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Cloning and characterization of 5'-upstream region of human phospholipase C-β2 gene

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Abbreviations: B2NR, Negative regulatory region of PLC-β2 promoter; B2PR, Positive regulatory region of PLC-β2 promoter; CAT, Chloramphenicol acetyl transferase; IP_3, Inositol 1,4,5-trisphosphate; PLC, Phospholipase C

Abstract

5'-upstream region of the phospholipase C-β2 gene, 810 bp, was cloned and characterized. S1 nuclease mapping and primer extension analyses revealed that a single transcriptional start site locates at 284 nucleotides upstream from the beginning of translation. The 5'-upstream region lacks both TATA motif and typical initiator sequence, but retains GC-rich segment. Two putative regulatory regions, a negative region (–636/–588) and a positive region (–98/–13) were identified in the upstream region of PLC-β2 gene. We suggest that the transcription of PLC-β2 may be regulated by binding of regulatory proteins to the negative and/or positive regulatory regions located in the upstream of the gene.

Keywords: phospholipase C-β2, promoter, cloning, transcription

Introduction

Phosphoinositide-specific phospholipase Cs (PLCs) mediate signals from the outside of the cells to various compartments of the cells. The enzymes catalyze the hydrolysis of phosphoinositol 4,5-bisphosphate (PIP_2) to two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1993). To date 11 distinct PLC isozymes are identified in mammalian tissues and cells. The PLCs were divided into four types, PLC-β, γ, δ and ε (Kelley *et al*., 2001). Each type is composed of more than two subtypes. PLC-β subtypes appear to have unique tissue distributions. PLC-β1 was detected in brain, while PLC-β3 was found in a variety of tissues and cell (Sekiya *et al*., 1999; Kim *et al*., 2000). PLC-β4 is expressed primarily in retina and cerebellum (Kim *et al.,* 1998). The expression of PLC-β2, however, is largely confined to hematopoietic lineage with a minor immunoreactivity in other tissues (Park *et al*., 1992; Jiang *et al*., 1997; Sekiya *et al*., 1999). The difference in tissue distributions reflects that the expression of each PLC-β subtype could be regulated differently. In this report, we have isolated and characterized the genomic clones including 5'-upstream region of PLC-β2 gene.

Materials and Methods

Isolation of genomic clone of 5'-upstream region of PLC-β2 gene and construction of deletion mutants for the region

A human genomic library in EMBL3 (Clonetech, Palo Alto, CA) was screened with a 600 bp *Acc*1 fragment of the human PLC-β2 cDNA as a probe (Park *et al*., 1992). One positive clone was rescreened and Southern-blotted with oligonucleotide probe (5' AATCTAGTTGC-TCTTATAGCCCCTG 3') located in 5'-untranslated region of the cDNA. Two fragments, an 810 bp AvaI fragment (-636/+175) and an 805 bp *Ban*I fragment (–621/+185), were selected and subcloned into an *Eco*RV site of pKS^+ (Stratagene, La Jolla, CA) named pB2A2 and pB2B5 respectively. Restriction enzyme mapping and nucleotide sequencing confirmed the correct inserts. A *Sal*I-*Sma*I fragment (850 bp) of pB2A2 was subcloned into *Sal*I-*Sma*I cut of E1b-TATA chloramphenicol acetyl transferase (CAT), resulting in pA2-636CAT. Nested deletion using *Exo*III and S1 nuclease (Promega, Madison, WI) after digestions with *Sph*I and *Cla*I was carried out to produce 5'-deletion mutants of the cloned region. The exact deletion was determined by dideoxy termination sequencing.

Cell Culture, transient transfection and CAT assays

Daudi (human Burkitt lymphoma) cells ($1\text{-}2 \times 10^7$) were electroporated with 20 μg of plasmid DNA as described (Lee *et al*., 1997). After 48 hrs of incubation, the cells were harvested and soluble, cytosolic extract was prepared by three cycles of freezing-thawing. The CAT activities were assayed with 100-150 μg of the extracts as described previously (Lee *et al*., 1997).

