

Development of Transgenic Chickens Expressing Bacterial β -Galactosidase

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Replication-defective retroviral vectors are efficient vehicles for the delivery of exogenous genes, and they may be used in the generation of transgenic animals. The replication-defective retroviral SNTZ vector carrying the *lacZ* gene with a nuclear localized signal was injected into the subgerminal cavity of freshly laid eggs. Subsequently, the eggs were allowed to hatch, and the chickens were screened for the *lacZ* gene by using the polymerase chain reaction. Eight of 15 male chickens that survived to sexual maturity contained the *lacZ* gene in their semen. Subsequently, these males were mated with wild-type female chickens. From one of the eight *lacZ*-positive G_0 males, two *lacZ*-positive male chickens were produced from a total of 224 G_1 progeny for a germline transmission rate of 0.89%. Both G_1 male chickens carrying the *lacZ* gene were mated with wild-type female chickens and 46.5% of the G_2 progeny contained the *lacZ* gene, which is consistent with the expected Mendelian 50% ratio for a heterozygous dominant allele. The product of the *lacZ* gene, nuclear localized β -galactosidase, was expressed in primary myoblast cultures derived from G_2 chickens, and it was also expressed in whole G_2 chicken embryos. *Developmental Dynamics* 226:439–445, 2003. © 2003 Wiley-Liss, Inc.

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INTRODUCTION

Transgenic animals have become important tools for biological research. In vertebrates, foreign DNA is routinely introduced into the genome by microinjection into newly fertilized zygotes (Gordon et al., 1980; Brinster et al., 1981; Costantini and Lacy, 1981; Wagner et al., 1981), through infection by using retroviral vectors (Jaenisch and Mintz, 1974; Crittenden and Salter, 1990), and by transplantation of embryonic stem cells (Robertson, 1991; Wheeler and Walters, 2001). Gene transfer in chickens is a more complicated process than in a mammalian system because of a large yolk. In addition, the chicken embryo contains ap-

proximately 50,000 cells before the egg is laid, making most standard mammalian gene transfer techniques difficult to use (Spratt and Haas, 1960).

Although a variety of approaches have been attempted to produce transgenic birds, only a few lines of transgenic chickens exist. The most successful method for generating transgenic birds has been the use of retroviral vectors (Salter et al., 1987; Petitte and Mozdziak, 2002). Bosselmann et al. (1989a,b, 1990) generated transgenic lines of chickens by using a replication-defective reticuloendotheliosis virus to overexpress the chicken growth hormone gene in embryos. Transgenic birds carrying

the *lacZ* gene have been produced by infecting primordial germ cells with a replication-defective spleen necrosis-derived retroviral vector encoding the *lacZ* gene and transplanting the transformed primordial germ cells into early recipient embryos (Vick et al., 1993). However, the previous investigators (Vick et al., 1993) never demonstrated β -galactosidase expression in their offspring. Similarly, Harvey et al. (2002) produced a line of transgenic chickens by using an avian leukosis-based retroviral vector, carrying the *lacZ* gene, but β -galactosidase expression was also not reported in their studies. Transgenic chickens carrying the *lacZ* gene were also pro-

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TABLE 1. Effect of Injection Procedures on Hatchability (% Hatch) and Effect of Injection Procedure on the Percentages of G_0 Chickens Carrying *lacZ* in Blood and Semen

Injection protocol	N	% Hatch (#)	% carrying <i>lacZ</i> in the blood (# positive/total # screened)	% carrying <i>lacZ</i> in the semen (# positive/total # screened)
Virus only ^a	66	36 (24)	10 (2/20)	80 (4/5)
Virus and cells ^b	54	19 (10)	0 (0/7)	40 (2/5)
Virus double injection ^c	40	35 (14)	0 (0/13)	40 (2/5)

N, number of eggs injected; % Hatch is the percentage of injected eggs that hatched; #, number;

