Development of Transgenic Chickens Expressing Human Parathormone Under the Control of a Ubiquitous Promoter by Using a Retrovirus Vector System[1]

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ABSTRACT Transgenic chickens, ubiquitously expressing a human protein, could be a very useful model system for studying the role of human proteins in embryonic development as well as for efficiently producing pharmaceutical drugs as bioreactors. Human parathormone (hPTH) secreted from parathyroid glands plays a significant role in calcium homeostasis and is an important therapeutic agent for the treatment of osteoporosis in humans. Here, by using a robust replication-defective Moloney murine leukemia virus-based retrovirus vector encapsidated with vesicular stomatitis virus G glycoprotein, we generated transgenic chickens expressing hPTH under the control of a ubiquitous Rous sarcoma virus promoter. The recombinant retrovirus was injected into the subgerminal cavity of freshly laid eggs at the blastodermal stage. After 21 d of incubation, 42 chicks hatched from 473 retrovirus-injected eggs. All 42 living chicks were found to express the vector-encoded hPTH gene in diverse organs, as revealed by PCR and reverse transcription-PCR analysis by using primer pairs specific for hPTH. Four days after hatching, 6 chicks died and 14 chicks showed phenotypic deformities. At 18 wk of age, only 3 G_0 chickens survived. They also released the hPTH hormone in their blood and transmitted the hPTH gene to G_1 embryos. However, although the embryos were alive at d 18 of incubation, none hatched. An electrochemiluminescence immunoassay further showed that the hPTH expression level was markedly elevated in mammalian cells infected by the retrovirus vector. Thus, we demonstrated that transgenic chickens, expressing a human protein under the control of a ubiquitous promoter, not only could be an efficient bioreactor for the production of pharmaceutical drugs, but also could be useful for studies on the role of human proteins in embryonic development. To our knowledge, this is the first report on the production of a human protein (hPTH) in transgenic chickens under the control of a ubiquitous promoter by using a replication-defective Moloney murine leukemia virus-based retrovirus vector system.

Key words: transgenic chicken, human parathormone, retrovirus vector, ubiquitous Rous sarcoma virus promoter

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INTRODUCTION

Transgenic chickens represent a useful model system for studies on embryonic development and as a low-cost, high-yield bioreactor for efficient production of human proteins of pharmaceutical importance because of their unique reproductive physiology, cost-effectiveness, and ease of protein purification (Chapman et al., 2005; Ishii and Mikawa, 2005). In the last 2 decades, several efficient methods have been developed to introduce exogenous genes into the chick embryo, including transfection of avian sperm (Nakanishi and Iritani, 1993), development of germ-line chimeras by using primordial germ cells (Watanabe et al., 1994) and blastodermal cells (Eyal-Giladi and Kochav, 1976; Bosselman et al., 1989), and development of embryonic stem cell lines (Zhu et al., 2005). Several demonstrations of germ-line transmission in chicks have been reported by using these methods with lentiviral (McGrew et al., 2004; Chapman et al., 2005; Lillico et al., 2007) and retroviral vectors (Harvey et al., 2002a; Harvey and Ivarie, 2003; Kwon et al., 2004; Koo et al., 2006). Of these, retroviral-mediated gene transfer has been a very successful gene transfer method in chickens (Mozdziak et al., 2003; Volkova et al., 2006). The efficacy of retrovirus vectors has been demonstrated by germ-line insertion of replication-competent as well as replication-defective retrovirus vectors (Shuman, 1991). Among these, the Moloney murine leukemia virus (**MoMLV**)-based retrovirus vector system has been the system most often used in gene transfer work (Kamihira et al., 2005). We have successfully used MoMLV-based retrovirus vectors for the

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production of transgenic avian (Kwon et al., 2004; Koo et al., 2004), porcine (Uhm et al., 2000, 2007; Choi et al., 2006), and bovine (Kim et al., 1993; Uhm et al., 2007) embryos. More recently, by using the MoMLV-based retrovirus vector, we successfully generated transgenic chicken lines that ubiquitously expressed high levels of enhanced green fluorescent protein and showed germline transmission of the inserted gene thereby, demonstrating its robustness (Koo et al., 2006).

Ubiquitous expression of human proteins in transgenic chickens could be a very useful model system for studying embryonic development as well as for efficiently producing pharmaceutical drugs as economical bioreactors (Chapman et al., 2005; Ishii and Mikawa, 2005). Recent reports of transgenic chickens include those with tissue-specific expression of human monoclonal antibodies (Kamihira et al., 2005; Zhu et al., 2005; Ivarie, 2006), human interferon-α2b (Rapp et al., 2003; Patel et al., 2007) or chimeric ScFv-Fc miniantibody, and human interferon-β1a (Lillico et al., 2007) in their egg white. Human parathormone (**hPTH**), secreted by the parathyroid gland, regulates calcium homeostasis and is an important pharmaceutical drug for the treatment of osteoporosis in humans (Neer et al. 2001). Ubiquitous expression of hPTH in transgenic chickens not only could be a very useful model system for determining its role during early embryonic development, but also could be a very economical bioreactor for effective production of hPTH. To our knowledge, however, there are no previous reports demonstrating successful production of transgenic avians expressing the hPTH gene under the control of a ubiquitous promoter. This study was therefore designed to explore the feasibility of producing hPTH-expressing transgenic chickens by using a robust MoMLV-based replication-defective retroviral vector system under the control of a ubiquitous Rous sarcoma virus (**RSV**) promoter.

