Successful Transfer of Exogenously Introduced Gene (*lacZ*/MiwZ and *lacZ*&GFP/*pkkv4-lacZ*) for Generations in Chicken

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Abstract. Exogenous genes (trial 1: *lacZ*/MiwZ and trial 2: *lacZ*&GFP/*pkkv4-lacZ*) introduced into germinal crescent region (GCR) of chicken embryos were confirmed to be successfully transferred to the gonads via primordial germ cells (PGCs) and remained for a long time after hatching. The injected DNA was also successfully migrated and incorporated into next generation (F1). The F1 chickens were raised until sexual maturation, then the female F1 chicken and normal males, the male F1 chicken and normal females were subjected to artificial insemination according to the routine methods. The collected fertilized eggs were incubated for 72h to obtain F2 generation and the F2 embryos were examined for the detection of introduced DNA. In trial 1, the embryos obtained from F1 female chicken were examined by X-gal staining method, and showed the expression of the *lacZ* gene. Furthermore, the presence of *lacZ* gene in the extracts from the embryos was also determined by polymerase chain reaction (PCR) analysis. However fertilized eggs obtained from F1 male chicken showed no expression and presence of the DNA. In trial 2, the expression of green fluorescent protein (GFP) gene in the F2 embryos was not detected under the fluorescent microscope. The embryos were, then, examined by X-gal staining method and no detection was observed. Finally, the presence of GFP gene in the embryo extracts was determined by PCR analysis, indicating the presence only in the embryos obtained from male F1 chicken. These results suggest that the exogenously introduced DNA was successfully transferred to the F2 generation of chicken.

Key words: Exogenous gene, Chicken, *lacZ*, GFP, Germinal crescent, Primordial germ cells.

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Since a complete culture system has been established for chicken embryos from the single cell stage to hatching [1], different trials have been done to introduce foreign genes into chicken embryos for the production of transgenic chicken. For example, using sperm cells as vectors [2] or microinjection methods [3-5]. In recent years, since the primordial germ cells (PGCs) are progenitor cells of spermatozoa or ova, it is useful to produce germline chimeric chickens, so PGCs have been used as vectors mainly [6-8]. In avian species,

PGCs concentrate in germinal crescent region (GCR) at early developmental stage [9, 10]. In our previous study, exogenous gene (*lacZ*/MiwZ) introduced into the GCR was successfully transferred to the gonad [11] and the DNA was remained for long time after hatching [12]. The DNA was also successfully transferred to the offspring [13]. On the other hand, another foreign gene (*lacZ*&GFP/*pkkv4-lacZ*) injected into the GCR was also detected in the embryos of offspring [14]. The existence of the transferred gene was detected by PCR analysis [14]. However, in our former experiments, the incorporation of exogenous gene into F2 generation has not been confirmed yet. In

this study, therefore, we tried to examine the incorporation of foreign genes into F2 generation of chicken.

Materials and Methods

Preparation of fertilized eggs

Fertilized eggs of Rhode Island Red were collected for 2 to 7 days after artificial insemination (AI).

Preparation of the DNA solution

In trial 1, the circular form MiwZ containing the *E. coli*- β -galactosidase (*lacZ*) gene under the control of β -action promoter [15] was employed. In trial 2, the circular form pkkv4-*lacZ* containing the *E. coli*- β -galactosidase (*lacZ*) gene under the control of CMV promoter and the green fluorescent protein (GFP) gene under the control of RSV promoter [14] was used.

In trial 1, the MiwZ DNA (6.25 μ g) was mixed with 22.5 μ l of transfection reagent (DOTAP/No1781995; Boehringer Mannheim, Germany), diluted up to 50 μ l with Hepes-buffered saline (HBS; 20 mM Hepes containing 150 mM NaCl, pH 7.4) in a polystyrene tube. In trial 2, the pkkv4-*lacZ* DNA (5.0 μ g) was mixed with 30 μ l of transfection reagent (DOTAP), diluted up to 150 μ l with HBS in a polystyrene tube. The transfection mixture was then incubated for 10–15 minutes at room temperature (22–25 C). A micropipet (G-1, Narishige, Tokyo, Japan), the tip of which was reformed to an outside diameter of about 40 μ m, was filled with the DNA solution prior to microinjection.

Microinjection of the DNA solution

Fertilized eggs were incubated at 38.5 C under a relative humidity at 60–70%. A window with 10–15 mm in a diameter was opened at the sharp edge of egg shell when embryos reached to the stage 9–11 [16] of the development. The DNA solution was injected into the GCR with the approximately 1.0 μ l in total volume. After the injection, the window was closed by adhesive tapes and the incubation was continued until hatching.

Preparation of fertilized eggs (F2) from F1 chicken

Hatched chicks were raised until the stage of sexual maturation. Female DNA-treated chicken

was mated with normal males to gain the fertilized eggs. The eggs were incubated at 37.8 C under a relative humidity of 60–70% until hatching. Hatched F1 chicks were raised until the stage of sexual maturation. Female F1 chickens were mated with normal males, and male F1 chickens were mated with normal females to obtain the fertilized eggs.

