Cock Spermatozoa Serve as the Gene Vector for Generation of Transgenic Chicken (*Gallus gallus*)*

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ABSTRACT : To evaluate the feasibility of using sperm-mediated gene transfer (SMGT) for carrying foreign gene into chicken oocyte, a reporter gene, CX-EGFP, was used in this study. The reporter gene was first mixed with liposome or liposome-like compound and the mixtures were further combined with ejaculated cock spermatozoa. The spermatozoa treated with liposome and CX-EGFP mixture was subsequently coincubated with DNaseI to remove the extra DNA which insured the authenticity of positive signals. The treated sperms were then subjected to transgene (reporter gene) existence analysis and artificial insemination of laying hens. Obtained results indicated that the spermatozoa were able to take-in the foreign DNA; which was confirmed by polymerase chain reaction and Southern blot analysis. In the following experiment, fresh ejaculated sperms were mixed with CX-EGFP-liposome or CX-EGFP-liposome-like complex then used for artificial insemination of each of six laying hens. Eggs laid between day-3 and day-7 post insemination were collected. Newly hatched chicks, two out of 53 from CX-EGFP/liposome treated group and two out of 21 from CX-EGFP/liposome-like treated group, were proven to be transgenic. This study suggests that SMGT is a powerful method for generating transgenic chickens. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 7 : 885-891)***

Key Words : Transgenic Chicken, Sperm-mediated, Gene Transfer

INTRODUCTION

Transgenesis is a powerful tool in terms of unveiling the basics of life science (Lee and piedrahita, 2003). Recently, transgenesis is also used in producing bioproducts (such as biomedicines). Large domestic animals, such as cows, goats and pigs, were made transgenic and producing designed proteins. In order to produce these bioproducts both high in quantity and quality. Combining the modern biotechnology and animal husbandry is the trend. Nonetheless, under the modern management, the chicken industry around the world has the highest production rate and economic value. In average, each laying hen can produce 260 eggs every month which can tightly meet the purpose stated previously. However, there is not yet an efficient technique for generating transgenic chicken. The existing studies have used following methods: (1) Infect the chicken embryo with a retrovirus vector (Salter et al., 1986; Thoraval et al., 1995; Iba, 2000). (2) Inject foreign DNA into the pronuclei of a fertilized egg by microinjection (Nakamura and Funahashi, 2001), (3) Transfect the early stage of chicken embryo with foreign DNA via liposomes (Longmuir et al., 2001). (4) Transfect the primordial germ cells (PGCs) with foreign DNA; and then certain PGCs are selected for fusion transplantation with chicken embryos (Han et al., 1994; Ebara and Fujihara, 2000; Han and Jeong, 2002), (5) Use spermatozoa as a gene vector to carry foreign DNA into oocyte during the fertilization process (Rottmann et al., 1992). All these methods had been used to generate transgenic poultry. However, the results have not been satisfactory due to various technical bottlenecks that remain to be overcome.

The spermatozoon is a male gamete with a haploid number of chromosomes. It is the final product of the spermatogenesis which starts with the spermatogonium that reside on the basal membrane of seminiferous tubule of testis and gone through the entire process of mitosis and meiosis. As far as embryo development is concerned, spermatozoa energize oocytes to disunite and become the key component of embryogenesis. From the viewpoint of hereditism, spermatozoa enable the number of somatic chromosome to become the same as the number of its parents. Therefore, spermatozoon play a vital role in determining the new number of hereditary substances. Brackett et al. (1971) discovered that sperm could be used as a gene vector. Their experiment indicated that the rabbit's sperm infected with the SV40 virus imported the DNA of SV40 into fertilized eggs through the process of fertilization. Since then, sperm from a number of species were found capable of binding foreign DNA spontaneously (Castro et al., 1991; Nakanishi and Iritani, 1993; Habrove et al., 1996;