MATERIALS AND METHODS

Cloning of the hPTH Gene

To clone the hPTH gene, RNA was isolated from human parathyroid adenomas immediately after surgery and was reverse transcribed to complementary DNA. A full-length 420-bp coding sequence of hPTH (GenBank Accession No. NM_000315) was then PCR amplified by using primer pairs (upstream: 5′-TCA GCA TCA GCT ACT AAC ATA CCT G-3′; downstream: 5′-CTG TTT TCA TTT TCA CTG GGA TT-3′) that flanked the full coding sequence (Figure 1). The PCR was run with a reaction mixture containing 50 pmol of hPTH primer, 0.2 m*M* deoxy nucleotide 5′-triphosphate, 1 m*M* $MgSO_4$, 5 U of avian myeloblastosis virus reverse transcriptase, 5 U of Tfl DNA polymerase, and avian myeloblastosis virus-Tfl 5× reaction buffer. The amplification profile was as follows: heating at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min (denaturation), 50°C for 1 min (annealing), and 72°C for 1 min (extension). After 35 amplification cycles, the samples were retained at 72°C for 7 min to ensure complete strand extension. Identification of the PCR product was reconfirmed by digestion of DNA bands with a diagnostic restriction enzyme and sequencing. The amplified product was subsequently cloned into the pGEM-T Easy vector (Promega, WI, Madison) according to the manufacturer's protocol and purified by using the Qiagen Maxiprep kit (Qiagen, Hilden, Germany) as described earlier (Park et al., 2004).

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AAAAGTCACCATTTAAGGGGTCTGCAGTCCAATTCATCAGTTGTCTTTAGTTTACTCAGC    60

                                                          N  1    2
ATCAGCTACTAACATACCTGAACGAAGATCTTGTTCTAAGACATTGTATGTGAAGATGAT   120

 P  A  K  D  N  A  K  V  N  I  W  N  L  A  I  C  F  L  T  K    22
ACCTGGAAAAGACATGGGTAAAGTTATGATTGTCATCTTGGGAATTTGTTTTCTTACAAA   180

 S  D  G  K  S  V  K  K  R  S  W  S  E  I  Q  L  N  N  N  L    42
ATCGGATGGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGGTTATGCATAAGGT   240

 G  K  N  L  N  S  N  K  R  V  E  W  L  R  K  K  L  Q  D  U    62
GGGAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCTGCAGGATGT   300

 N  N  F  V  A  L  G  A  P  L  A  P  R  D  A  G  S  Q  R  P    82
GCACAATTTTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTCCCAGAGGCC   360

 R  K  K  K  D  K  W  L  W  K  S  N  E  K  S  L  G  K  A  D   102
CCGAAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGGAGAGGCAGA  420

 K  A  D  V  N  V  L  T  K  A  K  S  Q  '     115
CAAAGCTGATGTGAATGTATTAACTAAAGCTAAATCCCAGTGAAAATGAAAACAGATATT   480

GTCAGAGTTCTGCTCTAGACAGTGTACGGCAACAATACATGCTGCTAATTCAAAGCTCTA   540

TTAAGATTTCCAAGTGCCAATATTTCTGATATAACAAACTACATGTAATCCATCACTAGC   600

CATGATAACTGCAATTTTAATTGATTATTCTGATTCCACTTTTATTCATTTGAGTTATTT   660

TAATTATCTTTTCTATTGTTTATTCTTTTTAAAGTATGTTATTGCATAATTTATAAAAGA   720

ATAAAATTGCACTTTTAAACCTCTCTTCTACCTTAAAATGTAAAACAAAAATGTAATGAT   780

CATAAGTCTAAATAAATGAAGTATTTCTCACTCAAAAAAAAAAAAAAA    828
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Figure 1. Nucleotide sequence and corresponding translated amino acid sequence of human parathormone mRNA. The sequence of primer pairs used for cloning complementary DNA are underlined.

Construction of the Retrovirus Vector and Virus Production

The retrovirus vector was constructed as described earlier with modifications (Kwon et al., 2004; Koo et al., 2006). Briefly, a plasmid (pLNRhPTHW) containing a retrovirus vector sequence was constructed by replacing the cytomegalovirus promoter of pLNCX (Miller and Rosman, 1989) with the fragment containing the RSV promoter, hPTH gene, and woodchuck hepatitis posttranscriptional regulatory element (**WPRE**) sequence. The RSV promoter was derived from pLXRN (Clontech, Palo Alto, CA), whereas the WPRE sequence of woodchuck hepatitis virus 2 genomic DNA (GenBank Accession No. M11082) was introduced following the strategy of Zufferey et al. (1999). Introduction of the WPRE sequence into the vector was done to boost expression of the transgene under control of the RSV promoter. A schematic representation of pLNRhPTHW is shown in Figure 2.

