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Genomic organization, promoter characterization and roles of Sp1 and AP-2 in the basal transcription of mouse *PDIP1* gene

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Abstract The mouse polymerase delta-interacting protein 1 gene, *PDIP1*, is mapped to chromosome 7F3 region, spans approximately 16.7 kb, and is organized into six exons. The transcription start site (TSS) was determined to be G, corresponding to position of 162-bp upstream of the translation start codon. The promoter region was found to lack TATA box or CCAAT box, instead, a CpG island was detected surrounding TSS. The region from −162 to +114 is required for basal transcriptional regulation of mouse *PDIP1* gene, contains two AP-2 and two Sp1 binding sites. The Sp1 site upstream of TSS activates, while the other Sp1 site and two AP-2 sites suppress the transcription activity of mouse *PDIP1* gene.

Keywords: Gene promoter; Gene transcription; *PDIP1*; Sp1; AP-2

1. Introduction

Human polymerase delta-interacting protein 1 (PDIP1) was demonstrated to co-localize with DNA polymerase δ (pol δ) and proliferating cell nuclear antigen (PCNA) at replication foci, physically and functionally interact with both proteins, and stimulate pol δ activity in the presence of PCNA [1]. The expression of human *PDIP1* gene has been detected in every tissue tested, including liver, colon, thymus, kidney, small intestine, placenta, lung and leukocyte.

We recently cloned two rat genes homologous to human *PDIP1*, termed rat *PDIP1* and rat tumor necrosis factor-induced protein 1 (*TNFAIP1*). The deduced protein of rat PDIP1 shares 92.7% identity with human PDIP1 protein while the rat PDIP1 and rat TNFAIP1 share 63.1% sequence identity with each other [2]. The PDIP1 family of proteins was quite conserved and distributed in various species of organisms including protozoan parasites (*Schistosoma japonicum*), *Caenorhabditis elegans*, insects (e.g., *Anopheles gambiae*, *Drosophila melanogaster*), fish (e.g., *Danio rerio*) and mammals. Both PDIP1 and TNFAIP1 proteins contain a conserved BTB/POZ domain at the N-terminus and a PCNA binding motif (QTKV-EFP) at the C-terminus of the proteins, respectively, and both proteins interact with PCNA and stimulate pol δ activity in a PCNA-dependent way [2].

Both human PDIP1 and TNFAIP1 are induced by tumor necrosis factor-alpha (TNF-α) [1,3]. TNF-α is a multifunctional cytokine which is involved in a variety of biological activities, such as apoptosis, proliferation, B cell activation, and some inflammatory responses [4]. One of TNF-α's functions is triggering hepatocyte proliferation during liver regeneration [5,6]. It has been shown that the activation of hepatocyte proliferation by TNF-α is mediated through an IL-6-dependent pathway, leading to induction of the transcription factor Stat3 and ultimately to DNA synthesis [7]. Giving the evidence that PDIP1 is inducible by TNF-α and IL-6, stimulates pol δ activity, it is quite possible that PDIP1 may play roles in liver regeneration by linking the TNF-α signal and DNA synthesis. Recently, Link et al. [8] engineered transgenic *C. elegans* Alzheimer's disease (AD) model to express the human amyloid peptide (Aβ). In this transgenic *C. elegans* model, expression of *TNFAIP1* gene in AD brain was found to be robustly induced, and the brain region with the least pathology had the highest expression of *TNFAIP1*, suggesting that the *TNFAIP1* plays protective roles during the process of developing AD [8]. These studies indicate that PDIP1 and related proteins play critical roles in both developmental and disease processes.

Till now, it has not been reported how the expression of *PDIP1* and related genes are regulated. As a first step to understand that, we cloned the cDNA and promoter of mouse *PDIP1* gene. We identified that transcription factors Sp1 and AP-2 are critical factors to regulate the basal transcription of mouse *PDIP1* gene.

2. Materials and methods

2.1. Materials

SMART™ rapid amplification of cDNA ends (RACE) cDNA amplification kit, pCMVβ, pTAL-Luc were purchased from Clontech (CA, USA). Luciferase assay system and primer extension kit were from Promega (WI, USA). CycleReader™ DNA sequencing kit was from MBI Fermentas (Lithuania). All restriction endonucleases and ligase were from TaKaRa (Dalian, China). DMEM was from Invitrogen/Gibco (CA, USA). Glutathione Sepharose beads and pGEX-4T-2

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Abbreviations: PDIP1, polymerase delta-interacting protein 1; PCNA, proliferating cell nuclear antigen; RACE, rapid amplification of cDNA ends; TSS, transcription start site; AP-2, activation protein-2; Sp1, specificity protein 1; EMSA, electrophoresis mobility shift assay

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were from Amersham Pharmacia (Freiburg, Germany), Sofast™ cationic polymer transfection Reagent was from Sunma Biotech Co (Xiamen, China). Sensicript™ reverse transcriptase and QIAquick™ nucleotide removal Kit was from Qiagen (Germany). All other reagents were of analytical grade.

