Volume 325, number 3, 210–214 FEBS 12622 July 1993


# Identification of a nuclear protein binding element within the rat brain protein kinase C γ promoter that is related to the developmental control of this gene

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Received 25 March 1993; revised version received 30 April 1993

Protein kinase C γ (PKC γ) is a brain-specific isozyme expressed at a high level in the adult but not in the fetal or newborn rat. At least seventeen nuclear protein binding sites within the 5′-flanking region extending from −1612 to +243 had been identified by DNase I footprinting analysis and gel mobility shift assays. Among them, one site, GAATTAATAGG, at −669 to −679 is protected from DNase I digestion by nuclear protein from newborn but not from the adult rat brain. The levels of this binding protein, as determined by gel mobility shift assay, were found inversely related to the levels of PKC γ in rat brain at different stages of development. These results suggest that this particular binding site may participate in the developmental regulation of PKC γ gene

Protein kinase C γ gene, Developmental regulation; DNA binding protein

## 1. INTRODUCTION

Protein kinase C (PKC) γ is one of the PKC isozymes specifically expressed in the adult brain [1]. During brain development, this kinase is expressed at a low level in the fetus and neonate one-week after birth and a rapid synthesis occurs between 2 and 3 weeks of age [2]. The mechanism that triggers the expression of PKC γ gene is unknown. Recently, the promoter region of rat PKC γ gene was cloned, sequenced, and mapped by transient expression assays [3]. A sequence of 163 base-pairs upstream from the transcriptional initiation site was found to possess full promoter activity in transient expression assays using non-neuronal 293 cells. This short region contains consensus recognition elements for the well characterized factors AP2 and Sp1 [4,5]. In addition, there are several sequence elements similar to *cis*-elements in other promoters that are located upstream from −163 [6]. The functional roles of all these potential *cis*-elements in regulating both the level of gene transcription and its tissue- and developmental stage-specific expression remain unknown.

Regulation of gene transcription is mediated in part through the interaction of a limited number of trans-acting nuclear factors with their respective target sequences located in the 5′-flanking region [7–9], within introns [10–12], and 3′ to their associated genes [13–15]. The complete genomic structures of the various PKC genes have not yet been determined. Human PKC β gene is believed to encompass more than 80 kb [16] and our preliminary results indicated that PKC γ gene is larger than 50 kb. The regulatory elements present in the introns and 3′-flanking regions of these genes cannot be defined until the structural organizations of these genes are characterized. In this study we have carried out DNase I footprinting and gel mobility shift assays with nuclear extracts from newborn and adult rat brains for the purpose of identifying any protein binding element in the 5′-flanking region that may be involved in the developmental regulation of PKC γ gene.

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*Abbreviations* PKC, protein kinase C; AP1, activator protein 1; AP2, activator protein 2, Sp1, stimulatory protein 1; CREB, cAMP regulatory element-binding protein; MLP, adenovirus type 2 major late promoter; C/EBP, CCAAT/enhancer binding protein; CAT, chloramphenicol acetyltransferase.

## 2. MATERIALS AND METHODS

The following items were obtained from the indicated sources. T4 DNA polymerase, T4 polynucleotide kinase, and DNA-modifying enzymes from Life Technologies Inc./Bethesda Research Laboratories; restriction enzyme *Nsi*I from New England Biolab; [γ-$^{32}$ P]ATP and [α-$^{35}$ S]dATP from Amersham Corp.; Poly(dI.dC) from Boehringer Mannheim Biochemical; DNase I from Pharmacia LKB Biotechnology Inc.; HPLC-purified synthetic oligonucleotides from Operon Technologies Inc. (Alameda, CA), and all other chemicals from Sigma.

### 2.1 *Preparation of oligonucleotides*

To generate short double-strand DNA regions containing a nucleoprotein binding site, purified oligonucleotides, dissolved in 10 mM


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Tris-HCl, pH 8.0, and 1 mM EDTA, were annealed by adding equal moles of complementary strands, heated at 80°C for 5 min and cooled slowly to room temperature. Sequences of each double-stranded DNA oligomer, with respect to PKC $\gamma$ promoter, were as following: B oligomer (+124 to +154); E oligomer (−63 to −30); F oligomer (−91 to −56); G oligomer (−113 to −87); K oligomer (−302 to −265); M oligomer (−684 to −664); and Q oligomer (−1522 to −1485). Other DNA probes contained consensus sequences corresponding to the binding sites of C/EBP [17], CREB [18], and AP1 [19].