Primer extension analysis

The primer used for extension was a synthetic oligonucleotide, corresponding to the antisense sequence (+106/+86) of the nucleotide in human PLC-β2 gene. The labeled primer was mixed with 50 μg of total RNA extracted from Daudi cells and coprecipitated with cold ethanol. The pellet was dissolved in 20 μl of hybridization buffer (80% formamide, 300 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA). The mixture was heated for 10 min at 75°C, incubated at 37°C overnight, then ethanol-precipitated. cDNA was prepared from the hybrids as described elsewhere (Lee *et al*., 1997). Extension was performed at 40°C for 75 min and the reaction was stopped by addition of 1 ml of 0.5 M EDTA and 1 ml of DNase-free RNase (Boeringer Mannheim, Mannheim, Germany), incubated for 30 min at 37°C, and extracted with phenol-chloroform. The elongated product was analyzed on 6% polyacrylamide sequencing gel with sequencing reaction products of the same primer.

S1 nuclease mapping analysis

The probe used for S1 nuclease protection was *Pvu*II/*Ava*I fragment (–210/+165) prepared from pB2B5. AvaI site of pB2B5 was 5' end-labeled with [γ-^{32}P]ATP and purified through agarose gel electrophoresis. The probe was hybridized with total RNA (100 μg) from Daudi cells in 40 μl of S1 hybridization solution (20 mM Tris, pH 7.4, 400 mM NaCl, 1 mM EDTA, 0.4% SDS, 75% formamide), heated for 15 min at 75°C and incubated at 49°C overnight. After addition of S1 mixture (salmon sperm DNA 2 mg 3 M NaCl, 33.2 mM $ZnSO_4$, 200 mM sodium acetate, pH 4.5, 200 units of S1 nuclease), the mixture was incubated at 37°C for 1 hr and the reaction was stopped by phenol-chloroform extraction. The protected fragment was fractionated on 6% polyacrylamide denaturing gel.

Gel mobility shift assay

For preparation of cell extract, 5×10^7 Daudi cells were suspended in a extraction buffer (20 mM Hepes, pH 7.9, 350 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin), were placed on ice for 10 min, and centrifuged. The resulting supernatant was collected and stored -70°C until use. The probes for gel shift assay were synthetic oligonucleotides, NS1 (–636/–606) and NS2 (–610/–587), and they were end-labeled with [α-^{32}P]dCTP by Klenow. The unreacted nucleotides were separated by gel filtration chromatography on Sepharose G-25 column. For binding assays, a various amounts of Daudi whole cell extracts were preincubated with 1 μg of poly (dI-dC) in the presence or absence of the indicated unlabeled DNA as a competitor for 5 min on ice in 13 μl of binding buffer (12 mM Hepes, pH, 7.9, 4 mM Tris, pH 7.9, 60 mM KCl, 12% glycerol, 1 mM EDTA, 1 mM DTT). Labeled probe (~40,000 cpm) was added to the reaction mixture and incubated at 15°C for 25 min. The resulting products were resolved on 5% polyacrylamide gel in Tris-borate-EDTA buffer.