^aVirus only, embryos were injected with 5 μ l of the concentrated retroviral stocks into the subgerminal cavity;

^bVirus and cells, embryos were injected with 5 μ l the concentrated retroviral stocks containing 500 SNTZ-producing cells;

^cVirus double injection, embryos from freshly laid eggs were injected with 5 μ l of the concentrated retroviral stocks into the subgerminal cavity with a pulled micropipette, and the same 40 embryos were injected 1 day after incubation with 4 injections of 3 μ l per injection of the retroviral stock into the germinal crescent.

duced by using a similar replication-defective avian leukosis-based retroviral vector (Thoraval et al., 1995). Unfortunately, β -galactosidase expression was only noted in cultures of embryonic fibroblasts from G_2 progeny, and expression was not reported in the entire embryo. By using microinjection of DNA into the germinal disk of newly fertilized eggs, Love et al. (1994) reported the production of a transgenic rooster harboring the *lacZ* gene, but there was no report of β -galactosidase expression. Overall, it does not appear that any of the transgenic birds carrying the *lacZ* gene have been used in any further studies, making it unlikely that they are available or that they are suitable for cell lineage analysis. Therefore, to our knowledge, there are no documented lines of chickens widely expressing β -galactosidase in existence that can be used to study embryonic development, making it a worthy endeavor to develop lines of chickens expressing a nuclear localized β -galactosidase.

Replication-defective retroviral vectors encoding bacterial β -galactosidase (the *lacZ* gene) have been used by several investigators as a stable heritable marker for cell lineage analysis during chick embryonic development (Price et al., 1987; Mikawa et al., 1991, 1992; Epstein et al., 1994; Cepko et al., 2000). In these previous studies, specific regions of the embryo have been infected with the retrovirus and the labeled

cells were identified at the appropriate time point. In one case, a replication-defective retroviral vector (SNTZ) was developed that encodes a nuclear-localized β -galactosidase (Mikawa et al., 1992). It has been demonstrated previously that the SNTZ vector can be produced with a high titer, it is replication incompetent, it is free of helper-virus, it is functional in a variety of cell types, it is capable of infecting a high number of avian cells, infection does not alter cell proliferation, and infection of embryos results in β -galactosidase expression at hatch (Mikawa et al., 1992). Therefore, it appears that the SNTZ retroviral vector would be an appropriate retroviral system for the generation of transgenic chickens expressing β -galactosidase. The objective of this study was to use the SNTZ vector to generate transgenic chickens that express nuclear localized β -galactosidase. The broader goal was to create a useful transgenic model system to provide a versatile tool for future studies aimed at gaining a deeper understanding of vertebrate embryonic development.

RESULTS

G_0 Chickens

Twenty-four of 66 injected embryos survived to hatch after 5 μ l of retroviral containing medium was injected into the subgerminal cavity of unincubated eggs. Ten of 54 embryos injected with the concen-

trated retrovirus containing medium and cells survived until hatching. Fourteen of 40 embryos injected with concentrated retroviral containing media on the day before initial incubation and again a day after incubation (two injections) survived until hatching. Therefore, it appears that survivability was maximized when retroviral-producing cells were omitted from the injection medium. Overall, 48 of the 160 originally injected embryos survived until hatching (~30%), illustrating that all injection and culture procedures result in a reasonable level of survivability/hatchability (Table 1). A total of 25 female chickens and 15 male chickens (40 total chickens) survived to sexual maturity. Two female G_0 chickens contained the *lacZ* gene in their blood, based upon the polymerase chain reaction (PCR). Ninety progeny from one G_0 blood *lacZ*-positive female chicken (G_0 female 1), and 101 progeny from a second G_0 blood *lacZ*-positive female chicken (G_0 female 2) were screened by means of PCR for the *lacZ* gene. Neither blood-positive female G_0 chicken produced any G_1 transgenic progeny. No G_0 males carried the *lacZ* gene in their blood, but 8 of the 15 (53%) G_0 males carried the *lacZ* gene in their semen (Fig. 1). A single injection of virus resulted in a greater number of birds containing the *lacZ* gene in their semen than injecting concentrated retroviral stocks with retroviral producing cells or from multiple injec-