2.2. *Plasmid construction*

Plasmids were constructed using standard methods [20]. An expression vector pGEM-7Zf(+) (Promega Corporation, Madison, WI) was used for construction of deletion mutants of PKC $\gamma$ promoter. The vector was first digested with *Hin*dIII and *Bam*HI for inserting the CAT coding region and SV40 polyadenylation signals, both of which are contained within a *Hin*dIII/*Bam*HI fragment derived from pSV2-CAT. The resulting plasmid was linearized with *Hin*dIII, and the 1.85 kb *Eco*RI/*Nco*I fragment (nucleotides −1612 to +243) of PKC $\gamma$ promoter [3] was inserted after all the restriction sites were made blunt-ended with T4 DNA polymerase. The junctions were sequenced to confirm the structure of the plasmid. To generate the sequential deletions originating at the 5′ end of the PKC $\gamma$ promoter, the recombinant plasmid was digested with *Cla*I and *Kpn*I and treated with exonuclease III according to the deletion protocol (Promega). End points of exonuclease III deletions were determined by dideoxy sequencing [21].

2.3. *Preparation of nuclear extracts*

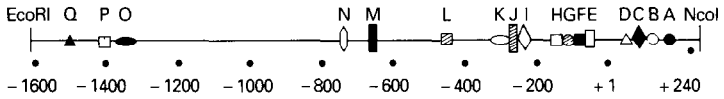
Adult (Sprague–Dawley, 200–250g) and newborn (3 days after birth) rat brain nuclear extracts were prepared essentially as described by Gorski et al. [22]. Briefly, fresh rat brains (10–15 g) in 30 ml of buffer (10 mM HEPES, pH 7.6, 25 mM KCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 2 M sucrose, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride) were homogenized with a motor-driven glass–teflon homogenizer. The homogenate was diluted to 85 ml with homogenization buffer, layered on top of a 10 ml cushion of homogenization buffer, and centrifuged at 24,000 rpm for 30 min at −2°C using a Beckman SW28 rotor. Nuclear pellets were resuspended in the homogenization buffer containing 20% glycerol and recentrifuged as above. Chromatin was removed and the nuclear extracts were further purified as described [22] and stored at −70°C in small aliquots. Protein concentrations were determined by the dye-binding method [23] using bovine serum albumin as standard.

2.4. *Preparation of end labeled DNA fragments for footprinting*

The probe for DNase I footprinting was labeled at a unique 5′ end of *Xba*I site present in the vector. DNA from different deletion mutants was digested with *Xba*I, dephosphorylated with phosphatase, and redigested with a second restriction enzyme *Sst*I, which cleaved a site within the PKC $\gamma$ promoter, or *Nsi*I, which cleaved a site within the vector. The desired DNA fragment was purified by electrophoresis on low melting agarose gel and end labeled with [$\gamma$-$^{32}$ P]ATP and T4 polynucleotide kinase

2.5. *DNase I footprinting*

Footprinting was performed with a modification of a published procedure [24]. The reaction mixture (50 $\mu$l) containing about 10 fmol of radiolabeled DNA probe, 0.5 $\mu$g of poly(dI·dC), 20 mM HEPES, pH 7.9, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), and 100 mM KCl was preincubated on ice for 15 min. After addition of nuclear extract, the mixtures were incubated on ice for additional 20 min, then at room temperature for 2 min. An equal volume (50 $\mu$l) of a solution containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$ and 8 ng of DNase I were added successively. After 1 min, the digestion was stopped by the addition of 100 $\mu$l of 1% SDS, 20 mM EDTA, and 50 $\mu$g/ml of tRNA. After phenol/chloroform extraction and ethanol precipitation, the samples were run on 8% polyacrylamide gels alongside a G+A track of the same DNA [25]. Control reactions were the same




| Binding Site | Position | Sequence |
|---|---|---|
| A | +168/+205 | TGGCCCCTATCGCCGTCCACCTGTTTCCTCAGAAAAA |
| B (Sp1) | +129/+149 | TGCCCTGCCCCTGTCCTTCCG |
| C | +84/+96 | GGTGCCGGGGGGT |
| D (AP2) | +45/+66 | GCTCTGACCCCACCCGCTTTCT |
| E (AP2) | −58/−35 | TGGCAAGCCTACCCCCACCCCCGA |
| F (Sp1) | −86/−61 | AAACTGAAACCCCGCCCCTTGGTGCT |
| G (AP2) | −108/−92 | TTGGGGGTGGGGGAACG |
| H | −154/−122 | GGGAGTGTGCACGTGGAGAGGAGGGAGGGGCAA |
| I | −248/−229 | GTGGGAGCCAAGATAACAGA |
| J | −260/−251 | AGGAAGATGG |
| K | −297/−272 | GATTCTGGCAAGACCCACACAAGCGG |
| L | −465/−442 | GGCATGGCACCAAGCCCAGAGGCT |
| M | −679/−669 | GAATTAATAGG |
| N | −741/−728 | CCCCCTTGCGACAG |
| O (MLP) | −1360/−1338 | GGTGGGGCGTGACCTGGGGGAGG |
| P | −1418/−1394 | GAACGGCTTCCTGCCAGAATGGACC |
| Q | −1517/−1490 | AGTTCTCCGTTGCGTTCCAAGAATAGAG |

