Identification and Characterization of Basal and Cyclic AMP Response Elements in the Promoter of the Rat GTP Cyclohydrolase I Gene*

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5812 base pairs of rat GTP cyclohydrolase I (GTPCH) 5′-flanking region were cloned and sequenced, and the transcription start site was determined for the gene in rat liver. Progressive deletion analysis using transient transfection assays of luciferase reporter constructs defined the core promoter as a highly conserved 142-base pair GC-rich sequence upstream from the cap site. DNase I footprint analysis of this region revealed (5′ → 3′) a Sp1/GC box, a noncanonical cAMP-response element (CRE), a CCAAT-box, and an E-box. Transcription from the core promoter in PC12 but not C6 or Rat2 cells was enhanced by incubation with 8-bromo-cyclic AMP. Mutagenesis showed that both the CRE and CCAAT-box independently contribute to basal and cAMP-dependent activity. The combined CRE and CCAAT-box cassette was also found to enhance basal transcription and confer cAMP sensitivity on a heterologous minimal promoter. The addition of the Sp1/GC box sequence to this minimal promoter construct inhibited basal transcription without affecting the cAMP response. EMSA showed that nuclear proteins from PC12 but not C6 or Rat2 cells bind the CRE as a complex containing activating transcription factor (ATF)-4 and CCAAT enhancer-binding protein β, while both PC12 and C6 cell nuclear extracts were recruited by the CCAAT-box as a complex containing nuclear factor Y. Overexpression of ATF-4 in PC12 cells was found to transactivate the GTPCH promoter response to cAMP. These studies suggest that the elements required for cell type-specific cAMP-dependent enhancement of gene transcription are located along the GTPCH core promoter and include the CRE and adjacent CCAAT-box and the proteins ATF-4, CCAAT enhancer-binding protein β, and nuclear factor Y.

GTP cyclohydrolase I (GTPCH); EC 3.5.4.16 is the first and rate-limiting enzyme in the biosynthesis of 5,6,7,8-tetrahydrobiopterin (BH4) (1), the required cofactor for tyrosine, tryptophan, and phenylalanine hydroxylase and the family of nitric oxide synthases (2–4). GTPCH is therefore absolutely necessary for the synthesis of the signaling molecules dopamine (DA), norepinephrine, epinephrine, serotonin, melatonin, and nitric oxide as well as the detoxification of the amino acid L-phenylalanine. GTPCH gene expression is normally restricted to select tissues and cell types and thus determines the BH4 phenotype (1, 5, 6). Moreover, expression of the GTPCH gene is highly dynamic, can be induced in cell types that do not normally express it, and is controlled by a growing number of signal transduction pathways that presumably converge on the GTPCH promoter (5–9). The genes encoding for human and mouse GTPCH have recently been cloned (10), and their 5′-flanking regions have been shown to promote transcription of heterologous reporter genes (11, 12). Nonetheless, virtually nothing is known regarding either the cis-acting elements or trans-acting factors that control the expression of GTPCH.

In the rat brain, GTPCH is localized to monoamine-containing neurons (6, 13, 14). The abundance of GTPCH mRNA (15) and protein (16) within these cells is very heterogeneous, with particularly low levels found within nigrostriatal DA neurons. Unlike many other cell types that contain GTPCH, DA neurons respond to increased levels of cAMP with a robust increase in GTPCH mRNA and BH4 content that is dependent upon gene transcription (17). The cellular specificity of this response suggests that transcription factor(s) other than or in addition to the relatively ubiquitous CREB (18) are involved in this process. BH4 synthesis within human nigrostriatal DA neurons is known to be selectively vulnerable to genetic mutations in GTPCH that cause hereditary progressive dystonia (19). Identification of the gene promoter elements and their cognate binding proteins that control basal and cAMP-dependent GTPCH transcription within DA neurons may thus be crucial to our understanding of this disorder as well as other diseases that involve this population of neurons. Toward this end, we have now cloned, sequenced, and begun to identify and characterize basal and cAMP response elements within 5812 bp of the 5′-flanking region of the rat GTPCH gene. These studies indicate that a noncanonical CRE and adjacent CCAAT-box that are located within the 142-bp core promoter region and recruit the transcription factors ATF-4, C/EBPβ, and NF-Y in vitro are both necessary and sufficient to confer sensitivity to cAMP on the GTPCH promoter.

NF-Y, nuclear factor Y; 8-Br-cAMP, 8-bromo-cyclic AMP; RSV, Rous sarcoma virus; UTR, untranslated region; TK, thymidine kinase; PIPES, 1,4-piperazinediethanesulfonic acid.

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‡ The abbreviations used are: GTPCH, GTP cyclohydrolase I; BH4, 5,6,7,8-tetrahydrobiopterin; DA, dopamine; C/EBP, CCAAT enhancer-binding protein; CRE, cAMP-response element; CREB, CRE-binding protein; CBP, CREB-binding protein; bp, base pair(s); kb, kilobase(s); TH, tyrosine hydroxylase; ATF, activating transcription factor; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid.