2.2. Rapid amplification cDNA ends

The 5′-RACE and 3′-RACE were performed using SMART RACE cDNA amplification kit according to the manufacturer's instruction. In brief, 1 μg of total RNA extracted from mouse liver tissue was reverse-transcribed with oligo (dT)-anchor primer, and with oligo (dT) primer and 5′-anchor oligonucleotide provided in the kit for 3′- and 5′-RACE, respectively. Then, 5′-RACE and 3′-RACE were performed with universal primer provided in the kit and gene-specific primer (GSP) (GSP for 5′-RACE: 5′-CTGGCACTCTCAGGCAGTGGCAC-3′, GSP for 3′-RACE: 5′-GACGATGAAGAGAACCGAGAGCACC-3′). To confirm the 5′- and 3′-RACE products, nested PCR was performed with nested universal primer provided in the kit and nested gene-specific primer (NGSP) (NGSP for 5′-RACE: 5′-CCCGCGTCTGTGAGCACTTCAAC-3′, NGSP for 3′-RACE: 5′-GCCATATCACCCACGATGAGCGTC-3′). The final products were cloned into pMD18-T vector and sequenced.

2.3. Primer extension

Primer extension assay was performed using primer extension kit according to the manufacturer's instruction. Briefly, an anti-sense primer (5′-CCACTCTCCACAGCTACTCCGC-3′) corresponding to the position from +33 to +54 was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled primer was incubated with 10 μg of total RNA from NIH3T3 or mouse liver for 30 min at 62 °C and then extended with AMV reverse transcriptase for 40 min at 42 °C. Simultaneously, sequencing reaction with the same primer as primer extension was performed using CycleReader™ DNA Sequencing kit. The primer extension product was analyzed on a sequencing gel next to the sequencing reaction product.

2.4. Cloning of the putative PDIP1 promoter and construction of deletion and mutant reporter plasmids

By searching mouse genome database using the cDNA sequence of *PDIP1* gene, we identified a mouse genomic chromosome 7 clone (NT_039435.2) comprising the whole *PDIP1* gene including the putative promoter. A 1185 bp of genomic region (−1071 to +114) upstream of the translation start codon ATG was amplified from mouse genomic DNA using primer pairs with *Kpn*I site (underlined) at the 5′ end of the forward primers and *Hin*dIII site at the 5′ end of reverse primers (Forward: 5′-GGGTACCGGTGACAGAGTTGGAGAAG-3′, Reverse: 5′-CAAGCTTGTCCGGTTCCCTGAGATC-3′). The amplified fragment was inserted upstream of the promoterless firefly luciferase gene in the *Kpn*I/*Hin*dIII sites of pTAL-Luc vector, denoted p(−1071/+114)-luc.

A series of unidirectional 5′-deletion constructs were generated through different restriction enzyme digestion and self-ligation of p(−1071/+114)-luc: p(−787/+114)-luc (*Pvu*II), p(−663/+114)-luc (*Sac*I), p(−293/+114)-luc (*Apa*LI), p(−162/+114)-luc (*Bam*HI), p(+6/+114)-luc (*Bss*HII). Site-directed mutagenesis of the p(−162/+114)-luc construct was performed by overlapping extension PCR [9]. Briefly, in the first round, two PCR reactions in parallel were performed using p(−162/+114)-luc construct as template: one with a wild-type sense primer corresponding to vector sequence upstream of insert (5′-CAAGTGCAGGTGCCAGAACAT-3′) and a mutated antisense primer, the other with a mutated sense primer (complementary to the mutated antisense primer) and a wild-type antisense primer corresponding to vector sequence downstream of insert (5′-GTCTTCCATG-GTGGCTTTACC-3′). The mixture of the above two PCR products was used as template for further PCR reaction using the wild-type sense primer and the wild-type antisense primer. The final PCR product was subcloned into upstream of the promoterless firefly luciferase gene at *Kpn*I/*Hin*dIII sites of pTAL-Luc vector. The mutated sense and antisense primers sequences used in site-directed mutagenesis are given in Table 1. The promoterless luciferase control reporter vector pLuc-control was constructed through deleting the TATA-like promoter by *Bgl*II/*Hin*dIII digestion and self-ligation of pTAL-Luc. All the above constructs were confirmed by DNA sequencing.

Table 1
Oligonucleotides used for promoter mutagenesis and electrophoretic mobility shift assay (EMSA)

Name	Sequence (5′–3′)	Purpose
WtAP-2(U)	CCCCAACCCGCAGGCTGGACT	EMSA
MtAP-2(U)	CCCCAACttGCAGaCTGGACT	Mutagenesis/EMSA
WtAP-2(D)	GACGCGGCCCTCGGCCTGGCC	EMSA
MtAP-2(D)	GACGCGGttCTCGaCCTGGCC	Mutagenesis/EMSA
WtSp1(U)	TCTTGGCTCCGCCCCCGAGACG	EMSA
MtSp1(U)	TCTTGGCTCCGatCCCGAGACG	Mutagenesis/EMSA
WtSp1(D)	GCGATGGGGCGGCTGGTGGACG	EMSA
MtSp1(D)	GCGATGGtaCGGCTGGTGGACG	Mutagenesis/EMSA
HMT	GATCGAACGGACCGCCCGCGGCCCGT	EMSA
SV40	ATTCGATCGGGGCGGGGCGAGC	EMSA

Note: Only the sense strands are shown.
Putative binding sites were underlined.
The lowercase letters denote mutated nucleotides.

