Germ-line Transmission of Pseudotyped Retroviral Vector in Chicken

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ABSTRACT : Using MLV (murine leukemia virus)-based retrovirus vectors encapsidated with VSV-G (vesicular stomatitis virus G glycoprotein), we tried to make transgenic chickens carrying the transferred genes in their chromosomes. Twenty one days after virus injection beneath the blastoderms of unincubated chicken embryos (stage Ⅹ, at laying), DNA isolated from the hatched chicks were analyzed by PCR with two sets of primers specific for *EGFP* (enhanced green fluorescence protein) gene or *Neo*^{R} (*E. coli* neomycin resistant) gene. Among sixty-seven embryos injected with retrovirus**,** four of them were identified to carry the *EGFP* genes in their genomes. Remarkably, one transgenic chick showed presence of the retrovirus vector sequences in all organs differentiated from one of endoderm, mesoderm, and ectoderm. Expression of *EGFP* gene was not detected, however, the stable germ line transmission of transgene was verified in spermatozoa from the founder chicken and 50% of F_1 progenies. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 1 : 27-32)***

Key Words : Retroviral Vector, Transgenic Chicken, Embryos, Germ Line Transmission

INTRODUCTION

Compared to the transgenic mammals, the production of transgenic chicken has several advantages including shorter generation time, lower expense, fecundity, etc. However, numerous trials for the transgenic chicken production has been huddled by characteristics of avian reproduction system different from mammals. The ovum is fertilized within 1 h after ovulation, then surrounded by several grams of albumin and eggshell. Most importantly, development of such early embryo initiates even in the reproductive organ of the female (Deeming et al., 1987; Perry, 1987), resulting in the embryo already consisting of around 60,000 morphologically undifferentiated pluripotent cells just after laying (Eyal-Giladi, 1984). For these reasons, one of the most critical factors in the successful production of transgenic chicken is an efficient gene transfer to the multiple blastodermal cells of the newly laid eggs designated as stage X (Eyal-Giladi and Kochav, 1976).

The transfer of foreign DNA into chicken genome has been attempted with either transfected blastodermal cells (Naito et al., 1991; Etches and Verrinder, 1997) or PGCs (Primordial Germ Cells) (Naito et al., 1994; Tsunekawa et al., 2000) into recipient embryos. As in productions of transgenic mammals, direct microinjection of exogenous DNA into the subgerminal cavity of fertilized avian ovum has also been reported (Sang and Perry, 1989; Perry et al., 1991). Other groups of researchers employ retrovirus-mediated gene transfer system for germline transmission of foreign DNA in chickens (Bosselman et al., 1990; Savva et al., 1991; Vick et al., 1993; Love et al., 1994; Thoraval et al., 1995; Harvey et al., 2002). Despite these demonstrations, however, the efficiency of transgenic poultry is still very low and needs further improvement. Problems involved in the approaches mentioned above are; technical difficulties in *in vitro* culture of pluripotent blastodermal cells and PGCs, and inefficient gene transfer system by direct DNA injection and retrovirus vector system.

In this report we used a new retrovirus vector system to transfer *EGFP* (enhanced green fluorescence protein) gene to the blastodermal cells of stage X chicken eggs. Until now most retrovirus vector systems used in the transgenic chicken production has been derived from avian retrovirus (Bosselman et al., 1990), while the virus vectors used in this study are based on MLV (murine leukemia virus) and designed to be packaged by VSV-G (vesicular stomatitis virus glycoprotein) enabling the progeny viruses to infect most of vertebrate cells (Burns et al., 1993). Advantages of this pseudotyped hybrid retrovirus vector system include availability of highly concentrated virus stock and decreased possibility of homologous recombination between the vector and the dormant endogenous retrovirus in the target cell. Except a chicken, transgenic cattle (Chan et al., 1998), fishes (Lin et al., 1994; Gaiano et al., 1996; Lu et al., 1997), and primates (Chan et al., 2001) have been produced by this retrovirus vector system. We present here an efficient way of transgenic chicken production by injecting the hybrid retrovirus stock beneath the blastoderm at stage X. These results could be helpful in establishing an

