Characterization of the 5′-Flanking Transcriptional Regulatory Region of Chicken Growth Hormone Gene

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A 1727-bp fragment of 5′-flanking region of chicken growth hormone (cGH) gene has been cloned and sequenced. Various lengths of the 5′-flanking region (122 to 1775 bp) was linked to a luciferase reporter gene, and its transcriptional regulation was examined by an *in vitro* transient transfection coupled with luciferase assay. Our results demonstrated that pituitary-specific transcription factor, Pit-1, is necessary and sufficient to confer a strong tissue-specific expression. Co-transfection with goldfish or chicken Pit-1 expression vectors significantly restored the luciferase expression in HeLa cells. Site-directed mutagenesis and mobility gel-shift assays further confirmed the position of the Pit-1 binding site at –113/–104. Moreover, a repressive thyroid hormone response element (TRE) was identified at –137/–74, and we propose that interactions between the TRE and Pit-1 sites may be required for its repressive effect.
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Key words: cGH 5′-flanking region; Pit-1; TRE; luciferase assay

Introduction

Growth hormone (GH), a polypeptide hormone synthesized in and secreted by the pituitary gland, affects a great variety of physiological parameters such as growth performance, carcass composition, and milk production (1–3). GH gene expression in pituitary somatotrophs depends on a pituitary-specific transcription factor, Pit-1 (also called GHF-1), which is responsible for tissue-specific expression of genes encoding GH (4), prolactin (PRL; Refs. 5, 6), thyroid-stimulating hormone (7), GH-releasing hormone receptor (8, 9), and Pit-1 genes (10–12). In human (hGH) and rat GH (rGH) genes, two Pit-1 binding sites have been located within the region –140/–70 (6, 13), whereas in chinook salmon, rainbow trout, and tilapia, three to five Pit-1 binding sites have been identified (14–16). Pit-1 plays a crucial role in GH gene expression in pituitary somatotrophs. Transfected Pit-1 is able to transactivate a co-transfected GH or PRL promoter in HeLa cells, suggesting that Pit-1 may be a limiting factor for GH synthesis (6, 17). Although it has been found that thyroid hormone receptor (TR) can act directly and independently on the promoter of rGH gene (18), Pit-1 can activate the rGH gene expression synergistically with TR (19). In the hGH gene, TR-binding sites have been located at the region –290/–129 (20). Although the sequence motifs of both human and rat thyroid hormone response elements (TRE) are conserved, they are inhibitory in hGH gene promoter. It has been suggested that binding of TR may overlap and cause a steric hindrance to the binding of the positively acting factors, Sp1 (–140/–116) and Pit-1 (–134/–106), respectively. Stimulatory and repressive TREs have also been identified in the third intron of rGH gene (21) and the proximal 3′-flanking untranslated region of the hGH gene (22).

In addition to Pit-1 and TRE, other regulatory elements, such as glucocorticoid response element (GRE) and cAMP response element (CRE), also play an important role in the regulation of GH gene expression. In the rGH gene, where two GREs have been identified, a mutation in the proximal element is sufficient to abolish the ability of mediating glucocorticoid (GC) induction of gene expression (23). GREs have also been identified in the intron 1 of the hGH gene, and an addition of a synthetic GC dexamethasone (Dex) elevates the GH release and GH mRNA level by 400% (24). Bernardini *et al.* (25) also demonstrated that GRE can act synergistically with cAMP on the rainbow trout GH gene. Two core CRE motifs (CGTCA) have been identified in the hGH gene promoter by mutational analysis (13), whereas the rGH gene that does not contain the CGTCA motif has been shown to be induced by an elevated cAMP level (26, 27), suggesting that human and rat GH promoters are regulated by different mechanisms upon cAMP induction. Although the tilapia GH promoter lacks the CGTCA motif and functional AP–2 elements, it is still upregulated by treatment with 8-Br-cAMP (16). cAMP

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stimulation of the tilapia GH promoter is found to be abolished upon mutation of the most proximal Pit-1 binding site, suggesting its cAMP responsiveness is mediated by the Pit-1 binding sites (16).

GH gene promoters in mammalian species have been well studied as compared to the chicken GH (cGH) gene promoter. In previous studies, only about 500 bp of the promoter region of cGH gene has been reported, which is too short for an extensive analysis on transcriptional regulation (28). In addition, Tanaka *et al.* (28) showed that the proximal promoter region of cGH gene shares only little homology with mammalian GH genes, suggesting that the transcriptional regulation of the cGH gene may be different from other mammalian GH genes. Therefore, the aim of the current investigation is to clarify the transcriptional regulation of the 5′-flanking region of the cGH gene by an *in vitro* transient transfection study. We show that the 5′-flanking region −137/+48, bearing a potential binding site for the pituitary-specific transcription factor Pit-1, is necessary and sufficient to confer a strong tissue-specific expression to a luciferase reporter gene. In addition, the position of Pit-1 binding site, which is located at the region −113/−104, is further confirmed by site-directed mutagenesis and mobility gel-shift assay. The results also demonstrate that the 5′-flanking regions −137/−74 and −1727/−1467 bear a suppressive TRE and a stimulatory GRE binding site, respectively. Therefore, the current studies provide further information on the mechanism of transcriptional regulation of GH gene in an avian species.

