ABSTRACT

BORWORNPINYO, SUPARERK. Production of Transgenic Chickens to Express Bacterial β-Galactosidase and the Subsequent Utilization of Lactose as a Feed Stuff. (Under the direction of Dr. James N. Petitte).

The main objective of this dissertation was to create transgenic chickens expressing β-galactosidase as a genetic marker for cell lineage studies. To generate the transgenic chickens, the replication-defective retroviral-based SNTZ vector carrying the *E. coli lacZ* gene encoding nuclear-localized β-galactosidase was injected into subgerminal cavity of stage X (EG & K) White Leghorn embryos. Eight of 15 male chicks that survived to sexual maturity were germline chimeric birds based on PCR screening for the *lacZ* sequences in their semen. One of the eight *lacZ*-positive G_0 roosters transmitted the *lacZ* gene to two male chicks from a total of 224 progeny (0.89%). From these two transgenic G_1 males, the *lacZ* gene was stably transmitted through 4 generations in an expected Mendelian pattern for a single dominant allele. The expression of β-galactosidase was detected in cultured myoblasts derived from 1-d-old chick muscle, entire embryos, and in a variety of examined tissue types from young and adult chickens. In the current study, the generated transgenic lines which stably inherited and expressed the reporter *lacZ* gene are the first report of transgenic birds that could provide an alternative ideal cell marker for cell lineage studies.

Qualitatively high expression of β-galactosidase was observed in villi of intestine. Potentially, the transgenic chickens that express β-galactosidase in the gastrointestinal tract could utilize lactose more efficiently. The second objective of

this study was to determine whether the transgenic chickens can improve lactose digestibility and its use as an energy source. Overall, when dietary lactose was increased from 5 to 10%, the transgenic chickens showed lactose digestibility approximately 10% better than those of non-transgenic chickens. This is the first report of using gene transfer technology to manipulate the chicken genome to utilize feed more efficiently for agricultural purposes.

PRODUCTION OF TRANSGENIC CHICKENS TO EXPRESS BACTERIAL β-GALACTOSIDASE AND THE SUBSEQUENT UTILIZATION OF LACTOSE AS A FEED STUFF

by

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DEDICATION

To

My grandparents, my parents, my wife, and

my beloved son Nicholas

BIOGRAPHY

Suparerk Borwornpinyo, son of Bunyong and Nopporn Borwornpinyo, was born on March 7, 1975. He finished his secondary education at Hor Pra School, Chiang Mai in 1988. He completed his high school education at Yupparaj Collage, Chiang Mai within two years. In June 1991, he entered Chiang Mai University and received his Bachelor of Science degree in Animal Science in March 1995. He continued his graduate study in Biotechnology at Mahidol University, Bangkok. In 1996, he received a Royal Thai Scholarship through the Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok to study abroad. In August 1997, he began a Master of Science program in Poultry Science at North Carolina State University under the guidance of Dr. James N. Petitte. He completed the requirements for the M.S. in July 2000. He continued a Physiology program for his doctoral degree under the supervision of Dr. James N. Petitte in January 2001, and earned the Ph.D. in August, 2006.

The author married Rattana Pirakittiworakol of Chonburi, Thailand on July 1999.

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My heart and my soul are given to my lovely son, Nicholas Borwornpinyo, who teaches me how to be a father. Live in peace my little angle.

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Gene transfer technology in animals is essential to basic scientific research, medicine and agriculture. Microinjection of DNA into the nucleus of newly fertilized zygotes is a widely used and successful method for delivering exogenous genes into mammalian host chromosomes. However, birds have different reproductive systems compared to mammals and require alternative strategies for the production of transgenic avian species. In domestic birds, newly fertilized ova are difficult to manipulate and impractical to return to form the egg inside the recipient hen oviduct. The most successful method of gene transfer in birds is to use retroviral vectors injected into the embryos following oviposition. The germline transmission rate of the founder birds is very low. Nevertheless, the transgenic lines demonstrate the stable integration and inheritance of the transgene to subsequent generations.

In this review, general avian reproduction and establishment of germ cells will be described for possible routes to gene transfer. Later, the current gene transfer methodologies will be summarized with a focus on retrovirus-mediated gene transfer. Finally, the application of transgenics to poultry will be discussed.

Avian embryonic development

An ovum, a female sex cell, in the domestic hen's ovary gradually accumulates yolk, which provides nutritive materials for the embryo and ovulation occurs approximately every 24 h. The nucleus and cytoplasm of the ovum is located at the surface of the yolk mass within a small white area called the germinal disc

(Perry, 1987). After ovulation, the ovum is immediately engulfed by the funnel-shaped infundibulum, the first portion of hen's oviduct where the spermatozoa, the male sex cells, fertilize the ovum. Fertilization in birds is polyspermic (Waddington *et al*., 1998). The forming zygote remains in this section approximately 15 min (Olsen and Neher, 1948). Subsequently the egg enters the magnum which secrets albumen that encases the yolk. The egg spends about 3 h in the magnum. After the magnum, the yolk together with the albumen capsule enters the isthmus where the inner and outer shell membranes are laid down and the first cleavage division of the fertilized egg occurs (Perry, 1987). Leaving the isthmus, the developing egg moves to the uterus, or the shell gland, where the eggshell forms. The egg spends the longest time in uterus than in any other portion of the hen's reproductive tract, about 18 to 21 h (Etches, 1996). For the period before oviposition, Eyal-Giladi and Kochav (1976) classified a series of normal stages describing the sequence of developmental events from the first cleavage up to primitive streak into 14 stages using Roman numerals. These events can be divided into three developmental periods: cleavage, formation of the area pellucida, and hypoblast formation. During 10 to 12 h in the uterus, the cytoplasmic mass of germinal disc is cleaving very rapidly to form an epithelial sheet 5 – 6 cells thick (E.G&K stage VI). The germ is now known as the blastoderm (Eyal-Giladi and Kochav, 1976). The blastoderm is separated from the underlying yolk mass by the subgerminal cavity. About 6 to 8 h before the egg is laid, the lower layers of the blastoderm start to detach and fall into the subgerminal cavity to form a transparent thinned-out area that marks the future posterior end of the embryo (Eyal-Giladi and Kochav, 1976). This process marks

the beginning of area pellucida formation. At about the time of oviposition the developing blastoderm appears to the naked eye as a whitish circular structure approximately 2 mm in diameter that is composed of two regions. The outer edge is opaque, and is called area opaca; and the inner layer is translucent from a shedding process, and is referred to as the area pellucida (Eyal-Giladi, 1993). The chick embryo at the time of oviposition is known as a stage X embryo (Eyal-Giladi and Kochav, 1976) and is composed of about 50,000 to 60,000 cells (Figure 1).

Upon incubation of the freshly laid egg a second layer of cells beneath the area pellucida starts to appear and is completed in a few hours (Eyal-Giladi and Koshov, 1976). This signals the process of hypoblast formation, which is completed at stage XIII (Eyal-Giladi and Kochav, 1976). When viewed ventrally, the hypoblast is smooth and has a well-defined border between area opaca and area pellucida (Eyal-Giladi and Kochav, 1976; Kochav *et al*., 1980; Balinsky, 1981, Eyal-Giladi, 1993). The cellular sheet of the area pellucida, the upper layer, is now known as an epiblast (Eyal-Giladi and Kochav, 1976; Balinsky, 1981; Eyal-Giladi, 1993). The resulting space between the two-layered blastoderm is a blastocoel, and the space between the hypoblast and the underlying yolk is the subgerminal cavity (Eyal-Giladi and Kochav, 1976). This marks the beginning of gastrulation, a process which gives rise to three germ layers: ectoderm, mesoderm and endoderm (Balinsky, 1981). The first visible sign of gastrulation is a thickening of epiblast that results in the primitive streak (Romanoff, 1960; Patten, 1971), marking the anterior-posterior axis where the embryo proper develops (Eyal-Giladi and Kochav, 1976; Balinsky, 1981; Eyal-Giladi, 1993). From this point on, the changing appearance of the embryo is

distinguished by the table of normal stages developed by Hamburger and Hamilton (1951), a system that is based on morphological characteristics. The stages are numbered from 1 to 46, from the pre-streak embryo to the newly hatched chick.

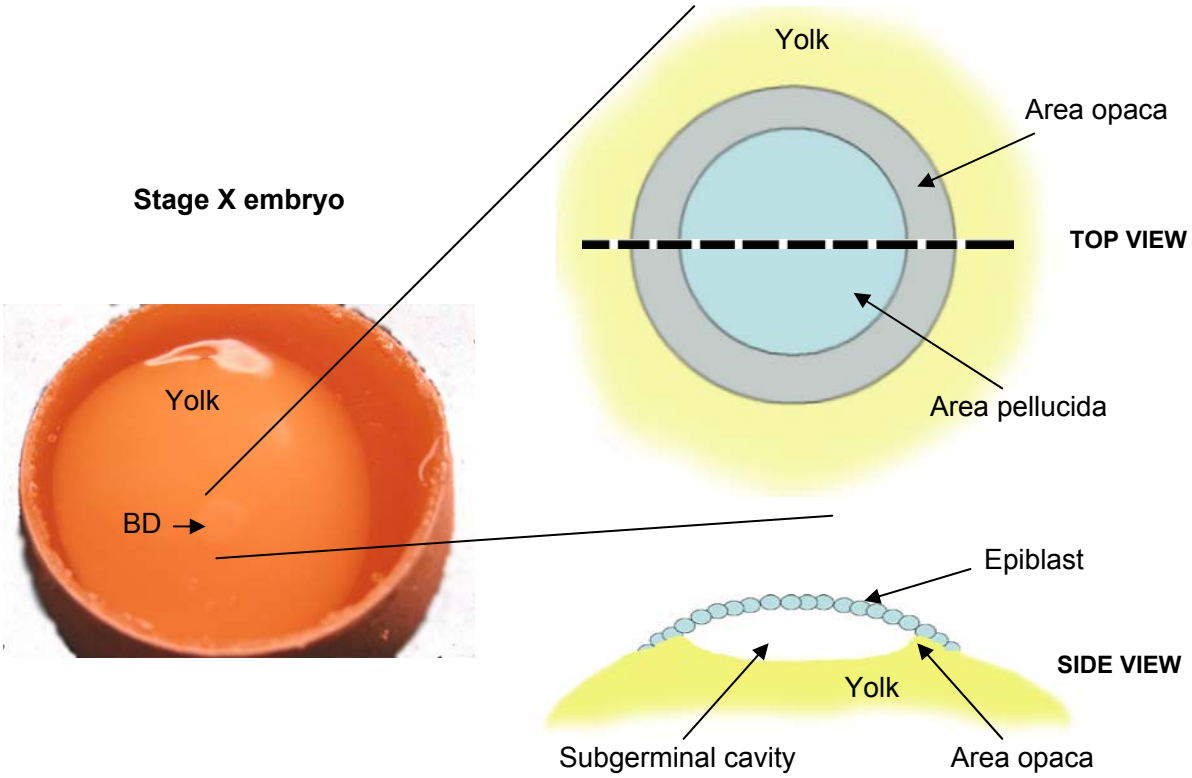


Figure 1. The basic structure of blastoderm (BD) at the time of oviposition. A freshly laid egg often contains an embryo at stage X (Eyal-Giladi and Kochav, 1976) characterized by a fully formed area pellucida (AP). Only the area opaca is in contact with yolk creating a subgerminal cavity beneath the area pellucida.

Germ cell formation

Avian germ cells develop early in embryogenesis and are the ultimate target of gene transfer for the production of transgenic chickens (Figure 2). Tsunekawa *et al.* (2000) has proposed that the chicken VASA protein, an RNA binding protein, could act as germplasm-containing materials for predetermining the formation of germ cells similar for that observed in Drosophophila. The chicken VASA protein can be detected histoimmunochemistically from early cleavage stages to the presumptive primordial germ cells (PGCs) of stage X embryos and later. The mechanism of the establishment of the germ line in birds is yet unknown. Primordial germ cells (PGCs), the precursors of spermatozoa and oocytes, in the early chicken embryo have been characterized by their morphological characteristics, their high glycogen content stained with periodic acid-Schiff (PAS) reaction, and the presence of cell surface antigens such as EMA-1 and SSEA-1 (D'Costa *et al*., 2001). Apprixmately 50 PGCs are first recognized in the stage X embryo based on immunostaining using anti-SSEA-1 and EMA-1 and later on the dorsal surface of the hypoblast (Karagenc *et al*., 1996). Then, PCGs migrate to an extraembryonic region anterior to the head fold, referred to as the germinal crescent that contains about 200 PGCs (Swift, 1914; Rogulska *et al*., 1971). From the germinal crescent region, PGCs imgrate into the newly formed vascular system and are passively carried to the vicinity of the germinal ridge through the extraembryonic circulation (Swift, 1914; Meyer, 1964). From the blood, PCGs actively migrate into the embryonic gonads (Kunawa *et al*., 1986; Nakamura *et al*., 1988; Urven *et al*., 1988).

These events suggest several ways to manipulate the avian genome. Many points of germ cell development can be accessed for gene delivery including mature oocytes/spermatozoa, the newly fertilized ova/zygotes, primordial germ cells during their early establishment, migration and colonization of gonad.

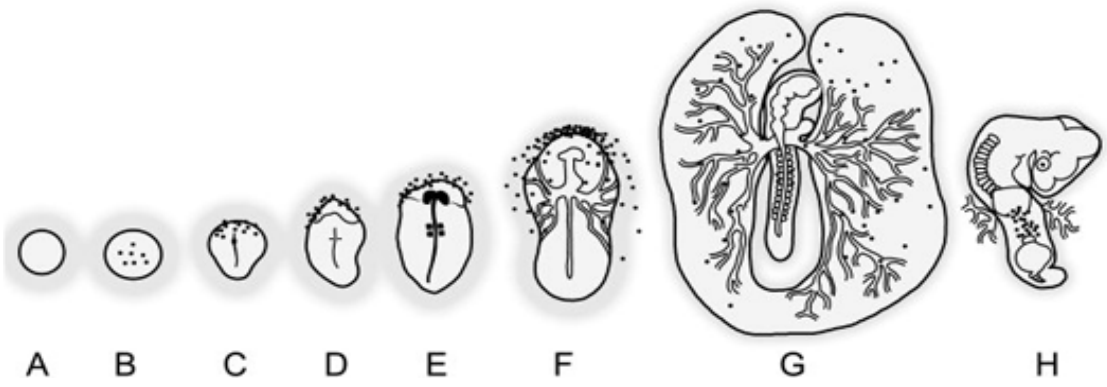


Figure 2. The developmental history of primordial germ cells from oviposition to their colonization of the genital ridge. Committed germ cells have not been identified in the stage X embryo (A) although some cells express a chicken homologue to VASA and begin to express SSEA-1. At stage XIII SSEA-1 marks a population of cells on the hypoblast that can give rise to germ cells (B). These SSEA-1 positive hypoblast cells move anteriorly during gastrulation and head fold stages (C-E) to from the germinal crescent described by Swift (1914). During the formation of blood islands and the vasculature (F), the germ cells enter embryonic circulation (G) until they colonize the gonadal rigde (H) (redrawn after Nieuwkoop and Sutasurya, 1979; with modification).

Target routes and gene transfer

Genetic manipulation of developing follicles

To introduce genetic material into chicken follicles, the ovary must be surgically exposed. Cioffi *et al.* (1994) injected a linearized DNA plasmid containing a bovine growth hormone (bGH) gene linked with the Rous sarcoma virus (RSV) long terminal repeat (LTR) into maturing ovarian follicles. This procedure did not affect subsequent fertilization and egg laying. Unfortunately, they found that the offspring produced in this experiment did not incorporate the bGH gene into the genome. Similarly, Shuman and Shoffner (1986) attempted to transfect unfertilized follicles with both replication-competent and replication-defective retroviral vectors. These studies showed that retroviral DNA vector sequences integrated in somatic cell genomes of examined embryos and chick tissues. However, germline transmission was not reported.

Microinjection of DNA into fertilized ova

Microinjection of DNA into pronuclei of fertilized ova collected after fertilization is a routine method for the generation of transgenic mice, rabbits, sheep, pigs and cattle (Niemann and Kues, 2003; Hunter *et al*., 2005). Subsequently, the injected embryos are returned to a surrogate mother for complete development. In chickens, fertilized ova are removed from the magnum of the oviduct at 2.5-2.75 h after the preceeding egg is laid. At this point, the yolk is covered with the small capsule of thick albumen. Before microinjection, the thick albumen has to be removed (Naito *et al*., 1991). DNA is injected into the cytoplasm of the germinal disc where male and female pronucleus is located. Brinster *et al*. (1981) showed that injection of plasmid

DNA directly into the pronuclei of mouse ova results in higher integration frequencies than cytoplasmic injection. However, the direct injection into pronuclei of an avian ovum is not possible because the cytoplasm is opaque with yolk spheres in the cytoplasm and the pronuclei are indistinguishable (Perry, 1987). It is impractical to return the injected ovum into hen oviduct to complete egg formation (Olsen and Neher, 1948). Hence, an alternative method has been devised to culture a fertilized chicken ovum before first cleavage to hatching (Perry, 1988). The method involves the use of surrogate shells as culture vessels and comprises three phases covering oviductal/blastoderm formation, oviposition/embryogenesis and postoviposition/embryonic growth through hatching. The hatching results were low (7%). Nevertheless, chicks hatched from the culture were healthy and grew to reproductive age. Through this *ex ovo* culture system, Sang and Perry (1989) injected linear DNA into the germinal disc of fertilized chick ova. After the injection, they found that the linear DNA molecules are ligated rapidly to form random concatamers of head-to-tail, head-to-head or tail-to-tail, and the injected DNA was gradually lost during the embryonic development. No evidence of DNA incorporation into the host genome was obtained (Sang and Perry, 1989). Perry *et al*. (1991) injected circular DNA carrying a *lacZ* gene into the fertilized chick ova. The gene expressed in mosaic pattern in later stages of development but the DNA persisted episomally. The first successful production of transgenic chickens based on DNA microinjection came from the work of Love *et al*. (1994). Love *et al*., (1994) created the first germline chimeric rooster with microinjection of plasmid DNA containing the bacterial *lacZ* gene. The rooster transmitted the transgene to 3.4% of

its offspring. These birds had been bred to show stable transmission of the transgene. However, expression of the transgene was not observed. The efficiency of chromosomal integration of injected DNA was improved by using a plasmid carrying an active *Drosophila* transposable element *mariner* (Sherman *et al*., 1998). About 27% of injected embryos contained *mariner* DNA. One of the sexually mature roosters transmitted mariner sequences at a frequency of 29%. This study indicated that the transposable element *mariner* can be designed as a vector for production of transgenic chickens. In addition, the efficiency of creating transgenic chickens can be improved by the further modifications of the *ex ovo* culture procedure (Naito *et al*., 1990).

Transfer of genetic materials via spermatozoa

Sperm-mediated gene transfer methods offering a direct access to the germ line are viewed as an easy and rapid way for generating transgenic animals (Freeman and Messer, 1985; Lavitrano *et al*., 1989; Shuman, 1991; Chang *et al*., 2002). Pandey and Patchell (1982) proposed a method of introducing genes into the fowl genome using sperm. Hens were inseminated with irradiated sperm carrying marker genes controlling egg and feather color and then followed by a second insemination of normal unirradiated semen. The irradiated spermatozoa are able to penetrate the ovum and then the fragmented DNA delivered from the irradiated sperm might be integrated into the fused male and female pronuclei or the zygotic nucleus. It was found that 3.5% of the progeny exhibited feather and egg color obtained from the donor irradiated sperm. This study was confirmed by Bumstead *et al*. (1987) who used major histocompatibility haplotyes as marker traits and showed

that 0.5% of the resulting progeny had been transformed with the introduced gene. Although the method appears simple and rapid, there are many drawbacks which make it impractical for chicken transgenesis. Many genes, wanted and unwanted, can be integrated simultaneously and randomly. The rate of gene transfer of desired traits is rare. Shoffner *et al*. (1990) could not demonstrate the gene transfer using the same technique. Finally, only genes from irradiated donor sperm which are homologous to the recipient genome can be transferred (Bumstead *et al*., 1987; Shoffner *et al*., 1990).

Attempting to transfer foreign genes into chicken genome, Gruenbaum *et al*. (1991) successfully produced transgenic chickens by insemination of hens with spermatozoa by simply incubating with recombinant DNA carrying the bacterial *lacZ* or chloramphenicol acethyl transferase (CAT) genes. They found that 30-60% of examined embryos and offspring contained the exogenous genes and express the transgenes in mosaic pattern. The founder chickens were able to transmit the transgenes to the next generation. However, Gavora *et al*. (1991) had no success in generating transgenic chickens using the similar method. This could result from that the sperm-bound foreign DNA materials are degraded by the deoxribonuclease in the seminal plasma or inside the hen oviduct before fertilization takes place (Cook *et al*., 1990; Rottmann *et al*., 1992).

To protect foreign DNA sequences carried by spermatozoa from DNase degradation, lipofection and electroporation have been used to internalize the DNA into sperm (Rottmann, *et al*., 1992; Nakanishi and Iritani, 1993; Squires and Drake, 1993). Nakanishi and Iritani (1993) found that lipofection and electroporation

increased the association of cloned DNA and sperm to 52%-60% compared to only 6% when the DNA was simply incubated with semen. Electroporation has shown to breakdown the acrosome and compromise fertility (Nakanishi and Iritani, 1993). On the other hand, lipofection maintains the ability of the spermatozoa to fertilize (Nakanishi and Iritani, 1993; Squires and Drake, 1993). Even though the transfer of the exogenous DNA into sperm was improved, no stable integration of the genes into host chromosome was found (Nakanishi and Iritani, 1993; Squires and Drake, 1993; 1997). In addition to lipofection and electroporation, a monoclonal antibody that interacts with the surface of sperm from many species has been used for the production of transgenic animals (Chang *et al*., 2002). The linker protein is a basic protein which electrostatically interacts with foreign DNA. With this sperm-mediated gene transfer, germline chimeric mice and pigs can be produced using *in vitro* fertilization and surgical oviduct fertilization, respectively. Founder mice transmitted the transgene to 33% of their offspring whereas the founder pigs transmitted the transgene to 38% of their progeny. The transgene was further demonstrated a FISH (fluorescence *in situ* Hybridization) to stably integrate into pig genome of second generation animals. Expression of the transgene was also be detected (Chang *et al*., 2002). The investigators found that the linker protein can interact with chicken sperm and could be used to generate transgenic chickens. However, successful production of transgenic chickens has not been reported using the antibody based sperm-mediated gene transfer.

Multicellular embryos

The embryos obtained from freshly laid eggs are the most convenient stage for manipulation of the avian embryo. The embryos at stage X contain a multi-celled blastoderm comprising of 50,000 cells in which only approximately 50 cells are presumptive germline precursors (Karagenc *et al*., 1996). Rosenblum and Chen (1995) used liposomes to transfer a DNA construct containg the firefly luciferase gene driven by a RSV promoter. The expression of luciferase was detected at high levels in 3-d-old embryos. Similarly, Muramatsu *et al*. (1997) attempted to use RSV/lacZ DNA complexed with either calcium-phosphate or liposome to inject into the embryos from the newly laid eggs. After 48 h post injection, the expression of the *lacZ* gene was observed. However these studies have not led to the successful production of the transgenic chickens.