Fig. 1. Summary and schematic representation of the nucleoprotein binding sites within the PKC $\gamma$ promoter. The nucleotide sequences mapped by DNase I footprinting analysis are given and the sequences similar or identical to the known binding elements are underlined.

as described above except that no nuclear extract was added and digestion was carried out with 2 or 4 ng of DNase I

2.6. *Gel mobility shift assay*

Oligonucleotides were labeled with [$\gamma$-$^{32}$ P]ATP and T4 polynucleotide kinase, then purified by Sephadex G-50 gel filtration chromatography. The reaction mixture (20 $\mu$l) containing 0.5 ng of labeled oligonucleotides, 0.5 $\mu$g of poly(dI.dC), 20 mM HEPES, pH 7 9, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol was preincubated on ice for 15 min in the presence or absence of 5- to 100-fold molar excess of unlabeled probe as a specific competitor, or oligonucleotides containing high affinity C/EBP binding site [17] as a non-specific competitor. The nuclear extract (10 $\mu$g) was added and incubated for additional 20 min on ice. Samples were run on 4% polyacrylamide gels in 0.5 × TBE buffer at 4°C. After the run, the gel was dried and exposed to X-ray film.

## 3. RESULTS AND DISCUSSION

Ten nested deletion mutants of PKC $\gamma$ promoter (nucleotides −1612 to +243) were generated for footprinting analysis. Each 5′-end labeled (coding strand) mutant DNA was incubated with adult or newborn rat brain nuclear extracts and then digested with DNase I. The nucleotide sequences of 17 protected regions and a schematic representation of the nuclear protein binding sites are summarized in Fig. 1. Both the adult and newborn rat brain nuclear extracts share 16 common binding sites and the latter binds an additional one, site M. The area between the cap and translational initiation site (+1 to +243) contain four protected regions: site A covers 38 nucleotides from +168 to +205, which do not contain any known binding element; site B (+129 to +149) contains the sequence CCCTGCCCC, which is similar to the SP1 recognition site CCCCGCCCC [5]; site C (+84 to +96) contains a sequence of GTGCCGGGG, which shares partial sequence similarity to the complementary Sp1 consensus element [5]; and site D (+45 to +66) contains CCCCACCCG, which is similar to the AP2 consensus sequence [26]. Four footprints were detected within a promoter DNA fragment spanning from −181 to +243: sites E (−58 to −35) and G (−108 to −92) share the AP2 consensus sequence of CCCCACCCC or its complementary GGGGTGGGG [26]; site F (−86 to −61) contains a nucleotide sequence matching the Sp1 consensus [5]; and site H covers 33 nucleotides (−154 to −122) without containing any known consensus sequence element. Four additional nuclear protein footprinting sites were detected at positions −248 to −229 (I), −260 to −251 (J), −297 to −272 (K) and −465 to −442 (L) using a PKC $\gamma$ promoter DNA fragment spanning from −493 to +243. These sites do not contain any consensus sequence elements to the known binding sites and site K, covering 26 nucleotides, has two hypersensitive sites at nucleotides −272 and −278. Two footprint regions, M and N, were observed between nucleotides −741 to −669. Both sites M (−679 to −669) and N (−741 to −728) do not correspond to any known sequence element. Site M, GAATTAATAGG, was protected by newborn rat brain nuclear extract but not by that of the adult brain (Fig. 2). The three extreme upstream binding sites O, P, and Q, extending from −1360 to −1338, −1418 to −1394, and −1517 to −1490, respectively, are interspersed by a segment of nearly 600 bp without any detectable protected region by footprint analysis. Site O contains a sequence, CGTGAC, identical to that of a binding site required for active transcription in human and mouse DNA polymerase-$\beta$, hamster 3-hydroxyl-3-methyl glutaryl coenzyme A reductase, and the adenovirus major late promoters (MLP) [27–29]. Mutation of this same 6-bp element greatly reduces the transcriptional activity of these three promoters. Both sites P and Q do not contain any known consensus sequence; there are two clear hypersensitive sites at position −1517 and −1509 within site Q.

