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The Production of Transgenic Bird expressing β-Galactosidase Based on A Spleen Necrosis Virus Retroviral Vector

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ABSTRACT

Replication-defective retroviral vectors are currently the most efficient gene transfer method for the production of transgenic animals. Transgenic animals have become important tools for biological research. In vertebrates, foreign DNA is routinely introduced into the genome by microinjection into newly fertilized zygotes. . Necrosis virus-based SNTZ vector carrying the E. coli lacZ gene designed to express a nuclear-localized β-galactosidase was injected into the genome. In this study, performance of the SNTZ vector carrying the E. coli lacZ gene to express a nuclear-localized β-galactosidase was injected into the subgerminal cavity of embryos collected from newly laid eggs will be reviewed.

Keywords: transgenic chickens, lacZ, β-galactosidase, retroviral vector.

INTRODUCTION

Of the current transgenesis methods for birds, retroviruses present the most effective gene delivery systems (Shuman and Shoffner, 1986; Shuman, 1991; Bosselmann et al., 1989, 1990, Petitte, 2002; Sang, 2004). Two types of avian retroviral vectors have been used: the replication competent virus and replication-defective virus.

The replication-competent retroviruses retain all genomic sequences necessary for producing infectious viral particles, integration, and self-replication from the host genome whereas the replication-defective viruses retain the packaging signal, sequences for the viral integration, and transcription initiation but most parts of the retroviral genes were removed and inserted with foreign DNA. Thus, the replication-defective retroviral vectors require the packaging cell lines to supply all viral proteins needed to assemble infectious particles. Once the retroviral RNA genome enteres the host, the viral RNA is reverse transcribed into double-stranded DNA

migrates to the host genome and is integrated as the provirus. At that point, provial DNA is only replicated along with the host genome (Shuman, 1991). The utilization of the replication-competent viruses to produce transgenic chickens is not practical because of the limited size of the inserted genes and the pathogenesis (Shuman, 1991; Wentworth and Wentworth, 2000).

The replication-defective retroviral vectors derived from the Avian Leukosis and Sarcoma Viruses (ALSV) and the Reticulo Endothelosis Viruses (REV) were used to produce transgenic chickens without the evidence of viremia and horizontal infection of helper viruses. Vick et al. (1993) produced transgenic birds with Avian Leukosis Virus-based vectors, but did not report the expression of the transgenes carrying neomycin resistance (Neor) and the lacZ gene. Thoroval et al. (1995) used the ALV-based vectors to produce the transgenic chickens with Neor and the lacZ gene, but the evaluation of the expression was limited to chicken embryonic fibroblasts (CEFs). A successful production of transgenic chickens expressing a reporter gene that could be useful for cell lineage has not been reported.

Mikawa et al. (1991, 1992a, 1992b) developed a SNV-based replication defective retroviral vector carrying a lacZ gene, called SNTZ, for cell lineage analysis of cardiac development. This retroviral vector can infect a wide variety of cell types in early chick embryos. Beta-galactosidase, the product of the lacZ gene, was functional in various cell types in various embryonic tissues and cell cultures. Furthermore, the lacZ gene contained a nuclear location sequence to concentrate activity in the nucleus. The lacZ gene was stably inherited and expressed by the progeny of the infected cells. Moreover, expression of the gene could be detected at hatch. Thus, it appears that the SNTZ vector would be an appropriate retroviral system potentially for the generation of transgenic chickens expressing β-galactosidase. The main objective of the study was to use SNV-based replication defective SNTZ retroviral vector developed from Mikawa et al. (1992b) to produce the transgenic chickens carrying the lacZ gene encoding nuclear-localized β-galactosidase. The goal of this study was to reviewed production transgenic chicken lines to stably inherit the lacZ gene and express in a variety of tissues throughout developmental stages which can be used as an ideal cell marker for cell lineage studies.

