Molecular cloning and characterization of $\alpha 1$ -soluble guanylyl cyclase gene promoter in rat pituitary cells

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Abstract

Soluble guanylyl cyclase is a cytosolic enzyme which catalyzes conversion of GTP to the second messenger cyclic GMP. The transcriptional regulation at the promoter levels of four soluble guanylyl cyclase subunits, termed $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$, is largely unknown. In this study, we identified the transcription start site of $\alpha 1$ -soluble guanylyl cyclase gene in rat pituitary cells and cloned the 3.5 kb 5'-promoter. Sequence analysis of this TATA-less promoter revealed the presence of several putative-binding sites for transcriptional factors, including CCAAT site at -41 to -32 and Sp1 site at -34 to -24 . Transfection of pituitary cells with constructs of variable lengths confirmed the relevance of different promoter regions in the control of transcriptional activity. Among them, the -49 to $+156$ region was critical for basal transcriptional activity. Electrophoretic mobility shift assay using nuclear proteins extracted from normal and immortalized pituitary cells indicated that the CCAAT/Sp1 site within the -49 to $+156$ region was able to specifically interact with CCAAT-binding factor and Sp1. These two sites were partly overlapped and both of them conferred stimulatory effects. The *in vivo* recruitment of CCAAT-binding factor and Sp1 was confirmed by chromatin immunoprecipitation. These results indicate that the composite CCAAT/Sp1 *cis*-element contributes to the expression of $\alpha 1$ -sGC subunit in resting pituitary cells.

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Introduction

Soluble guanylyl cyclase (sGC; E.C.4.6.1.2) catalyzes the formation of cGMP, an intracellular messenger that activates several downstream effectors, including ion channels, protein kinase G, and phosphodiesterases (Garbers *et al.* 1994). This signaling pathway regulates a broad spectrum of physiological processes, such as smooth muscle relaxation, platelet aggregation, and neuronal cell mobility. Based on cDNA cloning, four sGC subunits have been identified and termed $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$. The human isoforms isolated from an adult brain library, termed $\alpha 3$ and $\beta 3$, represent the human homologues of rat and bovine $\alpha 1$ and $\beta 1$ (Zabel *et al.* 1998). The $\alpha 1$ and $\alpha 2$ subunits are interchangeable in terms of sGC activity when coexpressed with $\beta 1$, but the $\alpha 1\beta 1$ heterodimer is most common in mammalian tissues (Hanafy *et al.* 2004). The dimer activity is regulated directly at protein level. Physiologically, the most relevant mode of such regulation is mediated by binding of nitric oxide (NO) to the heme group of sGC (Humbert *et al.* 1990). The enzyme is also subjected to phosphorylation by protein kinase, src-like kinases, and protein kinases C and G (Pyriochou & Papapetropoulos 2005). Protein–protein interactions also contribute to the direct control of sGC activity, since documented by interaction between the chaperonin containing t-complex polypeptide subunit η and $\beta 1$ -sGC, which leads to inhibition of the enzyme activity (Hanafy *et al.* 2004).

Several reports also indicate that the regulation of sGC activity occurs indirectly, at transcriptional and post-transcriptional levels, affecting the availability of sGC subunits and the total number of functional heterodimers. For example, the steady-state sGC subunits mRNA levels are reduced by treatments with estrogens (Krumenacker *et al.* 2001), lipopolysaccharide (Pedraza *et al.* 2003), and β -amyloid peptides (Baltrons *et al.* 2002). In human mesangial cells, collagen type I can also reduce the steady-state $\beta 1$ -sGC mRNA levels and such reduction occurs at transcriptional level (De Frutos *et al.* 2005). On the other hand, the AU-rich elements identified in the 3'-untranslated region of $\alpha 1$ - and $\beta 1$ -sGC mRNAs provide sites for post-transcriptional regulation by cAMP (Kloss *et al.* 2004). Differential expression of mRNAs for α and β -sGC also influences the formation of functional heterodimers. For example, in pituitary cells, the mRNA levels (Budworth *et al.* 1999) and the protein levels (Kostic *et al.* 2004) for $\alpha 1$ -sGC are lower than $\beta 1$ -sGC. Consistent with these findings, the overexpression of $\alpha 1$ -sGC alone in immortalized pituitary cells is sufficient to make several fold increase in NO-stimulated cGMP production (Kostic *et al.* 2004).

Changes in the mRNA expression levels of sGC subunits have also been reported in several disease models. In aortic tissue from spontaneously hypertensive rats, the vasodilator response to an NO donor was markedly attenuated compared with normotensive rats

(Bauersachs *et al.* 1998). The detailed analysis of hypertensive animals revealed that $\alpha 1$ - and $\beta 1$ -sGC mRNA levels, as well as $\beta 1$ -sGC protein levels, were reduced significantly, indicating that vasodilator dysfunction is related to the sGC gene expression (Kloss *et al.* 2000). In deoxycorticosterone acetate-salt model of rat hypertension, the protein expression level of $\beta 1$ -sGC was also decreased in the inner medulla (Taylor *et al.* 2003). In addition, the protein levels of $\beta 1$ -sGC were reduced in reactive astrocytes obtained from Alzheimer, Creutzfeldt-Jakob, and multiple sclerosis patients (Baltrons *et al.* 2004), whereas an upregulation of sGC was observed in aortas from rats with chronic heart failure (Bauersachs *et al.* 1999). Therefore, studies on the transcriptional regulation of sGC are not only important for understanding the mechanism of sGC gene expression, but are also clinically relevant.