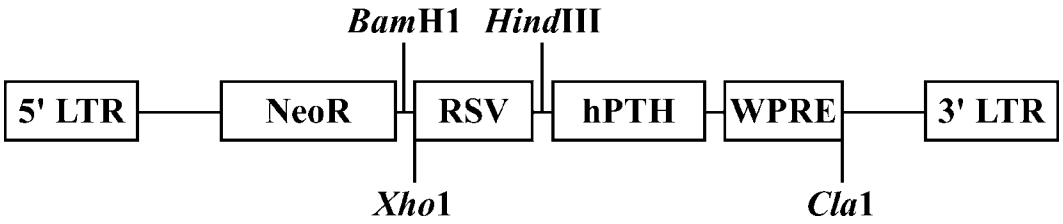

Figure 2. Structure of the LNRhPTHW provirus. LTR = long terminal repeat; NeoR = neomycin resistance gene; RSV = Rous sarcoma virus promoter; hPTH = human parathormone gene; WPRE = woodchuck hepatitis posttranscriptional regulatory element. Drawing is not to scale.

Retrovirus-producing cells were constructed as follows: initially, PG13 cells (Miller et al., 1991) were transiently transfected with pLNRhPTHW, following which LNRhPTHW viruses were harvested and applied to 293mGPHY cells as described earlier (Kim et al., 2001). PG13 are retrovirus packaging cells characterized by expression of the Gibbon ape leukemia virus envelope gene, whereas 293mGPHY cells have been designed to express the gag and pol genes of MoMLV. The 293mGPHY cells infected with LNRGW were selected with G418 (800 μg/mL) for 2 wk and the resultant G418R (or neomycin-resistant) cells were transfected with pHCMV-G to express vesicular stomatitis virus G (**VSV-G**) protein. Viruses were harvested 48 h posttransfection. All cells, including virus-producing cells, were grown at 37°C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (**DMEM**; Gibco BRL, Grand Island, NY) with 4.5 g/L of glucose supplemented with 10% (vol/vol) of fetal bovine serum (**FBS**), 100 μg/mL of penicillin, and 100 μg/mL of streptomycin. The virus-containing medium harvested from the virus-producing cells was centrifugally concentrated to 1/1,000 of the original volume and filtered through a 0.45-μm pore size filter. The virus titer of the concentrated stock was greater than 1×10^9 NeoR (neomycin-resistant) cfu/mL for both NIH 3T3 and primary cultures of chickens fetal fibroblast cells (data not shown).

Microinjection of Retrovirus Vector into Chickens Eggs

Microinjection of the retrovirus into fertile chicken eggs was carried out as described earlier (Koo et al., 2004). Briefly, fertilized eggs (stage X embryo according to the classification of Eyal-Giladi and Kochav, 1976) were obtained from Hyline brown laying hens that were artificially inseminated in groups once a week with semen from Hyline brown males. Only eggs of 62 ± 3 g weight and of normal shape were used in the experiments. These eggs were positioned with their sides facing upward for 8 h at room temperature to fix the blastoderm position. After swabbing the shell with 70% alcohol, a 4×4 mm^2-sized window was made in the equatorial plane of the eggshell by using a fine drill, followed by removal of the small shell membrane inside the window with fine forceps and a surgical blade.

Three microliters of DMEM (DMEM control group; n = 480) or DMEM containing concentrated virus (hPTH-injected group; n = 473) was injected into the central part of the subgerminal cavity by using a microinjection pipette. To increase infectivity, polybrene (10 μg/mL) was added to the virus medium. The injection pipette was drawn from a Pyrex glass tube with an inner diameter of 80 μm at the tip. After injection, the window was sealed with Parafilm. Nonmanipulated (control group; n = 870) or windowed but nonmicroinjected (windowed group; n = 410) eggs were used as controls for comparison.

Incubation of Microinjected Eggs

After microinjection, the sealed eggs were incubated at 37.7°C and 60% RH with a rocking motion every 2 h through a 90° angle for 18 d, following which they were further incubated at 36.7°C and 75% RH without rocking until hatching. The age of an egg was based on days postincubation (e.g., the day of microinjection is referred to as d 0). Eggs were candled on d 9 and 18.

