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Unanticipated Gene Deletion in the Transgenic Chicken Employing Ovalbumin Promoter for Oviduct Specific Expression

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ABSTRACT

Transgenic chickens have been spotlighted as an highly potent bioreactor for their fecundity, short generation time, and eggs associated with mass production of protein. In this study, we generated transgenic chickens exhibiting oviduct specific expression of human growth hormone fused to human transferrin for oral administration. Gene of the modified growth hormone located at downstream ovalbumin promoter (~3.6 kb) was introduced to stage X blastodermal cell employing retrovirus vector system. Several transgenic chickens were successfully generated. However, genomic analyses showed unexpected deletion within the transgene. The modification of the transgene seemed to occur during germ cell formation because the deletion was detected only from the sperm DNA of the G0 founder animal. There was no evidence of deletion in the somatic cell DNA samples of the same chicken. Consequently, same pattern of the deletion was confirmed in both somatic and germ cells of the G1 progeny.

(Key words : Ovalbumin promoter, Transgenic chicken, Retrovirus vector, Gene deletion)

INTRODUCTION

Transgenic chickens have been considered as highly potent bioreactors for their fecundity and short generation time (Ivarie, 2003). Various embryonic stages can be easily observed and manipulated by a number of methods. Furthermore, compared to other mammals higher similarity between human and chicken proteins especially in glycosylation patterns has been reported (Raju *et al*., 2000). Above all things, natural sterile microenvironment of the egg system and inherent protease inhibitors in eggs are believed to provide optimal condition as it stabilize the biological activity of foreign proteins (Mozdziak and Petitte, 2004; Rapp *et al*., 2003). Two methods, retrovirus-mediated gene transfer (Thoraval *et al*., 1995) and direct DNA transfection (Muramatsu *et al*., 1997) are mainly considered to transfer foreign genes to blastodermal stem cells at stage X (Eyal-Giladi and Kochav, 1976). Especially, retrovirus vector system has been more preferred for its technical ease and effectiveness of gene transfer. Until now, several successful generation of transgenic chickens using retrovirus vector has been reported, including human erythropoietin (Koo *et al*., 2010), human interferon α-2b (Rapp *et al*., 2003), human interferon β-1a (Lillico *et al*., 2007) as well as monoclonal antibodies (Kamihira *et al*., 2005). In this study, we performed an experiment using ovalbumin promoter enabling oviduct specific expression of the human modified growth hormone within ovalbumin that comprises majority of egg white proteins. We carried out vector construction, virus preparation, and injection of concentrated virus stock into stage X embryonic stem cell of eggs. As the result. we could detect two G0 transgenic chickens through PCR reaction. One of them mated with normal hen resulting in production of G1 progenies. Four months later when G1 was matured enough to collect sperm, we took the samples of sperm and blood from G0 and G1 chickens, then tested whether the integrated sequence was well preserved or not. Through several tests, we found that the integrated transgene was shortened mainly by deletion of most ovalbumin promoter region. Furthermore, DNA sequencing showed that this phenomenon has nothing to do with previously known splicing donor/acceptor interaction sites.