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Isolation and Sequencing of the Rat GTPCH 5′-Flanking Region—Approximately 106 plaques from a Harlan Sprague Dawley rat testis genomic library (Lambda DASH II; Strategene, La Jolla, CA) were screened by hybridization with a randomly primed 866-bp cDNA containing the entire rat GTPCH coding sequence (20). Two positive plaques were purified by repeated plating and hybridization, and their DNA was identified as the correct templates in PCR screening reactions. DNA from one of the positive plaques produced a PCR reaction using 1.7GTPCHluc as template and end-labeled primer was run simultaneously on the same gel. Gels were dried and cpm of probe. S1 nuclease digestion was carried out for 90 min at 37 °C in 20 mM EDTA, pH 8.0, and 50 m g/ml yeast tRNA. Labeled coding and noncoding strands were chemically purified by centrifugation through CsCl or on QIAGEN columns (QIAGEN, Chatsworth, CA).

Reporter Plasmid Construction—p6GTPCH5SZ was digested with Nco I and the 5.8-kb insert was isolated and cloned in both directions into the Nco I site of the luciferase-based reporter vector pGBluc (Promega, Madison, WI) to produce 5.8GTPCHluc. Both strands of p6GTPCH5SZ were sequenced by primer walking from a chain termination technique. This analysis indicated that the insert was approximately 15 kb in size. Digestion of the 15-kb insert with Nco I yielded fragments of approximately 6 and 9 kb. Southern blot analysis of this Nco I digest with the 47/23 5′-UTR probe showed that the 6-kb fragment contained GTPCH 5′-flanking sequence. This 6 kb was subcloned into the Nco I site of pGEM5Z to produce p6GTPCH5SZ. Both strands of p6GTPCH5SZ were sequenced by primer walking from a chain termination technique. This analysis revealed that the 6-kb insert was actually 5812 bp in size and, as predicted, contained at its 3′-end the entire 127 bp of the rat GTPCH 5′-UTR sequence including the initiation codon.

Site-directed Mutagenesis—Mutations of the CRE and CCAAT elements were introduced into 0.27GTPCHluc by overlap extension (21). The primers used for mutagenesis (mutations are underlined) were as follows: 

\[
5′-CTCGGCAAGGGCGCCATGAGGTC-3′; \quad CRE\text{mut} \text{F} \\
5′-GTCGAGATGTGCTCCTGGTATAGGGCC-3′; \quad CRE\text{mut} R
\]

Two sets of primary PCR reactions were run using the CRE\text{mut} (+) plus CRE\text{mut} (−) and CRE\text{mut} (−) plus CRE\text{mut} (+) primer pairs. The secondary PCR reaction was carried out using the combined gel-purified primary PCR products as templates. PCRs were hot started at 95 °C for 1 min and then continued for 30-1 min cycles at 95, 60, and 72 °C, with a final extension at 72 °C for 7 min. The final PCR product was digested with NoI and MluI and cloned back into the pG3 basic vector to produce 0.27GTPCHlucCRE\text{mut}. The identical strategy was used for mutation of the CCAAT-box alone or in combination with the mutated CRE, 0.27GTPCHluc or 0.27GTPCHlucCRE\text{mut} DNA were used as templates for these primary PCRs to produce 0.27GTPCHluc

\[
\text{CAAT} \quad \text{and } 0.27GTPCHlucCRE\text{mut, respectively. All mutations were confirmed by sequence analysis.}
\]

Cell Culture, Transfection, and Enzyme Assays—Cultures of PC12 cells were transfected in Dulbecco's modified Eagle's medium, 7,5 fetal calf serum, and 7,5% horse serum. Cultures of C6 glioma cells were maintained in Dulbecco's modified Eagle's medium, 1% fetal calf serum, and 5% fetal calf serum. Cultures of Rat2 fibroblast cells were maintained in Dulbecco's modified Eagle's medium and 10% fetal calf serum. All cultures contained penicillin/streptomycin and were incubated at 37 °C in a humidified atmosphere of 10% CO2. Cells were seeded onto poly-l-lysine-coated 16-mm dishes 24 h prior to transfection and grown to approximately 80% confluency. Cells were typically transfected with 0.45 m g of experimental DNA and 0.01–0.05 m g of pRSVgal or pcMVgal DNA using LipofectAMINE (Life Technologies, Inc.). For comparisons across constructs that differed in size, molar amounts of experimental DNA were equalized by the addition of pGEM5Z DNA. For co-transfection experiments, 0.3 m g of 0.27GTPCHluc DNA was transfected along with 0.05–0.15 m g of pCG-hATF4 (cytomegalovirus-driven human ATF-4, a gift from Dr. T. Haji, Ohio State University) or empty vector DNA along with 0.05–0.15 m g of pRSVgal DNA. 18 h later, 5 m x 8-Bromo-dGMP in growth-conditioned media was added. Cultures were harvested 7–18 h later in lysis buffer and assayed for luciferase (Promega, Madison, WI) and β-galactosidase (CLONTECH Laboratories Inc., Palo Alto, CA) activities as described by the manufacturers. Parallel experiments using β-galactosidase enzyme histochemistry showed that transfection efficiencies were similar for each cell line (15–25%; data not shown). Luciferase activity was divided by β-galactosidase activity to correct for transfection efficiency and was expressed as relative luciferase activity.

Nuclear Extracts—Large scale PC12 nuclear extracts used for DNase I protection analysis were prepared by the method of Dignam (22). Small scale nuclear extracts from PC12, C6, and Rat2 cells grown in 100-mm dishes were prepared for use in EMSA analysis by the method of Sweese and Faller (23). Protein content was determined by the method of Bradford (24).