Assay of Transgenic Chickens

Genomic DNA (**gDNA**) isolated from the wings and toes of surviving chickens and various organs (brain, thigh muscle, breast muscle, testis, lung, liver, proventriculus, intestine, cloaca, and oviduct) of dead chicks were analyzed for genomic integration of hPTH in transgenic chickens by PCR analysis as described earlier (Koo et al., 2006). Briefly, gDNA was extracted from transgenic and nontransgenic (control) chickens by using a Genomic DNA Purification kit (Promega). Primer sets specific for the hPTH gene were designed based on the nucleotide sequences of the hPTH gene (GenBank Accession No. NM_000315) in the NCBI database. The upstream (5′-CGATGGAGAGAGTAGAATGG-3′) and downstream (5′-CATTTTCACTGGGATTTAGC -3′) primer sequences for detection of the hPTH gene corresponded to nucleotide sequences at the 257 to 277 and 488 to 468 positions, respectively, to predict an amplified DNA fragment of 212 bp. As a control, PCR of the glyceraldehyde 3-phosphate dehydrogenase gene was also performed by using the primer set 5′-ACGCCATCACTATCTTCCAG-3′ and 5′-CAGCCTTCACTACCCTCTTG-3′, yielding a 1,445-bp DNA fragment including an intron. Each reaction mixture contained 1 μg of genomic DNA extract, 100 pmol of each primer, 5 μL of 10× PCR buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* of each deoxy nucleotide 5′-triphosphate, and 2.5 U of

Table 1. Survival and hatching rate (mean ± SEM) of manipulated chicken eggs[1]

Group	Eggs (n)	Survival rate		Hatching rate
		d 9	d 18	
Control	870	93.1[a] ± 1.5 (803)	88.5[a] ± 1.9 (757)	83.1[a] ± 2.9 (698)
Windowed	410	90.3[a] ± 1.0 (369)	81.4[a] ± 1.1 (332)	70.1[a] ± 1.6 (287)
Dulbecco's modified Eagle's medium injected	480	87.5[a] ± 2.1 (419)	71.7[a] ± 2.8 (345)	42.7[b] ± 2.0 (204)
Human parathormone injected	473	66.8[b] ± 1.2 (473)	22.6[b] ± 1.5 (110)	8.3[c] ± 2.0 (42)

[a–c]Values with different superscripts within a column differ ($P < 0.05$).

[1]Values in parentheses indicate the number of eggs

Taq polymerase (Promega), and the reaction volume was made up to 50 μL. Initial denaturation was done at 94°C for 5 min, followed by 40 cycles of PCR amplification. The amplification profile consisted of the following 3 steps: 94°C for 30 s (denaturation), 50°C for 30 s (annealing), and 72°C for 30 s (extension). After 40 amplification cycles, the samples were retained at 72°C for 7 min to ensure complete strand extension. Identification of the PCR product was reconfirmed by digestion of DNA bands with a diagnostic restriction enzyme.

Messenger RNA extracted from the wings and toes of surviving chickens and various organs of dead chicks was analyzed for expression of integrated hPTH in transgenic chickens by RT-PCR analysis. The mRNA was extracted from transgenic and nontransgenic chickens by using a Dynabeads RNA Direct kit (Dynal Asa, Oslo, Norway), and complementary DNA was synthesized by RT Premix (AccuPower RT Premix, Bioneer, Daejon, South Korea) according to the manufacturer's instructions. The PCR was run as described above.

Blood collected from the jugular veins of transgenic chickens was analyzed for estimation of the hPTH hormone level by commercial immunoradiometric parathyroid hormone assays (Samkwang Medical Laboratory, Seoul, Korea).

Isolation and Infection of Mammalian Cells with Retrovirus Vector

The effectiveness and robustness of the retrovirus vector for the hPTH gene was further validated in a mammalian cell culture system by using porcine fetal fibroblast cells as a model. The porcine fetal fibroblast cells were prepared as described earlier (Gupta et al., 2007). Briefly, primary fetal fibroblasts were isolated from fetuses at 35 d of gestation and cultured on 60-mm tissue culture dishes (Falcon BD, BD Biosciences, Franklin Lakes, NJ) in DMEM supplemented with 10% (vol/vol) FBS at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After 7 d of culture, cells were trypsinized and resuspended in DMEM supplemented with 10% (vol/vol) FBS. Cells were routinely maintained in 50-mL tissue culture flasks (Falcon BD, BD Biosciences) up to 2 to 7 passages. Cultured cells were then infected with retrovirus vector as described earlier (Uhm et al., 2007). Briefly, 3 mL of virus-containing medium (filtered through a 0.45-mm pore size filter) and polybrene (5 mg/mL of final concentration) were added to the target cells, which had been plated on the previous day. Target cells were exposed to the mixture for 1 h. The virus-containing medium was harvested from virus-producing cells that had been fed with nonselection medium (DMEM supplemented with 10% FBS) on the previous day. Following 1 d of culture after infection, infected cells were trypsinized and split in the nonselection medium. Selection medium (DMEM supplemented with 10% FBS and 600 μg/mL of G418) was added on the next day after splitting, and selection was performed for 2 wk with a change of culture medium every 3 d.

Assay of Retrovirus Vector-Infected Mammalian Cells for Expression of hPTH

After selection, infected cells were cultured in G418-free DMEM for 24 h and subjected to quantitative estimation of hPTH by electrochemiluminescence immunoassay (Sanchez-Carbayo et al., 1999). The cells were also analyzed for the mRNA transcript level of hPTH as described above.

