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Promoter paper

Characterization of the human UDP-galactose:ceramide galactosyltransferase gene promoter[1]

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Abstract

UDP-galactose:ceramide galactosyltransferase (CGT, EC 2.4.1.45) is a key enzyme in the biosynthesis of galactocerebroside, the most abundant glycosphingolipid in the myelin sheath. An 8 kb fragment upstream from the transcription initiation site of CGT gene was isolated from a human genomic DNA library. Primer extension analysis revealed a single transcription initiation site 329 bp upstream from the ATG start codon. Neither a consensus TATA nor a CCAAT box was identified in the proximity to the transcription start site; however, this region contains a high GC content and multiple putative regulatory elements. To investigate the transcriptional regulation of CGT, a series of 5′ deletion constructs of the 5′-flanking region were generated and cloned upstream from the luciferase reporter gene. By comparing promoter activity in the human oligodendroglioma (HOG) and human neuroblastoma (LAN-5) cell lines, we found that the CGT promoter functions in a cell type-specific manner. Three positive *cis*-acting regulatory regions were identified, including a proximal region at −292/−256 which contains the potential binding sites for known transcription factors (TFs) such as Ets and SP1 (GC box), a distal region at −747/−688 comprising a number of binding sites such as the ERE half-site, NF1-like, TGGCA-BP, and CRE, and a third positive *cis*-acting region distally localized at −1325/−1083 consisting of binding sites for TFs such as nitrogen regulatory, TCF-1, TGGCA-BP, NF-IL6, CF1, bHLH, NF1-like, GATA, and γ-IRE. A negative *cis*-acting domain localized in a far distal region at −1594/−1326 was also identified. Our results suggest the presence of both positive and negative *cis*-regulatory regions essential for the cell-specific expression in the TATA-less promoter of the human CGT gene.

Keywords: Uridine diphosphate-galactose:ceramide galactosyltransferase; Promoter; Transcription; Gene expression; Myelination

1. Introduction

The vertebrate nervous system is endowed by axons ensheathed with myelin, a multilamellar membrane extended from oligodendrocytes (OL) in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) [1]. The specialized structure of myelin is known to exhibit a low capacitance, thus contributing to the axonal insulation that facilitates transmission of nerve impulses by saltatory conduction [2]. Galactocerebroside (GalC), from which sulfogalactocerebroside or sulfatide is derived, is the most abundant lipid component of the myelin sheath, constituting more than 20% of the total lipid [1,3–5]. GalC consists of two molecular classes, one with 2-hydroxylated ceramide and the other with non-hydroxylated ceramide, which may be synthesized at different intracellular compartments [6]. The biosynthesis of GalC is achieved by the key enzyme UDP-galactose:ceramide galactosyltransferase (CGT, EC 2.4.1.45), which catalyzes the

Abbreviations: bHLH, basic helix-loop-helix; bp, base pair(s); CF1, common factor 1; CGT, UDP-galactose:ceramide galactosyltransferase; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CNS, central nervous system; CRE, cyclic AMP response element; DMEM, Dulbecco's modified Eagle's medium; ERE half-site, estrogen response element half-site; FBS, fetal bovine serum; GalC, galactocerebroside; γ-IRE, interferon-γ response element; GluC, glucocerebroside; GFAP, glial fibrillary acidic protein; GS, glutamine synthase; HOG, human oligodendroglioma; kb, kilobase pair(s); LAN-5, human neuroblastoma; Luc, luciferase; MBP, myelin basic protein; NF1-like, nuclear factor 1-like; NF-IL6, interleukin-6-regulated nuclear factor; OL, oligodendrocyte; PCR, polymerase chain reaction; PNS, peripheral nervous system; RPMI, Roswell Park Memorial Institute; SP1, specificity protein 1; TCF-1, T cell factor-1; TF, transcription factor; TGGCA-BP, TGGCA-binding protein; UDP, uridine diphosphate

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[1] The nucleotide sequence data for this study have been deposited in GenBank under accession No. AF285880.