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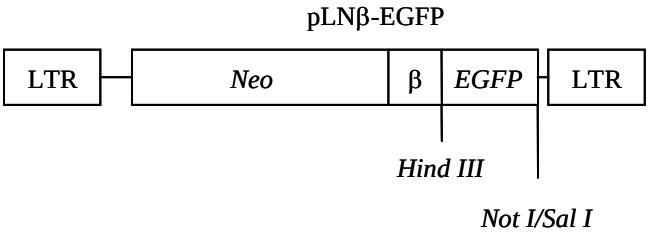

Figure 1. Structure of pLNβ-EGFP. The pLNβ-EGFP was constructed by replacing the 3.2 kb *Hind III- Sal I* fragment of *LacZ* gene of the pLNβZ (Kim et al., 1993) with the *EGFP* gene derived from 780 bp *Hind III- Not I* fragment of pEGFP-N1 purchased from Clontech. LTR, long terminal repeat; *Neo*, *E. coli* neomycin resistant gene; β, rat β-actin promoter; *EGFP*, enhanced green fluorescent protein gene. Drawing is not to scale.

effective method for the production of transgenic chicken.

MATERIALS AND METHODS

Windowed egg (injection of viral vector)

Fertilized eggs (Stage X embryo according to the classification of Eyal-Giladi and Kochave, 1976) were obtained from ISA brown laying hens group artificially inseminated once a week with semen from ISA brown males. Only 60±3 g weight, normal shaped eggs were used in the experiments. These eggs were positioned on their side-up for 8 h at room temperature in order to fix the blastoderm position. After swabbing the shell with 70% alcohol, 4.5×4.5 mm^2 sized windowed was made in the equatorial plane of the eggshell by using fine drill followed by removal of the small shell membrane (4×4 mm^2) inside the window with a fine forceps and a surgical blade.

Ten μl of Dulbecco's Modified Eagle Medium (DMEM) containing retroviral vector was injected into the center of disc by using micro injection pipette. To increase infectivity, polybrene (10 μl/ml) were added to the virus medium. The injection pipette was drawn from pyrex glass tube with inner diameter at the tip of 80 μm. After retroviral vector was injected into subgerminal cavity, the window was sealed with parafilm three times.

Egg incubation

After microinjection, the sealed eggs were incubated at 37.7°C and 55% relative humidity with a rocking motion every 2 h through 90 angle for 18 days, then the eggs were further incubated at 37.5°C and 75% relative humidity without rocking motion until hatching. The age of an egg is based on the post-incubation days (e.g., the day of microinjection is referred to as a day 0), and the eggs were candled at day 10 and 18.

Retroviral vector

Retroviral vectors were produced from the 293 mGPHY cells (Kim et al., 2001) after introductions of LNβ-EGFP (Figure 1) recombinant retroviruses and plasmid pHCMV-G (Burns et al., 1993) kindly provided from Dr. J. C. Burns. 293 mGPHY cells were designed to express the *gag* and *pol* genes of MoMLV (moloney murine leukemia virus), and pHCMV-G was used to express VSV-G protein from the 293 mGPHY cells. Introduction of LNβ-EGFP recombinant retroviruses to the 293 mGPHY cells were accomplished by infection of 293 mGPHY cells with LN-EGFP produced from PG13 cells (packaging cell line characterized by expression of Gibbon ape leukemia virus envelope gene) (Miller et al., 1991; Kim et al., 1993). Following the infection with LNβ*EGFP*, the cells were selected with G418 (800 μl/ml) for two weeks, then the resultant $G418^R$ cells were transfected with pHCMV-G to express VSV-G protein. Viruses were harvested 48 h post-transfection. All cells including final virus-producing cells were grown under DMEM with 4.5 g/l of glucose (Gibco BRL, 12800) supplemented with fetal calf serum (10%), penicillin (100 μ /ml), and streptomycin (100 μg/ml) in 37°C, 5% CO_2 incubator. The virus-containing medium harvested from the virus-producing cells was centrifugally concentrated to 1/200 of the original volume and filtered through 0.45 μm pore-size filter.

Genomic DNA purification

Each wing tip was obtained by cutting a small portion from the edge of the wing, and various tissue samples were prepared from a dead chicken. Sperm collection from the male chicken was made following abdomal massage method. Ejaculated semen was collected by using 1 ml disposable syringe. Each wing tip (1 g) and semen (300 μl) was incubated with 600 μl of lysis buffer (0.1 mg/ml proteinase K, 1 M Tris-HCl <pH 8.5>, 10% SDS, 0.5 M EDTA <pH 8.0>, and 5 M NaCl) at 60°C overnight. DNA was extracted once with phenol and twice with phenol:chloroform (1:1) and once with chloroform. After ethanol precipitation, nucleic acid pellet was dissolved in 200 μl of TE buffer and digested with heated pancreatic ribonuclease (RNase) for 60 min at 37°C.