Materials and Methods

Library Screening and Sequencing of 5′-Flanking Region of cGH Gene. Two digoxigenin (DIG)-labeled probes located at the 5′-flanking region (PR primers) and intron 1 (PM3 primers) of the cGH gene, as mentioned elsewhere (29), were generated by PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, Mannheim, Germany) using the primers 5′-TGCAGTGGATCCAAGCAA-3′ (PR1 forward), 5′-TACCTGGAGCCATT-CCTG-3′ (PR1 reverse), 5′-ATCCCCAGGCAAACATCCTC-3′ (PM3 forward), and 5′-CCTCGACATCCAGCTCACAT-3′ (PM3 reverse). The polymerase chain reaction (PCR) conditions comprised an initial denaturation step of 95°C for 4 mins, 35 cycles of denaturation at 94°C for 30 secs, annealing at 60°C for 45 secs, and an extension step at 72°C for 90 secs. The PCR products were then loaded in a 0.8% gel and purified by GENECLEAN II Kit, BIO 101 (Qbiogene, Vista, CA). Library screening of the chicken genomic phage library (Clontech Laboratories, Inc., Palo Alto, CA) was performed with the two PCR DIG-labeled probes. Phage λDNA was prepared and purified as mentioned in Lambda Library Protocol Handbook (Clontech Laboratories, Inc).

The 5′-flanking region of cGH gene was amplified by PCR using the primers of 5′-CTGCTTCTCATAGAGTCTTGCAGACAAACTGCGCAAC-3′ (5′ LD-Insert Screening Amplimer, Clontech), 5′-TGAACACTCGTCCGAGAATAACGAGTGGAT-CTGGGTC-3′ (3′ LD-Insert Screening Amplimer, Clontech), and 5′-CAGCATTGGAACACCCAT-3′ (PROREV2, located in intron 1 of cGH gene). Fifty pmole of each pair of primer was added to 50 μl of reaction mix containing 1X PCR buffer with $MgCl_2$ (Roche Molecular Biochemicals), 10 m*M* dNTP, 1.5 units Enzyme mix Expand High Fidelity (Roche Molecular Biochemicals), and 0.5 μl of λDNA as a template. The PCR condition used comprised an initial denaturation step of 95°C for 2 mins, 35 cycles of denaturation at 94°C for 30 secs, annealing at 55°C for 45 secs, and an extension step at 72°C for 3 mins. PCR products were gel purified by GENECLEAN II Kit (BIO101) and used for constructing the reporter plasmid.

Reporter Gene Construction. Fragments of various sizes containing 5′-flanking region of cGH gene promoter were generated by PCR using appropriate primers with specific adaptor (Table 1). Amplified fragments were then digested with appropriate restriction enzymes (*Xho*I, *Sst*I, and *Hind*III) I and subcloned into the pGL3-Basic vector (Promega, Madison, WI). The 5′-deletion constructs of the pGL-(−74/+48, −137/+48, −187/+48, −253/+48, −282/+48, −489/+48, −863/+48, −1210/+48, −1467/+48, and −1727/+48) contain 74, 137, 187, 253, 282, 489, 863, 1210, 1467, and 1727 bp of the 5′-flanking region of the cGH gene and 48 bp of transcribed sequence fused to the Luciferase gene. The constructs pGL-(−1727/−74, −1727/−333, and −1727/−863) were constructed from 3′-deletion method. The structure of all of the constructs was confirmed by endonuclease digestion and nucleotide sequencing. Plasmids were extracted by the kit CONCERT Rapid Plasmid Miniprep System (Life Technologies, Carlsbad, CA) and sequenced bidirectionally by sequence analyzer ABI PRISM 310 (Perkin Elmer, Norwalk, CT) to ensure the sequence is free of error.

Site-Directed Mutagenesis of Potential Pit-1 Binding Sites. Primers were designed to introduce desired mutation to the potential Pit-1 binding sites. The PCR mixture contains 150 ng of each oligonucleotide (Mut-110 pit-1F, Mut-110 pit-1R, Mut-530 pit-1F, and Mut-530 pit-1R), 10 ng of template plasmid DNA, 200 μ*M* dNTPs, 2.5 units *pfu* DNA polymerase (Clontech), and the 10X buffer supplied with the polymerase in a total volume of 50 μl. PCR was performed under the following conditions: denaturation at 95°C for 30 secs, annealing at 55°C for 1 min, and extension at 68°C for 2 mins per kb template.

Five microliters of the PCR product was then examined by agarose gel electrophoresis. The remaining 45 μl PCR product was digested with 20 units *Dpn*I for 2 hrs at 37°C. Five microliters of the digested DNA were then transformed into 200 μl competent XL-1 Blue bacterial cells (Stratagene, La Jolla, CA). Plasmids were extracted by the kit CONCERT Rapid Plasmid Miniprep System (Life Technologies) and sequenced bidirectionally by sequence

Table 1. Primers Used in the Construction of the Deletion Constructs[a]