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transfer of a galactosyl residue from UDP-galactose to ceramide [7]. Studies on brain galactolipid synthesis in different strains of mice have suggested that an increased accumulation of GalC is likely contributed by an enhanced activity of CGT [8,9]. Because of their abundance, together with the results of a number of antibody perturbation studies, these galactolipids have been suggested to play a crucial role in the formation and maintenance of the myelin sheath [4,10–18]. Furthermore, they are shown to be involved in many cellular events such as signal transduction [14,19], OL development [20,21], and axono-glial interaction [22–24], and oligodendroglia membrane sheet outgrowth and calcium flux [13,19,25]. In addition to neural organs, GalC is also found in low concentrations in extraneural organs such as kidney [26,27], gastrointestinal tract [28], liver [29], testis [30], and milk [31].

The gene encoding CGT has been elucidated in mouse [32,33], rat [34–36] and human [37,38]. The importance of GalC function in the nervous system has been independently demonstrated in CGT-deficient mouse models [39,40]. These animals are surprisingly capable of forming 'pseudo-myelin' which contains a high concentration of glucocerebroside (GluC) instead of GalC, and they exhibit tremoring, ataxia, hind limb paralysis, and vacuolization of the ventral region of the spinal cord due to the lack of stability and insulating capacity of the pseudo-myelin structure. These characteristics are similar to the pathological conditions found in humans with demyelinating disorders such as multiple sclerosis. However, virtually nothing is known about the molecular regulatory mechanisms of CGT gene expression in humans.

The expression of myelin-specific genes is highly regulated and coordinated during myelination. GalC in OL as well as in myelin is expressed in a development-dependent manner [41–43]. In the rat CNS, CGT mRNA expression appears at birth which corresponds to the onset of myelination, peaks around postnatal day 20, and continues to be expressed abundantly until at least 65 days postnatally [35,36]. This highly developmentally regulated and tissue-specific expression pattern is in accord with the myelination profile, suggesting that the CGT gene expression may be one of the key molecular events directly involved in myelination.

Several groups have reported the cloning and functional characterization of myelin-specific gene promoters and found many ubiquitous as well as specific *cis*- and *trans*-acting factors that function in a tissue-specific and/or developmentally regulated fashion [44]. Although the comprehensive characterization of the CGT gene promoter has not been documented, the mouse CGT proximal promoter has been reported recently to function in a tissue-specific manner [45]. To date, the human CGT promoter analysis has never been elucidated. In the present study, we have sequenced and characterized the promoter region of the human CGT gene, and further identified the putative *cis*-acting regulatory elements responsible for the functional promoter activity. Two human cell lines derived from two distinct lineages, the oligodendroglioma (HOG) [46] and the neuroblastoma (LAN-5) [47] cells, were utilized as CNS cell models in which to perform transient transfection experiments. HOG cells were selected because they exhibit many properties that are characteristic of oligodendroglial cells, including the expression of a 15 kDa form of myelin basic protein (MBP), GalC and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), but not glial fibrillary acidic protein (GFAP) and glutamine synthase (GS) [46]. In contrast, LAN-5 cells derived from human neuroblastoma were used because of their non-glial origin.

2. Isolation of the 5′-flanking region and identification of regulatory elements

To obtain the 5′-flanking region of the human CGT gene, the human placental λ Fix II genomic library (Stratagene, La Jolla, CA) was screened by polymerase chain reaction (PCR) using primers specific to the 5′ end of the human CGT cDNA coding region and confirmed by hybridization with the *Eco*RI fragment of the CGT-pCR 3.1 plasmid as described previously [38]. After the correspond-

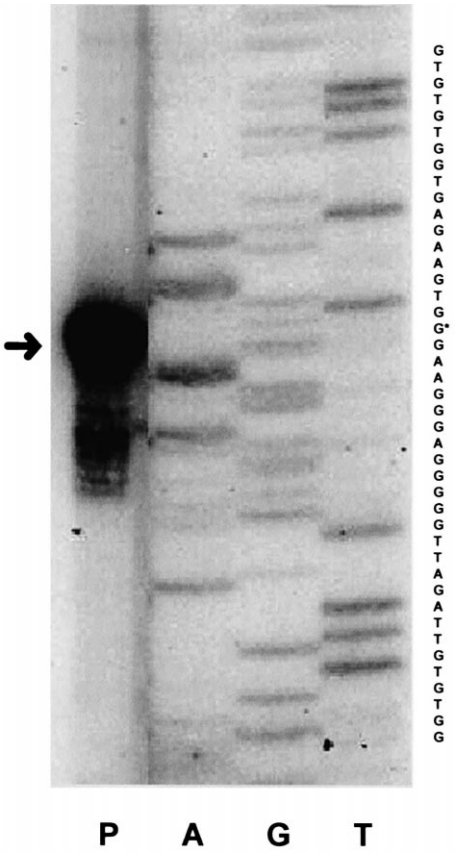


Fig. 1. Mapping of the transcription initiation site by primer extension analysis. The extended product (P) is indicated by an arrowhead and shown together with the nucleotide sequence. Positions for the nucleotides A, G, and T are displayed.