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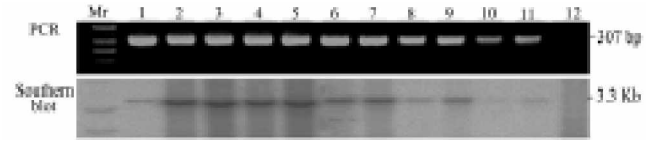

Figure 1. Evidence of transgene associated with chicken spermatozoa gDNA samples that had been pre-incubated with CX-EGFP/liposome complex were confirmed with a 307 bp and 3.3 Kb specific fragment of the transgene amplified after PCR and by Southern analysis. Lane 1: positive control; Lane 2: 1×10^8 sperm/ml+naked CX-EGFP; Lane 3: 1×10^8 sperm/ml+CX-EGFP/liposome complex; Lane 4: 1×10^8 sperm/ml+naked CX-EGFP with DNaseI treatment; Lane 5: 1×10^6 sperm/ml+CX-EGFP/liposome complex with DNaseI treatment; Lane 6: 1×10^6 sperm/ml+naked CX-EGFP with DNaseI treatment; Lane 7: 1×10^6 sperm/ml+CX-EGFP/liposome complex with DNaseI treatment. Lane 8. 1×10^4 sperm/ml+naked CX-EGFP with DNaseI treatment; Lane 9: 1×10^4 sperm/ml+CX-EGFP/liposome complex with DNaseI treatment; Lane 10: 1×10^2 sperm+naked CX-EGFP with DNaseI treatment; Lane 11: 1×10^2 sperm+CX-EGFP/liposome complex with DNaseI treatment. Lane 12: native control.

Lavitrano et al., 1999).

The purpose of this study was to examine the possibility of generating transgenic chicken using sperm vector with or without the assistance of liposomes. This includes that using the restriction enzyme/protamine/DOTAP (Ni et al., 1999; Clark and Civetta, 2000; Shemesh et al., 2000) and the liposome-like material to transfer the foreign DNA. At the same time, we also like to verified if these additional materials can improve the efficiency of foreign DNA entering the spermatozoa nuclei.

MATERIALS AND METHODS

Preparation of reporter gene/transgene

The reporter gene used in this study is pCX-EGFP (Okabe et al., 1997; Ikawa et al., 1999). Briefly, this plasmid DNA was digested with *Hin*dIII and *Apa*LI and yielded a 3.3 Kb fragment, CX-EGFP. The obtained DNA fragment was further purified and ready for making transgene mixture.

DNA binding ability analysis for cock spermatozoa

Sperms were collected from 6 sexually mature white leghorn males; washed with PBS and centrifuged at 500×g for 3 minutes for 3 times. The sperms then were resuspended in semen diluents and reached the final concentration of 2×10^8 sperms/ml. Five hundred microliter of sperms were further incubated with (A) 10 μg CX-EGFP, (B) liposome complex containing 10 μg CX-EGFP and (C) 500 μl of semen diluents at 18°C for 1.5 h. These solutions were spun again and supernatant was removed. Then 1 ml of PBS was added to each sample. The treatment A and treatment B were then preceded to a serial dilution according to dilution factor 10^0, 10^{-2}, 10^{-4} and 10^{-6}. Next, 150 unit of DNaseI was added and incubated at 37°C for 1 h. Genomic DNA was extracted from the treated spermatozoa nuclei.

Preparation the mixture of CX-EGFP-liposome and CX-EGFP-liposome-like complex with cock spermatozoa for artificial insemination

Cock spermatozoa were collected prior to the experiments. The mixture of reporter gene and liposome or liposome-like compound was prepared as described by Birchall et al. (2000) and Shemech et al. (2000). Briefly, for each unit of sperms (10^8 sperms), 10 μg of purified DNA fragment was slowly added to 20 μl of protamine sulfate (1 mg/ml) and incubated at 18°C for 10 minutes. Then 30 μg of DOTAP (Sigma, USA) was added and incubate for additional 10 minutes. At the same time, 100 units of restriction enzyme *Hin*dIII was mixed with 10 μg of DOTAP then incubated for 10 minutes. This enzyme mixture was later gently added to the DNA-protamine sulfate solution and continued the incubation for another 10 minutes. Finally, the sperm was combined with the solution and ready to use.