Results

Cloning and sequence analysis of the 5'-flanking region of the PLC-β2 gene

The 5'-flanking region of the PLC-β2 gene was cloned by screening a human genome library. The schematic diagram for the cloned region was illustrated in Figure 1. An AvaI fragment was subcloned into pKS$^+$ vector. DNA sequencing revealed that the *Ava*1 fragment contains 636 bp of 5'-upstream region and 175 bp of 5'-untranslated region. To identify the site for transcription initiation, we carried out S1 nuclease mapping and primer extension analyses. The probe used for S1 nuclease mapping was the *Pvu*II/*Ava*I fragment (Figure 1 and Figure 2A). When this fragment was hybridized to the total RNA from Daudi cells, 175 nucleotides of Daudi RNA was protected from the S1 nuclease, indicating the transcription initiation site is located at 175 bp-upstream of the *Ava*I site. The transcription start site was confirmed by primer extension analysis using end-labeled primer oligo A, which is denoted with underline in Figure 5. The extension products generated by reverse transcription from the labeled primer, oligo A, were 144 and 151 nucleotides long (Figure 2C) suggesting there are two transcription start sites. Since the sequence of oligo A is located at 31 nucleotides upstream of *Ava*I site of the probe for S1 nuclease mapping, 144 nucleotides fragment was identified as a product from major transcription start site. The major transcription site, then, is located at 284 nucleotides upstream from the translation initiation codon. The transcription start site is denoted by the arrowhead in Figure 2C and shown by +1 in Figure 5.

Regulatory region responsible for transcription of PLC-β2 gene

The 5'-upstream sequence near the transcription start site lacks both TATA box and typical initiator sequence (Smale and Baltimore, 1989). Interestingly the proximal region of 5'-upstream region of PLC-β2 (–100 to –1) is GC-rich (69%) and has multiple GC-boxes (Blake *et al*., 1990). A number of potential regulatory elements exist in this region (Table 1). To determine whether the 5'-upstream region of the human PLC-β2 gene has transcriptional regulatory region, series of deletion mutants of the region were generated. The resulting promoter/reporter gene constructs were transiently transfected in human Daudi Burkitt lymphoma cells and CAT activities were measured. The effects of deletions were diagramed in Figure 3. There was a dramatic increase of CAT activity in the deletion of –636 to –588 bp in the 5'-

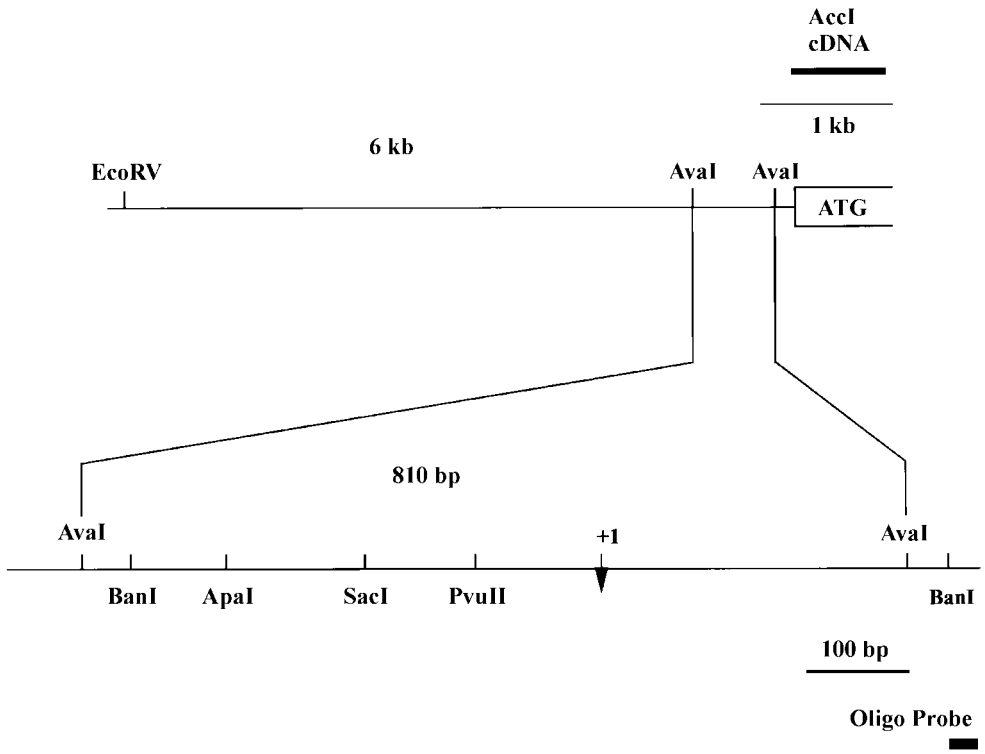

Figure 1. Schematic diagram of human genomic DNA segment, 5'-upstream region of PLC-β2. The top, thick line indicates a 600 bp cDNA probe. The oligonucleotide probe for screening genomic clone is described in bottom, thick line. Partial restriction map is described in the expanded view of the 811 bp *Ava*I fragment. Arrow indicates the transcription start site of the gene.