2.5. Computer analysis

The promoter region was predicted by PromoterInspector program (www.genomatix.de) and Proscan (www-bimas.cit.nih.gov). Transcription factor binding sites were predicted by PromoterInspector program and MatInspector program (www.genomatix.de), which uses TRANSFAC matrices (core similarity 1.0 and matrix similarity 0.9). The presence of CpG islands was analyzed with the EMBOSS program CpGplot (www.ebi.ac.uk). According the program, a CpG island is defined as stretches of DNA in which the average percentage of G plus C nucleotides is over 50%, the calculated observed/expected CpG ratio is over 0.6 and the conditions hold for a minimum of 200 bases.

2.6. Cell culture, transient transfection and luciferase reporter assays

NIH3T3 or HepG2 cells were grown in DMEM supplemented with 10% newborn calf serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 incubator. Cells were seeded in 35 mm dish and transfected at 70% confluence using Sofast™ Cationic Polymer Transfection Reagent according to the manufacturer's manual. Briefly, 1.5 μg of luciferase reporter plasmid were co-transfected the cells with 0.5 μg of β-galactosidase expression vector pCMVβ which was used as an internal control to normalize the transfection efficiency. At 36 h after transfection, the cells were lysed to measure the luciferase activity using the Luciferase Assay System. The luciferase activities were normalized according to β-galactosidase activities.

2.7. Plasmids construction and expression of mouse Sp1 and AP-2α

The coding sequence of mouse Sp1 was amplified from mouse spleen cDNA using primer pair with *Eco*RI site (underlined) at the 5′ end of the forward primers and *Sal*I site at the 5′ end of reverse primers (Forward: 5′-AGAATTCCTGCCACCATGAGCGACCA-3′, Reverse: 5′-CGTCGACCTAATCTTAGAAACCATTGCCACTG-3′), cloned into pCMV-HA and pGEX-4T-2 at *Eco*RI/*Sal*I, respectively. The coding sequence of mouse AP-2α was amplified from mouse brain cDNA using primer pair (Forward: 5′-GGAATTCAAATGCTTTGGAAACTGACG-3′, Reverse: 5′-GCTCGAGGAGAGCCTCACTTTCTGTG-3′), cloned into pCMV-HA and pGEX-4T-2 at *Eco*RI/*Xho*I sites, respectively. The resulting constructs were sequenced to confirm the orientation and sequence. The pCMV-Sp1 and pCMV-AP2α were used to express Sp1 and AP-2α in mammalian cells. The pGEX-4T-2-Sp1 and pGEX-4T-2-AP2α were transformed into *Escherichia coli* BL21, respectively, and GST-fusion proteins were expressed with IPTG induction and purified by Glutathione Sepharose beads.

2.8. Electrophoretic gel mobility shift assays

The oligonucleotides containing wild-type or mutated putative transcription factor binding site were synthesized (Table 1). The forward oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified using the QIAquick Nucleotide Removal Kit.

The purified labeled forward oligonucleotide and unlabeled reverse oligonucleotide were combined and heated to 95 °C for 10 min and then cooled slowly to room temperature. Binding reactions (15 μl) contained 5 pmol labeled probe, 10 mM HEPES (pH 8.0), 60 mM KCl, 4 mM MgCl2, 0.1 mM EDTA, 100 μg/ml BSA, 0.25 mM DTT, 10% glycerol and 50 pmol purified recombinant protein, were incubated for 20 min at 37 °C, and then resolved on 6% non-denaturation polyacrylamide gels. Gels were dried, exposed to Kodak X-ray film at −80 °C, and signals were detected by autoradiography. Competition reactions were performed by preincubating the recombinant protein with 10- or 50-fold molar excess of unlabeled competitor DNA prior to addition of the radioactive probe. The wild-type consensus sequences of AP-2 binding site at human metallothionein IIa promoter and Sp1 binding site at SV40 early promoter [10] were synthesized as competitor DNA (Table 1).

3. Results

3.1. Cloning of mouse PDIP1 cDNA

Through searching human *PDIP1* cDNA sequence against mouse EST database, we found two overlapping EST sequences (GenBank Accession Nos: AI849007 and AA546545), which shared significant homology with human *PDIP1* and could be assembled into a contig of 1006 bp. To confirm the sequence of the contig, PCR was performed using liver cDNA and a pair of primers (Forward: 5′-GTTGA-AGTGCTCACAGACGC-3′, Reverse: 5′-AGGTCAGTCCT-TGAAGACAATC-3′) corresponding to the 5′-end sequence of AI849007 and 3′-end sequence of AA546545, respectively. The amplification product was cloned into pMD18-T vector and sequenced. Based on the sequence of the contig, we designed 5′- and 3′-RACE primers to extend 5′- and 3′-end sequences by RACE-PCR. Then, the 5′-flanking sequence, 3′-flanking sequence and the contig sequence were assembled into the full-length cDNA of mouse *PDIP1*.