DNase I Footprint Analysis—p6GTPCHluc was digested with NoI or MluI, and the resulting overhangs were filled in with Klenow and [α-32P]dCTP (3,000 Ci/mmol) followed by a chase reaction with unlabeled nucleotides. The NoI digest was then treated with MluI, and the MluI digest was treated with NoI in order to label only the coding or noncoding strands, respectively. Probes were then purified by acrylamide gel electrophoresis, dialyzed, phenol-extracted, and concentrated by ethanol precipitation. To 20 m l of reaction buffer (12.5 m M HEPES-KOH, pH 7.9, 10% glycerol, 100 m M KC1, 1 m M EDTA, 1 m M dithiothreitol, 1 m g of poly(dI-dC), and 5 m g of actinomycin D in protein solution) on ice was added 20 m l of nuclear extract containing up to 150 m g of protein. The binding reaction was initiated by the addition of 10 m l of reaction buffer containing approximately 20,000 cpm of 32P-labeled probe, and the incubation continued for 20 min at room temperature. Alternatively, studies using competing oligonucleotides included a 120-min incubation on ice prior to the addition of probe and then an additional 20 min at room temperature. Samples were then incubated at room temperature for 1 min and a 1:10 dilution of NoI buffer (4 m M ammonium acetate, 4.5, 4.5 m M ZnSO4, 10 m g of salmon sperm DNA, and 300 units of NoI) to a dilution approximately 50% of the labeled DNA probe both in the presence and absence of binding protein. The RQ1 reaction was terminated by the addition of stop buffer (400 m M sodium acetate, 0.2% SDS, 10 m M EDTA, 50 m g/ml yeast tRNA, and 20 m g/ml proteinase K). Samples were then incubated at 55 °C, phenol-extracted, and ethanol-potentiated. Labeled coding and noncoding strands were chemically sequenced (25) to yield combined purines (G + A) and pyrimidines (T + A).
C), which were then run alongside the DNase I-treated samples on a 6% acrylamide, 8 M urea gel. Gels were dried and exposed to x-ray film at 280 °C.

**Electrophoretic Mobility Shift Assay—** Single-stranded complementary oligonucleotides were annealed and end-labeled with [γ-32P]ATP (3,000 Ci/mmol) and T4 kinase. To 13 ml of reaction buffer (12.5 mM HEPES-KOH, pH 7.9, 10% glycerol, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mg of poly(dI-dC), 5 mg of acetylated bovine serum albumin) on ice was added 5 μl of nuclear extract containing up to 5 mg of protein. The binding reaction was initiated by the addition of 2 μl of reaction buffer containing approximately 50,000 cpm of end-labeled oligonucleotide and continued for 20 min at room temperature. Antibodies (4 μg; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or competing double-stranded oligonucleotides were added to the reaction buffer before protein and 120 or 30 min, respectively, prior to the addition of labeled oligonucleotide. Samples were then returned to ice, and the entire volume was loaded onto a 5% acrylamide gel containing 1% glycerol. Running buffer was 1× TBE containing 1% glycerol. Gels were dried and exposed to x-ray film overnight at 280 °C.

**RESULTS**

Isolation of the Rat GTPCH 5′-Flanking Sequence and Determination of the Transcription Start Site—A combined strategy of Southern analysis, exon-based PCR, and restriction digestion was used to identify and clone 5.812 kb of rat GTPCH 5′-flanking sequence up to and including the translation initiation codon. A three-way alignment of the rat sequence with GTPCH 5′-flanking sequence currently available for the mouse and human genes showed a high degree of homology surrounding the previously established transcription start sites (10, 11) (Fig. 1). Because the human and mouse start sites are reportedly different, however, the start site for rat GTPCH was determined using S1 nuclease protection analysis (Fig. 2A).

**Fig. 1. Nucleotide sequence and organization of the proximal 5′-flanking region of the rat, mouse, and human GTPCH genes.** Transcription start sites are indicated by circled and boldface letters. Putative protein binding sites determined by computer analysis are identified by boxes and named below the sequence. Actual protein binding sites determined by DNase I footprint and EMSA analysis are labeled Domain 1–4 and are drawn as lines above the sequence. Numbering is based upon the distance from the rat transcription start site at T. This sequence corresponds to the 142 bp at the 5′-end of the 0.27GTPCHluc construct.

| Species | Nucleotide Sequence |
|---------|---------------------|
| Rat     | CTC GGGAGGCGCG      |
|         |                    |
| mouse   | CTC CGGAGGCGCG      |
|         |                    |
| human   | CTC CCGGGCGCGCG    |

**Fig. 2. Determination of the rat GTPCH transcription start site in rat liver.** A, a schematic diagram of the S1 nuclease digestion paradigm. B, 5 or 20 μg of mRNA isolated from rat liver or striated muscle, respectively, were hybridized with a single stranded DNA probe complementary to genomic GTPCH sequence between positions –239 and –23. Following digestion with S1 nuclease, samples were analyzed alongside a sequencing ladder generated by the –47 primer using 1.7GTPCHluc DNA as template. Only liver mRNA was able to protect the probe from S1 nuclease digestion (right arrow). Only a single protected band was detected, and this band aligned with an A at position –129 of the sequencing ladder (left arrow). A transcription start site for rat liver GTPCH can thus be assigned to the T 129 bp upstream from the initiation codon.