Nevertheless, the most successful method for gene delivery into the chicken chromosomes is the use of retroviral vectors. The following sections describe the unique life cycle of the retroviruses that make retroviral vectors successful for the generation of transgenic birds.

Biology of Retroviruses

Virion structure and classification

Retroviruses, originally known as RNA tumor viruses, are spherical particles with a diameter ranging from 80 to 130 nm (Varmus, 1988; Luciw and Leung, 1992; Coffin *et al*., 1997). The RNA genomes complex with viral-coded proteins derived from *gag* and *pol* genes. This nucleoprotein core is also referred to as the capsid.

The nucleoprotein complex is surrounded by a lipid bilayer envelop, which contains the glycoprotein encoded by a viral *env* gene. Analysis of the chemical composition of retroviruses shows that the virions are (by weight) about 1-2% RNA, about 35% lipids, and about 65% proteins.

Each virus particle contains two identical molecules of RNA genomes which are 7-12 kb in size, linear, single-stranded, nonsegmented and positive in polarity. The virion RNA as well as viral mRNA has a cap structure at the 5′, 7-methylguanosine in a 5′-5′ linkage via a triphosphate to a second 2′-O-methylated nucleotide and poly-A tail at the 3’ end from about 100 to 200 bases in length. Cap structures and poly-A tails are characteristic of cellular mRNA and posttranscriptionally modifided by host-cell enzymes (Luciw and Leung, 1992).

The retroviral genome can be divided functionally into *trans*- and *cis*-acting sequences (Figure 3). *Trans*-acting elements are composed of the coding domains, wheras the *cis*-acting sequenes are involved in the processes of reverse transcription, integration, viral gene expression, and viral assembly (Weiss *et al*., 1992; Coffin *et al*., 1997). All retroviruses contain three major coding domains of *trans*-acting sequences, namely, *gag*, *pol*, and *env*. Gene *gag* directs the synthesis of internal virion proteins that form the matrix (MA), the capsid (CA), and the nucleoprotein structures. Gene *pol* encodes the RNA-dependent DNA polymerase or reverse transcriptase (RT) and integrase (IN). Gene *env* encodes the surface envelope glycoproteins which consist of the surface (SU) and transmembrane (TM) components. An additional smaller coding domain present in all retroviruses is *pro*, which directs the synthesis of the virion protease. Simple retroviruses usually

contain only this elementary information, whereas complex retroviruses carry information coding for additional regulatory nonvirion proteins derived from multiple spliced messages (Coffin *et al*., 1997; Sherman and Greene, 2002).

Cis-acting elements play important roles in reverse transcription, integration, synthesis of viral RNA, and virion packaging (Coffin *et al*., 1997). A short repeated sequence, designated R, is located at each end of the genome, and R varies from 15-80 bases, depending on the viruses. During reverse transcription, this repeat provides a means to transfer newly initiated DNA strands from the 5′ end to the 3′ end of viral RNA.

At the 5′ end, a unique sequence, designated U5 ranging from 80 to 100 bases, is located between R and the site for attachment of a host-cell tRNA, called primer binding site (PBS), which fuctions as a primer for viral DNA synthesis. U5 sequences form stem-loop arrangements which are required for efficient initiation of reverse transcription. In the mechanism of reverse transcription, R and U5 become part of the flanking LTRs in the linear viral DNA. The PBS is followed by an untranslated leader (L) sequence (ranging from 150 to 200 bases) that precedes the initiation codon for *gag*. A sequence element within L, designated the encapsidation (E) or packaging (ψ) sequence, plays an important role in assembling genomic RNA into virions. Other important *cis*-acting sequences in genomic viral RNA include splice donors (SD) and splice acceptors (SA) which are involved in processing of viral RNA transcripts (Luciw and Leung, 1992).

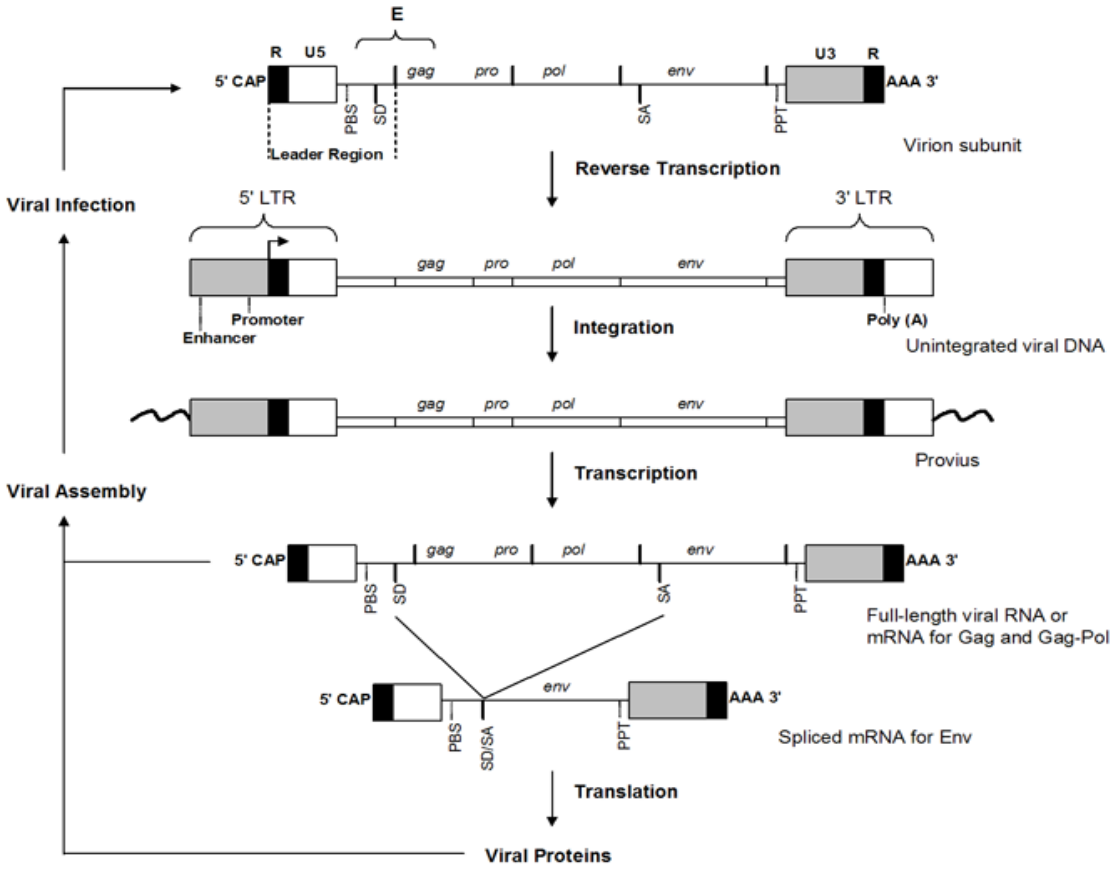


Figure 3. Diagram of the flow of genetic information during the retroviral life cyle. After viral infection and penetration into the host cell, the virion RNA genome is reversed transcribed to unintegrated doulble-stranded DNA with the formation of two long terminal repeats (LTRs). The genetic organization of viral DNA genome consists of *cis*- and *trans*-elements. The *cis*-elements consist of: (1) the LTR at each end of the viral DNA is subdived into U3, R and U5 which contain the transcriptional enhancer, promoter and polyA signal (poly (A)); (2) primer binding site (PBS) and polypurine tract (PPT); (3) the packaging signal (E); (4) splice donor site (SD) and splice acceptor site (SA). The *tran*-elements are the coding regions of the viral genes *gag*, *pro*, *pol* and *env*. The viral DNA is integrated into the host chromosome (indicated as the curved lines at each end of LTRs) to form the provirus. The provirus is transcribed by the promoters in the 5′ U3 to viral genome or full-length mRNA by host enzymes. The full-length mRNA is further spliced into mRNA for Env. These mRNAs are translated to viral proteins that are assembled at the cell membrane with the genomic RNA to form an infectious virus particle (modified from Crittenden, 1991).

At the 3′ portion of the viral genome, a short sequence about 15 bases long and rich in purines is located downstream from *env* and immediately precedes U3. This sequence serves to initiate the synthesis of plus-strand viral DNA and is designated the polypurine tract (PPT). U3 is a unique sequence extending from PPT to R at the 3′ end of the viral genome and ranging from about 200 bases to 1 kb. U3 sequence contains promoter and enhancer elements that control transcriptional initiation of the integrated provirus by cellular RNA polymerase II.

Retroviruses are subdivided into seven groups defined by evolutionary relatedness (Coffin *et al*., 1997). Five of these groups represent retroviruses formerly known as oncoviruses; the genus *Alpharetrovirus* (i.e., Avian leukosis virus (ALV), and Rous sarcoma virus (RSV)), *Betaretrovirus* (i.e., Mouse mammary tumor virus (MMTV)), *Gammaretrovirus* (i.e., Murine leukemia virus (MLV)), *Deltaretrovirus* (i.e., Human T-cell leukemia virus (HTLV) and Bovine leukemia virus (BLV)), *Epsilonretrovirus* (i.e., Walleye dermal sarcoma virus (WDSV)), and the other two groups are *Lentivirus* (i.e., Human immunodeficiency virus 1(HIV-1)) and *Spumavirus* (i.e., Chimpanzee foamy virus (CFV)). All oncogenic groups except the human T-cell leukemia virus and bovine leukemia virus (HTLV-BLV) are simple retroviruses. The deltaretroviruses, lentiviruses and spumavirues are complex retroviruses. In addition, retroviruses can be classified according to virion morphology and intracellular forms which are revealed by electron microscopy. Four types of viral particles, which are type A, B, C, and D particles, have been described (Luciw and Leung, 1992; Coffin *et al*., 1997). The members of the *Alpharetrovirus* and *Gammaretrovirus* genera, which assemble their immature capsids at the plasma

membrane, are classified as C-type viruses. Members of the *Betaretrovirus* genus in contrast assemble A-type particles (immature capsids) in the cytoplasm which then bud with either B-type or D-type morphology.

Overview of retroviral replication

The unique feature of the retrovirus family is its mechanisms of replication which includes reverse transcription step and the virion RNA into linear double-stranded DNA and the subsequent integration of the DNA into the host-cell chromosomes (Figure 3) (Varmus and Brown, 1989; Shuman 1991; Coffin *et al*., 1997). The life cycle of retroviruses is divided into two distinct phases. The early phase of replication involves binding of a virion to a receptor on the cell surface. Attachment to a receptor and entry are mediated by the viral surface *env* glycoprotein which leads to the fusion of viral and cellular membranes, and the virion core is released into the cell cytoplasm (Andreadis *et al*., 2000). Inside the cell cytoplasm, the retroviral core immediately undergoes a partial and progressive disassembly, known as uncoating, that leads to the generation of subviral particles called reverse-transcription complexes (RTCs) and pre-integration complexes (PICs). Viral enzymes RT and IN remain associated with genomic viral RNA in the form of a nucleoprotein complex (Cullen, 2001; Bukrinsky *et al*., 1993; Nisole and Saïb, 2004). The reverse-transcription of genomic viral RNA into duplex linear DNA takes place in the nucleoprotein complex in the cell cytoplasm. The model that describes the mechanism of retroviral reverse transcription requires two strand transfers or jumps to produce one molecule of double-stranded linear viral DNA from one or two single-stranded RNA genomes contained within a virion. These strand

transfers also involve displacement of preexisting sequences. The first DNA strand to be synthesized is complementary to the viral genome and is designated the negative (or minus) viral DNA strand. Subsequently, the positive (or plus) viral DNA strand is synthesized on the negative-strand DNA template. The LTRs flanking the viral genes of the unintegrated DNA molecules are produced in the reverse transcription process (Luciw and Leung, 1992). An unintegrated retrovirus of all oncogenic groups cannot enter the host nucleus unless the nuclear membrane disintegrates during cell replication (Miller *et al*., 1990) except for lentiviruses where an unintegrated viral DNA molecule is actively transported to the nucleus (Miller *et al*., 1997; Sherman and Greene, 2002). After gaining access to the host nucleus, both ends of viral DNA are covalently linked to host-cell DNA mediated by IN to generate a provirus. The provirus begins and ends with short inverted repeats that terminate in the dinucleotides 5′ TG:::CA 3′. In addition, as a result of integration, direct repeats of 4-6 bp in host-cell DNA flank on either side of the integrated provirus whose length is characteristic of the virus (e.g. 6 bp for ASV; 5 bp for HIV-1; 4 bp for MoMLV) (Varmus and Brown, 1989). Once integration of the viral genome is complete, the provirus is maintained in the cell just like any cellular gene.

Recently, from genome-wide surveys of integration sites of HIV-1 and MLV, it has been shown that MLV prefers to integrate in regions surrounding the transcription start site of genes, and that preference drops off with increasing distance from that location in either direction (Schroder *et al*., 2002; Wu *et al*., 2003). HIV-1 has a very strong preference for landing inside genes, but there is no precise location in the gene that is preferred. Integration preference for HIV-1 drops off

dramatically once outside the gene boundary. Corroborating this is the observation that MLV prefers to integrate near CpG islands, which are commonly associated with the transcription start sites of genes. HIV-1 shows no such preference for CpG islands. These results demonstrate that although both MLV and HIV prefer actively transcribed regions, they have clear differences in the fine details of integration selection (Wu and Burgess, 2004).

The late phase begins with the expression of viral genes from the provirus and continues through the release and maturation of progeny virions. A cellular RNA polymerase uses the viral promoter/enhancer in the 5′ LTR to initiate transcription of proviral DNA (Hirose and Manley, 2000). Transciption of the provirus generates spliced and unspliced mRNAs and full-length progeny RNA genomes (Crittenden, 1991; Coffin *et al*., 1997). Full-length and spliced viral RNA molecules are transported to the cytoplasm. Both species of viral RNA are translated on cellular ribosomes into virion polyproteins; however, full-length progeny RNA molecules also interact with virion polyproteins and are assembled into immature virus particles. This process is accommodated by the packaging signal, sometimes referred to as “ψ” or “E”, which is generally located near the 5′ end of the viral genome between the splice donor and the *gag* start codon. Because it is typically located within an intron, the packaging signal is removed during splicing, restricting only full-length viral transcripts and not spliced transcripts (containing only *env*) tobe incorporated into progeny virions (Boris-Lawrie *et al*., 2001). By budding through the cell plasma membrane, these particles acquire a lipid bilayer membrane that contains *env* glycoprotein. Budding of the viruses is followed by proteolytic

cleavage of virion polyproteins by a viral protease and cellular proteases to produce mature and fully infectious viral particles (Varmus and Brown, 1989; Luciw and Leung, 1992).

Retroviruses as gene transfer systems

As described in the previous section, retroviruses have naturally entered the germ cells and are transmitted as endogenous viruses in the chicken (Varmus, 1988). Because of the unique life cycle of retroviruses, viruses have been genetically manipulated and used as vectors of gene delivery into the chicken genomes. In the first of development of retroviral vectors, the gene to be transferred is inserted into the retroviral genome. Next, the retroviral particles carrying the retroviral vectors are generated and harvested. Lastly, the harvested retroviral vector-containing viruses are infected into the host cell. Upon the infection, the gene of interest carried by the retroviral genome is directly integrated into the host genomic chromosomes. Two classes of vector have been used: replication-competent and replication-defective retroviruses.

Replication-competent vectors retain their *trans*-(structural viral genes) and *cis*-(regular elements for expression, integration, and packaging) sequences and additionally carry the transferred gene. These vectors have an advantage in that they are able to undergo multiple rounds of infection and spread from cell to cell producing a high rate of infection that does not require a high titer of virus particles. The disadvantages of these vectors are the limited size of the inserted gene that can

be introduced (about 2 to 2.5 kb) and the disease associated with chronic viral infection (Shuman, 1991; Ronfort *et al*., 1997).

Replication-defective vectors are modified by removing all trans-sequences or some *cis*-elements and replacing those with the transferred gene so that they are unable to replicate once integrated into the host genomes in the first round of their life cycle (Shuman, 1991; Sang, 2004). Thus the production of replication-defective vector virus that is capable of completing one round of life cycle, i.e. infection and integration, requires the assistance of the helper plasmid which is genetically constructed to provide all viral structural proteins necessary for assembly of infectious replication-defective viral particles, reverse transcription of the RNA vector genome, and integration of the intermediate double-stranded viral vector DNA into the host chromosomes. To reduce the risk of producing helper virus (replication-competent virus), the encapsidation and other *cis*-acting sequences are removed as much as possible. The replication-defective viral vector plasmid is transfected into the helper cells which in turn produce the infectious particles containing the retroviral vectors. These replication-defective vectors are preferred over the replication-competent vectors for gene deliver into the chicken because they are viewed as the safer systems. In addition, a larger size of the inserted gene can be introduced (about 8 to 10 kb). However, the main disadvantage of using a replication-defective vector is that the efficiency of gene transfer is dependent on the titer of the viral stock produced. The production of transgenic chickens using the replication-defective retroviral vectors are mainly based on the infection of the viral stocks into or near the blastoderm of unincubated eggs through a small window made in the

shell of the freshly laid egg. Even though the technique is quite straightforward and the eggs are easily obtained, germline transfection is relatively low. This is because the embryo at this stage contains approximately 50,000 cells and only 25-50 cells are presumptive germline precursors (Karagenc *et al*., 1996). The injected embryos which survive to hatch are mosaic animals.

The first germline transgenic chickens were produced from using replication-competent retroviral vectors. Salter *et al*. (1986) injected avian leukosis virus (ALV) near the blastoderm of laid eggs obtained from line 0 hens (a White Leghorn strain free from endogenous viral sequences). Salter *et al*. (1987) twenty-one viremic transgenic males identified by Southern analysis in the first generation (G_1) were found to transmit the proviral insertions to the G_2 generation as expected with Mendelian inheritance. Two proviral inserts, *alv6* and *alv13*, were not found to produce infectious viruses but expressed the viral envelop glycoprotein. Salter and Crittenden (1989) reported that the birds carrying *alv6* and *alv13* were resistant to infection by ALV subgroup A. They suggested that the insertion of a dominant resistant gene was introduced and functions through viral interference.

Chen *et al.* (1990) produced transgenic chickens using replication-competent vectors carrying the bovine growth hormone (bGH) gene. The vectors were derived from Rous sarcoma virus (RSV) Schmidt-Ruppin A strain. The transgenic chicks were demonstrated to resist to the infection from subgroup A but not the corresponding subgroup B similar to that described by Salter and Crittenden (1989). Moreover they found that two transgenic males expressed the bGH gene.

Replication-defective vectors derived from reticuloendotheliosis virus (REV) and avian leukosis virus (ALV) were developed and used to produce transgenic chickens (Bosselman *et al*., 1989; Thoraval *et al*., 1995; Harvey *et al*., 2002; McKrew *et al*., 2004). Bosselman *et al*. (1989) reported the production of germline transgenic chickens using a REV vector carrying the Tn5 neomycin resistance (NPT II) gene driven by the viral long terminal repeat (LTR) promoter injected underneath the embryos collected from the newly laid eggs. Thirty-eight percent of the total 2,599 injected eggs hatched. Approximately 8% of male birds contained the transgene in the semen and transmitted the gene with rates ranging from 2% to 8%. Brinskin *et al.* (1990) reported the expression of progeny from these transgenic lines and found that the provirus was actively transcribed from the LTR in all tissues analyzed. Thoraval *et al.* (1995) injected replication-defective vectors (NL-B) derived from avian leukosis viruses carrying the *Neo*^{r} selectable marker and the *E. coli lacZ* gene directly beneath the unincubated chicken embryo blastoderm. One of the 16 males that hatched (6.25%) was positive for the transgene in the semen. The rooster passed the transgene to his G_1 progeny at a frequency of 2.7%. The expression of the *Neo*^{r}*/lacZ* was limited to cultured chicken embryo fibroblasts derived from transgenic embryos of the G_2 progeny. Harvey *et al*. (2002) used similar vectors as described by Thoraval *et al*. (1995) to generate transgenic chickens. Of the total 546 injected embryos, 126 (23%) embryos hatched. Sperm DNA from 56 roosters were screened for the transgene using real-time PCR and only 5% of these roosters were germline positive for the transgene. These 3 males transmitted the transgene to their offspring at rate of 0.7%. Hence, transmission rate of the germline chimeric

birds generated using retroviral vectors injected into the blastoderms obtained from the newly laid eggs is very low. This can be overcome by using retroviruses pseudotyped with vesicular somatitis virus G protein (VSV-G). Mizuarai *et al*. (2001) reported 80% germline transmission from G_0 quail injected with Moloney murine leukemia virus (MoMLV) pseudotyped with VSV-G protein. Similarly, McGrew *et al*. (2004) generated G_0 roosters that transmitted the transgenes to offspring with rates ranging from 4% to 45% based on an equine infectious anaemia virus (EIAV) vector 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.

Currently, retroviral based vectors injected into the subgerminal cavity of embryos obtained from newly laid eggs are the most successful method of gene transfer for generating transgenic chickens. However, many limitations of using retrovirus vectors are encountered: the problem of germline mosaicism, limited size of inserted transferred gene, and random site of proviral integration which influences the expression of the transgene and endogenous genes. One alternative could be embryonic stem cell (ES) that would allow sophisticated modifications of the cells in the cultures, prior to the generation transgenic birds.

Embryonic stem (ES) cells/primordial germ cells (PGCs)

It has been demonstrated that chicken blastodermal cells freshly isolated from the area pellucida of stage X (Eyal-Giladi and Kochav, 1976) embryos contribute to all somatic tissues and the germline when transferred to the subgerminal cavity of

the same stage recipient embryos (Petitte *et al*., 1990, Carsience *et al*., 1993; Kagami *et al*., 1995). Recently, van der Lavoir *et al*. (2006) successfully developed long-term culture of chicken embryonic stem (ES) cells (6 months) derived from the area pellucida of stage X embryos. They showed the modification of the stem cell genome could be performed and the transformed chicken stem cells were transplanted to the recipient embryos. Unfortunately, germline chimeras were not produced from the study. The ability of the long-term cultured blastodermal cells derived from stage X embryos failed to either incorporate or differentiate to germ cells (Etches *et al*., 1996, Pain *et al.*, 1996; Petitte and Mozdziak., 2002).

Primodial germ cells (PGCs), precursors of gametes, are considered a direct route for the production of transgenic birds. PGCs isolated from vascular veins during the establishment of embryonic circulation (Tajima *et al*., 1993), from germinal crescent (Vick *et al*., 1993), and from embryonic gonads (Han *et al*., 2002) are able to generated germline chimeras when transferred to the recipient embryos. Vick *et al.* (1993) were able to produce transgenic chickens by infecting freshly isolated blood-borne PGCs and gonadal PGCs with replication-defective virus vectors carrying *neo^r/lacZ gene* derived from a defective spleen necrosis virus and a defective avian leukosis virus *neo^r/lacZ gene*. After viral infection, PGCs were injected into the vasculature of stage 15 recipient embryos. Germline male chimeras were obtained and transmitted the transgene to their progeny at the frequency of 2% to 4%. However, they did not demonstrate expression of the transgene in transgenic animals.