However, the knowledge on promoter of sGC genes and regulation of the enzyme activity at transcriptional and post-transcriptional levels is very deficient. At the present time, the genomic organization of mouse (Sharina *et al.* 2000) and medaka fish (Mikami *et al.* 1999) $\alpha 1$ - and $\beta 1$ -sGC subunits, and human $\beta 1$ -sGC subunit (Sharina *et al.* 2003) has been reported. Among the mammalian species, only the mouse $\alpha 1$ -sGC promoter has been cloned, but its *cis*-elements have not been experimentally identified (Vazquez-Padron *et al.* 2004). Furthermore, the finding that $\alpha 1$ -sGC gene for mouse has only 56 and 45% homology with predicted sequences for rat and human genes respectively (Vazquez-Padron *et al.* 2004), clearly indicates a need for cloning of promoter regions in other species in order to understand the variability in their structures and regulation of transcriptional activity. Here, we describe the promoter region of rat $\alpha 1$ -sGC gene. Using pituitary tissue, we identified the transcriptional start site and cloned a 3.5 kb upstream promoter of the $\alpha 1$ -sGC subunit. We also identified numerous binding sites for transcriptional factors by means of sequential analysis of the $\alpha 1$ -sGC promoter region. Partly overlapping *cis*-elements CCAAT and Sp1 were further identified by the electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP), whereas the site-directed mutagenesis study indicated that this site regulates the expression of $\alpha 1$ -sGC gene in resting cells.

Materials and methods

Materials

Medium 199, Ham's F12K medium, Opti-MEM, penicillin, horse serum, streptomycin, and gentamicin stock solution were purchased from Invitrogen. Fetal bovine serum was obtained from Biosource International (Camarillo, CA, USA). DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville,

IA, USA) and the nucleotide sequences are listed in Table 1 and Fig. 6. Antibodies against CCAAT-binding factor (CBF)-A (SC-13045), CCAAT/enhancer-binding protein β (SC-746), nuclear factor-1 (NF-1) (SC-870), and Sp1 (SC-14027) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other reagents were obtained from Sigma, if not otherwise specified.

Cell cultures

Anterior pituitaries were removed from adult female Sprague-Dawley rats obtained from Tacoma Farms (Germantown, NY, USA) and dispersed as described previously (Kostic *et al.* 2002). Dispersed cells were cultured for 24 h in medium 199 containing Earle's salts, sodium bicarbonate, 10% horse serum, and penicillin (100 U/ml) and streptomycin (100 μ g/ml). Lacto-somatotroph GH₃ immortalized cells and MH1C1 liver cells were maintained in Ham's F12K medium supplemented with 1.5 g/l sodium bicarbonate, 15% heat-inactivated horse serum, 2.5% fetal bovine serum, and gentamicin (100 μ g/ml).

Identification of the transcription start site of $\alpha 1$ -sGC gene

Total RNA was extracted from normal anterior pituitary cells using Trizol Reagent (Invitrogen) and mRNA was enriched by PolyATtract mRNA Isolation Systems (Promega). Identification of the transcription start site of $\alpha 1$ -sGC gene was performed using the SMART RACE kit (Clontech) with minor modifications. Primers sGCaRT, RaceOuter, and RaceInner (Table 1) were designed according to the mRNA sequence of $\alpha 1$ -sGC deposited in Genbank (BC085746). Primers BD SMART II A oligo, Universal Primer A Mix, and Nested Universal Primer A were provided in the kit. The sGC first strand cDNA was synthesized by the gene-specific primer sGCaRT and BD SMART II A oligo. Then PCR was conducted with the Universal Primer A Mix and the gene-specific primer RaceOuter. Nested PCR was conducted with the Nested Universal Primer A and the gene-specific primer RaceInner. The PCR product was subcloned by TOPO TA cloning kit (Invitrogen). Plasmid DNA was prepared by QIAprep Spin Miniprep kit (Qiagen) and sequenced by Veritas (Rockville, MD, USA). The distance between transcription start site and start codon was calculated based on cDNA sequence (Lorens *et al.* 1993, Okita *et al.* 2003, Salgado *et al.* 2004). The boundary of exons I and II was defined by aligning cDNA sequence of $\alpha 1$ -sGC with the genomic DNA in the corresponding region retrieved from rat genome reference database (<http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html>).

Table 1 Primer sequences used in this study

	Position	Sequence	Annotation
Primer name			
sGC α RT	770 to 793	AGGAGCGTGCTGAAGCTGTTGAGA	
RaceOuter	550 to 577	GCCAATGTTCTCTGAAGTGCAAGGTTCA	
RaceInner	337 to 364	GGAACCTGACCAAGGAGCCAGTAAGGAGA	
Pt(−3K)	−3529 to −3502	TCAGGT ACC TCAACACGAAGAAAGAGATAATAAGCAC	KpnI site
Pt(−2K)	−2250 to −2224	TTTGGT ACC AGACAGTGAGATTGACAAGCCATTAGC	KpnI site
Pt(−875)	−875 to −848	AAGGGT ACC TGGATCCTGTAGCTGGCTTTCTTGGCAT	KpnI site
Pt(−719)	−719 to −702	CTAGGT ACC CGCTCTACCACTGAGCTA	KpnI site
Pt(−599)	−599 to −582	AGTGGT ACC AGAGTCGATGATGAGTCA	KpnI site
Pt(−516)	−516 to −500	AAAGGT ACC AGGAGTAAGTGGAGTGT	KpnI site
Pt(−278)	−278 to −258	CTTGGT ACC GAGTAGATTAGCCTCAAAGCT	KpnI site
Pt(−165)	−165 to −149	GTCGGT ACC AGCTAGTACGAGAGAAA	KpnI site
Pt(−49)	−49 to −32	TTGGT ACC TTGGAGGACAGCGATTGG	KpnI site
Pt(156)R	137 to 156	AACTCGAGCATCCGGGCGCAGGTGTCTT	XhoI site
CAAT/Sp1UP	−50 to −18	TTTGGAGGAC AGCGATTGGGCGGGGAGCAGGAC	CCAAT/Sp1
CAAT/Sp1DN	−50 to −18	GTCCTGCT CCCCGCCCAATCGCTGTCCTCCAAA	CCAAT/Sp1
MutCCAAT/Sp1UP	−50 to −18	TTTGGAGGACAGCGA AAAAGC GGGAGCAGGAC	Mutated site
MutCCAAT/Sp1DN	−50 to −18	GTCCTGCT CCCCGCTTTTTG CTGTCCTCCAAA	Mutated site
IPt(−3K)UP	−3506 to −3486	AGCACAAAACCTCCAGACTCA	
IPt(−3K)DN	−3411 to −3392	GGAACACCCCACTACACAAT	
IPt(−1K)UP	−1395 to −1377	CTCTATAGGAACCCACAGCACAGC	
IPt(−1K)DN	−1281 to −1258	TGAATAAAGGTCCACAGGCAGGTA	
IPt(−165)UP	−165 to −145	AGCTAGTACGAGAGAAAAGGT	
IPt(−18)DN	−40 to −18	GTCCTGCT CCCCGCCCAATCGCT	
IPt(−63)UP	−63 to −42	CTGGCTTTGGTGGTTTGGAGGA	
IPt(59)DN	40 to 59	CCTGGAGGGCTGTGTGTGAG	
RTGC1	1133 to 1156	TCTTGTCTTAGGGTCGCCGTGTG	
RTGC2	1276 to 1299	TGCCCTCAGCTTTCCCAACCTCTT	
RTactin1	312 to 335	CAACTGGGACGATATGGAGAAGAT	Template: NM_031144
RTactin2	493 to 516	AGAGGCATACAGGGACAACACAGC	

