Rapid Sex Identification of Chicken by Fluorescence *In Situ* Hybridization Using a W Chromosome-specific DNA Probe

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ABSTRACT : It has been known that the sex of chicken cells can be most accurately identified by fluorescence *in situ* hybridization (FISH). However, the presently available FISH has not been widely used for sex identification, because the procedures for cell preparation and FISH itself are complicated and time-consuming. The present study was undertaken to test a rapid FISH procedure for sexing chicken. A FISH probe was simultaneously synthesized and labeled with digoxigenin by polymerase chain reaction (PCR) targeting a 416 bp segment of the 717 bp *Xho*I family fragment which is repeated over 10 thousand times exclusively in the W chromosome. Sexing by FISH was performed on cytological preparations of early embryos, adult lymphocytes and feather pulps of newly hatched chicks. The DNA probe hybridized to all types of uncultured interphase as well as metaphase female but not male cells that had been examined. Moreover, consistent with the known site of the *Xho*I family, the hybridization signal was localized to the pericentromeric region of the W chromosome. We, therefore, conclude that the present PCR-based FISH can be used as a rapid and reliable sex identification procedure for chicken. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 11 : 1531-1535)***

Key Words : Chicken, W Chromosome, *Xho*I family, PCR, FISH, Sexing

INTRODUCTION

The identification of the sex of chicken or chicken cells has not only biological significance, especially in transgenic technology and embryological studies, but also has industrial significance. The sex of chicken cell has been identified mostly by polymerase chain reaction (PCR) or fluorescence *in situ* hybridization (FISH) targeting a W chromosome-specific repetitive DNA sequence (Clinton, 1994; Petitte and Kegelmeyer, 1995) such as *Xho*I or *Eco*RI family which altogether occupies 65% of the total W chromosome DNA (Kodama et al., 1987; Mizuno and Macgregor, 1998; Suka et al., 1993). Using PCR protocol, the unique sequences such as CHD-W (chromo-helicase-DNA binding protein gene) and EE0.6 (0.6 kb *Eco*RI fragment) also have been utilized for the identification of sex in chickens (Ogawa et al., 1997; Griffiths et al., 1998; Itoh et al., 2001). Although the unique sequence has less false positive, it is hard to apply on FISH. However, large copy number of *Xho*I family may give more advantage. Klein and Ellendorf (2000) evaluated the sexing accuracy of the PCR method targeting an approximately 400 bp partial *Xho*I family fragment with different amounts of template genomic DNA ranging from 10 pg to 1 ng and with different numbers of cycles between 20 and 50. These workers obtained a high accuracy (7.6% false-positive rate) of the sex diagnosis at their optimum conditions, but the overall procedure was rather stringent and laborious for a routine lab practice. Their PCR accuracy as well as the specificity of the PCR product was variable depending on the preparation procedure and amount of template DNA and the number of PCR cycles used after 20 cycles. They also performed FISH using the PCR product as probe for chicken sex diagnosis and concluded that FISH is better than PCR in terms of the accuracy. However, their FISH analysis, which was performed on metaphase cells following several days of in vitro culture, was too time-consuming for a routine lab procedure. The present study was therefore undertaken to test a rapid PCR-based FISH procedure for sexing various types of uncultured and cultured chicken cells.

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Received May 14, 2002; Accepted July 5, 2002

MATERIALS AND METHODS

Amplification by PCR of the *Xho*I family DNA segment of chicken DNA

Genomic DNA was extracted from female chicken lymphocytes using the Wizard DNA extraction kit (Promega, Madison, WI, USA) following the manufacturer's instructions. A PCR targeting a 416 bp segment within the 717 bp repetitive *Xho*I family DNA fragment (Kodama et al., 1987) was performed using the w-5 (5'-CCCAAATATAACACGCTTCACT-3') and w-3 (5'-GAAATGAATTATTTTCTGGCGAC-3') primer pair (Clinton, 1994). The PCR mixture contained 50 ng genomic DNA, 20 pmol of each primer, 12.5 μmol of each of the

four deoxyribonucleoside triphosphates and 1 unit *Taq* polymerase (Takara, Kyoto, Japan) in a total volume of 50 µl reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$). Reaction conditions were: initial denaturation for 5 min at 94°C; 30 cycles at 94°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec; and final extension for 5 min at 72°C. The PCR product was ligated onto a TA-cloning vector pCR2·1 (Invitrogen Life Technologies, San Diego, CA, USA) following the manufacturer's instructions. The nucleotide sequence of the insert was determined by the dideoxy chain-termination method (Sanger et al., 1977) using PRISM BigDye terminator mix, an automated DNA sequencer (ABI PRISM 377) and ABI PRISM Sequencing Software Version 3.0 (all from PE Biosystems, Foster City, CA, USA). The resulting sequence was compared with known sequences registered in GenBank using the BLAST program of the National Center for Biotechnology Information.

