韓國家畜繁殖學會誌 18(3) : 175~182(1994)
Korean J. Animal Reprod.

# Stable Expression of hGH Transgene in the Milk of Transgenic Mice

**Lee, C.S., S.J. Kim, Y.M. Han, D.Y. Yu and K.K. Lee**
Genetic Engineering Research Institute, KIST

# 유즙내 사람 성장 호르몬을 분비하는 형질전환생쥐의 형질 유전성에 관한 연구

**이철상 · 김선정 · 한용만 · 유대열 · 이경광**
한국과학기술연구원 유전공학연구소

## 요 약

Rat $\beta$-casein 유전자와 사람 성장호르몬 (hGH) 유전자의 융합유전자를 생쥐 수정란의 웅성전핵에 미세주입하여 형질전환생쥐 (transgenic mouse) 6계통을 확립하였다. 이들로부터 사람성장호르몬 (hGH) 유전자의 발현 여부를 조사한 결과, 6계통중 4계통의 생쥐 유즙에서 hGH가 2~900 ng/ml 수준으로 발현되고 있었으며, 혈중에서는 hGH가 검출되지 않았다. 따라서 이들 형질전환생쥐에서 사람 성장호르몬이 유선특이적으로 발현, 분비되고 있음을 알 수 있었다. 한편, 사람성장호르몬의 발현이 확인된 두 계통 (ChGH2-2, CChGH2)의 형질전환생쥐를 대상으로 하여 세대별, 산차별로 유즙내 사람성장호르몬의 함량을 조사한 결과, 3세대에 걸쳐, 또한 제1세대에서의 세차례 반복된 비유기에도 유즙내 사람성장호르몬은 지속적으로 분비되고 있었다. 이상의 결과는 형질전환생쥐에서의 외래유전자의 발현성은 반복되는 비유기와 여러 세대에 걸쳐 안정적으로 유지됨을 보여 주고 있다.

(Key word : rat $\beta$-casein, human growth hormone, trasgenic mice)

## I. INTRODUCTION

Transgenic animals capable of secreting pharmaceutical proteins into their milk have been pursued as a new mass production system (Gordon et al., 1987 ; Hennighausen, 1990). This system is based on the fact that the synthesis of a pharmaceutical protein can be directed to mammary gland of the animal by combining a regulatory element of the milk protein gene with the coding sequence of a pharmaceutically valuabel gene. As a model system, a variety of transgenic mice secreting foreign proteins such as tPA(Pittius et al, 1988), $\alpha_1$-antitrypsin (Archibald et al., 1990) and urokinase (Meade et al., 1990) in their milk were reported. Recently, this technology has been successfully applied to producting transgenic livestocks including sheep (Wright et al., 1991), goat (Ebert et al., 1991), pig (Wall et al., 1991) and cow (Krimpenfort et al., 1991). The results suggest that the mass production of pharmaceutical proteins may be feasible through the mammary glands of transgenic livestocks.

The rat $\beta$-casein gene encodes the principal murine casein and is mainly expressed during adult mammary development from virgin to mid-

This study was supported by a grant(N80231) from the Korea Ministry of Science and Technology.

lactation period (Hobbs et al., 1982). Transgenic mice, harboring the entire rat β-casein gene along with 3.5 kilobasepairs (kb) of 5' and 3.0 kb of 3' flanking sequence, expressed the transgene predominantly in the mammary gland during lactating period (Lee et al., 1988). The regulatory sequence (−2,300/+490 or −524/+490) of rat β-casein gene was sufficient to induce mammary gland specific expression of rat β-casein/CAT fusion genes in transgenic mice (Lee et al., 1989). Furthermore, the rat β-casein (−524/+490) fragment directed high level expression of bovine α and β subunit of follicle stimulating hormone in milk of transgenic mice and the expression was further increased by placing long terminal repeat sequence of mouse mammary tumor virus carrying four glucocorticoid response element sequences at its promoter region (Greenberg et al., 1991). These reports suggest that high level expression of the transgene in transgenic mice could be induced by a modificaiton of transcriptional regulatory elements.

