Identification of Transferred Chicken Germ Cells in Quail Gonad and Semen by Amplification of Chicken-Specific PCR Products

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A polymerase chain reaction (PCR) primer set for a chicken microsatellite locus on the Z chromosome, LEI0171 (GenBank Accession No. X85538), amplified a 363 bp fragment of genomic DNA in chickens (*Gallus domesticus*) but not in quail (*Coturnix japonica*). A concentration of at least 10 pg/ μ l of chicken DNA in the reaction mixture resulted in detectable amplification of the PCR product. Circulating primordial germ cells (PGCs) of chick embryos were transfused into quail embryos, and the chicken-genome-specific PCR product was observed in the chick-PGC-transfused chimeric quail when DNA samples were extracted from whole day-6 embryos, day-10 and -15 embryonic gonads, gonads of 5-day chicks, and the semen of adults.

Key words : microsatellite DNA ; chicken ; PCR ; primordial germ cells ; quail

Introduction

Primordial germ cells (PGCs) are the first identifiable progenitors of gametes and provide the only cellular continuity between generations. Unlike the PGCs in amphibians and mammals, PGCs in birds and reptiles migrate primarily by means of the bloodstream (Gilbert, 2000).

Recently, interspecific transfers of circulating PGCs have been attempted from turkeys to chickens (Reynaud, 1976), chickens to quail (Yasuda *et al.*, 1992 ; Ono *et al.*, 1998 b) and quail to chickens (Ono *et al.*, 1996 ; Ono *et al.*, 1998 a). If the transferred PGCs differentiate into functional gametes in the gonadal tissue of the recipient birds, offspring of the donor bird's species could be recovered by mating of germline chimeras. Thus, transfer of PGCs could play an important role in the preservation of endangered species (Tajima *et al.*, 1993,1998 ; Naito *et al.*, 1994 ; Fujihara *et al.*, 1999).

In order to identify the interspecifically transferred germ cells in the recipient animals, reliable markers that can distinguish between cells of exogenous and endogenous origin are required. We have evaluated 3 monoclonal antibodies (mAbs), QCR 1, QB2 and 2C9, for their ability to distinguish between chicken and quail PGCs in

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chimeric embryos (Ono *et al.*, 1996 ; 1998 ab). QCR1 and QB2 are quail-specific mAbs and 2C9 is a chick-specific mAb showing developmental-stage-specific staining. These mAbs unfortunately did not label germ cells in adult testicular tissues and spermatozoa (Ono *et al.*, 1998 a). We were able to show that transfused quail PGCs settled in the gonads of chick embryos and hatchlings ; however, we failed to detect donor-derived offspring born from interspecific germline chimeras (Ono *et al.*, 1998 a).

A polymerase chain reaction (PCR) primer set that distinguished between DNA of donor-derived germ cells and that of recipient cells would be useful as well as convenient for obtaining direct evidence that interspecifically transferred germ cells are present in the recipient animals. Sexing can be performed by PCR using a primer set which amplifies a W chromosome-specific DNA sequence (Clinton, 1994 ; Petitte and Kegelmeyer 1995 ; Simkiss *et al.*, 1996). In the present study, isolated chick PGCs were transferred into quail embryos, and the chimeric quail were raised to sexual maturity. We developed a system that distinguishes between donor chicken and recipient quail genomes by means of PCR, and we used this system to identify donor chicken-derived germ cells in the recipient quail.

Materials and Methods

Animals

Fertilized eggs of wild-type plumage strains of Japanese quail (*Coturnix japonica*) maintained in our laboratory and those of White Leghorn (Aichi-line) chickens (*Gallus domesticus*) obtained from Aichi Livestock and Poultry Breeding Center (Anjo) were used throughout the study. Incubation of eggs was done under normal conditions (Li *et al.*, 2001 a) and the staging of embryonic development was determined using the standards of Hamburger and Hamilton (1951).