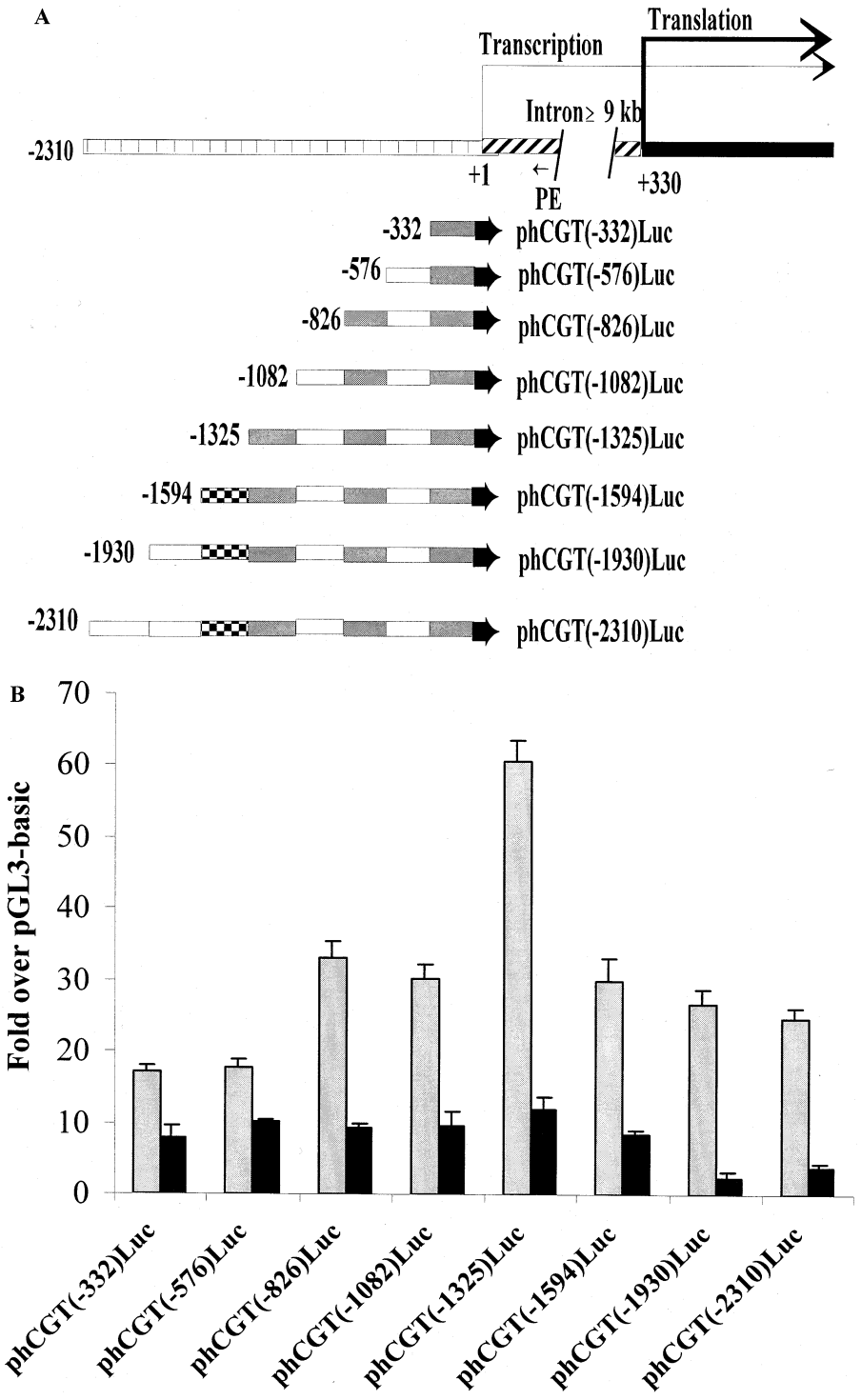


Fig. 2. (A) A schematic representation of the structure of the 2.3 kb human CGT gene promoter and its sequentially deleted fragments inserted upstream from the luciferase coding region (indicated as a black arrow at the 3′ end of each insert) in the pGL3-basic vector (Promega). Coordinates are given with respect to the transcription initiation site (+1) of the spliced mRNA. PE represents a 21-mer antisense oligonucleotide primer used in the primer extension analysis. The translation start site is indicated (+330). The numbers indicate the positions of the 5′ ends of the deletion constructs. Locations of the positive regulatory and negative regulatory regions are indicated by gray boxes and dotted box, respectively. (B) Functional activity of the 5′ deletion promoter constructs in HOG (gray bars) and LAN-5 (black bars) cells. A series of consecutive approx. 250 bp deletions of the CGT promoter were co-transfected into the cells with pHook-2 *lacZ* (Invitrogen). The luciferase activities of each deletion construct were normalized to the β-galactosidase activities. Activities are expressed as fold induction over pGL3-basic vector. Each value represents the mean ± S.D. of at least three independent sets of transfection experiments. Each set was performed in triplicate.

ing 17 kb clone was isolated, an 8 kb *Eco*RI fragment was recloned into pCR II vector (Invitrogen, Calsbad, CA), and the sequence of the CGT 5′-flanking fragment in the pCR II-CGT plasmid was obtained using CGT-specific primers [38]. The sequence information was analyzed for putative regulatory elements by searching the Genetics Computer Group's database (version 9.1, Madison, WI). Neither