

## Coexpression of Exogenous *hLYZ/hLF* Genes in Bovine Mammary Gland Epithelial Cells

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**Abstract:** Expression sequences of human Lactoferrin (LF) and Internal Ribozyme Entry Site (IRES) were successfully connected by double enzyme digestion and directed cloning and recombination plasmid pIL was constructed. Mammary gland specific expression plasmid pEBHIL of human lysozyme and human LF was constructed by BamHI restrictive enzyme digestion. Plasmid pEBHIL was introduced to cow mammary gland epithelial cells after liposome transfection. Positive cells were determined by G418 and PCR after EGFP expression observed by fluorescent microscope and proliferated and induced by hormone. The results of Western Blotting analysis on cell supernatant showed that transfected cells had expressed and secreted human lysozyme (MW 14.7 ku) and human LTF (MW 40 ku).

**Key words:** Human lysozyme, human lactoferrin, Green Fluorescent Protein (EGFP), transgenic, bovine mammary gland cells

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### INTRODUCTION

Some kinds of exogenous genes can express normally in transgenic animals including genes which code proteins. Therefore, it is possible that mammary gland can be used as bioreactor to produce rare medical proteins in a large scale. A few years ago, this possibility came true. Now, several activity proteins can be produced by bioreactor (Seymour *et al.*, 2004).

Human Lysozyme (hLYZ) can protect multi-cellular organisms from invasion of some kinds of microbes and has the functions of anti-infection, anti-tumor and immunologic regulation. Therefore, this enzyme is of great value in medicine and food industry (Rong and Ling, 1999). Although, natural hLYZ can be extracted directly from human milk, neutrophilic granulocytes and urine, the production is small. Moreover, the source of raw material is scarce, inefficient and difficult to extract. By now, *hLYZ* gene which has been obtained by such approaches as artificial synthesis or cDNA prepared from human tissues can express normally in prokaryotes and many eukaryotes such as *E. coli*, mouse (Yan *et al.*, 2005) and chicken (Zhu *et al.*, 2006). Human Lactoferrin (hLF) is an iron-binding protein widely existed in secretions such as milk and saliva. It is a double-globule shape composed

of a peptide chain has a molecular weight of 77kD (Nuijens *et al.*, 1997). hLF is capable of combining with many biomacromolecules such as heparin, polysaccharides and hLYZ (Van Berkel *et al.*, 1997). Studies *in vitro* proved that hLF can inhibit even kill many microbes such as gram-negative and gram-positive bacteria, aerobes, anaerobes, fungi and viruses and this function is proposed that hLF can bind Fe<sup>2+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> competing with pathogenic microbes, disturb latter carbohydrate metabolism. In addition, hLF can combine with other anti-microbial substances such as lysozyme so as to inhibit or kill pathogenic microbes. With increasing demands of milk products, the quality of milk product has attracted more and more concerns and mammary bioreactors are exploited in lactoferrin production to greatly improve milk product qualities (Van Berkel *et al.*, 2002).

Internal Ribosome Entry Site (IRES) (Ghettas *et al.*, 1991) is capable of translating two open reading frames of one mRNA simultaneously and its transcription is able to simultaneously translate two transcriptions in the downstream and upstream of IRES during the translation. As IRES can be connected with many related genes, they can be independently translated into a single-chain mRNA; in this way, many IRES sequence-linked genes

with same functions are safeguarded capable of independently expressing themselves and taking synergized effects. Many double-gene expression vectors have been constructed by exploiting IRES sequence in recent years (Liu *et al.*, 2004) and this makes it possible to get two exogenous genes simultaneously expressed by means of mammary bio-reactor.

In order to probe the effects of human lysozyme and human lactoferrin, the study constructed a common expression vector of lysozyme and human lactoferrin by linking lysozyme and human lactoferrin to one vector by means of IRES sequence and examined the expression of the common expression in mammary gland epithelia.