Meanings of bold cases are shown in the annotation column. The numbers in the position column represent the distance to the transcriptional start site.

Cloning the 5'-promoter of $\alpha 1$ -sGC gene and generation of luciferase deletion constructs

Genomic DNA was extracted from rat anterior pituitaries by Wizard Genomic DNA purification kit (Promega). To amplify the promoter, PCR was conducted using genomic DNA as template and Pt(156)R and Pt(−3K) as primers (Table 1). The downstream primer Pt(156)R was designed according to the SMART RACE result and incorporates an XhoI site. The upstream primer Pt(−3K) was designed according to the genomic DNA sequence flanking $\alpha 1$ -sGC gene and incorporates a KpnI site. The PCR product was digested by XhoI and KpnI sequentially, and subcloned into the XhoI/KpnI site of pGL3.Basic. The obtained promoter clone was termed pGL3.(−3K). Based on the pGL3.(−3K) sequence, primers Pt(−2K), Pt(−875), Pt(−719), Pt(−599), Pt(−516), Pt(−278), Pt(−165), and Pt(−49) were designed to prepare deletion constructs pGL3.(−2K), pGL3.(−875), pGL3.(−719), pGL3.(−599), pGL3.(−516), pGL3.(−278), pGL3.(−165), and pGL3.(−49) respectively (Table 1). In combination with Pt(156)R, these primers were able to amplify decreased length of $\alpha 1$ -sGC promoter. Identities of these promoter constructs were confirmed by sequencing.

Reverse transcription-PCR

Total RNAs were extracted from the 19th and 40th passages of GH₃ cells and the 55th passage of MH1C1 cells using Trizol Reagent (Invitrogen). One microgram RNA was treated with amplification grade DNase I and primed with oligo(dT)₂₀ primer to synthesize cDNA using SuperScript III first-strand synthesis supermix (Invitrogen). In mocking experiments, reverse transcription was conducted in the absence of SuperScript III. Fifty microliters of the PCR mixtures contained 1 μ l cDNA, 1 μ l of each primer (10 μ M), 3 μ l H₂O, and 45 μ l Platinum blue PCR SuperMix (Invitrogen). PCR was initiated with a denature step at 94 °C for 30 s, followed with 35 amplification cycles consisted of denature at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. PCR products were stored at 4 °C until use.

Transient transfection and dual-luciferase assays

One day before transfection, GH₃ and MH1C1 cells were plated in 96-well plates with a density of 0.04 million cells per well. Then 0.19 μ g constructs carrying 5'-promoter of $\alpha 1$ -sGC gene and firefly luciferase were

mixed with 0.01 μ g renilla luciferase construct pRL-CMV (Promega). The DNA mixture was combined with 0.6 μ l lipofectamine 2000 (Invitrogen) in 50 μ l OptiMEM medium according to the manufacturer's instructions. In these studies, pRL-CMV was used as an internal control to monitor the transfection efficiency between experimental groups. Transfection mixture with lipofectamine 2000 was incubated for 20 min at room temperature and added to GH₃ cells in 100 μ l OptiMEM medium. After 6-h incubation, the transfection medium was replaced with Ham's F12K medium supplemented with 1.5 g/l sodium bicarbonate, 15% heat-inactivated horse serum, 2.5% fetal bovine serum, and gentamicin (100 μ g/ml). Following 48-h incubation, medium was aspirated and cells were incubated in Ham's F12K medium supplemented with 1.5 g/l sodium bicarbonate and 0.1% BSA for an additional 24 h. The measurement of luciferase activity was done using the Dual-Luciferase Reporter Assay System (Promega). Transfected cells were washed in 1 $\times$ PBS twice and then dissolved in 5 μ l passive lysis buffer (Promega). For measuring the firefly luciferase activity, 20 μ l cell lysate were mixed with 50 μ l Luciferase Assay Reagent II and then 50 μ l Stop and Glo Reagent (Promega) were added. Luminescence signal was measured with a 5 s delay for a period of 10 s in a Mithras LB 940 multimode reader (Berthold Technologies, Bad Wildbad, Germany).

