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Functional characterization of the promoter region of the mouse protein kinase C γ gene

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Abstract Promoter activity of protein kinase C (PKC) γ gene was analysed by chloramphenicol acetyltransferase (CAT) assay using extracts from the cells transfected with various fusion constructs containing the 5′-flanking region of the mouse PKC γ gene and CAT gene. Transient expression experiments in PC12 cells revealed that the upstream region of 87 bp from the transcriptional initiation site was sufficient for promoter activity. The region containing nucleotides 87 upstream from the transcriptional initiation site was shown to silence CAT activity in Balb/c3T3 cells, in which mRNA of PKC γ was not detected, suggesting that this region might contain a transcriptional regulatory element for the cell type-specific expression of the PKC γ gene.

Key words: PKC γ; Neuronal expression; Promoter; PC12 cell

1. Introduction

PKC, the major cellular receptor for diacylglycerol and tumor-promoting phorbol esters, has been implicated in the regulation of a variety of cell responses including membrane function, secretion of hormones and neurotransmitters, gene expression, and cell proliferation and differentiation. Recent biochemical experiments and molecular cloning have revealed that PKC consists of multiple subspecies [1,2]. These PKC subspecies show distinct expression patterns from one another. In particular, PKC γ is distributed exclusively in the central nervous system [3]. Analysis of the promoter region of PKC γ is important for understanding the regulation of the tissue and cell type-specific expression of PKC γ. Here we report the functional characterization of the mouse PKC γ promoter.

2. Materials and methods

2.1. Library screening and DNA sequencing

Two oligonucleotide primers were synthesized on the basis of rat PKC γ cDNA [4]. The sense primer was 5′-GGCCATGGC(G/T)GGTCTGGG(G/T)CC-3′ (corresponding to numbers +237 to +257) and the antisense primer was 5′-CA(G/T)ATGAAGTC(A/G)GT(A/G)CAGTG(A/G)CTGCA-3′ (corresponding to numbers +386 to +412). PCR amplification was performed using these primers and mouse genomic DNA (30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min). After amplification, a 190 bp DNA fragment was labeled by the random-oligolabelling reaction, then used as a probe. A mouse genomic DNA library (Clontech Laboratories) was screened using nitrocellulose filters. Hybridization was carried out for 16 h at 42°C in 50% formamide, 5 × SSPE (1 × SSPE: 180 mM NaCl, 10 mM sodium phosphate and 1 mM EDTA, pH 7.7), 5 × Denhardt's solution (1 × Denhardt's solution: 0.02% polyvinylpyrolidone, 0.02% Ficoll and 0.02% bovine serum albumin), 0.1% SDS, 100 μl/ml heat-denatured salmon sperm DNA and the labeled probe. The filters were washed twice for 3 min at 25°C in 2 × SSC containing 0.1% SDS, and then twice for 15 min in 0.2 × SSC containing 0.1% SDS. The filters were autoradiographed onto Kodak X-AR films at −80°C with intensifying screens. Nucleotide sequences were determined by the enzymatic chain termination method using Sequenase (USB).

2.2. Northern blot analysis

Total RNA was isolated according to the guanidinium isothiocyanate/CsCl method [5] and then poly(A)$^+$ RNA was purified by oligo(dT)-cellulose column chromatography (Pharmacia). The poly(A)$^+$ RNA (10 μg) was electrophoresed on 1.5% agarose gel containing 16% formaldehyde and transferred to a nitrocellulose filter. A 3.0 kbp *Eco*RI fragment containing rat PKC γ cDNA labeled with [α-^{32}P]dCTP was used as a probe. Hybridization and autoradiography were carried out as described above.

