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PROMOTER REGION OF THE RAT PHOSPHOLIPASE C-γ1 GENE+

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SUMMARY : We have cloned the upstream region of the rat phospholipase C-γ1 gene and characterized its promoter activity. The 5'-upstream sequence is highly rich in GC, enriched with CpG dinucleotides and lacks a TATA motif. This sequence also contains many putative binding sites for regulatory proteins. Primer extension and RNase protection assay demonstrated a single transcriptional start site located at 103 nucleotides upstream of the ATG start codon. The transcriptional activities of various 5'-deleted fragments, fused with chloramphenicol acetyltransferase gene, were examined after transfection into C2C12 myoblast. Multiple positive and negative regulatory sites were observed.

Many types of cells respond to external stimuli by activating a phospholipase C(PLC) which hydrolyzes phosphatidylinositol 4,5-bisphosphate to two second messengers, inositol trisphosphate and diacylglycerol (1,2). A number of distinct PLC isozymes have been purified from a variety of mammalian tissues, and several forms have been cloned and sequenced. Comparison of deduced amino acid sequences has revealed that mammalian PLCs can be divided into three types (PLC-β, PLC-γ and PLC-δ), and that each type contains more than one subtype (3). These PLC isoforms appear to be activated by distinct mechanisms that initiate the cascade of molecular events leading to cellular response. The PLC-γ1 was found to be activated by the growth factor receptors with intrinsic tyrosine kinase activity (4). A higher expression of PLC-γ1 was observed in primary humman breast carcinomas and in human skins with hyperproliferative epidermal conditions than in normal tissues (5,6). Moreover, there are some evidences that PLC-γ1 expression may be regulated during animal development (7,8). These observations indicated that the PLC-γ1 expression can be under control of cell proliferation and differentiation. Thus, it is essential to characterize the molecular mechanism

+Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. L14476.

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Abbreviations used are: PLC, phosphatidylinositol-specific phospholipase C; CAT, chloramphenicol acetyltransferase; EDTA, ethylenediaminetetraacetic acid; M-MLV, Moloney-murine leukemia virus; WT1, Wilms' tumor predisposition gene 1; RARE, retinoic acid response element; TRE, thyroid hormone response element; EGF, epidermal growth factor; EGR-1, early growth response-1.

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of PLC-γ1 expression to elucidate its physiological role during cell proliferation and differentiation. In this study, we cloned and characterized the 5'-regulatory region of the rat PLC-γ1 gene.

MATERIALS AND METHODS

Isolation of genomic clone. A rat genomic library in EMBL3 (Clonetech, Palo Alto, CA) was screened by using a 0.4 kb EcoRI fragment of the rat brain PLC-γ1 cDNA as a probe (9). To identify the 5'-region, the positive clones were further analyzed by Southern blotting with the oligonucleotide(5'-GCTCGCCTCCCGGGCCGCCGCCGCCGCC-3') from the 5'-untranslated region of the rat PLC-γ1 cDNA. From the selected genomic clones, 1.2 kb XhoI fragment was subcloned into pBluescript KS (Stratagene, La Jolla, CA), named as pRCγ1X2. The sequence of the 1.2 kb XhoI fragment was determined by Erase-a-base sequencing system from Promega (Madison,WI) in both directions. DNA sequencing was performed by the dideoxy chain termination method (10). The sequence of the GC-compressed region was confirmed using dITP instead of dGTP.

Construction of plasmids. A CAT expression vector, pXCAT, containing no promoter activity, was prepared from E1b-CAT by the removal of E1b-TATA box (11). To construct the chimeric PLCγ1/CAT expression plasmid, 1.1 kb XhoI/NaeI fragment was cloned into pXCAT at 56 bp upstream from CAT gene and designated pPLCγ1034CAT. All 5'-deleted expression plasmids were derived from pPLCγ1034CAT by sequential deletion from 5' to 3' using Erase-a-base system from Promega, according to manufacturer's instructions. DNA sequencing was carried out to identify the exact sites of the deletions.

Cell culture conditions. C_2C_{12} cells (mouse skeletal myoblast line) were maintained at low density to prevent fusion in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 15% fetal bovine serum (FBS; GIBCO BRL) and 1mM sodium pyruvate (GIBCO BRL). PC-12 cells (rat pheochromocytoma line) were cultured in DMEM with 7% horse serum and 7% FBS. All cells were obtained from the American Type Culture Collection (Rockville, MD) and propagated in a humidified incubator at 37 °C in 5 % CO_2.

