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A Long-day-stimulus Induced the Expression of c-Fos-like Molecules in the Hypothalamus of Japanese quail

Keiko Okano[1#], Toshiyuki Okano[2], Tadashi Oishi[1], Yoshitaka Fukada[2*]

[1]Graduate School of Human Culture, Nara Women's University, Nara 630-8506, Japan, [2]Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, Tokyo 113-0033, Japan.

In birds, the photoperiodic seasonal breeding involves encephalic photoreception at the initial step of triggering the well-known endocrinal cascade. Especially in Japanese quail (*Coturnix coturnix japonica*), the reproductive neuroendocrine function responds to a single long day, and hypothalamic regions are known to be important for the reproductive response. However, little is known about where and how the light and time signals are integrated to detect daylength information and transduced to the endocrinal responses. To gain insights into this issue, we are interested in the c-Fos expression in the hypothalamus of the Japanese quail. Meddle and Follett (1997) previously identified two hypothalamic regions where c-Fos-like immunoreactivities were induced in response to a long day by using an antibody to carboxyl terminal region of human c-Fos (Lys^{347}-Leu^{367}). In the present study, we used a different anti-c-Fos antibody recognizing a region from Lys^{128} to Ala^{152} of human c-Fos, and found in long-day- stimulated quails many c-Fos-like immunoreactive nuclei localizing within two regions, nucleus anterior medialis hypothalami and nucleus periventricularis hypothalami, which are distinct from those identified in the previous study. Then, we focused on the difference in the cross-reactivities of the antibodies used, and determined the whole coding sequence of quail c-Fos to compare the antigenic sequences of the two antibodies with the amino acid sequence of quail c-Fos. We found that the antibody we used would recognize quail c-Fos more specifically than that used in the previous study.

Key words: photoperiodism, c-Fos, encephalic photoreception, Japanese quail

INTRODUCTION

To study the mechanism of photoperiodic response, Japanese quail (*Coturnix coturnix japonica*) has been used as an excellent animal model for its highly sensitive photoperiodicity [1]. Previously, Meddle and Follett [2] have shown that c-Fos-like immuno-reactivity (IR) increased in two hypothalamic regions in the quail brain, the infundibular nucleus (IN) and median eminence (ME) in response to a long-day photoperiod (LD). *c-fos* gene is an immediate early gene involved in cellular responses common to a variety of extracellular stimuli [3]. In this study, we detected the induction of c-Fos-like IR in the other areas of the quail brain by using anti-c-Fos antibody recognizing a region from Lys^{128} to Ala^{152} of c-Fos. To persuade the reason for the difference between the previous report [2] and the present study, we determined the whole coding sequence of quail c-Fos to compare the antigenic sequences of the two antibodies.

*To whom correspondence should be addressed.
E-mail: sfukada@mail.ecc.u-tokyo.ac.jp
#present address: Dept. Appld. Phys. Chem & Dept. Univ. Electro-Communications, Chofu, Tokyo, Japan

MATERIALS AND METHODS

Animals

Male Japanese quails at the age of 6 weeks were purchased from a commercial poulterer. They were divided into five groups (termed L15, L18, D15, D18 and L6), maintained under short-day photoperiod (SD, 8:16 hr light/dark) for 5 weeks, and subjected to the following light-treatment at 11 weeks of age. Groups L15 and L18 were transferred from SD cycles to the constant light and the animals were killed 15 hr and 18 hr after the light-on, respectively (n=4 for each group). Groups D15 and D18 were kept in SD and the animals were killed 7 hr and 10 hr after the light-off (15 hr and 18 hr after the light-on), respectively (n=4 for each group). Group L6 was kept in SD and the animals were killed 6 hr after the light-on

(n=2). Animals used in this study were treated in accordance with the guideline of The University of Tokyo.

Immunocytochemistry

Twenty-μm-thick sections were cut out from the frozen brain, fixed with 4% paraformaldehyde dissolved in 0.2 M Na-phosphate (pH7.4) for 30 min. After washing with PBS (10 mM Na-phosphate, 140 mM NaCl, 1m M $MgCl_2$, pH 7.4), the sections were pretreated with a blocking solution (PBS containing 0.02% TritonX-100 and 1% goat normal serum) for 1 hr and incubated with a primary antibody at 4 °C overnight. The primary antibody used was anti- c-Fos antibody (sc-253; 1:2,000 Santa Cruz Biotechnology Inc., USA) corresponding to the amino acids 128-152 of human c-Fos. The sections were treated with a secondary antibody (biotinylated anti-rabbit IgG; Vector Laboratories, USA) for 1 hr. Immunolabeling was detected by using a Vectastain Elite ABC kit (Vector Laboratories) and the positive signals were visualized by diaminobenzidine.

Cloning and sequencing

The coding sequence of quail *c-fos* was determined by reverse transcription-polymerase chain reaction (PCR) using total RNA extracted from the quail cerebellum and by PCR using quail genomic DNA (K. Okano et al., unpublished).