In this study, it was ivestigated whether diffent 3' flanking sequences for transcriptional termination might affect the expression level of hGH transgenes in transgenic mice. In addition, heritability of transgenic phenotype to their progeny and stability of the expression in their life span were examined by measuring the expression level of hGH in the milk of transgenic mice.

## Ⅱ. MATERIALS AND METHODS

### 1. Construction of transgenes

To construct three different expression vectors, pC, pCS and pCC, 2.8 kb EcoRI fragment was liberated from rat β-casein genomic clone B14 which was kindly provided by Dr. J.M. Rosen (Jones et al., 1985). The EcoRI fragment (−2,300/+487) that comprises 5' flanking region 2.3 kb), first exon and first intron (0.45 kb) was subcloned into the EcoRI site of pSP70 vector (Promega, USA). It was designated as pC. For construction of pCS vector, 0.15 kb *HpaI/BamHI* fragment of $pSV_2CAT$ containing SV 40 poly (A) signal sequence was ligated to *EcoRV/Bgl II* site of pC vector. pCC vector was made by combining pC vector with 3.2 kb *Stu I/BamHI* fragment of B14 that consisted of the last intron, exon and 3' flankin region (2.2 kb) of rat β-casein gene.

For the expression of human growth hormone gene, three vectors (pChGH, pCShGH and pCChGH) were constructed as follows. To construct pChGH vector, 2.1 kb *BamHI/EcoRI* human growth hormone (hGH) gene fragment including entire hGH gene and 3' flanking region (0.5 kb) was filled with dATP, dTTP and dGTP by Klenow and then ligated to *ClaI/EcoRV* site of pC vector after Klenow filling-in reaction with dCTP. pCShGH and pCChGH were constructed by ligating 1.5 kb *BamHI/SmaI* fragment of hGH gene, which is free of poly (A) signal region, to ClaI site of pCS or pCC vector after filling-in reaction, respectively.

Three fragments, 4.9 kb(ChGH), 4.5 kb(CShGH) and 6.5 kb(CChGH) of β-casein/hGH fusion genes were released from each pChGH, pCShGH and pCChGH by *XhoI/Hpa I* digestion and purified by agarose gel electrophoresis and CsC1 gradient ultracentrifugation (Hogan er al., 1986) for pronuclear injection.

### 2. Generation and screening of transgenic mice

The purified DNA was microinjected to the pronucleus of fertilized eggs obtained from $F_1$ (C57BL/6 × CBA) females after mating with $F_1$ males. The injected eggs were transferred to pseudopregnant ICR female mice using a stan-

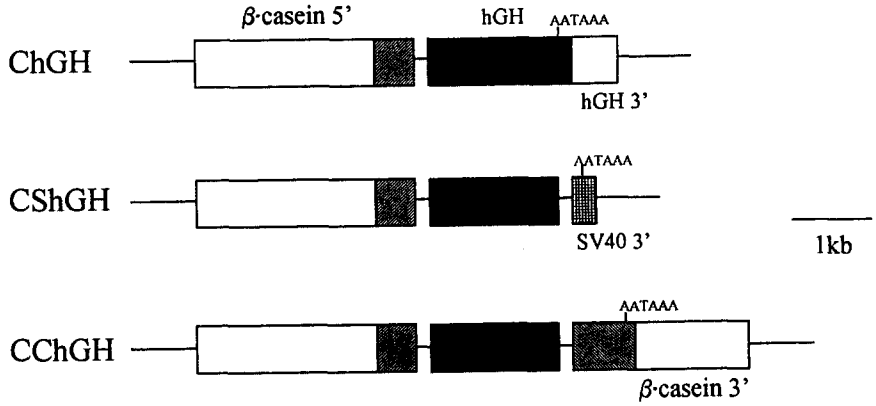


**Fig. 1. Constructs of rat β-casein/hGH hybrid genes for microinjection. The 2.8kb transcriptional regulatory sequence of rat β-casein gene was linked to hGH gene. ChGH consisted of structural hGH gene and its 3' flanking region. in CShGH, SV 40 poly(A) signal sequence was used for transcriptional termination. Transcriptional termination in CChGH was regulated by 2.2 kb 3' regulatory region of rat β-casein gene comprising last intron, exon and 3' flanking sequence. Symbols : □, flanking sequences of rat β-casein or hGH gene ; ▨, rat β-casein fist exon and intron ; ▧, rat β-casein last intron and exon ; ▩, SV 40 sequence for transcriptional termination ; ■, 5 exons and 4 introns of hGH structural gene.**