## MATERIALS AND METHODS

**Plasmids and strains:** Plasmid pMD 18-T vector was purchased from Takara Biotechnology (Dalian) Co., Ltd. (Takara, Dalian, China); plasmid pGEM-T easy vector from Promage (Shanghai, China); pIRES-Neo3 from Clontech; Recombinant plasmid pLTF with cDNA sequence of human Lysozyme (hLYZ) and mammary gland specific expression vector pEBH carrying 5'-regulation region and 3'-regulation region of  $\beta$ -CASEIN of milk cow, cDNA sequence of human Lactoferrin (hLF) and neomycin resistance gene, green fluorescent protein reporter gene and host bacterium DH5 $\alpha$  were taken from the storage of the research institute in which the researchers worked. Taq plus DNA polymerase, 10 $\times$ PCR Buffer and dNTP mixture were purchased from Dingguo Biotechnology Co., Ltd. (Dingguo, Beijing, China); restriction enzyme and marker from MetaBios Inc. (Victoria, Canada); T4 DNA Ligase and Wizard Plus Minipreps DNA Purification System from Promega company (Promega, USA); H.Q and Q. Gel extraction kit and Plasmid Rapid Isolation Kit from U-gene Biotechnologie Co., Ltd. (Anhui, China).

**Cloning, purification, sub-cloning and testing of hLF and IRES:** On purpose of ensuring the linkage of hLF to IRES, XbaI, restriction enzyme digestion sites were separately joined on to 5'-end of upstream primer and 5'-end of downstream primer of hLF and separately labeled as LsX and LaNB; BamHI and NheI/XbaI were separately joined on to 5'-ends of the upstream primer and downstream primer of IRES; the sequences of the primers were as follows:

LsX 5' TCTAGA CAGACCGCAGACATGAAACT 3',  
XbaI  
LaNB 5' GCTAGC GGATCC AATCCCCACCTTCA  
GCAG3',  
NheI BamHI

IsB 5' GGATCC GGCCTGTAAACCGTCGTACGTC  
TC 3',  
BamHI  
IaNX 5' GCTAGC TCTAGA GGTTGTGGCAAGCTTA  
TCATCGTGTTTT 3'  
NheI XbaI

The PCR amplification with pLTF as the template adopted the following reaction system and protocol: the reaction system was composed of 5  $\mu$ L 10 $\times$ PCR Buffer, 4  $\mu$ L dNTP mixture (10  $\mu$ mol L<sup>-1</sup>) 1  $\mu$ L LsX (1.0  $\mu$ mol L<sup>-1</sup>), 1  $\mu$ L LaNB (10  $\mu$ mol L<sup>-1</sup>), 1  $\mu$ L Taq plus DNA polymerase (5 U  $\mu$ L<sup>-1</sup>), 1  $\mu$ L plasmid pLTF and 37  $\mu$ L ultra-pure water and the reaction protocol proceeded as follows: after having denaturing at 94 $^{\circ}$ C for 3 min, denature for 30 sec then anneal at 50 $^{\circ}$ C for 45 sec and finally extend at 72 $^{\circ}$ C for 2 min and 30 sec; next to repeat the sequence 30 times and then extend at 72 $^{\circ}$ C for 10 min. The PCR amplification with pIRES-Neo3 as the template adopted the following reaction system and protocol: the reaction system was composed of 5  $\mu$ L 10 $\times$ PCR Buffer, 4  $\mu$ L dNTP mixture (10  $\mu$ mol L<sup>-1</sup>), 1  $\mu$ L IsB (10  $\mu$ mol L<sup>-1</sup>) 1  $\mu$ L IaNX (10  $\mu$ mol L<sup>-1</sup>), 1  $\mu$ L Taq plus DNA polymerase (5 U  $\mu$ L<sup>-1</sup>), 1  $\mu$ L plasmid pIRES-Neo3 and 37  $\mu$ L ultra-pure water and the reaction protocol proceeded as follows: after having denatured 94 $^{\circ}$ C for 3 min, denature at 94 $^{\circ}$ C for 20 sec, anneal at 50 $^{\circ}$ C for 45 sec, extend at 72 $^{\circ}$ C for 1 min; next repeat the sequence 30 times and finally extend at 72 $^{\circ}$ C for 10 min.

PCR products of hLF and IRES were run through electrophoresis and then the target segments were extracted according to the procedure described in the use manual of the Gel Extraction Kit. The isolated products were separately cloned to T sites of pGEM-T easy vector and pMD 18-T vector to transform competent *E. coli* DH5 $\alpha$  and then white positive colonies were screened; recombinant plasmids of hLF and IRES (named as pHLF and pIRES) were extracted with the white colonies. After their plasmid extraction, pHLF was tested by XbaI/NheI digestion and the insertion direction of pIRES into the target segments were tested by EcoRI digestion so as to find out inversely-linked plasmid.