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MATERIALS AND METHODS

Construction of Retrovirus Vector and Virus Production

The plasmid pLNOv36-hGH-helical-hTf-W we used in this study was constructed by switching CMV promoter of pLNCX retrovirus vector (Miller and Rosman, 1989) with a fragment consisting of chicken ovalbumin promoter, hGH-helical-hTf, and woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Fig. 1). The WPRE sequence (GenBank accession number M-11082) was introduced following the strategy of Zufferey *et al* (1999). About 3.6 kb of ovalbumin promoter fragment was obtained by manipulation of upstream region of chicken ovalbumin gene (Lillico *et al*., 2007), and a fragment of hGH-helical-hTf was prepared by fusion of human growth hormone gene (651 bp) and human transferrin gene (2037 bp, Genbank accession no. NM_001063.3) through short helical sequence (22 bp). Fig. 1 shows schematic representation of pLNOv36-hGH-helical-hTf-W. In constructing retrovirus producing cells, we used our established protocol (Kim, 2002). Briefly, PT67 packaging cells (purchased from Clontech) were transiently transfected with pLNOv36-hGH-helical-hTf-W, and LNOv36-hGH-helical-hTf-W viruses harvested from the transfected cells were added to the culture of GP2-293 cells (purchased from Clontech). PT67 cells are retrovirus packaging cells characterized by expression of the Gibbon ape leukemia virus envelope gene and gag and pol genes of the MoMLV (Moloney murine leukemia virus), whereas GP2-293 cells have been designed to express olny the gag and pol genes of the MoMLV. The GP2-293 cells infected with LNOv36-hGH- helical-hTf-W were selected with G418 (600 μg/ml) for 2 weeks and the resultant $G418^R$ (or Neomycin-resistant) cells were transfected with pVSV-G (purchased from Clontech) to provide vesicular stomatitis virus glycoprotein that can package the retroviral RNA genome. Viruses were harvested 48 hours post-transfection. All cells, including virus-producing cells, were grown at 37℃ in a 5% CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L of glucose (GibcoBRL, USA)) and supplemented with fetal calf serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml). The virus-containing medium harvested from the virus-producing cells was centrifugally concentrated to 1/1,000 of the original volume and filtered through a 0.45 μm pore-sized filter. The virus titer of the concentrated stock was 1×10^9 Neo^R cfu/ml (neomycin-resistant colony-forming unit/ml) on both NIH3T3 cells and primary cultures of chicken embryonic fibroblast cells (data not shown).

In this experiment, only fertilized eggs weighing among 60 to 65 were used. Selected eggs were incubated for 5 hours prior to virus infection. Meanwhile surrogate egg shells were prepared by decanting egg whites and yolks through 33 mm in diameter holes made on the edge. To minimize contamination, all egg shells were washed with sterile water. Whole egg contents of each pre-incubated fertilized egg were collected in a 100 mm petridish, and flip the yolk upside down using sterilized spoon. Five μl of concentrated virus stock in DMEM supplemented with polybrene (10 μg/ml) was injected into the central part of the blastoderm. After transferring the injected egg contents into the surrogate shell, the marginal space was filled with egg white before sealing the hole with plastic wrap. The sealed eggs were incubated at 37.5℃ and 60% relative humidity with a 90° rocking motion every 15 min for 3 days. After three days of incubation, the embryos were transferred to larger empty recipient eggshells through a 42-mm circular window before second incubation at 37℃ and 70% relative humidity with a rocking motion every 30 min for additional 15 days. At 20 or 20.5 days of incubation, the plastic wrap was replaced with a 60 mm petridish lid and the eggs were allowed to hatch without rocking motion.

PCR Analysis

Genomic DNA was extracted from chicken muscle ti-

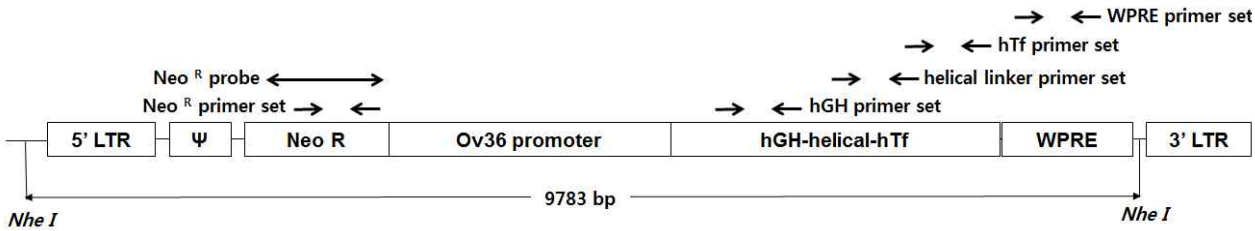

Fig. 1. Structure of the LN-Ov36-hGH-helical-hTf-W provirus. LTR, long terminal repeat; Neo^R, Neomycin-resistant gene; Ov36, ~3.6 kb ovalbumin promoter; hGH-helical-hTf, human growth hormone gene fused to human transferrin gene by helically structured linker; WPRE, woodchuck hepatitis post-transcriptional regulatory element gene. The approximate positions of the probes for Southern blotting and of the PCR primer sets were indicated as arrows with two (probes) or one head (primer), respectively. Enzymatic digestion of the provirus with *Nhe* I separates 9783 base pair fragment. Drawing is not to scale.