The full length cDNA contains an open reading frame encoding 329 amino acids. Amino acid alignment revealed 98.8% identity between mouse *PDIP1* and rat *PDIP1*, 91.8% identity between mouse *PDIP1* and human *PDIP1*. The sequence data of mouse *PDIP1* are deposited in GenBank with Accession No: AF534881.

3.2. Genomic structure and transcription start site of mouse PDIP1

To determine the genomic structure of mouse *PDIP1*, the mouse genome database was searched with mouse *PDIP1* cDNA. BLAST analysis showed that mouse *PDIP1* is mapped to chromosome 7F3 region, spans approximately 16.7 kb, and is organized into six exons. The first exon contains 5′-untranslated region and translation initiation site. The exon 2 and exon 3 are separated by the largest intron (>10 kb). Exon 1–6 encode the open reading frame, exon 6 also encodes the 3′-untranslated long sequence of mouse *PDIP1* (Fig. 1). The polyadenylation signals (AATAAA) was identified in the untranslated region of the last exon. All exon–intron boundaries were defined to conform to the GT/AG splice donor/receptor rule [11] (Table 2). The mouse, rat and human *PDIP1* genes are of similar size, have exons of the same length, and possess the same splice junction sites (Fig. 1).

Primer extension assay was performed to determine the transcription start site (TSS). An anti-sense primer located in 1st exon was end-labeled with [γ-^{32}P]ATP, extended with AMV reverse transcriptase and total RNA from NIH3T3 or mouse liver. A 54-bp band was detected in both primer extension products using RNA of NIH3T3 or liver. Simultaneously, sequencing reaction with the same primer as primer extension determined that the TSS is G, corresponding to position of 162-bp upstream of the translation start codon (Fig. 2).

3.3. Identification of transcriptional regulatory region of mouse PDIP1 gene

A 1185-bp of genomic region upstream of the translation start codon ATG was amplified and inserted into the promoterless luciferase reporter gene, denoted p(−1071/+114)-luc. Through different restriction enzyme digestion and self-ligation of p(−1071/+114)-luc, a series of unidirectional

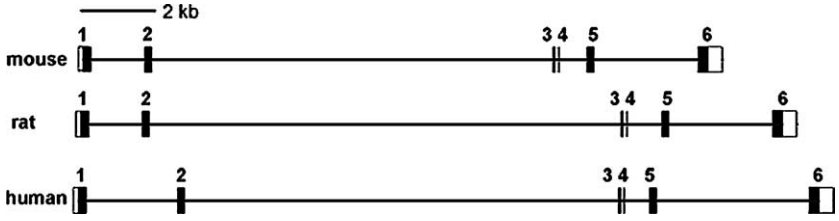

Fig. 1. The genomic organization of mouse, rat and human *PDIP1* genes. Exons are indicated by boxes. Solid boxes indicate coding region, open boxes represent the 5′- and 3′-untranslated regions.

Table 2
Exon–intron boundaries of the mouse *PDIP1* gene

Exon number	Exon size (bp)	Splice acceptor site	Splice donor site	Intron size (kb)
1	406		GACGCGGGAG**gt**atgcgtgc	1.334
2	170	accccacc**ag**GTTGGGTGCT	GGCGCTGCAG**gt**gagagccc	10.530
3	90	tcctctcc**ag**CAAAAAAGGG	CACCTCCAAG**gt**gaggccct	0.088
4	53	ctccctac**ag**CCTGTGGTGA	CTTACACCAG**gt**gagcgggg	0.583
5	196	tggttctc**ag**CACTTCAGAT	GCAGACCAAG**gt**cagagggg	2.542
6	747	cctttgc**ag**GTGGAATTCC		

Note: The exonic and intronic sequences are upper and lower case, respectively.
Each intron begins with a GT and ends with an AG.

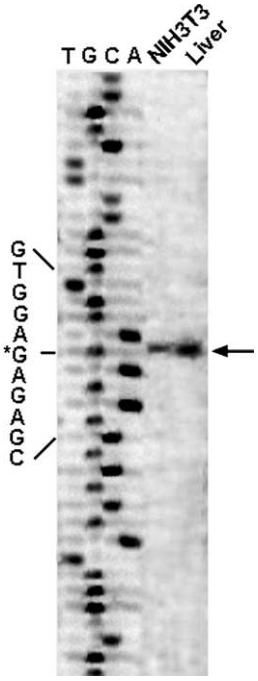

Fig. 2. Mapping of the transcription start site of the mouse *PDIP1* gene. ^{32}P-labeled primer was annealed to the total RNA from NIH3T3 cells or liver tissue and extended with reverse transcriptase. The DNA sequence ladder was obtained using the same primer and separated on the same gel. The extended product is indicated by the arrow.