MATERIALS AND METHODS

The replication-defective retroviral vector was derived from SNV, except for the splice acceptor sequences, which were derived from avian reticuloendotheliosis virus strain A (REV-A), a virus which has 98% nucleotide sequence homology to SNV (Dougherty and Temin, 1986) (Figure 2). Untegrated linear viral SNV DNA is approximately 8.3 kbp long (Chen et al., 1981) (Figure 1).

The plasmid pJD214 was constructed to lack the 5 end of the 5'LTR and the 3' end of the 3' LTR (Dougherty and Temin, 1986). The gag, pol, and env sequences were removed, replaced a pUC12 polylinker and ligated into the pBR322 at EcoRI/BamHI sites resulting in only 1.45 kbp in length.

The pJD214 was modified for convenience with future cloning by removing one PstI site located within the ampicilin-resistance gene with PvuI and BglI and inserted the PvuI-BglI of pGEM4 (Figure 2). The new plasmid construct was called pJDp- (Mikawa et al., 1992a). A fragment containing the SV40 early region promoter and enhancer sequenes were isolated from pMSG

(Pharmacia, Piscataway, NJ) and inseterted into the BamHI/HindIII sites of pGEM4 (Promega, Madison, WI). Then the insert was isolated with EcoRI/HindIII. A HindIII/EcoRI fragment containing the neomycinresistance gene with a splice donor and polyA signal was isolated from pSV2neo (Southern and Berg, 1982). These two fragments were then co-ligated into the EcoRI site of pJDp- (Figure 4). The new plasmid DNA construct, called pSN, encodes the SV40 promoter and neo-resistance gene in the same orientation as the SNV long terminal repeat (Mikawa et al., 1992b) (Figure 3).

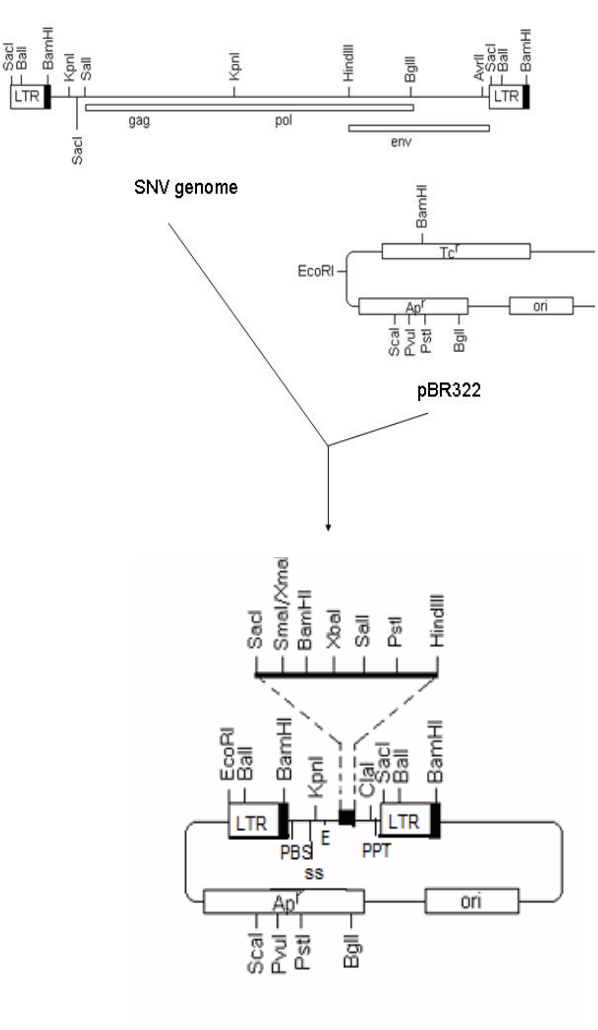

Figure 1. Vector construction and deduced restriction map of pJD214. The sequences required in cis for viral replication are the primer binding site (PBS), polypurine tract (PPT), and encapsidation sequence (E). Other abbreviations: SS, splice site and LTR, long terminal repeat. The multiple cloning sites were derived from the polylinker of pUC12. The virus RNA derived from pJD214 is 950 bp long. The unintegrated SNV DNA is approximately 8.3 kbp in length.