Preparations of metaphase spreads and interphase nuclei from embryonic cells and adult lymphocytes

Fertilized eggs of Single Comb White Leghorn hens were incubated for 18 h at 41°C. Then, before they were harvested, 100 µg colchicine (Sigma Chemical Co., Saint Louis, MO, USA) was injected into each egg through the air sac and further incubated for 1.5 h (Sohn et al., 1995). After breaking the shell and vitelline membrane, the embryonic disc was removed from each egg yolk and transferred into a 15 ml centrifuge tube. The cells were incubated for 20 min at 37.5°C in 5 ml of a hypotonic solution (3 parts of 0.3% sodium citrate and 1 part of newborn calf serum, v/v). The swollen embryonic disc cells were fixed in a methanol/acetic acid (3:1) fixative for 30 min followed by centrifugation for 10 min at 150×g. After repeating the above fixation procedure three times, the embryonic disc cells were dropped onto glass slides and air-dried overnight on a 65°C slide warmer. G-banding was performed following the procedures described elsewhere (Seabright, 1971; Sohn et al., 2000).

Metaphase chromosomes from lymphocyte cultures of adult chickens were prepared as described previously (Sohn and Ryu, 1999). In brief, a 4 ml 1:1 mixture of heparinized blood and RPMI 1640 culture medium was gently layered on top of a 3 ml cushion of 5.7% Ficoll (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) in a 15 ml tube and centrifuged for 35 min at 300×g. After removing the plasma layer from the top, the buffy coat layer containing mostly lymphocytes was transferred to a T-25 culture flask containing 10 ml RPMI 1640 supplemented with 1% penicillin-streptomycin, 2% pokeweed mitogen and 15% fetal bovine serum (v/v, all from Invitrogen Life Technologies, San Diego, CA, USA). The lymphocyte culture was incubated for 72 h at 37°C in an atmosphere of 5% CO_2 and 95% air. The metaphases were enriched by adding 2 µg colcemid 1 h before the end of the incubation period. The chromosome preparations were made following the procedure just described.

Interphase nuclei from uncultured embryonic disc cells and lymphocytes from adult chickens were prepared basically following the procedure described above, except for the culture procedures and colchicine/colcemid treatment.

Preparation of cells from feather pulps

Feather pulps were obtained from 16 newly hatched Single Comb White Leghorn chicks by squeezing the content of the feather stalk with a pair of forceps into 15 ml centrifuge tubes containing 5 ml RPMI 1640. The cells were separated from the pulp by vigorous pipetting followed by centrifugation for 10 min at 150×g. The precipitated cells were treated with a hypotonic solution (distilled water and new born calf serum, 3:1), fixed in a methanol and acetic acid mixture (3:1 by volume), dropped onto slides and air-dried as described above. The sex of the cells was determined by FISH using our newly generated W chromosome-specific DNA probe and this was confirmed by visual inspection of the internal genitalia of the respective chicks.

FISH with W chromosome-specific DNA probe

A 416 bp DNA fragment was simultaneously labeled with digoxigenin (Dig)-dUTP (Boehringer-Mannheim, Indianapolis, IN, USA) and amplified by PCR using the w-5 and w-3 primer pair and female chicken genomic DNA as template following the manufacturer's instruction. The FISH procedure was slightly modified from that of Kobayashi et al. (1998). Briefly, 10 µl hybridization buffer (2×SSC, 50% formamide and 10% dextran sulfate) containing 100 ng Dig-labeled probe was applied onto the cytological preparations, covered with a coverslip and sealed with rubber cement. The specimens and probe were denatured for 8 min on a heating plate at 70°C and were then allowed to hybridize for 1 h at 37°C. The slides were washed with 2×SSC at 72°C for 5 min and subsequently with PN buffer [7 mM sodium phosphate, pH 8.0, containing 0.1% Nonidet 40] for 2 min at room temperature and finally air-dried for 10 min. The air-dried slides were incubated for 5 min at 37°C with 20 µl of 0.5 µg anti-Dig-fluorescein isothiocyanate (FITC) conjugate/ml PN buffer containing 5% non-fat dry milk (Boehringer Mannheim). They were then washed with PN buffer, counter-stained with propidium iodide and examined under a fluorescence microscope (Model AX-70, Olympus, Tokyo, Japan) equipped with a WIB 523 nm-pass filter. The images were captured by photomicroscope (PM-30, Olympus, Tokyo,

Japan) with ASA 400 color film (Eastman Kodak, Rochester, NY, USA). From each sample slide a minimum of 50 cells were evaluated for the W chromosome-specific signal.