PCR analysis

All primers were designed based on the sequences of the cloning vector (pLNCX and pEGFP-N1) available from Genbank. Briefly, The upstream primer for Neo^R (5`-ATTCCGATCTGATCAAGAGAC-3`) corresponds to nucleotides 1616-1636 of the pLNCX and the downstream one (5`-TTTCCACCATGATATTCGGCA-3`) represents the reverse complement of nucleotides 2253-2273. This primer pair predicts an amplified DNA fragment of 638 bp. For *EGFP* gene, the upstream primer (5`-GAGCGCAC CATCTTCTTCAAGGAC-3`) and the downstream one (5`-

Table 1. Viability and hatching rates of manipulated chicken embryos

Treatment[1]	No. of embryos	No. (%) of embryos surviving for			No. (%) of chicks carrying retrovirus vector sequences[2]
		10 days	18 days	21 days	
Control	30	28 (93)[a]	28 (93)[a]	28 (93)[a]	-
DMEM only	44	40 (91)[a]	38 (86)[a]	16 (36)[b]	-
DMEM with virus	67	57 (85)[a]	19 (28)[b]	5 (7)[c]	4 (6)

[1] Embryos injected with either medium only or medium carrying retrovirus vector are designated with "DMEM only" or "DMEM with virus" respectively. "Control" refers to the eggs undergone neither window processing nor injection.
[2] Detection of retrovirus vector sequence was done by PCR analysis using EGFP primer set.
[a, b, c] Statistical analyses were carried out by Student's t-test, and means with different superscripts within the same colums were significantly different ($p<0.05$).

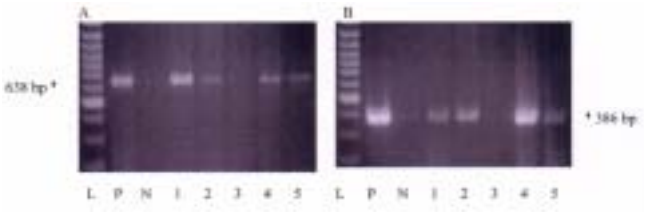

Figure 2. PCR analyses of the chicks hatched after the manipulation of retrovirus vector infection. Genomic DNA was isolated from virus-producing cells (P), wing tips of a non-manipulated chick (N) and of five hatched chicks after retrovirus vector injection (1-5), then subjected to PCR amplification using primers specific for *Neo*^{R} (A) or *EFGP* (B) gene. DNA size marker (L) is 100 bp DNA ladder. The size (bp) of the amplified product is indicated by an arrow head.

AACTCCAGCAGGACCATGTGATCG-3`) were derived from the pEGFP-N1 nucleotide sequences of 964-987 and 1,326-1,349, respectively. This primer pair predicts an amplified DNA fragment of 386 bp. The identification of PCR product was reconfirmed by digestion of DNA bands with a diagnostic restriction enzyme.

RESULTS

Retroviral vector injection

Ten μl of DMEM only or DMEM harvested from retrovirus-producing cells was injected into chicken blastoderm and candling was conducted at 10 and 18 days post incubation. The viability of the day 10 chicken embryos injected with the medium containing LNβ-EGFP retroviral vector was 85% (Table 1). It was not significantly different from both groups of DMEM only (91%) and non-injection control (93%). The viability at day 18 (28%) and the hatchability (7%) of retrovirus injection group were, however, significantly lower than those of DMEM only group (86 and 36%) and non-injection control (93% both). Among five hatched chicks from sixty-seven eggs injected with the medium containing retrovirus vectors, four chicks showed *EGFP* gene integration.

Genomic DNA analyses

PCR analyses were performed with the genomic DNA isolated from the wing tips of five chicks hatched after retrovirus microinjection. DNA purified from the virus-producing cells and DNA from wingtip of chick hatched from non-injected embryo was used as positive and negative controls, respectively. Among five chicks analyzed, four genomic DNA samples showed both 638 bp fragment for the *Neo*^{R} gene and 386 bp one for the *EGFP* gene as predicted (Figure 2).

