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Effects of Introns on the Regulation of Porcine Growth Hormone Expression

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Abstract: It has been widely acknowledged that introns can influence the efficiency of gene expression. Most transcriptional regulatory elements are located within non-coding DNA. The aim of the study was to study introns affecting porcine Growth Hormone (pGH) expression levels. A program was developed for mammalian cells (PK15 cells) by measuring the enhancement effect on GH expression of different introns inserted with otherwise identical plasmids. After selection with G418, quantitative Polymerase Chain Reaction (qRT-PCR) assays was performed to determine pGH mRNA expression levels. Western blotting was used to analyze pGH protein expression. The qPCR and Western blot results revealed that various introns have different effects on pGH expression efficiency. The recombinant *pGH* gene with introns 3 and 4 had the highest expression activity. In this study, researchers have provided the first insights into the function of pGH introns, establishing a foundation for the recognition and better utilization of these introns.

Key words: Intron, porcine growth hormone, pGH, gene expression, plasmids

INTRODUCTION

The expression of eukaryotic genes requires multiple steps to achieve the synthesis of the correct amounts of encoded proteins. This remarkable finding regarding introns in the late 1970s (Evans *et al*., 1977) has resulted in the evolutionary origin and functional roles of introns being intensely studied. There is abundant evidence that introns can play important roles in gene regulation via alternative splicing and missense-mediated decay. Many studies report that the presence of introns significantly enhances gene expression in mammalian cultured cell lines, transgenic mice, insects and plants (Buchman and Berg, 1988; Callis *et al*., 1987; Choi *et al*., 1991; Nott *et al*., 2003; Parra *et al*., 2011). Much research has found that introns affect gene expression through participation in transcriptional and post-transcriptional mechanisms (Maniatis and Reed, 2002; Morello *et al*., 2002; Orphanides and Reinberg, 2002; Proudfoot *et al*., 2002; Rose, 2002). Introns affect transcription, polyadenylation, mRNA export, translational efficiency and mRNA stability (Le Hir *et al*., 2003). Some mechanisms of introns are suited to enhancing gene expression, with studies showing that intron composition, length and position within a transcription unit were all relevant. As an example, an early study demonstrated that the efficiency of expression of a foreign gene, containing introns, in transgenic mice was 10-100 fold higher than a gene lacking introns (Brinster *et al*., 1988). Similarly when compared with corresponding cDNA fragments, genomic intron-containing fragments delayed the onset and diminished the efficiency of transitive silencing of a secondary target (Vermeersch *et al*., 2010).

The protein porcine Growth Hormone (pGH) is a hormone composed of 190 amino acids that is synthesized and secreted by cells in the anterior pituitary gland. The whole gene including introns is 2231 nucleotides long, containing 5 exons and four introns (Vize and Wells, 1987). It is a major participant in controlling several complex physiological processes including growth and metabolism (Muskens *et al*., 2000).

In this study, different recombinant plasmids were constructed containing the GH coding sequence interrupted by each single intron or a combination of 2 or 3 introns as well as the whole genomic sequence with all introns. *PGH* gene expression was evaluated in selected clones by RT-PCR and by Western blot analysis of protein extracts and new data on the enhancing effects of introns on porcine GH expression in transfected cell cultures were presented.

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MATERIALS AND METHODS

Generation of recombinant pGH overexpression vectors with different introns: The pGH cDNA was synthesized by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) using total RNA and a one step RT-PCR protocol (TaKaRa, Dalian, China). A specific pGH DNA genomic was amplified by PCR (TaKaRa, Dalian, China). The initial PCR program used involved a thermal cycling profile with an initial incubation at 98°C for 5 sec, followed by 68°C for 90 sec this was repeated for 30 cycles. The primers used were a pGH sense primer (5'-ATA AGC TTC CAC CAT GGC TGC AGG CAA GTG CC-3') and an anti-sense primer (5'-CGT CTA GAC CAG CAA CTA GAA GGC ACA GCT GCT-3'). To obtain the pGH cDNA, the PCR program used was 98°C for 5 sec, followed by 68°C for 50 sec, repeated over 30 cycles. This reaction utilized a pGH sense primer (5'-ATA AGC TTC CAC CAT GGC TGC AGG CCC TCG GAC C-3') and an anti-sense primer (5'-CGT CTA GAC CAG CAA CTA GAA GGC ACA GCT GCT-3'). Restriction enzyme sites for Hind III and Xba I were incorporated into the forward and reverse primers, respectively. Purified PCR products were ligated into the pcDNA 3.1 (+) vector which researchers designated pcDNA-GH cDNA and pcDNA-GH genomic for the cDNA and gene, respectively (Fig. 1 and 2).

