Improved Expression of Muscle-derived Growth Hormone Releasing Hormone from α-Skeletal-muscle Actin Enhancer/Cytomegalovirus Hybrid Enhancer/Promoter

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ABSTRACT : Growth hormone-releasing hormone (GHRH), a hypothalamic neuropeptide can stimulate the growth hormone secretion from the anterior pituitary. In this study, a porcine GHRH expression plasmid pHC-GHRH was used to enhance growth performance through ectopic expressions in muscle tissues of rats. Rats injected with the plasmid of pHC-GHRH and pCMV-GHRH exhibited cumulative weight gains 6.4% and 1% greater than controls. During a 5-day period, significant weight gain differences were observed as follows compared with that of control: during 5-10 days post-injection (DPI) period, the group pHC-GHRH on average 14.5% heavier than controls, 40.73 ± 0.88 g vs. 35.57 ± 1.23 g (p = 0.0023); during 10-15 DPI period, the group pHC-GHRH on average 13.6% heavier than controls, 37.49 ± 2.85 g vs. 33.00 ± 1.56 g (p = 0.0146); during 15-20 DPI period, the group pHC-GHRH on average 17.8% heavier than controls, 25.64 ± 1.39 g vs. 21.77 ± 1.27 g (p<0.05). In addition, plasmids-treated rats maintained higher serum IGF-I than controls. Significant differences of IGF-I were observed on 13 DPI and on 40 DPI in pHC-GHRH group compared with that of controls. This was accomplished through the use of an improved expression cassette that included the cytomegalovirus (CMV) immediate early enhancer/promoter in combination with a 1.5-kilobase portion of porcine α-skeletal muscle actin promoter.
(**Key Words** : GHRH, Plasmid DNA, Hybrid Enhancer/Promoter, Growth, Rat, CMV)

INTRODUCTION

Growth hormone (GH) enhances protein synthesis, lypolysis, and epiphyseal growth, and is implicated in the regulation of the immune system (Carrel and Allen, 2000). In domestic livestock, GH stimulates milk production, increases feed-to-milk conversion, and sustains growth, primarily by increasing lean body mass (Etherton and Bauman, 1998; Lapierre et al., 1999), and increases overall feed efficiency. The regulated, distinctively pulsatile pattern of GH secretion from the anterior pituitary is under the control through stimulation by growth hormone releasing hormone (GHRH) and inhibition by stomatostatin, both hypothalamic hormones (Scanlon et al., 1996), and possibly stimulation by ghrelin (Kojima et al., 1999). Although GHRH protein delivery entrains and stimulates normal cyclical GH secretion (Corpas et al., 1993), the short halflife of the molecule *in vivo* requires frequent (1 to 3 times per day) intravenous, subcutaneous, or intranasal (at a

300-fold higher dose) administrations. Thus, recombinant GHRH administration is not practical. Extracranially secreted GHRH, as a mature or a truncated polypeptide, is often biologically active (Thorner et al., 1984), and a low level of serum GHRH stimulates GH secretion (Corpas et al., 1993).

Plasmid DNA vectors coding for GHRH gene has been used in a number of different animal species, including mice and pigs (Draghia-Akli et al., 1997; Draghia-Akli et al., 1999). In most direct plasmid DNA gene transfer tests, skeletal muscle is a preferred target tissue because of its long life span, ease of accessibility for intra-muscular injections and transduction by circular DNA plasmids, and large capacity for protein synthesis and secretion. Skeletal muscle borne plasmids have been expressed efficiently over months or years in treated animal (Davis et al., 1993; Tripathy et al., 1996). To increase the plasmid expression, Bupivacaine, a kind of local anesthetic, can be used to selectively destroy myofibers and to help enhance muscle cell regeneration, and to help plasmid DNA to transfer into muscle cell, where a functional protein can be produced using the cellular transcriptional and translational

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Table 1. Schematic maps of three plasmids