Primer	Sequence
*Hind*III – Rev	5′-TACC**AAGCTT**GGAGAGTTGCTCAGGTGT-3′
*Hind*III – 74Rev	5′-ATCC**AAGCTT**CCACCTCTCCATATCCCC-3′
*Hind*III – 333Rev	5′-ATCC**AAGCTT**CCTGGTTTTTCTGCCCCA-3′
*Hind*III – 863Rev	5′-AATC**AAGCTT**CAAGAGCAGCATCATCAC-3′
*Hind*III – 489F	5′-TTCT**AAGCTT**GCAGTGGATCCAAGCAA-3′
*Sst*I – Rev	5′-TGAA**GAGCTC**CTGGAGCCATTCCTGCC-3′
*Sst*I – 74F	5′-TACC**GAGCTC**AGTGATCACGAGCACCC-3′
*Sst*I – 137F	5′-ATCC**GAGCTC**AATGAGGTAGCACCATGG-3′
*Sst*I – 187F	5′-GACC**GAGCTC**CCCAGGCAAACATCCTC-3′
*Sst*I – 253F	5′-GAAA**GAGCTC**ATGGTGATAAAACCTCTGGT-3′
*Sst*I – 282F	5′-GACC**GAGCTC**ACAGAACAGATTTGGGAT-3′
*Sst*I – 333F	5′-CAAT**GAGCTC**CAGGAAAATCAGTGGATTTT-3′
*Sst*I – 387F	5′-GACC**GAGCTC**AAGTCCCCTGCACTGCCCT-3′
*Sst*I – 489F	5′-GAAT**GAGCTC**TGCAGTGGATCCAAGCAA-3′
*Xho*I – 605F	5′-TTCT**CTCGAG**TTTGTTCTCATTCACGTCA-3′
*Xho*I – 678F	5′-TTAC**CTCGAG**TCCTCTGCTTAATCCAGA-3′
*Xho*I – 863F	5′-TTAC**CTCGAG**AAATCAGAGCCAAACCA-3′
*Xho*I – 1210F	5′-TTAC**CTCGAG**CATGCCATGCTTTTACTG-3′
*Xho*I – 1467F	5′-TTAC**CTCGAG**GTTACTGAGCGTCATGC-3′
*Xho*I – 1727F	5′-TTAC**CTCGAG**CCTGGTTTGTATCCCACC-3′

[a] Restriction enzymes sites *Xho*I (**CTCGAG**), *Sst*I (**GAGCTC**), or *Hind*III (**AAGCTT**) were added for sticky-end cloning.

analyzer ABI PRISM 310 to ensure the sequence is free of error.

Cell Culture and Transfection Experiments. GH4 (Pit-1-expressing rat pituitary cell line) and HeLa cells were maintained in Ham's F-10 medium (Life Technologies) and Dulbecco's minimal essential medium (DMEM), respectively, supplemented with 10% (v/v) fetal calf serum and 1% (v/v) antibiotic antimycotic. Cells were incubated at 37°C with 5% CO_2 and were plated in 6-well plates 1 day prior to transfection at density 2×10^6 (GH4) and 3×10^5 (HeLa) cells per well. A mixture containing 2.5 μg of promoter-luciferase construct, 30 ng of pRL-TK (Promega, Madison, WI), and 8 μg of DOSPER Liposomal Transfection Reagent (Roche Molecular Biochemicals) was prepared, and transfection was performed following the manufacturer's protocol. After 6 hrs of incubation, fresh medium containing 10% (v/v) fetal calf serum was replaced, and the cells were incubated for another day. For the studies on Pit-1, HeLa cells were co-transfected with 300 ng of goldfish or chicken Pit-1 in addition to the said DNA plasmids and grown for an additional 24 hrs. Effects of thyroid hormone (T3) and glucocorticoid (GC) were examined by supplementing transfected GH4 cells in hormone-free medium with 10^{-10} to 10^{-7} *M* 3,3,5-triiodo-L-thyronine and 10^{-7} *M* dexamethasone after transfection. Cells were incubated for another 24 hrs before being harvested for luciferase assay. All cellular lysate were collected with 500 μl 1X lysis buffer (Promega), and luciferase activity was assayed immediately after harvest. All experimental groups were carried out in triplicate, and the pRL-TK was used as an internal control to normalize transfection efficiency. A promoterless pGL3-Basic vector was also included as a control in all transfection experiments.

Luciferase Assays. Luciferase activity was determined by the kit Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations. Luminescence reader Lumat LB9507 (EG&G, Berthold, Germany) with automatic injector system was used, and it was programmed to perform a 2-sec premeasurement delay, followed by a 10-sec measurement period for each reporter assay. Firefly Luciferase activity was assayed by injecting 100 μl Luciferase Assay Reagent II (Promega) to 20 μl passive lysis buffer lysate in a 12 × 75 mm tube (Iwaki Glass). Renilla Luciferase activity was then assayed by injecting 100 μl Stop & Glo Reagent (Promega) to the tube.

Statistical Analysis. The effect of the 3,3′,5-triiodo-L-thyronine and Dex on luciferase activity was compared by ANOVA using the program Instat Ver. 2.05 (GraphPad software Inc., San Diego, CA). Differences were considered significant if $P < 0.05$. All data are expressed as means ± SE.

Nuclear Extract Preparation. Nuclear extracts were prepared from GH4 and HeLa cells (30). Protein concentrations of the extracts were determined by Bradford assay (31).

Electrophoretic Mobility Shift Assay. Synthetic double-stranded oligonucleotides, corresponding to regions between −118 and −97 and between −546 and −525, were end-labeled using [γ^{32}P]ATP and T4 polynucleotide kinase (Amersham, Buckinghamshire, England). Electrophoretic mobility assays were performed using 1 μg of either GH4 or HeLa cell nuclear extract, 400 ng of poly(dI-dC), and 5000–10000 cpm of ^{32}P-labeled oligonucleotides in 20 m*M* HEPES, 100 m*M* KCl, 0.03 m*M* $MgCl_2$, 8% glycerol, and 0.1 m*M* DTT. The resulting DNA-protein complexes were resolved by electrophoresis on a 5% polyacrylamide gel

A)

Motif	Start	End	Sequence
AG-rich region	-1603	-1495	\
Pit-1	-541	-534	AGCTTCAT
TATA Box	-149	-143	TATAAAT
TRE	-137	-128	CAATGAGGTA
Pit-1	-111	-104	ATCTGCAT
TATA Box	-24	-30	TATATAA

B)