**Constructions, transformation and testing of recombinant plasmid pIL:** The reaction systems of the two enzyme digestions were prepared according to the use manual of the restriction enzymes and their details were as follows: for the two reaction systems, the temperatures were set at 37 $^{\circ}$ C and the enzyme digestions lasted for 6-12 h. The digestion results were tested by 0.8% agar gel electrophoresis. After having been bathed in water bath at 80 $^{\circ}$ C for 20 min, small hLF segments were

extracted with pLTF and large IRES segments were extracted with pIRES. The detailed extractions were done according to the use manual of the Gel Extraction Kits. The products that were formed overnight by connecting the small and large segments were used to transform competent *E. coli* DH5 $\alpha$  and the Petri dishes of DH5 $\alpha$  were placed upside down and kept at 37°C for 12-16 h for its incubation then white parts of its colonies were delimited and stored as well as being oscillated; the plasmids were extracted from the white parts with plasmid rapid isolation kit and named as pIL. pIL was tested by XbaI digestion and its primers LsX and LaNB were tested by PCR. Then, the test results were tested by 0.8% agar gel electrophoresis. The results were recorded by photographing and in written form.

**Construction, purification and concentration determination of the mammary gland specific hLYZ/hLTF expression vector:** After pEBH and pIL had been digested with BamH I, ebh and il, large segments of the digestion were separately extracted and connected by means of T4 DNA Ligase and the connected segments were transformed into DH5 $\alpha$ ; after the plasmids were extracted from the transformed DH5 $\alpha$ , they were tested by BamH I digestion; in the meantime, they were PCR tested with the primers HLYs/HLYa as well as LsX/LaNB. The construction sketch of pEBHIL is presented as follows: In order to obtain endotoxin-free plasmids for facilitating cell transfection, the recombinant plasmids were purified by means of the Wizard Plus Minipreps DNA Purification System developed by Promega and then the ODs of their DNA samples were measured with ultraviolet spectrophotometer at the wavelengths of 260 and 280 nm then the concentrations and purities of the DNA samples and the concentrations of the purified DNA through spectrophotometry were calculated. The plasmids were stored at -20°C for cell transfection (Fig. 1).

**Tissue nubble culture and resistance testing of mammary gland epithelia of milk cow:** Mammary gland epithelia of milk cow were cultured on 60 mm culture boards by tissue nubble culturing. After the mammary gland epithelia had been purified, they were evenly placed on the 24-hole boards and cultured at 37°C, 5% carbon dioxide and a saturated humidity for 24 h and then G418 was added to the mammary gland epithelia. The concentrations of G418 were separated set at 100, 200, 300, 400, 500, 600, 700, 800, 1000 and 1200  $\mu\text{g mL}^{-1}$ . Alive cells were counted every 24 h. The screening concentration of G418 was defined the concentrations of G418 at which all alive cells died on the seventh culture day.

