Expression of exogenous DNA in the gonads of chimaeric chicken embryos produced by transfer of primordial germ cells transfected *in vitro* and subsequent fate of the introduced DNA

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The expression and fate of exogenous DNA (*lacZ* gene), introduced into the gonads of chimaeric embryos and chickens that had been produced by transfer of primordial germ cells (PGCs) transfected *in vitro*, were examined. PGCs obtained from embryonic blood were transfected *in vitro* by lipofection and transferred to the partially sterilized recipient embryos. Expression of the *lacZ* gene was observed in the gonads of chimaeric embryos incubated for 3 days after the PGC injection (71.2%, 37/52). Introduction of the *lacZ* gene into the gonads of chimaeric embryos was confirmed by PCR analysis. The percentage of embryos with gonads positive for the *lacZ* gene was 95% (38/40) after 3 days of incubation after the PGC injection. The *lacZ* gene, however, appeared to persist episomally but was gradually lost during embryonic development. After 17 days of incubation after the PGC injection, the *lacZ* gene was detected in only 14.3% (3/21) of the embryos examined. Although the *lacZ* gene was detected in the gonads of two hatched chicks (11.1%), it was not detected in the gonads of chimaeric chickens at sexual maturity. Offspring derived from the lipofected PGCs were obtained from the chimaeric chickens at frequencies of 12.1–69.9% in males and 71.6–97.6% in females. The technique developed in the present work could be used to test the expression of exogenous DNA in the gonads of early chicken embryos and should facilitate the production of transgenic chickens.

Introduction

The production of transgenic chickens is useful for biological research as well as for the genetic improvement of poultry. Although many transgenic mammals have been produced successfully (Wall, 1996), production of transgenic chickens still faces considerable difficulties because of the yolk-laden structure of the ovum and the unique reproductive system of birds (Perry and Sang, 1993; Sang, 1994; Naito, 1996, 1997). Various attempts have been made to produce transgenic chickens; however, non-retroviral methods are preferable for applying the transgenic techniques to practical poultry breeding programmes. DNA transfer into chickens has been achieved by microinjection into the germinal disc of the fertilized ovum (Naito *et al.*, 1994a), transfection of the blastoderm (stage X, Eyal-Giladi and Kochav, 1976) *in ovo* (Kopchick *et al.*, 1991) and ballistic transfection of primordial germ cells (PGCs) *in ovo* (Li *et al.*, 1995). However, the DNA introduced into chickens persisted episomally and disappeared during the life of the chickens. Integration of exogenous DNA into the chromosomes of chickens has recently been achieved by the microinjection method (Love *et al.*, 1994). Although the efficiency of germline transmission and chromosomal

integration of the introduced DNA are reasonable, the number of fertilized ova that can be manipulated in each experimental session by this method is limited.

For modifying a specific location of the chicken genome, it is necessary to establish a culture of stem cells maintained *in vitro*. However, to date, it has not been possible to establish stem cells that give rise to viable offspring by way of germline chimaeric chickens. Blastodermal cells (stage X, Eyal-Giladi and Kochav, 1976) or PGCs cultured for only a few days *in vitro* have been incorporated into the germline of recipient embryos (Chang, 1995; Etches *et al.*, 1996; Pain *et al.*, 1996). Transfected blastodermal cells were injected into recipient embryos at the same developmental stage and the expression of an introduced *lacZ* gene was observed after incubation for 2–3 days (Brazolot *et al.*, 1991; Fraser *et al.*, 1993); but it was unclear whether the successfully transfected blastodermal cells could enter the germline of recipient embryos.

We have been trying to develop a technique for gene transfer into chickens by way of PGCs, because PGCs are progenitor cells of ova or spermatozoa and should ensure the transmission of genetic modifications to the next generation. Since avian PGCs circulate temporarily in the bloodstream before migrating into the germinal ridges (Kuwana, 1993), PGCs in the blood circulation were transfected *in vivo* by lipofection (Watanabe *et al.*, 1994). The average number of

Received 21 August 1997.