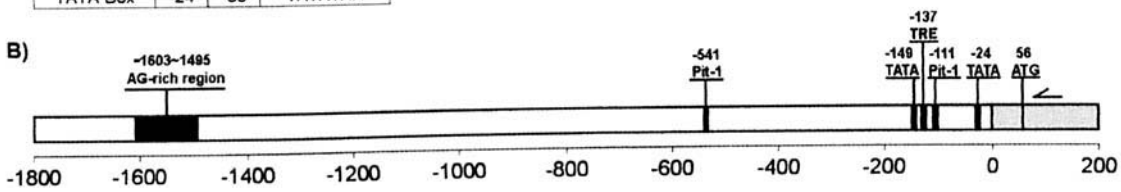


Figure 1. The chicken growth hormone (cGH) 5'-flanking region and its putative transcription binding sites. (A) The sequence and positions of the putative transcription binding sites. (B) The cGH 5'-flanking region. The position 0 on the scale bar indicates the transcription start site. The area shaded in gray represents the transcripted region. The area highlighted in black represents the putative transcription binding sites, including TATA (potential TATA box), TRE (thyroid hormone response element), Pit-1, and an AG-rich region. The position of primer PROREV2 is represented by an arrow. These sequence data have been submitted to the GenBank databases under accession number AF404827.

using 0.5X TBE as running buffer. In competitive experiments, 10-fold, 25-fold, or 50-fold molar excess of unlabeled double-strand oligonucleotides were used.

Results

Analysis on the DNA Sequence of 5'-Flanking Region of cGH Gene. A total of 1727 bp of the 5'-flanking region of the cGH gene was isolated from chicken genomic library and sequenced. Potential transcription factor binding sites of the cGH gene were analyzed by the Web sites http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html and http://wwwiti.cs.uni-magdeburg.de/~grabe/alibaba2/ (Fig. 1). The results revealed two putative binding sites of the pituitary-specific transcription factor Pit-1 at –113/–104 (proximal site) and –541/–533 (distal site), which match the proposed consensus teleost/avian Pit-1 binding site (T/A)NCTNCAT (32). A potential TRE and an AG-rich region were also identified and located at –137/–128 and –1604/–1494 respectively (Fig. 1).

Characterization of Chicken GH 5'-Flanking Region in GH4 and HeLa Cells. Functional study of the 5'-flanking region (~1.8 kb) of the cGH gene was conducted and found to be transcriptionally activated by transient transfection coupled with luciferase assay using rat pituitary GH4 cell line and nonpituitary HeLa cell line (Fig. 2A). To localize the cis-acting DNA elements within the 5'-flanking region of cGH, various 5'- and 3'-deletion mutants were constructed and analyzed.

Our results showed that in GH4 cells, the plasmid constructs containing the 5'-flanking region of 187 bp exhibit a maximum luciferase activity (Fig. 2A). A further reduction in the regulatory sequence led to a decrease in the luciferase activity (Fig. 2A), suggesting the region –187/+48 contains the minimal sequence for full promoter activity. A significant drop of luciferase activity was observed when the construct –489/+48 was cloned in a reverse fashion, confirming that the cGH gene promoter acts in an orientation-dependent manner, a typical property of promoters (Fig. 2A). Moreover, no luciferase activity was obtained with the plasmid construct –1727/–74, suggesting that the TATA box at –24 bp is crucial for the stimulatory effect of this fragment (Fig. 2A). The transcriptional regulations of the 5'-flanking region of the cGH gene were further studied by performing 3'-deletion on the region –1727/+48. An increase in luciferase activity was only observed with the construct –1727/–333, but neither with the constructs –1727/–863 nor –1727/–74 in GH4 cells, implying that an additional regulatory element is present within the region between –863 and –333 and/or within the region between –333 and –74. This potential site could be an enhancer within the region of –863 and –333 (Fig. 2A). Alternatively, it could be a transcriptionally inhibitory element present within the region of –333 and –74. On the other hand, no significant changes in the luciferase activity were observed in HeLa cells transfected with various constructs of the 5'-flanking region of the cGH gene, indicating that the transcription factor Pit-1, which is exclusively expressed in the pituitary cells, acts as a regulatory element within the 1727 bp of the 5'-flanking region of the cGH gene.

Promoter Activity of cGH Gene Is Dependent on Pit-1. Two putative Pit-1 binding sites have been found in the regions –113/–104 and –541/–533. Effect of Pit-1 on the promoter activity of promoter constructs was studied by co-transfection with expression vectors encoding goldfish (gf Pit-1) or chicken pituitary-specific transcription factor Pit-1 (cPit-1) in Hela cells. No stimulation in lucifersase activity was observed in the absence of Pit-1 (Fig. 2A), whereas both gf Pit-1 and cPit-1 were able to restore the luciferase activity in HeLa cells, but in different magnitudes (Fig. 2A).

To determine whether the proximal (–113/–104) and the distal Pit-1 binding sites (–541/–533) are both functional, luciferase activities were measured with the full-length construct (–1727/+48) with mutations at the proximal, the distal, or both Pit-1 binding sites, respectively. The results showed that luciferase activities were decreased