RESULTS AND DISCUSSION

Synthesis of a chicken W chromosome-specific DNA probe

An approximately 400 bp segment of the 717 bp W chromosome-specific *Xho*I family fragment was synthesized by PCR using female chicken genomic DNA as template (Figure 1, lane 1). Clinton (1994) previously reported the PCR-amplification of the *Xho*I family fragment in chicken using PCR primers with the same sequences as used in this study. However, the author reported that the size of the PCR product was only 315 bp, which may be a miscalculated value. In line with this, Klein and Ellendorf (2000) detected a calculated 415 bp PCR product using the same primers. To make a W chromosome-specific FISH probe, the approximately 400 bp segment of the *Xho*I family was labeled with digoxigenin (Dig) by PCR using Dig-dUTP. The resulting product appeared longer than 500 bp because of the Dig conjugation (Figure 1, lane 2).

As is shown in Figure 2, our resulting sequence was 416 bp long. The base sequence of the 416 bp PCR product (GenBank accession number AF461428) exhibited a 400/416 (96%) identity with that of a *Xho*I family fragment (Kodama et al., 1987, GenBank accession number X06548). The sixteen aberrant bases included a deleted base, an inserted base, eight bases which are different from the X06548 sequence but are identical with either sequence of two other reported *Xho*I family clones (Kodama et al., 1987, GenBank accession numbers M24754 and M24755), and six bases that are not identical to any of the previously reported sequences. Taken together, these results indicate that an authentic segment of the *Xho*I family fragment specific for female chicken W chromosome was synthesized by our present PCR protocol.

FISH on metaphase embryonic cells and lymphocytes

Following FISH on the metaphase chromosome spreads with the 416 bp Dig-labeled probe, a hybridization signal on the W chromosome was detected in female embryonic cells (Figure 3a) and adult lymphocytes (Figure 3b), but not

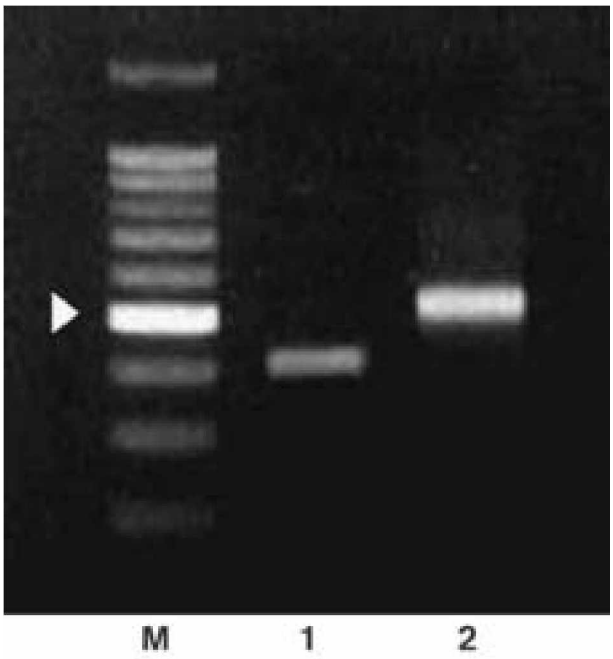

Figure 1. Agarose gel electrophoresis and ethidium bromide staining following PCR-amplification of approximately 400 bp segment of the 717 bp *Xho*I family fragment of a female chicken genomic DNA. M denotes the DNA size markers (100 bp ladders). Lane 1, PCR product of partial *Xho*I DNA (approximately 400 bp). Lane 2, The partial *Xho*I DNA labeled with digoxigenin (Dig) during PCR using Dig-dUTP. Note the labeled and unlabeled PCR products above and below the 500 bp DNA marker (*arrow head*), respectively.

Figure 2. Nucleotide sequence comparison between the PCR product and the chicken *Xho*I family fragment. The nucleotide sequence of the 416 bp PCR product in Figure 1 (upper sequence; GenBank accession no. AF461428) was aligned with a known sequence of the 717 bp chicken *Xho*I family fragment (lower sequence; GenBank accession no. X06548). Nucleotide numbers are indicated in the margin, with the first base of the PCR product arbitrarily assigned #1. Shaded are the w-5 and w-3 PCR primer sequences. The homology between these two sequences was 96%.

in male cells (not shown). The frequency of metaphase cells that exhibited the W chromosome-specific DNA signal was above 80% per female slide (>50 cells/slide), regardless of the cell type. Klein and Ellendorf (2000) have reported that the *Xho*I family sequence was present on some autosomes as well as on the W chromosome as determined by FISH in chicken, although the frequency of the autosomal hybridization was estimated to be less than one thousandth that of the W chromosome. In our hands, the probe signal was confined to the W chromosome only and no autosomal segment or the Z chromosome registered any signal. Moreover, upon examination at higher magnification of the W chromosome (Figure 3c), the probe signal was detected in the pericentromeric region, which is in agreement with a previously reported result (Saitoh et al., 1991; Saitoh and Mizuno, 1992).