Electrophoretic mobility shift assay

Nuclear proteins were extracted from dispersed normal and GH₃ pituitary cells using a NucBuster Protein Extraction Kit (Novagen, Madison, WI, USA). EMSA was conducted using a DIG gel shift kit (Roche Applied Science). Briefly, equal molar of CCAAT/Sp1UP and CCAAT/Sp1DN, or MutCCAAT/Sp1UP and MutCCAAT/Sp1DN were mixed and incubated at 95 °C for 10 min. After slowly cooling down to room temperature, double-stranded (ds) oligonucleotides formed by annealing of the respective primers were diluted with TEN buffer (10 mM Tris-Cl, 1 mM EDTA, and 0.1 M NaCl (pH 8.0)) to a final concentration of 3.85 pmol/ μ l. Labeling reaction was done in a final volume of 20 μ l containing 1 μ l ds-oligonucleotides, 9 μ l water, 4 μ l 5 $\times$ labeling buffer, 4 μ l CoCl₂ solution, 1 μ l DIG-ddUTP, and 1 μ l terminal deoxynucleotide transferase. The reaction mixture was incubated at 37 °C for 15 min and chilled on ice immediately. Labeling reaction was stopped by 2 μ l EDTA (2 mM). The labeled ds-oligonucleotides were diluted to 0.155 pmol/ μ l by adding 3 μ l double distilled water. For gel shift analysis, a small aliquot was further diluted to 15.5 fmol/ μ l. In the gel shift assay, 8 μ g nuclear extract from GH₃ cells or normal pituitary cells were incubated with 4 μ l binding buffer (5 mM EDTA, 50 mM (NH₄)₂SO₄, 5 mM dithiothreitol

(DTT), 1% Tween 20 (v/v), 150 mM KCl, 100 mM Hepes (pH 7.6)), 1 μ l poly(dI-dC), and 1 μ l poly-L-lysine. Double distilled water was added to a final reaction volume of 18 μ l. In the competition and supershift assays, we added unlabeled ds-oligonucleotides and antibodies in the reaction mixture respectively. After 5-min incubation at room temperature, 2 μ l DIG-labeled ds-oligonucleotides were added into the reaction mixture and incubated for another 30 min. After adding 5 μ l loading dye, the reaction mixture was size-fractionated in a 6% DNA retardation gel (Invitrogen) in 0.5 $\times$ TBE buffer and transblotted onto a positively charged nylon membrane for 2 h using an XCell II Blot Module (Invitrogen). The membrane was then u.v.-crosslinked using a Stratalinker 2400 (Stratagene, La Jolla, CA, USA), rinsed in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20 (pH 7.5)) for 5 min, and incubated for 30 min in blocking solution (1% blocking reagent in maleic acid buffer). Following incubation with anti-DIG-AP (1:10 000) in blocking solution for 30 min, the membrane was washed 2 $\times$ 15 min in washing buffer and equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl (pH 9.5)) for 5 min. Chemiluminescence signals were detected with CSPD as the substrate in X-ray film.

DNA affinity precipitation assay

Ds-probes were generated by annealing biotin-labeled upstream oligonucleotides (Fig. 6) with the respective reverse-complemented oligonucleotides. Binding mixtures contained 10 μ l ds-probes (10 μ M), 60 μ g nuclear protein extracted from GH₃ cells, 80 μ l of 5 $\times$ binding buffer (5 mM EDTA, 50 mM (NH₄)₂SO₄, 5 mM DTT, 1% Tween 20, 150 mM KCl, and 100 mM Hepes (pH 7.6)), and water to 400 μ l. After incubation at 4 °C for 1 h, 25 μ l Tetralink tetrameric avidin resin (Promega) was added to capture biotin-labeled DNA-protein complex. Resins were washed in 1 $\times$ binding buffer for five times and proteins were eluted in 25 μ l Novex Tris-glycine SDS sample buffer (2 $\times$; Invitrogen). Eluted proteins were analyzed by Western blot.

Site-directed mutagenesis

The construct pGL3.(–516) was subjected to site-directed mutagenesis by the QuikChange Site-Directed kit (Stratagene) and high fidelity *PfuTurbo* DNA polymerase (Stratagene). Reaction mixture was composed of 5 μ l of 10 $\times$ reaction buffer, 20 ng DNA template, 1 μ l of 10 mM dNTPs, 1 μ l *PfuTurbo* polymerase, and 140 ng PCR primers in a final volume of 50 μ l. The reaction was initiated with a denaturing step at 95 °C for 30 s, followed by PCR amplification with denaturation at 95 °C for 30 s, annealing at 55 °C for

1 min, and extension at 68 °C for 6 min for a total of 16 cycles. Subsequently, the reaction mixture was cooled on ice and 1 μ l DpnI was added to digest the original DNA template for 2 h at 37 °C. The PCR products were then precipitated with ethanol and used for transformation in XL1-Blue Supercompetent cells. Mutation was confirmed by DNA sequencing.

Chromatin immunoprecipitation assay

This assay was performed using ChIPs assay kit from Upstate (Waltham, MA, USA). Dispersed rat anterior pituitary normal cells and GH₃ cells were cross-linked with 1% formaldehyde and suspended in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.1)). Lysates were then sonicated to shear DNA and diluted tenfold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, and 16.7 mM Tris-HCl (pH 8.1)). The resultant chromatin was immunocleared with salmon sperm DNA/protein A agarose. Subsequently, 5 μ l antibody against CBF/A (SC-13045) or Sp1(SC-14027) was incubated overnight with the chromatin at 4 °C. The antibody/chromatin complex was pulled down by incubating with salmon sperm DNA/protein A agarose for 1 h. Agarose was precipitated by gentle centrifugation and washed sequentially with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% IGELPAL-CA630, 1% sodium deoxycholic acid, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1)), and 1× TE. Histone complex was eluted in elution buffer (1% SDS and 0.1 M NaHCO₃) and heated at 65 °C for 6 h to reverse formaldehyde cross-linking. Samples were then treated with protease K, followed by phenol extraction and ethanol precipitation. The DNA pellet was dissolved in 50 μ l Tris-EDTA buffer, and then 1 μ l DNA was used for PCR with 36 cycles of amplification. PCR amplification of soluble chromatin prior to immunoprecipitation was used as an input control.

Data transformation and statistical analysis

Promoter activities were expressed in firefly luciferase activity normalized against renilla luciferase activity in transfected GH₃ and MH1Cl cells. The normalization was conducted to minimize the potential problems of variations in transfection efficiency among groups. Data presented were expressed as mean $\pm$ S.E.M. and analyzed using Student's *t*-test. The differences were considered significant at *P* < 0.05.