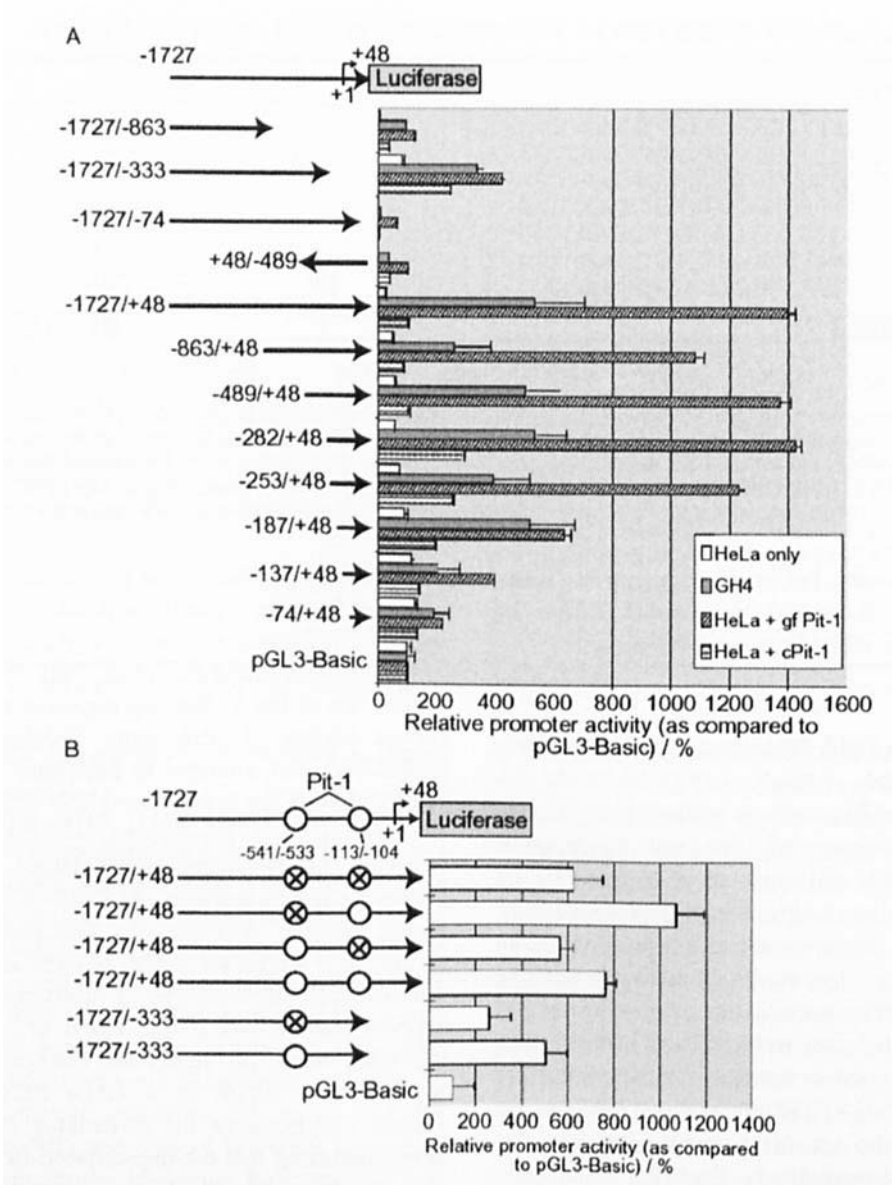


Figure 2. The chicken growth hormone (cGH) promoter activity is dependent on Pit-1. (A) The promoter activity of cGH gene in HeLa cells, GH4 cells, and HeLa cells (in the presence of goldfish and chicken Pit-1). Co-transfection of gf Pit-1 or cPit-1 in HeLa cells restores the expression level of cGH promoter, showing that Pit-1 plays an important role in pituitary cell specific expression (number of sample, $n = 9$). In addition, co-transfection of Pit-1 significantly restores the luciferase activity in HeLa cells ($n = 9$). (B) The luciferase activity of constructs –1727/+48 and –1727/–333 with mutated Pit-1 sites in GH4 cells ($n = 9$). Wild-type Pit-1 site is shown as a circle, whereas the mutated site is represented by a circle with a cross. (C) Gel-shift assay of a DNA fragment (–118/–97) containing the proximal Pit-1 site.

from 800% to 600% when both or only the proximal Pit-1 binding sites were mutated, respectively, indicating that the proximal Pit-1 site is functional (Fig. 2B). On the other hand, an increase in luciferase activity from 800% to 1000% was observed on the construct with the mutated distal Pit-1 binding site, suggesting that the distal Pit-1 binding sites may act in a repressive manner or it may be nonfunctional. The basal promoter activity was observed when both Pit-1 sites were mutated, implying the presence of Pit-1 sites with unconsensus sequence or other enhancer elements within the region. In order to obtain more information about the regulatory role of the distal Pit-1 binding site, site-directed mutagenesis assays were performed on the constructs containing the region –1727/–333, which contains the potential distal Pit-1 site only. Interestingly, mutation in the distal Pit-1 site caused a decrease in luciferase activity, suggesting that the distal Pit-1 site can act as a repressor only in the presence of the region –333/+48.

GH4 Cell Nuclear Proteins Bind Specifically to the Proximal Pit-1 Binding Site. In order to test whether the Pit-1 can actually bind to the proximal and the distal Pit-1 sites, gel-shift assays were performed with the nuclear extract of the HeLa and GH4 cells, respectively. A specific band-shift was observed in the gel mobility shift assay when a radioactive-labeled oligonucleotide –118/–97, which contains the proximal putative Pit-1 site, was

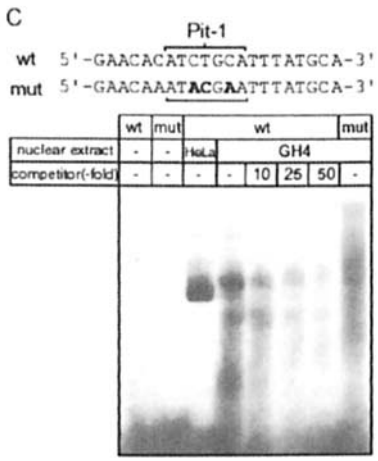


Figure 2. Continued.

incubated with nuclear proteins from rat pituitary GH4 cells (Fig. 2C). The intensity of the shifted band was significantly reduced by the addition of a nonradioactive oligonucleotide containing the same region –118/–97 (Fig. 2C). A smeary lane was observed when the potential proximal Pit-1 site (i.e., ATCTGCAT) was mutated to ATACGAAT (Fig. 2C), implying that the specific binding of Pit-1 to the proximal Pit-1 site is lost in the mutated oligonucleotides. A specific shifted band was also obtained in the HeLa cell extracts, but its identity remains to be elucidated. A gel mobility shift assay was also performed on the potential distal Pit-1 site. However, no shifted band was observed, indicating that this site is nonfunctional.