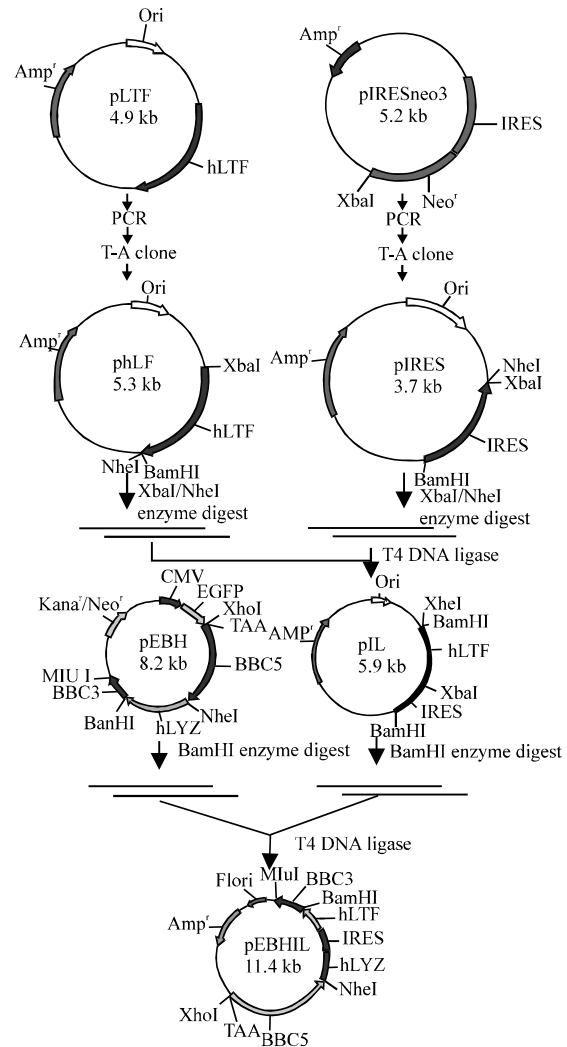


Fig. 1: Construction schematic drawing of pEBHIL

**Expression of liposome-mediated vector pEBHIF in mammary gland epithelia of mild cows:** About 1 day before transfection, mammary gland epithelia that were morphologically well developed and vigorous were screened and adjusted to have a concentration of  $1 \times 10^6$  cells  $\text{mL}^{-1}$ . And then they were placed on the 24 hole culture boards and cultured at 37°C, 5% carbon dioxide and a saturated humidity. When they grew to the density ranging within 70-90% they were cultured in serum-free and antibiotic-free culture solution. The transfection procedure was the one described in the use manual of Lipofectamine.

**Culture solution 1:** On the 24-hole culture boards, 50  $\mu\text{L}$  antibiotic-free and serum-free culture medium was added into each hole to dilute 3  $\mu\text{L}$  Lipofectamine 2000. Within 5 min after Lipofectamine 2000 dilution, the diluted

Lipofectamine 2000 was mixed with diluted DNA (prolonged incubation could result in decreased DNA concentrations).

**Culture solution 2:** About 50  $\mu\text{L}$  antibiotic-free and serum-free culture medium was added into each hole to dilute 2.0~2.5  $\mu\text{g}$  DNA and then the two were thoroughly mixed. Culture solutions 1 and 2 were lightly and thoroughly mixed and their mixtures were kept at room temperature for 20 min then the solution of well-coated liposome/DNA was carefully added in drops into the holes of the 24 hole culture boards and the mixtures in the holes were slightly oscillated to become thoroughly uniform; after this, the mixtures were incubated in a incubator with its temperature at  $37^\circ\text{C}$  and its  $\text{CO}_2$  concentration at 5% for 6-12 h. Next, D/F12, a medium containing 10% FBS, took the place of the original culture medium and then the incubation continued until the cells grew to occupy all the room spaces of the holes; after this, the cells were subcultured.

**Screening, sub-culturing and PCR testing of the positive cells:** After 48 h transfection, G418 parent solution was added into the Petri dishes until the final G418 concentration reached  $600 \mu\text{g mL}^{-1}$ ; the cells were cultured and screened at this concentration for 1 week and then subcultured with G418 concentration reduced to  $300 \mu\text{g mL}^{-1}$ . Genomic DNA of stable positive cells screened were extracted and amplified by PCR to sequence the cDNAs of human Lysozyme (hLYZ) and human Lactoferrin (hLF).

**Introduced cellular expression and its product testing:** PCR-positive monoclonal cells were chosen and multiplied in great quantities and then they were mixed with insulin ( $10 \mu\text{g mL}^{-1}$ ) hydrocortisone ( $20 \mu\text{g mL}^{-1}$ ) and prolactin ( $1 \mu\text{g mL}^{-1}$ ) and incubated in their specially designed culture solution. About 2 days later, the supernatant of the culture solution was taken and centrifuged at  $1000 \text{ r min}^{-1}$  for 10 min; next, it was concentrated by means of dialysis bag and stored at  $-20^\circ\text{C}$ . Afterwards, Western blotting was adopted to test the expression of human lysozyme and human LF.

## RESULTS AND DISCUSSION

**Results of IRES and hLTF amplification by PCR:** About 986 bp IRES segments and 2255 bp hLTF segments were obtained in the amplification of IRES and hLTF by PCR which were the expected results. The results are shown in Fig. 2.

**Test results of phLF and pIRES by restriction enzyme digestion:** Recombinant plasmid phLF was digested with

XbaI/NheI and then about 3.0 kb segments and about 2.2 kb segments were obtained which were the expected segments. Recombinant plasmid pIRES was digested with EcoR I and then about 2.6 kb segments and about 1.0 kb segments were obtained; they proved inversely connected thus applicable to future vector construction. The results are shown in Fig. 3.