DNA transfection and CAT assay. PLC-γ1/CAT chimeric plasmids were transfected into C_2C_{12} cells at 30-50 % confluency in 100 mm culture dishes using the calcium phosphate coprecipitation method. Ten μg of CAT plasmid was cotransfected with 3 μg of pCH110, β-galactosidase expression plasmid (Pharmacia LKB Biotechlogy, Piscataway, NJ). A β-galactosidase activity was measured as an internal control to normalize for transfection efficiency. After exposure to precipitate for 12-16 hr, the cells were washed with phosphate-buffered saline and fresh media was then added. The cells were harvested 48 hr later and the CAT activity was determined by the method reported in Kim et al.(12).

RNase protection assay. The RNase protection assay was carried out by using a kit from Ambion Inc (Austin, TX). Antisense RNA probe was synthesized from a BamHI-digested pRCγ1X2 using T3 RNA polymerase and [α-^{32}P]UTP. The riboprobe ($1X10^6$ cpm) was coprecipitated with 5 μg of the rat brain mRNA or yeast tRNA. Hybridization was performed in 80 % deionized formamide, 100 mM sodium citrate, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA at 45 °C overnight. Unprotected RNAs were digested with RNaseA/RNaseT1 mixture. Resulting RNA fragments were analyzed on an 8 M urea sequencing gel with M13 sequencing ladder as a standard.

Primer extension analysis. Twenty μg of total RNA from PC-12 cells was hybridized with end-labelled primer, G1(5'-GTGCCCACCTCGAGGCTGCGGCAGAGG-3'), in the same solution used in RNase protection assay. The sequence of G1 is complementary to bases 72-98 downstream from the start codon of rat PLC-γ1. Hybridization mixture was heated at 75 °C for 10 min and then incubated at 37 °C overnight. After synthesis of the cDNA with M-MLV reverse transcriptase (200u, GIBCO BRL), the product was analyzed on the 6% denaturing polyacrylamide gel.

RESULTS AND DISCUSSION

Five PLC-γ1 clones were selected from a rat genomic library by using the 5'-most 0.4 kb fragment of the rat brain PLC-γ1 cDNA, generated by EcoRI digestion, as a probe (9). The upstream fragment of PLC-γ1 gene was identified with the oligonucleotide probe corresponding to the 5'-untranslated region. From a 8 kb EcoRI fragment, 1.2 kb XhoI fragment which was hybridized with the oligonucleotide was isolated and analyzed. A schematic representation of 1.2 kb genomic fragment is shown in Fig.1A, displaying 5'-flanking region, restriction enzyme sites and the transcriptional initiation site.

The transcriptional start site was determined by RNase protection assay. A ^{32}P-labelled antisense RNA, produced from PLC-γ1 gene in vitro, was hybridized with rat brain mRNA

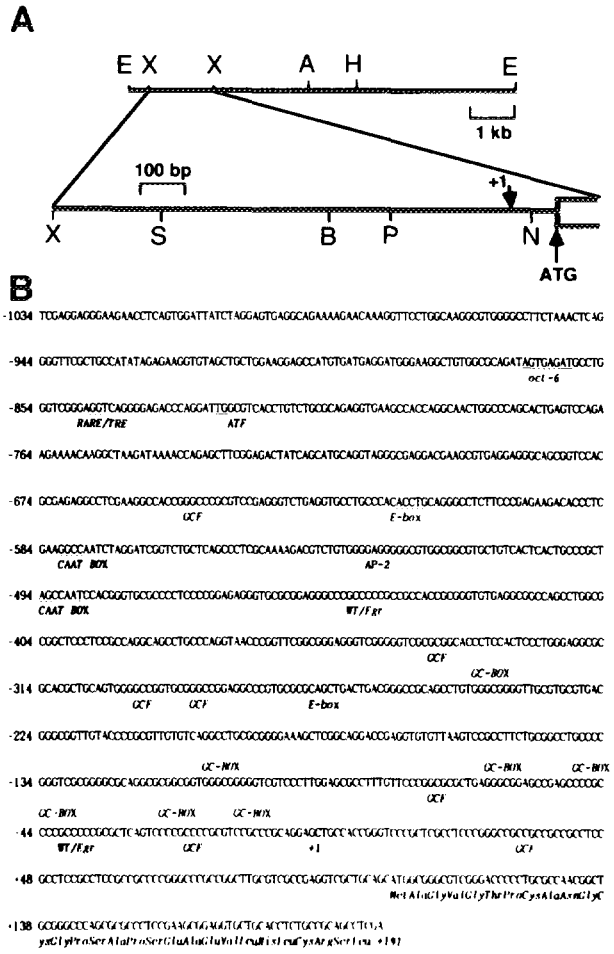

Fig.1. Restriction map and nucleotide sequence of the 5'-region of the rat PLC-γ1 gene. A) Restriction map of the genomic clones. Top, the 8 kb EcoRI fragment; Bottom, the 1.2 kb XhoI fragment derived from the EcoRI fragment. E, EcoRI; X, XhoI; A, ApaI; H, HindIII; S, SphI; B, BstEII; P, PvuII; N, NaeI. Cap site and translational initiation codon are indicated with arrows. B) Nucleotide sequence of the 1.2 kb XhoI fragment. Cap site is denoted as +1. N-terminal amino acid sequence is written in italic letter. Boxed or overlined sequences represent putative transcriptional regulatory elements.

and digested with RNaseA/RNaseT1 mixture. Only one protected fragment (191 bp), which was not seen in the nonspecific hybridization with tRNA, was detected. The result suggested that the transcription initiation site is 103 bases upstream of AUG start codon. A primer extension analysis using total RNA from PC-12 cells confirmed the same transcriptional start site (Fig.2).