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be a number of unique restriction sites as well as those sites that were used to generate the luciferase reporter deletion constructs. Molar amounts of experimental DNA were equalized by the addition of pGEM5Z DNA. Molar amounts of experimental DNA were equalized by the addition of pGEM5Z DNA. The next day, 5 mM 8-Br-cAMP dissolved in growth-conditioned media was added, and the cultures continued for another 24 h, when cells were lysed and assayed for luciferase and β-galactosidase activities. Luciferase activity was divided by β-galactosidase activity to correct for transfection efficiency and was expressed as relative luciferase activity. Data are the mean ± S.E. of three independent experiments each determined in triplicate.

### Fig. 3. Functional analysis of the GTPCH promoter in PC12, C6, and Rat2 cells.

**A**. A restriction map of the 5.812 kb of GTPCH 5′-flanking sequence showing a number of unique restriction sites as well as those sites that were used to generate the luciferase reporter deletion constructs. 3.6GTPCHLuc (HindIII), 1.8GTPCHLuc (NheI), and 0.27GTPCHLuc (Apal). B. Cells were transfected with GTPCHLuc and pRSVgal DNA. Molar amounts of experimental DNA were equalized by the addition of pGEM5Z DNA. The next day, 5 mM 8-Br-cAMP dissolved in growth-conditioned media was added, and the cultures continued for another 24 h, when cells were lysed and assayed for luciferase and β-galactosidase activities. Luciferase activity was divided by β-galactosidase activity to correct for transfection efficiency and was expressed as relative luciferase activity. Data are the mean ± S.E. of three independent experiments each determined in triplicate.

**Functional Characterization of the GTPCH 5′-Flanking Region**—DNA containing the reporter construct 5.8GTPCHLuc was transiently transfected into PC12 cells, which normally express GTPCH mRNA (6), or C6 or Rat2 cells, which normally do not (9). These studies showed that transcription from this chimeric gene was roughly equal in each cell line and up to 20-fold greater than produced by the promoterless pGL3Luc or the inverted 5.8GTPCHLuc construct (Fig. 3B). This suggests that DNA elements responsible for restricting expression of the endogenous GTPCH gene to PC12 cells are either not located within the 5.812 kb of 5′-flanking sequence or cannot be detected using transient transfection assays. Progressive deletions of the 5.8-kb construct revealed that the minimal promoter sequence necessary to sustain transcription in each cell line is located within 0.27GTPCHLuc, which contains 142 bp upstream from the cap site and the entire 128 bp of GTPCH mRNA 5′-UTR (+142 to –128; Fig. 1). Deletion analysis also detected a stepwise increase in transcription as 5′-flanking sequence was eliminated. This suggests that control elements that normally repress transcription may be found upstream from the core promoter.

PC12 cells transfected with 5.8GTPCHLuc responded to 8-Br-cAMP treatment with a 4–7-fold increase in relative luciferase activity (Fig. 3B). This contrasts with the 1.3–1.9-fold increase observed for the empty pGL3Luc vector or the INV5.8GTPCHLuc construct. Deletion of sequence up to the Apal site (Fig. 3A) did not diminish the response to 8-Br-cAMP. The cis-acting elements required for the cAMP-dependent activation of GTPCH transcription are thus located within the 142-bp core promoter contained within 0.27GTPCHLuc and presented in Fig. 1. In contrast to PC12 cells, transcription in C6 and Rat2 cells was either unaltered or slightly decreased following treatment with 8-Br-cAMP (Fig. 3B). The trans-acting factors(s) required for the cAMP-dependent enhancement of GTPCH transcription are therefore found in PC12 cells, which normally express this gene, but not in two cell lines that do not.

**DNase I Footprint Analysis of the GTPCH Core Promoter Region Reveals Four Protein Binding Domains**—A computerized search of the 142-bp GTPCH core promoter for putative cis-acting elements that may be responsible for the stimulation of transcription by cAMP was undertaken using the TFMATRIX transcription factor binding site profile data base (26). This search revealed an asymmetrical or partial CRE (TGACG-) palindrome located between positions –104 and –97 that differed from the CRE palindrome (TGACGTCA) at positions 6 and 7 of the octomer (Fig. 1). Computer analysis also revealed a number of other putative protein binding motifs including three overlapping Sp1/GC box elements. Footprint analysis thus established a general agreement between putative and actual protein binding sites. Notably missing were protected sequences corresponding to Sp1/GC box sites between positions –110 and –137, a CCAAT-box between positions –82 and –86, a TATA-like sequence at positions –45 to –39, and an E-box located at positions –17 to –12. DNase I footprint analysis of the core promoter using nuclear extracts prepared from untreated PC12 cells detected three distinct protein binding domains (Fig. 4). Domain 1 is a 26-bp region that spans from position +7 to –19 and includes the transcription start site and the E-box (Fig. 1). Domain 1 also contains two hypersensitive sites; a C at position –19 of the coding strand and a G at position –7 of the noncoding strand. Domain 2 is a 29-bp region that spans from position –97 to –69 and is centered upon the CCAAT-box. Domain 4 (numbered out of order; see below) is a 22-bp region that is 86% GC, spans from position –132 to –111 and is centered upon the repeating Sp1/GC box elements. Footprint analysis thus established a general agreement between putative and actual protein binding sites. Notably missing were protected sequences associated with the TATA-like ATAAAAA element and the partial CRE. Incubation of core promoter DNA with 1 μg of recombinant human CREB-1 bZip domain (amino acids 254–
produced a weak footprint, referred to as domain 3, that spanned from position 2106 to 2107 (Fig. 5). This footprint corresponds to the CRE predicted by computer analysis and overlaps the 59 boundary of domain 2.

