Co-expression of IRES-mediated hG-CSF cDNA and hGH Gene under the Control of Goat beta-Casein Promoter

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ABSTRACT : We developed a novel dicistronic system for the expression of target cDNA sequences in the milk of transgenic animals using goat beta-casein/hGH fusion construct, pGbc5.5hGH (Lee, 2006) and internal ribosome entry site (IRES) sequences of encephalomyocarditis virus (EMCV). Granulocyte colony-stimulating factor (hG-CSF) cDNA was linked to 3' untranslated region of hGH gene in the pGbc5.5hGH via EMCV IRES sequences. Transgenic mice were generated by microinjection and transgene expression was examined in the milk and mammary gland of transgenic mice at 10 days of lactation. Northern blot analysis showed that hGH gene and hG-CSF cDNA were transcribed as a single dicistronic mRNA. The hG-CSF and hGH proteins were independently translated from the dicistronic mRNA and secreted into the milk of transgenic mice. The highest concentration of hG-CSF and hGH in the milk of transgenic mice were 237 ㎍/㎖ and 8,990 ㎍/㎖, respectively. In contrast, another hG-CSF expression cassette, in which hG-CSF genomic sequences were inserted into a commercial milk-specific expression vector (pBC1), generated a lower level (91 ㎍/㎖) of hG-CSF expression in the milk of transgenic mice. These results demonstrated that the novel pGbc5.5hGH-based dicistronic construct could be useful for an efficient cDNA expression in the milk of transgenic animals.

Key words : cDNA, IRES, beta-Casein, Dicistronic, Milk, Transgenic mice.

INTRODUCTION

Functional transgenesis have enabled farm animals to produce large amount of recombinant proteins into their milk, thus referred as bioreactors. The cow and goat have been preferred to use as bioreactors because of their high milk yield. Several studies have reported generation of transgenic cow and goat producing tissue plasminogen activator (Ebert et al., 1991; 1994; Shen et al., 2007), lactoferrin (van Berkel et al., 2002), hG-CSF (Ko et al., 2000), and butyrylcholinesterase (Huang et al., 2007). Currently, a recombinant antithrombin III from goat milk was first approved in Europe and USA to use for human as a drug.

To generate transgenic animal, cloning of target gene from the genome and construction of fusion gene construct with mammary gland-specific regulatory sequences are essentially required procedures. As a target gene, cDNA sequences are beneficial for the reason of their short size, allowing making it easy to isolate from the genome and to insert those into the defined regulatory sequences of expression construct. However, expression levels from cDNA sequences was usually lower than those from full genomic sequences (Drohan et al., 1994; Barash et al., 1996; Colman et al., 1996). The efficient expression from full genomic sequences is thought due to the presence of enhancers within introns. For this reason, the first and last few genomic introns have been frequently included to the expression cassettes, such as a commercial pBC1 (InVitrogen, USA), together with mammary gland-specific regulatory sequences.

Previously, we reported inefficient expression of hG-CSF cDNA in the milk of transgenic goat under the control of goat beta-casein promoter although the first intron of goat

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beta-casein gene was included in the expression construct (Ko et al., 2000). To improve hG-CSF expression, recently we carried out co-injection of goat beta-casein/hG-CSF construct together with a highly expressing goat beta-casein/hGH construct (Oh & Lee, 2009). We expected that the weak hG-CSF expression could be rescued by the co-integrated highly expressing hGH gene in double transgenic mice (Clark et al., 1992; 1994). However, there was no improvement of hG-CSF expression in both mRNA and protein levels although goat beta-casein/hGH expression was maintained at high level in the double transgenic mouse. Consequently, we designed another novel strategy to improve hG-CSF expression in mRNA level by transcriptional coupling of the weak hG-CSF expression with the strong hGH expression via internal ribosome entry site (IRES) mediation.