RESULTS AND DISCUSSION

We found many c-Fos-like immunoreactive nuclei within two distinct regions, nucleus anterior medialis hypothalami (AM) and nucleus periventricularis hypothalami (PHN), in LD-stimulated animals (L15 [Fig. 1] and L18 [not shown]). We found no c-Fos-like IRs within these regions in dark kept animals (D15 and D18, not shown), and only a few nuclei were weakly immunoreactive in L6 animals (not shown). These results indicate that c-Fos-like IRs were induced in the AM and PHN not by light in daytime but by light extended to the early night. Incubation of sections with the affinity-purified rabbit IgG (sc-2027; Santa Cruz Biotechnology Inc.) instead of the anti-c-Fos antibody resulted in substantially no staining (not shown). As well as in the AM and PHN, c-Fos-like IRs were observed in various regions of the brain: hyperstriatum ventrale, nucleus accumbens, nucleus dorsolateralis anterior thalami, stratum griseum,

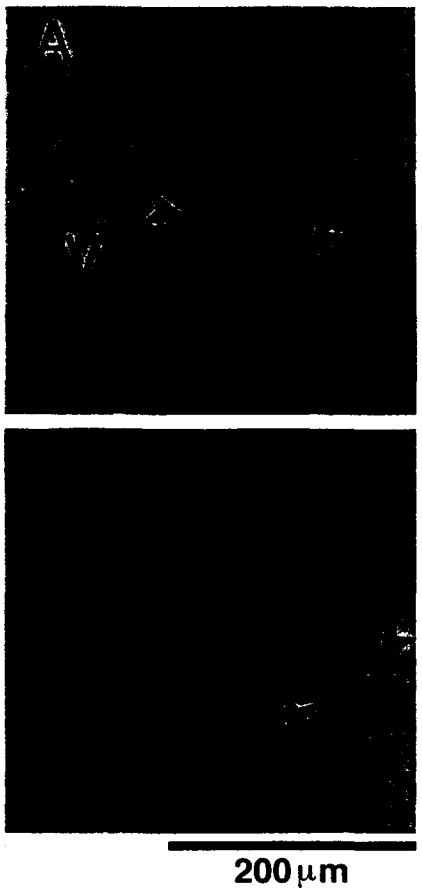

Figure 1. c-Fos immunopositive structures in the nucleus anterior medialis hypothalami (AM; panel A) and nucleus periventricularis hypothalami (PHN; panel B) of the quail brain. Intense immuno-reactive nuclei (arrowheads) were observed in the long-day-stimulated quails (group L15). V, third ventricle

substantia grisea centralis, IN and ME. However, the c-Fos-like IRs in these areas were not induced in a LD-specific manner. In contrast to the present observations, Meddle and Follett [2] had examined the LD-stimulated quails using a different anti-c-Fos antibody (9/3 antibody) developed by Sharp et al. [4], and shown that c-Fos-like IRs increased in the IN and ME where we did not find the log-day-induced c-Fos-like IRs.

To persuade the reason for the discrepancy between the previous report [2] and the present observation, we focused on the difference in the cross-reactivities (recognition sequences) of the antibodies used. The anti-c-Fos antibody used in our study was raised against 25 amino acids polypeptide near the basic region of

human c-Fos. On the other hand, the 9/3 antibody was raised against 20 amino acid-polypeptide (KGSSSNEP SSDSLSSPLLAL) that matches the carboxyl terminus of chicken c-Fos (KGSSSNEPSS DSLSSPTLLAL) except for the lack of one amino acid underlined.

In order to compare the antigenic sequences of the two antibodies with the amino acid sequence of quail c-Fos, we determined the coding nucleotide sequence of quail c-Fos cDNA. Quail c-Fos amino acid sequence was deduced, and it exactly matched the antigenic sequence of the sc-253 antibody. Thus, it seemed most likely that the molecule induced by LD stimulus in the AM and PHN is quail c-Fos. In the antigenic region of sc-253, however, other Fos family proteins (e.g. Fra-1 and Fra-2) retain amino acid sequences similar to the antigenic peptide, implying that sc-253 may cross-react with the other related proteins. In the antigenic region of 9/3 antibody, quail c-Fos also has the same amino acid sequences to the chicken c-Fos. However, 9/3 antibody was curiously developed against a 20-amino-acid peptide whose sequence lacks one amino acid in comparison with the carboxyl-terminal region of chicken c-Fos [4]. Thus the affinity of 9/3 antibody to chicken (and quail) c-Fos may not be higher than sc-253 antibody. In the carboxyl-terminal antigenic region, the amino acid sequence is conserved among several Fos-related proteins like the antigenic region of sc-253. Therefore it is also possible that 9/3 antibody cross-reacts with the other Fos-related proteins. Taking these considerations into account, we assume a possibility that the LD-induced IRs detected by 9/3 antibody may include IRs for another Fos-related protein. Alternatively, any differences in the experimental conditions including the strain of birds, ages, and stress may explain the apparent contradiction. Present cloning of quail *c-fos* gene will help further characterization of the LD-induced c-Fos-like IRs by approaches other than immunocytochemisty such as ribonuclease protection analysis and *in situ* hybridization.

In the quail, hypothalamic regions including the AM and PHN are essential for reproductive responses, and lesions of these sites totally block photoperiodic gonadal growth [5-8]. Also, electrical stimulation of the hypothalamus of immature birds induced the serection of GnRH [6] or LH [8]. It is hence possible that *c-fos* may trigger the photoperiodic induction of the neuroendocrine secretion, though there is no direct evidence for the involvement of *c-fos* in the photoperiodic response of birds. Not only Fos but also Jun family proteins, potential heterodimerizing partner of c-Fos, would be a fruitful target for the approach to the photoperiodic response in birds.

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