Vector	Different components of the plasmid constructs
pHC-GHRH	PSkaa pro→ CMVpro/enh+1/2 intron A→ PSkaa 1/2intron1→ GHRH cDNA→ PSkaa polyA→ Amp
pCMV-GHRH	CMV pro/enh→ CMV intronA→ GHRH cDNA→ PSkaa polyA→ Kan
pHC-control	PSkaa pro→ CMVpro/enh +1/2 intron A→ PSkaa 1/2intron1→ PSkaa polyA→ Amp

PSkaa-porcine skeletal muscle alpha actin; pro: Promoter; enh: Enhancer; Amp: Ampicilin resistance; Kan: Kanamycin resistance.

machinery (Akiyama et al., 1992). In this study, we demonstrated that expression of muscle-derived GHRH could be enhanced by a combination of porcine α-skeletal actin and CMV enhancer/promoter sequences driving gene expression.

MATERIALS AND METHODS

Construction of myogenic expression vectors and purification

The backbone vector pGEM-A5f3f was derived from pGEM-5zf (Promega, USA). The pGEM-A5f3f contains a 2.587 bp Nco I/Mlu I fragment, which includes a 1,849 bp porcine skeletal muscle α-actin (PSkaa) 5'-flanking region, the first exon and the first intron of PSkaa (Reecy et al., 1996), and contains a 613 bp Sac I/Nsi I fragment of the 3' flanking region of PSkaa. Then pGEM-A5f3f was used to ligate with the fragment of 286 bp GHRH cDNAs (including a 62 bp segment of human GH 5' UTR, a 93 bp segment of human GHRH leading peptide, and a 123 bp porcine GHRH cDNA) to create pG-A5f3f-GHRH (pH 6). Construct pHC-GHRH was based on the plasmid pH 6 and contained a 957 bp CMV enhancer/promoter and partial CMV intron A from pUMVC1-hpAP (Aldevron, USA). The 957 bp fragment replaced the Xho I/Bbe I segment of PSkaa 5'-flanking region (-84 ~ +422) in pH 6 to construct a chimeric CMV/α-actin enhancer/promoter and chimeric CMV/α-actin intron. Construct pCMV-GHRH was constructed by replacing the EcoR I /Bgl II segment in pUMVC1-hpAP with an 888 bp segment of GHRH cDNA and 3' flanking region of PSkaa in pH 6. Construct pHC-control was constructed as pHC-GHRH without GHRH cDNA.

The constructs of the plasmids are shown in Table 1. All plasmid DNA preparations were made using fermentor culture and purified by the Nucleobond AX Kit (Endotoxin Free) from Macherey Nagel (Germany). DNA preparations in phosphate buffered saline (PH 7.4, 10 mmol/L), were sterile filtered (0.22 μm), and shown to pass the TAL test for pyrogen content (<50 EU/mg).

Intramuscular injection of expression vectors in rats

Sprague-Dawley (SD) male SPF rats (4 weeks old) (Beijing Vital River Laboratory Animal Technology Company Ltd., P. R. China) were housed and cared for under specific pathogen--free conditions in the animal facility of the Institute for Space Medical Engineering

(Beijing, China). Animal experimentations were performed in accordance with guidelines of institutional medical or animal research committees for the care and use of laboratory animals, under environmental condition of 12 h light/12 h darkness and free access to food and water (room temperature). Rats were separated into three groups randomly (n = 25/group). On day 5 before plasmid injection, the left quadriceps muscle of rats (120-140 g of body weight) was injected with 100 μl of 0.75% bupivacaine hydrochloride (Grounds, 1991; Danko et al., 1994) in saline solution. On day 0, the animals were weighed, and regenerating muscle was injected with 200 μg of plasmids in 200 μl phosphate-buffered saline (PBS, pH 7.4). The animals were weighed and killed 5 to 40 days later. Blood samples were collected after the animal was anesthetized, centrifuged after being held overnight at 4°C, and stored at -80°C before analysis.