Transfer of chick PGCs to quail embryos

Chick embryonic blood was collected according to a previously reported protocol (Li *et al.*, 2001 a). PGCs were enriched by Ficoll density gradient centrifugation as described by Yasuda *et al.* (1992) with minor modifications. Briefly, pooled blood was dispersed in medium FCS/L15 (Leibovitz's L-15 medium (Sigma Chemical Co., St. Louis, MO) containing 10% fetal calf serum (FCS, Nippon Bio-Test Lab. Inc., Tokyo)), and centrifuged at 200 G for 3–5 min. The pellet was then dispersed in 250–300 μ l of 5.0% Ficoll (F9378, Sigma Chemical Co.) in FCS/L15, and overlaid on 800 μ l of 16.0% Ficoll in FCS/L15. After centrifugation at 800 G for 25 min, 400–600 μ l of the PGC-rich fraction located in the layer between 16.0% and 5.0% Ficoll was collected and dispersed in 1000 μ l of FCS/L15, and Ficoll was removed by repeated centrifugation at 200G for 5 min. The concentration of the PGCs was estimated using a haemocytometer viewed under a phase contrast microscope, and a suspension of 150–200 PGCs/ μ l in FCS/L15 was obtained.

Quail embryos at stage 14–15 were used as recipients. To reduce the number of endogenous PGCs, some embryos were exposed to soft X-rays (Li *et al.*, 2001 b). The irradiated embryos were cultured at 37.6°C and 70% relative humidity without tilting for about 1 hr prior to PGC-injection.

One microliter of chick PGC suspension (150–200 PGCs) was transfused into the marginal vein of the quail embryo and the perforation was sealed according to the method described by Yasuda *et al.* (1992). The transfused embryos were cultured *in vitro* in System Q3 (Ono *et al.*, 1994) until day 6, 10, or 15 of incubation, or until hatching.

Preparation of genomic DNA

Genomic DNA samples of chick PGC-transfused quail were extracted from whole day-6 embryos, gonadal tissues of day-10 and -15 embryos and 5-day chicks, semen of adult males, and from the epididymis and/or vas deferens from dead chimeras. DNA samples were also extracted from blood and semen of normal chickens and quail. The DNA was extracted using a DNeasy Tissue Kit (Qiagen, Tokyo) according to the manufacturer's instructions or using phenol-chloroform (Ausubel *et al.*, 1999). The DNA concentration was determined spectrophotometrically at 260 nm.

Identification of chicken germ cells in quail

Information about the primer sequences was obtained from the web site of the US Poultry Genome Project (<http://poultry.mph.msu.edu/resources/ms-tot.htm>). To distinguish between chicken and quail genomic DNA by PCR, a primer set for microsatellite locus LEI0171 (GenBank Accession No. X85538) on the Z chromosome (Schmid *et al.*, 2000) was used. The LEI0171 primer sequences 5'-GAGTG TAGACAGTAGTGTATC-3' and 5'-CTCAGGGCACCATTTTCACTG-3' were used as forward and reverse primers, respectively. The PCR was performed in a 25- μ l mixture consisting of 0.25–25 ng of DNA template, 2.5 μ l of 10X Ex Taq™ buffer; 2 μ l of dNTP mixture (2.5 mM each), each primer at 0.25 μ M, 0.075 units of EX Taq™ (TaKaRa EX Taq™ PCR kit, Takara, Tokyo) with 32 cycles of denaturation at 94°C for 30 sec, annealing at 56.5°C for 30 sec and extension at 72°C for 30 sec, with a preliminary denaturation at 94°C for 5 min and a final extension at 72°C for 5 min using a PC-801 Program Temp Control System (Astec, Fukuoka). Ten microliters of the PCR mixture were electrophoresed on a 1.5% agarose gel. The gel image was scanned (GT-9500, Epson, Tokyo) and the signal of the PCR products was enhanced with Photoshop software (Adobe Systems, Inc., Tokyo) to examine faint bands. The PCR products taken from Aichi-line chickens (1 female and 2 males) were sequenced using a model 377 automated DNA sequencer (ABI, Chiba).

Statistical analysis

Data were analyzed statistically with chi-square test (Snedecor and Cochran, 1989). Differences were regarded as significant at $p < 0.05$.

Results

We first examined the PCR products amplified from DNA samples extracted from chicken and quail blood (Fig. 1). Genomic DNA of male and female quail was not amplified with this primer set under these condition. By contrast, that of male and female chickens was amplified. At 10 pg/ μ l or higher concentrations, chicken DNA was amplified to a detectable level. The PCR product was also detectable when chicken DNA was mixed with quail DNA. Fig. 2 shows the nucleotide sequence of