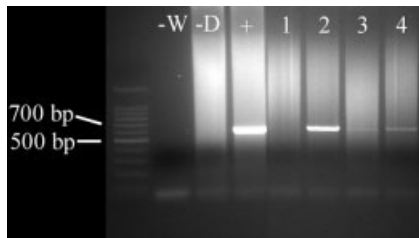


Fig. 1. Polymerase chain reaction analysis of the semen from G_0 chickens. -W is a negative control using a water blank. -D is a negative control using DNA from wild-type chickens. + is a positive control reaction containing the *lacZ* gene from the pmwZ plasmid (Kadokawa et al., 1990). Lane 1 represents a negative bird; lanes 2-4 represent positive birds. All positive lanes contain a 588-bp fragment.

tions of retroviral stocks (Table 1), suggesting that a single retroviral delivery regimen was an efficient retroviral gene transfer procedure. Seven G_0 males did not produce any *lacZ*-positive G_1 progeny, based upon PCR analysis of blood. However, one G_0 male produced two progeny from a total of 224 offspring that contained the *lacZ* gene in the blood for a germ line transmission rate of 0.89% (Table 2).

G_1 Chickens

Both G_1 progeny containing the *lacZ* gene in their blood were males, and as expected, they contained the *lacZ* gene in their semen (Fig. 2). The G_1 males were mated with wild-type White Leghorn female chickens. The first male produced 44 offspring. Twenty of the 44 offspring (45%) contained *lacZ* in their blood, based upon PCR screening. Similarly, the second male produced 25 total offspring. Twelve of the 25 total offspring (48%) contained *lacZ* in their blood based upon PCR screening. Therefore, the ratios of G_1 progeny containing the *lacZ* gene in the blood are consistent with the expected Mendelian ratio of 50% for a heterozygous dominant allele. Chi-square analysis revealed that there was no significant difference between the G_2 offspring and the expected 50% Mendelian ratio ($P < 0.05$).

β -Galactosidase Expression

The first experiment demonstrating β -galactosidase expression in G_2

chickens was to isolate myoblasts from the pectoralis thoracicus muscle to show that *E. coli* β -galactosidase is expressed in the myogenic satellite cell population after in vitro culture. Myoblasts from three of six G_2 chickens expressed β -galactosidase in vitro (Fig. 3). Subsequently, β -galactosidase expression was evaluated in other G_2 embryos, and the embryos also expressed β -galactosidase (Fig. 4).

DISCUSSION

The aim of this study was to develop transgenic chickens that may be used in future studies to gain insight into embryonic development of the chick. It has been shown by means of PCR that we have produced G_0 , G_1 , and G_2 chickens that contain a *lacZ* fragment in the blood. It has been demonstrated that the *lacZ* gene is inherited from the G_1 to the G_2 generation in an expected Mendelian pattern for a heterozygous dominant allele. It has been shown that proliferating myoblast cultures from *lacZ*-positive chickens express nuclear localized β -galactosidase and that similar β -galactosidase expression could be detected in G_2 embryos. Therefore, transgenic chickens expressing nuclear-located β -galactosidase have been produced, and the expression of β -galactosidase suggests that these birds are a viable long-term tool for developmental biology research.

The first step in generating the transgenic chickens was to infect unincubated eggs with SNTZ retroviral containing media. Our procedures produced a relatively high titer of virus that was delivered to the unincubated embryos. There were no overt differences in gene transfer between injecting the concentrated retrovirus and delivering the concentrated retrovirus along with the viral producing cells or performing multiple injections with the retrovirus. Therefore, it appears that the most efficient way to produce transgenic chickens by using the SNTZ retroviral system is a single injection of the concentrated retroviral stock to unincubated eggs.