RESULTS AND DISCUSSION

This study was designed to explore the feasibility of producing hPTH-expressing transgenic chickens by using a robust MoMLV-based replication-defective retroviral vector system under the control of a ubiquitous RSV promoter. The MoMLV-based retrovirus vector system was chosen because of its robustness, as documented earlier (Kim et al., 1993, 2001; Uhm et al., 2000, 2007; Kim, 2002; Koo et al., 2004, 2006; Kwon et al., 2004; Choi et al., 2006). A VSV-G pseudotyped retrovirus packaging cell line, designed to provide gag and pol proteins of MoMLV, and VSV-G protein instead of retrovirus env protein (Figure 2), was used to obtain a highly concentrated virus stock and to minimize the possibility of replication-competent virus production from the retrovirus vector system and from the transgenic chickens caused by less homology between the sequences of the murine retrovirus and the endogenous avian retrovirus (Burns et al., 1993; Kim, 2002). This retrovirus vector system was combined with the WPRE sequence to boost expression of the transgene under the control of a ubiquitous RSV promoter (Zufferey et al., 1999). The RSV internal promoter was chosen to control hPTH gene expression because the MoMLV long terminal repeat promoter was reported to be inactive

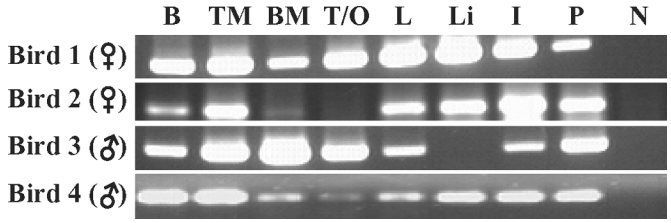

Figure 3. Detection of human parathormone (hPTH) genes in G_0 transgenic chickens. Genomic DNA were isolated from the brain (B), thigh muscle (TM), breast muscle (BM), testis (T), lung (L), liver (Li), intestine (I), and oviduct (O) of transgenic chickens and subjected to PCR amplification by using primer pairs specific for the hPTH gene. Analysis of only 3 representative transgenic birds is shown here. For positive (lane P) and negative (lane N) controls, genomic DNA isolated from virus packaging cells and nontransgenic chickens, respectively, were used.

when mouse embryos were infected with retrovirus at the early embryonic stage (Savatier et al., 1990). Moreover, Mizuarai et al. (2001) reported inactivity of the MoMLV long terminal repeat promoter, but successful expression of the NeoR marker gene under the control of the RSV promoter in a transgenic quail model.

A 3-μL quantity of concentrated virus solution was injected into the subgerminal cavity of the chicken blastoderm to obtain approximately 80 viruses/cell. Based on candling, the survival rate of hPTH-injected eggs on d 9 postincubation was 66.8 ± 1.2%, which was significantly lower than those of the noninjected (93.1 ± 1.5%), windowed (90.3 ± 1.0%), and DMEM-injected (87.5 ± 2.1%) groups. Similarly, survival rates on d 18 of retrovirus-injected eggs (22.6 ± 1.5) were significantly lower than those in other groups (Table 1). Of 473 retrovirus-injected eggs, 42 chicks hatched. The hatching rate (8.3 ± 2.0%) was much lower than those of the DMEM-injected (42.7 ± 2.0%), windowed (70.1 ± 1.6%), or noninjected control eggs (83.1 ± 2.9%). This suggests that the low survival and hatching rates observed in retrovirus-injected embryos may be due to the cytotoxic effect of hPTH gene expression or the retrovirus itself and not to the manipulation procedure.

All 42 chicks, hatched from 473 eggs injected with the medium containing retrovirus vectors, contained the hPTH gene in various body parts, including the brain, thigh muscle, breast muscle, testis, lung, liver, intestine, and oviduct, as revealed by the appearance of distinct 212-bp amplicons upon PCR analysis of gDNA isolated from the transgenic chickens (Figure 3). The gDNA purified from the virus-producing cells and from chicks hatched from noninjected embryos were used as positive and negative controls, respectively. This suggested that the gene transfer method used was effective. We also observed expression of the hPTH gene in various organs, including the brain, thigh muscle, breast muscle, testis, lung, liver, intestine, and oviduct, of all transgenic chickens, as shown by RT-PCR (Figure 4). However, the level of gene expression was apparently different among the organs examined (Figure 4). This difference in gene expression among different organs may be due to differences in the copy number or chromosomal integration locus of the provirus (Lois et al., 2002) or mosaicism. In this study, retrovirus injection was performed at stage X of embryonic development, at which the embryo contains ~60,000 morphologically undifferentiated pluripotent cells that are destined to differentiate into different organs. Failure of the injected retrovirus to infect all of these cells might have resulted in mosaicism (Koo et al., 2004).

Four days after hatching, 6 chicks died and 14 chicks showed phenotypic deformities such as deformed legs, swollen joints, shorter and thicker shank bones, and difficulty in walking and flexing the joints. By 18 wk after hatching, only 3 chickens (1 male and 2 females) survived. Examination of live chickens for the level of hPTH in their blood indicated 643.3 ± 5.9, 630.0 ± 4.2, and 823.0 ± 3.6 ng/dL of hPTH, further confirming expression of the transgene. Mating of these transgenic G_0 birds resulted in fertilized embryos that were live on d 9 and 18, as revealed by candling. However, on d 21 none of the eggs hatched and all embryos died soon after assisted hatching. Polymerase chain reaction analysis of tissue samples from these G_1 chicken embryos, obtained by assisted hatching on d 21, showed clear bands of hPTH in various organs, suggesting the germ-line transmission of the transgene (Figure 5).