Gene expression analysis by RT-PCR

Total RNAs from the injected muscles were extracted using TRIzol reagent (Invitrogen, USA), then treated by DNaseI and 1 μg of total RNA was used in the reverse transcriptase reaction, using M-MLVRT (Life Technology Inc.) according to the manufacturer's instructions. In the negative control (-) reaction, the reverse transcriptase was not included. Specific primers were used to amplify a 465 bp fragment of all plasmids with the sense primer: 5' ATGCCACTCTGGGTGTTCTT 3' and the antisense primer: 5' AGCATAACAGAATGGCTGAG 3'. The PCR reaction was carried out with an initial denaturation at 94°C for 5 min; 30 cycles of 94°C, 20 s; 55°C, 20 s; 72°C, 20 s, and a final extension at 72°C for 7 min in 15 μl volume. A 630 bp fragment of rat β-actin cDNA as a control was performed as described before (Raff et al., 1997).

Rat serum IGF-I concentrations

Rat serum IGF-I concentrations were measured using the ELISA kit (Boster Inc., China) according to the manufacturer's instruction. Blood samples for assaying were taken on day 5, day13, day 23 and day 40 after plasmid injections.

Statistical analysis

Data were analysed with the General Linear Models Procedure of SAS software (ver. 7.0, SAS Institute. Inc. USA). Values are expressed as means±standard errors of the
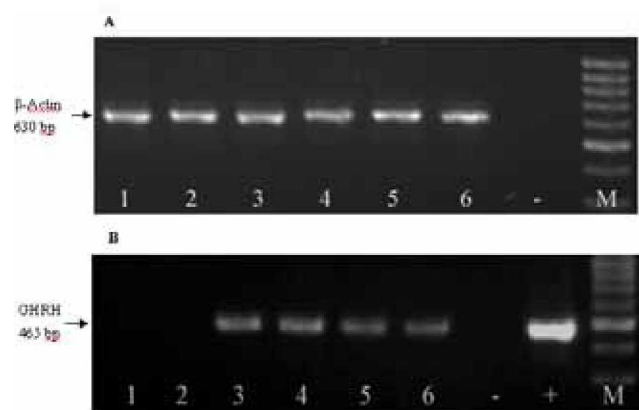
Figure 1. Expression of GHRH and β-actin was assayed by RT-PCR in treated muscle of rats on day 23 and 40 after injection of pHC-GHRH, pCMV-GHRH and pHC-con. A: PCR reaction using rat β-actin specific primers. 1-2: control on day 23 and 40 respectively; 3-4: pHC-GHRH on day 23 and 40 respectively; 5-6: pCMV-GHRH on day 23 and 40 respectively; -: negative control (not reverse transcribed). B: PCR reaction using GHRH cDNA specific primers. 1-2: control on day 23 and 40 respectively; 3-4: pHC-GHRH on day 23 and 40 respectively; 5-6: pCMV-GHRH on day 23 and 40, respectively; -: negative control (not reverse transcribed), +: positive control (template plasmid).

mean (SEM). The statistical significance of differences between plasmid DNA injection groups and the control group was evaluated independently with Duncan's t-test for multiple comparisons, and preceded by the analysis of variance (ANOVA). A p-value of less than 0.05 was considered to be significant.

RESULTS

Tissue expression of p-GHRH post-injection

The expressions of all plasmids *in vivo* were assayed by RT-PCR with RNA extracted from injected muscles (Figure 1). Total RNA was treated by DNase I to eliminate any contamination from the injected plasmid and 1 μg of total RNA was used in the reverse transcriptase reaction. RT-PCR analysis showed that the *in vivo* expression of the plasmids (465 bp PCR fragment) still could be detected even on 40 DPI when amplified with specific primer pairs. A 630 bp PCR fragment of rat cytoskeleton β-actin was used as a control. The efficiency of DNaseI treatment was determined by using RNA from tissue which had not been reverse transcribed and no amplification was observed.