**Test results of recombinant plasmid pIL:** Recombinant plasmid pIL was digested with XbaI and about 5.8 kb segments were consequently obtained. The segments were PCR amplified with primers LsX and LaNB and about 3.2 bp segments were consequently obtained which were the expected segments (Fig. 4).

**Test results of recombinant plasmid pEBHIL:** Recombinant plasmid pEBHIL was digested with BamH I

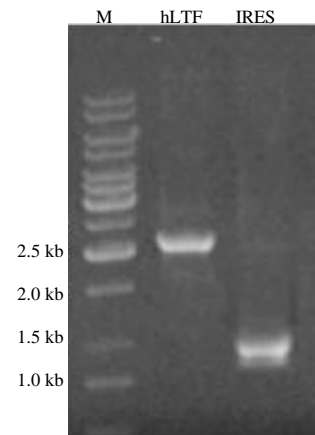


Fig. 2: PCR results of pIRES and phLTF (M, DNA Marker, hLTF, PCR product of phLTF, IRES, PCR product of pIRES-NEO+)

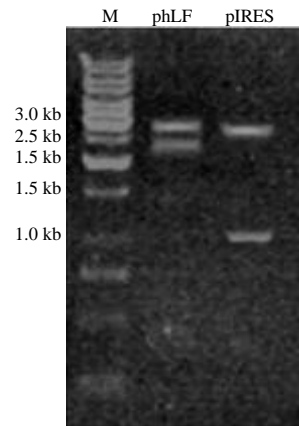


Fig. 3: Result of phLTF and pIRES (M, DNA Markers; phLF, result from enzyme digest of phLF; pIRES, result from enzyme digest of pIRES)

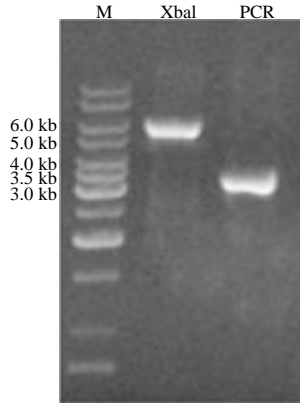


Fig. 4: Result from enzyme digest of pIL (M, DNA Marker; XbaI, result from enzyme digest of pIL; PCR, PCR product of pIL)

and about 8.2 kb segments and about 3.2 kb segments were consequently obtained. The segments amplified with primers (HLYs/HLYa) and (LsB/LaNB) were 0.85 and 3.2 kb, respectively which were the expected ones (Fig. 5). The primers of HLYs/HLYa were showed as following:

HLYs 5'GCACTCTGACCTAGCAGTCAACATG 3'  
 HLYa 5'GCAGGAGAATGGCGTGAACC 3'

**Resistance test results:** It was necessary for G 418 to enrich neomycin-positive resistance cells during their transfection and thus the G418 concentration most applicable to EMT6 cells had to be screened in advance. The cells were divided into equal parts and cultured at different G418 concentrations and alive cells of different parts were counted; all the cells cultured at the G418 concentration of 600  $\mu\text{g mL}^{-1}$  died out within about 7 days so that this concentration could be adopted to screen transgenic cells as a high-stress approach and the the G418 concentration of 300  $\mu\text{g mL}^{-1}$  was adopted in conducting sustaining culture of the cell clones.

**EGFP test results of transfected cells:** After having been transfected, the positive cells were screened and sub-cultured for 10 days and then their EGFP expressions were examined by fluorescence microscopy (Fig. 6).

**PCR results of the positive cells:** Genomic DNA of stable positive cells screened were extracted and amplified with special primers (LsB/LaNB and HLYs/HLYa) and about 3.2 and 0.85 kb segments were obtained which were the expected segments (Fig. 7) indicating that target DNA had been integrated into the genome of the cells.