PGCs positive for the *lacZ* gene per embryo after 2–3 days of the treatment was 2.1 in 29 embryos analysed and the efficiency of successful transfection of PGCs was 0.1–1.4%. For precise manipulation of PGCs *in vitro*, PGCs were collected from embryonic blood and then transferred into the bloodstream of recipient embryos of a different breed to produce germline chimaeric chickens (Tajima *et al.*, 1993). Germline chimaeric chickens with a high transmission rate (77.3–96.8%) of donor-derived gametes have been produced by increasing the number of donor PGCs and by partially sterilizing the recipient embryos (Naito *et al.*, 1994b; 1998). Also, PGCs obtained from embryonic blood have been frozen successfully in liquid nitrogen (-196°C), and subsequently produced viable offspring by way of germline chimaeric chickens (Naito *et al.*, 1994c). Thus, we are close to establishing an efficient system for giving rise to viable offspring from transfected PGCs by way of germline chimaeric chickens.

The present study was carried out to examine the expression of exogenous DNA in the gonads of chimaeric embryos produced by transfer of PGCs transfected *in vitro* and to determine the subsequent fate of the introduced DNA in the gonads of chimaeric embryos and hatched chickens.

Materials and Methods

Preparation of fertilized eggs

Fertilized eggs of White Leghorn and Barred Plymouth Rock chickens were obtained, by artificial insemination, from the genetic stocks maintained at the National Institute of Animal Industry.

Preparation of donor PGCs

Fertilized White Leghorn eggs were incubated at 38°C , relative humidity 60%, in air for about 53 h in a forced air incubator (P-008; Showa Incubator Laboratory, Yono-shi, Saitama) and blood was collected from the dorsal aorta of embryos at stages 13–15 (Hamburger and Hamilton, 1951) using a fine glass micropipette. PGCs were concentrated by a Ficoll-density gradient centrifugation method (Yasuda *et al.*, 1992) and the PGC-rich fraction (PGCs constituted 60% of the cells in this fraction) was dispersed in 500 μl culture medium (Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL Life Technologies, NY) supplemented with 10% fetal bovine serum (Flow Laboratories, McLean, VA)).

Transfection of PGCs in vitro

The plasmid DNA used for the present experiment was pAcZ, which contains *Escherichia coli lacZ* gene under the control of the chicken β -actin gene promoter (Naito *et al.*, 1994a). Transfection of PGCs was carried out by lipofection using a cationic lipid, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethylsulfate (DOTAP, No. 1781995; Boehringer Mannheim Biochemica, Mannheim). Four microlitres of DOTAP solution ($1\text{ }\mu\text{g }\mu\text{l}^{-1}$) were first diluted with 20 μl HBS (20 mmol Hepes l^{-1} , 150 mmol NaCl l^{-1} , pH 7.4) in a 6 ml polystyrene culture tube (code 8567; Stockwell

Scientific, Walnut, CA), mixed with 3.4 μl of plasmid DNA at a concentration of $294\text{ ng }\mu\text{l}^{-1}$ and then incubated for 60 min at room temperature. The collected donor PGCs were dispersed in 500 μl culture medium and then added to the DOTAP:DNA solution. The cap of the culture cube was tightly sealed and incubated for 5 h at 38°C , with no humidified condition, in air. The PGCs were then washed with culture medium, dispersed in 100 μl fresh culture medium and placed in a plastic dish (code 3001; Falcon, Becton Dickinson Co., Lincoln Park, NJ). Control PGCs were lipofected in a similar manner but without the pAcZ.

Closed, circular-form plasmid DNA was used when the *lacZ* gene expression was examined in the gonads of embryos incubated for 3 days after the PGC injection, and linear form plasmid DNA, digested with *Bam*HI, was used when the fate of the *lacZ* gene was examined in the gonads of chimaeric embryos, at various days of incubation after the PGC injection, and in the gonads of the hatched chickens.