The percentage of surviving embryos after the injection of retroviral

supernatant (30%) was approximately the same (35.5%; Harvey et al., 2002) or higher (2.3%; Thoraval et al., 1995) than previous reports. The success of our G_0 production may be tied to the surrogate egg shell system, where embryos are cultured in a surrogate chicken egg shell for the first 3 days of incubation (Perry, 1988). Subsequently, the embryos are cultured in a surrogate turkey egg shell until hatching (Rowlett and Simkiss, 1987). Similarly, we achieved a reasonable percentage of G_0 chickens containing *lacZ* in the semen (53%). There was no correlation between the presence of the *lacZ* gene in the blood and the presence of the *lacZ* gene in the germinal tissues, which is consistent with previous reports (Thoraval et al., 1995). It was noted that two G_0 hens were *lacZ*-positive in the blood. A total of 101 progeny were screened from one G_0 blood *lacZ*-positive hen, and 90 progeny were screened from the second G_0 blood *lacZ*-positive hen. All progeny from these hens were *lacZ*-negative. Similarly, all eight male chickens containing the *lacZ* gene in their semen failed to contain the *lacZ* gene in the blood. Therefore, it appears that there is no correlation between transgenic gene detection in the blood and germ line transgenic gene detection in the G_0 birds.

The germ-line transmission rate from the G_0 to the G_1 generation was very low. It was necessary to screen approximately 1,639 offspring from 8 sires to discover 2 *lacZ*-positive progeny. However, it should be noted that one sire produced the two *lacZ*-positive offspring from a total of 224 offspring for a germ-line transmission rate of 0.89%. Although the germ-line transmission rate from the G_0 to G_1 generation was very low, it was consistent with the results of other investigators (Bosselman et al., 1990; Thoraval et al., 1995; Harvey et al., 2002). Clearly, the G_0 birds were mosaic for *lacZ*, and the integration of the *lacZ* gene did not occur in all germ cells. Once the *lacZ*-positive G_1 generation was established, the *lacZ* gene was transferred from the G_1 to the G_2 generation at approximately the expected Mendelian ratio. It appears that the

TABLE 2. PCR Screening of the Progeny from G₀ Male Chickens Carrying the *lacZ* Gene in Their Semen^a

G ₀ Males carrying <i>lacZ</i> in their semen	Number of G ₁ chicks PCR screened for <i>lacZ</i>	G ₁ Chicks carrying <i>lacZ</i> in their blood
1	224 ^b	2
2	252	0
3	14 ^b	0
4	241	0
5	365	0
6	195	0
7	152	0
8	196	0

^aPCR, polymerase chain reaction.

^bScreening of the progeny from these two sires was terminated because the birds died.

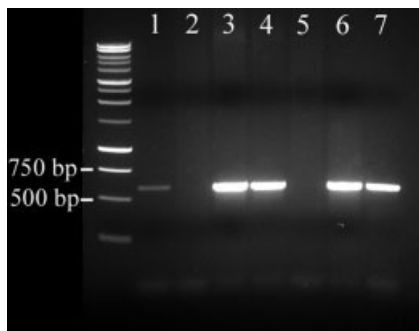


Fig. 2. Polymerase chain reaction analysis of G₁ chickens containing the *lacZ* gene. Lane 1 represents a positive control reaction containing the *lacZ* gene from the pmwZ plasmid (Kadokawa et al., 1990). Lanes 2 and 5 represent a DNA sample from a wild-type chicken. Lane 3 represents a DNA sample from the blood of the first G₁-positive male chicken. Lane 4 represents a DNA sample from the blood of the second G₁-positive male chicken. Lane 6 represents a DNA sample from the semen of the first G₁-positive male chicken. Lane 7 represents a DNA sample from the semen of the second G₁-positive male chicken. All positive lanes contain a 588-bp fragment.

gene insertions did not adversely affect the transgenic chickens because all transgenic flocks were healthy. Furthermore, previous work with the retroviral vectors used in this study (Mikawa et al., 1991, 1992) have indicated that infected cells are completely free of any replication competent retrovirus. Furthermore, no replication competent virus was revealed in any of the myoblast cultures from transgenic animals (see Experimental Procedures section).