Park *et al*. (2003) isolated PGCs from embryonic gonads and cultured them for a short time (7 d) before injection into the stage 17 (H&H) recipient embryos via the dorsal aorta. They successfully generated the germline chimeas inheriting the feather coloring from the donor PGCs. However, they did not attempt to produce transgenic chickens through this route.

Long-term PGCs cultures would be preferred as an efficient means for genetic manipulation. Moreover long-term cultures of PGCs have yet to be proven to be able to migrate to the recipient gonads.

Applications of transgenics to poultry

Practical applications in chicken transgenesis have not yet to be as in mammalian speices. This is because the lack of successful production of transgenic birds due to the differences in reproductive systems renders the proven successful methods in mammals fail when adapted to chicken. In addition, long-term cultures of true embryonic stem cells or embryonic germ cells have not been established. Nevertheless, the generated transgenic chickens have shown potential from gene transfer technology to be useful for the medical and agricultural purposes. Salter and Crittenden (1989) demonstrated that transgenic chickens can be produced to be resistant to infectious disease. The production of therapeutic proteins into the chicken eggs is highly anticipated. At present, only small amounts of exogenous proteins can be produced and deposited into the albumen (Harvey *et al*., 2002; Harvey and Ivarie, 2003; Rapp *et al*., 2003) and into serum and eggs (Kamihira *et al*., 2005).

STATEMENT OF THE PROBLEM

Generally, retrovirus-mediated gene transfer of exogenous genes into chicken cells is widely used as an effective technique of analysis in developmental biology (Iba, 2000). The virus-mediated method, which allows integration of reporter genes into chromosomes of host cells acting as a genetic marker, in conjuection with transplantation and chimeric studies offers a superior means of ensuring faithful inheritance by all progeny over any length of time. Various organogenesis systems such as limbs (Yokouchi *et al*., 1995) and central nervous system (Hemond and Glover, 1993; Homburger and Fekete, 1996) have been studied using retroviral vectors. However, to perform virus-mediated gene transfer for the developmental studies, a high titer virus is nessessary.

Alternatively, transgenic chickens that express β-galactosidase using replication-defective retroviral vectors carrying the *E. coli lacZ* as a stable cell marker would be a useful tool for lineage analysis. Ideally, existing retroviral vectors containing the *lacZ* gene that highly and ubiquitously express the transgene in tissues of chicks and adults birds would be a good candidate vector.

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 (Neo^r) and the *lacZ* gene. Thoroval *et al*. (1995) used the ALV-based vectors to produce transgenic chickens with Neo^r and the *lacZ* gene, but the evaluation of the expression was limited to chicken embryonic fibroblasts (CEFs).

Mikawa *et al*. (1991, 1992) developed a replication-defective retroviral vector, derived from spleen necrosis virus (SNV), carrying a *lacZ* gene, called SNTZ, for cell lineage analysis of cardiac development. The retroviral vector can infect a wide variety of cell types in early chick embryos. Beta-galactosidase, which the expression is concentrated in an infected host nucleus, was functional in various cell types in various embryonic tissues and cell cultures. The *lacZ* gene stably inherited and continued to express in the progeny of the infected cells. Moreover, expression of the gene can be detected at hatch.

Thus, it appears that the SNTZ vector would be an appropriate retroviral system 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*. (1992) to produce the transgenic chickens carrying the *lacZ* gene encoding nuclear-localized β-galactosidase as an effective cell marking system for cell lineage analysis.

Potentially, the creation of transgenic chickens to express β-galactosidase in gastrointestinal tract would increase their ability to utilize lactose more efficiently. The second objective of this study was to determine whether the transgenic chickens can improve lactose digestivibility and use as an energy source.

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CHAPTER 2:

The Production of Transgenic Chickens Expressing β-Galactosidase Based on A Spleen Necrosis Virus Retroviral Vector

ABSTRACT

Replication-defective retroviral vectors are currently the most efficient gene transfer method for the production of transgenic chickens. In this study, the replication-defective spleen necrosis virus-based SNTZ vector carrying the *E. coli lacZ* gene designed to express a nuclear-localized β-galactosidase was injected into the subgerminal cavity of embryos collected from newly laid eggs. The injected embryos were cultured to hatch using a surrogate eggshell culture system. Eight of 15 sexually mature males were *lacZ* positive in their semen based on PCR screening. Only one G_0 rooster transferred the *lacZ* gene to two males of 224 progeny, giving 0.89% germline transmission rate. The two transgenic G_1 male birds were demonstrated to stably transmit the *lacZ* transgene to next generation and at least up to G_4, which was used to generate transgenic chicken lines for the *lacZ* gene. In addition, the expression of the β-galactosidase was observed in cultured myoblasts, entire embryos and in adults in various tissues based on X-gal staining. In the current study, the generated transgenic lines which stably inherit and express the reporter *lacZ* gene are the first report of transgenic birds that could provide an alternative ideal cell marker for cell lineage studies.

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

INTRODUCTION

Cell lineage analysis reveals morphogenic movement and cell fate diversification during embryogenesis. Cell lineage analysis is approached by marking individual cells or a group of cells, tracing their migration, and determining their destination and differentiation. Successful interpretation of the results requires cell markers to unambiguously distinguish between the marked and unmarked cells along the course of developement. In birds, many cell tagging methods have been applied such as carbon particles (Spratt, 1946), tritiated thymidine (Weston, 1963; Rosenquist, 1971), and lipophillic vital dyes (Selleck and Stern, 1991; Garcia-Martinez and Schoenwolf, 1995). However, these extrinsic markers allow only short-term studies because they are lost over time and diluted through cell division (Sane, 1989). The chick-quail chromatin marker is used for long-term studies because of its stable inheritance to all daughter cells. In chimeras as a result of interspecific grafting experiments, the heterochromatin in the interphase nuclei of quail cells are easily differentiated from chick cells in tissue sections stained with a DNA stain (Le Douarin, 1973). The main disadvantage of the chick-quail marker method is the difference in genetic background which might pose problems in variable gene expression among cells in a tissue of a chimera resulting in abnormal cellular behavior (West, 1984). Chick and quail have different a rate of development (quails hatch after 16 days of incubation whereas chicks hatch after 21 days). In addition, xenogenic tissue grafts made in the embryo are rejected after hatch (Le Douarin, 1988), making the chick-quail tagging method inappropriate for cell lineage analysis

throughout development and in adults. These problems can be solved by the creation of a chimera made by grafts between the same species (allografts) in which the donor grafts were infected with a replication-defective retrovirus carrying a reporter gene (Fekete and Cepko, 1993; Stocker *et al*., 1993). Nevertheless, several drawbacks of the use of the retroviral vectors still exist. First, viral stocks need to be generated on the day of use (Ishii *et al*., 2004). Second, random integration of provirus in the host cells could disturb normal gene expression (Ishii and Mikawa, 2005). Third, infection occurs only in dividing cells (Miller *et al*., 1990).

An alternative approach to cell markers that circumvents the difficulties mentioned is the production of germline transgenic birds expressing a reporter gene for intraspecific grafting experiments. 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, 1989, 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 (Neo^r) and the *lacZ* gene. Thoroval *et al*. (1995) used the ALV-based vectors to produce the transgenic chickens with Neo^r 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 establish transgenic chicken lines to stably inherit the *lacZ* gene and express in a variety of tissues througthout developmental stages which can be used as an ideal cell marker for cell lineage studies.

MATERIALS AND METHODS

Breeding strategy of generating transgenic birds

Figure 1 illustrates general steps in the production of transgenic chickens in the current study. The retroviral vectors are harvested and injected into a prospective G_0 embryo before incubation. The injected embryos are cultured in a surrogate eggshell and hatch at approximately 21 days of incubation. After hatching, it takes another 17-20 weeks for the birds to reach sexual maturity. Semen can be collected and screened for the transgene. In general, the positive G_0 roosters are preferably used in mating program for the production of transgenic G_1 progeny carrying the transgene from many females. This step is inefficient due to the mosaic status of the germline of G_0 birds. Subsequently, the transgenic G_1

offspring will take another 21 weeks to reach sexual maturity and at least another 3 to 4 weeks until there is sufficient G_2 progeny to demonstrate Mendelian inheritance.

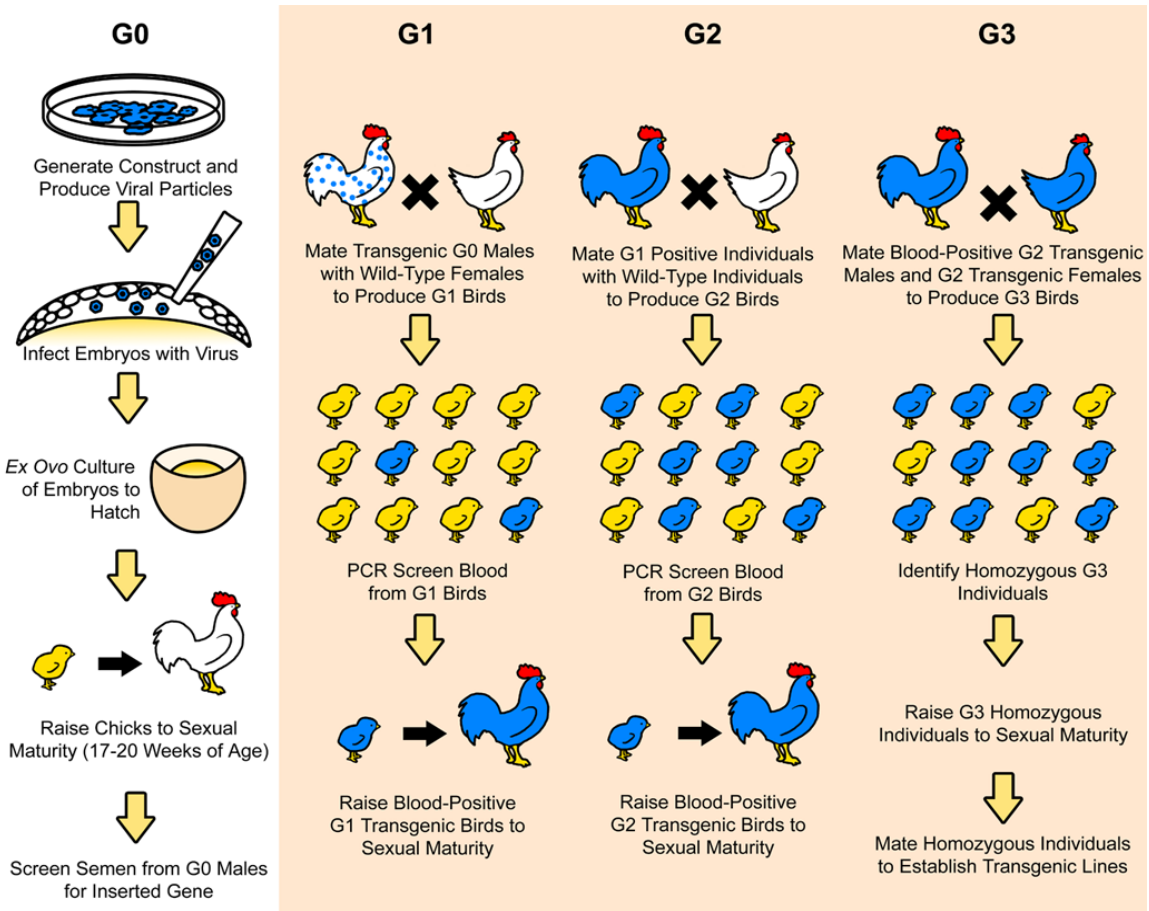


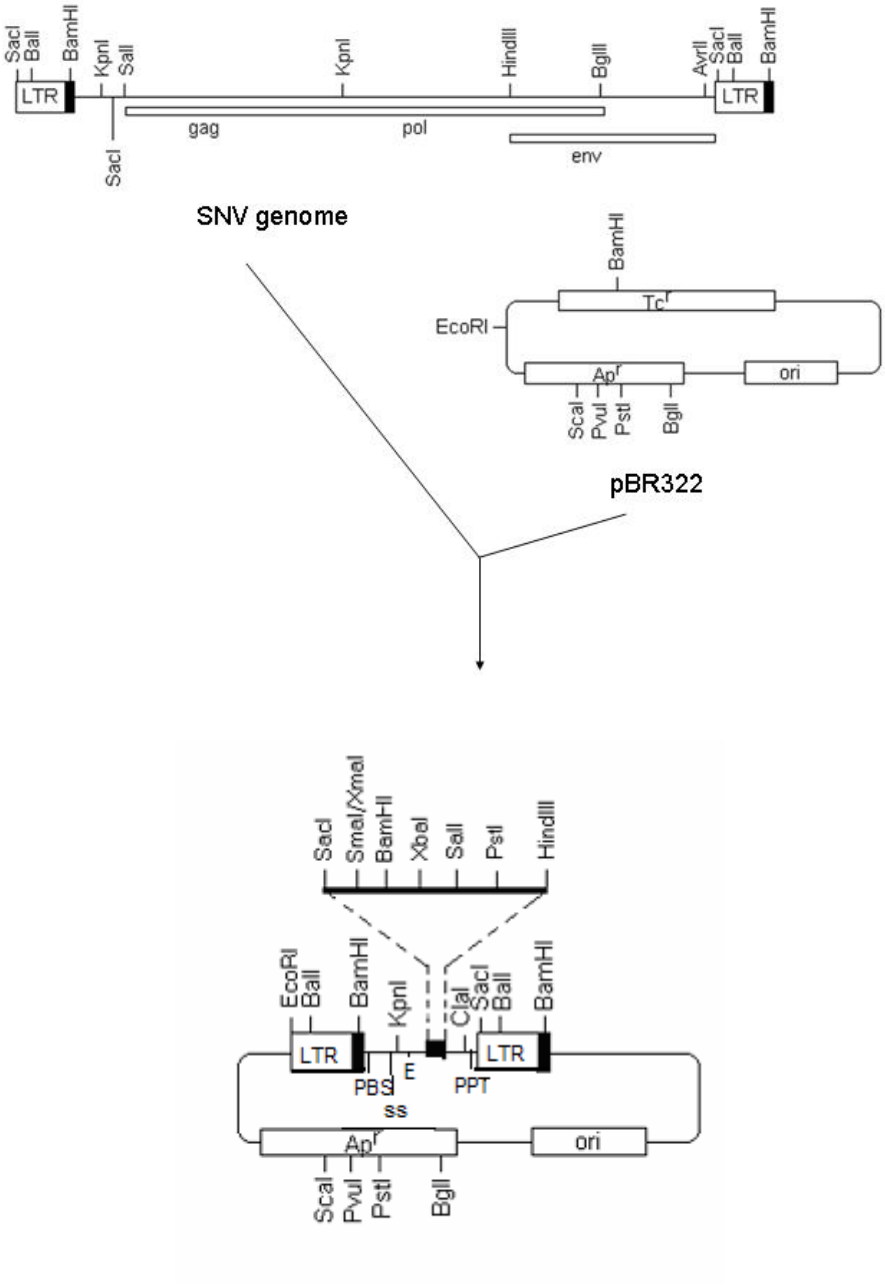
Figure 1. General steps in the production and establishment of transgenic chicken lines carrying a bacterial *lacZ* gene expressing β-galactosidase. Generation 0 (G_0) involves the construction and production of retroviral vectors and the infection of embryos followed *ex ovo* culture, hatching, and screening of putative mosaics for breeding. A G_0 male depicted having blue dots is a germline chimeric rooster. Chicks or adult chickens depicted having blue color are transgenic birds (taken from Mozdziak and Petitte, 2004).

Construction and deduced restriction map of pSNTZ

The replication-defective retroviral vector used in this study was derived from SNV, except for the splice acceptor sequences, which were derived from avain 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 2). 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 *Eco*RI/*Bam*HI 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 3). 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 neomycin-resistance 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 4).



pJD214

Figure 2. 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.

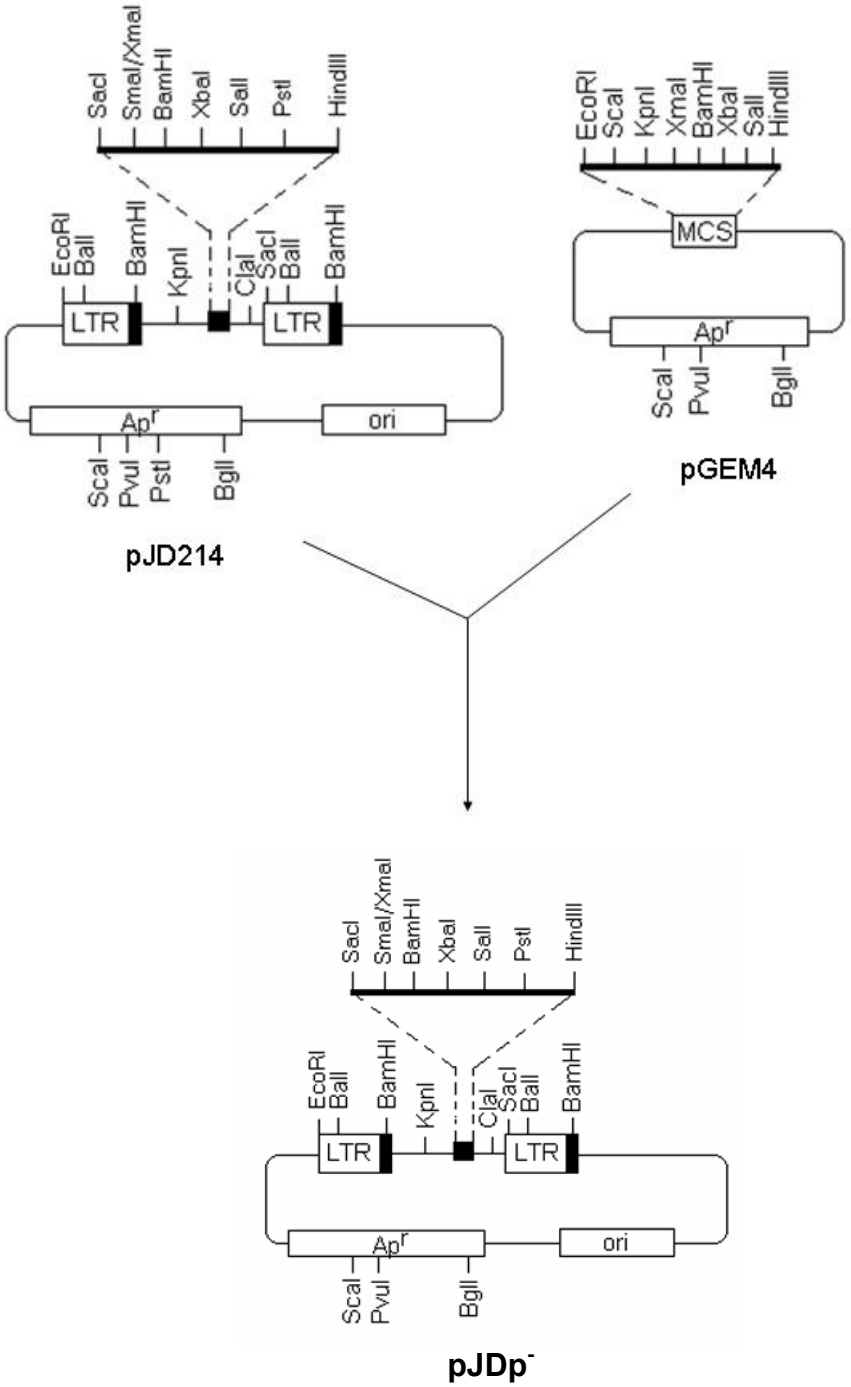


Figure 3. Vector construction and deduced restriction map of pJDp$^-$. The *cis* sequences of pJD214 illustrated in Figure 2. The pGEM4 was obtained from Promega Corporation.