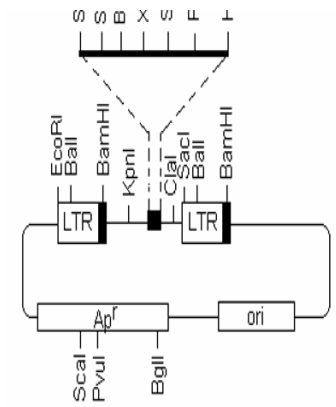

Figure 2. Vector construction and deduced restriction map of pJDp-. The cis sequences of pJD214 illustrated in Figure 2. The pGEM4 was obtained from Promega Corporation.

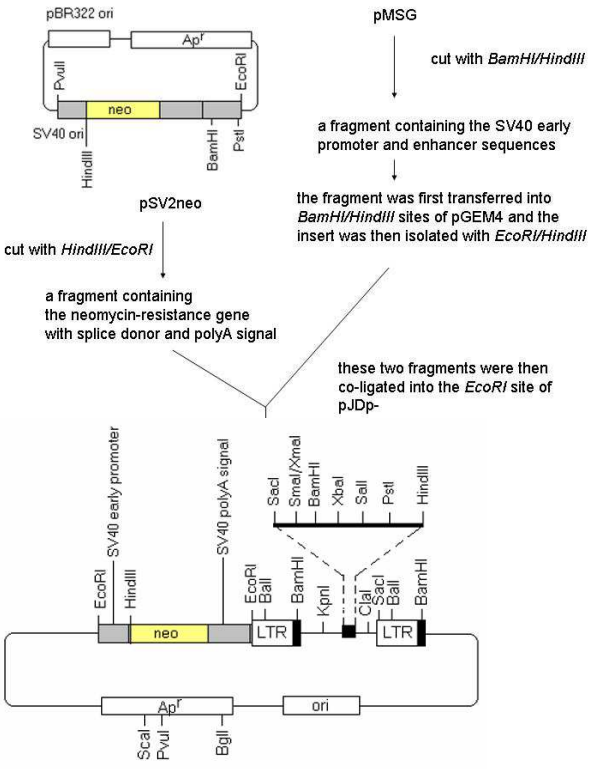

Figure 3. Vector construction and deduced restriction map of pSN.

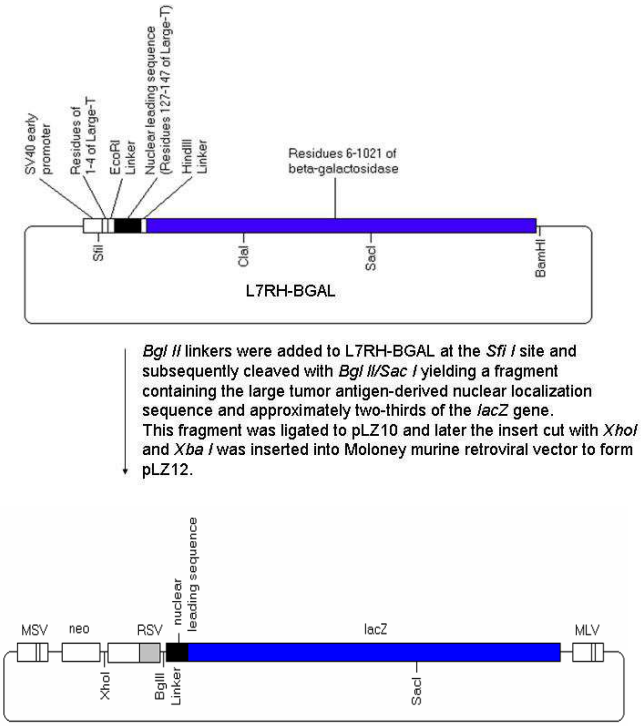

Figure4. Vector construction and deduced restriction map of pLZ12.

