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≪**Research Note**≫

A Simple Method for Sexing Chicken Embryos at Stage X

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A simple method for sexing chicken embryos was devised using DNA prepared from blastodermal cells. A cluster of cells was collected from the area opaca of a stage X blastoderm, and DNA was prepared by the boiling method (10-minute boiling and 3-minute centrifugation). DNA was also prepared by the extraction method (DNA extraction kit) as a control. PCR analysis was carried out using the prepared DNA, and the sex of embryos was clearly identified by both the boiling and extraction method. This simple and rapid method of embryo sexing using blastodermal cells isolated from the area opaca enables us to identify the sex of a large number of chicken embryos in a limited time.

Key words : blastodermal cell, chicken embryo, embryo culture, PCR, sexing

Introduction

The recent development of chicken embryo manipulation techniques has made it possible to produce same-sex and mixed-sex germline chimaeric chickens (Kagami *et al.,* 1995, 1997 ; Naito *et al.,* 1999, 2001). Such chickens can be produced by the transfer of stage X (Eyal-Giladi and Kochav, 1976) blastodermal cells or primordial germ cells (for review see Naito, 2003 a, b ; Naito and Kuwana, 2003). In these cases, sex combinations of donor primordial germ cells and recipient embryos are important for the normal development of donor-derived germ cells in recipient gonads. When the sex of the donor primordial germ cells and recipient embryo are the same, the transferred primordial germ cells differentiate normally in the recipient gonads, giving rise to viable offspring. However, when the sex of the donor primordial germ cells and recipient embryo are different, differentiation of the former in the recipient gonads was impeded, and few donor-derived offspring were obtained from the chimaeric chickens (Naito *et al.,* 1999, 2001).

In chickens, the male is the homogametic sex (ZZ) and the female is the heterogametic sex (ZW). Sexing chicken embryos can, therefore, be carried out by

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detecting W chromosome-specific repeating sequences (Mizuno *et al.,* 1993 ; Clinton, 1994). When studying germ cell differentiation in same-sex and mixed-sex germline chimaeric chickens, it is necessary to quickly identify the sex of donor primordial germ cells and recipient embryos. In chickens, sexing embryos can be carried out using collected cells from the stage X blastoderm (Kagami *et al.,* 1997 ; Naito *et al.,* 1999, 2001 ; Klein and Ellendorff, 1998, 2000). In this study, we developed a simple and rapid method of identifying the sex of chicken embryos at stage X.

Materials and Methods

Collection of stage X blastodermal cells and embryo culture

Fertilised White Leghorn eggs (stage X) were broken, and the contents were put into glass vessels. After the thick albumen capsule was removed from the yolk, the blastoderm was positioned on top of the yolk. A fine needle (27G×3/4 ; Terumo, Tokyo) connected to a micropipette was inserted underneath the blastoderm layer and a cluster of about 700 cells was sucked from the area opaca of the blastoderm (Kagami *et al.,* 1997 ; Naito *et al.,* 1999, 2001). The collected cells were used for DNA extraction and for sexing embryos. The manipulated embryos (yolks) were transferred into host eggshells, filled with thin albumen, sealed with cling film, and secured with plastic rings and elastic bands (Perry, 1988 ; Naito *et al.,* 1990). The reconstituted eggs were incubated at 38℃ for 3 days and examined for the viability of embryos. Some of the surviving embryos were transferred to large host eggshells, incubated for a further 7 days, and the sex was determined from the morphology of the gonads. Intact embryos were also incubated in the same manner as controls.

DNA extraction and PCR analysis

The collected blastodermal cells were dispersed in 20 μl of a sample preparation reagent (PrepMan™ Ultra ; Applied Biosystems, U.S.A.) and boiled at 100℃ for 10 minutes in a dry block bath (EB-303 ; As-One, Tokyo). The cell suspension was cooled to 4℃, centrifuged at 16,000×g for 3 minutes, and 5 μl (405 ng DNA, on the average) of the supernatant was used for PCR analysis. For comparison, DNA was extracted from the collected blastodermal cells using a DNA extraction kit (SepaGene™ ; Sanko Junyaku, Tokyo) according to the manufacturer's instructions. The extracted DNA was dissolved in a 20 μl Tris-EDTA buffer (10 mM Tris-HCl, 0.25 mM EDTA, pH 8.0), and 5 μl (288 ng DNA, on the average) of the DNA samples was used for PCR analysis.

PCR analysis for sexing was carried out using a programmable thermal controller (Model 9700 ; Perkin Elmer, U.S.A.). The PCR reactions were performed in 50 μl of 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 200 μM dNTPs, 0.5 μM primers and 1 U *Taq* polymerase (R-001B ; Takara, Tokyo). The sequences of the primers for detecting W chromosome-specific repeating sequences were : 5′-CCC AAA TAT AAC ACG CTT CAC T-3′, 5′-GAA ATG AAT TAT TTT CTG GCG AC-3′ (Clinton, 1994 ; Clinton *et al.,* 2001). Control PCR reactions were carried out in the same sample (a single tube assay) to ensure the presence of genomic DNA using primers : 5′-AGC TCT TTC TCG ATT CCG TG-3′, 5′-GGG TAG ACA CAA GCT GAG CC-3′ designed to amplify the chicken 18S ribosomal gene (Clinton *et al.,* 2001).