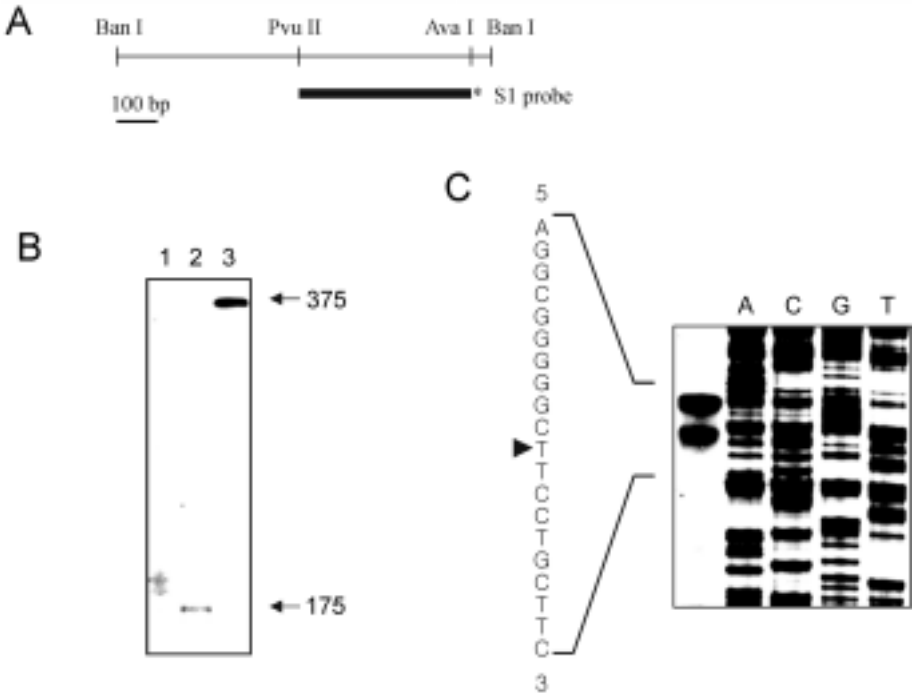

Figure 2. Identification of the transcription start site of the human PLC-β2 gene. **A**. The restriction map of pB2B5. **B**. S1 nuclease mapping analysis. *Pvu*II/*Ava*I fragment described in **A** was used as a probe. Lane 1, control products with yeast tRNA, lane 2, the protected fragments with Daudi RNA, lane 3, the S1 probe. Protected fragments containing 175 bases are indicated by arrow. **C**. Primer extension analysis, ^{32}P-labeled oligo A was hybridized to 50 μg of total RNA from Daudi cells. The dideoxy-sequencing ladder of the same strand is shown. The arrowhead indicates a putative major transcription start site.

Table 1. Consensus sequences for transcription factors in the 5'-upstream region of PLC-β2 gene