2.3. Plasmid construction and CAT assay

To construct the mouse PKC γ promoter-CAT fusion plasmid, a 1.8 kbp fragment of the mouse PKC γ 5′-flanking region (−1571 to +234) was inserted at the *Hin*dIII site in pSV00CAT (Nippon gene) in both forward and reverse orientations, and named pRCAT1 and pRCATR, respectively. Several deletions of the 5′-flanking region of the mouse PKC γ gene were generated using a Kilo-Sequencing Deletion Kit (Takara Shuzo). Fusion constructs were transfected into PC12 cells and Balb/c3T3 cells by Lipofectamine (Gibco BRL) and into C6 cells by Lipofectin (Gibco BRL). After incubation for 48–72 h, cells were harvested and CAT activity was measured [6]. CAT enzyme reaction was performed at 37°C for 2 h using 0.25 μCi [^{14}C]chloramphenicol, 5 mM acetyl-CoA and the cell extract. Acetylated reaction products were resolved by TLC analysis using a TLC plate (Merck) and a 95% $CHCl_3$/5% methanol mobile phase, and then autoradiographed. As an internal control for differences in transfection efficiency, plasmid pTB1328 containing the alkaline phosphatase gene was co-transfected [7]. Alkaline phosphatase activity in conditioned medium from the transfected cells was determined based on the increase in light absorbance at 405 nm which accompanies the hydrolysis of *p*-nitrophenyl phosphate [8].

3. Results and discussion

First, the expression of PKC γ mRNA was examined in rodent cell lines. The transcripts were analyzed in a rat pheochromocytoma cell line PC12, a non-neural cell line Balb/c3T3, and a glial cell line C6. As shown by Northern blot analysis in Fig. 1, about 3 kb of PKC γ mRNA was detected in PC12 cells, whereas it was undetectable in both Balb/c3T3 and C6 cells. Therefore, PC12 cells were used as a cell line for analysis of the expression of the PKC γ gene.

Next, the 5′-flanking region of the mouse PKC γ gene was

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Abbreviations: bp, base pair(s); kb, kilobase(s); PCR, polymerase chain reaction.

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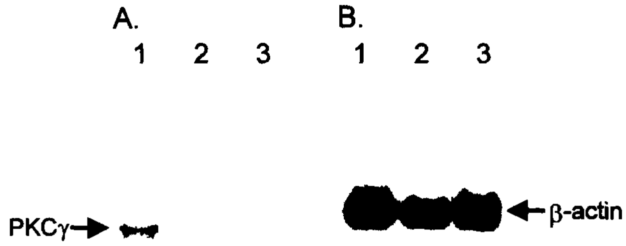

Fig. 1. Northern blot analysis of PKC γ mRNA. Poly(A)$^+$ RNA from PC12, Balb/c3T3 and C6 cells (10 μg each) was electrophoresed and hybridized consecutively with probes of rat PKC γ cDNA (A) and β-actin (B). Lanes: 1 = PC12; 2 = Balb/c3T3; 3 = C6.

cloned in order to identify the functional regulatory region of the mouse PKC γ gene. Nucleotide sequence analysis of the positive clone indicated that a 2 kbp *Eco*RI-*Pst*I fragment contained the 5′-flanking region and the first exon of the mouse PKC γ gene. As shown in Fig. 2, alignment of the sequences of mouse, rat and human revealed that the PKC γ 5′-flanking region demonstrated high homology among these three species. The transcriptional initiation site was mapped at the corresponding position to that of the rat PKC γ gene [9] (data not shown). A search for canonical control sequences upstream from the transcriptional initiation site revealed that the region lacks classical control sequences, e.g. TATA and CAAT boxes, at the usual positions, − 25 to − 30 bp and − 50 to − 100 bp, respectively. Thus, there seemed to be other *cis*-elements in this region interacting with transcriptional factors to initiate transcription. To search for potential binding sites for transcriptional factors, the 'SSCAN 33' computer program was used as described by Prestridge [10]. Together with the results of the nucleoprotein binding sites within the rat PKC γ promoter region mapped by DNase I footprinting analysis in the previous report [11], the mouse PKC γ 5′-flanking region was indicated to possess some candidates for the binding sites of transcriptional factors, including AP2 and SP1 as shown in Fig. 2.