Results

Identification of $\alpha 1$ -sGC transcription start site

Primer extension is a common method used to map the transcription start site. However, premature termination and intrinsic terminal transferase activity of reverse transcriptase make this method ineffective in some experiments (Gong & Ge 2000). Since SMART RACE can reduce these problems (Gong & Ge 2000), it was used in this study to identify the transcription start site of $\alpha 1$ -sGC gene. Pituitary mRNA was transcribed to cDNA with a gene-specific primer sGCART in the presence of BD SMART II A oligonucleotides and 5'-CDS primer. Amplification of cDNA with primers Universal Primer A Mix and RaceOuter produced a specific band with a size about 600 bp. Using primers Nested Universal Primer A and RaceInner, a specific band with a size around 400 bp was obtained (data not shown) and subcloned into the TOPO TA cloning vector. Sequencing of five clones revealed that they possessed different 5'-ends (data not shown). The most upstream 5'-end, located at 287 bp upstream of the start codon, was assigned as position +1 (Fig. 1). The respective clone contains the longest 5'-untranslated region (UTR) among other rat $\alpha 1$ -sGC cDNA sequences (BC085746, NM_017090) and gene sequence (AF327645) deposited in the Genbank. Alignment between the cDNA sequence and the genome sequence of $\alpha 1$ -sGC gene indicated, it has a 183 bp exon I and a 356 bp exon II. In exon I, two nucleotides were different between cDNA sequence in this report and that in rat genome, possibly reflecting the presence of single nucleotide polymorphism (Fig. 1). The start codon is located in the exon II (Fig. 1).

Exon I

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ggaggagtgaggaggactcggagcagcgggggttctcacacacagccot
ccaggcggtcctcctccagtgctcgcagcccgaccccaaggcggaaga
gcagcagtgctcagcccggggagtcctcgaagcgaagacacctgcgc
      +1
      agcctg
      Start of BC085746
cggatgccctggcctccgtgaccgcatcatg

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Exon II

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atgctggggccactcgtgtctttgagtcagtagaagcagatcttcatca
gtccacatcaaacaccggctataaaggaggaaaccactgccaagctcca
ggaacaccATGttctgcaggaagttcaagatctcaagatcacggggg
agtgtcctttctccttactggctccttggtcagggttccatcggagccaa
tagaggaggtggccggaggtctctgagagctgccaggccactctgccca
cctgccaggaggtttgctgagaatgcagaaggagtcacccccaagaa
agacaagccgcaacagagtcctacacacaccctggcagagagtattg
gcaagctcattttccagag

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Figure 1 Nucleotide sequence of the exons I and II of $\alpha 1$ -sGC gene from rat anterior pituitary. The identified transcriptional start site is underlined and denoted as +1. Nucleotides in exon I that mismatch with rat genome sequence were shown in italic bold. The position of the transcriptional start site previously reported (BC085746) is indicated by an arrow and the translational start codon in exon II is shown in capital bold.

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comparable $\alpha 1$ -sGC mRNA levels was detected in two passages of GH₃ cells (Fig. 3A), a feature that makes these cells a favorable model in $\alpha 1$ -sGC studies. Furthermore, primers RTGC1 and RTGC2 were unable to amplify product using cDNA from MH1C1, which was unlikely caused by cDNA quality, because primers RTactin1 and RTactin2 (Table 1) amplified a strong PCR band (Fig. 3A). Therefore, MH1C1 cells represent a valuable control cell model for studies on $\alpha 1$ -sGC mRNA expression.

In 19th passage of GH₃ cells, the construct pGL3.(−3K), carrying 3529 bp promoter sequence of $\alpha 1$ -sGC, has a similar promoter activity with the control vector pGL3.Basic. Deletion of the promoter fragments up to −49 in length induced biphasic changes in transcriptional activity. The deletion between −3529 and −2250 (construct pGL3.(−2K)) increased the promoter activity, indicating the presence of a negative

element(s) in this region. Further deletions until −516 have only a marginal effect on the promoter activity, indicating that the region between −2250 and −516 is not critical for basal sGC promote activity. Deletion of −516 to −278 and −278 to −165 sequences reduced and enhanced the promoter activity more than twofold respectively, indicating the presence of stimulatory and inhibitory elements in these regions (Fig. 3B). Deletion of the most proximal −49 region reduced the promoter activity more than sixfold, implying that this region is critical for the basal activity of $\alpha 1$ -sGC promoter. We observed no significant difference among all promoter constructs examined in 19th and 40th passages of GH₃ cells. In general, the $\alpha 1$ -sGC promoter activities MH1C1 paralleled those observed in GH₃ cells, but the amplitudes of responses were smaller (Fig. 3B).

Characterization of CCAAT/Sp1 transcription-binding sites

The most proximal −49 region contains the putative CCAAT/Sp1 *cis*-element (Fig. 2). To investigate the role of CCAAT/Sp1 site in basal promoter activity of $\alpha 1$ -sGC, DNA–protein interaction was studied by EMSA. The synthesized oligonucleotides, CCAAT/Sp1UP and CCAAT/Sp1DN, located between −50 and −18 (Table 1), encompassed the CCAAT/Sp1 element completely. The annealed ds-oligonucleotide was named dsCCAAT/Sp1. Using DIG-labeled dsCCAAT/Sp1 and nuclear extract from GH₃ cells, EMSA revealed the presence of specific band in addition to the free probe (Fig. 4A, lane 1). The specific band was eliminated by the competition of unlabeled dsCCAAT/Sp1 in a dose-dependent manner (Fig. 4A, lanes 2–4). Competition assay was also conducted using mutated dsCCAAT/Sp1. Analysis done by a transcription element search system (<http://www.cbil.upenn.edu/tess/>) indicated that this mutation renders the dsCCAAT/Sp1 loss of pattern of CCAAT and Sp1 elements. In this case, the competition with mutated dsCCAAT/Sp1 did not change the signal of the specific band (Fig. 4A, lanes 5–7). When nuclear proteins from normal anterior pituitary cells were used, one strong shifted band was observed (Fig. 4B, lane 1). This band was reduced by unlabeled dsCCAAT/Sp1 in a dose-dependent manner (Fig. 4B, lanes 2–4), but was not affected when the core sequence of dsCCAAT/Sp1 was mutated (Fig. 4B, lanes 5–7).