dard procedure (Hogan et al., 1986). To identify transgenic mice, purified tail genomic DNA (10 μg) was fractionated by electrophoresis on a 0.8 % agarose gel after *BamHI* digestion and transferred to nitrocellulose membrane. After baking at 80°C for 2 hours, the membrane was applied to Southern hybridization using $^{32}$P-labeled *PvuII* fragment of hGH gene as a probe. (Hogan et al., 1986 ; Southern, 1975 ; Sambrook et al., 1989). The copy number of the transgene and transmission frequency of the transgenic mice were determined by using slot blot analysis (Hogan et al., 1986 ; Kafatos et al., 1979).

### 3. Collection of milk and blood

Milk and blood were collected from transgenic and non-transgenic nursing mice on the 11 day of lactation. The mothers were separated from their pups and 4 hours later, injected intraperitioneally with sodium pentobarbital (18 mg/30 g of body weight, Somnopentyl, Pitman Moore, USA) and oxytocin (10 units/mouse, Sigma, USA). Milk cellection was accomplished by gentle suction with hand-controlled vacuum pump form mammary gland (Mityvac, Nalge, USA) and blood was collected from tail. Skim milk and serum were isolated by a brief centrifugation and then stored at −20°C for hGH radioimmunoassay(RIA). The concentration of human growth hormone in milk and serum was determined by a heterologous double-antibody RIA using a commercial hGH RIA kit (Incstar, USA). This kit quantifies hGH at concentrations ranging form 1 to 30 ng/ml.

## Ⅲ. RESULTS

### 1. Production of transgenic mice carrying rat β-casein/hGH fusion gene

Total 1870 pronuclear stage eggs were microinjected with three linearezed DNA fragments (ChGH, CShGH and CChGH). Of them, 1273

**Table 1. Profiles of transgenic mice.**

| Transgenic mice | Fertility /sex | Transmission rate | Copy number | hGH concentration (ng/ml) | |
|---|---|---|---|---|---|
| | | | | Milk | Serum |
| ChGH1 | sterile / ♀ | | | | ND[1] |
| ChGH2-1 | fertile / ♂ | 12/16 | 1 | ND | ND |
| ChGH2-2 | | (segreate) | 1 | 160 | ND |
| CShGH1 | fertile / ♀ | 2/4 | 1 | 80 | ND |
| CShGH2 | fertile / ♂ | 0/12 | | | |
| CShGH3 | fertile / ♂ | 5/11 | 2 | 2 | ND |
| CChGH1 | fertile / ♂ | 0/24 | | | |
| CChGH2 | fertile / ♀ | 4/8 | 12 | 900 | ND |
| CChGH3 | fertile / ♀ | 3/15 | 2 | ND | ND |
| Non-transgenic control | | | | ND | ND |

[1] ND, not detectable ; the hGH concentration was lower than 1ng/ml, below the detectable level by using the hGH RIA Kit.

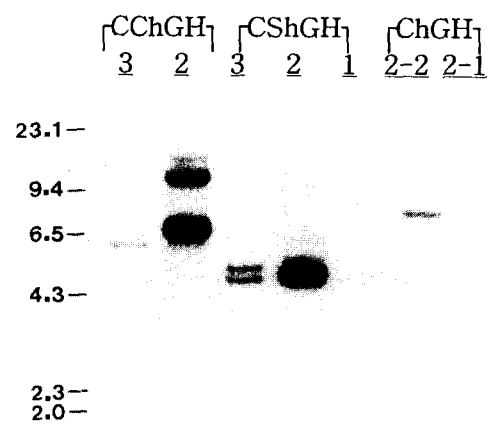


**Fig. 2. Southern hybridization of genomic tail DNAs isolated from transgenic mice. Purified tail DNA (10 μg) was digested with *BamHI*, which is unique restriction site of the transgenes, fractionated by electrophoresis on a 0.8% agarose gel, transferred to nitrocellulose membrane, and hybridized to $^{32}$P-labeled *PvuII* fragments of hGH gene.**