Table 1. Primer sequences for PCR analysis

Gene	Primer sequence	Annealing temperature (℃)	Product size (bp)
hGH	F-5'TTATCCAGGCTTTTTGACAACGCT3' R-5'GTTTGGATGCCTTCCTCTAGGTCC3'	57	353
hTf	F-5'GTTGCTTGTGTGAAGAAAGCTTCC3' R-5'AGCTGGTTCATCTGGAAGCCACTA3'	57	227
helical linker	F-5'CTACCAGGAGTTTGAAGAAGCCTA3' R-5'CCTCTAGGTCCTTTAGGAGGTCAT3'	57	275
Neo^R	F-5'ATGATCTGGACGAAGAGCATCAGG3' R-5'ATATCACGGGTAGCC AACGCTATG3'	57	217
WPRE	F-5'GGATACGCTGCTTTAATGCCTTTG3' R-5'CGACAACACCACGGAATTGTCAGT3'	60	315

ssue and blood using a genomic DNA purification kit (Promega, USA). For PCR analysis, primer sets were designed for the *hGH, hTf, helical linker, WPRE, Neo^R* genes based on the nucleotide sequence of pLNOv36-hGH-helical-hTf-W. The forward and reverse primer pairs correspond to the pLNOv36-hGH-helical- hTf-W nucleotide sequences of 6304-6658 (for *hGH*), 8785- 9012 (for *hTf*), 7939-8215 (for *helical linker*), 9325-9641 (for *WPRE*), and 1790-2007 (for *Neo^R*). Each reaction mixture consisting of 1 μg of genomic DNA extract, 50 pmol of each primer, 5 μl 10XPCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP was added with water to bring the total reaction volume to 50 μl. The mixture was heated to 94℃ for 5 min prior to the addition of 2.5 U of Taq polymerase (Promega, USA). The amplification profile consisted of 94℃ for 30s (denaturation), 54℃ for 30s (annealing), and 72℃ for 30s (extension). After 35 amplification cycles, the samples were retained at 72 ℃ for 7 min to ensure complete strand extension. The sequences of each primer sets are listed on Table 1.

Southern Blot Analysis

For Southern blot analysis, genomic DNA (12 μg) was digested with *Nhe* I, and separated on a 0.8% agarose gel. The probe corresponding to *Neo^R* DNA fragment (729 base pair) was synthesized using the PCR DIG Probe Synthesis kit (Roche, Basel, Switzerland) with the primer set of 5'-AAGCTATTCGGCTATGACTG-3' (upstream) and 5'-AAGAAGGCGATAGAAGGCGA-3' (downstream). The resulting probe was labeled with digoxin alkaline phosphatase and purified by agarose gel electrophoresis before hybridization. Detection of labeled DNA on the positively charged nylon membrane was performed using a DIG luminescent detection kit (Roche, Germany).

DNA Sequencing of Genomic Regions Flanking the Provirus

Genomic DNA was amplified with PCR using *Neo^R* forward primer and *WPRE* reverse primer. Amplified DNA was electrophorsed through 1% agrose gel for 15 minutes with 120 voltage. DNA was eluted using Gel Extraction Kit (Dok Do Prep™ ELPIS BIOTECH, Korea). After cloning the extracted DNA fragment into the TA cloning vector kit (RBC Bioscience, Taiwan), the sequencing was performed by BIONICS CORPORATION (Seoul, Korea).

RESULTS

PCR Analysis of Blood and Sperm of G0 Transgenic Chicken

After injection of highly concentrated LNOv36-hGH-helical-hTf-W virus into the subgerminal cavity of the chicken blastoderm, 36 of 163 eggs were hatched. Thirteen females and two males were identified as transgenic chickens by blood PCR analysis (data not shown). Six months later, to assure germline transmission, we tested sperm genomic DNA of two male transgenic chickens (Chicken numbers 41 and 42). PCR analysis determined only one chicken (chicken #41) being transgenic. Interestingly, however, profile of the PCR bands were different depending on the source of the genomic DNA: PCR amplification of blood DNA showed all bands expected, while all genes except *Neo^R* gene were missing from sperm DNA samples (Fig. 2-A).