Identification of transcriptional factors bound to CCAAT/Sp1 site

To identify the transcriptional factors bound to the CCAAT/Sp1 site, the supershift analysis was conducted. In this experiment, antibodies against CBF/A,

Figure 3 Promoter activity of $\alpha 1$ -sGC deletion constructs. (A) Expression of $\alpha 1$ -sGC mRNAs in GH₃ immortalized pituitary and MH1C1 liver cells. PCRs were conducted using $\alpha 1$ -sGC specific primers and β -actin specific primers in the absence (−) or presence (+) of reverse transcription (RT). (B) Transient transfection analysis of deletion constructs. GH₃ and MH1C1 cells were cotransfected with renilla luciferase construct pRL-CMV and firefly luciferase reporter constructs containing different size promoter fragments. Promoter activities for each construct were presented as a ratio of relative light units between firefly luciferase and renilla luciferase. Results are means $\pm$ S.E.M. ($n=8$).

CCAAT/enhancer-binding protein, NF-1, and Sp1 were used. Three of these transcriptional factors, CBF/A, CCAAT/enhancer-binding protein, and NF-1, can bind with CCAAT element (Mantovani 1999). Using nuclear extract from GH₃ cells, antibody against CBF/A reduced the specific signal of shifted bands, indicating the presence CBF/A in the CCAAT/Sp1–nuclear protein complex (Fig. 5A, lane CBF/A). On the other

hand, when antibody against Sp1 was used, a strong supershift band was observed (Fig. 5A, lane Sp1). Antibodies against CCAAT/enhancer-binding protein and NF-1 did not change the shifted bands (Fig. 5A,

Figure 4 Analysis of protein complexes formed between putative CCAAT/Sp1 oligonucleotides and nuclear extracts from (A) GH₃ and (B) normal pituitary cells. Eight micrograms nuclear extracts were incubated with DIG-labeled putative CCAAT/Sp1 oligonucleotides in the absence of competitor (lane 1) and in the presence of different folds of unlabeled CCAAT/Sp1 oligonucleotides (lanes 2–4) or unlabeled mutated CCAAT/Sp1 oligonucleotides (lanes 5–7). Horizontal arrows indicate specific binding.

Figure 5 Identification of transcription factors binding with CCAAT/Sp1 oligonucleotides in (A) GH₃ and (B) normal pituitary cells. Eight micrograms nuclear extracts were incubated with DIG-labeled putative CCAAT/Sp1 oligonucleotides in the absence of antibody (lane Control) or in the presence of different antibodies (lanes 2–5 in panel A and lanes 2–6 in panel B). Shifted bands are indicated with arrows. To confirm that binding sites for CBF/A and Sp1 are overlapping, 8 μ g nuclear extracts were also incubated with DIG-labeled mutated CCAAT/Sp1 oligonucleotides (lane 6 in panel A). C/EBP, CCAAT/enhancer-binding protein; NF-1, nuclear factor-1.

lanes C/EBP and NF-1). When labeled mutated dsCCAAT/Sp1 was used in EMSA, no shifted band was observed, indicating that the binding sites for Sp1 and CBF/A were overlapping (Fig. 5A, lane 6). Similar results were observed when nuclear extracts from the normal cells from rat pituitary were used in the assay (Fig. 5B). Furthermore, antibodies against CBF/A and Sp1 have additive effects on the supershift result (Fig. 5B, lane CBF/A+Sp1), indicating CBF/A and Sp1 can independently bind to the CCAAT/Sp1 element.

Delineate nucleotides required for CBF and Sp1 binding

To precisely delineate nucleotides required for CBF/A and Sp1 binding, DNA affinity precipitation assay was conducted using a series of mutated ds-oligonucleotides, termed mutants 1–8 (Fig. 6, upper panel). The upstream oligonucleotides of wild type has the same sequence as primer CCAAT/Sp1UP, located at –50 to –18, and mutant 5 in this experiment has the same sequence as the above-mentioned primer MutCCAAT/Sp1UP. When Sp1 antibody was used, signals in mutants 5–8 were weaker than that in the wild type (Fig. 6, panel Sp1), indicating that the region between –35 and –26 was important for Sp1 binding. On the other hand, when CBF/A antibody was used, signals in mutants 3–6 were much weaker than that in the wild type (Fig. 6, panel CBF/A), indicating that the region between –39 and –30 was important for CBF binding. Clearly, the CCAAT and Sp1 *cis*-elements are partly overlapping because mutation in the region between –35 and –30 reduced the affinity for both CBF/A and Sp1 binding (Fig. 6).

Inhibition of transcription by mutation of CCAAT/Sp1 element

To clarify the potential role of CCAAT and Sp1 *cis*-element in basal promoter activity of $\alpha 1$ -sGC gene, mutagenesis analysis was conducted. The CCAAT and Sp1 sites in construct pGL3.(–516) was mutated to pGL3.(–516Mut3), pGL3.(–516Mut5), pGL3.(–516Mut7) according to the sequences of mutant 3, 5, and 7 respectively. Mutant 3 has a reduced affinity to CBF/A but not to Sp1, whereas mutant 7 has a reduced affinity to Sp1 but not to CBF/A (Fig. 6). Mutant 5 has the weakest affinity to both CBF/A and Sp1 (Fig. 6). Comparing with the promoter activity of pGH3.(–516), all three mutant constructs had a weaker promoter activity, demonstrating that both CCAAT and Sp1 *cis*-elements were stimulatory to the basal activity of $\alpha 1$ -sGC promoter (Fig. 7).