In the previous study [3], we predicted that PKC $\gamma$ promoter contained CREB and AP1 binding sites at −582 to −573 and −270 to −264, respectively. However, we did not detect any protection of these two predicted binding sites using DNase I footprinting assay. To as-

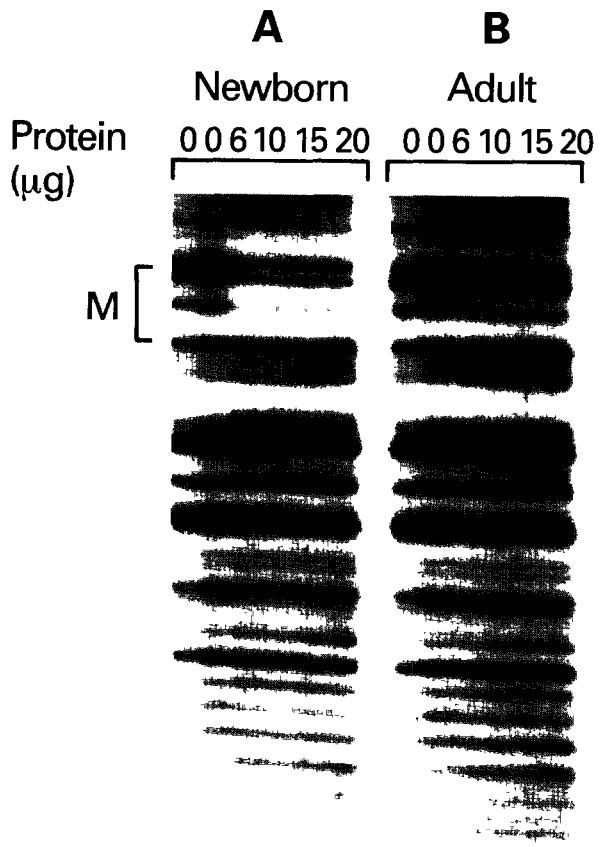



Fig. 2. DNase I footprint analysis of PKC $\gamma$ promoter fragment −812 to +243. Footprint analysis was performed with various amounts of newborn and adult rat brain nuclear extracts. Labeling of DNA fragment, binding reactions, and DNaseI footprinting were carried out as described in section 2. Footprint of site M (−679 to −669) is shown. The two lanes marked '0' are control reactions without nuclear extract using 2 and 4 ng of DNase I.