a TATA box nor a CCAAT box was present in the proximal promoter region. However, the 5′-flanking region contained a high GC content and a number of putative *cis*-acting elements for known transcription factors (TFs). These data provide a reasonable starting point to determine the promoter elements and TFs involved in the regulation of human CGT gene expression.

3. Total cellular RNA isolation and primer extension assay

Total cellular RNA was isolated from N-370 FG cells, a hybrid tumor cell line derived from human fetal glioma and oligodendroglial cells which express GalC (21.5% of the total glycolipids) [38,48] using Trizol reagent (Gibco, Gaithersburg, MD) following the manufacturer's instructions. The 5′ end of human CGT mRNA was determined as follows: a 21 nucleotide antisense primer PE (5′-GCGAGTCTGAGTGTGTGTCTG-3′) corresponding to positions +183 to +203 of the human CGT cDNA [38] was 5′ end-labeled with [^{32}P]ATP and T4 polynucleotide kinase. The radiolabeled primer (10^6 cpm) was hybridized to 10 μg of total RNA from N-370 FG cells and extended with Superscript II reverse transcriptase (Gibco). The resulting extended product was separated using 6% denaturing sequencing gel electrophoresis adjacent to the sequencing reactions (Sequenase, Amersham Life Science, Cleveland, OH) of pCR II-CGT plasmid primed with the same primer as the primer extension reaction. Results were visualized after autoradiography for at least 24 h. As depicted in Fig. 1, the extended product was mapped to the DNA sequence at a position 329 bp upstream from the ATG start codon.

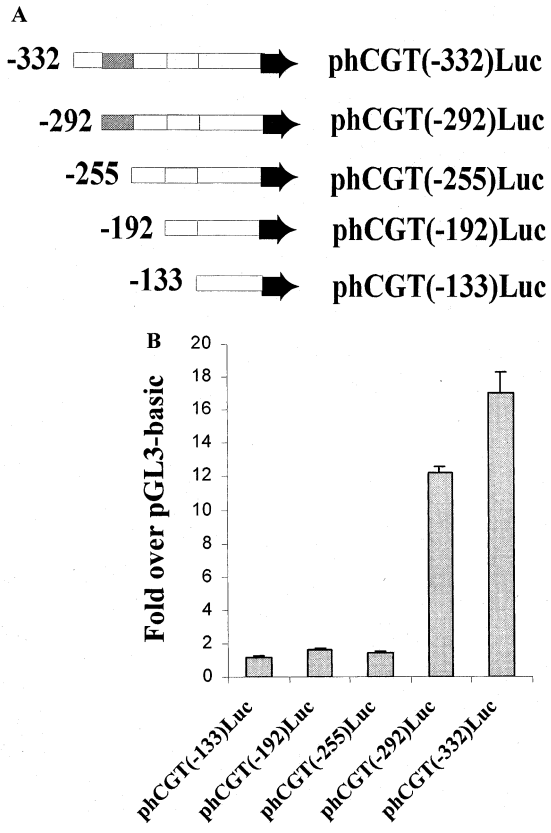
4. Deletion constructs of luciferase reporter for promoter analysis