Nuclear extracts prepared from PC12 cells contain numerous CRE-binding proteins (see below) yet fail to footprint the GT-PCH CRE. DNase I protection analysis using subsaturating amounts of nuclear extract and domain-specific competing oligonucleotides was used next to determine whether this failure could be the result of occupation of the overlapping domain 2 and adjacent domain 4 by their cognate binding proteins. Incubation of PC12 nuclear extracts with a 500-fold molar excess of double-stranded oligonucleotide centered upon the GTPCH CCAAT-box (Fig. 5A; GTPCHccaat) was able to compete away domain 2 without affecting domain 1 or 4 or exposing domain 3 to protein binding (Fig. 5B). This competition was specific in that a double-stranded oligonucleotide containing a mutated form of the GTPCH CCAAT-box (Fig. 5A; GTPCHccaat mt) failed to inhibit the formation of domain 2. The addition of a molar excess of a double-stranded oligonucleotide centered upon the GTPCH CRE (Fig. 5A; GTPCHcre) or the consensual CRE of the rat tyrosine hydroxylase promoter (Fig. 5A; THcre; Ref. 27), both of which act to scavenge CRE-binding protein(s) present in the extract (see below), did not enhance the formation of domain 2 or 4 (Fig. 5B). Incubation with a double-stranded oligonucleotide containing a canonical Sp1 element (Fig. 5A) was able to compete away domain 4 without affecting domain 1 or 2 or exposing domain 3 to protein binding (Fig. 5B).
Binding to the GTPCH CRE was further characterized by comparison with the canonical TH CRE. EMSA using PC12 nuclear extracts and the THcre as probe produced three major complexes, with the smallest being the most abundant (Fig. 6C). Competition with a 100- and 1000- but not a 10-fold molar excess of unlabeled GTPCHcre reduced the formation of each of these complexes. When present at high concentrations, GTPCHcre thus has the potential to bind the same nuclear proteins recruited by THcre. Incubation with unlabeled THcre at a 10-fold excess approximated the competition produced by a 100-fold excess of GTPCHcre. This observation and the data presented in Fig. 6B indicate that PC12 nuclear proteins have at least a 10-fold greater affinity for the canonical TH CRE than for the GTPCH CRE sequence.

Nuclear extracts from control and 8-Br-cAMP-treated PC12, C6, and Rat2 cells were next analyzed by EMSA to determine whether GTPCH CRE binding activity is correlated with the cellular specificity of the cAMP response. Fig. 7B shows that extracts from untreated C6 and Rat2 cells express little GTPCHcre binding activity but bind avidly to the THcre. GTPCHcre binding activity was induced, however, in C6 and Rat2 extracts following a 24-h treatment with 8-Br-cAMP. In contrast, major differences in binding across treatments were not detected when these same nuclear extracts were analyzed using the THcre probe (Fig. 7B). These data further indicate that the GTPCH CRE can recruit a CRE-binding protein(s) that is normally found in PC12 but not C6 or Rat2 cells.

**Mutagenesis of the CRE Element Inhibits Basal and cAMP-dependent Transcription**—Three bases within the CRE were simultaneously mutated to produce the reporter construct 0.27GTPCHlucCREmt (Fig. 8A), a G to A at position –105, a C to T at position –103, and a G to T at position –102 (Fig. 8A). These bases were chosen for mutagenesis because methylation interference has shown that each is involved in ATF-1 binding to DNA (28). Nonetheless, EMSA using the GTPCHcremt oligonucleotide showed that binding was severely decreased but not eliminated by these mutations (Fig. 8B). Furthermore, while competition experiments demonstrated a reduced potency, the GTPCHcremt oligonucleotide still retained the ability to compete with the wild type oligonucleotide. These results suggest that the DNA binding specificity of the protein(s) that interacts with the GTPCH CRE is different from that established for CREB and ATF-1. Transient transfection experiments using the 0.27GTPCHlucCREmt construct demonstrated that these mutations of the CRE decreased basal transcription by 50% (Fig. 9A). The enhancement of transcription by 8-Br-cAMP was also reduced to 3-fold from the 4.9-fold observed for the wild type construct (Fig. 9A). While these results may be explained by the lower affinity of the mutated CRE for the GTPCH CRE-binding protein(s), it is more likely that some other cis-acting element(s) within the core promoter, such as the adjacent Sp1/GC- or CCAAT-boxes, may also participate in mediating the cAMP enhancement of GTPCH transcription.