The efficacy of our retrovirus vector system was further tested in mammalian cells. Electrochemiluminescence immunoassay of the culture medium harvested from porcine fetal fibroblast cells infected with the retrovirus vector harboring hPTH genes showed 17,830 pg/mL of hPTH compared with 18.2 pg/mL in nontransfected controls. Reverse transcription-PCR analysis of mRNA extracted from these cells further confirmed the success of gene expression (data not shown). These results therefore demonstrate the production of hPTH transgenic chickens and reinforce the superiority of the retrovirus vector system over other available avian retrovirus vector systems (Kim et al., 2001; Harvey et al., 2002a; Kim, 2002; Mozdziak et al., 2003; Koo et al., 2004, 2006; Kwon et al., 2004). The low hatching ability and viability of transgenic chickens might be due to expression of hPTH. The parathyroid hormone in humans and animals regulates bone mineralization by mobilizing calcium among the bones, intes-

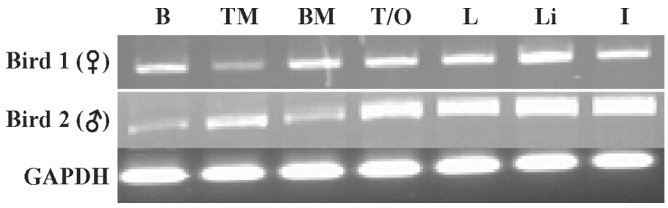

Figure 4. Detection of human parathormone (hPTH) transcripts in G_0 transgenic chickens. Messenger RNA was extracted from the brain (B), thigh muscle (TM), breast muscle (BM), testis (T), lung (L), liver (Li), intestine (I), and oviduct (O) of transgenic chickens and subjected to reverse transcription-PCR amplification by using primer pairs specific for the hPTH transcript. Analysis of only 2 representative transgenic birds is shown here. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard.

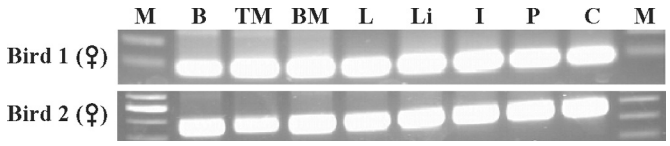

Figure 5. Detection of human parathormone (hPTH) genes in G_1 transgenic chickens. Genomic DNA was isolated from the brain (B), thigh muscle (TM), breast muscle (BM), lung (L), liver (Li), intestine (I), proventriculus (P), and cloaca (C) of transgenic chickens and subjected to PCR amplification by using primer pairs specific for the hPTH gene. Analysis of only 2 representative transgenic birds is shown here. M = 100-bp ladder.

tines, kidneys, and blood. Hyperparathyroidism in humans results in lower fertility and bone deformities (Osmolski et al., 2006; Silverberg and Bilezikian, 2006) similar to those we observed in hPTH transgenic chickens. Interestingly, the level of hPTH in the blood of G_0 transgenic chickens was lower than the levels recorded in media harvested from infected porcine fibroblast cells. This difference may be due to rapid clearance of circulating hPTH by the chicken liver or to differences in the copy number or chromosomal integration locus of the provirus (Lois et al., 2002). If the latter is the case, blood from G_1 transgenic chickens might show a higher expression level.

In conclusion, by using replication-defective retrovirus vector encapsidated with VSV-G glycoprotein, expression of the hPTH gene under a ubiquitous RSV promoter was achieved in chickens. However, low hatching ability, high mortality, and phenotypic deformities were observed in hPTH-expressing transgenic chickens. The significance of this work stems from the fact that it is the first report on the production of a transgenic chicken expressing the hPTH gene under the control of a ubiquitous promoter using a robust replication-defective MoMLV replication-defective retrovirus vector system. This approach can be applied to create useful transgenic model systems for further studies on the role of human proteins in embryonic development and for the efficient production of transgenic chickens as bioreactors of pharmaceutical drugs.

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REFERENCES

Bosselman, R. A., R. Y. Hsu, T. Boggs, S. Hu, J. Bruszewski, S. Ou, L. Kozar, F. Martin, C. Green, F. Jacobsen, M. Nicolson, J. A. Schultz, K. M. Semon, W. Rishell, and R. G. Stewart. 1989. Germline transmission of exogenous genes in the chickens. Science 243:533–535.

Burns, J. C., T. Friedmann, W. Driever, M. Burrascano, and J. K. Yee. 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc. Natl. Acad. Sci. USA 90:8033–8037.