G₂ progeny from transgenic G₁ male chicks were killed and myoblasts were isolated and evaluated

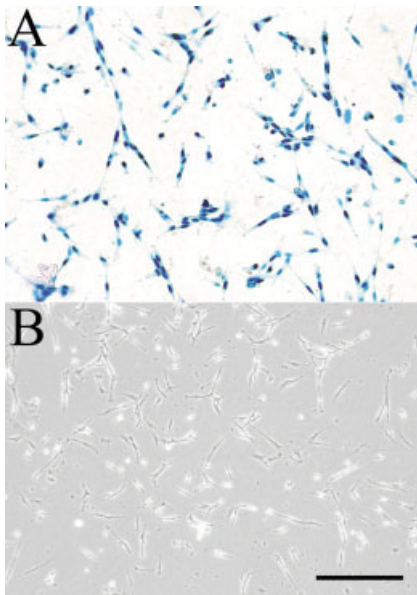


Fig. 3. Myoblast cultures from day old chickens. **A:** Brightfield photomicrograph of primary myoblast cultures from *lacZ*-positive chickens. Cells with stained nuclei represent β -galactosidase-positive cells. **B:** Phase-contrast photomicrograph of primary myoblast cultures from *lacZ*-negative chickens. Scale bar = 150 μ m in B (applies to A,B).

in vitro. Of the six chicks that were used in the initial experiment, three were found to contain β -galactosidase (X-gal)-positive cells. We believe that this is the first demonstration of a *lacZ*/ β -galactosidase transgene transferred to and expressed in a chicken muscle. It appears that the *lacZ* gene is expressed in the G₂ progeny and that myoblasts can be used in cell culture studies and retain their β -galactosidase-positive phenotype, making them a useful model to

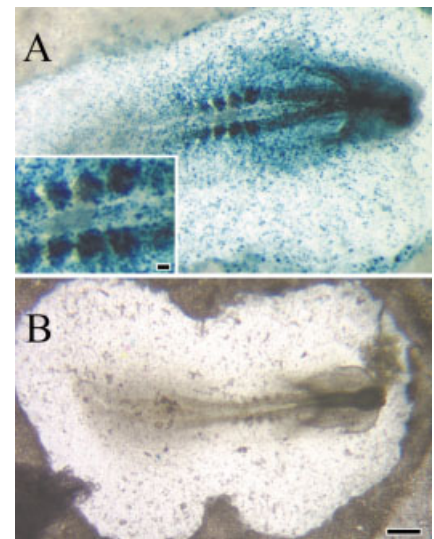


Fig. 4. Embryos (Stage 8; Hamburger and Hamilton, 1951) from (A) β -galactosidase-positive transgenic chickens and from (B) wild-type β -galactosidase-negative chickens. Inset in A shows staining in the somites of β -galactosidase-positive transgenic chickens. Scale bar = 50 μ m in inset, 300 μ m in B (applies to A,B).

understand myogenesis. Similarly, it was found that both G₁ males produced G₂ progeny that expressed β -galactosidase throughout the embryo. It appears that nuclear located β -galactosidase is expressed throughout the embryo and that we have generated a useful model that may be used in embryonic cell lineage studies in ovo and in vitro.