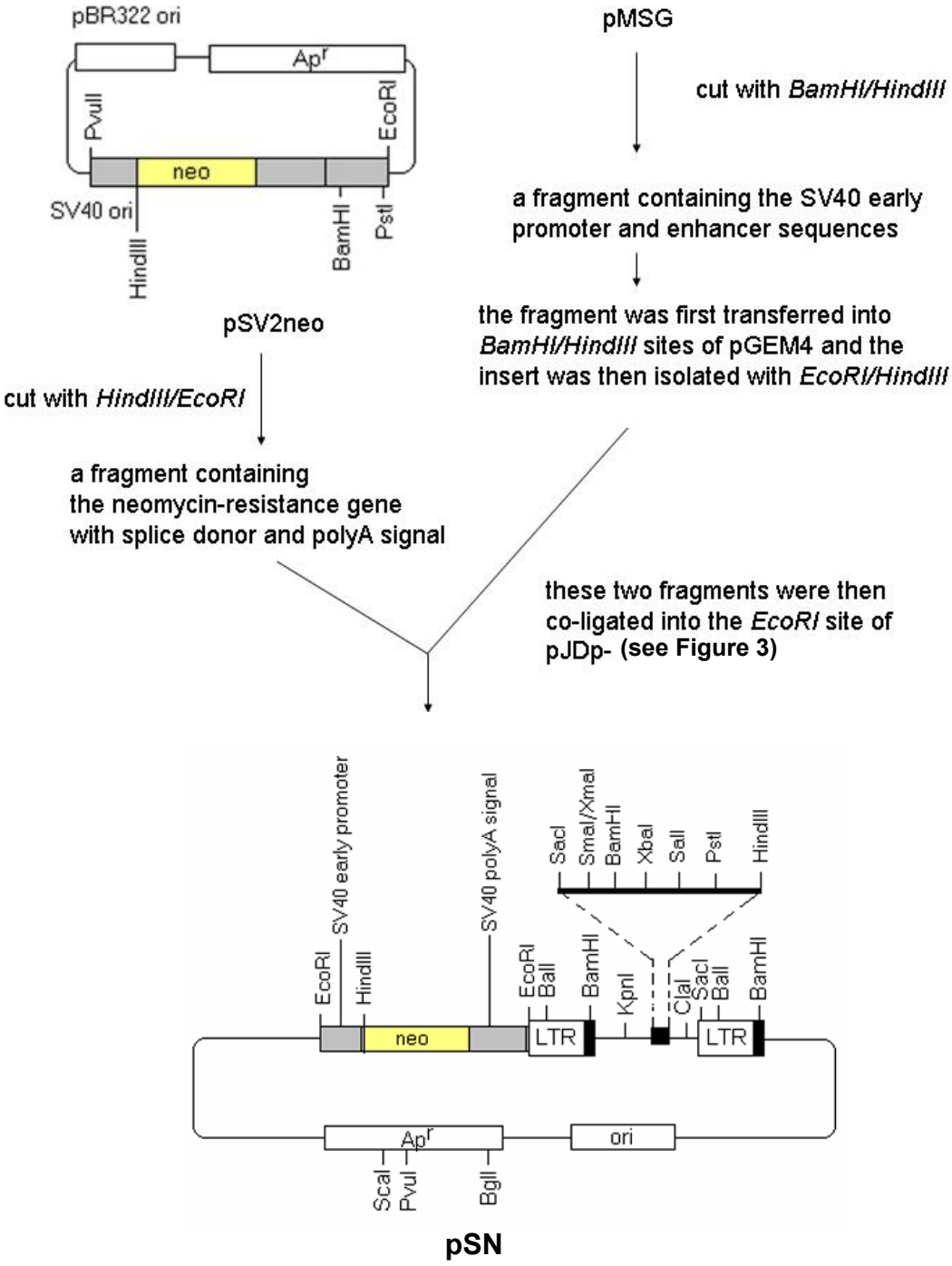
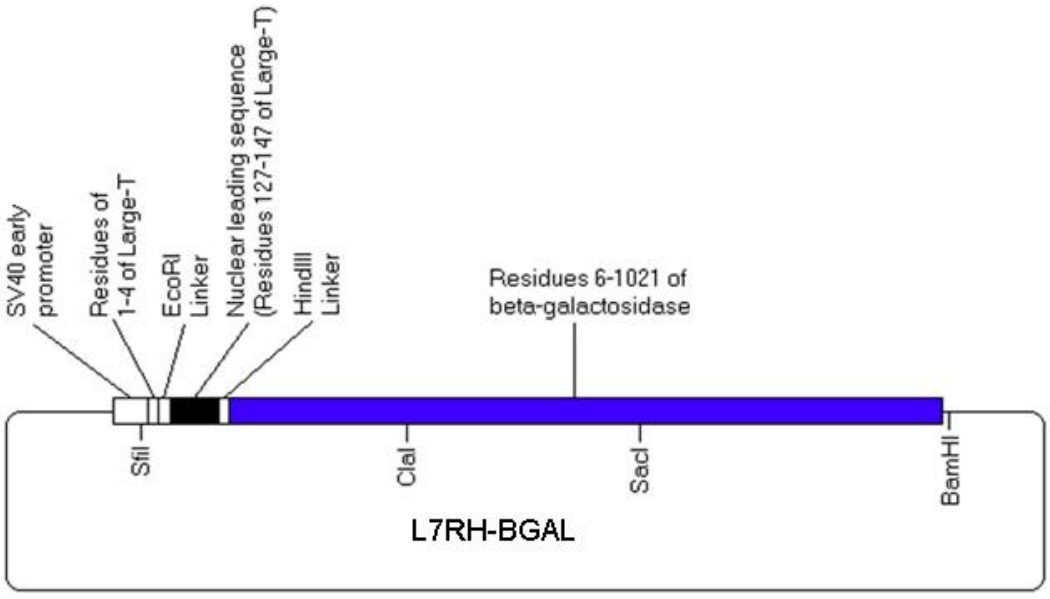


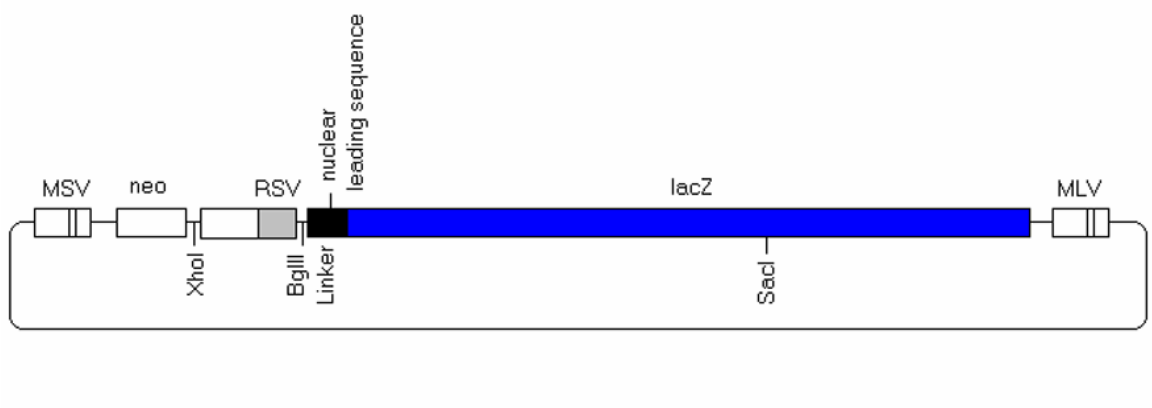
Figure 4. Vector construction and deduced restriction map of pSN.

The *lacZ* sequences fused to the nuclear leading or localization sequence (NLS) of SV40 large T antigen was isolated from the plasmid LZ12. The construction of the plasmid LZ12 was described by Galileo et al. (1990) (Figure 5).

The 5' end of 2-kb fusion sequence of pLZ12 for the *lacZ* gene was isolated by Bgl II/Sst I digestion and ligated into the Bam HI/Sst I sites of pGEM4. The insert was then removed from the vector with Xba I/Sst I. The 1-kbp fragment of the 3' end of *lacZ* was cut from pMC1987 (Pharmacia) by Sst I/Sal I digestion. These two fragments were ligated into pSN at Xba I/Sal I site to form pSNTZ (Mikawa *et al*., 1992b) (Figure 6). The pSNTZ is about 7.9 kbp.

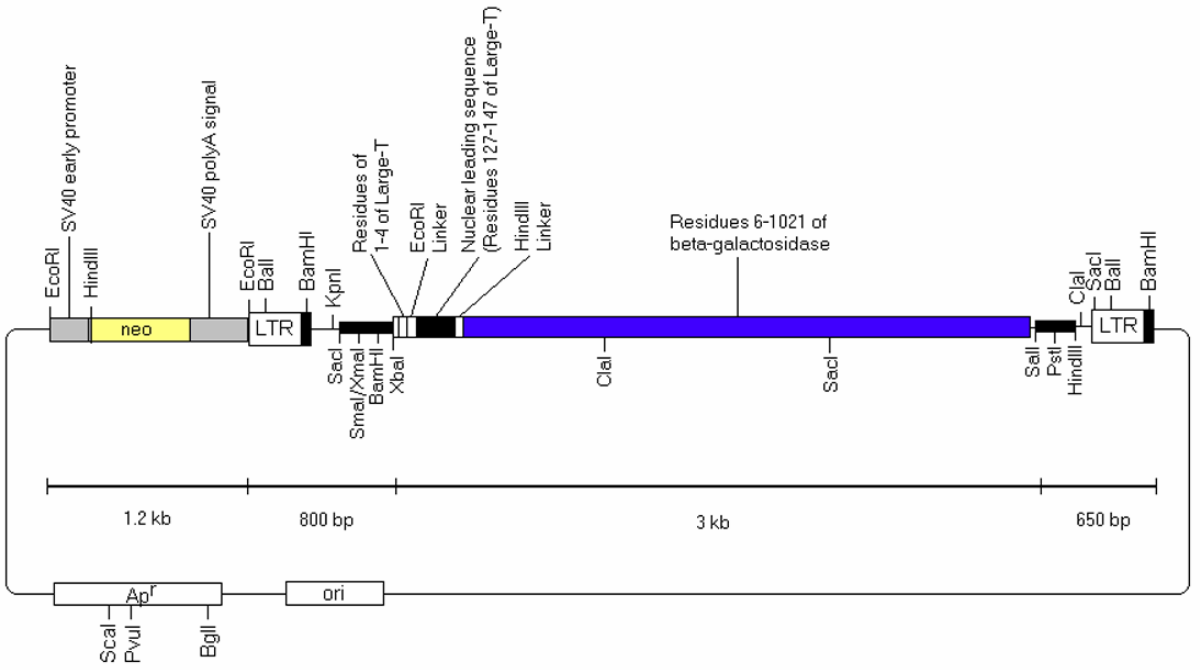


Bgl II linkers were added to L7RH-BGAL at the *Sfi I* site and subsequently cleaved with *Bgl II/Sac I* yielding a fragment containing the large tumor antigen-derived nuclear localization sequence and approximately two-thirds of the *lacZ* gene. This fragment was ligated to pLZ10 and later the insert cut with *XhoI* and *Xba I* was inserted into Moloney murine retroviral vector to form pLZ12.



pLZ12

Figure 5. Vector construction and deduced restriction map of pLZ12.



pSNTZ

Figure 6. Vector construction and deduced restriction map of pSNTZ. The *cis* elements of the retroviral sequences were illustrated in Figure 2.

Production of Retroviral Virions Containing SNTZ

The created pSNTZ vector encoding *lacZ* was transfected into the SNV packaging cell lines, D17.2G, which provide the products of *gag*, *pol*, and *env* gene in trans witout 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[1], and 1% penicillin-streptomycin[2].

[1] Fisher Scientific, Pittsburge, PA

[2] Life Technologies, Rockville, MD

Once the packaging cells became 90% confluent, they were maintained in retrovirus producing medium (DMEM supplemented with 1% fetal bovine serum and 1% penicillin-streptomycin) for 24 hr before the medium was harvested for viral transfection of chick embryos. Subsequently, SNTZ retrovirus was harvested from the culture supernatants (50 ml from 10 100-mm culture plates) of the D17.2G SNTZ-producing packaging cell lines was concentrated by using a stirred cell apparatus containing a membrane filter[3] to a volume of approximately 500 µl. After concentration, the retroviral virions were microcentrifuged for 3 min at 15,000 × g.

Viral titers were determined by infecting D17 canine fibroblastic cells with concentrated retrovirus in the presence of 1 µg/ml polybrene. Forty-eight h after transfection, cells were fixed with 4% paraformaldehyde in PBS for 25 min at 4°C, washed with PBS, and then incubated with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 16 mM potassium ferrocyanide, 16 mM potassium ferricyanide, 2 mM $MgCl_2$ in PBS pH 7.2 for overnight at room temperature. The number of blue (X-Gal)-nuclear stained cells in each culture plate was evaluated and used to determine the retroviral titers. The titers of the concentrated retroviral stocks ranged from 2.5×10^6 to 2×10^7 virions/ml.

[3] YM100, Millipore Corp., Bedford, MA

Retroviral Transfection and Surrogate Egg Shell Culture

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. The parent flocks were maintained at the North Carolina State University Poultry Unit.

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 (Figure 7A). The recipient chicken eggshells were prepared using chicken eggs which were 3 to 4 g heavier than the donor eggs, and a 32-mm window was made at the sharp end. Before injection of retroviral particles, all transferred embryos were positioned uppermost at the opening window. Three viral injection protocols were employed in this study: injection of stage X embryos, double injection of stage X and germinal crescent, and injection of stage X with D17.2G SNTZ-producing cells. In the first method, the stage X embryos were injected with 5 µl of the concentrated retroviral stocks containing polybrene (100 µg/ml) into the subgerminal cavity with a pulled micropipette. The micropipettes with 50 µm in diameter were pulled with a vertical micropipette puller[4] and connected with a rubber hose as seen in Figure 1B during injection.

[4] Vertical Pipette Puller Model 700C, David Kopf Instruments, Tujunga, CA 91042

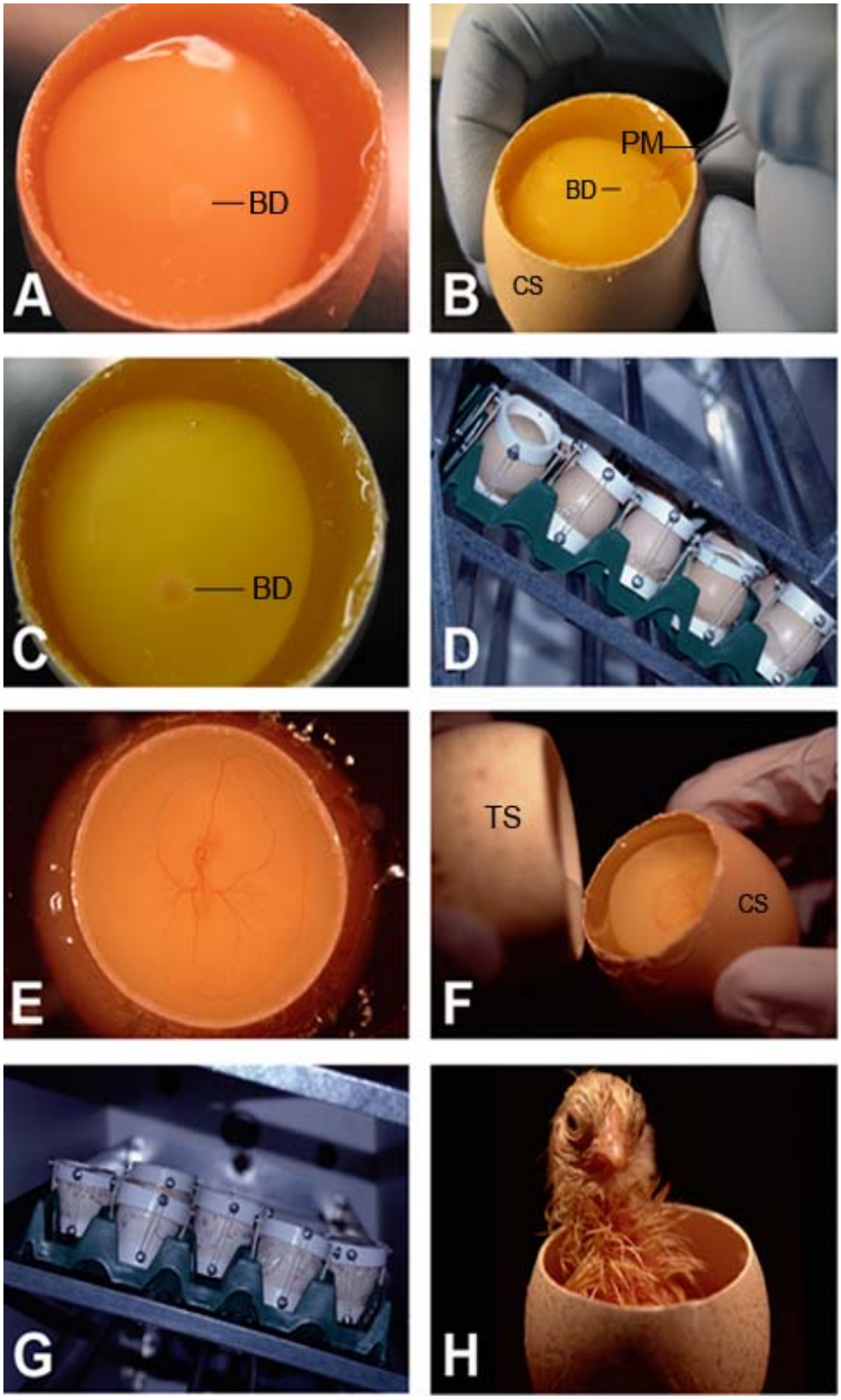
The micropipette needle was pointed near the center of are pellucida and then gently pushed into the subgerminal cavity, and the viral solution was released gradually into the cavity (Figure 7B). The successful injection was revealed by the pink color of the viral solution spreading all over the area pellucida underneath the blastoderm without any leakage from the injection point (Figure 7C). In the second injection method, the stage X embryos were injected with 5 µl of the concentrated retroviral stocks containing 500 D17.2G SNTZ-producing cells and polybrene (100 µg/ml) with a pulled micropipette. Finally for the double injection, the first injection was made as described as in the first injection method in which the stage-X embryos were injected with 5 µl of the concentrated retroviral stocks containing polybrene (100 µg/ml) into the subgerminal cavity with a pulled micropipette, and then the embryo cultures were sealed at the window with plastic cling film and incubated at 37.5°C for 24 h. For the second injection, the 24-h incubated embryos were injected again with 4 injections of 3 µl of the concentrated retroviral stocks containing polybrene (100 µg/ml) per injection into the germinal crescent. All the injected embryos were sealed at the opening window with Saran Wrap®[5] and incubated at 37.5°C with the window side down for the total for 3 d (Figure 7D).

[5] Dow Chemical, Midland, MI

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 (Figure 7E, 7F). The recipient turkey eggshells were prepared from using turkey eggs which 40 g heavier than the chicken donor eggs and an 8-cm window was made at the blunt end. After transfer, the window in the turkey eggshell was sealed with Handi Wrap®[6] and incubated at 37.5°C with the window up until hatch (Figure 7G, 7H). Borwornpinyo *et al*. (2005) showed that the use of Saran Wrap® and Handi Wrap® in combination as covering materials for the surrogate cultures resulted in the best hatchability. The hatched chicks were wing-banded for identification, and grown to sexual maturity. The birds generated from viral injection formed the G_0 chimeric founder chickens.

[6] Dow Chemical, Midland, MI

FIGURE 7. Viral injection and surrogate eggshell cultures for the production of germline chimeric G_0 birds carrying a *lacZ* gene. (A) A stage X embryo collected from a newly-laid donor chicken eggs; BD: blastoderm. (B) Viral injection is performed by using a pulled micropipette (PM) containing retroviral stocks (seen as pink solution inside the pulled micropipette) injected into the subgerminal cavity of the embryo; CS: chicken recipient surrogate eggshell. (C) A successful injection is viewed as having retroviral-concentrated solution filling inside the subgerminal cavity without any leakage of the solution. (D) After injection, the embryo culture is sealed and incubated with its window down. (E) After 3 d of incubation, the injected embryo develops normally. (F) The 3-d-old embryo is transferred into a surrogate turkey eggshell (TS). (G) After transfer, the embryo culture is sealed and incubated with its window up. (H) The chick hatches at 21 d of incubation. This chick forms G_0 generation (Figure D, E, F, G and H were taken from Borwornpinyo *et al*., 2005).


BD
A
PM
BD
CS
B
BD
C
D
E
TS
CS
F
G
H

DNA Isolation

Blood

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 $MgCl_2$, 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.

Semen

Genomic DNA was isolated from chicken semen based on the procedures from Thoraval *et al.* (1995) and Afanssieff *et al*. (1996). Approximately 190 µl of freshly collected semen was rinsed 2 twice in 400 µl PBS (phosphate buffer saline) by centrifugation at 620 × g for 20 min at room temperature.

After centrifugation, 400 µl SLB buffer (10 nM EDTA, 150 mM NaCl, 10 mM Tris-HCl (pH 7.6), and 0.2% SDS) was added to semen pellets in a 1.5 ml microtube and then the mixture was homogenized using a hand-held homogenizer[7]. Eight µl Proteinase-K (10 mg/ml) was added to homogenized semen and incubated at room temperature overnight. After incubation, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added into the nucleic acid suspension. The 1.5 ml microtube containing the mixture was inverted gently to mix until an emulsion was formed, and then microcentrifuged at 1,600 × g for 4 min at room temperature. After centrifugation, the upper aqueous layer was transferred into a new 1.5 ml microtube. The phenol:chloroform:isoamyl alcohol extraction was repeated for 3 times until no protein was visible at the interphase layer. After phenol:chloroform:isoamyl alcohol extraction, approximately 100 µl of the upper aqueous layer was recovered and an equal volume of chloroform:isoamyl alcohol (24:1) was added to precipitate some residual proteins by centrifugation at 1,600 × g for 4 min at room temperature. After centrifugation, the clean nucleic acid solution was transferred into a new 1.5 ml microtube, and exactly 2 volumes of cold ethanol were added to precipitate the genomic semen DNA. The aggregated DNA was microcentrifuged at 1,600 × g for 10 min at 4°C. The DNA pellets were air dried and resuspended in 150 µl Tris-EDTA buffer.

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[7] Pellet Pestle® Motor, Kontes

Chick Comb

A comb was clipped from a newly hatched chick, kept in a 0.5 ml microtube, and stored at 4°C. The comb was 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 enzymatic digestion, 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.

Polymerase Chain Reaction (PCR) Screening for the *LacZ* gene

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 thermocycler[8]. A 25 µl reaction volume containing 200 ng of DNA samples, 1.5 mM $MgCl_2$, 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.

[8] PTC-200, MJ Reseach, Waltham, MA

Germline Transmission

G_0 Chimeric Founder Chickens

DNA samples isolated from either blood or semen of G_0 founder chickens were analyzed for the *lacZ* DNA, and the *lacZ*-positive G_0 founder chickens were used for germline transmission determination. Germline transmission of G_0 founder chickens was determined from the cross breeding with wild-type White Leghorn birds. The offspring produced were analyzed for the *lacZ* gene and formed the G_1 transgenic lines.

G_1 and G_2 Transgenic Lines

Germline transmission of G_1 birds was determined from cross breeding with wild-type White Leghorn birds. DNA isolated from blood from the generated offspring were analyzed for the *lacZ* gene, and the *lacZ*-positive birds formed the G_2 transgenic lines. To determine germline transmission of G_2 transgenic lines, inter-mating between G_2 transgenic birds was employed.

G_3 Transgenic Lines

Four hundred and fifty two day-one chicks were hatched from hemizygous G_3 x hemizygous G_3. Each chick was neck-tagged and wing-banded. Then, their combs were clipped and used for DNA extraction for genotype determination. Genotyping used a multiplex PCR based on procedures described by Mozdziak *et al*. (2006). Two primers (the forward primer 5′-CACGCACTAACATCCAATTCCC-3′ and the reverse primer 5′-TCCACAGCACAAGCACTATTCC-3′) were designed based on the predicted chicken genomic sequences flanking the provirus and one primer (the reverse primer 5′-CCTCTTCGCTATTACGCCAG-3′) was designed

specific to the *lacZ* sequence. Multiplex PCR was performed in a 20-µl reaction containing 0.2 mM dNTPs, 10 pmol each of the 3 primers, PCR buffer[9], 1 × MasterAmp PCR Enhancer[10], 1.5 U of Taq polymerase, and DNA samples. The PCR thermal cycle was at 94°C for 3 min for initial denaturation, followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 70°C for 2.5 min, and the final extension at 70°C for 10 min. The predicted size of the amplification products for a wild-type non-transgenic bird was a single 522-bp fragment specific to chicken genomic DNA. For a heterozygous transgenic chick, 2 predicted amplified products were generated. The first product indicative of a transgenic allele was approximately 1,200-bp fragments amplified from the *lacZ*-specific reverse primer and the chicken genome-specific forward primer. The second product was the predicted 522-bp fragments indicative of a non-transgenic allele. The amplified products from a homozygous chick were the only 1,200-bp fragment indicative of 2 transgenic alleles.

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[9] Takara Mirus Bio, Madison, WI,

[10] Epicentre, Madison, WI

Expression of β-Galactosidase

Embryos

Embryos isolated from incubated eggs at various times were fixed at 4°C with 2% formaldehyde and 0.2% glutaraldehyde in PBS pH 7.4 for 30 min, rinsed in PBS twice, and incubated in X-Gal solution containing 1 mg/ml X-Gal (in DMF), 5 mM potassium ferrocyanide, 5 mM postassium ferricyanide, 2 mM $MgCl_2$ and 0.02% Triton-X 100 in PBS pH 7.4 in the dark at 37°C for overnight.

Whole Mount Tissues

Various tissue types isolated from transgenic and wild-type birds were fixed at at 4°C with 2% formaldehyde and 0.2% glutaraldehyde in PBS pH 7.4 for 30 min, rinsed in PBS twice, and incubated in X-Gal solution containing 1 mg/ml X-Gal (in DMF), 5 mM potassium ferrocyanide, 5 mM postassium ferricyanide, 2 mM $MgCl_2$ and 0.02% Triton-X 100 in PBS pH 7.4 in the dark at 37°C for overnight.