Chapman, S. C., A. Lawson, W. C. Macarthur, R. J. Wiese, R. H. Loechel, M. Burgos-Trinidad, J. K. Wakefield, R. Ramabhadran, T. J. Mauch, and G. C. Schoenwolf. 2005. Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. Development 132:935–940.

Choi, B. R., B. C. Koo, K. S. Ahn, M. S. Kwon, J. H. Kim, S. K. Cho, K. M. Kim, J. H. Kang, H. Shim, H. Lee, S. J. Uhm, H. T. Lee, and T. Kim. 2006. Tetracycline-inducible gene expression in nuclear transfer embryos derived from porcine fetal fibroblasts transformed with retrovirus vectors. Mol. Reprod. Dev. 73:1221–1229.

Eyal-Giladi, H., and S. Kochav. 1976. From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. Dev. Biol. 49:321–337.

Gupta, M. K., S. J. Uhm, and H. T. Lee. 2007. Differential but beneficial effect of phytohemagglutinin on efficiency of in vitro porcine embryo production by somatic cell nuclear transfer or in vitro fertilization. Mol. Reprod. Dev. doi:10.1002/mrd.20720.

Harvey, A. J., and R. Ivarie. 2003. Validating the hen as a bioreactor for the production of exogenous proteins in egg white. Poult. Sci. 82:927–930.

Harvey, A. J., G. Speksnijder, L. R. Baugh, J. A. Morris, and R. Ivarie. 2002a. Consistent production of transgenic chickens using replication-deficient retroviral vectors and high-throughput screening procedures. Poult. Sci. 81:202–212.

Harvey, A. J., G. Speksnijder, L. R. Baugh, J. A. Morris, and R. Ivarie. 2002b. Expression of exogenous protein in the egg white of transgenic chickens. Nat. Biotechnol. 20:396–399.

Ishii, Y., and T. Mikawa. 2005. Somatic transgenesis in the avian model system. Birth Defects Res. C, Embryo Today 75:19–27.

Ivarie, R. 2006. Competitive bioreactor hens on the horizon. Trends Biotechnol. 24:99–101.

Kamihira, M., K. Ono, K. Esaka, K. Nishijima, R. Kigaku, H. Komatsu, T. Yamashita, K. Kyogoku, and S. Iijima. 2005. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. J. Virol. 79:10864–10874.

Kim, T. 2002. Retrovirus-mediated gene transfer. Pages 173–193 in Transgenic Animal Technology. 2nd ed. C. A. Pinkert, ed. Academic Press, San Diego, CA.

Kim, T., Y. M. Lee, H. T. Lee, Y. T. Heo, H.-C. Yom, M. S. Kwon, B. C. Koo, K. Whang, and K. S. Roh. 2001. Expression of the *E. coli* LacZ Gene in chicken embryos using replication defective retroviral vectors packaged with vesicular stomatitis virus G glycoprotein envelopes. Asian-australas. J. Anim. Sci. 14:163–169.

Kim, T., M. L. Leibfried-Rutledge, and N. L. First. 1993. Gene transfer in bovine blastocysts using replication-defective retroviral vectors packaged with Gibbon ape leukemia virus envelopes. Mol. Reprod. Dev. 35:105–113.

Koo, B. C., M. S. Kwon, B. R. Choi, J. H. Kim, S. K. Cho, S. H. Sohn, E. J. Cho, H. T. Lee, W. Chang, I. Jeon, J. K. Park, J. B. Park, and T. Kim. 2006. Production of germline transgenic chickens expressing enhanced green fluorescent protein using a MoMLV-based retrovirus vector. FASEB J. 13:2251–2260.

Koo, B. C., M. S. Kwon, B. R. Choi, H. T. Lee, H. J. Choi, J. H. Kim, N. H. Kim, I. Jeon, W. Chang, and T. Kim. 2004. Retrovirus-mediated gene transfer and expression of EGFP in chickens. Mol. Reprod. Dev. 68:429–434.

Kwon, M. S., B. C. Koo, B. R. Choi, H. T. Lee, Y. T. Kim, W. S. Ryu, H. Shim, J. H. Kim, N. H. Kim, and T. Kim. 2004. Development of transgenic chickens expressing enhanced green fluorescent protein. Biochem. Biophys. Res. Commun. 320:442–448.

Lillico, S. G., A. Sherman, M. J. McGrew, C. D. Robertson, J. Smith, C. Haslam, P. Barnard, P. A. Radcliffe, K. A. Mitrophanous, E. A. Elliot, and H. M. Sang. 2007. Oviduct-specific expression of two therapeutic proteins in transgenic hens. Proc. Natl. Acad. Sci. USA 104:1771–1776.

Lois, C., E. J. Hong, S. Pease, E. J. Brown, and D. Baltimore. 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 295:868–872.

McGrew, M. J., A. Sherman, F. M. Ellard, S. G. Lillico, H. J. Gilhooley, A. J. Kingsman, K. A. Mitrophanous, and H. Sang. 2004. Efficient production of germline transgenic chickens using lentiviral vectors. EMBO Rep. 5:728–733.