**Mutagenesis of the CCAAT-box Element Inhibits Basal and cAMP-dependent Transcription**—EMSA was used first to establish that nuclear proteins from control and 8-Br-cAMP-treated PC12 are recruited by a double-stranded oligonucleotide that is centered on the GTPCH CCAAT-box and does not contain the CRE (Fig. 5C; GTPCHccaat). Depending upon the preparation and electrophoresis time, these studies detected one large and one or two smaller binding complexes, the formation of which required nuclear protein, was effectively competed by unlabeled GTPCHccaat, and generally appeared unaffected by treatment with 8-Br-cAMP. In addition, unlike binding to the GTPCH CRE, extracts from control and 8-Br-cAMP-treated PC12, C6, and Rat2 cells were next analyzed by EMSA to determine whether GTPCH CRE binding activity is correlated with the cellular specificity of the cAMP response.
cAMP-treated C6 cells bound the CCAAT-box (Fig. 8C). Two bases within the CCAAT pentamer were mutated to produce the reporter construct 0.27GTPCHlucCCAATmt; a C to A at position ~86 and an A to T at position ~84. Methylation interference has shown that either one or both of these bases are involved in the binding of the transcription factor NF-Y, CP2, or NF-1 to the CCAAT-box motif (29, 30). This requirement was corroborated by EMSA using the mutated oligonucleotide as probe (GTPCHccaatmt; Fig. 8C). Transient transfection experiments with the 0.27GTPCHlucCCAAT mt construct demonstrated that these mutations decrease basal transcription by 22% (Fig. 9A). The enhancement of transcription by 8-Br-cAMP was also reduced to 3.5-fold from the 4.9-fold observed for the wild type construct. The CCAAT-box element thus contributes to basal and cAMP-dependent GTPCH transcription, although to a somewhat lesser extent than does the CRE.

Simultaneous Mutagenesis of Both the CRE and CCAAT-box Elements Eliminates the Response to cAMP—A reporter construct was next prepared in which both the CRE and the CCAAT-box were mutated as described to produce 0.27GTPCHlucCREmtCCAATmt. Transfection with this construct decreased basal levels of transcription by 66% (Fig. 9A) or approximately the sum of the declines in basal activity produced by the individual mutations alone. Furthermore, these combined mutations completely eliminated the response of the GTPCH promoter to cAMP. The CRE and adjacent CCAAT-box are thus necessary to confer a major proportion of basal promoter activity as well as the cAMP-dependent enhancement of GTPCH gene transcription.
not respond to cAMP with an increase in luciferase activity (Fig. 9B). Moreover, the addition to this construct of 30 bp of 5’ sequence that includes domain 4 and contains the Sp1/GC box element (−142 to −63 bp; GTPCHsp1/cre/ccaatTKluc) had little effect on basal or cAMP-dependent transcription (Fig. 9B). In contrast, when a single copy of the CRE and CCAAT-box cassette was placed in the correct orientation upstream of the SV40 minimal early promoter (GTPCHcre/ccaatSV40luc) a 3-fold stimulation in basal activity and a 3.6-fold increase in cAMP-dependent transcription were observed (Fig. 9B). The addition of the Sp1/GC box 5’ to this construct substantially reduced basal luciferase activity, although the -fold response to cAMP was maintained and possibly even enhanced. When combined with the data obtained by mutagenesis, these observations demonstrate that the CRE and CCAAT-box cassette is necessary and sufficient to enhance basal and cAMP-dependent transcription from the rat GTPCH promoter. Moreover, occupation of the 5’ Sp1/GC box was shown to inhibit CRE-dependent and CCAAT-box-dependent basal activity without negatively affecting the cAMP response that is mediated by the same combined element.

Protein Binding to the CRE—EMSA using PC12 nuclear extracts and antibodies specific to five members of the CREB/ATF family revealed that ATF-4 and, to a much lesser degree, CREB-1 are recruited by the GTPCHcre oligonucleotide (Fig. 7C). Anti-ATF-4 was found to reproducibly decrease the intensity of both the major and minor binding complexes and to supershift two distinct bands. When using the THcre probe, anti-CREB-1 produced a supershift of the middle binding complex, whereas anti-ATF-4 shifted two faint bands without dramatically altering overall band intensity. While ATF-4 does not heterodimerize with CREB, it is known to form heterodimers with the bZip transcription factors c-Jun, c-Fos, Fra-1, C/EBPβ, and C/EBPδ (31, 34). Like anti-ATF-4, anti-C/EBPδ was observed here to decrease the intensity of the GTPCHcre major and minor bands and to supershift the binding complex (Fig. 7C). In contrast, antibodies specific to C/EBPα or epitopes common to c-Fos, FosB, Fra-1, and Fra-2 or common to c-Jun, JunB, and JunD were without effect (Fig. 7C). The GTPCHcre binding complex formed by PC12 nuclear extracts thus includes the transcription factors ATF-4 and C/EBPβ.

Overexpression of ATF-4 Transactivates the Response to 8-Br-cAMP—Expression of endogenous CREB does not appear to correlate with either the in vivo GTPCH promoter response to 8-Br-cAMP or the in vitro binding of nuclear extracts to the GTPCHcre. These observations along with the binding of ATF-4 to the GTPCH CRE suggest that ATF-4 in intact PC12 cells is able to mediate the response of the GTPCH promoter to cAMP. In order to investigate this, PC12 cells were co-transfected with a fixed amount of 0.27GTPCHluc reporter DNA and varying amounts of DNA expressing ATF-4 under the control of the cytomegalovirus viral promoter. Overexpression of ATF-4 was found to increase the transcriptional response to cAMP in a DNA concentration-dependent manner, demonstrating that ATF-4 is able to transactivate the GTPCH promoter in intact cells (Fig. 9C).