Thyroid Hormone Suppression of cGH-Luc in GH4 Cells Is Mediated by the 5′-Flanking Region –137/–74. Preliminary sequencing data showed that a putative thyroid hormone responsive element (TRE) is located at the 5′-flanking region –137/–128, which is highly conserved between tGH and cGH promoters (Fig. 3). In order to test if the TRE in the cGH promoter is functional, a series of constructs containing various 5′-deletions of the cGH promoter fused to the luciferase reporter gene were transiently transfected into GH4 cells and incubated with 3,3,5-triiodo-L-thyronine (the final concentration of 100 n*M*) for 24 hrs before the luciferase activity was determined. As shown in Figure 4A, all constructs except the construct –74/+48 and the promoter-less pGL3-Basic were suppressed by 3,3′,5-triiodo-L-thyronine by 50%–60% ($P < 0.05$), indicating that the 3,3′,5-triiodo-L-thyronine exerts its effect on the cGH promoter. Moreover, the suppressive effect of 3,3′,5-triiodo-L-thyronine was still observed when the final concentration of 3,3′,5-triiodo-L-thyronine was decreased to 10 n*M*. As a control, the pGL-3-Basic promoter-less vector showed no sufficient suppression with the addition of 10 n*M* of 3,3′,5-triiodo-L-thyronine (Fig. 4B). Therefore, we conclude that a repressive TRE is located between the region –137 and –74 bp.

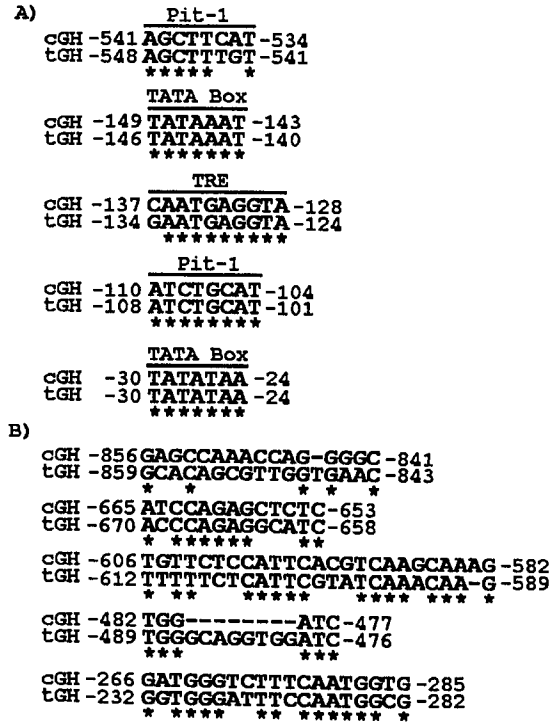
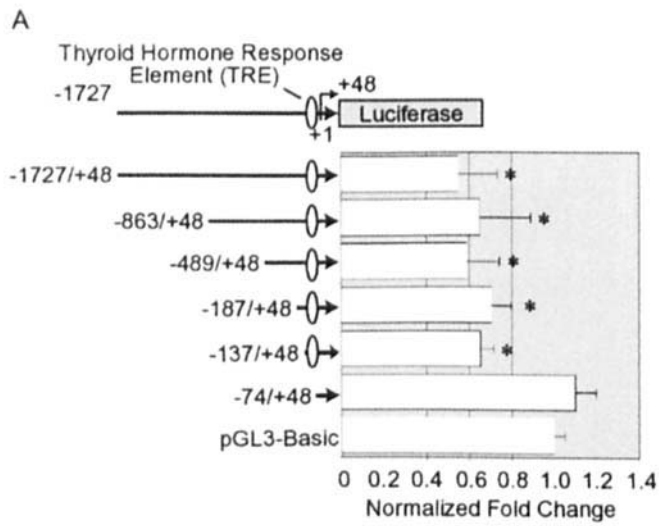


Figure 3. Alignment of the 5′-flanking region of cGH and tGH. Only (A) putative transcription binding sites and (B) sites with low homology (<50%) are shown. The 5′-flanking region of cGH gene is highly conserved with that of tGH gene. Conserved sites are marked with an asterisk (*). The proximal Pit-1 and TRE binding sites are highly conserved between the species.

Glucocorticoid Induction of cGH-Luc in GH4 Cells Is Mediated by the 5′-Flanking Region –1727/–1467. Although no GRE has been identified in the cGH genes by the sequence analysis, it has been reported in the promoter of GH genes in several other organisms. Thus, it is tempting to test if there is any effect of glucocorticoid (GC) on cGH promoter expression. To characterize the hormonal response of the cGH to GC, cGH-Luc constructs were tested for their responsiveness against synthetic GC dexamethasone. cGH-Luc constructs were transiently transfected into GH4 cells and incubated with Dex (final concentration of 100 n*M*). As shown in Figure 5, the construct –1727/+48 is the only one exhibiting a 2-fold increase in luciferase activity upon the induction of Dex ($P < 0.05$, as compared to all other constructs), showing there is a GRE located at the region between 1727 and 1467 bp upstream of the transcriptional start site.