Preparation of blood genomic DNA

Chicken blood was collected from the *Vena cutanea ulnaris*. A small puncture was made using a 23 gauge needles and 10 μl of blood was pipetted into a clean tube. Genomic DNA was extracted from each blood samples.

Verification of transgene existence and expression

Polymerase chain reaction : The primer was located inside EGFP; forward primer (5` TGAACCGCATCGAGCTG AAGGG 3`) and reverse primer (5` TCCAGCAGGACCATGTGATCGC 3`). This primer set will give a 307 bp fragment. The polymerase chain reaction program was started at 94°C for 5 minutes, then denatured at 94°C for 30 seconds, annealed at 67°C for 15 seconds and elongated at 72°C for 20 seconds. This procedure was repeated 10 times. Then the fragment was denatured at 94°C for 30 seconds, annealed at 66°C for 15 seconds and elongated at 72°C for 20 seconds. This procedure was repeated 25 times.

Southern blot analysis : The method as modified by Koetsier et al. (1993) was used. Estimated 15 μg of genomic DNA was digested by the restriction enzyme, fractionated on 1.1% agarose gels and transfer to nylon membranes. The EGFP gene specific probe was labeled with ^{32}P-dCTP using the Rediprime™ II Random Prime Labelling System (Amersham Pharmacia Biotech, UK). Followed by serious of washes and the membranes were

Table 1. Effect of liposome-like and/or liposome treatment on the transgenic efficiency in transgenic chicken generation via the sperm-mediated strategy for gene transfer

Strategy used for gene transfer	No. of hens inseminated	No. of chicken hatched	No. (%) of chick carry foreign DNA
Liposome-like	6	21	2 (9.5)
Liposome	6	53	2 (3.7)
Control	2	13	0

exposed to the image processing system (Bio-imaging Analyzer, BAS-1500, FUJIX, Japan) and signal was recorded.

Frozen section : The method as modified by Fejzo and Slamon (2001) was used. Chick embryo tissue were collected and embedded in OCT (Tissue-Tek, USA) compound and stored at -70°C. Frozen sections were made (Leica 1800, Germany) and visualized with a microscope under ultraviolet light (Leica DM IRB, Germany: EGFP filter #41020, CHROMA, USA).

RESULTS AND DISCUSSION

The experimental results demonstrated that cock sperm was capable of binding foreign DNA, and that the efficiency of foreign DNA entering spermatozoa nuclei could be greatly enhanced with the presence of liposome.

Figure 1 shown the sperm genomic DNA treated with DNaseI analyzed by polymerase chain reaction and Southern blot analysis. As observed in the Southern blot analysis, the 3.3 Kb DNA fragments were acquired from the sperms that did not incubated with DNaseI (Lane 2 and 3), regardless if such sperms had been processed by liposome or not. The sperms did treated with DNaseI (Lane 4 and 5), regardless whether such sperms had been processed by liposome or not, presented a sharp band at 3.3 Kb. When DNaseI processed the treatments with various sperm concentration, the band turned lighter as the sperm concentration decreased, regardless whether the treatment had been processed by liposome or not. Nevertheless, the treatment still could hybrid the signals, which meant that the foreign DNA was capable of entering into the spermatozoa. As shown by the results, the sperms processed by liposome transfection (Lane 5, 7, 9 and 11) presented the band with signals that were stronger than the band produced by the treatment co-incubated by spermatozoa and foreign DNA (Land 4, 6, 8 and 10). It was therefore concluded that the spermatozoa of a chicken were capable of carrying foreign DNA and that the liposome enhanced the amount of foreign DNA entering the spermatozoa. However, it is well known that the genome of spermatozoa is tightly packed and would be very difficult for any foreign DNA to integrate. Furthermore, the nucleus of sperm is the actual organelle entering the oocyte during the process of