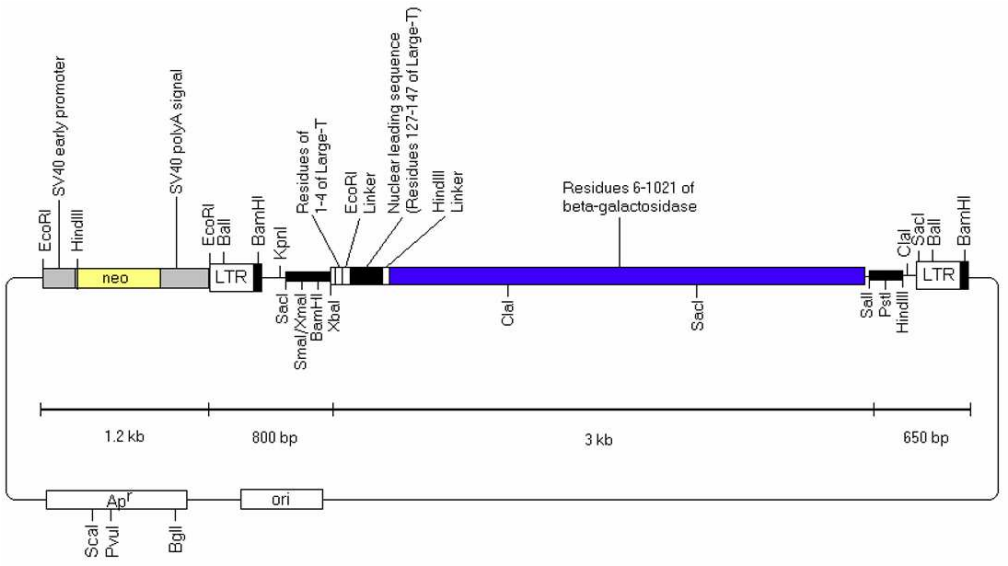

Figure 5. Vector construction and deduced restriction map of pSNTZ. The cis elements of the retroviral sequences were illustrated in Figure 1.

Production of Retroviral Virions Containing SNTZ

The created pSNTZ vector encoding lacZ was transfected into the SNV packaging cell lines, D17.2G, which provides the products of gag, pol, and env gene in trans without producing infectious virus as a result of the deletion of E (Dougherty and Temin, 1988). The D17.2G SNTZ-producing cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 7% fetal bovine serum, and 1% penicillin-streptomycin.

All the embryos used for retroviral transfection were obtained from freshly laid unincubated fertile White Leghorn eggs which were at about stage X (Eyal-Giladi and Kochav, 1976) with approximately 50,000 cells per embryo.

Embryo cultures were performed based on the procedures of Perry (1988) and modified by Borwornpinyo (2000) and Borwornpinyo et al. (2005). The embryos from the newly laid eggs (donor eggs) were transferred into recipient chicken eggshells through the window cut at the sharp end.

After 3 d of incubation, the embryos and whole culture contents were transferred into new recipient turkey eggshells through the window cut at the blunt end .The birds generated from viral injection formed the G0 chimeric founder chickens.

DNA isolation

Genomic DNA isolation from chicken blood was modified from Petitte et al. (1994). First, 5 µl of whole blood was diluted with 45 µl PBS (1:10 dilution) in a 0.5 ml microtube, and then mixed with 100 µl lysis buffer (10mM Tris HCl pH 7.5, 5 mM MgCl2, 0.32 M sucrose, 1% Triton X-100) by vortexing. The mixture was microcentrifuged at 1,200 × g for 20 sec to pellet the nuclei of erytrocytes and the supernatant was discarded. After removing the supernatant, the pellets of nuclei were incubated in 100 µl TEN buffer (10 mM Tris HCl pH 8.0, 2 mM EDTA, 400 mM NaCl, 1% SDS, and 12 µl Proteinase-K (20 mg/ml) at 50°C for overnight. After incubation, 50 µl saturated NaCl was added to precipitate proteins and microcentrifuged at 1,200 × g for 15 min. The supernatant was transferred into a 1.5-ml microtube. Two volumes of ethanol were added to the tube and microcentrifuged at 1,600 × g for 15 min to precipitate DNA. The recovered DNA was air dried and resuspended in 50 µl Tris-EDTA buffer.