5′-deletion constructs were generated (Fig. 3A). All the above constructs were transfected, respectively, into NIH3T3 cells and their luciferase activities were determined. As shown in Fig. 3B, the longest construct p(−1071/+114)-luc displayed a higher promoter activity in NIH3T3 than that of the promoterless luciferase control plasmid, pLuc-control. Deletion extending to position −787 resulted in reduction of 22.3% of promoter activity. In contrast, deletion to −663 resulted in significant increase of promoter activity. These results suggested that positive regulatory site(s) existed in the region from −1071 to −787, and negative regulatory site(s) in the region from −787 to −663. Further deletion extending to −293 and −162 gradually decreased the promoter activity, but the promoter activities were still high: the promoter activities of constructs p(−293/+114)-luc and p(−162/+114)-luc were 121.0% and 118.7% of that of longest construct p(−1071/+114)-luc, respectively. However, deletion of the sequence from −162 to +6, the promoter activity dramatically decreased, only 8% of that of p(−1071/+114)-luc. All these results show that the isolated 1185 bp genomic fragment (−1071 to +114) of mouse *PDIP1* gene contains a functional promoter and that the core sequence of the basal promoter is localized in the region −162 to +114.

3.4. Identification of transcription factors potentially regulating mouse PDIP proximal promoter activity

To identify the putative cis-regulatory elements of the basal promoter region of mouse *PDIP1* gene, the 5′-flanking sequence upstream of translation initiation site was inspected by computer analysis. No TATA box or CCAAT box was found in the promoter region, instead, a CpG island was detected surrounding TSS from nucleotide −279 to +107, which spans 386 bp long with 65.3% of GC content. Similar to mouse gene, the 5′-flanking sequences of the rat and human *PDIP1* genes also lack TATA-box, possess CpG islands.

A search for transcription factors potentially regulating *PDIP1* gene expression was performed using the MatInspector program. We found two consensus binding sites for Sp1 (−68 to −63, and +15 to +20), and two consensus binding sites for AP-2 (−123 to −115 and −52 to −44) in the basal promoter region (Fig. 3A).

Through alignment of the 5′-flanking sequences-positions −171 to +70 (relative to mouse TSS, i.e. −333 to −93 relative to mouse translation initiation site) of mouse *PDIP1*, rat *PDIP1* and human *PDIP1* promoters, we found that the two Sp1 sites are perfectly conserved regarding the sequences and relative positions (Fig. 3C). However, though we could identify two AP-2 binding sites in mouse and human promoters in this region, their relative position are not highly conserved. The two AP-2 binding sites in mouse promoter are located at positions −285 to −277 and −214 to −206 relative mouse translation initiation site, while those potential sites in human promoter are located at positions −299 to −291 and −91 to −83 relative to human translation initiation site. We could not identify the AP-2 binding sites in this region of rat promoter. By further looking upstream sequences of rat promoter, we could identify two potential AP-2 binding sites at positions −579 to −571 and −544 to −536 relative to rat translation initiation site. These results indicate that the regulatory mechanisms for the basal transcription among the mouse, human and rat *PDIP1* genes may be conserved.

To further define the sequence elements recognized by Sp1 and AP-2, we conducted electrophoretic gel mobility shift assays (EMSA) using ^{32}P-labeled probes corresponding to the potential Sp1 and AP-2 binding motifs in mouse *PDIP1* promoter. As shown in Fig. 4A, both the wild-type oligonucleotides wtAP-2(U) (corresponding to sequence from −129 to −109) and wtAP-2(D) (corresponding to sequence from −58 to −38) produced retarded bands with purified recombinant AP-2-α protein, while the mutated oligonuleotides mtAP-2(U) and mtAP-2(D) did not produce retarded bands, suggesting that oligonucleotides wtAP-2(U) and wtAP-2(D) was capable of binding recombinant AP-2α. Specificity of the binding was confirmed by competition with excess wild-type consensus sequence (hMt) of AP-2 binding site at hMtIIa promoter. While 10-fold molar excess unlabeled oligonucleotides Mt-AP-2 were added, the DNA-protein complex disappeared.

The binding of recombinant Sp1 protein to the consensus Sp1 binding sequence in mouse *PDIP1* proximal promoter was also demonstrated by EMSA (Fig. 4B). The results showed that wild-type oligonucleotides wtSp1(U) and wtSp1(D) were capable of binding to recombinant Sp1 protein, but mutated oligonucleotides mtSp1(U) and mtSp1(D) were not.

3.5. Sp1(U) site upstream of TSS activates, whereas Sp1(D) site and two AP-2 sites suppresses PDIP1 transcription

To test the functional significance of Sp1 and AP-2 binding, various reporter plasmids containing site-specific mutation in consensus binding sites of Sp1 and AP-2 were transfected into NIH3T3 and their activities compared with reporter expressing the normal sequence p(−162 to +114)-luc. Mutation of the Sp1 binding site upstream of TSS resulted in a 43.56% loss of