Promoter of growth by myogenic expression vector

Weights of these animals were measured over 40 days to assess whether growth rate was affected by the injection of these constructs. The growth rate from groups injected with plasmids was augmented over 40 days (Figure 2A). The total average gains of body weight from the groups
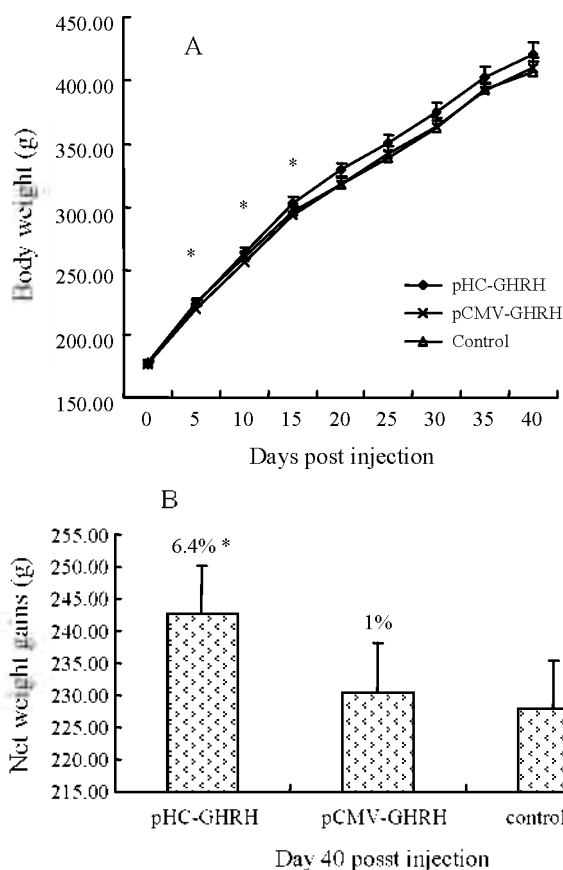

Figure 2. Increase in body weight after a single dose plasmid DNA injection in the regenerating left quadriceps muscle of rats. A: Body weight of rats in plasmids injected groups (n = 25). Significant weight gains differences were observed during 5-10 DPI period, 10-15 DPI period, and 15-20 DPI period respectively of pHC-GHRH group compared with control, (*p<0.05). B: Cumulative weight gains of rats on day 40 post injection in plasmids injected group (n = 25). Rate weight gain (%) = (total weight gain in treated group-total weight gain in control group)/ total weight gain in control ×100%. Rate weight gains of pHC-GHRH and pCMV-GHRH groups relative to control were 6.4% and 1% respectively. And also, significant difference of cumulative weight gain was observed of pHC-GHRH compared with control, (*p<0.05). Values are mean±SE.

pHC-GHRH and pCMV-GHRH injections were greater than controls (242.64±8.31 g, 230.51±7.44 g vs. 227.99±3.2 g, respectively) (Figure 2B). These results are consistent with the expression of constructs as well as the released IGF-I activities in serum and suggested that GHRH gene in plasmids can effectively augment the biological growth of animal by *in vivo* inoculation.