Transient transfection experiments were performed using various expression plasmids containing fragments of the human CGT promoter upstream from the firefly luciferase coding region in the promoterless luciferase expression vector, pGL3-basic (Promega, Madison, WI). A series of 5′ deletion fragments of the human CGT 5′-flanking region, each differing in size approx. 250 bp (Fig. 2A), were amplified by PCR using the 8 kb promoter region of human CGT cloned into pCR II plasmid as a template. PCR fragments were generated using different forward primers (−332F/*Nhe*I, −576F/*Nhe*I, −826F/*Nhe*I, −1082F/*Nhe*I, −1325F/*Nhe*I, −1594F/*Nhe*I, −1930F/*Nhe*I, and −2310F/*Nhe*I) and the same reverse primer (−46R/*Nhe*I), each designed with an *Nhe*I-containing tail. Therefore, all fragments varied in their 5′ ends, but all of their 3′ ends terminated 46 nucleotides upstream from the transcription start site (+1). All oligonucleotide primers used in this study are listed in Table 1. PCR amplification was performed following a procedure described elsewhere [38,49,50]. PCR products were digested with *Nhe*I to create the cohesive ends, purified, and then ligated into the *Nhe*I site upstream from the luciferase coding region in the pGL3-basic vector. In this study, we dedicated our investigation to the first two fragments containing promoter activity as observed from the promoter assays using the first set of eight 5′ deletion constructs.

Table 1
Oligonucleotide sequences and their locations used to generate promoter fragments of the human CGT gene

Name	Oligonucleotide sequence[a] (5′→3′)	Position[b]
−46R/*Nhe*I	caaaGCTAGCATCACTCGCCTCTGACTG	−46/−68
−332N/*Nhe*I	caaaGCTAGCGGGCACAGGGACTACGTG	−332/−315
−576N/*Nhe*I	caaaGCTAGCCCCAAACTCGCGTTTTTTAAGGC	−576/−554
−826N/*Nhe*I	caaaGCTAGCCAATCTACCTCAGGCGCTC	−826/−808
−1082N/*Nhe*I	caaaGCTAGCCCTATTTCTGAATCTCACTTTGG	−1082/−1060
−1325N/*Nhe*I	caaaGCTAGCGGCTGTGAAAATATTCCCTGC	−1325/−1305
−1594N/*Nhe*I	caaaGCTAGCGGACAAGGATGTGGTGAAAC	−1594/−1575
−1930N/*Nhe*I	caaaGCTAGCGCCAATAATGTTACCAATTAAAGG	−1930/−1907
−2310N/*Nhe*I	caaaGCTAGCAGGAGTCTGATCCCAGAGTTAATGG	−2310/−2286
−292N/*Mlu*I	ccg**ACGCGT**GACACGCCTCGCAAAGAGG	−292/−274
−255N/*Mlu*I	ccg**ACGCGT**GCCGTTGTCAGAGTTCGC	−255/−235
−192N/*Mlu*I	ccg**ACGCGT**AGCCAACCGCGCTCAGCGGAC	−192/−172
−133N/*Mlu*I	ccg**ACGCGT**GCGAGCGCGTGGTACGAAG	−133/−114
−786N/*Mlu*I	ccg**ACGCGT**TCTGAGGCACCACAGAGAG	−786/−768
−747N/*Mlu*I	ccg**ACGCGT**TTGCTGGTCAAGACGCCAAC	−747/−728
−687N/*Mlu*I	ccg**ACGCGT**ATAAAGTCGCCACAGGCTCA	−687/−668
−628N/*Mlu*I	ccg**ACGCGT**AGAGGAGGGATGTTATTCA	−628/−610

[a]Lower case denotes additional nucleotides flanking the restriction site for improving the cleavage close to the end of DNA fragments. The *Nhe*I and *Mlu*I sites are indicated as underlined and bold letters, respectively.
[b]Nucleotide positions relative to the transcription start site (+1) of the human CGT gene.