PCR screening

The presence of the *lacZ* gene in the offspring determineusing PCR . Genomic DNA isolated from various sources as described above was subjected to 35 cycles of polymerase chain reaction (PCR) for the presence of the E. coli lacZ gene. The 3-steps pattern involved denaturation at 95°C for 30 sec, primer annealing at 54°C for 1 min and extension at 72°C for 1 min using a thermocycler8.

A 25 µl reaction volume containing 200 ng of DNA samples, 1.5 mM MgCl2, 0.8 U of Taq polymerase, 1 µM of dNTPs and 1 µM of each primer, the forward primer 5'-TTCTGTATGAACGGTCTGGTC-3', and the reverse primer 5'-ACTTACGCCAATGTCGTTATC-3' was used to amplified a 588-bp fragment specific to the lacZ gene. Subsequently, the 588-bp amplified products were fractionated through a 1.5% agarose gel and visualized with ethidium bromide.

β -Galactosidase Expression

Experiment demonstrate β -galactosidase express in G2 chickens was to isolate myoblasts from the pectoralis thoracicus muscle to show that E. coli β -galactosidase is expressed in the myogenic satellite cell population after in vitro culture. Myoblasts from three of six G2 chickens express β -galactosidase in vitro (Fig.6). β -galactosidase expression in other G2 embryos, and the embryos also express β -galactosidase (Fig. 7).

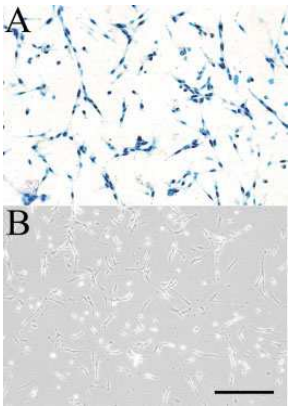

Figure 6. Myoblast cultures from day old chickens.
A: Bright field photomicrograph of primary myoblast cultures from lacZ–positive chickens. Cells with stained nuclei represent β –galactosidase –positive cells.
B:Phase-contrast photomicrograph of primary myoblast cultures from lacZ –negative chickens. Scale bar = 150 µm in B (applies to A,B).

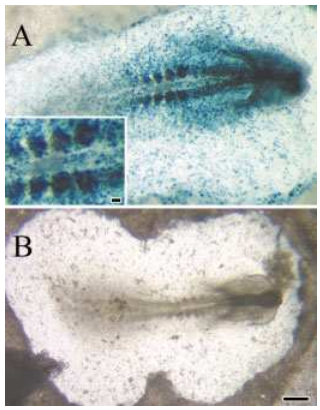

Figure 7. Embryos (Stage 8; Hamburger and Hamilton, 1951)
From (A) β -alactosidase–positive transgenic chickens and from (B) wild-type β -alactosidase–negative chickens.
Inset in A shows staining in the somites of β -galactosidase-positive transgenic chickens. Scale bar = 50 µm in inset, 300 µm in B (applies to A.B).

Staining embryos.

Embryos fix at 4°C with 2% formaldehyde and 0.2% glutaraldehyde in PBS pH 7.4 for 30 min, rinsed in PBS, and incubate in X-Gal solution (1 mg/ml X-Gal PBS pH 7.4, 5mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2,0.2% Triton X-100) overnight in the dark at 37°C. Subsequently, the embryos wash with PBS and stored in 70% ethanol before microscopic evaluation.