The majority of mRNA is recognized to ribosome through methylguanosine capped 5'end, and cap-binding complex proteins to promote translation initiation, thus known as cap-dependent initiation mechanism. However, some mRNAs, particularly in viruses, bypass the conventional scanning mechanism by IRES elements, which are long and highly structured sequences, possessing the ability to initiate an efficient cap-independent protein synthesis (Holcik et al., 2000; Sonenberg & Hinnebusch, 2009). The IRES elements were first discovered in the picornaviruses encephalomyocarditis virus (EMCV) (Jang et al., 1988), and currently those IRES have been widely used in experiments and pharmaceutical applications to express protein in eukaryotic cells (Bochkov & Palmenberg, 2006). These features of IRES led us to examine whether the weak cDNA expression could be improved by coupling it with a strong goat beta-casein/hGH expression via IRES element. We constructed an expression vector, in which IRES-coupled hG-CSF cDNA sequences were inserted into 3'untranslated sequences of hGH, possibly allowing that goat-beta casein promoter drives synthesis of dicistronic mRNA. Here we report that a novel strategy, which is IRES-mediated translation system under control of goat beta-casein/hGH construct, is effective for increasing the expression of cDNA sequences in transgenic mice.

MATERIALS AND METHODS

1. PCR Cloning of IRES and hG-CSF cDNA Sequences

PCR amplification was carried out with a Taq DNA polymerase possessing a proofreading activity (ExTaq; Takara). EMCV IRES was amplified from pIRESneo vector (Clontech) using *Eco*RI-linked forward primer 5'-AAGAATTCTGCA TCTAGGGCGGCCAATT-3' and *Nco*I-linked reverse primer 5'-GGCCATGGTATCATCGTGTTTTTCAAAG-3'. The hG-CSF cDNA was cloned using total RNAs isolated from mammary gland epithelial cells, which were collected by brief centrifugation of milk of transgenic goat expressing hG-CSF (Ko et al., 2000). Briefly, the cell pellet was lysed in Trizol reagent (InVitrogen), and subsequently the total RNA pellet was dissolved in DEPC-treated water. The reverse transcription was carried out with SuperScript™ first-strand synthesis system (InVitrogen), and the hG-CSF cDNA was synthesized by PCR using *Nco*I-linked forward primer, 5'-GACCCATGGCTGGACCTGCCA-3' and *Eco*RV-linked reverse primer 5'-GGGATATCTCAGGGCTGGGCAA-3'.

2. Construction of a Dicistronic pGbc-hGH-IRES-hGCSF Vector

Cloned IRES segment and hG-CSF cDNA sequences were together inserted into *Eco*RI and *Eco*RV of a pBluscript II vector (designated as pIRES-hGCSF). Subsequently, 3' untranslated region (3' UTR) of hGH gene containing poly(A) signal sequences was excised by *Pvu*II/*Xho*I digestion and then inserted into the downstream from IRES-hGCSF. Sequences containing intron III through exon V of hGH gene were synthesized by PCR using a primer set (5'-AACCCG GGCAGCACAGCCAAT-3' and 5'-CAGAATTCCAGCTAGAAG CCA-3') and inserted into the *Sma*I/*Eco*RI sites in pIRES-hGCSF (designated as p⊿hGH-IRES-hGCSF). Next, *Sma*I/*Xho*I insert fragments of p⊿hGH-IRES-hGCSF were isolated

and inserted into the pGbc5.5hGH (Lee, 2006) by replacing the sequences corresponding to the hGH gene, which was designated as pGbc-hGH-IRES-hGCSF (Fig. 1A). To test hG-CSF expression from its genomic sequences in a commercially available expression cassette, genomic sequences of hG-CSF gene were isolated from pGbc-hGCSF (Ko et al., 2000) by *Eco*RV and *Xba*I digestion and inserted into *Xho*I site of the pBC1 (InVitrogen) after modifying their ends to *Xho*I/*Sal*I using pSP73 vector (Promega), which was designated as pBC1hGCSF (Fig. 1B).

3. Generation of Transgenic Mice

For microinjection, the insert fragments were isolated from pGbc-hGH-IRES-hGCSF and pBC1hGCSF by digestion with *Not*I/*Xho*I and *Sal*I/*Not*I, respectively, as shown in Fig. 1. The concentration of each DNA solution was adjusted to 4 ng/㎖ in Tris-Cl/EDTA buffer (pH 8.0). Transgenic mice were generated by DNA injection into the pronuclei of mouse zygotes. Transgenic founders were screened by PCR analysis using tissue lysates of ears (Chen & Evans, 1993).