Tissue Cryosections

Tissues were dissected from 5-wk-old G_3 transgenic and wild-type birds. For intestinal tissue samples, chyme was flushed out of the lumen with PBS using a 3-ml syringe. All dissected tissues were cut to 0.5 cm in size, and rinsed with PBS twice. Next, tissues were fixed with 2% paraformaldehyde, 0.2% glutaraldehyde, and 0.02% Triton-X 100 in PBS pH 7.4 at 4°C for 30 min. The fixed tissues were washed 3 times for 20 min/wash with PBS. After washing, the tissues were infiltrated with 20% sucrose in PBS for about 2 h at 4°C until the tissues sank into the bottom of container. The infiltrated tissues were then embedded in an aluminum foil mold which contained embedding gel (2 portions of 20% sucrose in PBS and 1

portion of OCT compound[11]) at 4°C for 30 min. After embedding, the tissues were snap frozen in isopentane cooled in liquid N_2 for 10 to 15 sec, and the frozen tissues were immediately taken into a cryostat chamber[12] set at -20°C. The frozen tissues were allowed to equilibrate with the chamber temperature for at least 20 min. The frozen tissues were sectioned at 10 µm, and laid on a charged slide. The tissue sections were air dried for 30 min and stained with X-Gal solution containing 1 mg/ml X-Gal (in DMF), 5 mM potassium ferrocyanide, 5 mM postassium ferricyanide, 2 mM $MgCl_2$ and 0.02% Triton-X 100 in PBS pH 7.4 in the dark at 37°C for at least 4 h. After staining, the sections were fixed with 2% paraformaldehyde for 5 min, washed twice with PBS, and mounted with aqueous mounting media.

Presence of Replication-Competent Virus in Transgenic Chicken Lines

To determine whether the generated transgenic chicken lines were free of replication-competent virus, chicken myoblasts were cultured from transgenic chickens and the culture media collected from the β-galactosidase-positive myoblast cultures were used to grow wild-type chicken myoblasts. Myoblasts were isolated from 1-d-old G_2 chickens using procedures modified from Mozdziak et al. (1996). First, samples from pectoralis thoracicus muscles of 1-d-old chicks positive for the *lacZ* gene were minced in Hanks' balanced salt solution and digested for 35 min with warm (37°C) 0.17% trypsin and 0.085% collagenase in Hanks' balanced salt solution for satellite cell liberation.

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[11] Tissue-Tek®, Sakura FineTek USA, Inc., Torrence, CA 90540

[12] UltraPro 5000 Cryostat, the Vibratome Company, St. Louis, MO 63134

After enzymatic digestion, the tissue was washed twice with DMEM, 15% fetal bovine serum, and 1% penicillin-streptomycin. The liberated cells were plated on 0.1% gelatin-coated plates. The cells were incubated for 5 d until the cultures became approximately 80% confluent. Next, the cultures were fixed in 4% paraformaldehyde and stained with X-Gal (1 mg/ml X-Gal, 16 mM potassium ferricyanide, 16 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS pH 7.2) at 37°C for overnight. All myoblasts were positive in the presence of X-Gal with blue nuclear staining. Lastly, the presence of any replication-competent virus was determined by removing cell culture supernatant from the myoblast cultures derived from β-galactosidase-positive chickens and placing on proliferating wild-type chicken myoblast cultures in the presence of polybrene (100 µg/ml).

Statistical Analysis

Chi-square analysis was used to determine whether the germline transmission rate from cross breeding between 2 transgenic G_1 males hemizygous for the *lacZ* gene and wild-type White Leghorn hens differed from the expected ratio 1:1 (transgenic:wild-type) based on the assumption of a single integration site of the provirus carrying the *lacZ* gene, inter-mating between hemizygous G_2 birds for the *lacZ* transgene differs from the expected ratio 3:1 (transgenic:wild-type), and inter-mating between hemizygous G_3 birds for the *lacZ* transgene differs from the expected Mendelain ratio 1:2:1 (homozygous:hemizygous:wild-type). Statements of statistical significance in all instances were based upon ($P < 0.05$).

RESULTS

Generation of chimeric G_0 chickens

Table 1 summarizes the effect of injection strategies on hatchability and the percentage of chimeric G_0 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 G_0 chicks, 25 females and 15 males, survived to sexual maturity. Two G_0 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 G_0 female and 90 progeny produced from the second G_0 female were screened for the *lacZ* gene by means of PCR in their blood (Table 2). Neither G_0 females generated *lacZ*-positive offspring. In addition, all blood *lacZ*-negative G_0 females did not produce *lacZ*-positive progeny in their blood. Eight of 15 G_0 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 G_0 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 G_0 male produced 2 blood *lacZ*-positive 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 G_0 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 only[1]	66	36 (24)	10 (2/20)[4]	80 (4/5)
Virus and cells[2]	54	19 (10)	0 (0/7)	40 (2/5)
Virus double injection[3]	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 G_0 females generated from retroviral injection[1]

Chimeric G_0 females	Number of G_1 chicks PCR screened for *lacZ*	G_1 chicks carrying *lacZ* in their blood
1[2]	101	0
2[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 G_0 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

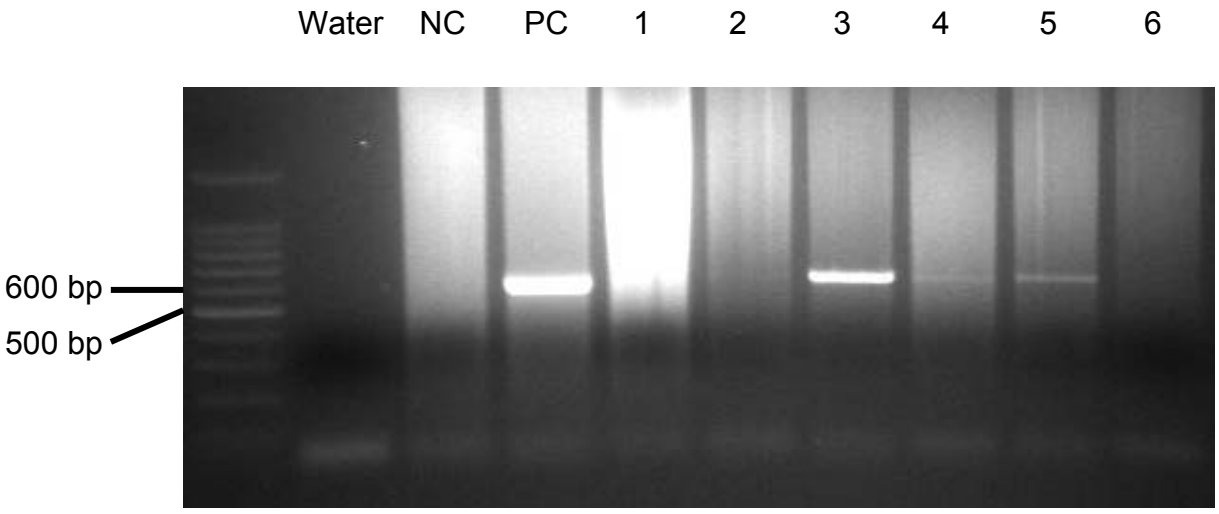


Figure 8. Polymerase chain reaction analysis of the semen from G_0 chickens. Water is a negative control using a water blank. NC is a negative control using DNA isolated from semen of wild-type chickens. PC is a positive control reaction containing the *lacZ* gene from pmiwZ plasmid (Kadokawa *et al*., 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 G_0 male chickens carrying the *lacZ* gene in their semen[1]

G_0 Males carrying *lacZ* in their semen	Number of G_1 chicks PCR screened for *lacZ*	G_1 chicks carrying *lacZ* in their blood
1[2]	224[3]	2[4]
2	252	0
3	14[3]	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 G_0 sire was 0.89% (2/224)

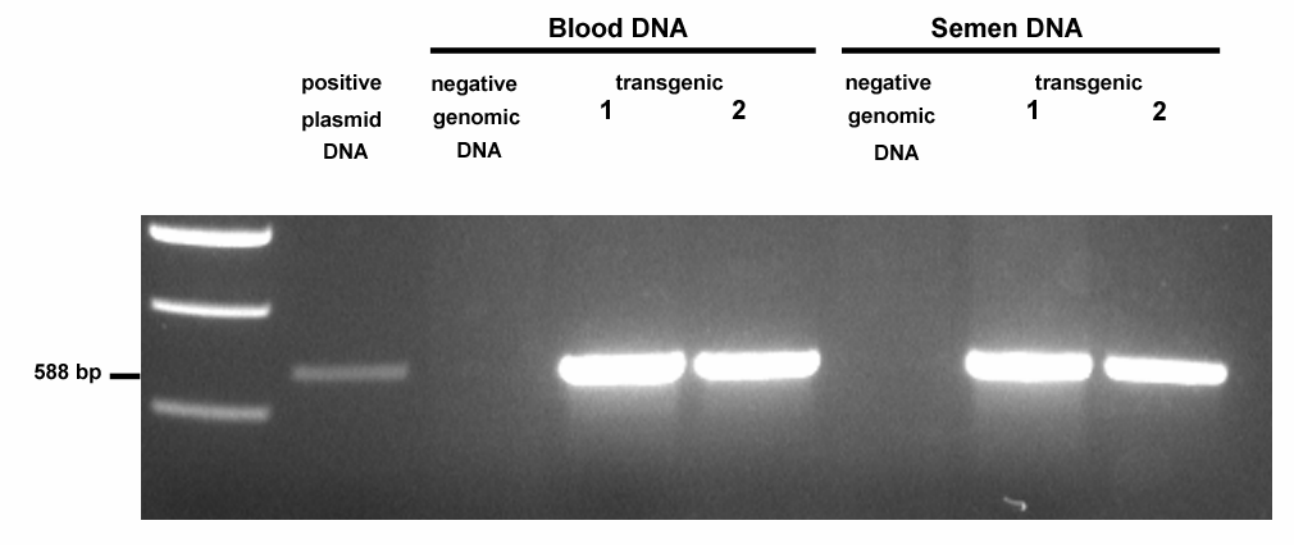


Figure 9. Polymerase chain reaction analysis of G_1 male chickens containing the *lacZ* gene. The positive plasmid DNA was pmiwZ plasmid coning *E. coli lacZ* gene (Kadokawa *et al*., 1990). The negative genomic DNA was chicken genomic DNA isolated from wild-type non-transgenic birds. Transgenic 1 referred to Precious-I transgenic G_1 male. Transgenic 2 referred to Precious-I transgenic G_1 male. All positive lanes contain a 588-bp fragment.

TABLE 4. Analysis of germline transmission in G_1 and G_2 transgenic chickens for the *lacZ* transgene[1].

Generation × Breeding strategy --- Transgenic lines and generation of offspring	PCR screening for the *lacZ* gene	Observed frequency	Expected Frequency	Probability[6]
$P1G_1$ male[2] × wild-type hens				
$P1G_2$	Transgenic[4]	27	31	0.3096
	Wild-type[5]	35	31	(Pr > Chi-square)
	Sample size	**62**	**62**	
$P2G_1$ male[3] × wild-type hens				
$P2G_2$	Transgenic	24	23	0.7681
	Wild-type	22	23	(Pr > Chi-square)
	Sample size	**46**	**46**	
$P1G_2$ males × $P1G_2$ females				
$P1G_3$	Transgenic	58	63.75	0.1498
	Wild-type	27	21.25	(Pr > Chi-square)
	Sample size	**85**	**85.00**	
$P2G_2$ males × $P2G_2$ females				
$P2G_3$	Transgenic	40	45.75	0.0891
	Wild-type	21	15.25	(Pr > Chi-square)
	Sample size	**61**	**61.00**	

[1] The presence of the *lacZ* gene was based on PCR (polymerase chain reaction) using DNA samples isolated from blood.
[2] P1 was referred to the first *lacZ*-positive G_1 male and used as a prefix for defining the transgenic line. P stands for "Precious".
[3] P2 was referred to the second *lacZ*-positive G_1 male and used as a prefix for defining the transgenic line. P stands for "Precious".
[4] Transgenic was referred to the chicken offspring which were determined to carry the *lacZ* in their genomic DNA.
[5] Wild-type was referred to the chicken offspring not carrying the *lacZ* gene in their genomic DNA.
[6] All chi-square analysis were based on 1 df (degree of freedom). Statements of statistical significance in all instances were based upon ($P < 0.05$).

Germline transmission in G_1 and G_2

The two blood *lacZ*-positive G_1 birds were males and grown to sexual maturity (Figure 9). Both G_1 males were also shown to carry the *lacZ* gene in their semen (Figure 9). This indicated that these two G_1 males are transgenic chickens in which

all of their somatic and germ cells contain the *lacZ* transgene in genomic DNA. The first *lacZ*-positive G_1 male was named Precious-1 and formed the transgenic line for later breeding program. The second *lacZ*-positive G_1 male was named Precious-2 and formed the second transgenic line for later breeding regimen. Precious-1 male was mated with wild-type White Leghorn hens and produced 62 offspring. Twenty-seven of the 62 offspring (45%) contained the *lacZ* gene in their blood, based upon PCR screening (Table 4). Precious-2 was mated with wild-type Leghorn hens and generated 46 progeny. Twenty-four of the 46 offspring (52%) were blood *lacZ*-positive chickens (Table 4). Chi-square analysis shows that the ratios of G_1 and G_2 progeny carrying the *lacZ* gene in their blood are consistent with the predicted Mendelian ratio of 50% for the heterozygous dominant allele ($P < 0.05$) in which only a single integration occurs in their genome (Table 4). For further analysis of a single integration site event in both transgenic chicken lines, G_3 progeny were generated from $P1G_2$ and $P2G_2$ generation with inter-mating regimen (Figure 1 and Table 4). $P1G_2$ transgenic males mated with $P1G_2$ transgenic females produced 85 offspring. Fifty-eight of the 85 $P1G_3$ progeny (68%) were *lacZ*-positive in their blood based upon PCR screening (Table 4). Similarly, 40 of 61 $P2G_3$ offspring (65%) were detected positive for the *lacZ* gene in their blood (Table 4). Chi-square analysis revealed that there was no significant difference between G_3 offspring carrying the *lacZ* gene and the expected 75% Mendelian ratio for the transgenic birds and 25% ratio for non-transgenic animals ($P < 0.05$, Table 4).

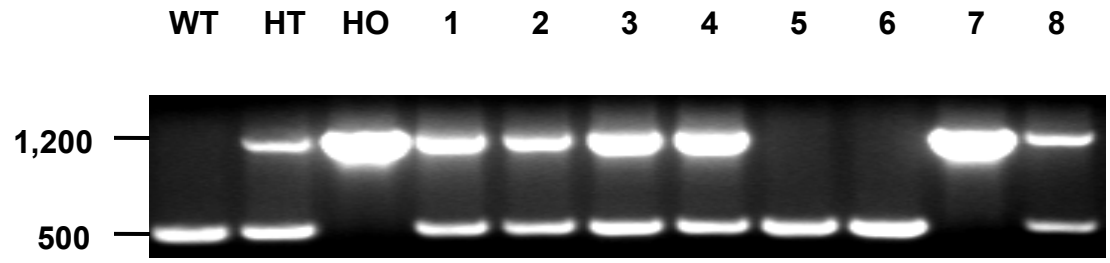


Figure 10. PCR genotyping. WT (wild-type) shows a single 500-bp fragment indicating a non-transgenic bird. HT (hemizygous) shows double bands of a 500-bp fragment and a 1,200-bp fragment indicating bird heterozygous for the *lacZ* gene. HO (homozygous) shows a single 1,200-bp fragment indicating a bird homozygous for the *lacZ* gene. Lanes 1, 2, 3, 4 and 8 are heterozygous for the *lacZ* gene. Lanes 5 and 6 are wild-type birds. Lane 7 is homozygous for the *lacZ* gene.

TABLE 5. Determination of germline transmission in G_3 transgenic chicken lines carrying the *lacZ* transgene as a heterozygous dominant allele.

Generation × Breeding strategy --- Transgenic lines and generation of offspring	Genotype	Observed frequency	Expected Frequency	Probability[5]
$P1G_3$ males[2] × $P1G_3$ females ---				
$P1G_4$	Homozygous	111	113	0.833 (Pr > Chi-square)
	Hemizygous	231	226	
	Wild-type[4]	110	113	
	Sample size	**452**	**452**	
$P2G_3$ males[3] × $P2G_3$ females ---				
$P1G_4$	Homozygous	14	19	0.1171 (Pr > Chi-square)
	Hemizygous	47	38	
	Wild-type	15	19	
	Sample size	**76**	**76**	

[1] The presence of the *lacZ* gene was based on PCR (polymerase chain reaction) using DNA samples isolated from blood.
[2] P1 was referred to the first *lacZ*-positive G_1 male and used as a prefix for defining the transgenic line. P stands for "Precious".
[3] P2 was referred to the second *lacZ*-positive G_1 male and used as a prefix for defining the transgenic line. P stands for "Precious".
[4] Wild-type was referred to the chicken offspring not carrying the *lacZ* gene in their genomic DNA.
[5] All chi-square analysis were based on 1 df (degree of freedom). Statements of statistical significance in all instances were based upon ($P < 0.05$).

Genotyping

Figure 10 illustrates a representative genotyping analysis in G_4 chicks based on the PCR protocol described by Mozdziak *et al*. (2006). $P1G_3$ and $P2G_3$ inter-mating produced G_4 progeny used for genotyping for the *lacZ* transgene. Inter-mating between $P1G_3$ transgenic birds generated a total of 452 offspring ($P1G_4$

chicks) (Table 5). One-hundred and one (25%) birds were homozygous, 231 chicks (51%) were hemizygous and 110 chicks were non-transgenic wild-type (24%). $P2G_3$ transgenic males mated with $P2G_3$ transgenic females produced 76 chicks. Similarly, 14 chicks (18%) were homozygous, 47 birds (62%) were hemizygous and 15 birds (20%) were wild-type. Chi-square analysis revealed that there was no significant difference ($P < 0.05$) between the genotypes of G_4 chicks and the expected Mendelian ratio of 1:2:1 (homozygous:hemizygous:wild-type) in both transgenic chicken lines (Table 5).

Expression of β-Galactosidase

Cultured myoblasts

Myoblasts isolated from the pectoralis thoracicus muscle of blood *lacZ*-positive 1-d-old chicks were cultured and stained with X-gal solution. Figure 6A illustrates that β-Galactosidase was expressed in the cultured myoblasts as seen in the blue nuclei of the *lacZ*-positive myoblasts. Beta-galactosidase was not expressed in cultured myoblasts isolated from the pectoralis thoracicus muscle of blood *lacZ*-negative 1-d-old chicks after stained with X-gal solution (Figure 11B).

Cell culture supernatant removed from myoblast cultures derived from β-galactosidase-positive chickens was placed on proliferating wild-type chicken myoblast cultures. After prolong culture, no β-galactosidase-positive cells were observed in the cultures derived from chickens that did not carry the *lacZ* gene.

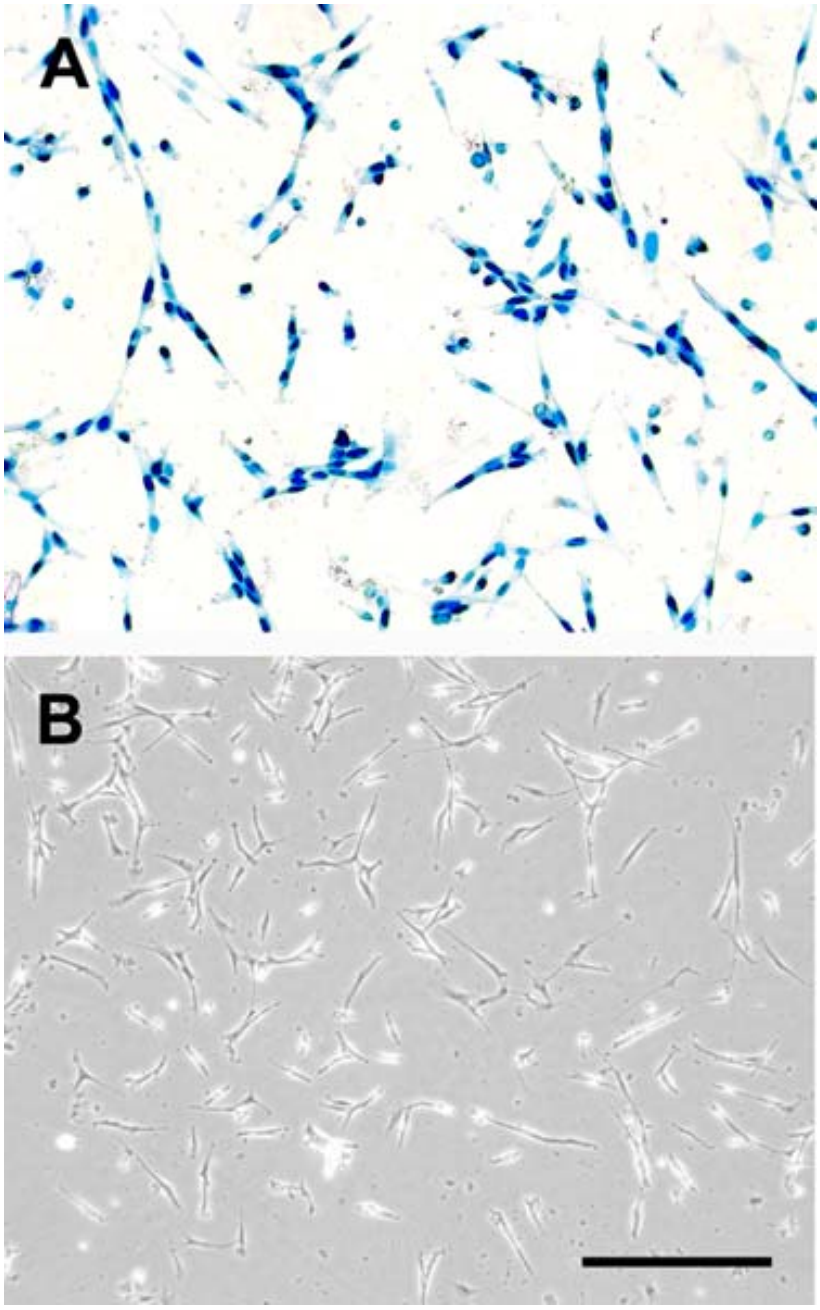


Figure 11. Myoblast cultures from 1-d-old G_2 chickens. (A) Brightfield photograph of primary myoblast cultures from *lacZ*-positive chickens. Cells with stained nuclei represent β-galactosidase-positive cells. (B) Phase-contrast photomicrograph of primary myoblast cultures from *lacZ*-negative chickens. Scale bar = 150 μm in B (applied to A, B)

Whole-mount embryos

β-galactosidase expression was evaluated in G_2 and G_3 embryos at various stages of development. Figure 12A illustrates the β-galactosidase expression can be observed in an entire stage-8 embryo. No β-galactosidase was expressed in a stage-8 wild-type embryo (Figure 12B). Similar β-galactosidase expression pattern can be revealed in stage-20 and stage-28 *lacZ*-positive embryos (Figure 13A, 13B). Stage-20 and stage-28 *lacZ*-negative control embryos did not express β-galactosidase (Figure 13A, 13B).