**SDS-PAGE results:** In the SDS-PAGE, the polyacrylamide gel demonstrated that in the 1st lane, there

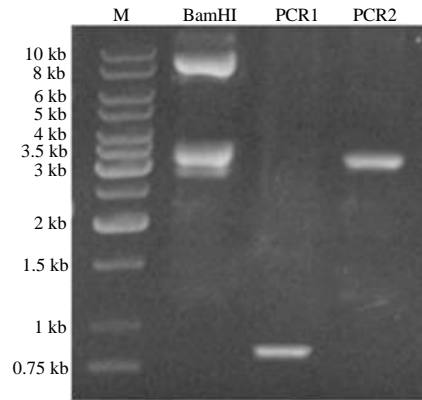


Fig. 5: Result from enzyme digest of pEBHIL [M, DNA Marker, BamHI, result from enzyme digest of pEBHIL, PCR1, PCR product from primer (HLYs/HLYa); PCR2, PCR product from primer (LsB /LaNB)]

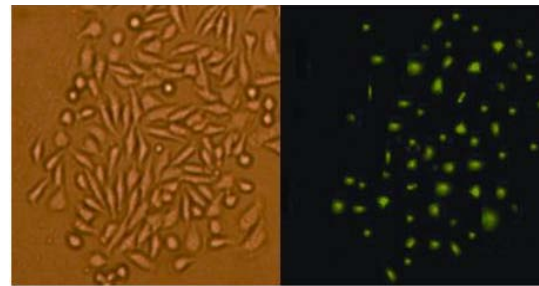


Fig. 6: Expression of EGFP on bovine mammary epithelial cells (left, the embryos under light microscope; right, the embryos under fluoroscope)

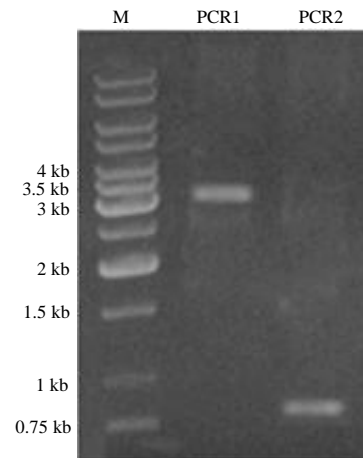


Fig. 7: PCR result of positive cells [M, DNA Marker; PCR1, PCR product from primer (LsB/LaNB); PCR2, PCR product from primer (HLYs/HLYa)]

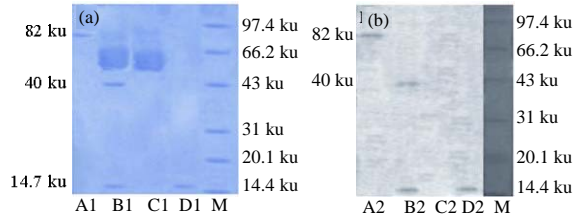


Fig. 8: a) SDS-PAGE results of expression products (left); b) results of Western blot analysis of expression products (right)

was a band ranging within 66-97 ku which stood for the standard sample of human Lactoferrin (hLF). The 2nd lane (test group 1) stood for the supernatant in the culture of mammary gland epithelia carrying transformed human Lactoferrin (hLF) gene and human lysozyme gene of cow. The 3rd lane (control group 1) stood for the culture solution of the cells carrying no transfected genes. The 4th lane stood for the standard sample of lysozyme. The 5th lane presented six clear bands which stood for the marker (Fig. 8a).

**Western blotting results:** Western blotting test indicated that human lysozyme and human Lactoferrin (hLF) were detected to exist and their molecular weights were 14.7 and 42 ku, respectively (Fig. 8b).

All previous researches have tried to express individual proteins (Wright *et al.*, 1991; Caver *et al.*, 1993) or >2 proteins by injecting different gene components together, thus sometimes making different genes integrated at the same sites (Clark, 1992); their disadvantages are that while one gene component is favored such as expression of the inhibitory substance to one endogenous protein, the expression of another protein is favored; their advantages is that the expressions of recombinant proteins with the same or similar functions such as human lysozyme and human lactoferrin with anti-microbial and anti-virus functions are of great importance. The simultaneous expressions of different proteins, for instance are favorable for the expression of another protein. Besides fusion protein expression, there are two common methods of getting many genes expressed in one vector: the first method is that two genes rely on their own promoters and the second is that all the genes share one promoter while they connect one another by means of IRES. On double-promoter vectors, it is sometimes difficult to get two target genes effectively expressed because the promoters interfere with each other and it is more liable for the genes in the downstream of 3'-end promoter to suffer this interference more than those in the downstream of 5'-end

promoter (Proudfoot, 1986). The process of IRES-mediated translation is independent, i.e., the same transcripts are transcribed into different proteins (Wallardo *et al.*, 1997). Hence, the study exploited  $\beta$ -casein promoter in combination with IRES hoping that IRES components were manipulated to replace internal promoters so as to overcome inter-promoter interference and then improve expression efficiency. In addition, the expressions of EGFP and target proteins are ensured to be non-fusion expressions because TAA, a termination code was joined on to 5'-end in the upstream of  $\beta$ -casein promoter.