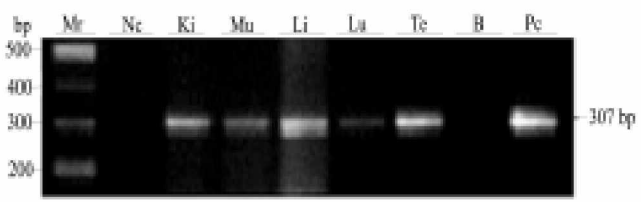

Figure 2. Evidence of transgene contained in transgenic chicken No. 92 generated after insemination with chicken spermatozoa that had been transfected by CX-EGFP/liposome complex; a 307 bp specific fragment of the transgene was presented in various tissues including kidney (Ki), muscle (Mu), liver (Li), lung (Lu) and testis (Te) etc. when each gDNA had been subjected to PCR control amplification. Nc: negative control; B: blank, Pc: positive control.

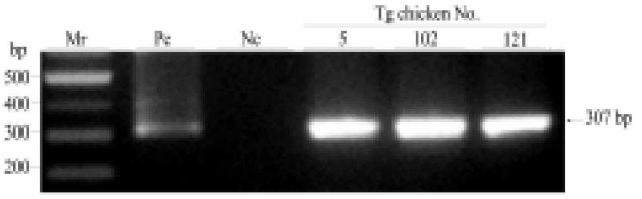

Figure 3. Evidence of transgene contained in transgenic chicken No. 5, 102 and 121 that were generated after insemination with chicken spermatozoa that had been transfected by CX-EGFP/liposome complex (No. 121) and/or CX-EGFP/liposome-like complex (No. 5, 102); a 307 bp specific fragment of the transgene was obtained when each gDNA extracted from blood samples had been subjected to PCR amplification. Pc: positive control; Nc: negative control.

fertilization. Those evidences suggest that the transgene was preserved inside the limited space of nucleus and integrated into the decondensed chromosomes at the fusion of two nuclei. This result is similar to the finding presented by Rottmann et al. (1992). According to the study conducted by Nakanishi and Iritani (1993), while spermatozoa coincubated with liposome, the binding position of foreign DNA and spermatozoa was no longer limited to the front of acrosome. The investigators further labeled the sperms with isotope, 52% of the sperms were labeled. When proceed with electroporation, the sperm mobility was not affected, but the fertility decreased, probably because the electroporation caused the damage of acrosome. Therefore, our subsequent experiments were focused on the transfection using liposome or liposome-like compound.

Table 1 presents the statistical results of hatchability of liposome treated spermatozoa when used in artificial insemination. Six laying hens were inseminated artificially with the spermatozoa transfected by CX-EGFP/liposome complex or CX-EGFP/liposome-like complex and then eggs were collected. During the experimental period, 66 and 32 eggs were collected independently. At the end, 53 and 21 chicks were hatched. The hatchability was 100.0%. Therefore, the fertility was not affected if hens were