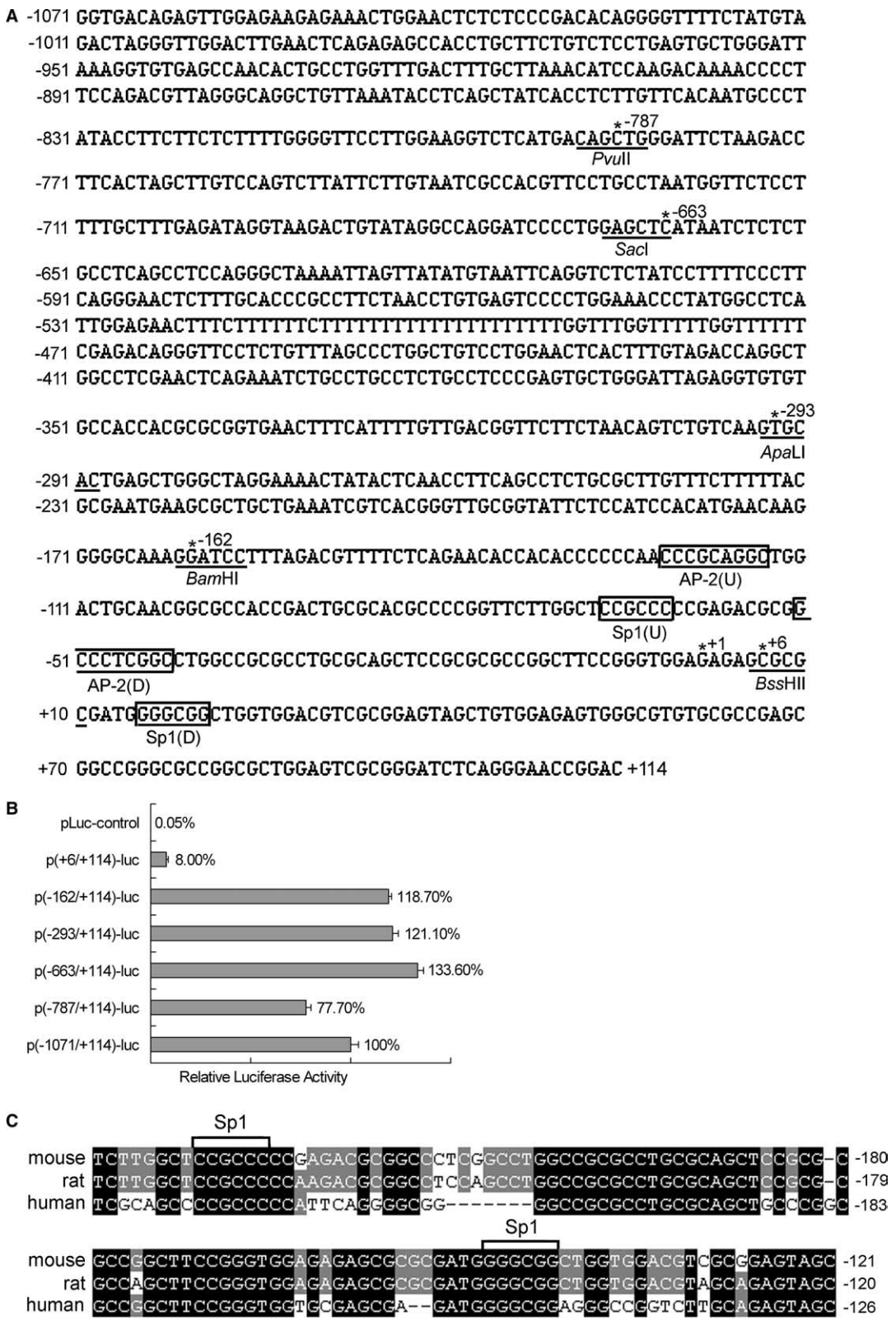

Fig. 3. (A) The sequence of 5′-flanking region (−1071 to +114) of mouse *PDIP1* gene. The sequences that match the consensus binding sites of known transcription factors were boxed while the restriction sites of enzymes used in deletion mutation were underlined. Numbers indicate the nucleotide position relative to the transcription start site. The transcription start site is G, denoted +1. (B) The plasmids containing sequentially deleted fragments of mouse *PDIP1* 5′-flanking region (−1071 to +114) were transfected into NIH3T3 cells. Luciferase activities were measures at 36 h post-transfection. Data (means ± S.D.) were represented as the percent activity relative to that observed in p(−1071/+114)-luc. (C) Alignment of the 5′-flanking sequences upstream of translation initiation sites of mouse *PDIP1*, rat *PDIP1* and human *PDIP1* genes. Numbers indicate the nucleotide position relative to the translation initiation site.