EXPERIMENTAL PROCEDURES

Virus Production

Construction of the replication-defective spleen necrosis virus-based

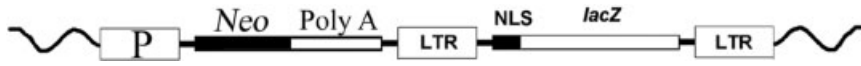


Fig. 5. SNTZ retroviral vector used to generate the transgenic chickens expressing β -galactosidase. The figure illustrates the constructs used to generate the D17.2G SNTZ line of transfected packaging cells. Straight lines indicate spleen necrosis virus sequences and wavy lines indicate bacterial sequences. LTR represents the spleen necrosis virus long terminal repeats. P represent the SV40 promoter. Neo represents the neomycin resistance gene. Poly A represents the SV40 polyA signal. NLS represents the nuclear localized signal. *lacZ* represents the *lacZ* gene. This figure is adapted from Mikawa et al., 1992.

SNTZ vector has been described previously by Mikawa et al. (1992). The SNTZ replication-defective retrovirus carrying a *lacZ* gene (Fig. 5) that expresses a nuclear-localized β -galactosidase was generated by using the D17.2G line of transfected packaging cells (Mikawa et al., 1992), which were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 7% fetal bovine serum (Fisher Scientific, Pittsburgh, PA), and 1% penicillin-streptomycin (Life Technologies, Rockville, MD; Mikawa et al., 1992). 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 the subsequent experiments. Subsequently, the retroviral containing medium (50 ml from ten 100-mm plates) was concentrated by using a stirred cell apparatus containing a membrane filter (YM100, Millipore Corp, Bedford, MA) to a volume of approximately 500 μ l. After concentration, the retrovirus was microfuged for 3 min at $15,000 \times g$.

Viral titers were determined by infecting D17 canine fibroblastic cells with appropriate concentrations of virus in the presence of 1 μ g/ml polybrene. Forty-eight hours after infecting cells with the concentrated retroviral containing supernatant, cells were fixed with 4% paraformaldehyde in PBS for 25 min at 4°C, washed with PBS, and 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 MgCl₂, PBS pH 7.2 overnight at room temperature. The number of blue (X-Gal)-stained cells in each culture dish was evaluated and used to determine the retroviral titer.

The titers of the concentrated retroviral stocks ranged from 2.5×10^6 to 2×10^7 virions/ml.

Surrogate Egg Shell Culture

Embryo culture procedures were based on the procedures of Perry (1988) and Rowlett and Simkiss (1987) with the modifications included below by Borwornpinyo (2000). Freshly laid unincubated fertile White Leghorn eggs containing approximately 50,000 cells were obtained from the North Carolina State University Poultry Flock. Subsequently, the embryos were transferred into a surrogate recipient chicken egg shell through a window cut in the sharp end of the egg. The recipient egg shells were obtained from chicken eggs that weighed 3 to 4 g heavier than the donor eggs. Subsequently, the 66 embryos were injected with 5 μ l of the concentrated retroviral stocks containing polybrene (100 μ g/ml) into the subgerminal cavity with a pulled micropipette. The injection was performed immediately after the embryos were transferred into the surrogate chicken egg shells. A total of 54 embryos were injected with 5 μ l of the concentrated retroviral stocks containing 500 D17.2G SNTZ-producing cells and polybrene (100 μ g/ml) with a pulled micropipette. The injection was performed immediately after the embryos were transferred into the surrogate chicken egg shells. Lastly, 40 embryos from freshly laid eggs were injected with 5 μ l of the concentrated retroviral stocks and polybrene (100 μ g/ml) into the subgerminal cavity with a pulled micropipette, and these same 40 embryos were injected 1 day after incubation with four injections of 3 μ l per injection into the germinal crescent. The first injection was performed immedi-

ately after the embryos were transferred into the surrogate chicken egg shells. The second injections were performed immediately before the embryos were transferred to the surrogate turkey egg shells (see below). The opening in the recipient egg shell was sealed with Saran-Wrap (Dow Chemical, Midland, MI), and it was placed in an egg incubator at 37.5°C. After 3 days of culture in the surrogate chicken egg shell, the embryos were transferred to surrogate turkey egg shells that were derived from turkey eggs that weighed 40 g heavier than the donor chicken eggs. The embryos were delivered to the recipient turkey egg shell through a window cut at the blunt end of the turkey egg shell. The window in the turkey egg shell was sealed with Handi-Wrap (Dow Chemical), and the embryos were incubated until hatch at a temperature of 37.5°C (Borwornpinyo, 2000). The use of Saran-Wrap and Handi-Wrap were empirically determined to be the best combination for embryo survivability.