RESULTS

Table 1 summarizes the effect of injection strategies on hatchability and the percentage of chimeric G0 chickens carrying the lacZ gene in blood and semen. When injected with the concentrated retroviral stocks into the subgerminal cavity, 24 embryos out of 66 injected embryos survived to hatch. Ten embryos out of 54 embryos injected with the concentrated retroviral-containing medium and cells survived to hatch. Fourteen of 40 embryos injected with the concentrated retroviral containing media into subgerminal cavity and again one day after incubation at the germinal crescent survived to hatch. Overall, thirty percent hatchability was obtained in the experiment (48 of the total 168 injected embryos survived to hatch).

It has been shown that embryo injection without retrovirus-producing cells resulted in the highest hatchability which accounted for thirty-six percent hatchability (24 of 66 injected embryos). Forty G0 chicks, 25 females and 15 males, survived to sexual maturity. Two G0 females carried lacZ gene in their blood DNA when using PCR screening. These two females resulted from a single injection of retroviral containing media. One-hundred-and-one progeny produced from one G0 female and 90 progeny produced from the second G0 female were screened for the lacZ gene by means of PCR in their blood (Table 2). Neither G0 females generated lacZ-positive offspring. In addition, all blood lacZ-negative G0 females did not produce lacZpositive progeny

in their blood. Eight of 15 G0 males (53%) carried the lacZ gene in their semen DNA, but none of them were blood lacZ-positive (Figure 8). A single injection of retroviral stocks resulted in a greater number of G0 males carrying the lacZ gene in their semen (Table 1). This suggests that a single injection of concentrated retrovirus into the subgerminal cavity of the stage X embryos collected from the newly-laid eggs are the most efficient procedure for retroviral infection and gene transfer. Only one semen lacZ-positive G0 male produced 2 blood lacZpositive chickens from a total 224 progeny which accounted for the 0.89% germline transmission rate (Table 3).

TABLE 1. Effect of injection procedures on hatchability (% hatch) and effect of injection procedure on the percentages of G0 chickens carrying lacZ gene in blood and semen

Injection procedure	N	% Hatch (#)	% carrying *lacZ* in the blood (# positive/ total # screened)	% carrying *lacZ* in the semen (# positive/ total # screened)
Virus only1	66	36(24)	10 (2/20)4	80 (4/5)
Virus and cells2	54	19(10)	0 (0/7)	40 (2/5)
Virus double injection3	40	35(14)	0 (0/13)	40 (2/5)

N, number of eggs injected; % Hatch is the percentage of injected eggs that hatched; #, number 1 Virus only, embryos collected from newly-laid eggs were injected with 5 µl of the concentrated retroviral stocks into the subgerminal cavity 2 Virus and cells, embryos collected from newly-laid eggs were injected with 5 µl of the concentrated retroviral stocks containing 500 SNTZ-producing cells into the subgerminal cavity 3 Virus double injection, embryos collected from newly-laid eggs were with 5 µl of the concentrated retroviral stocks into the subgerminal cavity, and the same embryos were again injected 1 d after inbubation with 4 injections of 3 µl per injection of the retroviral stocks into the germinal crescent 4 These 2 lacZ-positive birds in blood were females

TABLE 2. PCR screening of the progeny from chimeric G0 females generated from retroviral injection1

Chimeric G0 females	Number of G1 chicks PCR screened for lacZ	G1 chicks carrying lacZ in their blood
12	101	0
2	90	0
3	66	0
4	88	0
5	32	0
6	22	0
7	24	0
8	49	0
9	55	0
10	22	0
11	40	0
12	65	0

1 PCR, polymerase chain reaction
2 These chimeric G0 hens were positive for the lacZ gene in their blood, and produced from the injection at stage X embryos collected from newly-laid eggs with concentrated virus

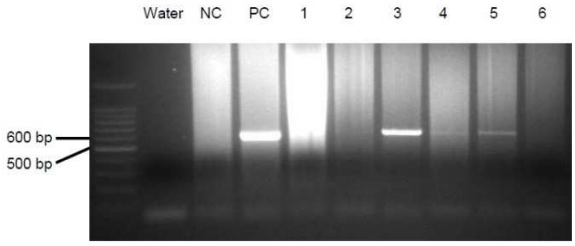

Figure 8. Polymerase chain reaction analysis of the semen from G0 chickens.
Water is a negative control using a water blank. NC is a negative control using DNA isolated from semen of wild-type chickens. PC is a positive control reaction containing the lacZ gene from pmiwZ plasmid (Kadokawa et al., 1990) spiked into semen DNA from wild-type chickens.
Lanes1, 2, and 6 represent negative birds; lanes 3, 4, and 5 represent positive birds. All positive lanes contain a 588-bp fragment.