The base composition analysis showed an unusual highness in GC-content and a frequent occurrence of CpG dinucleotides in the upstream region of PLC-γ1 gene. A bulk of vertebrate DNA contains 40% G+C, and the occurrence of CpG is one fifth of GpC (13). However, the GC-content in the proximal region of PLC-γ1 promoter, from -134 to -1(where +1 is transcriptional initiation site), is 82%, and CpG/GpC ratio in this region is 1.00. Since the CpG islands are often found in the 5'-terminal regions of housekeeping genes, spanning the 5'-flanking sequences and proximal exons, it is suggested that the CpG islands of the PLC-γ1 promoter can act as the regulatory region of the PLC-γ1 gene expression.

DNA sequence of the 5'-flanking region of PLC-γ1 gene and potential regulatory sites are shown in Fig.1B. There is no TATA-box around the transcriptional initiation site. However, seven GC-boxes, known as the Sp-1 binding sites (14), are clustered between -245 and +75. Multiple GC-boxes and the lack of a TATA box are the characteristics of the promoters of several genes important in the regulation of cell growth or DNA synthesis, such as transforming growth factor-β1 (12), c-Ha-*ras* (15), nerve growth factor (16), epidermal growth factor receptor (17), and DNA polymerase β (18). In this type of promoter, the GC-box seems to play a role in the entry of the basic transcription machinery (19). Although a number of promoters which lack TATA box or initiator sequence have several transcriptional

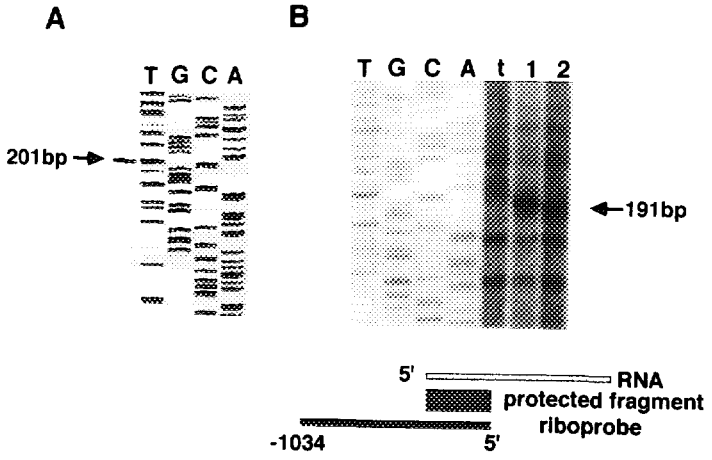

Fig.2. Determination of the transcriptional initiation site. The transcriptional start site was determined by primer extension (A) and RNase protection assay (B). Lanes, A, C, G, and T, are M13 sequence ladders, used as size marker. Resulting fragments are indicated with arrows and the sizes are expressed as base pairs. A) 5'-labelled oligonucleotide primer was hybridized with 20 μg of total RNA from PC-12 cells. B) Antisense riboprobe was hybridized with 5μg or 2μg of rat brain mRNA (lanes 1 and 2, respectively) or 20 μg of tRNA (lane t), and then digested with RNase mixture.

initiation sites, PLC-γ1 turned out to contain a single transcriptional start site. This suggests that there must be a strict control mechanism for entry of transcription machinery and that novel sequence and/or multiple GC-boxes could be involved in the determination of the transcriptional initiation site of PLC-γ1. In addition to the Sp-1 sites, other GC-rich regulatory sequences, such as AP-2 (20) and GCF (21), are scattered in the 5'-upstream region. The enhancer elements which are regulated developmentally, such as E-box (22), oct-6 (23) and RARE/TRE (24), are also present.