Chloramphenicol acetyltransferase (CAT) assay was performed in order to examine the functional promoter activity of the 5′-flanking region of the mouse PKC γ gene. A reporting construct, containing the 1.8 kbp DNA fragment from − 1571 to + 234, linked to the CAT-reporting gene (pRCAT1) was first investigated. As shown in Fig. 3, the extract from pRCAT1-transfected PC12 cells displayed positive CAT activity, whereas no activity was detected in the extracts from pRCAT1-transfected Balb/c3T3 and C6 cells. These results indicate that the 1.8 kbp DNA fragment contains the functional promoter, which is active in the cells expressing the PKC γ gene.

To characterize further the upstream sequences of PKC γ that promote expression of the CAT gene in a manner specific to cell types, a series of 5′-deletions of the 1.8 kbp PKC γ promoter fragment were carried out (Fig. 4A). These shorter constructs were transfected into PC12 and Balb/c3T3 cells, and CAT assay was performed. As shown in Fig. 4B, the CAT activity did not change significantly, except for + 104/CAT plasmid in PC12 cells. These results suggest that the 5′-flanking region of 1.8 kbp of the mouse PKC γ gene does not contain an apparent enhancer element, and that the short span of − 87 bp upstream from the transcriptional initiation site is sufficient to support the transcriptional activity in this transient expression assay. Some candidates for the binding sites of transcriptional factors were found in this short span as described above (Fig. 2). Therefore, these *cis*-elements might act as positive regulatory elements for the basal promoter activity of the PKC γ gene. Furthermore, CAT activities in the extracts from Balb/c3T3 cells transfected with − 1571 bp/CAT and − 196 bp/CAT were quite low. However, the activity with − 87 bp/CAT construct was enhanced significantly (Fig. 4B, b, lane 4). These results suggest that a silencer-like element exists in this region

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Mouse GATGACTGACTGAC-------TGGGAGAAAGGGGGTGGTCAAGGCTCGGAGGACGTCCCCTCCTTGCATAGAGCCTGCGGAATGGAAGTGTGCATGTGGAGAGGA-----GGGAGGGGCA -119
Rat   GATAACAGACTGGAATGTATTTGGGAGAAAGGGGGTGGACAAGGCTTGGAGGACGTCCCCTCCTTGCATAGAGCTCGGGCAATGGGAGTGTGCACGTGGAGAGGA-----GGGAGGGGCA
Human GATGACAGATTGCA-------GGGTGGTGGGGGGGAG--CCAGGCTCAGAGGATGCCCCTCCCTCCAGCCAGGCCCCCGGAGTGGGTGTGTGCACGTGTGGGGGGCGGGCAGGGAGGACA
      GAT AC GA TG          GG  G   GGGGG G  C AGGCT  GAGGA G CCC  CCT        GC    G A TGG  GTGTGCA GTG  G GG        GG  GG CA

                                              Sp1                            AP2
Mouse TCTGTCCCGG--------G-----CGGGGGGGGGGGGCTTTAAACTGAAACCCCGCCCCTTGGTCGTCATGGCAACGCCTTCCCCCACCCCCGACTTCTACATTTCAGCAGGTGCTGAGA  -12
Rat   ACTGTCCGGGATTCTTGGG-----GGTGGGGGAACGCCTTTAAACTGAAACCCCGCCCCTTGGTGCTCATGGCAA-GCCTACCCCCACCCCCGACTTCTAC-TTTCAGCAGGTGCGATAG
Human TTTGTCCCGT--------GTCTCCGGGAGGGGAGCGCCTTTAAGCCGAAACCCGCCCTCTCGGTCGTCCTG-CAA-CGCCTCCCCCACCCGGGGCTCCCACATTTCAGCAGGTGCCGGAG
       TGTCC G          G       G  GGGG   G CTTTAA C GAAACCC  CC CT GGT  TC TG CAA   C  CCCCCACCC  G CT C AC TTTCAGCAGGTGC