Plasmids pcDNA-GH cDNA and pcDNA-GH genomic were digested with Pfo I (Fermentas), Nar I (NEB) and Tth111 I (NEB) in separate reactions. Following recombination, another seven vectors were constructed: pcDNA-GH1 (pGH with intron 1); pcDNA-GH2 (pGH with intron 2); pcDNA-GH3 (pGH with intron 3); pcDNA-GH4 (pGH with intron 4); pcDNA-GH12 (pGH with introns 1 and 2); pcDNA-GH34 (pGH with introns 3 and 4) and pcDNA-GH123 (pGH with introns 1-3). The vector overexpressing pGH containing different introns was constructed via the ligation of different fragments from the vectors mentioned above. It was then transfected into *Escherichia coli* DH5α and selected by sequencing.

Cell culture and transfection: The porcine PK15 cell line was purchased from the China Center for Type Culture Collection. Cells were grown in Eagle's minimal essential medium, supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, at 37°C/5% CO_2. Cells were divided into 10 groups with each group transfected separately with the following plasmids: pcDNA-GH1 (GH1), pcDNA-GH2 (GH2), pcDNA-GH3 (GH3), pcDNA-GH4 (GH4), pcDNA-GH12 (GH12), pcDNA-GH34 (GH34), pcDNA-GH123 (GH123), pcDNA-GH cDNA and pcDNA-GH genomic, pcDNA3.1 (+) as a control. Transient transfection of the PK15 cell line was performed

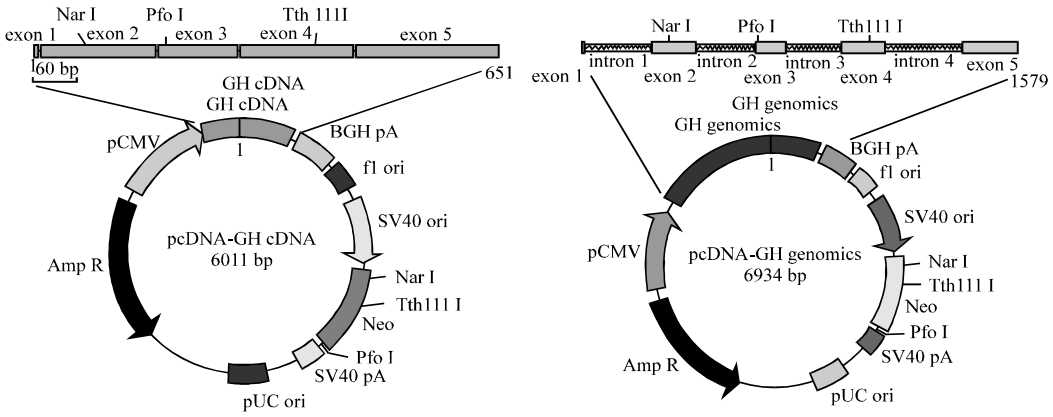

Fig. 1: Schematic diagram of vector

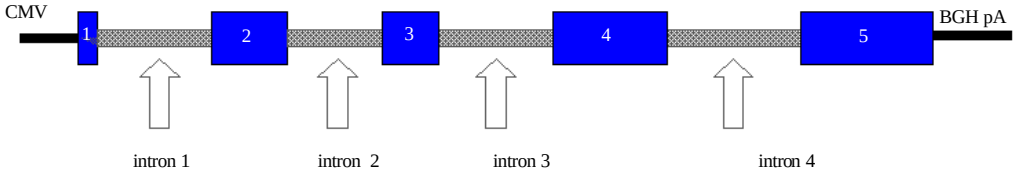

Fig. 2: Effect of PGH introns in their natural location. For constructs in different vectors, introns were inserted into the intronless gene by substituting regions from the genomic copy for comparable regions of intronless gene through the use of convenient restriction site

at 90% confluency using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The pcDNA3.1 (+) vector was used as negative control. Each plasmid was transfected in triplicate in at least three independent experiments. After selective culture in medium with G418 (1 g L^{-1}), the stable cell clones were used for RNA and protein extraction.