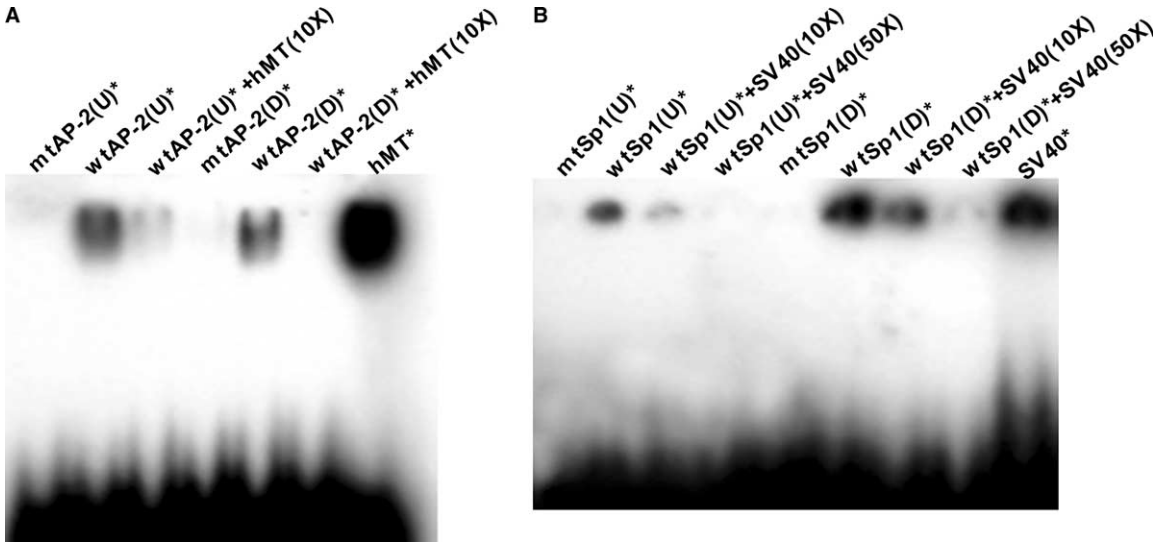

Fig. 4. Electrophoretic gel mobility shift assays. The fusion protein (A: GST-AP-2α, B: GST-Sp1) was incubated with a ^{32}P-labeled oligonucleotide (indicated by asterisk). The protein-DNA complexes were confirmed by competition with unlabeled AP-2 consensus sequence in human metallothionein IIa promoter (hMT) or unlabeled Sp1 consensus sequence in SV40 promoter (SV40).

reporter activity compared to wild-type promoter p(−162/+114)-luc, while mutation of the Sp1 binding site downstream of TSS increased promoter activity by 25.71%. Mutation of the two Sp1 sites decreased the luciferase activity by 32.73%. These results suggested that Sp1(U) site activates promoter activity, whereas Sp1(D) suppresses promoter activity (Fig. 5A).

Mutations of single or both AP-2 sites increased luciferase activities, suggesting that both AP-2 sites are negative regulation regions of mouse *PDIP1* promoter (Fig. 5A). To further confirm the function of AP-2 in transcriptional regulatory of mouse *PDIP1*, we co-transfected AP-2-deficient cells HepG2 with *PDIP1* promoter plasmid p(−162/+114)-luc and pCMV-AP-2 plasmid, the promoter activities decrease with the increasing of pCMV-AP-2 (Fig. 5B).

4. Discussion

In the report, we have cloned the full-length cDNA of mouse *PDIP1* gene, characterized its genomic structure and functional minimal promoter. The mouse, rat and human *PDIP1* genes are very similar in their overall genomic organization and promoter structure. The 5′-flanking regions of mouse, rat and human *PDIP1* genes lack TATA box or Inr element, while contain a conserved cluster of CpG dinucleotides, two Sp1 binding sites, and two AP-2 binding sites in common, suggesting that mouse, rat and human *PDIP1* genes have common regulatory mechanisms. The GC-rich and TATA-less promoters have been found predominantly in ubiquitously expressed genes, and this structure is considered to be characteristic of housekeeping genes [12,13]. The human *PDIP1* was found to be ubiquitously expressed gene, but it could be up-regulated by TNF-α [1]. Actually, several recent results have demonstrated that GC-rich TATA-less promoters could be highly regulated [14,15]. The regulation of such promoter is poorly understood. It has been shown that GC-rich promoter regions typically lack TATA or DPE core promoter elements, but contain multiple GC box motifs that are bound by Sp1 and related transcription factors [16,17]. Although Sp1 is ubiquitously expressed, some evidence suggests that Sp1 expression [18], binding affinity [19] and post-transcriptional modifications [20] may be modulated, and Sp1 is involved in transcription of many cell-type specific genes, including the monocytic specific gene *CD14* [21], the liver specific α1 acid glycoprotein gene [22]. The presence of Sp1 binding sites in CpG islands is particularly important, and the degree of activation from Sp1 tends to be stronger in the context of TATA-less promoters than TATA-containing promoters [23]. Not only does Sp1 contribute to the maintenance of hypomethylated state of CpG islands [16,17], but it may also plays a critical role in the assembly of the transcription start complex [24]. Our study demonstrated the importance of Sp1 in regulating *PDIP1* gene expression for several reasons. First, two Sp1 binding sequences were identified in the proximal promoter region of mouse *PDIP1* gene. Second, EMSA results showed that Sp1 was capable of binding to both Sp1 sites. Third, point mutation introduced at the Sp1-binding sites resulted in significant change in promoter activity. Especially, site-mutation of Sp1(U) site significantly decreased promoter activity by 43.56%, indicating that the Sp1(U) site plays a critical role for basal promoter activity of the mouse *PDIP1* gene. However, the Sp1(D) site downstream of TSS negatively regulates the promoter activity.