                 *                                                   AP2
Mouse GCGAACGTCCC---GCCGCCGCCCGTGCCTGCGGCTCCTTGATGCCCCAGCCTTC---AGCTCTG--ACCCCACCCGCTTTCTCCCC-GGTCGGTGCTGCCCGTGCCGGGGTGT--TGCT  +98
Rat   C--GACGTCCC---GCCGCCGCCCGTGCCTGCCG-TCCTAGATGCCCCAGTCTTCTACTGCTCTG--ACCCCACCCGCTTTCTCCCG-GCTCGGTACAGCTGGTGCCGGGGGGTGCTGCT
Human CTGGAGCTCCCACCGCCGCCGCCCGTGCCTCCGGCTGCCGGC-GCCCCTGCCTTT---GGCTCTTCCTCCCCACTCGCCCGCTCCCCCTGGCGGAGCCGGCGCGCCCGGGGTGC--CGCT
         A   TCCC    GCCGCCGCCCGTGCCT C G T C   G   GCCCC G CTT       GCTCT     CCCCAC CGC    CTCCC      CGG   C G       CCGGGG+G     GCT

Mouse TTCTGCCGTGTGC--TGTGCACCGTTAGGTGCCTTGCCCCTGTCCTTCCTATCTCAGAGTCTGCGGAGT-CCTCCTACCG------------------------CCGTCCACCTGTTTCC +191
Rat   TTCTGCCCTGCGC--TGCGCACCGTTAG-TGCCCTGCCCCTGTCCTTCCGATCTCAGAGTCTGCGGAGTGGCCCCTATCG------------------------CCGTCCACCTGTTTCC
Human CCCTGCCTGGCGCGCTCCGCACCTGGAGGTGCCTTGCCCCTCTCCTGCCCACCTCGGAATTTCCCTGTG-GCTCCTTTGATCCTTCGAGTCTCCAGCTCCTCTCCCTTCCACCTGTTTCC
       CTGCC   G GC   T   GCACC    AG TGCC  TGCCCCT  TCCT  CC A CTC  GA T  T C         C CCT                               CC  TCCACCTGTTTCC

Mouse TC-GGAAAAAGGCCAGCTCGTGATCCCTTCTGCGTTCCTGGGGCCATG  +238
Rat   TCAGAAAAAAGGCCAGCTCGTGATCCCTGCTGCGTTCCTGGGGCCATG
Human CC-CAAGAAAGGCAGGATCCTGGTCCCTGCTACGTTTCTGGGGCCATG
       C   A AAAGGC   G TC  TG  TCCCT  CT  CGTT  CTGGGGCCATG
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Fig. 2. Alignment of the nucleotide sequence of the mouse, rat and human PKC γ 5′-flanking region. The nucleotide number indicated for the mouse sequence is relative to the transcriptional initiation site denoted by an asterisk. Gaps introduced to maximize homology are shown as dashes. Translational initiation codon ATGs are underlined. SP1 and AP2 binding sequences are denoted by italics. The consensus sequence derived is shown at the bottom. The nucleotide sequence of the 5′-flanking region of the mouse PKC γ gene from the clone isolated in this study was exactly the same as that registered in GeneBank under accession number X65720. Rat and human sequences were from [9] and GeneBank accession number X62533, respectively.

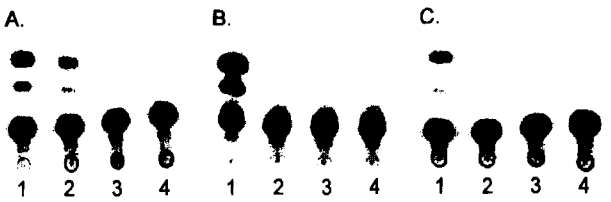

Fig. 3. CAT activity in the extracts of PC12, Balb/c3T3 and C6 cells transfected with the PKC γ promoter-CAT fusion genes. CAT assay was performed on transient transfectants generated in PC12 (A), Balb/c3T3 (B) and C6 (C). Lanes: 1 = pSV2CAT plasmid; 2 = pRCAT1 plasmid; 3 = pRCATR plasmid; 4 = pSV00CAT plasmid. Essentially identical results were obtained in five independent experiments.

upstream from −87, and that expression of the PKC γ gene is regulated negatively in most types of cells except for neuronal cells.