TABLE 3. PCR screening of the progeny from G0 male chickens carrying the lacZ gene in their semen1

G0 Males carrying lacZ in their semen	Number of G1 chicks PCR screened for lacZ	G1 chicks carrying lacZ in their blood
12	2243	24
2	252	0
3	143	0
4	241	0
5	365	0
6	195	0
7	152	0
8	196	0

1 PCR, polymerase chain reaction
2 This germline chimeric sires were produced from the injection at a stage X embryo collected from newly-laid eggs with concentrated virus
3 Screening of the progeny from these two sires was terminated because the birds died
4 Germline transmission rate of this G0 sire was 0.89% (2/224)

DISCUSSION

This study attempted to show the efficiency of retroviral gene delivery into germline precursors by varying different methods of retroviral injection. Regardless of injection procedures, no overt differences were observed in gene transfer between injecting the concentrated retrovirus and delivering the concentrated retrovirus along with the virus-producing cell or performing multiple injections with retrovirus. Thus, a single injection of the concentrated SNTZ retrovirus into the subgerminal cavity of stage-X embryos (Eyal-Giladi and Kochav, 1976) containing approximately 50,000 cells appears to be the most efficient method for producing chimeric germline transgenic chicken founders.

Overall hatchability of the injected embryos in many experiment was 30% which was approximately the same reports (38%, 36%, and 27% hatchability obtained from Bosselman et al. (1989), Harvey et al. (2002), and McGrew et al.(2004), respectively). Nevertheless, the percentage hatchability in Mozdziak(2003) study was relatively higher than those obtained from Thoraval et al. (1995) and Chapman et al. (2005) who reported 2.3% and 4% hatchability of viral injected embryos, respectively. The acceptable hatchability obtained in Mozdziak(2003) study was mainly due to the surrogate eggshell system (Perry, 1988) with modifications (Borwornpinyo, 2000, Borwornpinyo et al. (2005) for improving survivability and hatchability.

In many studies, no correlation between the presence of the lacZ gene detected in the blood (somatic cells) and the sperm or ova (germ cells) were observed (Thoraval et al., 1995; McGrew et al., 2004). In the Mozdziak(2003) experiment, two blood lacZ-positive hens were generated

from the retroviral injection and together produced 191 G1 offspring when mated with wild-type roosters. None of the progeny carried the lacZ in the genomic DNA based on PCR screening. On the other hand, eight roosters were found lacZpositive in their semen DNA, but their blood DNA samples were lacZ-negative.

Gene transfer into the germline using retroviral infection into embryos obtained from newly laid eggs is not efficient. Mozdziak et al(2003) showed, a total of 1,639 offspring generated from mating 8 semen lacZ-positive roosters and wild-type hens had to be screened in order to detect 2 lacZ positive G1 chicks. Notably, only one rooster was shown to transmit the lacZ gene to the two G2 transgenic chicks which accounted for 0.89% (2/224). The low germline transmission rates were consistent with previous reports (Bosselman et al., 1989, 1990; Harvey et al., 2002; Chapman et al., 2005).

However, the efficiency of gene transfer into germline precursors based replicationdefective vectors can be improved by infecting the unincubated embryos with retrovirus pseudotyped with vesicular stomatitis virus envelop protein (VSV-G).

Mizuarai et al. (2001) reported that 80% germline transmission rate was obtained from the G0 quail injected with Moloney murine leukemia virus (MoMLV) pseudotyped with VSV-G protein. Similarly, McGrew et al. (2004) generated G0 roosters that transmitted the transgene to their offspring with rates ranging from 4% to 45% based using equine infectious anaemia virus (EIAV) vectors pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G). In addition, relatively high retroviral titers can be produced when using the VSV-G-pseudotyped retroviral vectors.