Fig. 1. Fragments produced after 32 cycles of PCR amplification of DNA from the blood of quail and chickens. Ten microliters of PCR products were applied to the gel. PCR conditions are described in the text. Lanes : 1, 100 bp ladder molecular size marker ; 2, female quail (1 ng/ μ l). Numbers in parenthesis indicate the concentration of the DNA template in a 25- μ l PCR mixture ; 3, male quail (1 ng/ μ l) ; 4, female chicken (1 ng/ μ l) ; 5, male chicken (1 ng/ μ l) ; 6, male chicken (100 pg/ μ l) ; 7, male chicken (10 pg/ μ l) ; 8, mixed DNA consisting of male chicken (100 pg/ μ l) and male quail (900 pg/ μ l) DNA ; 9, mixed DNA consisting of male chicken (10 pg/ μ l) and male quail (990 pg/ μ l) DNA ; 10, mixed DNA consisting of male chicken (10 pg/ μ l) and male quail (1 ng/ μ l) DNA. B is an enhanced image of A processed with Adobe Photoshop.

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1  CTCAGGGCAC CATTTCAC TGGTATAAGCA TATCAGCACT TAATCTATGT
51  ATAAGGAGTA CTATGGGGCT CTTTAGGACA ACTCACA CTT CAACCACAAA
101 AACACATTCA TTTAGCAGCT GTTGTTACAT AAAAGCGAAC TGTTTTAATT
151 TAATGAAAAC ACAAATAAAA ACATATTACA TTATGCAACT GTGTTTTTCA
201 TTAAAATGTA CCTGATGTAC ACACACACAC ACACACACAC ACACACACAC
251 ACACACACAA AAAGTGGTGA AAATTGGCTG TATATGCTAA GCTAATTTAA
301 GTGTTGCAAA AATGTAAAAT ATCTTGTAAC TGCCTTCATA TTGATACACT
351 ACTGTCTACA CTC

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Fig. 2. Nucleotide sequence of the amplified region of microsatellite locus LEI0171 on the Z chromosome in Aichi-line chickens. Underlined sequences are the primer regions.

the amplified region of the chicken DNA. All three samples had identical 363-bp sequences.

We next examined the PCR products of DNA samples extracted from gonadal

Fig. 3. Fragments produced after 32 cycles of PCR amplification of gonadal DNA from chick-PGC-transfused quail. Ten microliters of PCR products were applied to the gel. PCR conditions are described in the text. Lanes: 1, 100 bp ladder molecular size marker; 2, day-10 chick embryo (1 ng/ μ l). Numbers in parenthesis indicate the concentration of the DNA template in a 25- μ l PCR mixture; 3, day-10 chick embryo (100 pg/ μ l); 4, day-10 chick embryo (10 pg/ μ l); 5, day-10 quail embryo (1 ng/ μ l); 6, day-6 chick-PGC-transfused quail embryo (1 ng/ μ l, whole embryo); 7, day-10 chick-PGC-transfused quail embryo (1 ng/ μ l); 8 and 9, day-15 chick-PGC-transfused quail embryo (1 ng/ μ l). B is an enhanced image of A processed with Adobe PhotoShop.

tissues of chick PGC-transfused quail (Fig. 3). Amplified products were detected for DNA from whole day-6 embryos, gonadal tissues of day-10 and -15 embryos and 5-day chicks (Figs. 3 and 4). The intensity of the signal in chimeric embryos was roughly one-hundredth of that in chick embryos.

Table 1 shows the hatchability of manipulated quail embryos. When quail embryos at stage 14–15 were cultured using System Q3, 41.8% of them hatched. Manipulation by irradiation, introduction of chick PGCs or both caused reduced hatchabilities of 23.1%, 20.5% and 11.7%, respectively.

Finally we examined the PCR products of DNA samples extracted from the semen of chick PGC-transfused quail. The expected PCR products were amplified from DNA extracted from the semen of chimeras (Fig. 4). The intensity of the signal in semen of chimeras was between one-hundredth and one-tenth of that in semen of chickens. The 363-bp band was also amplified from the DNA samples extracted from the vas deferens and epididymis of chimeras (data not shown).

Table 2 shows the fraction of chick-PGC-transfused quail that expressed the PCR products of the chicken-specific DNA. At all stages examined, germ cell chimeras were observed in the irradiated and non-irradiated groups, but there was no significant difference between the two groups.