Putative element	Consensus sequence	PLC-β2 sequence	Location
AP2	$CCC^{A}/_{C}N^{G}/_{C}{}^{G}/_{C}{}^{G}/_{C}$	CCCACCCC	–355/–343
			–138/–131
CTCF	CCCTC	CCCTC	–244/–240
			–185/–181
E2A	$^{G}/_{A}$CAGNTG	GCAGGTG	–460/–454
Ets-1	$^{G}/_{C}{}^{A}/_{C}$GGA$^{A}/_{T}$G$^{T}/_{C}$	GAGGAAGC	–434/–427
		CAGGAAGT	–65/–58
GCF	$^{C}/_{G}$CG$^{C}/_{G}{}^{C}/_{G}{}^{C}/_{G}$C	GCGCCGC	–617/–610
NF-IL6	T$^{T}/_{G}$NNGNAA$^{T}/_{G}$	TTGAGAAAG	–425/–417
Oct-2	CTCATGA	CTCATGA	–114/–108
PEA3	AGGAA$^{G}/_{A}$	AGGAAG	–433/–428
			–78/–73
			–64/–59
PuF	GGGTGGG	GGGTGGG	–44/–26
Sp1	$^{G}/_{T}{}^{G}/_{A}$GGC$^{G}/_{T}{}^{G}/_{A}{}^{G}/_{T}$	GGGGCGGG	–27/–20
		GAGGCGGG	–11/–3
TCF-1	$^{C}/_{A}$A$^{C}/_{A}$AG	CAAAG	–503/–499
		CACAG	–154/–150
TCF-2a	$^{G}/_{C}$AGGAAG$^{T}/_{C}$	GAGGAAGC	–434/–427
		CAGGAAGT	–45/–38

Potent transcriptional factor binding sites found within 5'-upstream region of PLC-β2 are listed. Consensus sequences for transcriptional factors were obtained from Faisst and Meyer (1992).

upstream region, suggesting that there is a negative regulatory element in the deletion. Further deletion up to –98 bp maintained the promoter activity comparable to that of pA2-588 CAT. On the contrary, there was a sharp drop of CAT activity between –93 and –13 bp, implying the existence of a positive regulatory element in that deletion. Further removal to +169 bp, then, having no transcription start site, abrogated the promoter activity completely. These results indicate that there are one positive and one negative element in the 5'-upstream region of the PLC-β2 gene. The negative regulatory region of PLC-β2 gene was designated as B2NR, extending from –636 to –588, and the positive regulatory element as B2PR is located between –98 and –13 bp 5'-upstream of the gene.

Identification of putative negative regulator of PLC-β2 gene

To identify proteins binding to B2NR, gel mobility shift assay was performed using radiolabeled oligonucleotides, NS1 (–636/–606) and NS2 (–610/–587). When whole cell extracts from Daudi cells were incubated with NS1, a single distinct band appeared (Figure 4). The presence of 50-fold excess unlabeled NS1 hindered the complex formation. The NS2 probe produced four distinct retarded bands (Figure 4B). All bands detected with NS2 seemed to be specific to the NS2, since 50 fold excess unlabeled NS2 competed for complex formation.

As shown in Figure 4, NS1 produced one band that had mobility similar to one of the bands produced by NS2. To test whether the complexes formed by two probes were the same identity, the cross-competition