4. Collection and Analysis of Milk and Blood Samples

Milk was obtained from transgenic mice at 10 days of lactation as described previously (Lee, 2006). The milk samples were kept at −80℃ before analyzing. Blood samples were collected from orbital sinus of mice at different developmental stages of mammary gland, virgin, 10 days, 14 days, and 18 days of pregnancy, and 10 days of lactation, following Hoff's protocol (Hoff, 2000). The concentrations of hGH in milk and blood samples were determined using radioimmunoassay (RIA) kit specific for human growth hormone (Daichii). The concentrations of hG-CSF in milk were determined using its specific ELISA kit (R&D systems) according to manufacturer's instructions. Western blot analysis was previously described in detail (Ko et al., 2000). The antibodies against hGH and hG-CSF were purchased from Abcam and Oncogene Research Products, respectively.

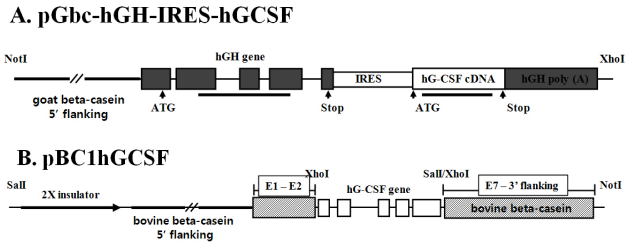

Fig. 1. The diagram of two hG-CSF expression constructs. A. The dicistronic pGbc-hGH-IRES-hGCSF construct. The IRES-coupled hG-CSF cDNA was inserted into untranslated region of exon V of hGH. The translation initiation codons and stop codons were indicated as arrow heads. The probes for northern blot analyses were represented under the construct as bold lines. B. pBC1hGCSF construct. hG-CSF genomic sequences including full coding region was inserted into multicloning site of pBC1 milk expression vector (InVitrogen, USA).

5. Northern Blot Hybridization

After milking at 10 days of lactation, the forth mammary gland of transgenic mice was taken out and homogenized in Trizol reagent (InVitrogen). Isolated total RNAs were melted in DEPC-treated water and then kept at −80℃ before analyzing. Total RNA samples (20 ㎍) were resolved on 1% agarose/formaldehyde gel and transferred to nylon membrane (Boehringer Mannheim). Northern hybridization was carried out with ^{32}P-labeled probes specific for the hGH and hG-CSF transcripts. The probes used for northern blot analysis were represented in Fig. 1.

RESULTS AND DISCUSSION

Four transgenic mice harboring the pGbc-hGH-IRES-hG CSF inserts were generated and three of them transmitted the transgene to next generation. Transgenic females were milked at 10 days of lactation and their mammary glands were taken out. To examine that hG-CSF could be translated by IRES mediation, we performed western blot analysis using the milk samples. The protein size of hG-CSF from pGbc-hGH-IRES-hGCSF and pBC1hGCSF were 22 kD, which was the same to the expected size of

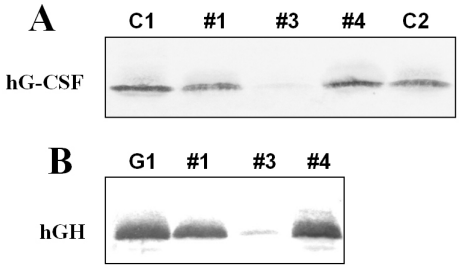

Fig. 2. Western blot analysis of milk hG-CSF and hGH from pGbc-hGH-IRES-hGCSF transgenic mice. The milk samples collected at 10 days of lactation were resolved on SDS-PAGE gel and transferred to membranes. A. The blot was incubated with hG-CSF specific antibody. The size of milk hG-CSF from pGbc-hGH-IRES-hGCSF mice (#1, #3 and #4) was identical to the controls, which were from pGbc5.5hGCSF (C1) and pBC1hGCSF consturct (C2). B. Western blot analysis was performed using hGH specific antibody. The size of hGH translated from the dicistronic construct (#1, #3 and #4) was identical to control from pGbc5.5hGH (G1).