Protein Binding to the CCAAT-box—EMSA using the GTPCHcrecaat probe demonstrated that antibodies directed against the C terminus of the A subunit of the heterotrimeric transcription factor NF-Y partially shift the large binding complex without altering the mobility of the smaller band (Fig. 7C). In contrast, antibodies directed against the C terminus of the NF-Y B subunit completely shift the upper band, while antibodies to the C terminus of NF-YC were without effect. Antibodies directed against another CCAAT-binding protein, NF-1, did not interact with either complex. These results indicate
that the GTPCHccaat oligonucleotide recruits the transcription factor NF-Y but that only the C terminus of the NF-Y B subunit of the NF-Y trimer is completely accessible to antibody binding.

**DISCUSSION**

Progressive deletion analysis of the 5812 bp of the rat GTPCH 5′-flanking sequence revealed that the first 142 bp upstream from the liver cap site contain the GTPCH core promoter and also mediate the cAMP-dependent enhancement of gene transcription. This 142-bp region is GC-rich (73%) and exhibits 90% homology with the mouse and 77% homology with the human GTPCH genes. The human GTPCH core promoter is also reported to lie within the first 211 bp upstream from the transcription start site (11). With the exception of the E-box, which is not present in the human sequence, each of the protein binding motifs detected in the rat promoter by *in vitro* footprinting is found in the mouse and human genes. Moreover, an additional DNA response element that is found in the human sequence but is absent from the mouse and rat promoters is a putative Sp1 binding motif strategically placed between the CRE and CCAAT-box. Whether this Sp1 site and/or the absence of the E-box are responsible for the significant differences in GTPCH expression between humans and rodents awaits determination (1).

The TATA-like ATAAAA sequence and its position and flanking bases are also completely conserved within the GTPCH core promoter, where the sequence may function to position the preinitiation complex (Fig. 1). Inasmuch as the rat, mouse, and human cap sites are all different, however, it would appear that this sequence serves only a weak positioning function within the GTPCH promoter. This conclusion is supported by the failure of PC12 nuclear extracts to footprint the ATAAAA sequence and, at least in the case of the mouse cap site, the lack of consensual distance expected between a bona fide TATA box and a transcription start site (31). Inasmuch as the recruitment of TFIID to the promoter is proposed to be an obligatory and probably limiting step in the formation of the preinitiation complex, the weak positioning of TBP by the ATAAAA sequence may actually contribute to GTPCH enhancer function. Indeed, the capacity of the CRE and CCAAT-box cassette to enhance cAMP-dependent transcription was only observed within the context of the GTPCH and SV40 minimal promoters, which, unlike the thymidine kinase minimal promoter, do not contain canonical TATA boxes.

The CRE and adjacent CCAAT-box each contribute independently to basal and cAMP-dependent transcription from the GTPCH promoter, although the contribution of the CRE to both activities is greater than that of the CCAAT-box. It would also appear that this combined sequence has the properties of an enhancer element. EMSA and co-transfection studies indicate that the transcription factors ATF-4, C/EBPβ, and NF-Y are the likely candidates recruited by the GTPCH CRE and CCAAT-box. ATF-4 seems to be found in many but not all tissues and cell lines (32, 33) and, in agreement with the data presented here, is found to be constitutively expressed along with C/EBPβ in PC12 cells (34, 35). Although originally characterized as a repressor (32), ATF-4 is now known to be a strong activator of transcription (34, 36), a role mediated by physical interactions of the N and C termini with the general transcription factors ATF-4, C/EBPβ, and NF-Y but that only the C terminus of the NF-Y B subunit of the NF-Y trimer is completely accessible to antibody binding.