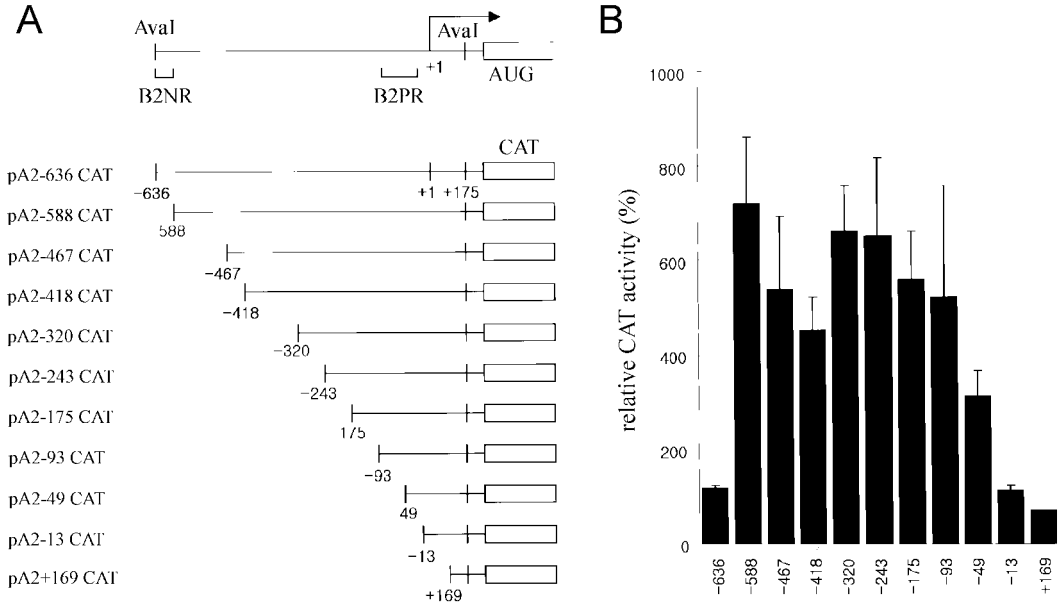

Figure 3. 5'-deletion mutants of human PLC-β2 gene and the promoter activity. **A**. Various deletion mutants of the 5'-upstream region of PLC-β2 gene are shown. Numbers are relative to the transcription start site. These PLC-β2 deleted fragments were fused with the coding region of bacterial chloramphenicol acetyl transferase (CAT) gene. Twenty μg of each deletion constructs were transfected by electroporation into human Daudi Burkitt lymphoma cells. CAT activities have been normalized for the β-galactosidase activity produced by co-transfected pCH110 plasmid. **B**. Corrected activities of each construct are shown as the percent activity relative to the mean activity of the longest promoter construct. Transfection was repeated at least twice, yielding reproducible results.