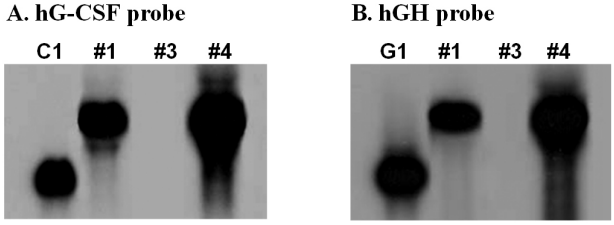

Fig. 3. Integrity of the dicistronic mRNA in the mammary gland of transgenic mice. Northern blot analysis was performed using specific hG-CSF probe (A) and hGH probe (B). Notably, the sizes of mRNAs from the dicistronic pGbc-hGH-IRES-hGCSF mice (#1, #3 and #4) were shown to be longer than those from the control mice pGbc5.5hGCSF (A, C1) and pGbc5.5hGH (B, G1).

the recombinant hG-CSF (Fig. 2A). Milk hGH from pGbc-hGH-IRES-hGCSF also showed normal protein size and its size was the same as that of pGbc5.5hGH (Lee, 2006) transgenic mouse (Fig. 2B). To ensure that milk hGH and hG-CSF were translated from single dicistronic mRNA, we carried out northern blot analysis. As shown figure 3A, the blot, hybridized with hG-CSF specific probe, showed that mRNA from the mammary gland of pGbc-hGH-IRES-hGCSF mice was larger than that from pGbc5.5 hGCSF mice. This feature was not changed in northern hybridization signals using hGH-specific probe (Fig. 3B). These results demonstrate that milk hGH and hG-CSF are translated from single dicistronic mRNA. Notably, the signal intensity of hG-CSF mRNA from the dicistronic transcripts was stronger than that from pGbc5.5hGCSF mice (Fig. 3A) and even comparable to that of hGH mRNA from a highly expressing pGbc5.5hGH mouse (Lee, 2006).

We measured hG-CSF and hGH concentrations in the milk samples of transgenic mice. Milk hG-CSF level from three independent pGbc-hGH-IRES-hGCSF lines was 10, 187 and 237 ㎍/㎖, respectively. However, Milk hG-CSF level from three pBC1hGCSF mouse lines was as low as 45, 91 ㎍/㎖, and even undetectable (Table 1). Notably, hG-CSF expression level from its cDNA sequences in pGbc-hGH-IRES-hGCSF was higher than those from pBC1hGCSF and pGbc5.5hGCSF mice (Oh & Lee, 2009), in which genomic sequences of hG-CSF were used for the construction, suggesting that increased milk hG-CSF level from pGbc-hGH-IRES-hGCSF mice was resulted from the increased transcriptional activity of pGbc-hGH-IRES-hGCSF in the transgenic mice.

We also measured milk hGH concentrations in pGbc-hGH-IRES-hGCSF mice (Table 1). The hGH level was 121, 6,723, and 8,990 ㎍/㎖, respectively. The milk hGH level was very similar to the expression profile of pGbc5.5hGH

Table 1. The concentrations of hG-CSF and hGH in the milk of transgenic mice

Transgenic lineage	Concentration of	
	hG-CSF (㎍/㎖)	hGH (㎍/㎖)
pGbc-hGH-IRES-hGCSF		
#1	187	6,723
#3	10	121
#4	237	8,990
pBC1hGCSF		
#1	91	
#2	45	
#4	Not detected	

mice (Lee, 2006; Oh & Lee, 2009). This means that the appending pIRES-hGCSF to pGbc5.5hGH construct resulted in an increase of hG-CSF mRNA synthesis under the transcriptional regulation of hGH expression without any perturbation in the transcriptional and translational processes from this dicistronic expression construct.

As regulatory sequences to drive hG-CSF gene expression, bovine alpha s_1-casein promoter appears preferable to goat beta-casein promoter. Actually, Dvorianchikov et al. (2005) reported hG-CSF expression at the level of 1,000 ㎍/㎖, and Zhang et al. (2001) reported its expression at level of 540 ㎍/㎖ using bovine alpha s_1-caseion promoter in their transgenic mice. Given that pGbc-hGH-IRES-hGCSF dicistronic construct was useful to increase hG-CSF cDNA expression in this study, bovine alpha s_1-caseion promoter coupled to hGH-IRES-hGCSF sequences could lead to much better performance in the hG-CSF expression from its transgenic animals.

