatids and elongated spermatid (stage IV) were still observed as the most advanced germ cells in some tubuli seminiferi in 2 of 5 birds (40%). In 3 other birds (60%), primary spermatocytes but none of round spermatids and sperm was observed (stage II-III).

Northern blot of protamine transcripts and changes of protamine mRNA levels in testis of the quail under different daylengths were shown in Fig. 6. Although a weak

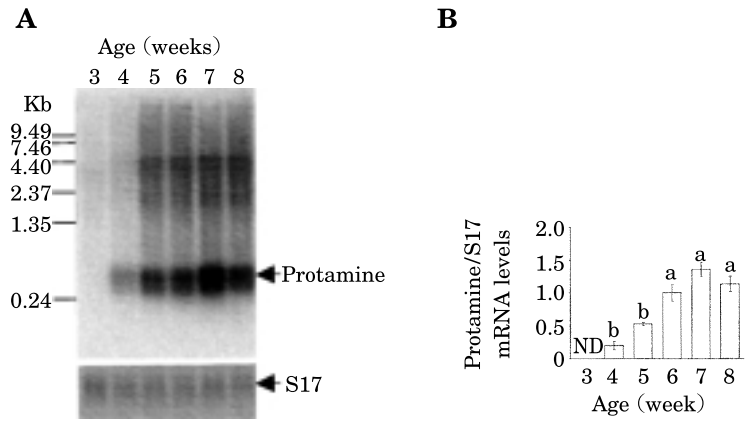

Fig. 3. Northern blot analysis of protamine mRNA expression in testes (A) and quantification of specific protamine mRNA signals (B). Results were represented as means±SEM (n=5). Means with different letters are significantly different (at $P<0.05$).

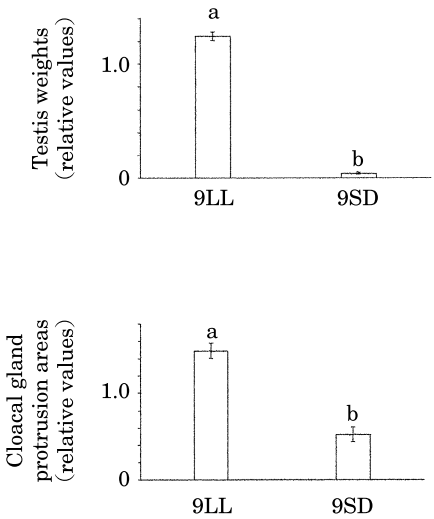

Fig. 4. Changes of testis weights and cloacal gland protrusion areas after different daylengths treatment in quail. Results were represented as means±SEM (n=5), relative value to mean of 6 weeks old. Means with different letters are significantly different (testis weights at $P<0.001$ and cloacal gland protrusion areas at $P<0.01$).

signal of protamine mRNA was still detected in 2 of 5 birds of 9SD group which displayed stage IV in the testis histology, the average protamine mRNA levels was markedly decreased when compared to those in 9LL group ($P<0.05$), because the 3 other birds did not show any protamine mRNA expression.

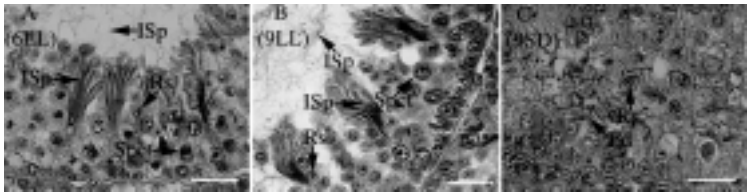

Fig. 5. Histological changes of testes of quail under different daylengths. A : 6LL, 6 weeks old ; B : 9LL, 9 weeks old in continuous light ; C : 9SD, 9 weeks old (quail kept in short day for 3 weeks from 6 weeks old). Other legends are same as in Fig. 2., except elongated spermatid (Ed). Scale bars＝20μm.