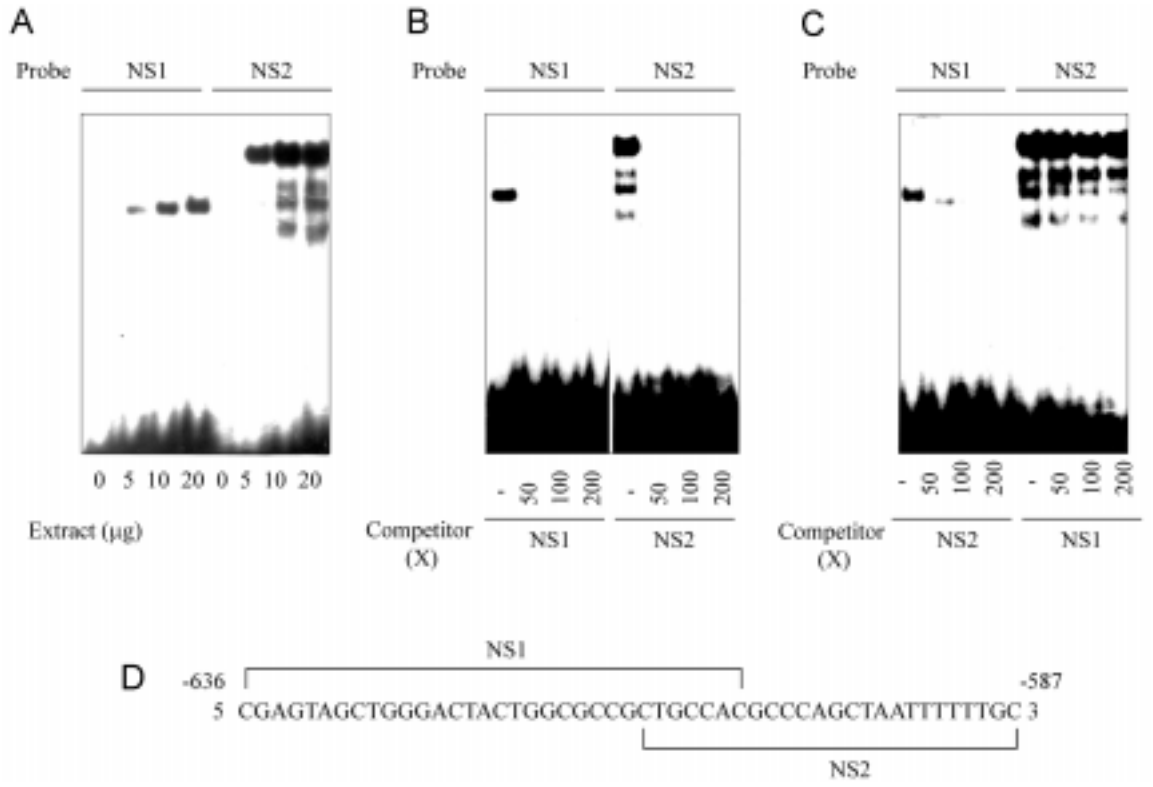

Figure 4. Gel mobility shift assays. **A-C**. Labeled fragments, NS1 and NS2, were incubated with the indicated amounts of Daudi whole-cell extracts in the absence or presence (50-, 100-, and 200-fold molar excess) of competitor as indicated. The samples were run on nondenaturing 5% polyacrylamide gels. **D.** The DNA sequence of oligonucleotide of B2NR.

experiment was carried out with the other oligonucleotide as a competitor. Inclusion of excess unlabeled NS2 in the NS1 reaction mixture competed for the complex formation. But unlabeled NS1 in 50-fold excess did not compete for the complex formation bound NS2 except two minor lower bans. These results suggest that several proteins that bind to this region specifically recognize B2NR.

Discussion

PLC-β2 gene expression is largely confined to hematopoietic lineage (Jiang *et al.,* 1997). Studies of the 5'-upstream region of PLC-β2 gene might provide a basis of assessing *cis*-elements and transcription factors involved in restricted expression of the gene. Here, we report the isolation and functional characterization of 5'-upstream from the AUG codon, which further supported by the promoter activities of the deletion constructs. The deletions of upstream region from –13 and downstream region from –49 abolished the promoter activity almost completely and suggest the presence of the entry site for the basic transcriptional machinery between –49 and –13 and agrees with the suggested transcription initiation site.

The primary sequence around transcription start site lacks either TATA motif or initiator sequences, but contains multiple GC-rich boxes. Within –100 bp from the transcription start site, two consensus sequences for Sp1 are identified, –25 and –11. Analysis of GC-boxes in the dihydrofolate reductase promoter revealed that the interactions between at least two of four GC boxes are required for transcription initiation (Blake *et al.*, 1990). In addition, in a model system of TATA-lacking promoters, Sp1 has been shown to mediate transcription initiation complex (Pugh and Tjian, 1991). Therefore, the specificity of selection of transcription start site of PLC-β2 gene might reside in the GC-boxes. Actually binding sequences for Sp1 have been identified in the proximal promoters of many cellular genes including PLC-β2 gene. In agreement with this suggestion, removal of downstream region from –49, which resulted in elimination of Sp1 sites, shut down the promoter activity.

Transfection study with deleted-promoter constructs revealed that two regulatory regions exist in PLC-β2 gene. A positive regulatory region (–98 to –13), B2PR, and a negative regulatory region (–636 to –588), B2NR, were identified. B2NR contains a binding site (5'-GCG-CCGC-3') for GCF, a suppressor of epidermal growth factor receptor (EGFR) gene (Kageyama and Pastan, 1989; Beguinot *et al.*, 1995). In B2PR there are many