Preparation of recipient embryos and PGC transfer

Recipient embryos were cultured in host egg shells, as described by Naito *et al.* (1990), to allow precise manipulation. Fertilized eggs of Barred Plymouth Rock recipient embryos (stage X, Eyal-Giladi and Kochav, 1976) were broken and the embryos (yolks) were transferred to small host egg shells. The reconstituted eggs were incubated at 38°C , relative humidity 60%, in air for about 53 h. When the embryos had reached stages 14–15 (Hamburger and Hamilton, 1951), blood (4–10 μl) was drawn, as much as possible from the dorsal aorta (to eliminate the endogenous PGCs), using a fine glass micropipette. The embryos were then incubated for a further 2–4 h before the PGC injection.

Three hundred, lipofected PGCs were picked up by a fine glass micropipette under a light microscope (total volume was about 1 μl). The PGCs were injected into the bloodstream of recipient embryos via the dorsal aorta. The manipulated embryos were then transferred to large host egg shells and incubated at 38°C , relative humidity 60%, in air for 3–17 days, or until hatching (Perry, 1988; Naito *et al.*, 1990).

Detection of the *lacZ* gene expression in the gonads of chimaeric embryos

Embryos cultured for 3 days after injection of PGCs were removed from the yolk, fixed with 1% (v/v) glutaraldehyde in DPBS(–) (Dulbecco's PBS without Ca^{2+} and Mg^{2+} , No. 21300-025; Gibco BRL Life Technologies, New York) for 30 min and then washed three times with gentle shaking in DPBS(–). The embryos were then incubated for 2 h at 37°C in a staining solution (pH 7.4) containing 0.05% (v/v) 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, Nakarai Tesque, Tokyo), 1 mmol $\text{MgCl}_2\text{ l}^{-1}$, 0.1% (v/v) Triton X, 3 mmol potassium ferrocyanide l^{-1} and 3 mmol potassium ferricyanide l^{-1} in DPBS(–) (Naito *et al.*, 1991).

Detection of the *lacZ* gene in the gonads of chimaeric embryos and chickens

Gonads were obtained from the embryos cultured for 3–19 days after injection of lipofected PGCs and homogenized.

Spermatozoa of the male germline chimaeric chickens and blood of donor-derived offspring from the female germline chimaeric chickens (owing to the difficulty of collecting ova from live female chickens) were obtained and washed with DPBS(-). DNA was then extracted from the gonadal cells, spermatozoa and blood cells using a DNA Extraction Kit (*SepaGene*[™], Sanko Junyaku, Tokyo). The extracted DNA was dissolved in TE buffer (10 mmol Tris-HCl l⁻¹, 0.25 mmol EDTA l⁻¹, pH 8.0) and PCR analysis was then performed on 100 ng DNA samples to detect the *lacZ* gene.

The PCR was carried out using a programmable thermal controller (Model 480, Perkin Elmer, Norwalk, CT). The PCR reactions were performed in 50 µl of 10 mmol Tris-HCl l⁻¹, 1.5 mmol MgCl₂ l⁻¹, 50 mmol KCl l⁻¹, 0.1 mg ml⁻¹ gelatin, 200 µmol dNTPs l⁻¹, 0.5 µmol primers l⁻¹ and 2.5 U *Taq* polymerase (No. R001B; Takara, Tokyo). The sequences of the primers for detecting the *lacZ* gene were: 5'-AGATGCACGGT TACGATGC-3', 5'-GGTCAAATTCAGACGGCAAACG-3' (Love *et al.*, 1994). For internal control, a sequence of the endogenous GAPDH gene was amplified using the primers: 5'-TGTGACTTCAATGGTGACA-3', 5'-CAGATCAGTTTC TATCAGC-3' (Love *et al.*, 1994). After an 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. PCR products were separated on a 2% agarose gel and the bands were visualized under UV light after ethidium bromide staining.