In order to identify the minimum promoter and the transcriptional control region of PLC-γ1 gene, the various chimeric PLC-γ1/CAT plasmids with 5'-serial deletions in the 5'-

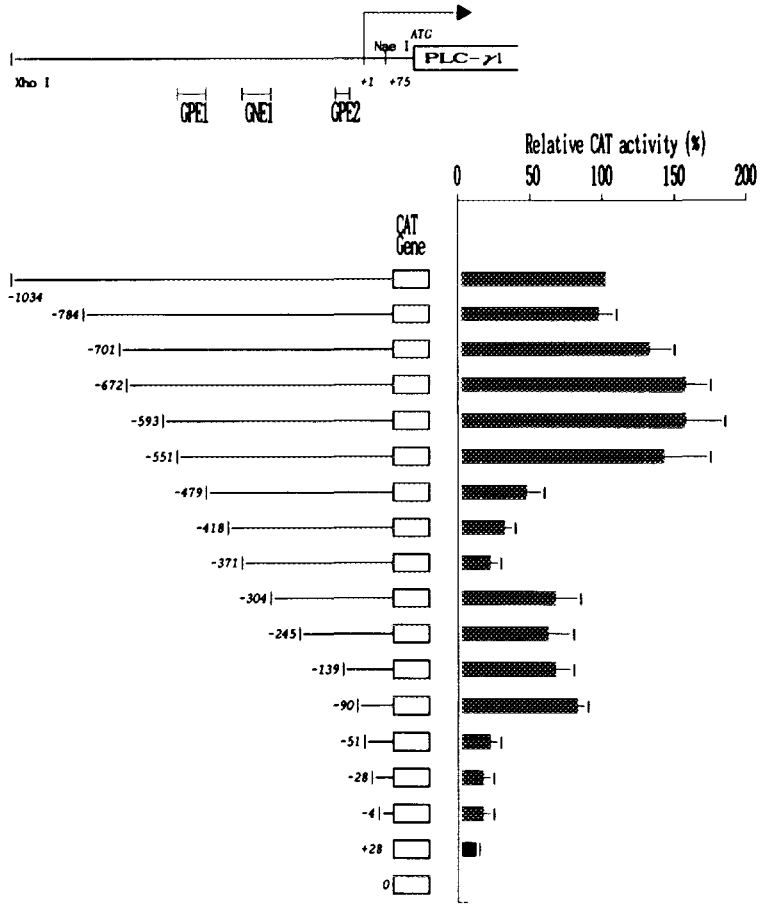

Fig.3. Basal activity of the 5'-flanking region of PLC-γ1 gene in C2C12 cells. The structures of the PLC-γ1/CAT plasmids are displayed on the left and transcriptional activity of each 5'-fragment is represented on the right. Each construct was transfected into C2C12 cells with β-galactosidase expression vector and after 48 hr of incubation CAT activities were measured. Transfection efficiencies were normalized with β-galactosidase activities. CAT activities are expressed as percentage of pPLCγ1034CAT. Transfections were repeated at least four times for all constructs. A diagram of the 5'-region of PLC-γ1 gene is shown at the top. Transcriptional start site is indicated by arrow. Opened rectangle displays the coding region of PLC-γ1 gene. GPE, positive regulatory element; GNE, negative regulatory element.

flaking region were constructed as described in the "Materials and Methods". Each plasmid together with β-galactosidase expression plasmid was introduced into C2C12 cells and then the promoter activity was determined 48 hours later. The relative activities of the constructs are shown in Fig.3. The construct with 551 bp 5'-flanking region exhibited almost the same promoter activity as that containing 1034 bp. This suggests a possibility that the minimal promoter of PLC-γ1 gene is 551 bp. Deletion from -551 to -479 reduced the promoter activity to 50 % of control. Further deletions to -371 bp showed continued reduction of the promoter activity to a minimum of 17 %. Therefore, it appears that there may be coorperation of more than one positive regulatory elements present between -551 and -371 bp. This region contains AP-2, CAAT (25) and WT1/EGR-1 binding sites (26). Especially, EGR-1 protein is known to be growth factor-inducible and WT1 may function by antagonizing the action of the growth factor-inducible EGR proteins (27). Therefore, there is the possibility that these factors (AP-2, CAAT and WT1/EGR-1) may be involved in the expression of PLC-γ1 gene during cell growth. The deletions from -371 to -304 bp restored the promoter activity to about 70% of control, suggesting that there may be a negative regulatory element between -371 and -304 bp. In this region, GCF binding site was found. The GCF is the suppressor of EGF receptor gene (21). Since PLC-γ1 was reported to be overexpressed in the similar manner with EGF receptor in breast cancer and hyperproliferative skin (5,6), it is possible that the common regulator(s) is involved in the control of expression of both genes.

In conclusion, we have cloned and analyzed the PLC-γ1 promoter. This promoter contains several consensus binding sites for Sp-1 protein and WT1/Egr-1 binding proteins etc., which may participate in the regulation of the PLC-γ1 gene expression. Functional analysis of the PLC-γ1 promoter revealed possible presence of multiple positive and negative cis-acting elements. Therefore, the characterization of the specific cis-acting elements and trans-acting factors involved in the regulation of the PLC-γ1 promoter will provide further insight into the mechanisms that control the expression of PLC-γ1 gene.

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