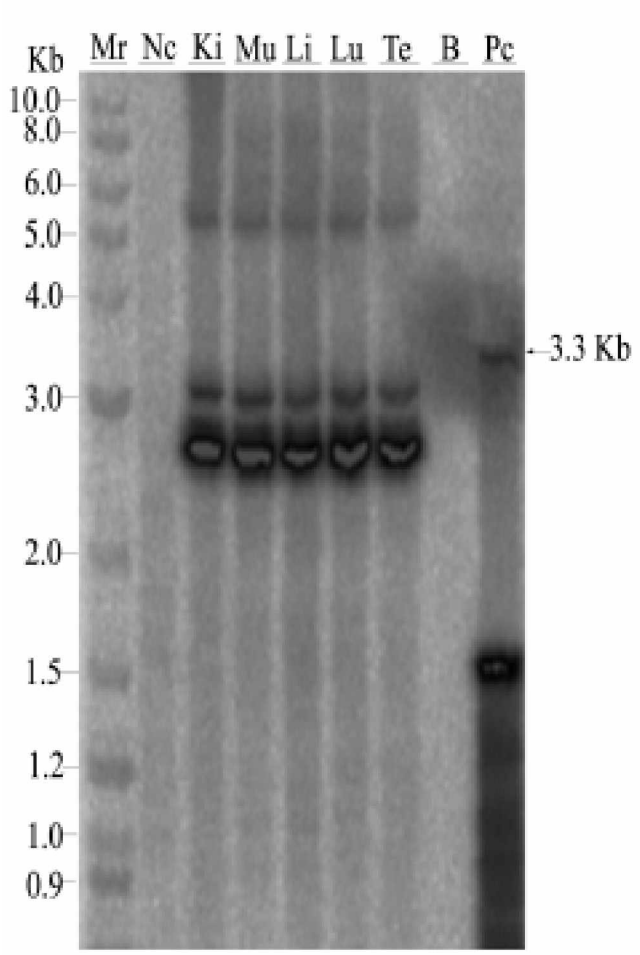

Figure 4. Southern blot evidence showing the transgene integrated within the genome of transgenic chicken No. 92 that were generated after insemination of a hen with chicken spermatozoa that had been transfected by CX-EGFP/liposome complex. Various tissues including kidney (Ki), muscle (Mu), liver (Li), lung (Lu) and testis (Te) etc. when each gDNA had been subjected to digestionwith *Hin*dIII & *Apa*LI before they were subjected to Southern analysis using a random priming probe for hybridization. Nc: negative control; B: blank, Pc: positive control.

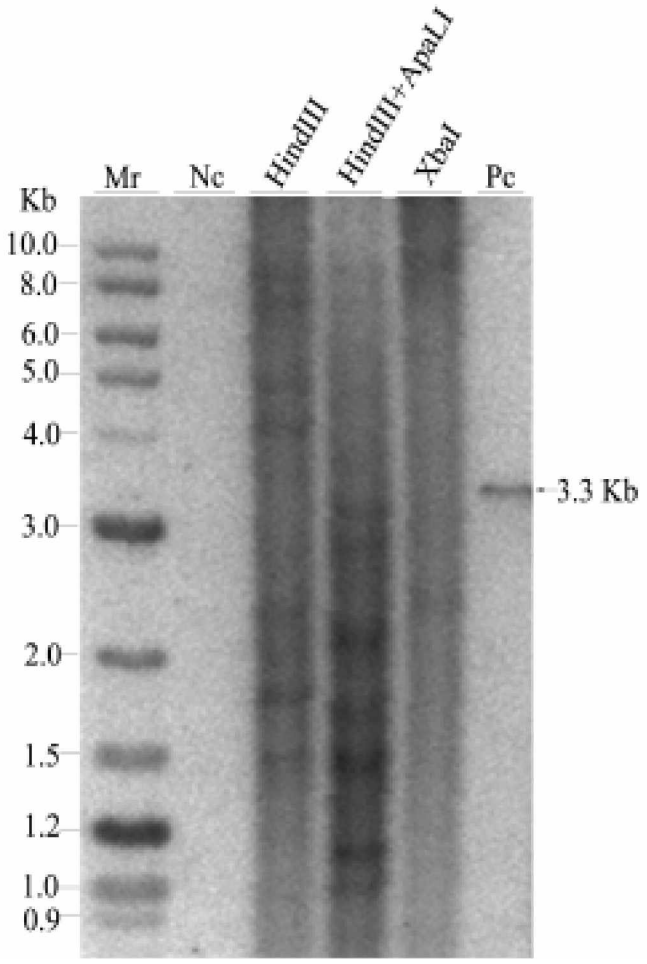

Figure 5. Southern blot evidence showing the transgene integrated within the genome of transgenic chicken No. 121 that were generated after insemination of hen with chicken spermatozoa that had been transfected by CX-EGFP/ liposome complex. Genomic DNA samples were digested with *Hin*dIII & *Apa*LI before they were subjected to Southern analysis using a random priming probe for hybridization. Pc: positive control; Nc: negative control.

artificially inseminated with the sperms processed by liposome or liposome-like complex. This result is similar to the findings of Nakanishi and Iritani (1993). We then compared the chicken spermatozoa without liposome transfection and the chicken spermatozoa with liposome transfection. As the results indicated, the mobility of chicken spermatozoa transfected with liposome drops slightly; and the hatchability for both treatments after artificial insemination was 90.0%. There was no significant difference between both treatments. The hatchability after electroporated treatment was only 55.0%, probably because the electroporated treatment caused more damage to the acrosome than the liposome complex did. Therefore, transfection using liposome is preferred due to the higher hatchability.