Miller, A. D., J. V. Garcia, N. Von Suhr, C. M. Lynch, C. Wilson, and M. V. Eiden. 1991. Construction and properties of retrovirus packaging cells based on Gibbon ape leukemia virus. J. Virol. 65:2220–2224.

Miller, A. D., and G. J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. Biotechniques 7:980–990.

Mizuarai, S., K. Ono, K. Yamaguchi, K. Nishijima, M. Kamihara, and S. Iijima. 2001. Production of transgenic quails with high frequency of germ-line transmission using VSV-G pseudotyped retroviral vector. Biochem. Biophys. Res. Commun. 286:456–463.

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

Nakanishi, A., and A. Iritani. 1993. Gene transfer in the chicken by sperm-mediated methods. Mol. Reprod. Dev. 36:258–261.

Neer, R. M., C. D. Arnaud, J. R. Zanchetta, R. Prince, G. A. Gaich, J. Y. Reginster, A. B. Hodsman, E. F. Eriksen, S. Ish-Shalom, H. K. Genant, O. Wang, and B. H. Mitlak. 2001. Effect of parathyroid hormone (1–34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N. Engl. J. Med. 344:1434–1441.

Osmolski, A., R. Osmolski, Z. Frenkiel, and G. Adamiak. 2006. Primary hyperparathyroidism—Case report and review of the literature. Otolaryngol. Pol. 60:93–96.

Park, S. M., S. J. Song, S. J. Uhm, S. G. Cho, S. P. Park, J. H. Lim, and H. T. Lee. 2004. Generation of embryonic stem cell derived transgenic mice by using tetraploid complementation. Asian-australas. J. Anim. Sci. 17:1641–1646.

Patel, T. B., E. Pequignot, S. H. Parker, M. C. Leavitt, H. E. Greenberg, and W. K. Kraft. 2007. Transgenic avian-derived recombinant human interferon-α2b (AVI-005) in healthy subjects: An open-label, single-dose, controlled study. Int. J. Clin. Pharmacol. Ther. 45:161–168.

Rapp, J. C., A. J. Harvey, G. L. Speksnijder, W. Hu, and R. Ivarie. 2003. Biologically active human interferon α-2b produced in the egg white of transgenic hens. Transgenic Res. 12:569–575.

Sanchez-Carbayo, M., M. Mauri, R. Alfayate, C. Miralles, and F. Soria. 1999. Analytical and clinical evaluation of TSH and thyroid hormones by electrochemiluminescent immunoassays. Clin. Biochem. 32:395–403.

Savatier, P., J. Morgenstern, and R. S. P. Beddington. 1990. Permissiveness to murine leukemia virus expression during preimplantation and early postimplantation mouse development. Development 109:655–665.

Shuman, R. M. 1991. Production of transgenic birds. Experientia 47:897–905.

Silverberg, S. J., and J. P. Bilezikian. 2006. The diagnosis and management of asymptomatic primary hyperparathyroidism. Nat. Clin. Pract. Endocrinol. Metab. 2:494–503.

Uhm, S. J., M. K. Gupta, T. Kim, and H. T. Lee. 2007. Expression of enhanced green fluorescent protein in porcine- and bovine-cloned embryos following interspecies somatic cell nuclear transfer of fibroblasts transfected by retrovirus vector. Mol. Reprod. Dev. doi:10.1002/mrd.20755.

Uhm, S. J., N. H. Kim, T. Kim, H. M. Chung, K. H. Chung, H. T. Lee, and K. S. Chung. 2000. Expression of enhanced green fluorescent protein (EGFP) and neomycin resistant (NeoR) genes in porcine embryos following nuclear transfer with porcine fetal fibroblasts transfected by retrovirus vector. Mol. Reprod. Dev. 57:331–337.

Volkova, N. A., N. A. Zinoveva, L. V. Volkova, and L. K. Ernst. 2006. Retroviral-mediated gene transfer as an effective tool for the in vitro genetic transformation of chickens embryonic cells and production of transgenic chickens. Genetika 42:84–88.

Watanabe, M., M. Naito, E. Sasaki, M. Sakurai, T. Kuwana, and T. Oishi. 1994. Liposome-mediated DNA transfer into chickens primordial germ cells in vivo. Mol. Reprod. Dev. 38:268–274.

Zhu, L., M. C. van de Lavoir, J. Albanese, D. O. Beenhouwer, P. M. Cardarelli, S. Cuison, D. F. Deng, S. Deshpande, J. H. Diamond, L. Green, E. L. Halk, B. S. Heyer, R. M. Kay, A. Kerchner, P. A. Leighton, C. M. Mather, S. L. Morrison, Z. L. Nikolov, D. B. Passmore, A. Pradas-Monne, B. T. Preston, V. S. Rangan, M. Shi, M. Srinivasan, S. G. White, P. Winters-Digiacinto, S. Wong, W. Zhou, and R. J. Etches. 2005. Production of human monoclonal antibody in eggs of chimeric chickens. Nat. Biotechnol. 23:1159–1169.

Zufferey, R., J. E. Donello, D. Trono, and T. J. Hope. 1999. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J. Virol. 73:2886–2892.