DNA Isolation

Genomic DNA was extracted from the blood of the chickens generated in this study by using a protocol modified from Petite et al. (1994). Briefly, blood was diluted 1:10 with PBS, mixed with lysis buffer (10 mM Tris HCl pH 7.5, 5 mM MgCl₂, 0.32 M sucrose, 1% Triton X-100), and microfuged for 15 sec; the supernatant was placed in a fresh tube. The DNA containing solution was mixed with sodium dodecyl sulfate and digested overnight with proteinase-K at 37°C with constant rotation. Subsequently, the protein was precipitated by using saturated NaCl, and the DNA was precipitated by using ethanol. All DNA was resuspended in Tris-EDTA buffer.

Genomic DNA was isolated from chicken semen by using procedures modified from Thoraval et al. (1995) and Afanassieff et al. (1996). Briefly, semen was diluted with PBS, incubated with proteinase K, and isolated from the protein by using phenol:chloroform:isoamyl alcohol extraction. Subsequently, the DNA

pellet was washed with 70% ethanol and resuspended in Tris-EDTA.

PCR Screening

The presence of the *lacZ* gene in the offspring was determined by using the polymerase chain reaction. Briefly, Taq polymerase (Fisher Scientific) was used to amplify a 588-bp fragment of *lacZ* by using the forward primer 5'-TTCTGTATGAACG-GTCTGGTC-3, and the reverse primer 5'-ACTTACGCCAATGTCGT-TATC-3. The DNA was amplified by using 35 cycles of 95°C for 30 sec, 54°C for 1 min, 72°C for 1 min by using a thermocycler (PTC-200, MJ Research, Waltham, MA). Subsequently, the amplification products were fractionated through a 1.5% agarose gel to reveal the presence of the 588-bp *lacZ* fragment.

β -Galactosidase Expression

Myoblasts were isolated from day-old G_2 chickens ($n = 6$) by using procedures modified from Mozdziaik et al. (1996). Briefly, samples from the pectoralis thoracicus muscle 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. After enzymatic digestion, the tissue was washed twice with DMEM, 15% fetal bovine serum (Fisher Scientific), and 1% penicillin-streptomycin. The liberated cells were plated on 0.1% gelatin-coated plates. The cultures were incubated for 5 days until the cultures became approximately 80% confluent. The cultures were fixed in 4% paraformaldehyde and stained with X-Gal (1 mg/ml X-Gal, 16 mM potassium ferrocyanide, 16 mM potassium ferricyanide, 2 mM $MgCl_2$, PBS pH 7.2) for overnight. Cultures were observed from each chicken. No negative cells were observed in the positive cultures, and no positive cells were observed in the negative cultures. Lastly, the presence of any replication competent virus was evaluated by removing cell culture supernatant from parallel myoblast cultures derived from β -galactosidase-positive chickens and placing

on proliferating wild-type chicken myoblast cultures. No β -galactosidase-positive cells were observed in the cultures derived from chickens that did not carry the *lacZ* gene.

Staining embryos.

Embryos were fixed at 4°C with 2% formaldehyde and 0.2% glutaraldehyde in PBS pH 7.4 for 30 min, rinsed in PBS, and incubated in X-Gal solution (1 mg/ml X-Gal PBS pH 7.4, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.2% Triton X-100) overnight in the dark at 37°C. Subsequently, the embryos were washed with PBS and stored in 70% ethanol before microscopic evaluation.

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