Detection of GFP gene expression in the embryos from F2 generation

In trial 2, the expression of GFP gene was observed under a fluorescent microscope. Fertilized eggs obtained from the F1 chickens were incubated at 37.8 C under a relative humidity of 60–70% for 72 h (stage 19–20 [16]). A window with 10–15 mm in a diameter was opened at the sharp edge of egg shell and the expression of DNA in the embryos were observed from the window under fluorescent microscope.

Detection of lacZ gene expression in the embryos from F2 generation

In trial 1, fertilized eggs obtained from the F1 chickens were incubated at 37.8 C under a relative humidity of 60–70% for 72 h (stage 19–20 [16]). Then, the embryos were removed from the yolk, washed with DPBS(-) (DULBECCO'S PBS TABLETS without Ca^{2+} and Mg^{2+} , No. 81726-001; Dainippon Pharmaceutical Co., Laboratory Products Division). The embryos were then fixed with 0.25% glutaraldehyde in DPBS (-) for 20 min at 4 C, washed three times with DPBS (-). The embryos were stained for 3–6 h or overnight at room temperature (22–25 C) with a staining solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.2 mM MgCl_2 , 0.1% TritonX-100 and 0.1% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, Sigma, USA) in DPBS (-) (pH 7.8).

In trial 2, after the detection of GFP expression, the embryos were removed from the yolk, washed with DPBS (-). The embryos were examined by the same X-gal staining method as mentioned above.

Detection of marker gene (lacZ and GFP) by PCR analysis

After X-gal staining, whole embryos were washed DPBS (-) and digested completely in lysis buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 100

Table 1. Detection of the foreign gene in F1 embryos

DNA-chicken No.	Number of positive for marker gene (%)
17 ()	7/42 (16.7)
18 ()	5/30 (16.7)
395 ()	7/34 (20.6)
398 ()	7/44 (15.9)
60 ()*	3/29 (10.3)
69 ()*	3/31 (9.7)
397 ()*	5/36 (13.9)
399 ()*	1/18 (5.5)

lacZ/MiwZ introduced chicken.

* *lacZ*&GFP/pkdv4-*lacZ* introduced chicken.

mM EDTA, 0.5% SDS and 0.5 ng/ml proteinaseK) at 50 C for overnight. The total DNA was extracted by the phenol-chloroform method. The PCR analysis was performed on 100 ng DNA sample in order to detect the presence of the marker gene. The sequences of the primers used for the detection of the *lacZ* gene were: 5'-AGA TGC ACG GTT ACG ATG C-3', 5'-GGT CAA ATT CAG ACG GCA AAC G-3' [17]. After initial denaturation step of 94 C for 2 min, 40 cycles of amplification were performed; DNA was denatured at 94 C for 30 s, annealed at 55 C for 30 s and extended at 72 C for 30 s. The reactions were then incubated at 72 C for 5 min. On the other hand, the sequences of the primers used for the detection of the GFP gene were: 5'-TCG TGA CCA CCC TGA CCT AC-3', 5'-TTC ACC TTG ATG CCG TTC TT-3' [14]. Forty cycles were performed: denaturation at 94 C for 1 min, annealing at 55 C for 1 min and extension at 72 C for 1 min. PCR products were separated on a 2% agarose gel and the bands were visualized under UV light after ethidium bromide staining.

Results

In trial 1, fertilized eggs (F2) obtained from F1 female chicken showed the expression of *lacZ* gene by X-gal staining (Table 2, Figs. 1-A, B). On the other hand, no expression was observed in control (Figs. 1-C, D). The introduced DNA was detected by PCR analysis in the extracts from the F2 embryo (Table 3, Fig. 2). In this experiment, the DNA band was also observed in the extracts from the embryos which indicated positive reaction to *lacZ* gene by

Table 2. Expression of *lacZ*/MiwZ in the embryos of F2 generation

F1 chicken No.	Fertility (%)	Expression of <i>lacZ</i> gene by X-gal staining (%)
97 ()	13/15 (86.7)	1/13 (7.7)
98 ()	15/17 (88.2)	1/15 (6.7)
90 ()	4/7 (57.1)	0/4 (0)

Semen from No. 90 male was diluted to be 1.0×10^8 /ml and artificial insemination (AI) was conducted to normal female chicken. Fertilized eggs were collected for 2 to 7 day after AI.

X-gal staining. On the other hand, fertilized eggs obtained from F1 male chicken showed no expression of *lacZ* gene in the embryos (Table 2) and the DNA was not detected by PCR analysis (Table 3).

In trial 2, fertilized eggs obtained from F1 female chicken denoted no expression of GFP gene in the embryos under a fluorescent microscope and no expression of *lacZ* gene was observed by X-gal staining (Table 4). The presence of introduced DNA was not also detected by PCR analysis (Table 5). Fertilized eggs obtained from the F1 male chicken expressed neither GFP nor *lacZ* gene (Table 4). However, the introduced DNA was detected by PCR analysis in the extracts from F2 embryos (Table 5, Fig. 2).