Tissues or blood genomic DNA was extracted from 53 and 21 chicks that hatched from liposome or liposome-like treatments and used in the EGFP gene specific fragment amplification. There were 2 chicks in each treatment barring the desired 307 bp fragment (Figures 2 and 3). The numbers of these chicks were No. 92, 121, 5 and 102. During the test period, chicks No. 92 and No.5 died at the 19th weeks of the age due to illness. Chick No. 5 was anatomized; later, its genomic DNA extracted from blood was used for the Southern blot analysis (Figure 6). It was possible that No. 5 chick died on a viral infection. The genomic DNA of kidney, muscle, liver, lung and testis of No. 92 chick was digested with restriction enzymes and proceed to the Southern blot analysis (Figure 4). The pattern shown in each tissues was the same. Therefore, it was concluded that the foreign DNA did insert into the genomic DNA of the embryos and stay integrated in the growing

chicks. However, the lengths of 3 hybridized fragments were approximately 2.6, 3.1 and 5.3 Kb, respectively. For the hybridized fragment which was shorter than expected, 3.3 Kb, it was assumed that certain sequences of the foreign DNA might have been degraded before the fragment inserted into the genome; or because part of transgene was lost due to the genome rearrangement . The 5.3 Kb hybrid was likely a result of integrated multiple copies of used reporter gene.

Figure 5 depicted the results of the blood genomic DNA of chick No. 121 that was generated by artificial insemination with spermatozoa coincubated with CX-EGFP/liposome complex. As shown in the subsequent Southern analysis, the genomic DNA cut with *Hin*dIII produced fragments ranging from 1.5 to 9.0 Kb; and the *Hin*dIII and *Apa*LI digestion then hybridized to DNA fragment ranging from 1.0 to 3.2 Kb. Nonetheless, the genomic DNA treated with *Xba*I generated a single fragment which was approximately 2.3 Kb long. The desired fragment size, 3.3 Kb, was missing in all reactions. There is not yet a concreted theory which can properly interpret such a phenomenon. It is said that foreign DNA has been cut a number of times inside the spermatozoa nuclei by enzymes and, consequently, resulted in a genome rearrangement (Tsai et al., 1997). Figure 6 contains the results of southern blot analysis of blood genomic DNA of chick No. 5 that was generated from the combination of sperms and CX-EGFP/liposome complex. Its genomic DNA was cut by *Hin*dIII, *Hin*dIII and *Apa*LI, and *Xba*I at various enzyme cutting sites. When the sample was digested with *Hin*dIII, the expected fragment and 3 longer fragments were obtained. The similar pattern was seen when cut with *Hin*dIII and *Apa*LI. Most likely, the truncated reporter gene might also have been integrated into genomic DNA and