Discussion

In the current study, approximately 1.8 kb of 5′-flanking region of cGH gene was cloned and sequenced from a chicken genomic library. The sequence characterized is much longer than the previously reported one (~500 bp) (28), which allows us to conduct various functional tests on this promoter region. Here, we find that there is a Pit-1 binding site at –113/–104, a TRE binding site at –137/–74,



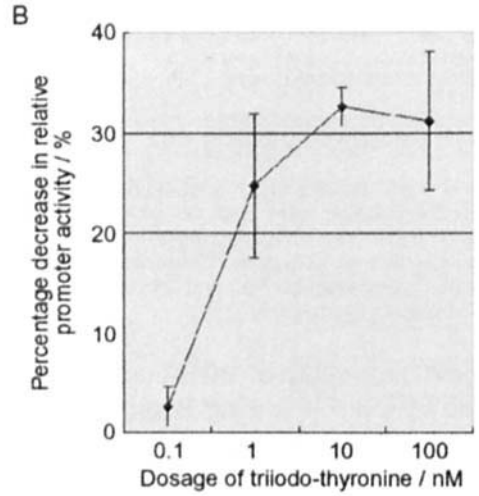


Figure 4. Thyroid hormone suppression of chicken growth hormone (cGH)-Luc in GH4 cells is mediated by the 5′-flanking region –137/–74. (A) Suppression of the cGH promoter activity with 3,3′,5-triiodo-L-thyronine (100 n*M* final concentration). A thyroid hormone response element (TRE) is located at the region –137/–74 and suppression to 60% ($P < 0.05$, shown by asterisk*) of luciferase activity is observed ($n = 9$). (B) Dose-response analysis of 3,3′,5-triiodo-L-thyronine (0.1, 1, 10, and 100 n*M*, final concentration) on luciferase activites of the construct –137/+48.

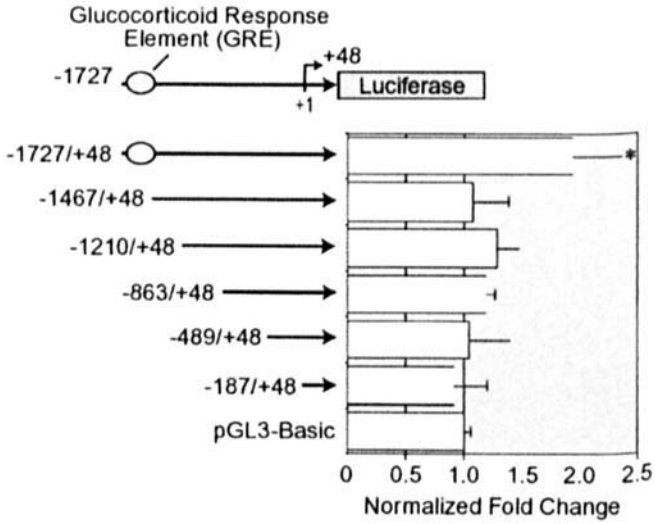


Figure 5. Glucocorticoid induction of chicken growth hormone (cGH)-Luc in GH4 cells is mediated by the 5′-flanking region –1727/–1467. Induction of the cGH promoter activity with dexamethasone (Dex). A glucocorticoid response element (GRE) is located in the region –1727/–1467 and a 2-fold stimulation ($P < 0.05$, shown by asterisk [*]) of luciferase activities is observed upon the addition of Dex ($n = 9$).

and a GRE binding site at –1727/–1467 within a 1.8-kb fragment upstream of the cGH gene (Fig. 6).

Comparison with the turkey counterpart shows an overall 90% sequence homology (33), and the binding sites of the proximal Pit-1 and TRE were highly conserved in both species (Fig. 3). This suggests that GH gene of both chicken and turkey might share similar regulatory mechanisms even though the 5′-flanking region of turkey has not been characterized yet. However, little homology with the mammalian GH promoter region (Fig. 6) suggests that the regulation of GH gene expression in avian species may be different from the mammalian one.

Using an *in vitro* luciferase assay, we found that Pit-1 is essential for the promoter activity of the cGH gene. Co-transfection experiments showed that both gf Pit-1 and cPit-1 were able to restore the luciferase activity in HeLa cells, although in different magnitudes (Fig. 2A). Pit-1 contains a DNA-binding POU domain and an N-terminal transactivation domain. The POU domain is divided into two well-conserved regions, separated by a poorly conserved linker region. The C-terminal half encodes a low-affinity DNA-binding domain called the POU-homeobox domain (POU_{HD}), whereas the N-terminal half, which is responsible for protein-protein interactions, confers high affinity to the POU domain called POU-specific domain (POU_S; Refs. 34, 35). Transcriptional activation is mediated by the less conserved domain at the N-terminus that is rich in serine and threonine residues: serine/threonine activation (STA) domain (34). Phylogenetic studies showed that all POU_S, STA-3, and POU_{HD} domains are well conserved between chicken and goldfish, sharing 90.5%, 70.9%, and 81.1% homology, respectively (Lam and Yu, personal communication). Therefore, it is expected that gf Pit-1 can activate the transcription of cGH promoter *in vitro*. In fact, our *in vitro* transfection experiment showed that cGH promoter, like its mammalian counterpart, also exhibited cell-type specific expression by the pituitary specific transcription factor Pit-1 and can be activated by gf Pit-1 or cPit-1. However, some discrepancies were seen in the transfection experiments. A maximum luciferase activity was observed with the plasmid construct –282/+48 in HeLa cells (with gf Pit-1) instead of with the construct –187/+48 that was observed in GH4 cells (Fig. 2A). This discrepancy may be due to the presence of another Pit-1 site with unknown sequence in the region –253/–187, to which the gf Pit-1 could bind and further activate the luciferase activity