It is the easiest and most effective cloning strategy of vector construction that two restriction enzymes are employed to digest exogenous segments and plasmid vectors and then the digestion products are connected. Although, the study successfully constructed the common expression vector of human lysozyme and human lactoferrin, the construction came across many problems. Firstly as the enzyme-digestion sites of pEBH, a currently existing vector had limitations, it was only able to digest well-connected segments of IRES and human lactoferrin with BamH I and then connected them with Pebh, thus increasing the necessity of its testing the connection direction. Secondly, considering the lengths of the segments and the limitation of enzyme-digestion sites of the cloning vectors, it could not but join IRES on to pMD 18-T Vector and then test the connection direction during the construction of recombinant plasmid pIL to see if the construction be adequate. If it joined IRES on to pGEM-T easy vector, although it could avoid the inconvenience of testing the connection direction, it was not favorable for the segments to be extracted and purified.

Because of its relatively favorable characteristics as high transfection rate, higher security, easy and convenient use, Liposome has been widely adopted in gene transformation systems without virus meditation (Ma, 2002). The compounds formed by positively charged liposome and vector DNA can avoid the digestion of vector DNA with restriction endonuclease and DNaseI and transform gene components that they carry into target cells through cellular endocytosis and fusion (Qian and Xiao, 1999).

The study transformed the mammary gland-specific expression vector of human lysozyme into breast carcinoma cells (EMT6) of mice by liposome transfection and then tested the cells with G418 proving the expression of the resistant genes in the expression vector. The examination by fluorescence microscopy showed that green fluorescence proteins were mainly expressed in the cytoplasm of transfected cells, thus proving the rationality of the design of the expression vector.

G418, a medicine similar to neomycin, kills cells by inhibiting ribosome functions and protein synthesis. But neomycin does not exert influence on eukaryotic cells and G418 has effect on bacterium and eukaryotic cells. Neo is the gene encoding 3'-phosphotransferase and the proteins that it expresses are able to degenerate neomycin and G418. During cell transfection, cell membrane is affected and antibiotics probably affect cells; considering the sterilizing efficacy of G418, some people have proposed that antibiotics should not be added to cell transfection.

Recently there have been many studies on double-gene expression but no reports about studies concerned with common expression of different proteins in mammary glands of transgenic milk cow. Some researchers separately constructed transgenic mice carrying transformed granulocyte colony-stimulating factor (G-CSF) or long acting tissue type plasminogen activator (La-tPA) by microinjection and bred double transgenic mice carrying both transformed G-CSF and La-tPA by crossing the mice carrying transformed G-CSF with the mice carrying transformed La-tPA under the circumstances that the mice carrying transformed G-CSF or La-tPA expressed G-CSF or La-tPA. Wei *et al.* (2005) constructed pIRES2-EGFP-GGF2, a double transgenic eukaryotic expression vector carrying both green fluorescence protein and glial growth factor 2, transformed it into spinal cord tissues in the chest part of rats by liposome transfection and got both EGFP and GGF2 expressed in the spinal cord tissues.

In bicistronic vectors, the expressions of exogenous genes in the downstream of IRES are 20~50% of the expressions of cap initiation translation genes indicating the post-transcription expressions are not equal (Kapturczak *et al.*, 2002). Western blotting is the first choice in testing if the expression products of one gene are right or comparatively examining the relative variations of the amounts of its expression products and that it can also be used to indicate the relative variations of target proteins. The study adopted Western blotting to test the expressions of human lysozyme and human Lactoferrin (hLF) in mammary gland epithelia of milk cow; in the study, the combination of the samples with the antibodies indicated that the cultured cells were able to express human lysozyme and human Lactoferrin (hLF) and but the expressions of human lysozyme and human lactoferrin could not quantitatively determined and thus needed further research.

## CONCLUSION

The study tried to show that during the construction of a mammary bioreactor, expression vectors should be

first employed to transfect *in vitro* cultured mammary gland epithelia and the expression levels of exogenous genes should be tested on cellular level to determine if the designs and effectiveness of expression vectors were proper; next, positive transgenic cells should be employed as the nucleus donors to produce transgenic animals. This also is a high-efficiency and high-precision method.

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