Figure 6 Delineation of nucleotides required for CBF/A and Sp1 binding. Sixty micrograms nuclear extracts from GH₃ cells were incubated with ds-oligonucleotides with biotin labeling on one strand. Bound proteins were pulled down by avidin resin and analyzed by western blot. Upper panel shows the biotin-labeled strand and mutated nucleotides were indicated in bold. WT, wild type; NC, negative control (the biotin-labeled scramble ds-oligonucleotides).

Figure 7 Effects of CCAAT/Sp1 site mutation on the promoter activity of $\alpha 1$ -sGC in GH₃ cells. Construct pGL3-sGC(–516) was used to prepare mutation constructs pGL3-sGC(Mutant 3), pGL3-sGC(Mutant 5), and pGL3-sGC(Mutant 7). GH₃ cells were transfected with renilla luciferase construct pRL-CMV and different firefly luciferase reporter constructs. Promoter activity for each construct was presented as the ratio of relative light units between firefly luciferase and renilla luciferase. Results are means $\pm$ S.E.M. ($n=8$) and asterisks indicate significant difference when compared with the wild-type pGL3-sGC(–516; $P<0.05$).

Recruitment of Sp1 and CBF/A to the promoter of $\alpha 1$ -sGC gene *in vivo*

To investigate whether both Sp1 and CBF/A can be recruited to the CCAAT/Sp1 element *in vivo*, ChIP was conducted in GH₃ and normal pituitary cells. Four primer pairs were designed to amplify different regions of $\alpha 1$ -sGC promoter. Primers IPt(−3K)UP and IPt(−3K)DN were used to amplify a 115 bp fragment located 3 kb upstream of CCAAT/Sp1 site, and primers IPt(−1K)UP and IPt(−1K)DN were used to amplify a 138 bp fragment located 1 kb upstream of CCAAT/Sp1 site (Table 1). On the other hand, primer pairs IPt(−165)UP and IPt(−18)DN, IPt(−63)UP, and IPt(59)DN cover the CCAAT/Sp1 region (Fig. 8, lower panel). PCR products were only analyzed in a qualitative manner. In GH₃ chromatin, all these primers amplified a specific product with the predicted size using the input DNA as the template (Fig. 8A, Input). When the chromatin was immunoprecipitated by antibody against the CBF/A, no PCR product was found using first primer pairs (Fig. 8A, CBF/A, lane 1), and only a weak signal appeared using second primer pairs (Fig. 8A, CBF/A, lane 2). In agreement with the presence of CBF/A binding in the region between −41 and −32, third and fourth primer pairs generated specific PCR products (Fig. 8A, CBF/A, lanes 3 and 4). When the chromatin was immunoprecipitated with antibody against Sp1, a strong PCR amplification was also observed using primer pairs covering the CCAAT/Sp1 element (Fig. 8A, Sp1, lanes 3 and 4). No or weak

PCR amplification was detected using primer pairs distal to the CCAAT/Sp1 element (Fig. 8A, Sp1, lanes 1 and 2). The PCR amplification was specific because no amplicon was observed when chromatin was precipitated with immunoglobulin G as the negative control (Fig. 8A, IgG). Similar results were observed in normal pituitary cells (Fig. 8B).

Discussion

Using normal cells from rat pituitary, we identified the transcription start site and cloned the 3.5 kb upstream promoter sequence of rat $\alpha 1$ -sGC subunit gene. For the first time, we also characterized the promoter of $\alpha 1$ -sGC subunit gene in this species. The data showed that the transcription start site of rat $\alpha 1$ -sGC is located at 287 bp upstream of the start codon in mRNA, which enabled us to define the boundary of 5'-UTR and promoter. Our sequence analysis indicated that the cloned promoter of rat $\alpha 1$ -sGC subunit belongs to the TATA-less promoters and possesses several putative transcription factor-binding sites, including Sp1, CCAAT, Wilms' tumor, and activator protein-1. The results further revealed that CCAAT box and Sp1 *cis*-element were crucial in the regulation of $\alpha 1$ -sGC promoter activity in this species. These elements may also provide a crosstalk mechanism between different components in the NO/sGC signaling pathway by regulating the transcription efficiency of $\beta 1$ -sGC and NO synthases.

Figure 8 Recruitment of CBF/A and Sp1 transcription factors to the $\alpha 1$ -sGC promoter in (A) GH₃ and (B) normal pituitary cells. Soluble chromatin from GH₃ cells or normal cells was precipitated with antibodies against CBF/A, Sp1, or preimmune rabbit immunoglobulin (IgG). The DNA size marker is labeled M. DNA regions analyzed in PCR (lanes 1–4) are schematically represented at the bottom. The predicted overlapping binding site for CBF/A and Sp1 is indicated by vertical dotted line.

The sequence analysis indicated that the cloned promoter of rat $\alpha 1$ -sGC subunit belong to the TATA-less promoters. The promoter of mouse $\alpha 1$ -sGC (Vazquez-Padron *et al.* 2004) and human $\beta 1$ -sGC (Sharina *et al.* 2003) subunit genes also lacks a TATA box. In general, the TATA-like element is crucial for the accurate transcription initiation in the protein-coding gene (Smale 1997). Consistently, our results also revealed the presence of different transcription initiation sites among different clones. However, a special scenario exists in medaka fish, where only a single transcriptional start site was identified in different tissues despite the absence of functional TATA-like element in the promoter of $\alpha 1$ -sGC (Yamamoto & Suzuki 2002).

Our transient transfection experiments confirmed that the cloned promoter of $\alpha 1$ -sGC subunit exhibits low activity in resting cells and identified elements that could contribute to it. The maximal transcription activity was obtained when a 599 bp promoter fragment was inserted in the luciferase construct. In another study, it was shown that the transcriptional activity increased twofold when a 1.6 kb mouse $\alpha 1$ -sGC promoter sequence was inserted in the promoterless construct and expressed in NIE-115 cells, whereas the transcriptional activity increased 4.6-fold when a 1.4 kb mouse $\beta 1$ -sGC promoter sequence was inserted (Sharina *et al.* 2000). Our deletion analysis demonstrated that a putative inhibitory element might exist in the region between -2 and -3 kb. In this region, several putative *cis*-elements were identified, which can bind with transcription factors, including the glucocorticoid receptor, hepatocyte NF-1, GATA, retinoid X receptor, and CCAAT/enhancer-binding protein. Notably, glucocorticoid receptor can interact with corepressors, such as nuclear receptor corepressor and silencing mediator of retinoid and thyroid receptors, and reduce gene expression (Wang *et al.* 2004).