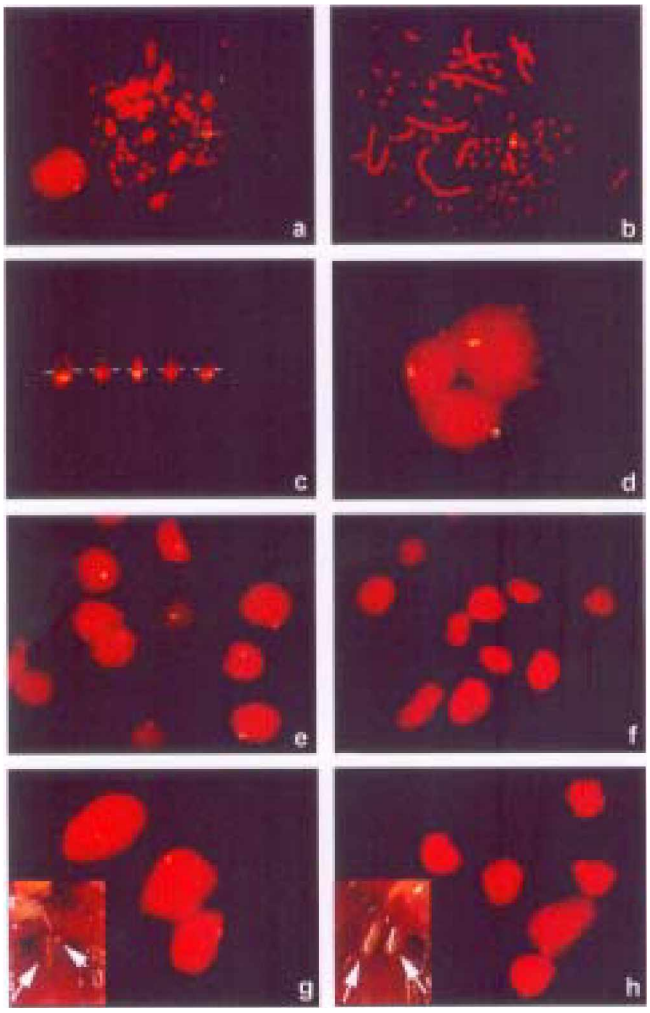

Figure 3. FISH on cytological preparations of various types of cultured and uncultured chicken cells using the 416 bp Dig-labeled *Xho*I family fragment as probe. The FISH was performed on cultured (a~c) and uncultured (d~h) cytological preparations; a and b, metaphase chromosome spread of a female embryonic cell (a) and of a female adult lymphocyte (b); c, magnified W chromosomes of female lymphocytes; d~f, female embryonic cells (d) and lymphocytes (e) and male lymphocytes (f); g and h, feather pulp cells of a newly hatched female chick (g) and a male chick (h) which was subsequently identified for sex by visual inspection of the gonad (*box*). Note the probe signal on the W chromosome. Also note a degenerating ovary (short arrow) and a developing ovary (long arrow) in the female chicken (g) and two developing testes (arrows) in the male (h). Shown are representative results.

FISH on uncultured interphase cells

Now that we have established the reliability of our FISH for sex identification of cultured metaphase cells in the previous experiment, we next set out to determine if the FISH probe would also hybridize to uncultured interphase cells in a W chromosome-specific manner. As anticipated, the present FISH probe bound to the W chromosomes of interphase nuclei of uncultured female embryonic cells (Figure 3d) and lymphocytes (Figure 3e), but not to uncultured male lymphocytes (Figure 3f). The sex of the cells was confirmed by standard karyotyping (Sohn et al., 2000; Sohn and Ryu, 1999) following several days of in vitro culture of the cells. Furthermore, the probe bound to uncultured feather pulp cells derived from newly hatched female (Figure 3g) but not male (Figure 3h) chicks. Of the 16 hatched chicks that were examined, eight exhibited the positive W-specific probe signal in their feather pulp. These eight chicks were subsequently identified as females by visual identification of the ovary (Figure 3 g, box). The other eight chicks, which were signal-negative, were males as indicated by the presence of testes (Figure 3 h, box). It is evident from these results that by using our FISH procedure, one can by-pass the time-consuming culture procedure for obtaining metaphase chromosome spreads and one can directly use uncultured interphase cells, without compromising the female-specificity.

In conclusion, our results indicate that the present FISH procedure can be used as a rapid and reliable procedure for sexing various types of uncultured as well as cultured cells of chicken including the newly hatched chicks in which cells other than the feather pulp cells are barely obtainable.

ACKNOWLEDGEMENTS

The authors thank Jill Hansen, Cellular Genetics Lab., M. D. Anderson Cancer Center, Houston, for critical review of the manuscript. This work was supported by the Korea Research Foundation Grant (KRF-2000-GA0014).

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