Muscle Tissue DNA PCR Analysis of G0 and G1 Transgenic Chickens

G0 chicken (#41) was mated with normal hen and one of twelve progenies was confirmed to be transgenic. However, as the G1 was reaching to 6 month old, both G0 and G1 chicken died for unknown rea-

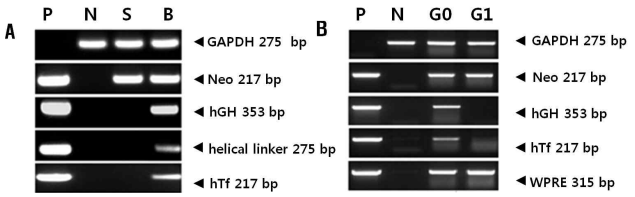

Fig. 2. (A) PCR analysis of G0 transgenic chicken (chicken #41). Genomic DNA samples were derived from sperm and blood. P, plasmid pLNOv36-hGH-helical-hTf-W; N, Normal chicken genomic DNA; S, sperm genomic DNA; B, blood genomic DNA. (B) PCR analysis of muscle genomic DNA of G0 and G1 transgenic chicken.

sons. PCR analysis of muscle genomic DNA isolated from frozen G0 transgenic chicken carcass showed same profile of bands as shown in the blood sample of same chicken ("B" lane of Fig. 2-A and G0 lane of Fig. 2-B). In case of muscle genomic DNA isolated from frozen G1 transgenic chicken carcass, however, some PCR bands were missing as shown in the sperm sample G0 transgenic chicken ("S" lane of Fig. 2-A and G1 lane of Fig. 2-B). We presume that the central part of transgene which encompasses much of Ov36 promoter and hGH-helical-hTf region was vanished in germline of G0 transgenic chicken.

Estimation of Transferred Gene Length

We performed Southern blotting to confirm the deleted region of the transgene in G1 muscle genomic DNA. As previously stated, genomic DNA (12 μg) was digested with *Nhe* I and then separated on a 0.8% agarose gel before applying Neo^R probe. Compared with P lane (positive control of diluted plasmid DNA) G1 lane

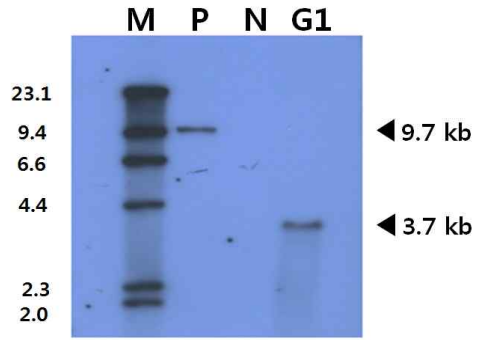

Fig. 3. Southern blot analysis of muscle genomic DNA isolated from G1 transgenic chicken. Three different DNA samples (P, plasmid DNA; N, Normal; G1, Transgenic G1 chicken) were digested with enzyme *Nhe* I. Neo^R probe was used to detect the bands.

shows a ~3.7 kb band, indicating deletion of around 6 kb (Fig. 3). We presumed that the gene deletion occurred over the regions of Ov36 promoter and hGH-helical-hTf.

Sequencing of the Transgene Integrated into G1 Muscle Genomic DNA

We conducted PCR amplification of muscle genomic DNA extracted from the muscle of G1 transgenic chicken using Neo^R (Forward) and *WPRE* (Reverse) primer set. The resulting PCR amplified fragment of around 1.4 kb was sequenced by BIONICS CORPORATION (Seoul, Korea) (Fig. 4-A). It has been known that splicesome recognize two dinucleotide sites; GT and AG as splicing donor and splicing acceptor, respectively. However, the sequencing data showed two separate splicing ev-