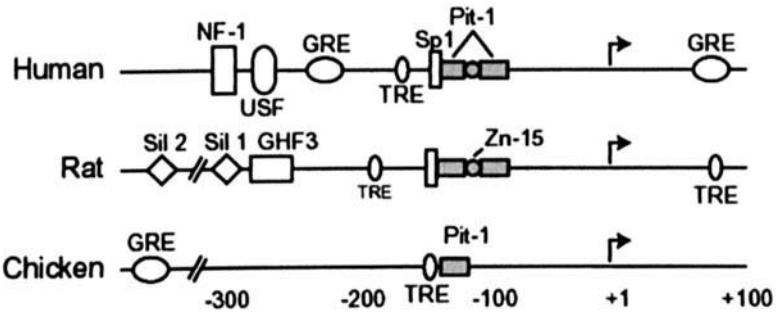


Figure 6. Comparison of proximal promoter region of growth hormone (GH) gene in different organisms. The promoter region of the chicken growth hormone (cGH) gene, as compared to the corresponding region of human and rat GH genes, shared only little homology. Abbreviations: NF-1, nuclear factor-1; USF, upstream stimulating factor; GRE, glucocorticoid response element; TRE, thyroid hormone response element; Sp1, stimulating protein 1; Pit-1, pituitary-specific transcription factor; Sil, silencer protein; GHF3, GHF3 factors; Zn-15, zinc-finger transcriptional factor.

efficiently. It is believed that in order for a Pit-1 to activate a promoter, two Pit-1s should be bound to it. Therefore, it is tempting to locate the Pit-1 site in future study, and further experiment is required to confirm this potential Pit-1 binding site.

Therefore, although gf Pit-1 could bind to the cGH promoter and activate the luciferase activity, its stimulation on the cGH promoter may not reflect the real case. In order to support the co-transfection data obtained by gf Pit-1, similar experiments were performed with cPit-1. Similar results were obtained, although the luciferase activity (a maximum of ~300% for the construct –282/+48) was increased to a lesser extent than that with the gf Pit-1. Moreover, no significant stimulation in the luciferase activity by cPit-1 was obtained for constructs –489/+48, –863/+48, –1727/+48. It may be due to the presence of a suppressor in the region –489 to –282, and further experiment is required for clarification.

In addition to the studies on the pituitary-specific transcription factor, other putative transcription elements, like TRE and GRE, were also conducted. Although no GRE binding site was identified in the 5′-flanking region of cGH gene by the sequence analysis, a 2-fold induction of luciferase activity was observed with the construct containing –1727/+48 of cGH gene. Interestingly, this potential GRE is located within the region –1727/–1467, partially overlapped with an AG-rich region. Whether this AG-rich region is responsible for the GC induction remains to be clarified in the future. However, it is possible that the GC, which may act as a modulator, plays a role in the regulation of GH gene expression, as observed in other animals.

TRE binding motifs have been identified in both rGH and hGH genes, and extensive studies have been carried out to study the effect of thyroid hormone to the GH gene expression. Earlier studies using chimeric plasmids containing various lengths of rGH gene and the 5′-flanking DNA fused to the coding region of dominant selectable marker gene *neo* showed that the region –235/+11 was responsible for thyroid hormone induction of rGH-neo RNA (36). *In vitro* transfection studies in GC cells also demonstrated that the DNA between –210 and –181 was essential for stimulation by 3,3′,5-triiodo-L-thyronine (37). It has been proposed that thyroid hormone acts *via* its receptor (TR) to enhance the function of the cell-specific element by forming a more "active" transcription complex that stimulates the level of gene expression (38). Although the TRE binding motifs identified in both human and rGH genes are well conserved, inhibitory effect was observed in hGH gene (39, 40, 41). In the hGH gene, two Pit-1 binding sites have been located at –134/–106 and –96/–70, in which the distal Pit-1 binding site partially overlapped with an Sp1 binding sites at –140/–116 (42). Previous studies demonstrated that binding activities of the Sp1 and the Pit-1 were mutually exclusive, and the binding of Sp1 could compensate partially for the decreased stimulation of transcription seen at low Pit-1 concentration (43), implying that both Sp1 and Pit-1 were essential for the expression of hGH gene. Binding of the TR at –290/–129 or –64/–44, respectively, might overlap with the binding of the Sp1 and Pit-1 or cause DNA bending, in which decrease in the transcription efficiency was observed (20, 39–41, 44, 45). The sequence analysis on the 5′-flanking region of the cGH gene indicated that a potential TRE is located at the region –137/–128. *In vitro* transient transfection studies with the addition of 3,3′,5-triiodo-L-thyronine confined a repressive TRE at the region –137/–74, in which up to 50%–60% suppression of pGL3-luciferase expression vector was observed. It was consistent with previous *in vivo* studies in cockerels and pullets, where feeding chickens with T3 or T4 significantly lowered plasma GH concentration (46). Moreover, in the gel mobility shift assay, it was demonstrated that a Pit-1 binding site, which was an important limiting factor for GH gene expression, was located at –113/–104. Therefore, the suppressive effect may be mediated by steric hindrance between the TR (–137/–128) and the Pit-1. The binding of TR may block the binding of Pit-1, which in turn suppresses the transcriptional expression of cGH gene.

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