Frequency of germline transmission of donor PGCs

White Leghorn are homozygous dominant (*I/I*) and Barred Plymouth Rock are homozygous recessive (*i/i*) for the autosomal pigment inhibitor gene. Chimaeric chickens that survived to sexual maturity were mated with Barred Plymouth Rock by artificial insemination and the feather colour of their offspring was examined. Since PGCs were transferred from White Leghorn to Barred Plymouth Rock, white chicks (*I/i*) from chimaeric chickens indicated that they were derived from the transferred White Leghorn PGCs and black chicks (*i/i*) indicated that they were derived from the endogenous Barred Plymouth Rock PGCs.

Results

Morphology of PGCs after lipofection

Freshly collected PGCs and lipofection-treated PGCs are shown (Fig. 1). No apparent difference was observed in morphology before and after lipofection.

Expression of the *lacZ* gene in the embryonic gonads

The expression of the *lacZ* gene in the gonads of embryos incubated for 3 days after the injection of PGCs transfected *in vitro* is shown (Fig. 2). Many, intensely blue-stained cells were observed in the right and left gonads (Fig. 2a) or mainly in the left gonad (Fig. 2b) of the chimaeric embryos. The

number of cells stained blue per embryonic gonad varied from a few to more than 50 (13.7 on average), and many of the blue spots consisted of several cells. Cells expressing the *lacZ* gene were distributed throughout the gonads of the chimaeric embryos. No gonadal staining was observed in the control embryos produced by the transfer of PGCs lipofected *in vitro* without pAcZ. Of 52 embryos examined, 37 (71.2%) embryos showed the expression of the *lacZ* gene in the right or left gonads and 15 (28.8%) embryos showed no expression of the *lacZ* gene.

Presence of the *lacZ* gene in the gonads of chimaeric embryos

DNA samples from the gonads of chimaeric embryos were analysed for the presence of the *lacZ* gene by PCR (Fig. 3). The number of embryos with gonads that expressed the *lacZ* gene out of the 209 manipulated embryos are shown (Table 1). The *lacZ* gene was detected in the majority of embryos examined after 3 days of incubation after the injection of the lipofected PGCs (total incubation time 5.5 days). However, the *lacZ* gene was detected in only a small proportion of the embryos examined after 17 days of incubation after the PGC injection (19.5 day incubated embryo). Although the percentages of embryos with gonads positive for the *lacZ* gene decreased gradually during development, the *lacZ* gene was detected in the gonads of two hatched chicks (11.1%, 2/18).

Presence of the *lacZ* gene in the germline of chimaeric chickens

DNA was extracted from the sperm samples of eight male germline chimaeric chickens and from the blood samples of 216 donor-derived offspring from three female germline chimaeric chickens, and analysed for the presence of the *lacZ* gene. In none of the DNA samples was the *lacZ* gene detected by PCR analysis.

Germline chimaerism obtained from progeny test

One hundred and seven White Leghorn embryos were injected with Barred Plymouth Rock PGCs transfected *in vitro* and 36 (33.6%) chicks were obtained. Male and female chickens that reached maturity and were tested were all germline chimaeras (Table 2). The proportion of germline transmission of donor PGCs was 12.1–69.9% (35.3% on average) in eight male chimaeric chickens and 71.6–97.6% (86.9% on average) in three female chimaeric chickens. Germline chimaerism of the male and female chimaeric chickens did not change markedly with increasing age.

Discussion

In the study described here, exogenous DNA (the *lacZ* gene) was successfully introduced into the gonads of chimaeric embryos by transferring PGCs that had been obtained from embryonic blood and transfected *in vitro* by lipofection. The expression of the *lacZ* gene was observed in the gonads of embryos incubated for 3 days after the PGC injection. In addition, the presence of the *lacZ* gene in the gonads of

Fig. 1. Chicken primordial germ cells (a) before and (b) after the lipofection treatment. Blood was collected from embryos at stages 13–15 and primordial germ cells (large cells with eccentrically placed nuclei and a considerable number of lipids in the cytoplasm) were concentrated by Ficoll-density gradient centrifugation. The small round cells are erythrocytes. Scale bar represents 20 μm .