The transcriptional regulation of many eukaryotic genes has been intensively studied during the last two decades, and tissue-specific enhancers and silencers have been identified. Tissue-specific enhancer elements for different cell types, e.g. liver [12], lymphoid cells [13], pancreas [14], and anterior pituitary [15] are well established. In addition, several silencer elements to regulate neuronal cell-specific expression were previously identified as follows; (i) the sequence from −60 to −34 in the rat type II sodium channel [16,17]; (ii) from −163 to −154 and −72 to −62 in rat peripherin [18]; (iii) from −73 to −62 in the mouse neurofilament [19]; (iv) from −231 to −211 in the human synapsin I gene [20]; (v) from −400 to −388 in the human dopamine β-hydroxylase; (vi) from −1014 to −1002 in the rat type II sodium channel; and (vii) from −1479 to −1471 in the rat SCG10. (i)–(iii) include a region that contains CCAGG, 5 bp common core sequence [21]. (iv) has a homologous sequence to (i) [22]. (v) has a silencer element homologous to (vi) and (vii) [23]. The sequence from −1571 to +234 bp of the mouse PKC γ gene contained neither the 5 bp CCAGG common core sequence nor another stretch homologous to the sequence mentioned above (iv)–(vii). Thus, a new type of repressive element might contribute to the neuron-specific expression of the PKC γ gene. Future studies, such as the precise structural analysis and identification of the *trans*-acting factors that interact with this element, are needed in order to elucidate the mechanism of the neuronal cell type-specific expression of the PKC γ gene.

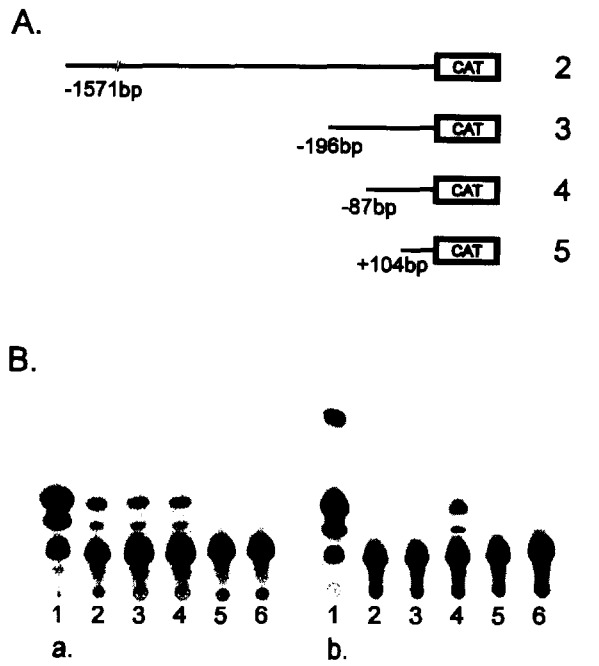

Fig. 4. Deletion analysis of the PKC γ promoter region. PC12 and Balb/c3T3 cells were transiently transfected with PKC γ promoter-CAT fusion genes. (A) Schematic representation of the 5′-deletion DNAs. Numbers indicate the correspondence with the lanes of CAT assay. (B) CAT activity with the 5′-deletion DNAs in PC12 (a) and Balb/c3T3 (b). Lanes: 1 = pSV2CAT plasmid; 2 = pRCAT1 plasmid (−1571 to +234); 3 = −196/CAT plasmid (−196 to +234); 4 = −87/CAT plasmid (−87 to +234); 5 = +104/CAT plasmid (+104 to +234); 6 = pSV00CAT plasmid. Essentially identical results were obtained in three independent experiments.

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