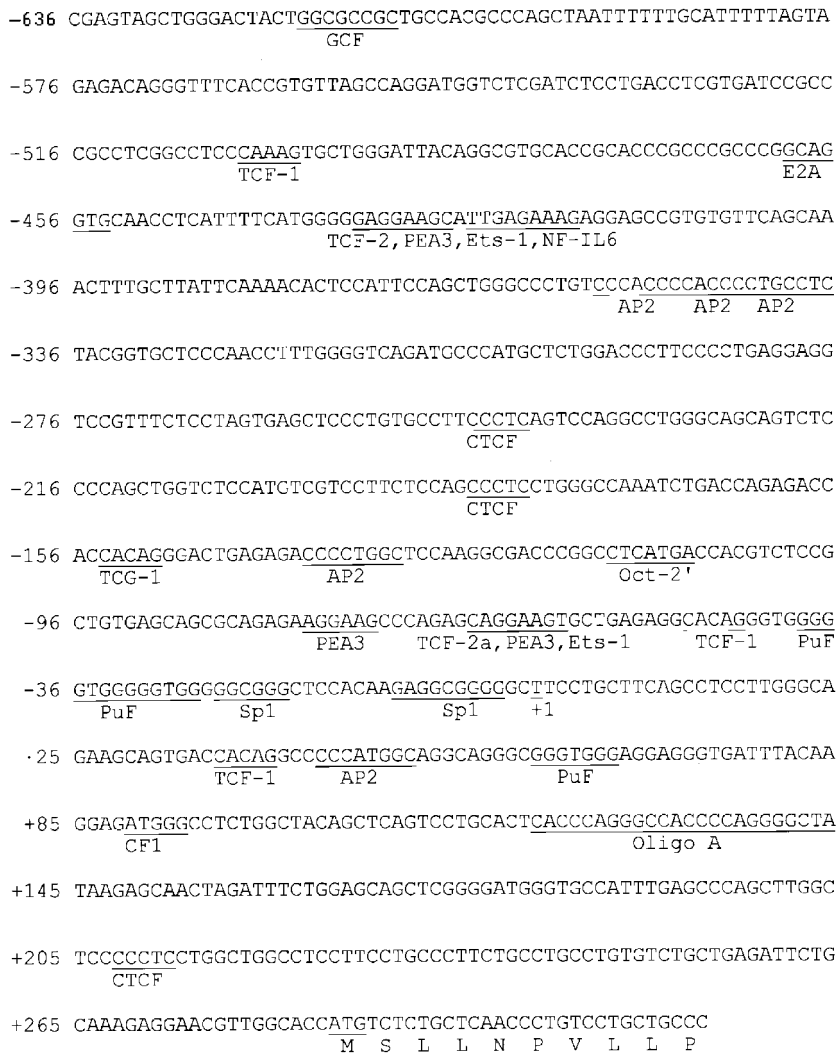

Figure 5. The nucleotide sequence of the 5'- and 3'-flanking region of the transcription start site of human PLC-β2. 5'-upstream region of the human PLC-β2 in Figure 1 was sequenced. The transcription start point identified by primer extension analysis is marked as +1. Various possible transcription factor-binding sites are underlined. Underline (oligo A) indicates the oligonucleotide probe used for primer extension analysis.

binding sites for transcription factors such as TCF-2a, PuF, and TCF-1, which are expressed in specific tissue types that express the PLC-β2. TCF-1 is expressed in T-cell specific fashion, which might play a role in the establishment of the mature T cell phenotypes (van de Wetering *et al.*, 1991). Also Ets has been reported to be expressed preferentially in lymphoid cells (Gunther *et al.*, 1990). Although the putative binding sites for transcription factors were identified in the regulatory regions of PLC-β2 gene, further characterization should be needed to elucidate the involvement of these factors in the transcriptional regulation of PLC-β2 gene.

The binding proteins for B2NR were identified by gel mobility shift assays. NS2 formed four protein-nucleotide complexes and cross-competition analysis revealed that at least two of the complexes were not competed with NS1. These results suggest that there are different binding proteins recognize this B2NR region and the repression of the transcription of PLC-β2 gene might be conferred by the interaction of these DNA-binding proteins.

From these results, it seems likely that the transcription of PLC-β2 gene might be under control of both positive and negative regulatory mechanisms and characterization of the transcription factors which bind to the regulatory regions would be the promising way to understand the regulation of PLC-β2 expression.

Acknowledgments

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