**FIG. 9. Transient transfection assays of GTPCH promoter constructs.** 18 h after transfection, PC12 cells cultures were incubated with or without 5 mM 8-Br-cAMP dissolved in conditioned medium, harvested 7 h later, and assayed for luciferase and β-galactosidase activity. All data are the mean ± S.E. of at least two independent experiments each determined in triplicate. **A**, site-directed mutagenesis of the GTPCH CRE and CCAAT box decreases basal and cAMP-dependent transcription. Cells were transfected with either the wild type 0.27GTPCHluc construct or constructs in which the CRE (0.27GTPCHluc CREmt), the CCAAT box (0.27GTPCHluc CCAATmt), or the CRE and CCAAT box (0.27GTPCHluc CREmt CCAATmt) have been mutated. The -fold increase in response to 8-Br-cAMP is presented in parentheses. **B**, the combined CRE and CCAAT-box element can enhance basal activity and confer sensitivity to cAMP on a minimal heterologous promoter. Cells were transfected with either 0.27GTPCHluc or the luciferase-based reporter plasmids TKluc or SV40luc containing either the GTPCH CRE and CCAAT-box (cre/ccaat; positions 112 to −63; see Fig. 1) or the Sp1/GC box, CRE, and CCAAT-box (sp1/cre/ccaat; positions −142 to −63) to produce the minimal promoter constructs GTPChcre/ccaatTKluc plus GTPCHsp1/cre/ccaatSV40luc, respectively. The -fold increase in response to 8-Br-cAMP is presented in parentheses. **C**, overexpression of ATF-4 enhances the response to 8-Br-cAMP. 0.3 μg of 0.27GTPCHluc was transfected along with 0.05−0.15 μg of pCG-hATF4 (ATF-4) or empty vector DNA along with 0.05 μg of β-galactosidase DNA. The -fold increase in response to 8-Br-cAMP is presented in parentheses.
transcription factors TBP, TFII B, and TFII F as well as the co-activator CBP (36). While ATF-4 does not heterodimerize with CREB-1, it does form heterodimers with a number of other bZip transcription factors including C/EBPβ (33, 37). The formation of ATF-4 and C/EBPβ heterodimers in vitro is known to modify the DNA binding properties of this complex so that noncanonical CREs like that found in the GTPCH promoter are recognized (33). Moreover, ATF-4 and C/EBPβ have been shown to interact on the Gadd153 (C/EBPγ, CHOP) promoter to enhance transcription in PC12 cells (34). This observation and the data presented here would seem to establish that ATF-4 and C/EBPβ can function as partners in the control of gene transcription. The other ATF-4 partner on the GTPCH CRE and CCAAT-box enhancer would appear to be NF-Y, a highly conserved and ubiquitous heterotrimeric transcription factor composed of A, B, and C subunits, all of which are required for high affinity DNA binding (29, 38, 39). Like ATF-4, NF-Y is able to mobilize histone acetyltransferase activity by interacting directly with CBP as well as p300/CBP-associated factor (40, 41). Also like ATF-4, the amino-terminal domain of C/EBPβ has been established to interact directly with CBP (42). The combination of ATF-4 and C/EBPβ arrayed along with NF-Y on the GTPCH core promoter should thus have enormous potential to affect transcription through the modification of chromatin structure.

Located immediately 5′ to the GTPCH noncanonical CRE is a protein binding domain 4, which is composed of three overlapping Sp1-like elements. When placed in the context of the SV40 minimal promoter, the addition of this Sp1-like element to the CRE and CCAAT-box cassette suppressed basal activity yet possibly even enhanced cAMP-dependent transcription. It would thus appear that the cognate proteins recruited by the Sp1/GC box are somehow capable of distinguishing between these two modes of CRE-dependent and CCAAT-box-dependent enhancer activities. Whether the Sp1/GC box acts similarly within the context of the GTPCH promoter awaits determination. Although the protein(s) recruited by this GC-rich sequence have not been identified, an oligonucleotide containing a canonical Sp1 binding motif was found to compete away domain 4 on the GTPCH promoter. This suggests that a member(s) of the Sp1 family of transcription factors, which includes Sp1, Sp3, and Sp4, may bind this element (43–46). Since Sp4 appears to be exclusively expressed in the brain (43), the most likely candidates found within PC12 cells, which are derived from the adrenal medulla, would seem to be Sp1 and/or Sp3. Unlike Sp1, Sp3 generally acts to repress rather than activate transcription (47–50) and may thus be responsible for the inhibition of basal transcription observed here. Alternatively, since Sp1 and C/EBPβ are reported to interact physically (51), this interaction may somehow serve to repress basal GTPCH promoter function without affecting the cAMP response.

Characterization of a number of promoters has shown that a CRE can act in concert with a CCAAT-box (52–55) or a CCAAT-box can act alone (40, 56) to enhance gene transcription in response to cAMP. In those cases where both motifs are involved, the CRE and CCAAT-box can be separated by as much as 10–20 bp or approximately one or two turns of the double helix. This spatial geometry is conserved in the rat, mouse, and human GTPCH promoters and lends support to our contention that both the CRE and CCAAT-box elements are required for basal and cAMP-dependent transcription. In promoters where it has been studied in detail, the phosphorylation of CREB-1 and/or ATF-1 by protein kinase A has been established to mediate that portion of the cAMP response contributed by the CRE, while the biological basis for the NF-Y and CCAAT-box component of the cAMP response remains a mystery. The case of the GTPCH promoter is further complicated by the observation that ATF-4 does not appear to be a substrate for protein kinase A (36). C/EBPβ is a substrate for protein kinase A, however, and upon phosphorylation in PC12 cells, it is shuttled from the cytoplasm to the nucleus (35). How then does cAMP stimulate GTPCH transcription through the CRE and adjacent CCAAT-box? A model that may be relevant, at least to the NF-Y and CCAAT-box component of the GTPCH promoter response, has recently been presented to explain the ability of protein kinase A to enhance transcription from a CCAAT-box alone (57). This model proposes that the NF-Y B subunit is a substrate for protein kinase A and that phosphorylation of NF-YB by protein kinase A enhances its ability to recruit NF-YC, NF-YA, p300/CBP-associated factor, and CBP to the CCAAT-box. The ubiquitous nature of NF-Y, however, and the fact that not all CCAAT-box elements mediate cAMP responsiveness argue that additional trans-acting factors such as ATF-4 and C/EBPβ, which can bind noncanonical and therefore cryptic cis-acting elements, may ultimately be found to be involved in this process.

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