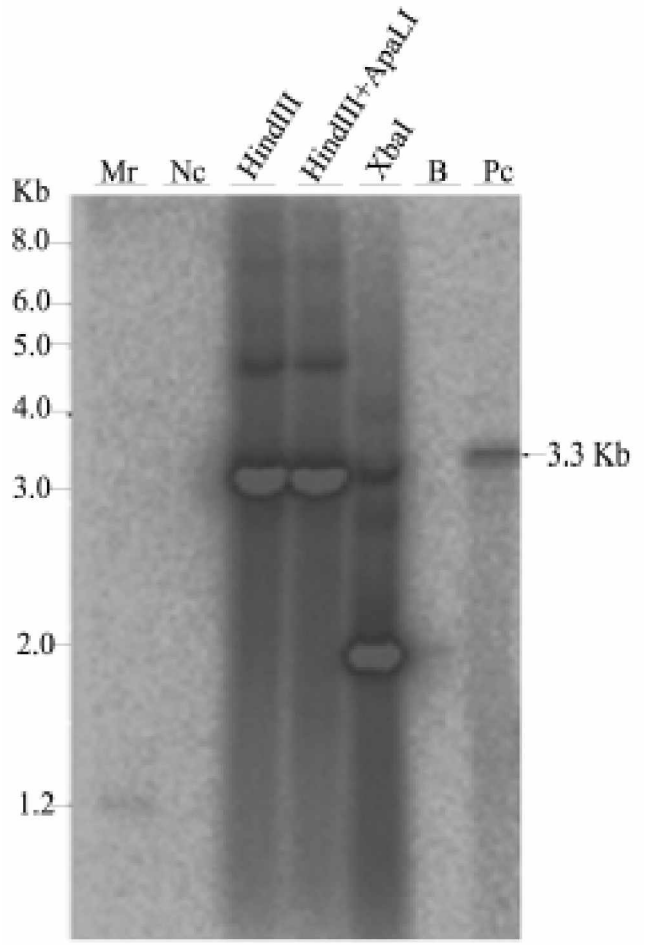

Figure 6. Southern blot evidence showing the transgene integrated within the genome of transgenic chicken No.5 that were generated after insemination of hen with chicken spermatozoa that had been transfected by ORF-EGFP/liposome complex. Genomic DNA samples were digested with *Hin*dIII & *Apa*LI before they were subjected to Southern analysis using a random priming probe for hybridization. Nc: negative control; B: blank; Pc: positive control.

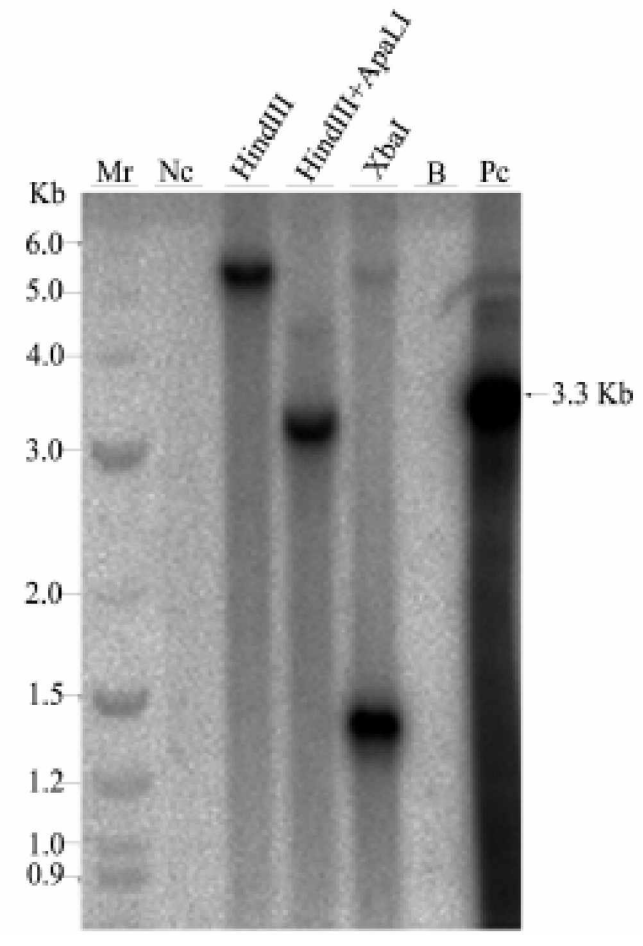

Figure 7. Southern blot evidence showing the transgene integrated within the genome of transgenic chicken No.102 that were generated after insemination of hen with chicken spermatozoa that had been transfected by CX-EGFP/ liposome complex. Genomic DNA samples were digested with *Hin*dIII & *Apa*LI before they were subjected to Southern analysis using a random priming probe for hybridization. Nc: negative control; B: blank; Pc: positive control.