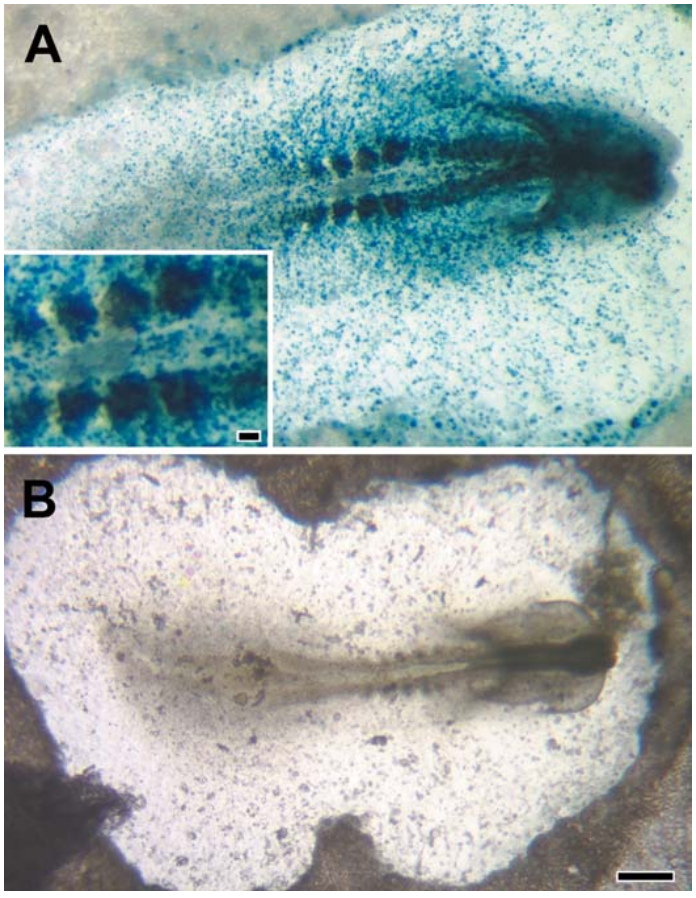


Figure 12. G_2 embryos (Stage 8: Hamburger and Hamilton, 1951) from (A) β-galactosidase-positive transgenic chickens and from (B) wild-type β-galactosidase-negative chickens. Inset in A shows staining in the somites of β-galactosidase-positive transgenic chickens. Scale bar = 50 μm in inset, 300 μm in B (applied to A, B)

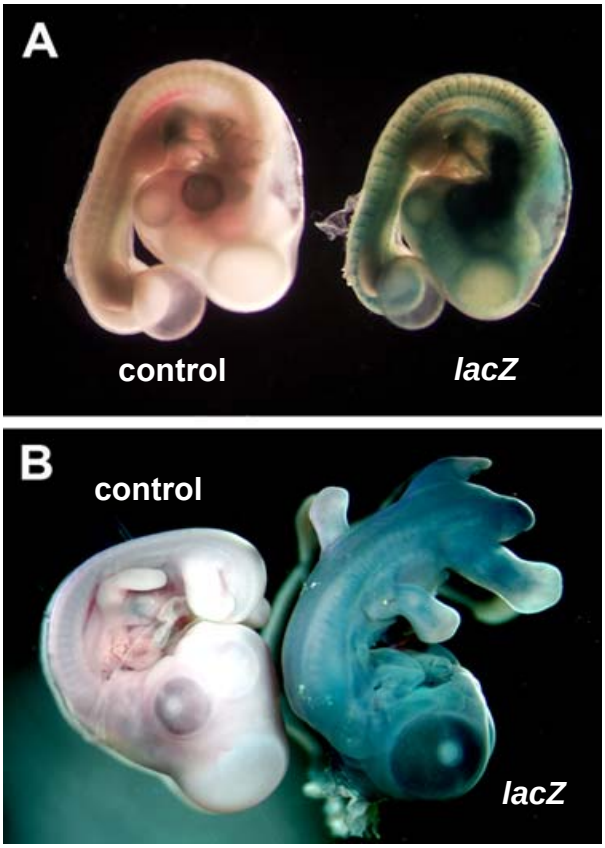


Figure 13. Whole mount of chick embryos stained with X-Gal. (A) Stage-20 G_3 embryos; control wild-type embryo, *lacZ* transgenic embryo. (B) Stage-28 G_3 embryos; control wild-type embryo, *lacZ* transgenic embryo.

Whole-mount tissues

β-galactosidase expression was observed in various tissues isolated from young and adult transgenic chickens for the *lacZ* gene. Various examined whole-mount tissues from blood *lacZ*-positive 16-d-old chicks including brain, gizzard, heart, intestine, muscle, and lung were observed to express β-galactosidase in nuclei (Figure 14, 15). However, the expression was not detected in all cells of tissue types based on X-gal staining. Highly expressed β-galactosidase can be seen in intestine, muscle and liver. No β-galactosidase was observed in tissues isolated from *lacZ*-negative 16-d-old chicks (Figure 14, 15). Intestine from non-transgenic chicks appeared blue after X-gal staining may be due to background staining of endogenous lactase activity (Figure 14D).

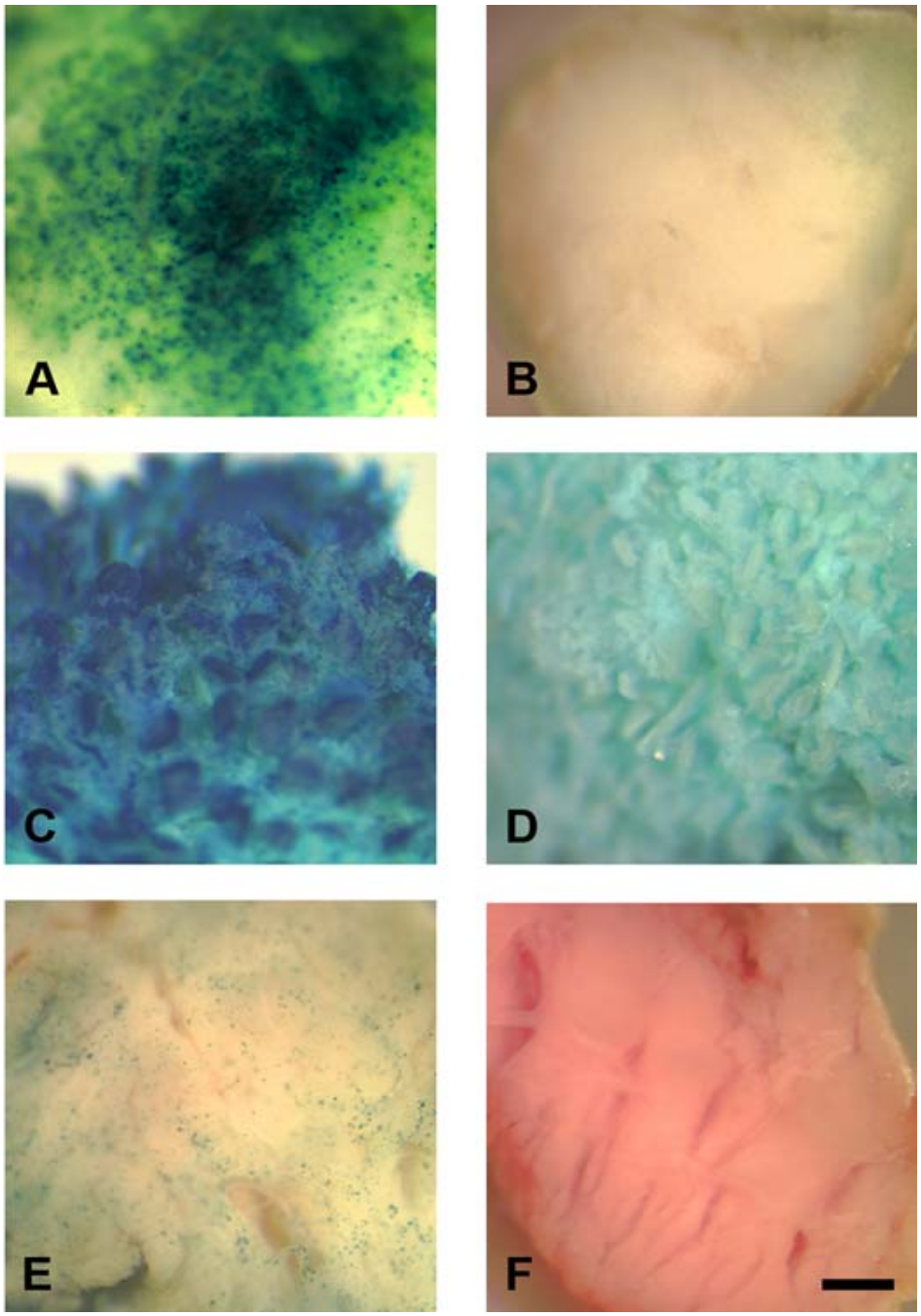


Figure 14. X-gal staining of whole-mount tissues of 16-d-old G3 chicks. (A) Brain tissue from a *lacZ*-positive chick. (B) Brain tissue from a *lacZ*-negative chick. (C) Small intestine from a *lacZ*-positive chick. (D) Small intestine from a *lacZ*-negative chick. (E) Heart tissue from a *lacZ*-positive chick. (F) Heart tissue from a *lacZ*-negative chick. Scale bar in F = 400 μm (applied to A, B, C, D, E)

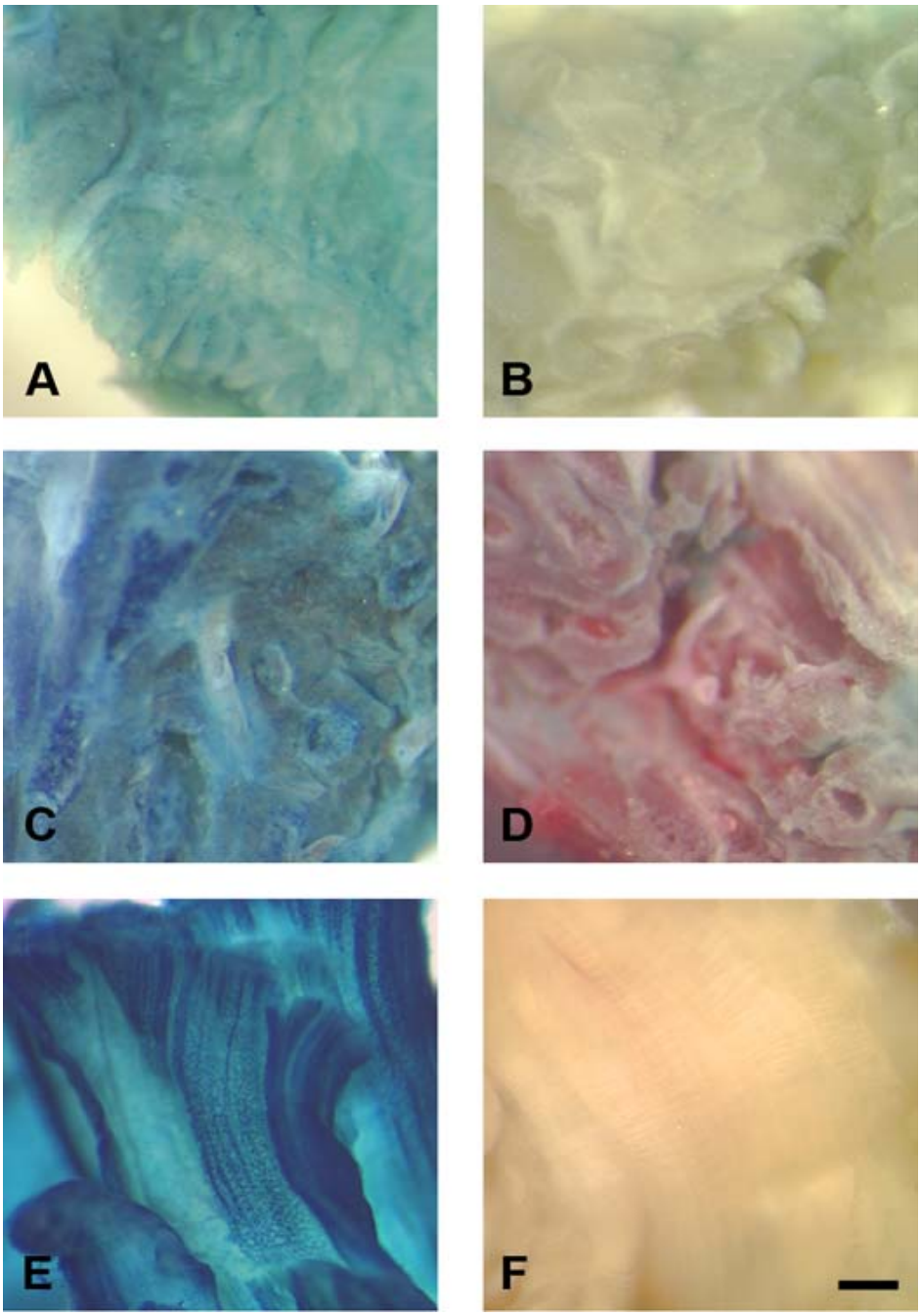


Figure 15. X-gal staining of whole-mount tissues of 16-d-old G3 chicks. (A) Gizzard from a *lacZ*-positive chick. (B) Gizzard from a *lacZ*-negative chick. (C) Lung tissue from a *lacZ*-positive chick. (D) Lung tissue from a *lacZ*-negative chick. (E) Muscle from a *lacZ*-positive chick. (F) Muscle from a *lacZ*-negative chick. Scale bar in F = 400 μm (applied to A, B, C, D, E)

Cryosections

In this study, β-galactosidase expression was evaluated from tissues isolated from 28-wk-old transgenic chickens, and was detected in all tissues examined including bursa, kidney, liver, muscle, spleen, and testes (Figure 16). Similarly observed in whole-mount tissues, expression was qualitatively higher for skeletal muscle and liver of the transgenic chickens compared with the other tissues (Figure 16 A, B).

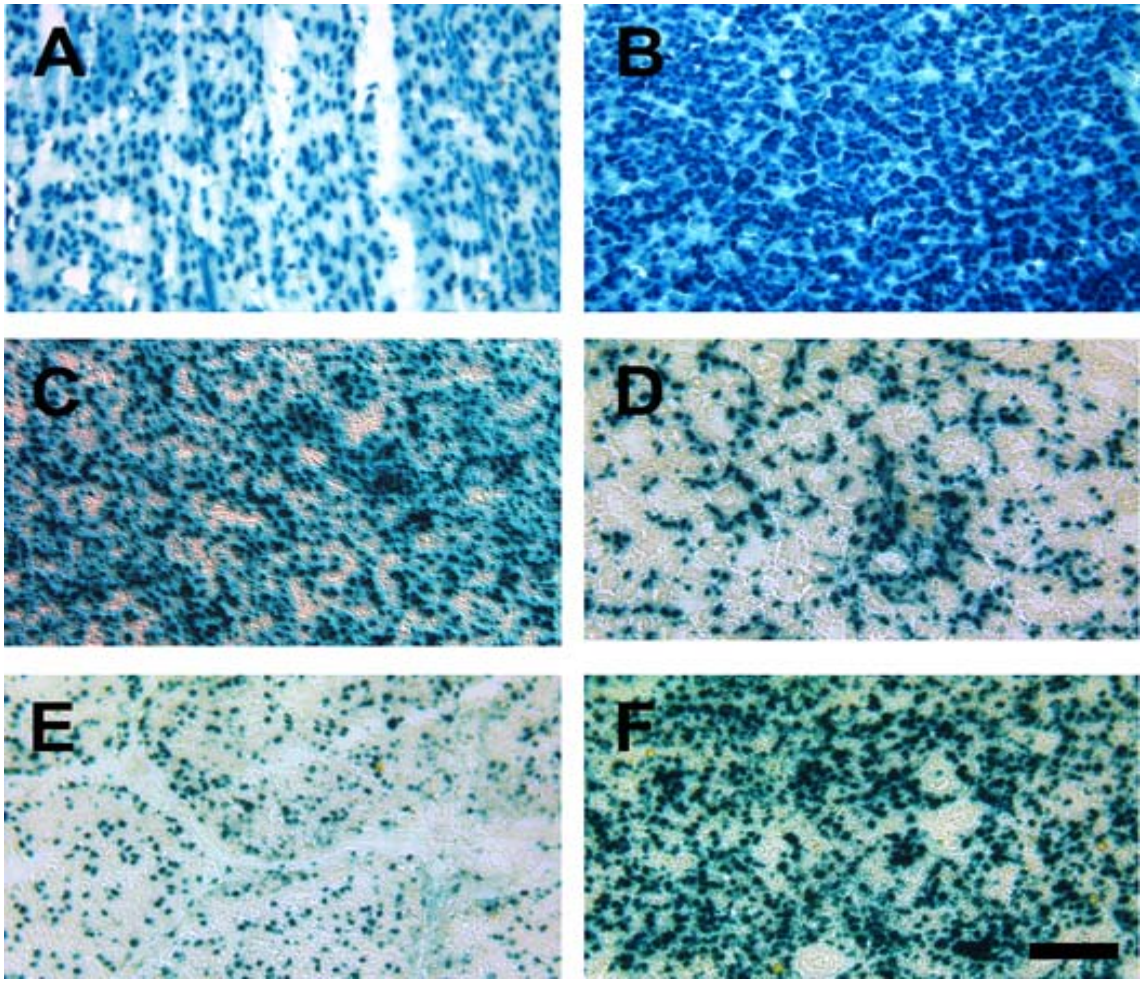


Figure 16. Cryosections from various tissues of adult transgenic G_3 chickens stained with X-Gal. (A) Muscle. (B) Liver. (C) Spleen. (D) Testes. (E) Bursa. (F) Kidney. Scale bar = 0.1 mm (applied to A, B, C, D, E, F)

DISCUSSION

This attempted to improve 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 this experiment was 30% which was approximately the same as previous 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 this 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 this 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 this study, no correlation between the presence of the *lacZ* gene detected in the blood (somatic cells) and the sperm or ova (germ cells) was observed. The findings are also in agreement with those previously reported (Thoraval *et al*., 1995;

McGrew *et al*., 2005). In the current experiment, two blood *lacZ*-positive hens were generated from the retroviral injection and together produced 191 G_1 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 *lacZ*-positive 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. In this study, 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 G_1 chicks. Notably, only one rooster was shown to transmit the *lacZ* gene to the two G_2 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 replication-defective 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 G_0 quail injected with Moloney murine leukemia virus (MoMLV) pseudotyped with VSV-G protein. Similarly, McGrew *et al*. (2004) generated G_0 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 this study, it is clear that the germline transmission rates of G_1, G_2, and G_3 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 G_0 sire. Recently, our research group (Mozdziak *et al*., 2006) studied the integration site and copy number of the *lacZ* provirus in both transgenic G_1 male founders and their G_2 offspring using Southern analysis. They found that these two G_1 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 cells containing the *lacZ* exhibit staining and the β-galactosidase is not transferred among cells. In the study, no helper viruses were produced indicating that the horizontal transmission is absent. 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. In the current study, it was shown that the expression of the *lacZ* gene

could be observed in all tissues from embryos and adult birds. The β-galactosidase activity can be detected in entire embryos from at least stages 8, 20, and 28 (Hamburger and Hamilton, 1951). G_3 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, the current study successfully produced transgenic birds as an alternative cell marker that could be used 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 transgenic chickens carrying the *lacZ* gene and expressing β-galactosidase have been generated and demonstrated to stably inherit and express the transgene at least to G_4. 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) develmentally neutral. This current study was the first report of generating transgenic birds containing the reporter *lacZ* gene ideally useful for long-term analysis of cell lineage studies.

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CHAPTER 3:

Determination of Apparent Lactose Digestibility from Transgenic Chicks Expressing Bacterial β-Galactosidase

ABSTRACT

Endogenous lactase activity in chickens is very low. Only trace amounts of lactase activity can be detected at brush border membranes. Utilization of dietary lactose in chickens is limited to bacterial fermentation. Transgenic chickens expressing β-galactosidase or bacterial lactase could potentially improve efficiency of lactose utilization. The objective of this study was to determine whether the transgenic birds have improved lactose digestibility when fed basal diets containing lactose. Lactase activity was found in all examined tissues of the digestive tract of the transgenic chickens, whereas lactase activity was not detected in non-transgenic, wild-type birds. It was found that the transgenic chickens exhibited greater lactose digestibility when fed 7.5% and 10% lactose in a diet compared to wild-type control birds. Overall, when fed 5 to 10% lactose in their diets, the transgenic chickens show lactose digestibility approximately 10% better compared to non-transgenic chickens. However, the greater lactose digestibility of the transgenic birds did not result in better growth performance. This could be due to the retroviral *lacZ* provirus inserting into neurotactin/fractalkine (CX3CL1) gene thereby compromising the physiology of the transgenic birds which could mask the beneficial effect on growth from greater lactose degradation. Nevertheless, this is the first report of using transgenesis technology to manipulate the chicken genome to utilize feed more efficiently.

Keywords: transgenic chickens, β-galactosidase, lactose digestibility, feed utilization

INTRODUCTION

Lactose is a major component of milk products. Many studies have considered the utilization of lactose by domestic birds in diets containing pure lactose or in milk by-products such as whey (Rutter *et al*., 1953; Fox and Briggs, 1959). Adding lactose or lactose-containing whey in poultry feed has many benefits for poultry production. Providing lactose in molting hens may enhance the resistance of *Salmonella enteritidis* colonization (Corrier *et al*., 1997). Deloach et al. (1990) reported that broiler chicks fed 5% whey effectively lowered *Salmonella typhimurium* numbers in the intestine. Supplementation of lactose at 2.5% improved growth performance in chickens (Gülşen *et al*., 2002). At most, chickens can tolerate 20% lactose in the feed. At higher levels, growth was impaired and diarrhea was pronounced (Rutter *et al*., 1953). The limitation of using lactose as a feed ingredient for poultry is due to inability of birds to secrete lactase and breakdown lactose to glucose and galactose (Sinddons, 1969; Siddon and Coats, 1972; Chotinsky *et al*., 2001). The availability of lactose as an energy source comes from the fermentation of lactose to lactic acid and volatile fatty acids by bacterial in the lower gut with most activity in ceaca (Siddons and Coats, 1972; Waldroup *et al*., 1992; Tellez *et al*., 1993; Hollister *et al*., 1994; Gülşen *et al*., 2002). In addition, the utilizable energy value for lactose is highly variable and depends upon the amount fed in test diets (Vohra, 1967).

In general, the use of whey in poultry diets is limited by its high lactose content (~80%). Improving the ability of poultry to utilize lactose would be of practical importance not only in increasing nutritional value of whey in feed

formulation but also alleviate the problem of its disposal as waste. Scott (1952) attempted to increase the whey in a basal diet by using whey sources varying in lactose. He found that this strategy considerably improved growth performance of the turkey poults. Transgenic chickens that express β-galactosidase activity in digestive systems could potentially utilize lactose as energy source. The objective of this study was to determine whether the transgenic birds exhibit improved lactose digestibility when fed with basal diets containing lactose.