eggs were transferred to pseudopregnant recipients and then 210 pups (16.5%) were born. Of 169 pups screened by Southern hybridization, 8 mice were identified as transgenic(Table 1). It was also revealed by Southern analysis that entire sequences of transgenes were integrated as multiple copies on their genomes in some transgenic lines (Fig. 2). Four of them were female and the others were male. One (ChGH1) of founder mice was infertile. 7 transgenic mice mated with fertile $F_1$ males or females gave birth to their progeny. But two founder mice (CShGH2, CChGH1) did not transmit their tansgenes to their $G_1$ (generation 1) progeny. ChGH2 line was segregated to two independent lines (ChGH2-1, ChGH2-2) because its transgene was integrated at two different loci. The copy number of the transgenes in the transgenic lines was between 1 and 12(data not shown).

### 2. Secretion of hGH into the milk of transgenic mice

The concentration of hGH in the milk of the transgenic mice is shown in Table 1. Two transgenic female founders (CShGH1, CChGH2) and transgenic daughters of two transgenic male

founders (ChGH2-CShGH3) secreted hGH into their milk at the levels of 2~900 ng/ml, whereas two lines (ChGH2-1, CChGH3) did not. However, hGH was not detected at all in sera of transgenic and non-transgenic mice as well as in milk of non-transgenic mice.

### 3. Inheritance and maintenance of the transgenic phenotype

Two transgenic lines (ChGH2-2 and CChGH2) secreting a considerable amount of hGH into their milk were subjected to an experiment to access the inheritance and maintenance of transgenic phenotype. They were bred for three generations and in their $G_1$ mice, pregnancy was induced three times repeatedly. The mean concentration of hGH in milk form each generations was 94, 78, 156 ng/ml in ChGH2-2 line and 355, 412, 592 ng/ml in CChGH2 line (Table 2). In each lactation period of the transgenic $G_1$ mice, hGH was secreted into milk at the levels of 94, 85, 72 ng/ml in ChGH2-2 and 355, 570, 206 ng/ml in CChGH2, respectively (Table 3).

## Ⅳ. DISCUSSION

Four transgenic line that secrete hGH into their milk were developed from 3 different vectors. In these transgenic lines, human growth hormone was detected in milk, but not in serum (Table 1). The results indicate that the regulatory region (−2,300/+487) of rat $\beta$-casein gene is sufficient for tissue-specific expression in transgenic mice and three types of flanking sequences in our vectors do not affect the tissue-specificity.

The expression levels observed were lower than those of other fusion gene expression systems including mouse whey acidic protein (mWAP)/hGH (Tojo et al., 1993), mWAP/human tissue plasminogen activator (Pittius et al., 1988), bovine $\beta$-lactoglobulin/human $\alpha_1$-antitrypsin (Archibald et al., 1990) and bovine $\alpha_{s1}$-casein/human urokinase (Meade et al., 1990) in transgenic mice. Our result is in agreement with those of Lee et al. (1988) and Bühler et al.

**Table 2. Human growth hormone concentration in the milk of transgenic mice in three successive generations**

| Transgenic lines | hGH concentration (ng/ml)[1] | | |
|---|---|---|---|
| | 1st generation | 2nd generation | 3rd generation |
| ChGH2-2 | 94 | 78 | 156 |
| CChGH3 | 355 | 412 | 592 |

[1] Each value represents the mean of the hGH concentration in milk collected from two individual mice in each generation.

**Table 3. Human growth hormone concentration in the milk of $G_1$ transgenic mice in repeated lactation periods**

| Transgenic lines | hGH concentration (ng/ml)[1] | | |
|---|---|---|---|
| | 1st lactation | 2nd lactation | 3rd lactation |
| ChGH2-2 | 94 | 85 | 72 |
| CChGH3 | 355 | 570 | 206 |

[1] Each value represents the mean of the hGH concentration in milk collected at each lactation period of two individual $G_1$ mice.