The expression of $\alpha 1$ -sGC mRNA is cell specific. In mice, the highest expression level of $\alpha 1$ -sGC mRNAs was observed in lung, and the lowest levels in liver, muscle, and ileum (Mergia *et al.* 2003). In the present study, RT-PCR was unable to detect $\alpha 1$ -sGC mRNA in a liver-derived cell line MH1C1. Furthermore, the activities of promoters more proximal to -2 kb in MH1C1 cells were much weaker than that in GH₃ cells, indicating that the lack of $\alpha 1$ -sGC expression in MH1C1 cells may partly result from reduced proximal promoter activity. However, the elements and the 3.5 kb region so far reported are not enough to achieve the cell-specific expression of $\alpha 1$ -sGC, since the promoter activity of pGL3. (-3 K) was similar in different cell lines. We have examined the possible involvement of DNA methylation and histone modifications in transcriptional regulation of $\alpha 1$ -sGC mRNA. Treatment with DNA demethylating reagent 5-azacytidine or histone deacetylase inhibitor trichostatin A did not change the $\alpha 1$ -sGC mRNA level in

GH₃ cells (data not shown), indicating that the epigenetic regulation does not contribute to the expression of $\alpha 1$ -sGC mRNA in GH₃ cells.

The coexpression of $\alpha 1$ - and $\beta 1$ -sGC subunits is required to obtain a functional enzyme, implying that the expression of both subunits is coordinated. As a matter of fact, the expression of $\alpha 1$ and $\beta 1$ subunits is tightly coregulated (Nedvetsky *et al.* 2002). However, variable mechanisms may account for coregulation of two subunits in different animal models. In medaka fish, $\alpha 1$ - and $\beta 1$ -sGC genes are 986 bp apart and organized in tandem in the genome. The expression of both subunits is directly coordinated by the 5'-upstream region of $\alpha 1$ -sGC subunit (Mikami *et al.* 1999). This is not the case with mammalian species. According to the National Center for Biotechnology Information database, $\alpha 1$ and $\beta 1$ subunits are separated by 60 and 43 kb in mouse and human respectively. In mouse, the extended region comprises 2% of the total chromosomal length (Sharina *et al.* 2000). In rat, genes for $\alpha 1$ - and $\beta 1$ -sGC subunits have been mapped to chromosome 2 and linked to known quantitative trait locus markers of salt-sensitive hypertension in the Dahl rats (Azam *et al.* 1998), and two subunits are separated by 19 kb (NCBI data).

The coordination of transcriptional regulation of two subunits in mammals may be achieved by sharing some common transcription factor-binding sites, such as CCAAT box. Proteins that can bind with CCAAT box include CBF, NF-1, CCAAT/enhancer-binding protein, and CCAAT displacement protein (Mantovani 1999). Furthermore, CBF is composed of three subunits, CBF/A, CBF/B, and CBF/C, which are required for DNA binding. The binding of CBF with CCAAT box can result in the disruption of nucleosome and transcription activation (Cousty *et al.* 2001). In the promoter of $\beta 1$ -sGC gene in human, the CCAAT box can bind with CBF and enhance promoter activity (Sharina *et al.* 2003). In this study, we also identified the CCAAT box in $\alpha 1$ -sGC promoter at region between -50 and -18 , which can interact with the transcription factor CBF. Moreover, the region between -39 and -30 is most critical for this interaction. Disruption of the CBF-CCAAT interaction resulted in twofold decrease in promoter activity. Therefore, CCAAT box conferred the stimulatory effect on both $\alpha 1$ - and $\beta 1$ -sGC promoter activities. The *in vivo* binding of CBF with this segment was confirmed by ChIP assay, providing a mechanism to coordinate the gene expression of $\alpha 1$ - and $\beta 1$ -sGC subunits. The binding of CCAAT displacement protein to this site was not examined, because it normally acts as an inhibitory factor (Nishio & Walsh 2004).

Our results also demonstrated that Sp1 is involved in the transcription regulation of $\alpha 1$ -sGC subunit gene. In this case, the region between -35 and -26 is important for the DNA-protein interaction. A mutant with reduced affinity to Sp1 exhibited lower promoter activity

than wild type. As the founding member of the family of zinc finger transcription factors, Sp1 participates in a wide variety of physiological processes, including the cell-cycle regulation and hormonal activity (Chu & Ferro 2005). In the NO/sGC signaling pathways, Sp1 is important for the basal activity of endothelial (Wu 2002) and neuronal (Saur *et al.* 2002, Bachir *et al.* 2003) NO synthases. Moreover, it is also involved in the regulation of human type I protein kinase G gene expression (Sellak *et al.* 2002). Together with the findings presented here, these observations indicated the importance of Sp1 in the NO/sGC signaling.

The binding sites for CBF and Sp1 are overlapping in the region between -35 and -30 , which implies the cooperation between CBF and Sp1 in the transcriptional regulation of $\alpha 1$ -sGC subunit gene. In our experiments, mutation of the overlapping region resulted in the highest reduction in the promoter activity. In the promoter of rat fatty acid synthase, an insulin-responsive element (IRE) was composed of a CCAAT element and a Sp1 element with 10 bp in distance. Using IRE as the probe, three kinds of DNA–protein complex were formed: IRE:Sp1, IRE:CBF, and IRE:Sp1:CBF. The cooperation of Sp1 and CBF increased the stability of DNA–protein complex (Roder *et al.* 1997). Because the distance between CBF and Sp1 seems to be critical for the synergism of CBF and Sp1 (Alimov *et al.* 2005), their relationship in the control of $\alpha 1$ -sGC subunit gene expression remains to be proven.

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