MATERIALS AND METHODS

Bird husbandry and management

All animal procedures used in this study were approved by North Carolina State University Institutional Animal Care and Use Committee. Four hundred and fifty two day-old chicks used in the experiment were the offspring from hemizygous P1G3 x hemizygous P1G3 transgenic chickens from the NCSU Blue1 line. Each chick was neck-tagged and wing-banded. Then, combs were clipped and used for DNA extraction for genotype and sex determination. A group of 20 chicks were placed in Petersime battery brooder cages (70 x 96 x 25 cm) in a temperature-controlled room up to 10 d of age. The wire floor of the cage had a 1.3 x 1.3 cm mesh A basal diet was formulated to contain 2,880 kcal/kg of apparent metabolizable energy (ME) and 207 g/kg of crude protein (Table 1). The basal diet was formulated based on the requirement of immature Leghorn-type chickens as recommended by NRC (1994). The birds were given a basal diet with 10% dextrose (Table 1) and water *ad libitum* through outside food and water troughs. The birds

were maintained under 24 h of artificial light. At 10 days of age, all chicks were weighed and separated according to determined genotypes and sexes.

Experimental design

The experiment was designed as a randomized complete block that compared 8 treatments comprising of 4 dietary lactose concentrations (0%, 5%, 7.5% and 10% of lactose in a basal diet) (Table 1) and 2 genotypes (wild-type and hemizygous for the *lacZ* transgene) with 3 batteries as a block effect. Each of 3 batteries was randomly arranged within an environmentally controlled room. All 8 treatments were assigned randomly in a 12-caged battery with 2 tier levels. Each cage was 70 x 96 x 25 cm dimension with 1.3 x 1.3 wire floor. Four 10-d-old males and 4 10-d-old females were distributed in each cage with a mean body weight (BW): of 97.37 ± 0.15 g (means ± SEM, n = 24) and with an individual food trough, water trough and stainless steel excreta collection tray (Table 4, 5). Each cage formed an experimental unit.

The birds were given the experimental diets (Table 1) and water *ad libitum* throughout 14 d feeding period (10 to 24 d of age). Celite^{TM}, insoluble ash (AIA), was added in all experimental diets at 1% and used as an ingestible reference marker to determine apparent lactose digestibility. The birds were maintained under 24 h of light. Individual body weights (body weight gain: BWG) and cage feed consumption were recorded at 17 and 24 d of age, and periodic and cumulative feed/gain (FCR) were calculated. Mortality was recorded.

TABLE 1. Ingredient composition (%) of corn-soybean meal diets with dietary lactose used in chick assays

Ingredients	Lactose level (%) in experimental diets			
	0[1]	5	7.5	10
Corn	50.50	50.50	50.50	50.50
Soybean meal (48% CP)	33.30	33.30	33.30	33.30
Dextrose[2]	10.00	5.00	2.50	
Lactose[3]		5.00	7.50	10.00
Poultry fat	1.00	1.00	1.00	1.00
Dical (18.5% P)	1.80	1.80	1.80	1.80
Limestone	1.20	1.20	1.20	1.20
Salt	0.60	0.60	0.60	0.60
NCSU mineral premix[4]	0.20	0.20	0.20	0.20
NCSU vitamin premix[5]	0.05	0.05	0.05	0.05
Choline choride 60%	0.20	0.20	0.20	0.20
DL-methionine	0.17	0.17	0.17	0.17
Acid insoluble ash (Celite™)[6]	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00
Calculated nutrient composition				
ME (Kcal/kg)	2880.00			
Crude protein (%)	20.70			
Lysine (%)	1.10			
Methionine (%)	0.48			
Methionine + Cystine (%)	0.80			
Calcium (%)	1.10			
Phosphorus (%)	0.49			
Sodium (%)	0.24			

[1] A basal diet
[2] Fisher Scientific, Fairlawn, NJ 07410
[3] Edible Lactose (Fine Grind), Davisco Foods International, Inc., Eden Prairie, MN 55344
[4] Supplies the following per kilogram of feed: zinc 120 mg; manganese 120 mg; iron 80 mg; iodine 2.5 mg; cobalt 1 mg
[5] Supplies the following per kilogram of feed: vitamin A 6,600 IU; vitamin D3 200 ICU; vitamin E 33 IU; vitamin B12 µg; riboflavin 6.6 mg; niacin 55 mg; d-pantothenate 11 mg; menadione 2 mg; thiamine 20 mg; pyridoxine 4 mg; biotin 126 µg; folic acid 1.1 mg
[6] Celite™, Celite Corp., Lampar, CA 93436

Excreta collection

Excreta were collected at 12-h intervals during last 3 consecutive days of the feeding period (22 to 24 d of age) to evaluate water content, apparent lactose and insoluble ash. Clean (free of obvious feather and feed contaminants) representative fecal samples were collected twice a day from the stainless steel collection trays underneath each cage. The tray was washed and dried with paper towels before it was returned to the cage. Excreta collected from each experimental unit (or cage) and each collection period was kept in a 0.94-l polyethylene bag[1]. All 8 bags of excreta samples in each collection period were kept together in a 4-l polyethylene bag assigned for a particular block. Then the excreta samples were stored at -20°C for 24 h before storage at -70°C for later analysis. Before analysis, the frozen excreta were thawed at 37°C for 5 h. The defrosted excreta from the same cage and battery were pooled together in a 4-l polyethylene bag. The pooled excreta were hand-massaged well to mix the excreta samples evenly. An approximate 2-g portion of the excreta was taken and kept in a 50-ml conical tube stored at -20°C for lactose analysis.

[1] Ziplock™, Freezer Bags, Dow Brand L. P., Indianapolis, IN 46268

Water Content of Excreta

Excreta moisture determinations were conducted for each experimental unit. Two samples of about 7 to 10 g of pooled and mixed excreta of each experimental unit were weighed in disposable aluminum dishes. The water content of the excreta were determined by drying with a forced-air convection oven[2] to constant weight at 70°C. The mean of 2 replicates was used to calculate the excreta water content and dry matter of each individual experimental unit.

Acid Insoluble Ash of Excreta and Feed

For the determination of the digestibility of animal diets, many indicator methods are commonly used as an alternative to the total collection method which is laborious and requires special equipment. In this study, acid insoluble ash (AIA) was used to determine lactose digestibility in chicks as described by Vogtmann *et al*. (1975).

An approximate 200-g portion of the pooled and mixed excreta from each experimental unit was blended with 100 ml deionized water, resulting in slurry. To prepare feed samples, an approximate 100-g portion of each experimental diet was blended with 200 ml deionized water to the slurry. The slurry was then adjusted to pH ≤ 5.4 using 0.1N sulfuric acid, poured into aluminum pie pans and placed in a forced-air convection oven at 70°C until completely dry. Dried excreta or feed was then ground in a blender[3] to fine power and stored in polyethylene storage bags at -20°C until analysis.

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[2] Blue-M, Model # DC-326F, Serial # DC-509, Blue M, Atlanta, GA

[3] Waring® Commercial Blender Model 31BL92, Waring Commercial, New Hartford, CT 06057

The moisture content of dried excreta or feed were determined by drying an approximate 5-g portion of pre-dried excreta and feed at 105°C in a forced-air convection oven for overnight. Acid insoluble ash recovery was performed according to the method described by Vogmann *et al*. (1975). In this method, 5 g of finely ground dried excreta or 10 g of finely ground dried feed were boiled in 100 ml 4N HCl for 30 min resulting in a slurry. The slurry was then filtered through ashless filter paper and washed with deionized water. The washed residue was free of acid. The residue and the filter were together put into a pre-ashed porcelain crucible and ashed directly at 600°C for 15 h. The determination of acid insoluble ash of each experimental unit excreta or experimental diet was done in duplicate. The acid insoluble ash was expressed per gram of dry matter.

Lactose in Excreta

Lactose was extracted from excreta as follows. Distilled water was added to the 50-ml conical tube containing the 2-g portion of the frozen excreta to a total of 40 ml. The excreta and water were incubated for 15 min at approximate 70°C and then vortexed periodically. After cooling to 20-25°C, the tube was centrifuged at 1000 × g for 5 min. After centrifugation, the supernatant was aliquoted into a fresh 50-ml conical tube and stored at 4°C for lactose assay. Lactose extracted in this near-boiling distilled water was measured enzymatically. In the lactose assay, lactose is hydrolyzed to glucose and beta-galactose in the presence of beta-galactosidase and water. Beta-galactose is then oxidized by nicotinamide-adenine dinucleotide to galactonic acid in the presence of beta-galactose dehydrogenase. The amount of reduced nicotinamide-adenine dinucleotide formed is stoichiometric with the amount

of lactose and was measured in a microplate reader[4] at 365 nm. Using a Lactose/D-galactose UV-method kit[5], the reagents, solutions and samples were scaled down to 10 times less than normally assayed to perform 320 tests per kit. A total of 12 assays were performed in a 96-well plate[6]. In each lactose assay for an individual 96-well plate, an internal standard was constructed from spiking pure lactose dissolved in distilled water into fecal supernatant from a wild-type bird to 5 different concentrations: 0.25, 0.5, 1.0, 2.0, and 4.0 g/l. Lactose assays were performed in duplicate for the standard solutions and in triplicate for sample solutions. Each assay contained samples from both genotypes at a given lactose level and a given block and control lactose samples with a known concentration (6.0 g/l) for determination of inter- and between-assay precision (see Table 1 in Appendix A for raw data of control lactose samples). The measurements were stopped when no increase in readings were observed. The constructed standard curves of all 12 lactose assays showed linearity with a coorelation coefficient of 0.9989 (see Figure 1 in Appendix A). The inter-assay coefficient of varience (CV) was 4.26% and the between-assay CV was 5.82%. Lactose samples extracted from the excreta were determined by interpolating to the generated linear equation in each individual microplate assay. The lactose amounts were based on a gram of dry matter.

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[4] Spectamax 250, Molecular Devices Corp., Sunnyvale, CA 94089

[5] Lactose/D-Galactose UV-method kit, R-Biopharm AG, Darmstadt, Germany

[6] 96-Well Flat Bottom Bacti Plate, Nalge Nunc Intn., Rochester, NY 14625

The lactose digestibility was calculated using the following equations (Hill and Anderson, 1958):

$Lactose_{diet}$ (g/g diet) = gram lactose per gram of dry matter

$Lactose_{excreta}$ (g/g diet) = gram lactose in excreta per gram diet dry matter

= gram lactose per gram excreta × (g AIA per gram diet/ g AIA per gram excreta)

Lactose retained = $Lactose_{diet}$ - $Lactose_{excreta}$

$$\text{Lactose digestibility (\%)} = \frac{Lactose_{diet} - Lactose_{excreta}}{Lactose_{diet}} \times 100$$

Statistical Analysis

The statistical model used for the analysis of dependent variables (BWG, FCR, water content in excreta and lactose digestibility) in this randomized complete block design was

$$Y_{ijk} = \mu + G_i + L_j + LG_{ij} + B_k + e_{ijk}$$

where Y_{ijk} is the individual observation per cage (or experimental unit); μ is the experimental mean; G_i is the genotype effect; L_j is the lactose effect; LG_{ij} is the interaction effect between genotype and lactose; B_k is the block effect; and e_{ijk} is the random error.

Data were subjected to statistical analysis using the General Linear Model (GLM) procedure of SAS software (SAS Institute, 1999), and the treatment means were compared using orthogonal contrasts.

Chi-square analysis was used to determine whether the germline transmission rate of inter-mating between hemizygous birds for the *lacZ* transgene

differs from the expected Mendelian ratio (1:2:1). Statements of statistical significance in all instances were based upon ($P \leq 0.05$).

RESULTS

Genotyping

A total of 452 day-old P1G4 chicks were produced from inter-mating between hemizygous P1G3 x hemizygous P1G3 parents. One hundred and 11 chicks (24.6%) were homozygous for the *lacZ* transgene, 110 (24.3%) were wild-type non-transgenic, and 231 (51.1%) were hemizygous for the *lacZ* transgene. The observed frequency of genotypes was consistent with the expected Mendelian ratio of 1:2:1 (Chi-square: $P > 0.9$) (Table 2). The proportions of genotypes separated by sex also showed the consistency with the expected Mendelian ratio (Chi-square: $P > 0.6$ and $P > 0.5$ for male and female respectively) (Table 2). In addition, this indicated that the integration of the *lacZ* trasgene in 2 alleles of homozygous embryos did not result in lethality.

TABLE 2. Determination of frequency of chicks for the *lacZ* transgene as expected Mendelian ratio (1:2:1; homozygous:hemizygous:wild-type) as separated by sex using Chi-square

Sex	Genotype	Observed frequency	Expected frequency	DF	Probability
Male	Heterozygous	101	104	2	0.6213 (Pr > Chi-square)
	Homozygous	58	52		
	Wild-type	49	52		
	Sample size	**208**	**208**		
Female	Heterozygous	130	122	2	0.4553 (Pr > Chi-square)
	Homozygous	53	61		
	Wild-type	61	61		
	Sample size	**244**	**244**		
Mixed	Heterozygous	231	226	2	0.8933 (Pr > Chi-square)
	Homozygous	111	113		
	Wild-type	110	113		
	Sample size	**452**	**452**		

TABLE 3. Determination of effects of dietary lactose on mortality rates in homozygous chicks for the *lacZ* transgene using Chi-square

Sex	Lactose (%)	Observed frequency	Expected frequency	DF	Probability
Lactose Effect					
Mixed	0	5	5	3	0.9402 (Pr > Chi-square)
	5	5	5		
	7.5	6	5		
	10	4	5		
	Sample size	**20**	**20**		

Mortality

During the feeding period from the age of d 10 to 24, a total of 20 (20.8%: 20 deaths out of 96 homozygous chicks) homozygous birds was reported (Table 3). Initially, a few birds displayed a sudden inability to stand but with full consciousness at the age of d 15 and then assumed normal activities. The symptoms periodically occurred for 2 to 3 d before the birds completely were unable to stand. After 6 to 7 d since the beginning of the incidence, the birds assumingly died from social competition. The majority of the homozygous chicks showed symtoms at the age of d 17. The level of dietary lactose included in the experimental diets was not a factor of the mortality in homozygous birds (Chi-square: $P > 0.9$). Due to the health problems of homozygous chicks for the *lacZ* transgene, they were not included in this study. All heterozygous chicks in the experiment were designated as transgenic birds (TG) and all non-transgenic chicks as wild-type birds (WT).

Growth Performance

The initial mean body weights at the age of d 10 of the birds fed basal diet (0% lactose) were not different among the experimental groups (Table 4, 5). The effects of supplementary lactose and genotype upon growth and feed efficiency of chicks may be seen in Table 4 and 5. During the first week (from 10-17 d of age), when lactose was fed at a level of 10% in the basal diet, the body weight gains of chicks averaged 4 to 6 g significantly smaller than that of chicks fed with 5%-7.5% lactose and unsupplemented control chicks, respectively ($P < 0.05$). The *lacZ* transgene had no effect upon growth of chicks fed with all experimental diets. However, during the second week of the feeding period (from the age of d 18 to 24),

the effects of graded lactose at 7.5% and 10% significantly lowered the average body weight gains of chicks by 7 to 10 g ($P < 0.05$). Chicks grown with 5% lactose diet showed an approximately 5 g reduction in the average body weight gain when compared to the control birds, but no statistical difference was found between the 2 groups. In addition during this period, the transgenic birds exhibited poorer growth than the wild-type control birds ($P < 0.05$). Over all in the entire experimental period (10-24 d of age), the effect of dietary lactose and genotype were observed similar to the response in the second feeding period ($P < 0.05$). The body weight gains decreased linearly with higher levels of lactose supplemented ($P < 0.05$). The transgenic birds were significantly smaller than the wild-type chicks ($P < 0.05$). The significant effect of the genotype on depressed growth was observed in birds fed not only with 10% supplemented lactose but also a basal diet containing no lactose ($P < 0.05$).

Feed conversion ratio measures the efficiency of utilization of feed intake nutrients (g) for body weight gain (g). In the first week of feeding period (10-17 d of age), the *lacZ* transgene and lactose levels did not affect feed efficiency. However, in the second and overall period of the feeding trial, the birds utilized feed poorer with 10% lactose in the diet ($P < 0.05$). At each level of lactose inclusions, both transgenic and non-transgenic birds had comparable FCR ratios except for the transgenic chicks exhibited lower efficiency of feed utilization than the control birds when fed with the basal diet containing no lactose in the second week of feeding period ($P < 0.05$).

TABLE 4. Effect of dietary lactose on body weight gain (BWG) of transgenic (TG) and wild-type (WT) chickens (10 to 24 d)[1]

Lactose (%)	Genotype	Reps	BW (d 10) (g/bird)	BWG (g/bird) 10-17 d	BWG (g/bird) 18-24 d	BWG (g/bird) 10-24 d
Genotype effect by lactose						
0	WT	3	98.30 ± 0.51	84.71 ± 0.74	99.91 ± 1.10	185.15 ± 0.97
0	TG	3	97.13 ± 0.07	84.67 ± 1.61	90.75 ± 1.44	175.42 ± 2.64
5	WT	3	97.25 ± 0.50	82.38 ± 2.97	93.21 ± 2.67	175.59 ± 4.69
5	TG	3	97.13 ± 0.14	82.59 ± 0.29	88.46 ± 1.63	171.04 ± 1.57
7.5	WT	3	97.54 ± 0.61	82.59 ± 1.17	92.42 ± 1.24	175.00 ± 2.10
7.5	TG	3	96.92 ± 0.15	81.63 ± 1.46	85.13 ± 0.83	166.75 ± 2.29
10	WT	3	97.71 ± 0.56	80.04 ± 1.06	92.59 ± 2.20	172.63 ± 1.33
10	TG	3	97.00 ± 0.26	76.96 ± 2.18	78.59 ± 5.12	155.54 ± 3.58
Main effect						
Genotype						
Wild-type		12		82.43 ± 0.89	94.53 ± 1.25[a]	177.09 ± 1.85[a]
Transgenic		12		81.46 ± 1.08	85.73 ± 1.83[b]	167.19 ± 2.49[b]
Lactose (%)						
0		6		84.69 ± 0.79[a]	95.33 ± 2.20[a]	180.29 ± 2.51[a]
5		6		82.48 ± 1.34[a]	90.84 ± 1.76[ab]	173.32 ± 2.44[b]
7.5		6		82.11 ± 0.86[a]	88.77 ± 1.76[b]	170.88 ± 2.31[b]
10		6		78.50 ± 1.29[b]	85.59 ± 4.00[b]	164.09 ± 4.19[c]
ANOVA						
R^2				0.55	0.76	0.83
		df		Probabilities		
Genotype		1		0.4262	0.0002	0.0001
Lactose		3		0.0180	0.0127	0.0003
Genotype × lactose		3		0.7550	0.3485	0.1796
Block		2		0.5272	0.7989	0.5189
Error		14				

[abc] Means within the same columns with no common superscript differ significantly ($p < 0.05$)

[1] Means ± SEM

TABLE 5. Effect of dietary lactose on feed conversion ratio (FCR) of transgenic (TG) and wild-type (WT) chickens (10 to 24 d)[1]

Lactose (%)	Genotype	Reps	BW (d 10) (g/bird)	FCR		
				10-17 d	18-24 d	10-24 d
Genotype effect by lactose						
0	WT	3	98.30 ± 0.51	2.15 ± 0.06	1.90 ± 0.07	2.02 ± 0.06
0	TG	3	97.13 ± 0.07	2.14 ± 0.14	2.29 ± 0.06	2.22 ± 0.09
5	WT	3	97.25 ± 0.50	2.16 ± 0.07	2.10 ± 0.04	2.13 ± 0.04
5	TG	3	97.13 ± 0.14	2.13 ± 0.04	2.18 ± 0.03	2.16 ± 0.03
7.5	WT	3	97.54 ± 0.61	2.19 ± 0.01	2.26 ± 0.08	2.22 ± 0.04
7.5	TG	3	96.92 ± 0.15	2.21 ± 0.03	2.31 ± 0.04	2.26 ± 0.04
10	WT	3	97.71 ± 0.56	2.24 ± 0.07	2.49 ± 0.05	2.37 ± 0.04
10	TG	3	97.00 ± 0.26	2.28 ± 0.09	2.60 ± 0.28	2.43 ± 0.14
Main effect						
Genotype						
Wild-type		12		2.18 ± 0.03	2.19 ± 0.07	2.19 ± 0.04
Transgenic		12		2.19 ± 0.04	2.34 ± 0.08	2.27 ± 0.05
Lactose (%)						
0		6		2.15 ± 0.07	2.10 ± 0.09[a]	2.12 ± 0.07[a]
5		6		2.15 ± 0.04	2.14 ± 0.03[a]	2.15 ± 0.02[a]
7.5		6		2.20 ± 0.02	2.28 ± 0.04[a]	2.24 ± 0.03[a]
10		6		2.26 ± 0.05	2.54 ± 0.13[b]	2.40 ± 0.06[b]
ANOVA						
R^2				0.29	0.67	0.68
		df		Probabilities		
Genotype		1		0.8989	0.0723	0.0990
Lactose		3		0.3697	0.0061	0.0035
Genotype × lactose		3		0.9652	0.4364	0.5472
Block		2		0.3863	0.5091	0.2602
Error		14				

[ab] Means within the same columns with no common superscript differ significantly ($p < 0.05$)

[1] Means ± SEM

Water Content of Excreta

Supplementation of 5, 7.5, and 10% lactose to the basal diet resulted in a linear increase in moisture content in the fecal excreta of the chicks by 1.2, 2.4, and 3.8%, respectively ($P < 0.05$). At diets containing 5 and 7.5% lactose, transgenic chicks showed a 2% lower level of excreta water contents than the control with a significant difference at 7.5% lactose ($P < 0.05$). In overall, the fecal excretion of transgenic chicks were lower in moisture content ($P < 0.05$)

Lactose Digestibility

There was suppression in lactose digestibility in a linear response as chicks fed with higher lactose included in the basal diet ($P < 0.05$). At 5% lactose level, transgenic chicks had lower digestibility of lactose compared to wild-type birds. However, the responses were reversed when chicks were fed with higher levels of lactose. Especially at the highest amounts of lactose (10%) in the experimental diet, transgenic birds showed greater ability to digest more lactose.