chimaeric embryos was confirmed by PCR analysis. The percentages of embryos positive for the *lacZ* gene, after 3 days of incubation after the PGC injection, was very high (95%); however, the *lacZ* gene was detected in only 14.3% of embryos examined after 17 days of incubation after the PGC injection. Nevertheless, the *lacZ* gene was still detectable in the gonads until the chimaeric embryos had hatched. However, upon sexual maturation, the *lacZ* gene was not detected in the gonads of chimaeric chickens, and was not transmitted to the next generation. Similar results have been obtained in *Xenopus*, in that DNA injected into the cytoplasm usually remains extrachromosomal during early cleavage stages, is replicated up to the blastula stage and is then gradually lost (Bendig, 1981; Rusconi and Schaffner, 1981). In the present experiment, the *lacZ* gene introduced into the embryonic gonads appears to be present episomally in the PGCs or germ cells, and is gradually lost during embryonic development.

As assessed by PCR, the *lacZ* gene was present in 95% of the embryos examined after 3 days of incubation after the PGC injection, while expression of the *lacZ* gene was observed in 71.2% of embryos examined at the same developmental stage. The difference in the frequency of presence and expression of

the *lacZ* gene suggests that not all the *lacZ* genes introduced into the embryonic gonads are expressed. In some of the successfully transfected PGCs, the *lacZ* gene may remain in the cytoplasm and therefore shows no expression. The average number of *lacZ* gene-positive cells in the gonads was 13.7 per embryo in the currently reported experiment and was much higher than that produced by *in vivo* transfection of PGCs (2.1 cells per embryo, Watanabe *et al.*, 1994). Thus, *in vitro* manipulation used in the present experiment was much more efficient than *in vivo* manipulation for the transfection of PGCs.

Germline transmission of exogenous DNA into the G1 generation has been achieved using PGCs (Vick and Simkiss, 1993). PGCs obtained from the germinal crescent region or from the blood of early-stage embryos were transfected with defective retroviruses and transferred to the recipient embryos to form chimaeras that grew to sexual maturity and produced offspring. Exogenous DNA was detected in some of the G1 offspring, as well as in the spermatozoa of the G0 chimaeric chickens. In the work described here, a similar procedure was applied to produce transgenic chickens, but germline transmission of exogenous DNA was not achieved. This was probably due to the low efficiency of chromosomal integration of

Fig. 2. Expression of the *lacZ* gene in the gonads of chimaeric chicken embryos 3 days after the injection of primordial germ cells transfected *in vitro*. Cells stained blue indicate the expression of the *lacZ* gene and are scattered in the developing (a) right and left gonads or (b) mainly in the left gonad of chimaeric embryos.

Fig. 3. Analysis of the chimaeric chicken embryos produced by transfer of primordial germ cells transfected *in vitro* with the plasmid DNA pAcZ. DNA was extracted from the gonads of chimaeric embryos and analysed by PCR to detect the *lacZ* gene of the plasmid and the endogenous GAPDH gene for internal control. Lanes 1 and 21: molecular size marker (PhiX174 DNA digested with *Hae* III restriction enzyme); lane 2: negative control (water); lane 3: positive control (pAcZ); lane 4: control DNA from intact chicken embryonic gonads. Lanes 5–20 show positive and negative embryos for the *lacZ* gene at various days after the primordial germ cell injection (lanes 5 and 6: 3 days; lanes 7 and 8: 4 days; lanes 9 and 10: 5 days; lanes 11 and 12: 7 days; lanes 13 and 14: 10 days; lanes 15 and 16: 13 days; lanes 17 and 18: 17 days; lanes 19 and 20: chicks at hatch).

exogenous DNA into PGCs by the lipofection method compared with the retrovirus vector method. All methods of transfection of isolated cells produce more transient than stable transfections. Although the construct was not found in the germline of mature birds, stable transfection may occur, but the frequency may be so low that a greater number of birds would

have to be examined before it would be detectable in the germline.