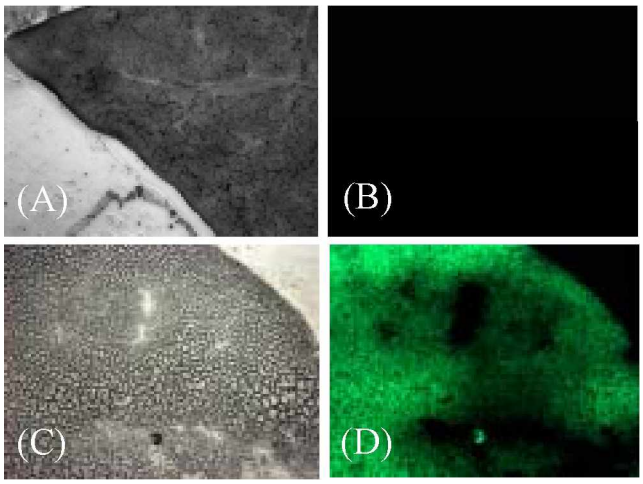

Figure 8. The expression of CX-EGFP gene in the chicken liver. (A) control, hen's liver observed under inverted microscope (10×5). (B) control, hen's liver observed under fluorescence microscope (10×5). (C) liver of 12 day chick embryo visualized with inverted microscopy (10×5) (D) liver of 12 day chick embryo visualized with fluorescence microscopy (10×5).

sticked to the intact fragment. When *Xba*I was used, the intacted and truncated forms were found. It is reasonable to hypothesize that the transgene, full lenth or damaged, might have formed a concatamer with head to tail in a randomized fashion. Figure 7 contains the results of blood genomic DNA of No. 102 chick that was artificially inseminated with spermatozoa which originated from the CX-EGFP/liposome complex treatment group. When it genomic DNA treated with *Hin*dIII, a fragment longer than 3.3 Kb was obtained; a fragment with length 3.3 Kb and another fragment with length 4.3 Kb was obtained via *Hin*dIII and *Apa*LI; and a fragment with length 1.4 Kb was obtained via *Xba*I treatment. It is thought that the transgene had integrated into the genomic DNA of the chick. At the protein level, we observed the expression of EGFP in the liver of 12 day chick embryo (Figure 8). This result confirmed that using sperm as a mediator for foreign DNA is possible in domestic chickens and successful. As indicated by the Southern blot analysis the hybridization patterns were complicated. In the study done by Tsai et al. (1995), the researchers generated transgenic Chinook salmon by electroporation, and using the same protocol in making transgenic Japanese abalone (Tsai et al., 1997). They found that the fragment hybridized via Southern blot analysis method was longer than expected and there were many similar fragments with varied lengths. According to Maione et al. (1997), the unexpected lengths of the fragments were probably a result from the reaction of endogenous nuclease, a spermatozoa nucleus enzyme, thereby rearranging the genomic DNA sequence and creating unexpected DNA fragments. The previous researches also stated when foreign DNA entered the ejaculated spermatozoa, the nuclear scaffold become dense; and thus enhancing the non-homologous recombination caused by the topoisomeraseII that resided inside the spermatozoa nuclei. This research demonstrated that certain sequences of the foreign DNA/transgene are permitted to insert into genome of the spermatozoa, which were the same as the DNA sequence that can be recognized by topoisomeraseII. Furthermore, topoisomeraseII, a DNA modifying enzyme, can easily create the rearrangement of foreign DNA and generate longer fragments (Zoraqi and Spadafora, 1997). The larger hybrids might be resulted from concatamer (Patil and Khoo, 1996).

In conclusion, transgenic animals generated via sperm vector probably result in the rearrangement of foreign DNA or missing DNA fragments. Thus, a number of unexpected fragments were generated and observed in the Southern blot analysis. This might explain why only few transgenic animals actually express the target protein (Kuznetsov et al., 2000).

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