IGF-I concentration in rat serum

In order to assess the effects of expression of plasmids, the levels of serum IGF-I in the plasmids-injected animals were evaluated and compared with the control-injected group (Figure 3). IGF-I values in pHC-GHRH--treated
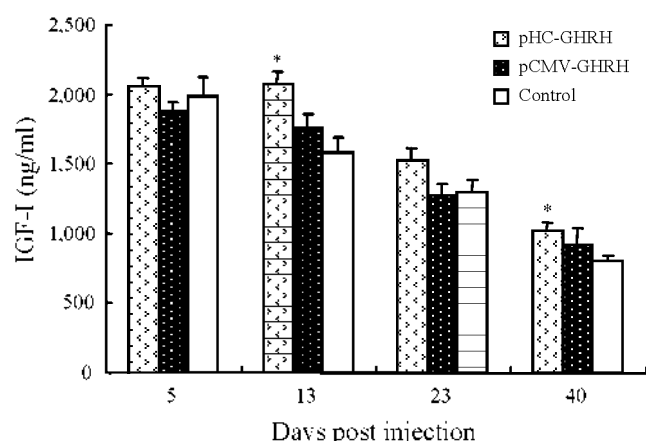
Figure 3. Serum IGF-I concentrations of rats measured after directly intra-muscular injection of pHC-GHRH, pCMV-GHRH and control. Significant differences were observed on day 13 and day 40 post injection of pHC-GHRH compared with controls, (* p<0.05). Values are mean±SE.

groups were maintained at a higher level than those of the control, though all values decreased with the time. Also, significance differences were seen in pHC-GHRH group relative to the control. On 13 DPI, 2,071.76±91.84 ng/ml of pHC-GHRH group vs. 1,577.33±117.13 ng/ml of control group (p = 0.0027); On 40 DPI, 1,016.45±66.93 ng/ml of pHC-GHRH group vs. 799.66±38.24 ng/ml of control group, (p = 0.0067).

DISCUSSION

The natural GHRH is expressed in the hypothalamus and passes to the pituitary by blood circulation triggering GH expression and secretion. In this research, we demonstrated the feasibility of delivering porcine GHRH expressed in muscle fibres via an expression plasmid. The mature GHRH secreted from muscle will go to the pituitary through blood circulation as natural GHRH does and will trigger GH expression and secretion. We found that the intra-muscular injection of pHC-GHRH resulted in higher body weight gain and IGF-I levels.

The CMV enhancer/promoter was considered as one of the strongest promoters, so we constructed one expression plasmid promoted by CMV promoter as a positive control. According to some reports, expression from the CMV promoter is clearly influenced by target tissue and vector sequences. It has been demonstrated that CMV promoter was shutdown in the context of a retroviral vector in muscle, but it could be prevented by addition of a muscle creatine kinase (mck) enhancer (Dai et al., 1992). In another study, it was shown that presence of one or multiple copies of mck enhancer in the vectors elevated the expression activity in myotubes by 4.5- to 19-fold over those without mck enhancer *in vitro* (Wang et al., 1996). Therefore, it was

possible that addition of a strong, muscle-specific element can improve the transcriptional activity of CMV promoter in muscle.

The pHC-GHRH containing the α-skeletal muscle actin/CMV promoter showed enhanced expression of GHRH in rats, compared with pCMV-GHRH. This might be because the addition of α-skeletal muscle actin enhancer sequences enhanced transcription activity of the CMV promoter in muscle cells. The α-skeletal muscle actin enhancer sequences were used in pHC-GHRH containing three serum response elements at bp-93, -166 and -209 (Taylor et al., 1988), a putative Sp1 binding site, and a binding site for bi-functional factor YY1 (Muscat and Kedes, 1987). In addition, conserved E-boxes at bp-280 (Davey et al., 1995) and a TEF-1 binding site (Mar and Ordhal, 1990) were included. This portion also contains the synergistic enhancer elements at -153 to -87 and -1,300 to -628 of the actin promoter. These data would suggest that muscle-specific up-regulated expression of the strong CMV enhancer/promoter element by α-skeletal muscle actin enhancer sequences in muscle fibers is far superior to expression from the CMV enhancer/promoter by itself. In sum, the synergistic effect on gene expression achieved by a combination of a CMV enhancer/promoter element with α-skeletal muscle actin enhancer sequences of gene transfer will be useful in future designs of expression cassettes, and the intra-muscular plasmid delivery may be efficacious in improving performance of domestic animals and constitute a step forward to human gene delivery.

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