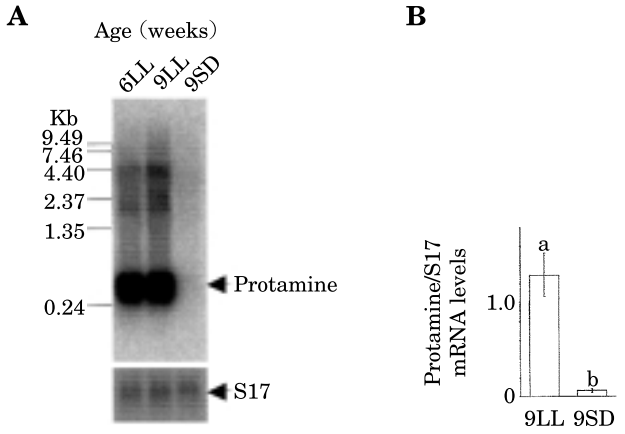

Fig. 6. Northern blot analysis of protamine mRNA expression in testes of quail under different daylengths (A), and quantification of specific protamine mRNA signals (B). Same legends for 6LL, 9LL and 9SD as in Fig. 5. Results were represented as means±SEM (n＝5) relative values to the mean of 6 weeks old birds (6LL). Means with different letters are significantly different (at $P<0.05$).

Discussion

The present study confirmed the marked increase in cloacal gland development as an androgen-dependent tissue between 3 and 5 weeks old. Synchronously, dramatic increase of testis weights occurred at this period. Basically our result was in agreement with the growing pattern of both parameters in quail in the previous study (Siopes and Wilson, 1975).

Histological analysis of gonadal development in the present study are comparable to the previous study reported by Mather and Wilson (1964). Spermatogenic activity progressed from stage II to stage VI between 3 and 5 weeks old. At 3 weeks no round spermatid was observed. Since there was no protamine mRNA signal was detected at 3 weeks, it may suggest that, in quail, protamine gene is not transcribed in

spermatogonia, and primary spermatocytes as shown by Northern blot analysis in the chicken (Oliva *et al.,* 1988). At 4 weeks old, all birds have progressive spermatogenic activity with sperm formation. At this time, round spermatid and sperm appeared and weak signals of protamine mRNA were first detected. Furthermore, the average protamine mRNA levels increased markedly at 6, 7, and 8 weeks old coincidently with full spermatogenic activity.

The experiment 2 of this study was undertaken to correlate the protamine mRNA expression with a drop in spermatogenic activity that was manipulated by short daylengths treatment. In addition to hormonal control (Yoshimura *et al.,* 2000 ; Maeda and Yoshimura, 2002), it has been established that quail daylengths is one of environmental factors important for reproduction in quail (Hashiguchi *et al.,* 1977 ; Kobayashi *et al.,* 1989). Plasma testosterone and dihydrotestosterone rapidly diminished in adult male quail when they were transferred from long to short days (Delville *et al.,* 1985) and decreases in daylengths causes regression of the testis weights (Sachs, 1967 ; Eroschenko, 1974 ; Delville *et al.,* 1985), area of cloacal gland (Sachs, 1967 ; Delville *et al.,* 1985), an androgen-dependent gland (Kaku *et al*, 1993), and spermatogenic activity (Eroschenko, 1974). Basically, our results confirmed this rule. After three weeks of reduced hours of daylengths, seminiferous tubules of regressed testes decreased complexity and the thickness of germinal epithelium. In the present study, all birds of 9SD group were out of reproductive stage since no sperm was observed in the testis. However, the levels of regression of spermatogenic activity were variable between stages II and IV among individual birds. Testes with lower spermatogenic activity (stage II-III) showed no protamine mRNA expression, whereas those with higher activity (stage IV) showed weak expression.

In conclusion, the present study suggests that testicular expression of protamine mRNA is positively and highly correlated with spermatogenic activity in quail.

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