C

(-292) (-256)

5' GACACGCCTCGCAAAGAGGGAAGAGTGGGCGGGGCCA 3'

Ets SP1 (GC box)

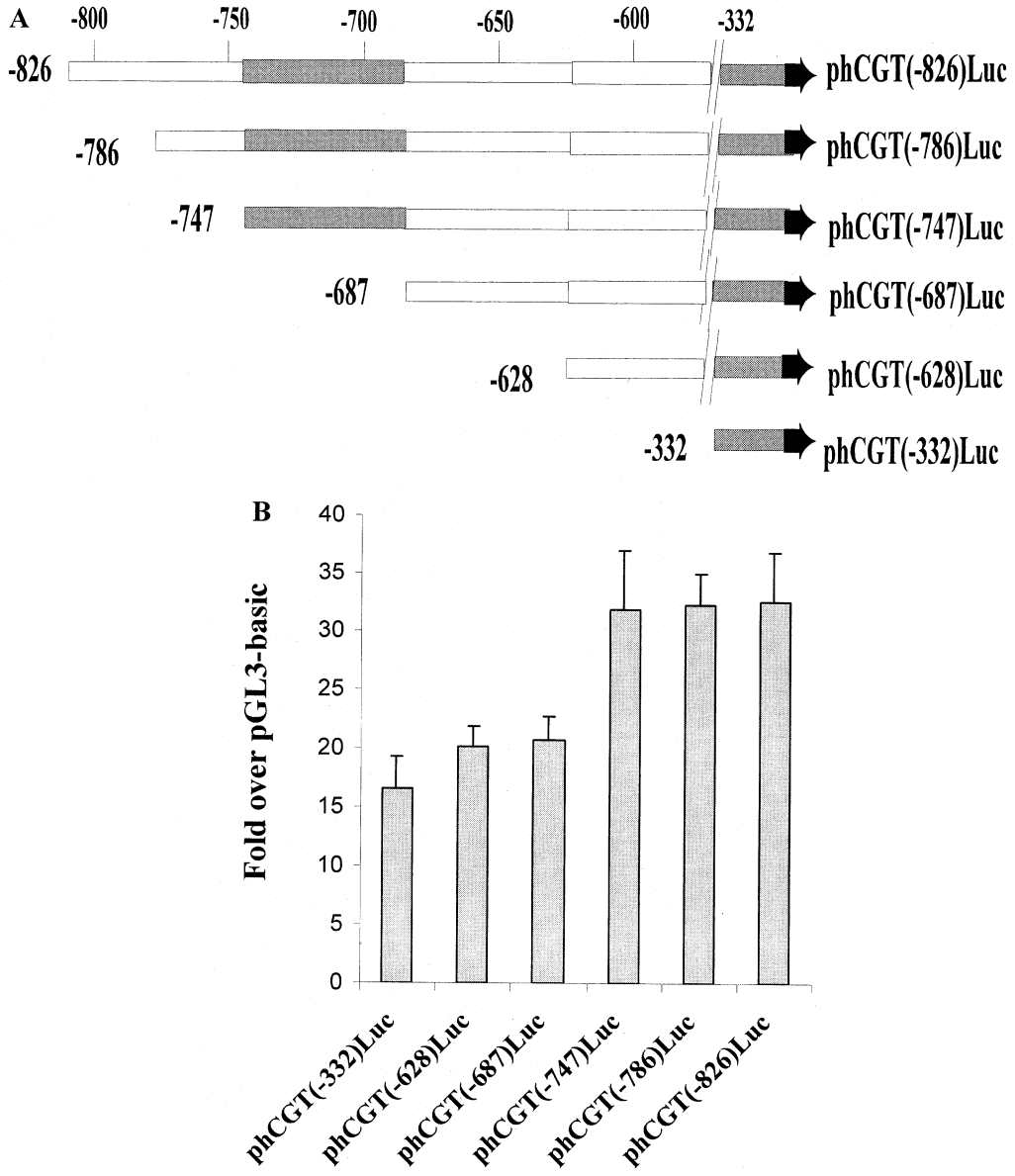
Fig. 3. (A) A schematic representation of the proximal human CGT gene promoter constructs which contain sequences spanning from −332 to −46 and its sequentially deleted fragments, each about 50 bp shorter, inserted upstream from the luciferase coding region (represented as a black arrow) in pGL3-basic plasmid. The numbers in relation to the transcription start site (+1) indicate the positions of the 5′ ends of the deletion constructs. Location of the positive regulatory region is indicated by a gray box. (B) Functional activity of the 5′ deletion proximal promoter constructs in HOG cells. The luciferase activities for HOG cells transfected with each promoter construct were normalized to the β-galactosidase activities. Activities are expressed as fold induction over that seen in cells transfected using the pGL3-basic vector. Each value represents the mean ± S.D. of at least three independent sets of transfection experiments. Each set was performed in triplicate. (C) The nucleotide sequence of the human CGT proximal gene promoter region spanning from −292 to −256. Putative recognition sites for known TFs are underlined.