After an initial denaturation step of 94℃ for 2 minutes, 25 cycles of amplification were performed ; DNA was denatured at 94℃ for 30 seconds, annealed at 56℃ for 30 seconds, and extended at 72℃ for 30 seconds. The samples were then incubated at 72℃ for 5 minutes. After amplification, 5 μl of the reaction product was separated on a 2% agarose gel and visualised under UV irradiation after ethidium bromide staining. A band of 415 bp was detected in females but not in males, and a band of 256 bp was detected for the control reaction.

Results

Viability of manipulated embryos

Viabilities of the manipulated and intact embryos cultured for 3 days are shown in Table 1. Most of them developed normally and showed a viability of 73.9% (193/261) at day 3 of incubation, which was about 20% lower than the controls (92.6%, 201/217).

Sex identification of embryos

Sexes of the embryos were clearly identified by PCR using the DNA prepared by the boiling method (Fig. 1). In addition, 256 bp products of the ribosomal gene primers were produced in both male and female embryos. The results of PCR analysis using the

Table 1. Viability of embryos at day 3 of incubation which were removed a cell cluster from the area opaca of the stage X blastoderm

Sex	Number of embryos treated	Embryos surviving at day 3 of incubation
Male	134	103 (76.9%)
Female	127	90 (70.9%)
Total	261	193 (73.9%)
Control	217	201 (92.6%)

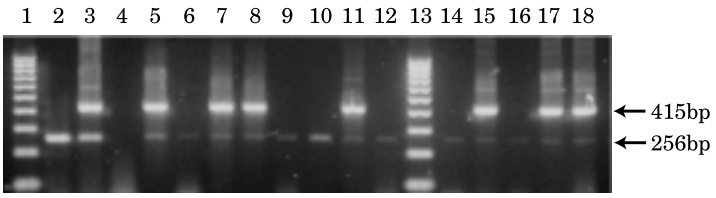

Fig. 1. PCR analysis for sexing chicken embryos using DNA prepared from stage X blestodermal cells. DNA was prepared from blastodermal cells by the boiling or extraction method (control) and analysed for the presence of the W chromosome-specific repeating sequences by PCR (415 bp). Endogenous 18S ribosomal gene was detected as an internal control (256 bp). Sex-specific and control reactions for PCR were carried out in a single tube. Lanes 1 : size marker, 2 : control (male), 3 : control (female), 4 : negative control (water), 5–12 : DNA prepared by boiling method, 13 : size marker, 14–18 : DNA prepared by DNA extraction kit.

DNA prepared by the DNA extraction kit are also shown in Fig. 1. Both sex-specific and control products were clearly identified. For sexing embryos, no apparent difference was observed in the PCR products between the two methods of DNA preparation. The sexes identified by PCR were identical to those examined by gonadal morphology.

Discussion

For the normal development of chicken embryos from stage X onwards, thick albumen can be replaced by thin albumen under culture conditions (Naito *et al.,* 1990). By removing the thick albumen capsule from the yolk, accesibility to the blastoderm layer underlying the yolk membrane is greatly facilitated. The stage X blastoderm is composed of a single layered area pellucida and a peripheral area opaca. The embryo proper arises mainly from the area pellucida, and the area opaca contributes only to extra-embryonic structures (Petitte *et al.,* 1999). When a cell cluster was removed from the central disc of the area pellucida, the viabilities of the manipulated embryos at day 3 of incubation were 50–60% (Naito *et al.,* 1999, 2001). Furthermore, a high incidence of twin embryos was observed (more than 20% of those surviving embryos), and this incidence further reduced the embryos that could be used for manipulation, because most twin embryos die by day 10 of incubation and do not hatch (Naito *et al.,* 1991, 1999, 2001). In this study, a cluster of cells was collected from the area opaca, and a high viability (73.9%) of embryos at day 3 of incubation was obtained without producing twin embryos. Because cells in the area opaca do not contribute to form the embryo proper, cell removal from the area opaca exerted only a minimum influence on embryonic development.

DNA for PCR analysis can easily be prepared from the blastodermal cells by the boiling method, i.e. a 10-minute boiling and 3-minute centrifugation. Sex-specific and control reactions for PCR can be carried out in a single tube by the method of Clinton *et al.* (2001). The PCR products were not different between the two methods of DNA preparation, boiling and extraction, and the sexes of embryos were clearly identified by both. This simple and rapid technique for sexing using blastodermal cells isolated from the area opaca enables us to identify the sex of large numbers of chicken embryos in a limited time. This technique of embryo sexing can also be used in studying sexual differentiation of embryos, germ cell differentiation in mixed-sex germline chimaeric chickens, *etc.*

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