Discussion

Previously, it has been reported that the expression of exogenous DNA introduced into chicken embryos was gradually decreased and was not transmitted to next generation [8]. The same tendency was observed in our previous reports, though exogenously introduced DNA was clearly expressed in offspring and the presence of DNA was clearly confirmed by PCR analysis [13, 14]. In the present studies, the introduced DNA was transferred to F2 generation and the DNA was clearly expressed (Figs. 1-A, B). We have reported that in the embryos of offspring, the expression of introduced DNA was not detected in the whole embryos but in some parts of them, however, DNA expression was observed in considerably wide section of embryos (Figs. 1-A, B) suggesting that the introduced DNA might not be silenced in the

Fig. 1. Expression of *lacZ* gene by X-gal staining in the embryos of F2 generation. (A): the embryo obtained from No.98 (). (B): the embryo obtained from No.97 (). (C, D): Control. Bar=715 μ m.

Table 3. Detection of *lacZ*/MiwZ by PCR analysis in the embryos of F2 generation

F1 chicken No.	Number of embryos positive for <i>lacZ</i> (%)
97 ()	2/13 (15.4)
98 ()	2/15 (11.8)
90 ()	0/4 (0)

F2 embryos.

On the other hand, in trial 1, some of the embryos having no positive reaction to *lacZ* observation by X-gal staining showed the presence of introduced DNA by the method of PCR analysis. In trial 2, the same results were obtained. Judging from these results, the expression of marker gene might be suppressed during the embryonic development. In our previous reports, the same tendency was observed in the embryos of offspring [14].

Fig. 2. Detection of foreign genes by PCR analysis in the embryos of F2 generation. Upper: *lacZ*/MiwZ, Lower: *lacZ*&GFP/*pkkv4-lacZ*. P: positive control, N: negative control, the number: samples.

Table 4. Expression of *lacZ*&GFP/*pkkv4-lacZ* in the embryos of F2 generation

F1 chicken No.	Fertility (%)	Expression of GFP gene (%)	Expression of <i>lacZ</i> gene by X-gal staining (%)
51 ()	16/20 (80.0)	0/16 (0)	0/16 (0)
85 ()	15/15 (100)	0/15 (0)	0/15 (0)
36 ()	1/7 (14.3)	0/1 (0)	0/1 (0)
84 ()	4/7 (57.1)	0/4 (0)	0/4 (0)

Semen from No. 36 and No. 84 males were diluted to be 1.0×10^8 /ml and artificial insemination (AI) was conducted to normal female chicken. Fertilized eggs were collected for 2 to 7day after AI.

Table 5. Detection of *lacZ*&GFP/*pkkv4-lacZ* by PCR analysis in the embryos of F2 generation

F1 chicken No.	Number of embryos positive for GFP (%)
51 ()	0/16 (0)
85 ()	0/15 (0)
36 ()	1/1 (100)
84 ()	2/4 (50.0)

In trials 1 and 2, no expression and no presence of marker genes were detected in some F1 chickens (No. 90, 51 and 85). No. 90, 51 and 85 were obtained from No. 18, 397 and 399 DNA-chickens, respectively. The F1 embryos incorporated exogenous genes were obtained from all DNA-chickens (Table 1). However, these F1 chickens might be wild type with no foreign genes. Therefore, no positive reaction might be detected in F2 embryos.

In the most previous studies on the introduction of exogenous gene into chicken, fertilized ovum (single-cell stage) was obtained from the magnum of the oviduct by a surgical operation [3, 4]. However, recently, the exogenous gene introduced into the central area of the germinal disc of freshly laid eggs was successfully incorporated into the gonads [5]. It has also been reported that the injection of foreign DNA into the GCR was successfully incorporated into PGCs and transferred into the gonads [11, 12]. Using these methods, therefore, enable us to avoid the problem that laying hens should be sacrificed for each experiment. In this study, the exogenous DNA introduced into GCR of early chicken embryos was transmitted to F2 generation as well as to offspring. The present method described here might be enable

us to produce transgenic chicken more effectively.

In case of the transgenic mice incorporated with GFP gene, green fluorescence was observed in various tissues [18, 19]. In these reports, they used β -actin promoter in recombinant plasmid. On the other hand, in this study, RSV promoter used, then, it seems that the difference of enhancer/promoter led to no expression of GFP gene. In trial 1, *lacZ* / *MiwZ* built-in β -actin promoter might be effective to detect the expression of DNA in F2 generation. Judging from these results, β -actin promoter may be useful for the detection of foreign gene expression. It may be needed to try to do the experiment using *pkkv4-lacZ* replaced RSV promoter with β -actin promoter. If chicken incorporated with GFP gene was produced, much more earlier detection of transgenic chicken may be possible by observing live embryos under a fluorescent microscope.

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