The PGCs lipofected *in vitro* were morphologically normal, successfully migrated into the germinal ridges and differentiated into ova or spermatozoa in the gonads of the chimaeric embryos and chickens. These gametes were functional and

Table 1. Presence of the *lacZ* gene introduced into the gonads of chimaeric chicken embryos by transfer of primordial germ cells transfected *in vitro* as determined by PCR

Days of incubation after injection*	Number of embryos manipulated	Number of embryos positive for <i>lacZ</i> (%)
3 (5.5)	40	38 (95.0)
4 (6.5)	22	15 (68.2)
5 (7.5)	12	8 (66.7)
7 (9.5)	23	8 (34.8)
10 (12.5)	21	4 (19.0)
13 (15.5)	52	9 (17.3)
17 (19.5)	21	3 (14.3)
Hatch (21.0)	18	2 (11.1)

*Days of incubation after the start of incubation from laid egg stage are shown in parentheses.

Table 2. Progeny test of chimaeric chickens produced by transfer to Barred Plymouth Rock of White Leghorn primordial germ cells transfected *in vitro*

Chimaera ID number	Number of chicks hatched	Number of white chicks*	Number of black chicks	White chicks* (%)
♂ 1	359	89	270	24.8
♂ 2	230	114	116	49.6
♂ 3	223	27	196	12.1
♂ 4	233	58	175	24.9
♂ 5	155	96	59	61.9
♂ 6	166	116	50	69.9
♂ 7	256	38	218	14.8
♂ 8	267	64	203	24.0
♀ 1	140	128	12	91.4
♀ 2	67	48	19	71.6
♀ 3	41	40	1	97.6

*Donor-derived offspring.

gave rise to viable offspring, although the majority of the DNA introduced into the gonads of chimaeric embryos disappeared during their embryonic development. The average proportion of donor-derived offspring from the germline chimaeric chickens produced by transfer of PGCs lipofected *in vitro* were 35.3% in males and 86.9% in females. The proportion of donor-derived offspring from the male chimaeric chickens was lower than that in our previous experiments (80.8%, Naito *et al.*, 1994b) in which germline chimaeric chickens were produced by transfer of freshly collected, non-transfected PGCs. However, the frequency of donor-derived offspring from the female chimaeric chickens showed only a slight decrease compared with our previous result (95.8%, Naito *et al.*, 1994b). Thus, the lipofection treatment of PGCs used in the present experiment only slightly affected the migration of PGCs into the germinal ridges and the subsequent development and differentiation into gametes in the chimaeric embryos and chickens.

PGCs in recipient embryos can be compromised by γ -radiation (Carsience *et al.*, 1993), or exposure to busulphan to obtain a high transmission rate of donor-derived gametes from germline chimaeric chickens (Aige-Gil and Simkiss, 1991). In the currently reported experiment, blood was drawn as much as possible from the recipient embryos, since PGCs circulate in the bloodstream (Naito *et al.*, 1994b). This alternative method was effective for decreasing the number of PGCs in the recipient embryos, and the donor-derived offspring from the chimaeric chickens were efficiently produced.

The technique described in the present study could be used to test the expression of exogenous DNA in the gonads of early chicken embryos. It may, however, be difficult to produce transgenic chickens routinely at the present level of transfection efficiency for PGCs and because of the restrictions on the number of PGCs that can be manipulated at one time (about 3000 PGCs). Integration efficiency of exogenous DNA into the host chromosome must be increased and the PGCs integrated with exogenous DNA must be selected and proliferated by culture *in vitro* to produce transgenic chickens by way of PGCs. Then, transgenic chickens could be produced efficiently. In addition, it would be possible to manipulate specific locations of the avian genome by homologous recombination using stem cells maintained *in vitro*, such as embryonic germ cells derived from PGCs.

The authors are grateful to the staff of the Poultry Management Section of the National Institute of Animal Industry for taking care of the birds. This study was supported by the Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of the Japanese Government.

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