(1990). They pointed out that the lack of a powerful 5' and/or 3' cis-acting regulatory β-casein sequence might contribute to the low expression of a gene construct. In spite of the low level of hGH expression in our transgenic mice, its specificity to milk suggests that rat β-casein promoter is useful for hGH production form transgenic animal, because it has been reported that ectopic expression of hGH causes adverse reproductive consequence (Tojo et al., 1993 ; Bartke et al., 1988). In this present study, only one transgenic mous(ChGH1) showed female infertility. But it would not be caused by ectopic expression of hGH because hGH was not detected in serum. Greenberg et al. (1991) reported that rat β-casein (−524/+490) fragment directed high level expression of heterodimeric bovine follicle stimulating hormone in milk of transgenic mice and the expression was further increased by placing long terminal repeat of mouse mammary tumor virus carrying four glucocoticoid element sequences at rat β-casein promoter. This means that if appropriate engineering is given to rat β-casein fusion gene, high level expression of transgenes can be achieved. In adition, the result that hGH expressing phenotype of our transgenic mice was stably inherited to their subsequent generations and maintained in their life span confirms the suefulness of transgenic technology for the mass production of pharmaceutical proteins.

In this study, we intended to show the effect of different 3' flanking sequences on hGH gene expression directed by rat β-casein promoter in transgenic mice. But it was impossible because only one or two transgenic lines were established from each three different gene constructs. Although the highest expression level (900 ng/ml) was set up in CChGH2 line carrying rat β-casein 3′ flanking region, it was not clear whether the highest expression was caused by 3′ flanking sequence of rat β-casein gene, copy number of the transgene or position effect. It is well known that the integration site heavily influcences the expression of a transgene in transgenic mice (Palmiter and Brinster, 1986). This phenomenon was clearly observed in this study. In two independent lines (ChGH2-1, ChGH2-2) segregated from ChGH2 founder mouse, only ChGH2-2 line expressed hGH in spite of same copy number of transgene. Therfore, to confirm the enhancing activity of rat β-casein 3′ flanking sequence on hGH gene expression, we are now trying to produce additional transgenic lines from three different gene constructs.

## V. SUMMARY

Six transgenic mouse lines that carry three different rat β-casein /hGH fusion gene constructs containing rat β-casein promoter, human growth hormone (hGH) gene and three different 3' flanking sequences were establised by pronuclear injection procedure. Four lines of them secreted 2~900 ng/ml of human growth hormone into the milk, but not into the serum. To assess the inheritance and maintenance of the transgenic phenotype, hGH concentrations were determined in the milk collected from the following three generations of founder mice and three successive lactation periods of their generation 1 ($G_1$) mice using two transgenic lines (ChGH2-2, CChGH2) that show a considerable expression of hGH their milk. These two transgenic lines stably secreted hGH into their milk through multiple generations and lactation periods.

## VI. REFERENCES

1. Archibald, A.L., M. McClenaghan, V. Hornsey and J.P. Simons. 1990. High-level ex-

pression of biologically active human $\alpha_1$-antitrypsin in the milk of transgenic mice. Proc. Natl. Acad. Sci., 87:5178-5182.