The stable integration and inheritance of transgenes is one of the most important aspects for the production of transgenic chicken lines. In Mozdziak et al(2003) study, it is clear that the germline transmission rates of G1, G2, and G3 transgenic birds are consistent with the expected Mendelain ratios indicating that the lacZ transgene acts as a hemizygous dominant allele and is stably inherited for both transgenic lines (P1 and P2) which were generated from the same G0 sire. Recently, Mozdziak research group (Mozdziak et al., 2006) studied the integration site and copy number of the lacZ provirus in both transgenic G1 male founders and their G2 offspring using Southern analysis. They found that these two G1 males contain a single copy and an identical integration site of the provirus suggesting that they are from the same germline precursor. The results confirmed that the germline transmission of transgenic chicken lines for the lacZ gene were consistent with the predicted Mendelian ratios.

Six criteria for an ideal cell tracer for studies of cell lineage were mentioned (McLaren, 1976; Oster-Granite and Gearhart, 1981). First, the expression of β-galactosidase was localized only in the nucleus of transgenic cells. Moreover, this nuclear-localized β-galactosidase alleviates the problem of endogenous activity when X-gal staining is applied. Second, the expression was cell autonomous in that only cell containing the lacZ exhibit staining and the β-galactosidase is not transferred among cells. Third, the lacZ gene is stably inherited and expresses β-galactosidase in subsequent cell division. Fourth, the expression of β-galactosidase was ubiquitous among both the internal and external tissues of the body. The β-galactosidase activity can be detected in entire embryos from at least stages 8, 20, and 28 (Hamburger and Hamilton, 1951). G3 birds

expressed the lacZ in all tissues examined including brain, bursa, gizzard, intestine, liver, lung, kidney, skeletal muscle, spleen, and testes. Fifth, the expression was easy to detect, both grossly and in histolocial sections using X-gal staining. However, the expression was not present in every cell based on the X-gal staining. This might be due to the low sensitivity of detection using X-gal staining (Couffinhal et al., 1997).

Immunohistochemistry staining using anti-β-galactosidase antibody drastically improved the detection of the positive cells carrying the lacZ gene (Couffinhal et al., 1997). In addition, in-situ polymerase chain reaction (in-situ PCR) could be used to localize all cells carrying a single copy of the lacZ gene in tissue sections (Komminoth and Long, 1993). Finally, the transgenic birds were normal throughout development indicating that they were developmentally neutral, not causing cell selection or influencing developmental processes. Recently, satellite cells isolated from skeletal muscle of transgenic adult chickens can be used to trace their fate after implantion into stage 14 chicken embryos (Mozdziak et al., 2006).

Furthermore, Zhang et al. (2006) have shown that hematopoietic stem cells (HSCs) have potential to differentiate into multiple non-blood cell lineages using parabiosis between quail and transgenic chick embryos. Thus, production transgenic birds as an alternative cell marker can be use in experiments in homospecific grafts for cell lineage studies and to confirm and validate the results obtained from the chick-quail cell tagging method. In addition, this stable genetic tagging is ideally suitable for long-term cell lineage analysis because the marker expression does not decline over time and the chimeras made in embryos between the same species and strains would survive throughout their entire lfe without immunological rejection.

CONCLUSION

In this study, the stages of production transgenic chickens carrying the lacZ gene and expressing β-galactosidase have been reviewed. The expression of β-galactosidase can be observed in all examined tissues from embryos and adults at different stages of development. The produced transgenic chickens could meet the six criteria for an ideal cell marker which would be (1) cell localized, not secreted extracellularly; (2) cell autonomous, not transferred between cells; (3) stable in all cells; (4) ubiquitous throughout development in all tissue types; (5) easy to detect, both grossly and in tissue sections, and (6) developmentally neutral.

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