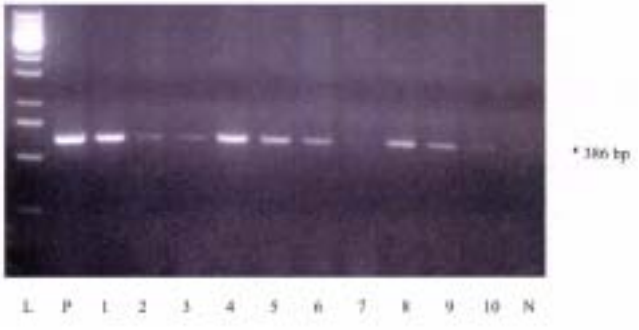

Figure 3. PCR-mediated detection of transgenes in various organs of a transgenic chick. A primer set specific for the *EGFP* gene was used and expected size (bp) of an amplified fragment is indicated by an arrow head. 100 bp DNA ladder was used for the sige marker (L). The source of each DNA sample is; virus-producing cells for positive control (P), liver (1), breast muscle (2), thigh muscle (3), lung (4), small intestine (5), proventriculus (6), brain (7), blood vessel (8), testis (9), ceca (10), and a wing tip of a non-manipulated chick for negative control (N).

Two weeks after hatching, one chick died of its weakness. Genomic DNA samples from ten different organs including liver, muscle of breast, muscle thigh, lung, small intestine, proventriculus, brain, blood vessel, testis, and ceca were prepared to determine mosaicism. As shown in Figure 3 all organs differentiated from one of ectoderm, mesoderm and endoderm carried retrovirus vector sequence in their genomic DNA.

Germ line integration of the retrovirus vector sequence

Four chicks (three females and one male) carrying foreign gene in their genome were brought up until they showed their puberty, and semen of one male chicken was collected. Volume of collected semen was 300 μl and sperm concentration was 22.3×10^8 /ml as normal range. As shown in Figure 4, PCR analysis using *EGFP*-specific primer set

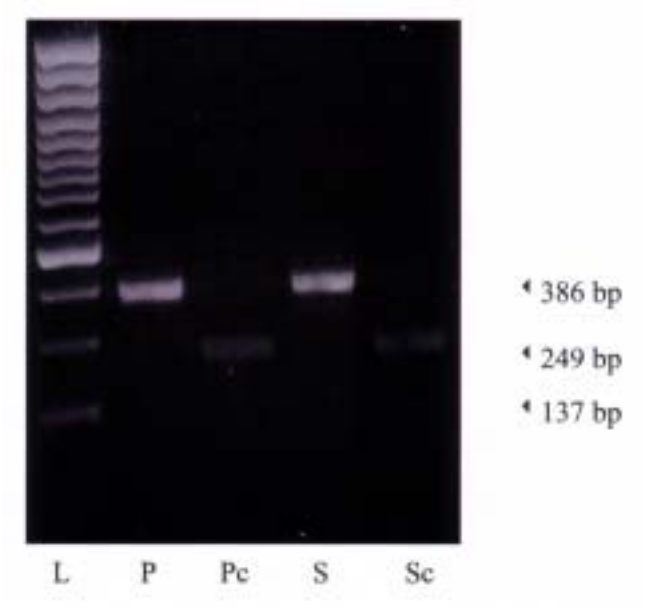

Figure 4. PCR analyses of sperm DNA collected from a transgenic male chicken. PCR was performed with a primer set specific for the *EGFP* gene. The PCR-amplified product was further confirmed by digestion with *Bsg I* restriction enzyme, resulting in a cleavage of 386 bp fragment to 249 and 137 bp fragments. L: 100 bp DNA ladder, P: PCR-amplified positive control (DNA from the virus-producing cells), Pc: *Bsg* Ⅰ digestion of PCR-amplified positive control, S: PCR-amplified sperm DNA, Sc: *Bsg* I digestion of PCR-amplified sperm DNA.

resulted in amplification of 386 bp-sized fragment, indicating germ line integration of retrovirus vector sequence. To further confirm this result, the amplified fragment was digested with *Bcg I* restriction enzyme and the fragment was digested into 249 bp and 137 bp bands as expected.

Ten offsprings were produced from a nontransgenic chicken artificially inseminated with the sperm of a transgenic male. Transmission of *EGFP* transgene to F_1 progeny was verified from five of ten chicks through PCR analyses of the wing tip samples (Figure 5).