In order to localize the positive *cis*-acting elements more precisely, we created two additional series of 5′ deletion constructs of DNA fragments, each differing in size approx. 50 bp. Using both the same template and reverse primer as above, PCR fragments were synthesized utilizing different forward primers, each tailed with an *Mlu*I site, specific to the proximal promoter region (−133F/*Mlu*I, −192F/*Mlu*I, −255F/*Mlu*I and −292F/*Mlu*I) and the distal promoter region (−628F/*Mlu*I, −687F/*Mlu*I, −747F/*Mlu*I and −786F/*Mlu*I) as shown in Fig. 3A and Fig. 4A, respectively. PCR products were digested with both *Mlu*I and *Nhe*I, and then unidirectionally cloned into the *Mlu*I-*Nhe*I sites of pGL3-basic. The integrity and orientation of all constructs were confirmed by restriction digestion and DNA sequencing.

5. Cell culture, transient transfection, and reporter gene assay

HOG (a generous gift of Dr. Glyn Dawson, University of Chicago, Chicago, IL) and LAN-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium, respectively. The media were supplemented with 10% fetal bovine serum (FBS), and cells were maintained at 37°C in a humidified atmosphere at 5% CO_2. Transient transfections were performed using LipofectAMINE (Gibco). Briefly, cultured cells at about 70% confluence (approx. 5×10^5 cells) in a six-well plate were co-transfected with 2 μg of each deletion construct and 0.2 μg of the pHook-2 *lacZ* vector (Invitrogen), which expresses β-galactosidase under the control of the CMV promoter, to monitor the transfection efficiency. In each transfection experiment, untransfected cells were included as controls for a background level of luciferase and β-galactosidase. The pGL3-basic was also transfected in parallel as a negative control. Forty-eight hours after transfection, cells were lysed in 200 μl of the lysis buffer (Tropix, Bedford, MA). The luciferase activities in the cell lysates were determined using a Luciferase Assay System (Promega) and normalized to β-galactosidase activities evaluated using a Luminescent β-galactosidase Detection Kit II (Clontech, Palo Alto, CA). Reporter assays were performed with 20 μl of lysate using a microplate luminometer TR717/Winglow (Tropix). The data represent the mean ± S.D. of three independent experiments. Triplicate transfections were performed in each experiment. Luciferase levels were reported as fold over the promoterless pGL3-basic.

As illustrated in Fig. 2B, transient transfections with eight 5′ deletion reporter constructs demonstrated that phCGT(−332)Luc, which contained the CGT sequence from −332 to −46, possessed minimal promoter activity, which was about 18-fold higher than that of the parent pGL3-basic. The distal 5′-flanking region in phCGT(−826)Luc conferred a considerable increase in the transcription activity, corresponding to approx. 2-fold luciferase activity over that obtained from either the phCGT(−332)Luc or phCGT(−576)Luc. The data suggest that the region from −826 to −577 may contain additional positive *cis*-acting regulatory element(s). Maximal transcription activity was observed in cells transfected with phCGT(−1325)Luc, suggesting that this dramatic increase in activity, corresponding to about 3-fold activity higher than that gained from phCGT(−332)Luc, is conferred by the additional regulatory region spanning nu-



C

(-747) (-688)

5' TTGCTGGTCAAGACGCCAACTTGGCAAGGTTATGCTGCTGGCAAAGGCAGATTCGTCAGA 3'