RNA preparation and qRT-PCR: Cellular RNA was extracted from the 10 cell groups using an RNeasy Mini kit (Qiagen) and treated with DNase using the Turbo DNA free kit (Bioteke, Beijing, China). First-strand cDNA was synthesized from 1 μg of total RNA isolated from different cells using Toyobo-ReverTra Ace (Toyobo, Tokyo, Japan) in accordance with the manufacturer's instructions. An aliquot of first-strand cDNA was then utilized for qRT-PCR. Sample loading and cycling conditions used for the qRT-PCRs was the same as those outlined in SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China). Amplification of the neomycin gene was conducted as an internal control. The reactions utilized the following pGH and neomycin primers: neomycin sense, 5'-CTG CCC ATT CGA CCA CCA AG-3'; neomycin anti-sense, 5'-GGA GCG GCG ATA CCG TAA AG-3'; pGH sense, 5'-AGC AGG GTC TTC ACC AAC AG-3' and pGH anti-sense, 5'-CTT AAG CAG CGC GTC ATC AC-3'.

Protein preparation and western blot: Total protein was extracted from the 10 cell groups using RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid disodium salt (EDTA), 1% Triton X-100, 0.1% dodecyl sodium sulfate (SDS), 1×Roche protease inhibitor cocktail). Fractions were solubilized in SDS sample buffer (100 mM TRIS-HCl pH 8.0, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue), heated at 100°C for 5 min and analyzed by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) using 12% polyacrylamide gels. Native PAGE was carried out using standard buffers lacking SDS or 2-mercaptoethanol. The gels were blotted onto nitrocellulose membranes overnight and membranes were then blocked for 1 h at room temperature in 3% skim milk and probed with polyclonal antibodies against pGH (1:300 dilution) for 1 h at room temperature in 3% skim milk. The blots were washed and subsequently incubated in the same buffer containing anti-rabbit immunoglobin conjugated with horseradish peroxidase (1:2500) for 1 h at room temperature. After extensive washing, the blots were developed with the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Meridian, USA). Densitometric measurements of band intensity on the blots were performed using Quantity One Software (Bio-Rad, USA).

RESULTS AND DISCUSSION

Detection of pGH expression by qRT-PCR: The qRT-PCR analysis of pGH expression was performed using β-actin as the housekeeping gene. The cells transfected with pcDNA3.1 (+) were the control group and cells transfected with the pGH overexpression vectors containing different introns were the experimental groups. The pGH mRNA expression level in experimental groups was markedly up-regulated compared with the control group. The pGH mRNA expression level for the recombinant pGH containing introns 3 and 4 (pGH34) was increased 1.637×10^3 (Fig. 3).

Detection of pGH protein expression: The expression profile of the pGH protein was studied using Western blotting. As can be seen in Fig. 4, pGH protein expression was dramatically increased in the experimental group when compared with the control. The results were almost with those observed for the qRT-PCR assays.

In the experiments, expression analysis was mainly performed at the protein level. However, RT-PCR data collected from intronless plasmids or plasmids carrying different introns, show a increase in PGH mRNA except GH2 that parallels that of PGH proteins thus confirming the general evidence that effect we can observed occurs at the transcriptional level. Muskens *et al.* (2000) found that parts of the genes are arranged as Inverted Repeats

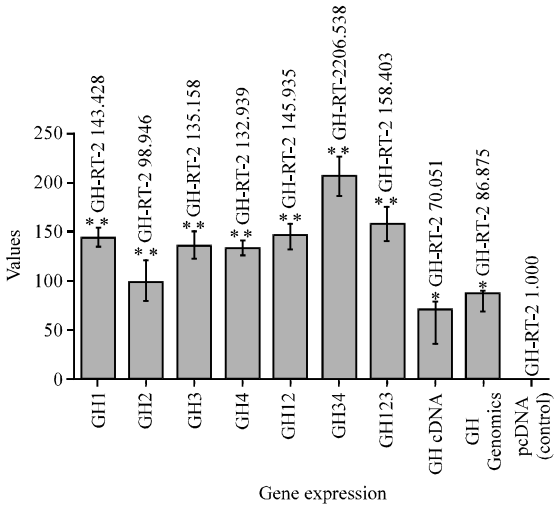

Fig. 3: pGH mRNA expression of different introns inserted with otherwise identical plasmids. The relative expression of pGH mRNA was compared with the internal control reference gene β-actin, the data represent the mean±SD from four independent experiments performed in duplicate (**p<0.01)

(IR) which are dominant silencing loci such as IRs can repress the expression of homologous genes elsewhere in the genome in trans which is usually associated with an increase in the level of DNA methylation (Muskens *et al*., 2000). And Lehmann (2004) have demonstrated that anything else but GAGA, a nonhistone protein complex reshapes chromatin structure, suggesting that it corresponds to a negative regulatory element. Interestingly, pGH2 consists of a 9 bp reverse repeat unit with the sequence GGAGAGGGG and a 7 bp reverse repeat unit with the sequence GAGAGGG and a 6 bp reverse repeat unit GAGGGG (Fig. 5), researchers can conclude that high density of GAF binding site may help recruit arrays of the GAF and play a role in the maintenance of such mostly heterochromatic regions in a post-transcriptionally repression state.