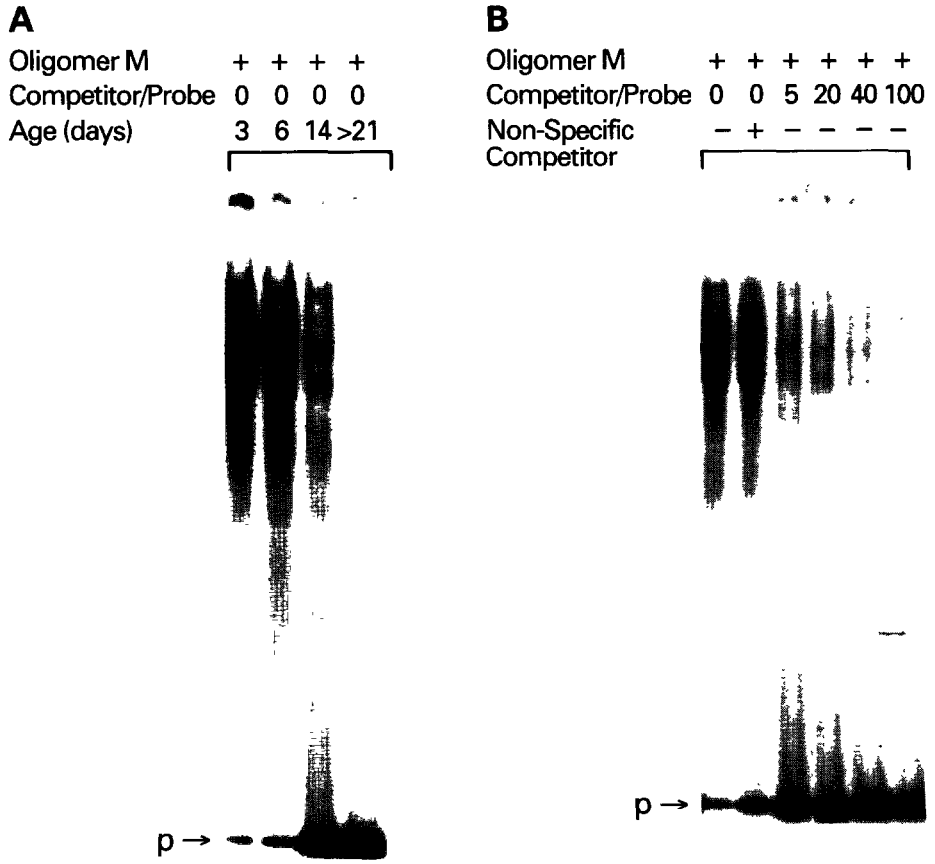



Fig. 3. Gel mobility shift assay of M *cis*-element with rat brain nuclear extracts at different stages of development and competition with oligonucleotides containing specific and non-specific elements. Nuclear extracts (10 $\mu$g each) of brain from different ages of rats were used for the assays (panel A). Arrows indicate the major specific retarded bands. Newborn rat brain (3-day-old rat) nuclear extracts were used for the competition experiments (panel B).

certain the results of footprinting assays, sequences corresponding to CREB and AP1 binding sites [18,19] were used for gel mobility shift assays. Incubation of the oligonucleotides containing CREB and AP1 consensus sequences with both newborn and adult rat brain nuclear extracts revealed the presence of retarded bands for each one of them and the bindings were competed efficiently with an excess of each of the respective unlabeled probe but not by a non-specific competitor (probe for C/EBP). Incubation of synthetic oligonucleotides corresponding to the predicted CREB (−588 to −568) and AP1 (−277 to −257) sites within rat PKC $\gamma$ gene with these nuclear extracts did not reveal any retarded band. These findings indicated that rat brain nuclear extracts do contain proteins recognizing CREB and AP1 sequences, and that our PKC $\gamma$ promoter sequence lacks functional binding sites for these two classes of DNA binding protein.

Several synthetic oligonucleotide probes corresponding to nuclear protein binding sites B, E, F, G, K, M, and Q (see section 2 for sequences) were also used to confirm the existence of these binding sites by gel mobility shift assays. Specific binding to each of these probes, with the exception of site M, were observed using both newborn and adult rat brain nuclear extracts (data not shown). Binding of site M probe to nuclear proteins present in the three- and six-day-old rats were evident but much less for the fourteen-day-old and undetectable for the three-week-old ones (Fig. 3). The binding motif of site M, GAATTAATAGG, harbors an imperfect palindrome; the factor could bind as a monomer or dimer as was observed for the double signals in the gel mobility shift assays. In addition, the ATTA motif is typical for homeo-box like protein binding site, TCAATTAAAT, of the POU-family factors [30]. Binding of site M probe to the newborn (three days old) rat brain nuclear proteins was competed away with specific probe but not by a non-specific probe such as that for C/EBP. The decrease in the site-M binding proteins in the developing rats is inversely related to the expression of PKC $\gamma$ [2], suggesting that these binding proteins may possess a negative regulatory function. This hypothesis is consistent with the findings that the majority of POU-family genes are expressed in distinct spatial and temporal patterns in the developing brain [31]. The site M-binding proteins in those PKC $\gamma$-nonexpressing tissues, such as liver and spleen, are at near constant levels in the nuclear extracts of 3-, 6-, and 14-day-old,

and the adult rat liver and spleen (data not shown). Since PKC $\gamma$ only expresses in the fully differentiated adult rat brain neurons but not those from fetal or newborn rat, we have not yet identified any cell culture expressing PKC $\gamma$ for testing the function of site M by deletion analysis.

Rat PKC $\gamma$ promoter contains several *cis*-elements clustered around the transcriptional initiation site (from −200 to +200), possibly, including two Sp1 (sites B and F), three AP2 (sites D, E, and G), and three other unidentified binding elements. The presence of these closely spaced binding sites could lead to an intimate interaction among bound proteins, as well as with RNA polymerase II. Several genes exhibiting tissue-specific expression, such as those found in liver and brain [24,32,33], have been shown to display a similar pattern of protein binding site complexity; for example, there are six SP1 binding sites have been identified in the SV40 promoter [5] and multiple AP2 binding sites in metallothionein IIA and growth hormone genes [34,35]. Transcriptional factor AP2 is responsive to both phorbol esters and cAMP for the induction of gene transcription. It seems that transcription of PKC $\gamma$ can be induced by treatment of phorbol esters, which activate PKC, or by forskolin, which raises the concentration of cAMP. It is also possible that activation of PKC $\alpha$ and $\beta$ during early stage of brain development may provide a positive signal for the transcription of PKC $\gamma$, which coexists with either one or both of the former two kinases in a variety of neurons in CNS. This possible control mechanism may contribute to the delayed expression of PKC $\gamma$ as compared to PKC $\alpha$ and $\beta$ during brain development [2].

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