Table 1. Hatchability of cultured embryos exposed to soft X-rays and/or injected with chick PGCs

Type of treatment	No. of embryos		Hatchability
	Cultured	Hatched	
None	134	56	41.8% ^a
X	104	24	23.1% ^b
FCS/L15	95	27	28.4% ^{bc}
PGC	151	31	20.5% ^{bcd}
X & FCS/L15	42	7	16.7% ^{bcd^e}
X & PGC	394	46	11.7% ^e

Embryos were transferred into System Q3 at stage 14-15 prior to the treatment. X, exposed to soft X-rays for 40 sec at stage 14-15 ; FCS/L15, injected with 1 μ l of 10% fetal calf serum in Leibovitz's L-15 medium ; PGC, injected with 1 μ l of chick PGC suspension in FCS/L15 (150-200 PGCs/ μ l). The hatchabilities marked with different letters are significantly different based on chi-square test ($P < 0.05$).

Fig. 4. Fragments produced after 32 cycles of PCR amplification of semen and gonadal DNA from chick-PGC-transfused quail. Ten microliters of PCR products were applied to the gel. PCR conditions are described in the text. Lanes: 1, 100 bp ladder molecular size marker; 2, quail (1 ng/ μ l); 3, chicken (1 ng/ μ l). Numbers in parenthesis indicate the concentration of the DNA template in a 25- μ l PCR mixture; 4, chicken (100 pg/ μ l); 5, chicken (10 pg/ μ l); 6 and 7, gonadal tissue of 5-day-old chick-PGC-transfused male quail (1 ng/ μ l); 8 and 9, semen of chick-PGC-transfused quail (1 ng/ μ l). B is an enhanced image of A processed with Adobe PhotoShop.

Table 2. The incidence of chicken-specific fragments in DNA from chimeric quail

Types of treatment	Day 6 embryo	Day 10 embryo	Day 15 embryo	5-day chick	Adult male	Total ¹⁾
None	40% (2/5)	40% (2/5)	40% (6/15)	43% (3/7)	25% (1/4)	39% (14/36)
X-rays	50% (3/6)	57% (4/7)	63% (10/16)	63% (5/8)	33% (4/12)	53% (26/49)

Numbers in parenthesis are expressed as no. of chimeras with amplified chicken genomic band per no. of chimeras observed. Genomic DNA samples of chick-PGC-transfused quail were extracted from whole day-6 embryos, gonadal tissues of day-10 and -15 embryos and 5-day chicks, and semen of adult males.

¹⁾ There was no significant difference based on chi-square test.

Discussion

We surveyed a number of primer sets originally designed for microsatellite loci of the chicken genome which were described in the web site of the US Poultry Genome Project. Among them we found that the primer set for microsatellite locus LEI0171 on the Z chromosome (GenBank Accession No. X85538 ; Schmid *et al.*, 2000) amplified a fragment of 363 bp in chicken (Aichi-line) genomic DNA but not in quail genomic DNA. This locus contains a polymorphic CA repeat region, and thus the length of the PCR product vary from 350 to 365 depending on the chicken line or breed (see web site at [http : //poultry.mph.msu.edu/resources/ms-tot.htm](http://poultry.mph.msu.edu/resources/ms-tot.htm)).

In the present study, this primer set was able to amplify the donor-chicken-derived DNA in the chimeric quail. A concentration of at least 10 pg/ μ l of chicken DNA in the PCR mixture was needed to detect the chicken-genome-specific band. The amount of DNA required would be reduced if a more sensitive PCR protocol and/or a more efficient primer set were available. A number of species-specific primer sets have been reported in poultry (Levin *et al.*, 1995 ; Liu *et al.*, 1996 ; Inoue-Murayama *et al.*, 1998 ; Pang *et al.*, 1999 ; Huang *et al.*, 1999 ; Kayang *et al.*, 2000) and these primers are candidates for our purposes. In the present study, the intensity of the chicken-DNA-specific signal in the gonads of chimeras was roughly one-hundredth of that in the gonads of chickens. This may be an over-estimation because a considerable amount of the recipient's somata was included in chimera's DNA samples, and the PCR protocol used was not designed for quantitative analysis. In our previous report we showed that 5.6% of gonadal PGCs were of donor chick origin in the chick-PGC-transfused quail embryos at stage 29 (Ono *et al.*, 1998 b). Semen consists of sperm, their precursor cells such as spermatids and spermatocytes, and seminal fluid, so that DNA samples extracted from the semen of chimeric quail are mainly derived from sperm and their precursor cells. Therefore, it is likely that the population of donor-derived germ cells is roughly between 1% and 10% of total cells in the semen of chimeras. As yet no information about whether these donor-derived germ cells are functional sperm or not.

In conclusion, we found that the PCR primer set used in this study provided an

excellent marker for distinguishing between chicken and quail DNA in chimeras, and with this marker we were able to show interspecific germ cells in the chimeras' semen.

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