In our knowledge, we first carried out a dicistronic construct using IRES sequences to target mammary gland for production of a pharmaceutical protein, and showed that the dicistronic hGH-IRES-hGCSF construct in transgenic mice lead to an increased expression from hG-CSF cDNA in their milks. However, its actual level was still very low when compared to hGH level. It could be postulated that IRES is inefficiently working in the mammary epithelial cells of transgenic animals. Indeed, several studies reported that translation efficiency of IRES-mediated second cistron varies considerably from one cell type to another type, thus occasionally leading to low level expression of protein (Borman et al., 1997; Mizuguchi et al., 2000; Wong et al., 2002). Although EMCV IRES, which have been used in this study, is known to generally direct higher translation activity than any other IRES or capped mRNA (Borman at al., 1995), it still remains elusive whether IRES is completely functional in the mammary epithelial cells.

Growth hormone has been widened to clinical application as a therapeutic drug for GH deficient children and adults, and to various clinical conditions, not necessarily related to short stature (Murray & Shalet, 2000; Krysiak et al., 2007). To find pertinent fusion constructs ensuring high level expression of hGH gene, various milk protein promoters have been tested in transgenic mice (Devinoy et al., 1994; Ninomiya et al., 1994; Lee et al., 1996; 1997; Fujiwara et al., 1999). Consequently, several transgenic mice highly expressing hGH by limited promoters were reported, however, those mice frequently showed reproductive failure due to high level presence of hGH in the circulating blood at non-lactating stage (Devinoy et al., 1994; Ninomiya et al., 1994; Fujiwara et al., 1999). Eventually, Lee (2006) reported that goat beta-casein promoter directs high level expression of hGH in almost all transgenic mice, and hGH expression in circulating blood is restricted to the lactating stage, thus leading to those fertile. The dicistronic construct in this study led to high level expression of the first cistronic hGH, and nevertheless, the mice carrying it were fertile, likely as pGbc5.5hGH mice (Lee, 2006). To examined whether the gene expression from the dicistronic construct is properly controlled under the goat beta-casein promoter during mammary gland development, we measured hGH levels in circulating bloods obtained from different reproductive stages of two pGbc-hGH-IRES-hGCSF mice (#1 and #4). We found that the hGH levels in bloods showed reproductive stage-dependent

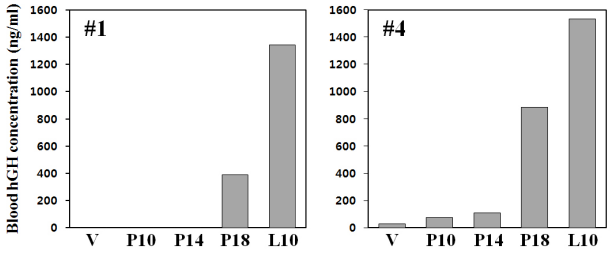

Fig. 4. Temporal regulation of hGH expression during development of mammary glands of the dicistronic pGbc-hGH-IRES-hGCSF mice (#1 and #4). Blood samples were collected from each of transgenic mice, at virgin (V), 10 days (P10), 14 days (P14), and 18 days (P18) of pregnancy, and 10 days of lactation (L10) stages, respectively. Concentrations of the hGH in blood were determined as described in materials and methods.

regulation in the mammary glands, showing very low level at virgin stage, and gradually increase at late pregnant and lactating stages (Fig. 4). This demonstrates that the transgene expression from the dicistronic pGbc-hGH-IRES-hGCSF construct is temporally regulated according to the developmental scheme of mammary gland in transgenic mice, similarly to the monocistroninc pGbc5.5hGH construct (Lee, 2006).

In conclusion, we first adopted IRES-mediated dicistronic construct in an attempt to improve target cDNA expression in the milk of transgenic mice and showed that the dicistronic pGbc-hGH-IRES-hGCSF construct lead to a moderately increased expression of hG-CSF in their milks.

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