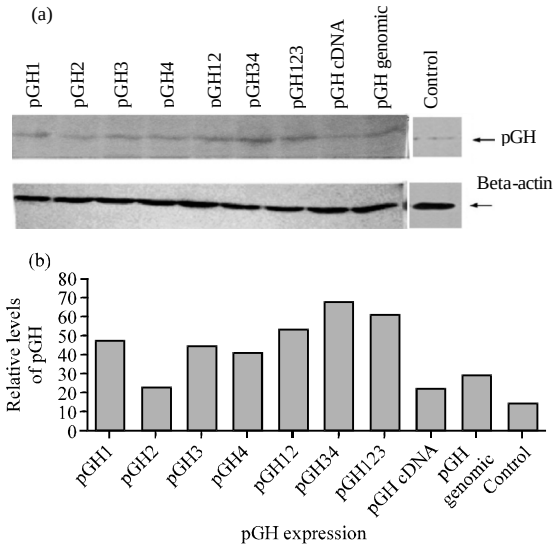

Fig. 4: Western blot analysis of pGH expression of different introns inserted with otherwise identical plasmids in different cell groups. pGH protein (50 mg per lane) obtained from different cell groups that were transfected with pGH overexpression vectors containing different introns. a) Hybridization with pGH and β-actin probes; b) a plot of the relative levels of pGH in different cell groups

In the present study, researchers investigated the potential of different introns to be involved in transcriptional regulation by acting as enhancer or repressor. The results revealed pGH expression efficiency was affected by different introns. Although at this point, the results do not provide a mechanism by which the effect of different introns occurs, several studies demonstrated the ability of introns to bind various factor and modulate gene expression. Morello *et al*. (2002) have shown that some element or components have existed in introns that can enhance or repress transcriptional activity. Different introns, even within the same gene can have different regulatory functions (Lehmann, 2004). The experimental values in this study differ slightly when compared with published data from rat studies (Morello *et al*., 2002). Similar to the research, it has been reported that the first intron of the rat growth hormone gene was essential for higher levels of expression. The third and fourth introns also had a positive effect on expression. The positive effect of introns might relate to the gene structure of the first intron in pGH. The splice sites of pGH introns, 5'-GC-AG-3', 5'-UA-GG-3' and 5'-GG-CA-3' were different from the common intron splice site, 5'-GU-AG-3' [15, 20]. Strikingly, pGH1, pGH3, pGH4 own a 6 bp repeat unit TCTCTC that can bind GAGA-binding Factor (GAF). GAF can bind $(GA)_n$ or $(CT)_n$ sequences and display activator/antirepressor activity.

Growth Hormone (GH) is a pluripotent vertebrate hormone produced by the hypophysis. Its major role is mediating somatic growth, muscle development, metabolic regulation and the like. The coding, promoter and both 5' and 3'-noncoding sequences of the *pGH* gene were found to be highly conserved when compared with earlierly sequenced genes coding for rat, human,

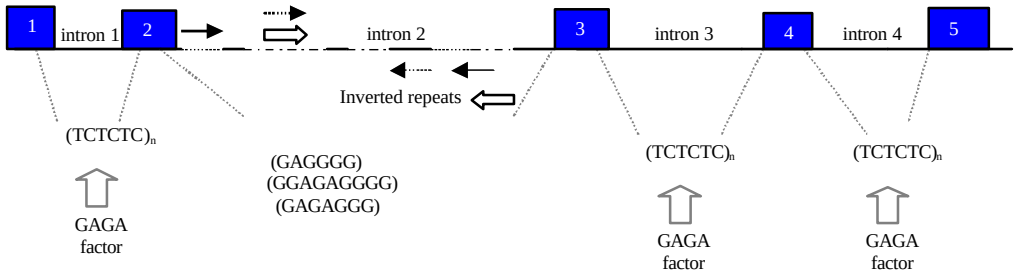

Fig. 5: Schematic presentation of the *pGH* gene organization and distinguishing feature. Shaded boxes, exons, numbers inside boxes denote exon number; pairs of dotted arrows, long arrows, empty arrows denotes inverted repeats, respectively

bovine and porcine GHS (Morello *et al*., 2002). The results from this study will help in understanding the function of GH introns. It is hoped that further studies will result in more effective use of the *pGH* gene in improving domestic breeding.

CONCLUSION

The results of this study suggest that introns can influence the efficiency of pGH expression. The recombinant *pGH* gene with introns 3 and 4 had the highest expression activity. This is believed that the introns can be used to regulate the pGH expression efficiency.

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