In the long GC-rich region of *PDIP1* promoter, we also identified two AP-2 binding sequences. Gel shift analysis using a radiolabelled probe corresponding to the potential AP-2 binding motif revealed direct binding of purified AP-2α protein to the AP-2 binding motif in *PDIP1* promoter. AP-2 is a tissue-specific transcription factor which has been implicated as a critical regulator of gene expression during mammalian development, differentiation, and carcinogenesis [25]. Although AP-2 was initially described as a transcriptional activator, it has been shown to regulate negatively the transcription of several genes, including K3 keratin [26], *MX1* [15], *PAR-1* [27]. Our results also demonstrate that AP-2 functions as repressor in transcriptional regulation of mouse *PDIP1*.

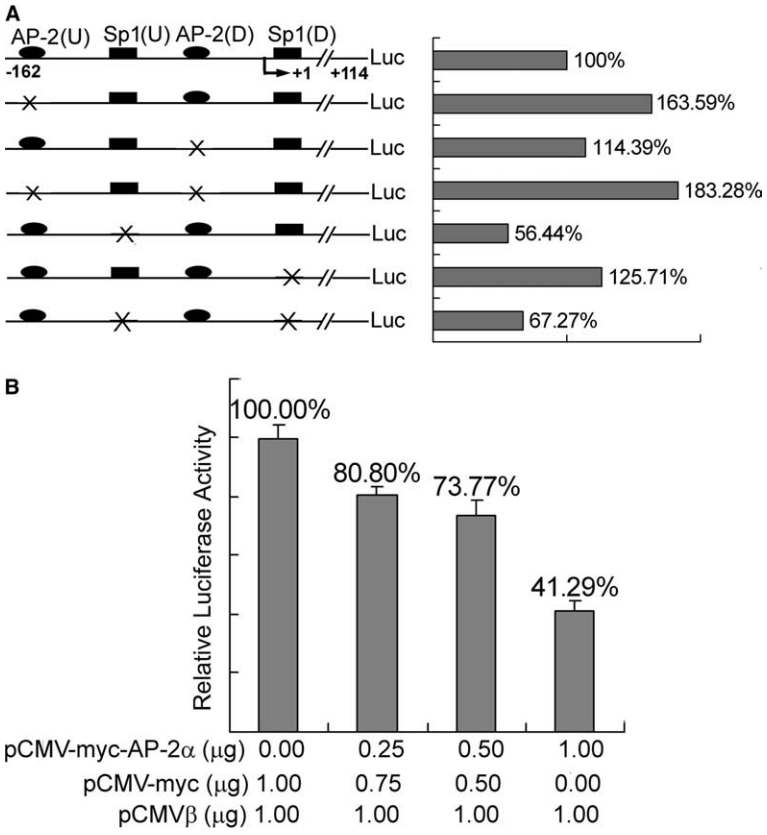

Fig. 5. Effect of Sp1 and AP-2 sites in proximal promoter to promoter activity of *PDIP1* gene. (A) Various site-mutations in Sp1 and AP-2 sites of *PDIP1* proximal promoter (−162 to +114 bp) were cloned into upstream of the promoterless firefly luciferase, then transfected the NIH3T3 cells. The binding sites of AP-2 and Sp1 were indicated by oval and box, respectively. The cross (X) indicates mutated site. (B) The wild-type *PDIP1* promoter plasmid p(−162/+114)-luc was co-transfected HepG2 with pCMV-AP-2α plasmid. Luciferase activities were measured at 36 h post-transfection. Data (means ± S.D.) were represented as the percent activity relative to that observed in wild-type proximal promoter.

Mutation of both AP-2 binding motifs increase promoter activity by 40.48% compared to wild-type promoter p(−162/+114)-luc, suggesting AP-2 suppresses promoter activity of mouse *PDIP1*. The function of AP-2 in transcription regulation was confirmed by co-transfection of AP-2 gene and *PDIP1* promoter, we co-transfected AP-2-deficient cell HepG2 with *PDIP1* promoter construct p(−162/+114)-luc and pCMV-AP-2α plasmid expressing AP-2α, the promoter activities decrease with the increasing of pCMV-AP-2α.

Our data demonstrate that transcription factors Sp1 and AP-2 are involved in transcription regulation of mouse *PDIP1*, a phenomenon commonly observed in mammalian TATA-less promoters [15,27–30], in which there are overlapping or adjacent Sp1 and AP-2 binding sites, with Sp1 activating and AP-2 repressing gene transcription. In rabbit corneal epithelial cells stimulated to differentiate, AP-2 is dramatically down-regulated, thus altering the Sp1:AP-2 ratio and favoring the activation of the K3 keratin gene by Sp1 in differential cells [26]. It may be that the relative ratio of Sp1 and AP-2 in a particular cell environment determines the activation and repression of mouse *PDIP1* gene. It has been shown that Sp1 is required for the activation of TNF-α-stimulated *ADAM17* [31] and *MCP-1* [32] gene. *PDIP1* is a TNF-α inducible protein [1], but it is unknown how the expression of *PDIP1* gene is regulated by TNF-α. We did not detect any significant change of *PDIP1* promoter activity in NIH3T3 cells after treatment with TNF-α (data not shown). We conclude that in the proximal promoter region we tested, there is no TNF-α responsible element. Obviously, further studies are needed to determine by what mechanism TNF-α induces the expression of *PDIP1*.

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