2. Bartke, A., R.W. Steger, S.L. Hodges, T.A. Parkining, T.J. Collins, J.S. Yun and T.E. Wagner. 1988. Infertility in transgenic female mice with human growth hormone expression : Evidence for luteal failure. J. exp. Zool., 248:121-124.
3. Bühler, T.A., T. Bruyere, D.F. Went, G. Stranzinger and K. Bürki. 1990. Rabbit $\beta$-casein promoter directs secretion of human interleukin-2 into the milk of transgenic rabbits Bio/Technology, 8:140-143.
4. Ebert, K., M.J.P. Selgrath, P. Ditullio, J. Denman, T.E. Smith, M.A. Memon, J.E. Schindler, G.M. Monastersky, J.A. Bitale and K. Gordon. 1991. Transgenic production of a variant of human tissue-type plasminogen activator in goat milk : Generation of transgenic goats and analysis of expression. Bio/Technology, 9:835-838.
5. Gordon, K., E. Lee, J.A. Vitale, A.E. Smith, H. Westphal and L. Hennighausen. 1987. Production of human tissue plasminogen activator in transgenic mouse milk. Bio/Technology, 5:1183-1187.
6. Greenberg, N.M., J.W. Anderson, A.J.W. Hsueh, K. Nishimori, J.J. Reeves, D.M. deAvila, D.N. Ward and J.M. Rosen. 1991. Expression of biologically active heterodimeric bovine follicle-stimulation hormone in milk of transgenic mice. Proc. Natl. Acad. Sci., 88:8327-8331.
7. Hennighausen, L. 1990. The mammary gland as a bioreactor : Production of foreign proteins in milk. Protein Expression and Putification, 1:3-8.
8. Hobbs, A.A., D.A. Richards, D.J. Kessler and J.M. Rosen. 1982. Complex hormonal regulation of rat casein gene expression. J. Biol. Chem., 257:3598-3605.
9. Hogan, B., F. Costantini and E. Lacy. 1986. Manipulating the mouse embryo, A laboratory manual. New York : Cold Spring Harbor Laboratory Press.
10. Jones, W.K., L.Y. Yu-Lee, S.M. Clift, J. Brown and J.M. Rosen. 1985. The rat casein multigene family. J. Biol. Chem., 260:7042-7050.
11. Kafatos, F.C., C.W. Jones and A. Efstratiadis. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res., 7:1541-1552.
12. Krimpenfort, P., A. Rademakers, W. Eyestone, A. Van der Schans, S. Vander Broek, P. Kooiman, E. Kootwijk, G. Platengurg, F. Pieper, R. Strijker and H. de Boer. 1991. Generation of transgenic dairy cattle using '*in vitro*' embryo production. Bio/Technology, 9:844-847.
13. Lee, K.F., F.J. DeMayo, S.H. Atiee and J. M. Rosen. 1988. Tissue-specific expression of the rat $\beta$-casein gene in transgenic mice. Nucleic Acids Res., 16:1027-1041.
14. Lee, K.F., S.H. Atiee and J.M. Rosen. 1989. Differential regulation of rat $\beta$-casein-chloramphenicol acetyl transferase fusion gene expression in transgenic mice. Mol. Cell. Biol., 9:560-565.
15. Meade, H., L. Gates, E. Lacy and N. Lonberg. 1990. Bovine $\alpha_{s1}$-casein gene sequences direct high level expression of active human urokinase in mouse milk. Bio/Technology, 8:443-446.
16. Palmiter, R.D. and R.L. Brinster. 1988. Germ-line transformation of mice. Ann. Rev. Genet., 20:465-477.
17. Pittius, C.W., L. Hennighausen, E. Lee, H. Westphal, E. Nicols, J. Vitale and K. Gordon. 1988. A milk protein gene promoter dir-

ects the expression of human plasminogen activator cDNA to the mammary gland in transgenic mice. Proc. Natl. Acad. Sci., 85:5874-5878.

18. Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular cloning, A laboratory manual. 2nd edition. New York : Cold Spring Harbor Laboratory Press.
19. Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., 98: 503-509.
20. Tojo, H., S. Tanaka, A. Matsuzawa, M. Takahashi and C. Tachi. 1993. Production and characterization of transgenic mice expression a hGH fusion gene driven by the promoter of mouse whey acidic protein (mWAP) putatively specific to mammary gland. J. Reprod. Dev., 39:145-163.
21. Wall, R.J., V.G. Pursel, A. Shamay, R.A. McKnight and C.W. Pittius and L. Hennighausen. 1991. High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. Proc. Natl. Acad. Sci., 88:1696-1700.
22. Wright, G., A. Carver, D. Cottom, D. Reeves, A. Scott, P. Simons, I. Wilmut, I. Garner and A. Colman. 1991. High level expression of active human $\alpha$-antitrypsin in the milk of transgenic sheep. Bio/Technology, 9:830-834.