DISCUSSION

In transgenic chicken production, several other retrovirus vector systems based on reticuloendothelial virus (REV) (Shuman and Shoffner, 1986; Savva et al., 1991), spleen necrosis virus (SNV) (Battula and Temin, 1977) or avian leukosis virus (ALV) (Thoraval et al., 1995) have been reported. One of the worrisome concerns of this retrovirus vector system is, however, production of replication-competent pathogenic viruses resulting from recombination with dormant endogenous retroviral sequence in the chicken. Another disadvantage of this kind of retrovirus vector system is production of mosaic chickens due to low virus titer and numerous blastodermal

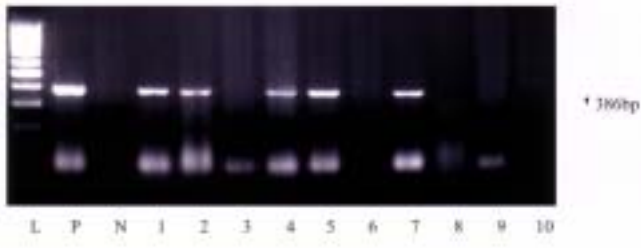

Figure 5. Detection of the *EGFP* gene in F_1 chicks by PCR. DNA samples were prepared from the virus-producing cells (P), from a wing tip of a chick produced from non-transgenic parents (N), and from the wing tips of ten F_1 progenies produced from a transgenic male chicken (1-10). L: 100 bp ladder.

cells in an stage X embryo. One of the solutions for these problems might be using a new retrovirus vector system producing MLV-based recombinant viruses packaged with VSV-G envelopes. The advantages of this new vector system over other retrovirus vector system mentioned above are: Firstly, the possibility of generation of replication-competent retrovirus from the transgenic chicken is very low because the virus vector system is derived from non-avian virus and homologous recombination is very unlikely. Secondary, titer of the retrovirus vectors encapsidated with VSV-G can be easily increased by centrifugal concentration, resulting in low degree of mosaicism. Except a chicken, transgenic cattle (Chan et al., 1998), fishes (Lin et al., 1994; Gaiano et al., 1996; Lu et al., 1997) and primates (Chan et al., 2001) have been produced by this retrovirus vector system.

In this study, to assess the applicability of the retrovirus vector system expressing VSV-G in transgenic chicken production, we injected concentrated retroviral vector stock into the blastodermal area of stage X chicken eggs. Among five hatched chicks from sixty-seven eggs injected with the medium containing retrovirus vectors, four chicks showed *EGFP* gene integration in their genome (Figure 2) and one male transmitted the transgene to the offsprings with 50% efficiency (Figure 5).

Compared to two groups of DMEM only-injection and non-injection control, the hatchability of eggs injected with 10 μl DMEM containing retroviral vector was much lower (36 and 93% vs. 7%, Table 1). Based on these data, the lower hatchability must be due to mechanical damage by blastodermal microinjection. We tested this possibility by reducing blastodermal injection volume from 10 to 2.5 μl and scored doubled hatchability (data not shown). On the other hand, it is evident that this reduction causes to decrease in gene transfer efficiency, but the problem can be overcome by using more concentrated virus stock. Thus, we used 200X concentrated virus stock in the experiments, but test of 1,000X concentrated one is undergoing.

Other possible explanations for low hatchability include

chromosomal damage during the integration of reverse transcribed retroviral vector sequence into the genome (Conlon et al., 1991), and cytotoxic effect of *EGFP* gene expression in the embryonic cells undergoing active differentiation (Perry et al., 1999). We did not test whether the integration of reverse transcribed retroviral vector sequence into genome caused chromosomal damage. However, it is very likely that expression of the *EGFP* gene during the embryo development is detrimental because no expression of the gene was observed from all the transgenic chickens. In the chicken fibroblasts infected with the same LNβ-EGFP retroviral vector expression of the *EGFP* genes was confirmed (data not shown), hence no expression of the gene in the transgenic chickens is not caused by virus vector system itself.

Even though we failed production of the transgenic chicken expressing the exogenous *EGFP* gene, integration of the transgene occurred in various organs including liver, muscle of breast, muscle of thigh, lung, small intestine, proventriculus, brain, blood vessel, testis, and ceca (Figure 3). Therefore, this result along with successful germ line transmission of the transgene (Figure 4 and 5) indicate that the VSV-G pseudotyped retrovirus vector system is very promising tool in transgenic chicken production.

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