TABLE 6. Effect of dietary lactose on water content in excreta of transgenic (TG) and wild-type (WT) chickens (22 to 24 d)[1]

Lactose (%)	Genotype	Reps	Water content (%)
Genotype effect by lactose			
0	WT	3	72.92 ± 0.32
0	TG	3	72.23 ± 0.84
5	WT	3	74.78 ± 0.42
5	TG	3	72.92 ± 1.19
7.5	WT	3	75.95 ± 0.24
7.5	TG	3	74.06 ± 0.30
10	WT	3	76.64 ± 0.73
10	TG	3	76.08 ± 0.74
Main effect			
Genotype			
Wild-type		12	75.07 ± 0.47[a]
Transgenic		12	73.84 ± 0.56[b]
Lactose (%)			
0		6	72.61 ± 0.42[a]
5		6	73.85 ± 0.70[ab]
7.5		6	75.01 ± 0.46[b]
10		6	76.36 ± 0.48[c]
ANOVA			
R^2			0.78
		df	---------- Probabilities ----------
Genotype		1	0.0134
Lactose		3	0.0002
Genotype × lactose		3	0.5602
Block		2	0.1212
Error		14	

[ab] Means within the same columns with no common superscript differ significantly ($p < 0.05$)
[1] Means ± SEM

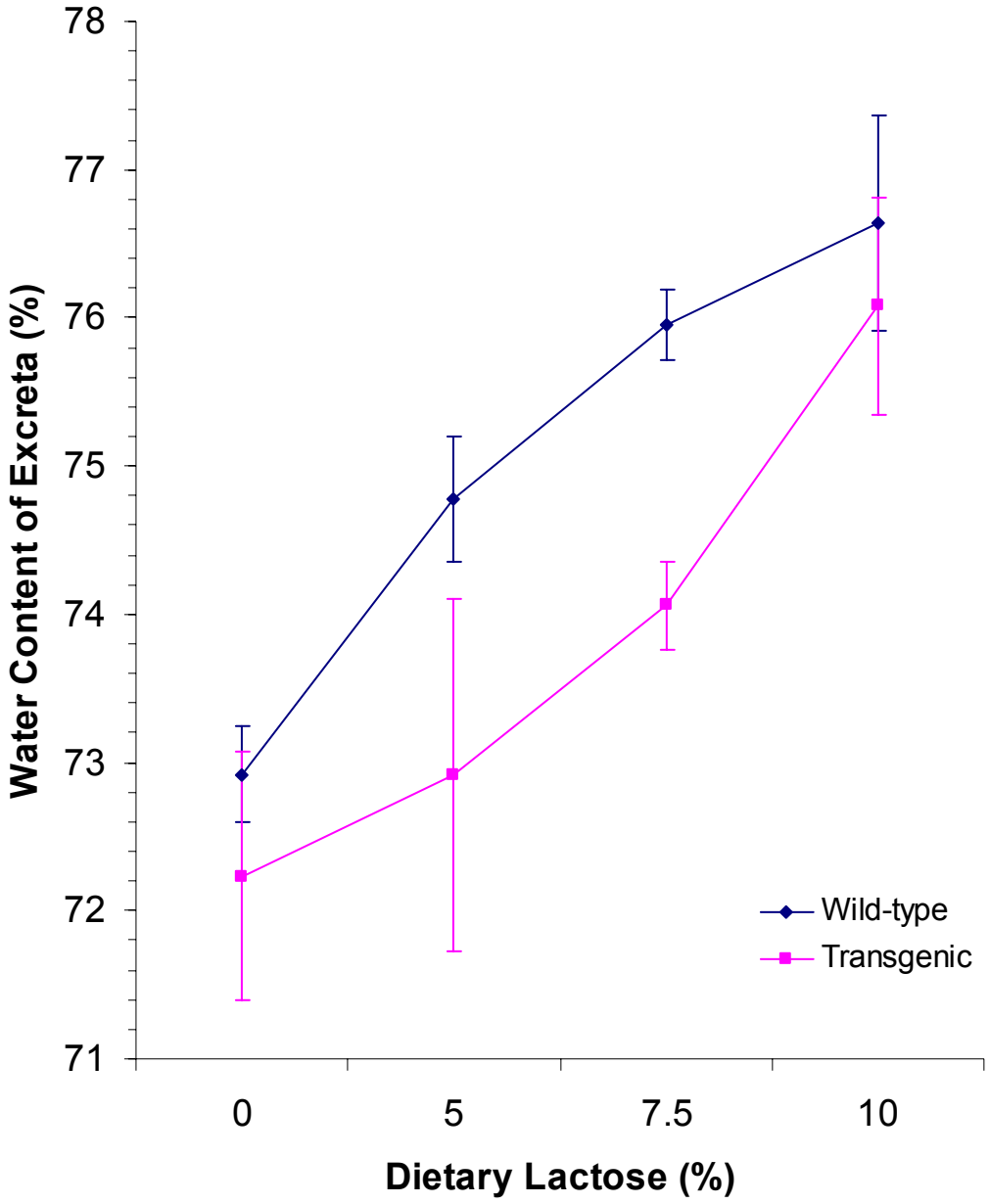


Figure 1. Water content of excreta (%) of transgenic and wild-type chicks fed dietary lactose (error bar: SEM)

TABLE 7. Effect of dietary lactose on lactose digestibility of transgenic (TG) and wild-type (WT) chickens (22 to 24 d)[1]

Lactose (%)	Genotype	Reps	Lactose digestibility (%)
Genotype effect by lactose			
5	WT	3	79.81 ± 3.03
5	TG	3	73.88 ± 2.57
7.5	WT	3	70.76 ± 3.92
7.5	TG	3	73.12 ± 4.15
10	WT	3	59.37 ± 1.11
10	TG	3	64.10 ± 0.37
Main effect			
Genotype			
Wild-type		9	69.98 ± 3.30
Transgenic		9	70.36 ± 2.11
Lactose (%)			
5		6	76.85 ± 2.22[a]
7.5		6	71.94 ± 2.61[a]
10		6	61.74 ± 1.18[b]
ANOVA			
R^2			0.78
		df	---------- Probabilities ----------
Genotype		1	0.8723
Lactose		2	0.0011
Genotype × lactose		2	0.1971
Block		2	0.3693
Error		10	

[ab] Means within the same columns with no common superscript differ significantly ($p < 0.05$)
[1] Means ± SEM

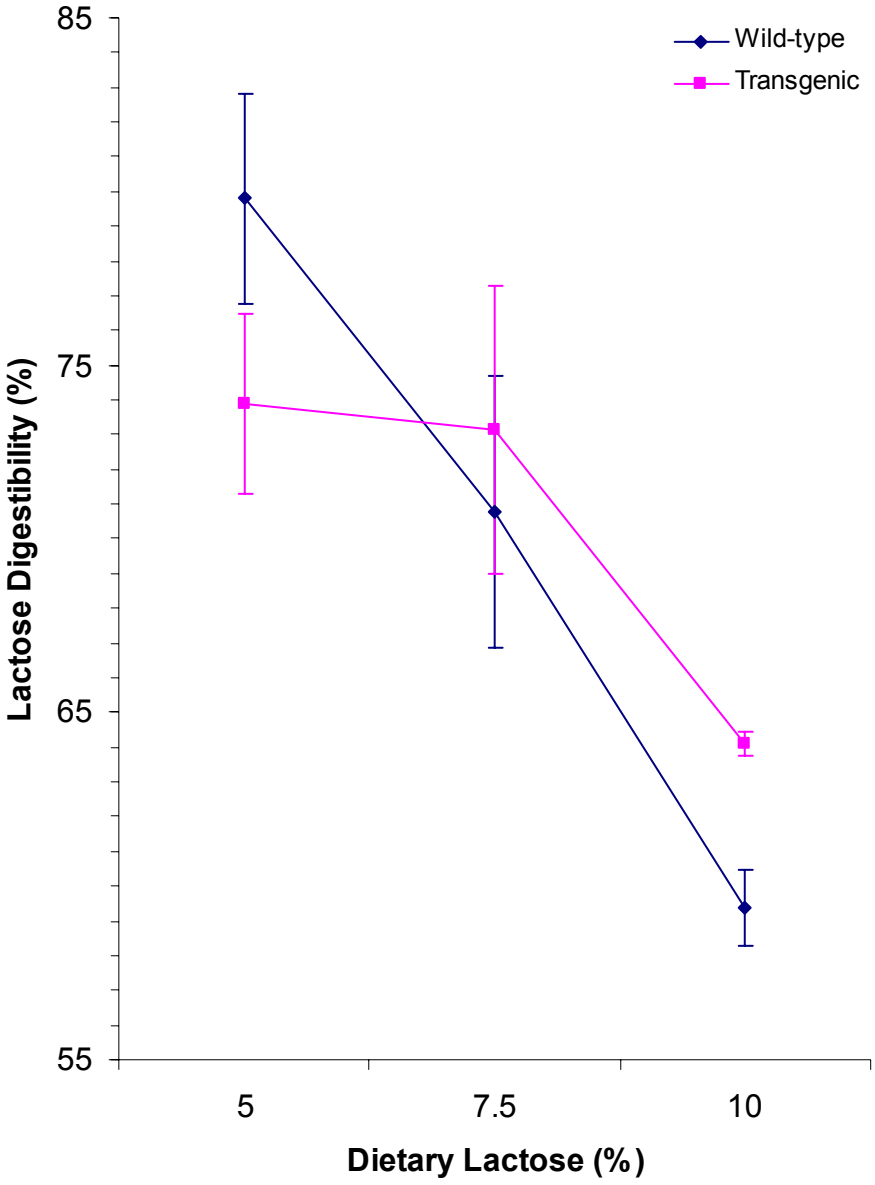


Figure 2. Lactose digestibility (%) of transgenic and wild-type chicks fed 3 levels of dietary lactose (error bar: SEM)

DISCUSSION

The growth performance of the birds fed diets supplemented with lactose was poorer than those fed with a basal diet containing no lactose. The effect was observed even with the lowest level of lactose (5%) and was more evident when the birds were grown on 10% (Table 4). These results are in contrast to those of Rutter *et al.* (1953), who reported that diets containing lactose up to 20% did not suppress the growth of the chicks, and of Fox and Briggs (1959), who reported that inclusion of 20% lactose in a purified ration promoted the growth of the chicks at 4 wk of age. However, Waldroup *et al.* (1992) showed that supplementation of 5% and 7.5% lactose in the diet of broilers had poorer growth performance. Similarly, Scott (1952) reported that lactose at 3.25% in the diet of turkeys was severely detrimental to growth.

Feed utilization was also impaired when the chicks were fed lactose. These results were consistent with those Waldroup *et al.* (1992), who reported that inclusion of graded lactose levels in 2.5% increments up to 7.5% showed lower feed efficiency in a linear manner. Sinddons and Coates (1972) and Chotinsky *et al*. (2001) reported that small intestine homogenates of chicks contained trace amounts of lactase activity. Furthermore, low lactase activity was found in purified brush border membrane (Kedinger *et al*., 1981). Brot-Laroche and Alvarado (1984) did not find lactase in enterocytes isolated from 15 d-old chick intestine. Brot-Laroche and Alvarado (1984) also found that the chicks limited lactose uptake through simple diffusion. Siddons and Coates (1972) reported that lactase activity was not detected

in intestinal contents indicating that the existing endogenous lactase is not secreted into the intestinal lumen. Thus lactose can be considered as indigestible soluble polysaccharide in chick small intestine. Van der Klis *et al*. (1993a) studied the effects of an indigestible soluble polysaccharide such as carboxymethylcellulose (CMC) on broiler performance. They found that the inclusion of CMC at 5% and 10% in the semi-synthetic diet showed a change in the viscosity in the intestinal lumen of the chickens and affected the absorption of some nutrients resulting in poorer growth of the birds (van der Klis *et al*., 1993b; Smits *et al*., 1998). Likewise, ingested lactose might decrease digestive efficiency and result in the adverse effect on growth of the chicks as seen in the findings.

The transgenic birds for the *lacZ* gene expressed β-galactosidase or lactase in homogenates prepared from the small intestine approximately 20 times higher than the wild-type control birds (Mozdziak *et al.*, 2003), but grew poorly when fed with supplemented lactose at any levels (Table 4). This could result from toxicity of galactose liberated from the exogenous lactase activity. Assuming that the transgenic birds can hydrolyze all the portions of digested lactose based on lactose digestibility data (Table 7), the generated galactose equivalent to 1.9, 2.7, and 3.1% in the basal diet when the chicks were fed with 5, 7.5, and 10% lactose, respectively. Douglas *et al*. (2003) found that 1-d-old male broiler chicks fed with basal diets containing galactose at 2 and 4% improved weight gains and feed utilization at the age of 21 d. Rutter *et al*. (1953) reported that the chicks can tolerate galactose up to 10% in the feed ration. Moreover, the transgenic birds appeared normal and showed no signs of galactose toxicity that have been described for the chick, such

as curled toes or convulsions (Dam, 1944; Rutter *et al*., 1953). Hence, the equivalent galactose levels could not be the factor affecting the poor growth performance in this study.

The negative effect on growth of the transgenic birds evidently began in the second period of lactose feeding which is coincident with the occurrence of constitutional symptoms and death in homozygous birds. Recently, Mozdziak *et al*. (2006) found that the transgenic chickens had the retroviral provirus carrying the *lacZ* gene integrated into the neurotactin/fractalkine (CX3CL1) gene. They reported that neurotactin RNA transcripts were amplified by reverse transcription/PCR (RT-PCR) in the brain from heterozygous and wild-type chicks but were absent from the homozygous brain tissue. Neurotactin has been shown to be involved in inflammatory processes in the mouse brain (Soriano *et al*., 2002) and induces antitumor immunity through chemoattraction and activation of T-cells and dendritic cells of murine bone marrow (Guo *et al*., 2003). The expression and function of this gene has not been studied in chicken. From observations in the current study, the chicks seemed susceptible to the lack of neurotactin at age 15-17 d according to the symptoms causing in the mortality of homozygous chicks and negative growth responses in hemizygous birds. Even though heterozygous birds expressed the neurotactin, the physiological amounts of the neurotactin could be compromised to some extent and could be a factor affecting the poorer growth in the transgenic chicks when compared to the wild-type siblings no matter how much lactose present in the diets.

The excreta water contents increased as the chicks ingested more lactose. Rutter *et al*. (1953) reported diarrhea was more pronounced with higher lactose in the feed. Fox and Briggs (1959) also found that when chicks fed 20% lactose the intestinal and cecal contents were more fluid than those of the control birds without lactose. Douglas *et al.* (2003) showed that the excreta appeared significantly watery and loose when the broiler chicks fed with lactose higher than 2%. Water excretions were proportional to the lactose levels (3 and 6%) in the diets for male broiler chicks (Carré *et al*., 1995). As mentioned previously, lactose has similar properties as soluble indigestible molecules such as carboxymethylcellulose (CMC). Van der Klis *et al.* (1993) reported that the viscosity in the supernatant of the chyme linearly increased in all intestinal segments as CMC added in the chick diets. The increased viscosity of the intestinal contents caused the reduction of net sodium absorption from the intestinal lumen in the birds which consumed CMC-containing diets. Sodium molecules are important to the mechanism of water transport in chicken. The rate of water absorption through the intestinal mucosa is decreased as the rate of sodium transport decreased resulting in the increase in moisture contents of the chyme. Thus, the birds drink more water in order to satisfy their water requirement. Chicks consumed indigestible polysaccharides such as β-D-glucans, pentosans, and galactomannan, which are generally found in a poultry diet containing guar meal, rye, wheat, and barley, had sticky or wet droppings (Gohl *et al*., 1978; Carré *et al*., 1995, Lee *et al*., 2003). These polysaccharides have also been associated with high viscosity of intestinal contents. Adding exogenous enzymes such as glucanase, pentotanase and mannanase to these highly viscous diets can alleviate the chyme

viscosity and in turn reduce the occurrence of wet droppings in the chicks (Pettersson and Åman, 1989; Lee *et al*., 2003; Taylor and Jones, 2004).

It was found that the water contents of excreta from the transgenic chicks expressing β-galactosidase fed lactose-supplemented diets were lower than those from control chicks (Table 6, Figure 1). These findings suggest that the transgenic birds were able to hydrolyze lactose by the actions of β-galactosidase which reduced the amounts of lactose in the gastrointestinal tracts, which in turn decreased the moisture contents of the excreta when compared to non-transgenic control birds by at most 2%. The effect of the *lacZ* transgene on the fecal water contents was not striking as expected from the greater lactase activity in the transgenic birds than the wild-type birds. However, the expressed β-galactosidase is localized to the nucleus of intestinal tissues and not secreted. Thus, the limited lactase activity of the transgenic birds would assumingly have come from the breakage of the gastrointestinal membranes and then the release of the enzyme into the lumen.

Lactose, a milk sugar, is not a natural nutrient for the birds. The endogenous lactase activity in the chicken digestive tracts is low and independent of lactose in the diet and after hatching only trace amounts of lactase activity can be detected (Freund *et al*., 1997; Chotinsky *et al*., 2001). Chickens limit lactose utilization through bacterial fermentation in lower gut segments particularly in ceca. The products of bacterial fermentation of lactose are lactic acid and volatile fatty acids which are absorbed by the tissues and metabolized as energy source (Hume *et al*., 1992). However, Vohra (1967) reported that metabolizable energy value for lactose is highly variable and depends upon the level used in the test diets. Fox and Briggs

(1959) found that only 50% of lactose can be retained by chicks fed lactose at 20%. Carré *et al.* (1995) found that chicks cannot digest great amounts of lactose, when the level of lactose is increased from 3 to 6% the lactose digestibility is reduced from 78 to 64%. The saturation of lactose fermentation in ceca may happen readily in birds. Similarly as in this study, it has been found in the non-transgenic control birds that lactose digestibility decreased from 80 to 60%, 20% decrease when the chicks increased lactose consumption from 5 to 10% in the basal diet (Table 7, Figure 2). However, the magnitude of the negative lactose digestibility is reduced when compared to the transgenic chicks fed with lactose in the same incremented amounts (Table 7, Figure 2). The lactose digestibility of the transgenic birds changes from 74 to 64% which is about a 10% reduction compared to 20% in wild-type birds. The lower reduction rate of lactose digestibility in transgenic birds clearly shows that the transgenic chicks have greater ability to utilize lactose which not only depends on bacterial fermentation limited by the number of colonized bacteria but also on the gene function of the introduced β-galactosidase.

CONCLUSION

The generated transgenic chickens carrying the bacterial *lacZ* transgene express functional β-galactosidase in all tissues of gastrointestinal tracts examined. It has been shown that the transgenic birds have a greater ability to digest lactose through the hydrolizing of lactose to galactose and glucose than those of non-transgenic wild-type chicks at least by 10%. However, the greater lactose

digestibility of the transgenic birds does not result in better growth performance. This could be explained that the retroviral *lacZ* provirus inserting into neurotactin/fractalkine (CX3CL1) gene and interfering its expression compromises the health of the transgenic birds which masks the beneficial effect on growth resulted from greater lactose degradation. Nevertheless, this is the first report of using transgenesis technology to manipulate the chicken genome to utilize feed more efficiently for agricultural purposes.

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CHAPTER 4: GENERAL CONCLUSIONS

Cell markers are an important tool for cell lineage studies in which the tagged cells can be traced along the course of development. Many cell marking methods have been applied such as carbon particles, tritiated thymidine and lipophillic vital dyes. However, these extrinsic markers allow only short-term studies. The chick-quail chromatin marker is used for long-term studies because of its stable inheritance to all daughter cells. However, tracing migration and determination of destination and differentiation of the marked cells in adults are hamperd by the immunological rejection of xenogenic tissue grafts. These problems can be solved by the creation of a chimera made by grafts between the same species (allografts) in which the donor grafts were infected with a replication-defective retrovirus carry a reporter gene. However, viral stocks need to be generated on the day of use.

An alternative approach to cell markers that circumvents the difficulties is the production of germline transgenic birds expressing a reporter gene for intraspecific grafting experiments. The established transgenic chickens expressing the nuclear-localized fulfilled all the requirements for an ideal cell marker. Recently, the transgenic chicken line has been used for cell fate of adult satellite cells after implantation into the host embryos and differentiation determination of hematopoietic stem cells (HSCs) using parabiosis.

In addition, the transgenic chickens exhibit high activity of lactase in the intestinal tract. The transgenic birds fed a basal diet containing dietary lactose had lower water content and higher lactose digestibility. This study indicated that the transgenic chickens were able to hydrolyze lactose *in vivo* and showed that the use

of gene transfer technology to manipulate the chicken genome could utilize feed more efficiently for agricultural purposes.

Appendix A

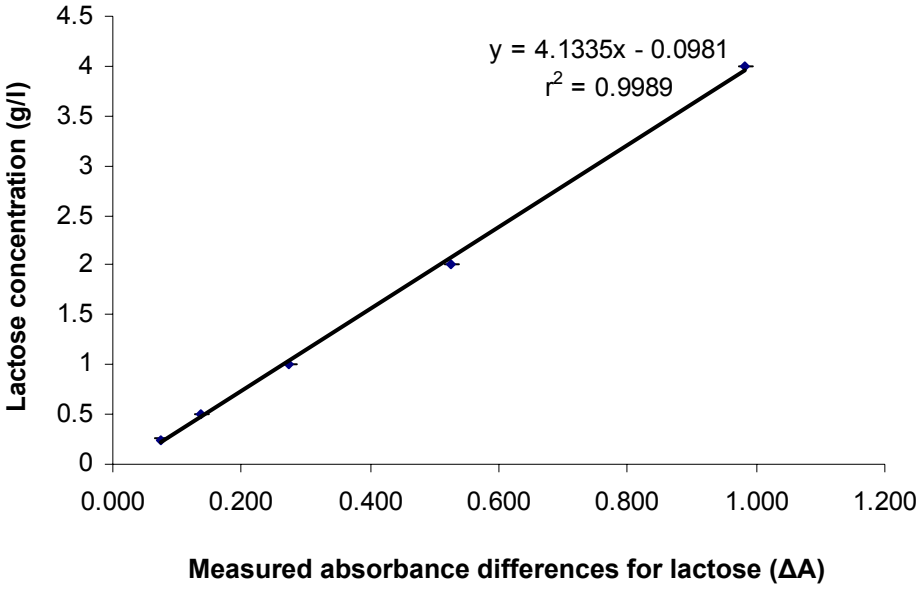


Figure 1. The representative lactose standard curve.

Table 1. Raw data of measured control lactose samples (g/l) for calculation of inter- and between-assay variation.

Assay plate	Lactose (%)	Block	Replication		
			1	2	3
1	0	1	0.58	0.60	0.63
2	0	2	0.58	0.59	0.55
3	0	3	0.56	0.55	0.59
4	5	1	0.56	0.58	0.55
5	5	2	0.51	0.55	0.58
6	5	3	0.53	0.61	0.66
7	7.5	1	0.67	0.61	0.57
8	7.5	2	0.56	0.55	0.54
9	7.5	3	0.56	0.57	0.58
10	10	1	0.50	0.58	0.58
11	10	2	0.70	0.55	0.58
12	10	3	0.61	0.58	0.56

Appendix B

Current publications resulting from dissertation in Chapter 2

Mozdziak, P. E., Borwornpinyo, S., McCoy, D. W., and Petitte, J. N. (2003). Development of transgenic chickens expressing β-galactosidase. *Dev. Dyn*., **226**, 439-445.

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Current publications resulting from dissertation in Chapter 3

Mozdziak, P. E., Pophal, S., Borwornpinyo, S., and Petitte, J. N. (2003). Transgenic chickens expressing β-galactosidase hydrolyze lactose in the intestine. *J. Nutr*., **133**, 3076-3079.