ERE half-site NF1-like TGGCA-BP TGGCA-BP CRE

Fig. 4. (A) A schematic representation of the distal human CGT gene promoter constructs which contain sequences spanning from −826 to −577 and its sequentially deleted fragments, each differing in size about 50 bp, inserted upstream from the luciferase coding region (represented as a black arrow) in pGL3-basic plasmid. The numbers in relation to the transcription start site (+1) indicate the positions of the 5′ ends of the deletion constructs. Location of the positive regulatory regions is indicated by a gray box. (B) Functional activity of the 5′ deletion distal promoter constructs in HOG cells. The luciferase activities for each reporter construct were normalized to the β-galactosidase activities. Activities are expressed as fold induction over that seen in the cells transfected using the pGL3-basic vector. Each value represents the mean ± S.D. of at least three sets of independent transfection experiments. Each set was performed in triplicate. (C) The nucleotide sequence of the human CGT distal gene promoter region spanning from −747 to −688. Putative recognition sites for known TFs are underlined.

cleotides −1325 to −1083 relative to the transcription start site. The inclusion between nucleotides −1594 and −1326, in the expression plasmid as phCGT(−1594)Luc, greatly reduced the activity from that seen in cells transfected with phCGT(−1325)Luc, suggesting the presence of a negative regulatory domain in this region. In promoter assays using LAN-5 cells (Fig. 2B), we found that the promoter activity observed in cells transfected with phCGT(−332)Luc was about 50% of that obtained from HOG cells using the same expression plasmid. The pres-

ence of additional nucleotides −333 to −2310 did not significantly alter the transcription activity, implicating that this basal activity is driven by the proximal region. The difference in promoter activities between HOG and LAN-5 cells suggests that the human CGT promoter functions in a cell-specific fashion.

In this report, we dedicated our study to the first two regions (proximal −332/−46 and distal −826/−577) containing the promoter activity in the HOG cells. In order to localize the positive *cis*-acting elements more precisely, we subsequently performed promoter assays using additional series of 5′ deletion constructs belonging to either the proximal or distal regions, each differing in size from each other about 50 bp (Fig. 3A and Fig. 4A, respectively). Constructs containing sequences −46 to −255 were unable to confer higher transcription activity than that seen in cells transfected with the parent pGL3-basic plasmid (Fig. 3B). Extending this region to contain the sequence up to nucleotide −292 as in phCGT(−292)Luc elevated the promoter activity almost to that seen in cells transfected with the full-length proximal promoter construct, phCGT(−332)Luc. Therefore, the proximal regulatory sequence was confined to the region between −292 and −256 bp, which contained the potential binding sites for known TFs such as Ets and specificity protein 1 (SP1) (GC box) (Fig. 3C). In the promoter assays using the distal promoter fragment constructs (Fig. 4B), the positive regulatory elements were localized to the region between −747 and −688 bp relative to the transcription start site. Putative binding sites for TFs such as estrogen response element (ERE) half-site, nuclear factor 1 (NF1)-like, TGGCA-binding protein (TGGCA-BP), and cyclic AMP response element (CRE) were found in this region (Fig. 4C). The observation that the presence of the third positive regulatory region from −1325 to −1083 was of great importance because it contributed to the highest transcription activity (Fig. 2B). Sequence analysis revealed that it consisted of numerous potential regulatory elements for TFs including nitrogen regulatory, T cell factor-1 (TCF-1), TGGCA-BP, interleukin-6-regulated nuclear factor (NF-IL6), common factor 1 (CF1), basic helix-loop-helix (bHLH), NF1-like, GATA, and interferon-γ response element (γ-IRE). To date, a thorough characterization using progressive 5′ deletions of this region is underway.

These results indicate that the human CGT promoter contains both positive and negative *cis*-acting elements as well as regulatory elements for its cell-specific expression. Three positive *cis*-acting regulatory regions are situated at −292/−256, −747/−688 and −1325/−1083 relative to the transcription start site, while one negative *cis*-acting region is present at −1594 to −1326. It is likely that cooperative transactivation through these regulatory elements is involved in the complicated transcriptional regulation of the human CGT gene.

In summary, this study has identified the transcription initiation site, sequenced the gene promoter, and demonstrated the functional promoter regions which contain *cis*-acting elements required for restricting the expression of the gene. The cloning and characterization of the human CGT gene promoter regions along with identification of the putative TFs involved in tissue- or cell type-specific expression offer the opportunity for future studies further defining the transcriptional regulation of the human CGT gene at the molecular level as well as using transgenic in vivo approaches.

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