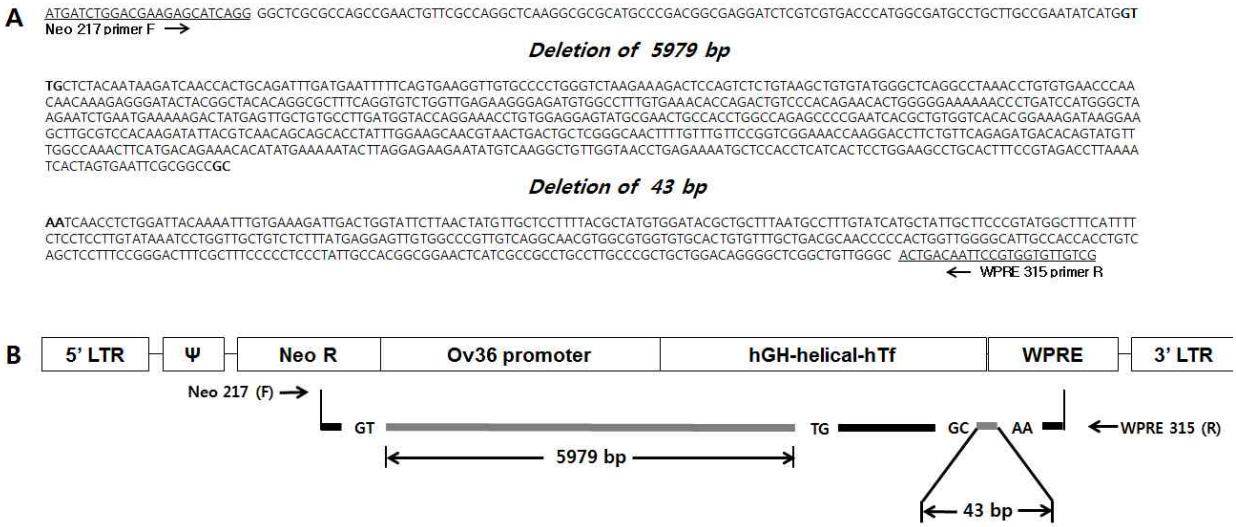

Fig. 4. (A) DNA sequencing data. Putative splicing donor and acceptor sites are marked in bold. (B) Schematic deleted region of LNOv36-hGH-helical-hTf-W provirus. The presumable deletion regions were shown in gray.

ents: One is between GT and TG, and the other between GC and AA. It was estimated that, deletion of as many as 6,022 bp occurred as summarized in Fig. 4-B.

DISCUSSION

In this study, we aimed to generate transgenic chickens using retrovirus vector which is composed of essential elements for efficient retrovirus production and ovalbumin promoter enabling oviduct specific expression of the human growth hormone gene modified by linking of the 3′ end to the human transferrin gene via helical linker sequence (Amet *et al*., 2009). Inserted *hTf* gene facilitates oral delivery of hGH-helical-hTf fusion protein (Amet *et al*., 2010). Thirty six eggs were hatched from 163 eggs undergone retrovirus-mediated gene tansfer. Among the progenies, 15 chickens were verified as transgenic chickens through PCR analysis of blood genomic DNA (13 females and 2 males). Sperm of two transgenic roosters (chicken numbers 41 and 42) were collected to perform genomic DNA PCR analysis. As the result, chicken #42 proved absence of transgene in the germ cell, while chicken #41 showed massive deletion within the transgene. Afterwards, we conducted muscle genomic DNA PCR analysis of G1 transgenic hen (offspring of chicken #41) then found same deletion as observed in #41 sperm genomic DNA gene. Through the Southern blotting of G1 muscle genomic DNA, we could estimate the length of truncated sequence being approximately 6 kb. Finally, we also tasked DNA sequencing to identify deletion of 6,022 bp. One interesting point to be considered is that deletion was observed only in the DNA isolated from sperm. No deletion was detected from the DNA of muscle cells of G0 founder chicken. Taken together, the most plausible explanation for the unexpected deletion might be due to unique features of germline cells, such as rapid proliferation, etc. It has been well known that the expression of partly deleted viral genome through recombination is superior to the expression of viral genome retaining whole sequence (Parr *et al*., 2009). The significance of this study may stems from that this is the first report on the retrovirus-mediated gene transfer system in which deletion of the transferred transgene occurs only in the germ cells, although exact mechanism of this phenomenon is yet to be identified. Considering that retrovirus vector has been regarded as one of the best gene transfer system in terms of transgene stability, more studies of the aberrant gene deletion mechanism must be done for future application of retrovirus vector system to the transgenic animal production.

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