Regulation of the human protein C inhibitor gene expression in HepG2 cells: role of Sp1 and AP2

Tatsuya HAYASHI, Masanobu USUI, Junji NISHIOKA, Zu Xun ZHANG and Koji SUZUKI[1]

Department of Molecular Pathobiology, Mie University School of Medicine, Tsu-city, Mie 514, Japan

Protein C inhibitor (PCI) is the plasma inhibitor of activated protein C, which is the main protease of the anticoagulant protein C pathway. In this study the transcriptional regulation of human PCI gene in the human hepatoma cell line, HepG2, was characterized by evaluating the transient expression of a luciferase reporter gene. The 5′ flanking region (residues −1587 to +2) of the PCI gene showed an adequate transcriptional activity, the maximum transcriptional activity being in a region between residues −452 and −94, which contains an Sp1-binding site, two AP2-binding sites and an inverted AP2-binding site. Transient expression assays with various deletion mutants and site-directed mutants showed that the Sp1-binding site (residues −302 to −294) has a potent promoter activity and that the upstream AP2-binding site (residues −350 to −343) has a potent enhancer activity; no activity was detected in the inverted (residues −413 to −404) and downstream (residues −136 to −127) AP2-binding sites. In addition, a region of the PCI gene (residues −452 to −414) containing the STATx-binding site, the A-activator (AA)-binding site, and the interferon α (IFN-α) response element, and another region of the PCI gene (residues −176 to −147) containing the GATA-1 and the IFN-γ response element showed potent silencer activities. Gel mobility-shift assays with various DNA fragments indicated that the Sp1-binding site, the upstream AP2-binding site, the AA-binding site and the IFN-γ response element interact with nuclear protein(s) of HepG2 cells. These findings suggest that the Sp1-binding site is the promoter, the AP2-binding site (residues −350 to −343) the enhancer, and both the AA-binding site and the IFN-γ response element are the silencers of human PCI gene expression in HepG2 cells.

INTRODUCTION

Protein C inhibitor (PCI), a multifunctional serine protease inhibitor, was initially isolated from human plasma as an inhibitor of activated protein C, which is the main active protease of the anticoagulant protein C pathway [1]. Subsequent studies showed that PCI also inhibits other serine proteases implicated in the blood coagulation system, such as thrombin [2], factor Xa [2], factor XIa [3], kallikreins [3], the thrombin–thrombomodulin complex [4] and proteases involved in the fertilization process such as the prostate-specific antigen [5] and the sperm protease acrosin [6]. The main source of plasma PCI is thought to be the liver; its plasma level is markedly decreased in patients with liver disease [7], and cells of the human hepatoma-derived cell line HepG2 were found to secrete significant amounts of PCI *in vitro* [8]. However, PCI mRNA was also detected in kidneys, prostate, testis, seminal vesicles and epididymal glands [9].

We previously reported that the 2.1 kb cDNA of the human PCI contains a 5′ non-coding region, a 19-residue signal peptide, a 387-residue mature protein, a stop codon and a long 3′ non-coding region of 839 bp [10]. Subsequent studies reported by Meijers and Chung [11] showed that the human PCI gene is composed of five exons and four introns and that its 5′ flanking region contains several *cis* elements (an Sp1-binding site, an AP2-binding site and an inverted AP2-binding site) but not the TATA box and CCAAT box sequences. We then confirmed this exon–intron organization and found that a 1.6 kb fragment of the 5′ flanking region of the PCI gene has two additional AP2-binding sites and expresses a significant promoter activity in HepG2 cells [12].

The gene organization of most plasma proteins of the serine protease inhibitor family has been reported [13–20] but the mechanism of their gene expression is not fully understood. A few studies have been reported regarding the proteins of the serine protease inhibitor family. For example, interferon γ (IFN-γ) was reported to enhance the expression of the C1 inhibitor gene [21], and the NFκB-binding site was found to be involved in the lipopolysaccharide-induced expression of the α_1-antitrypsin gene [22]. Recently it has been reported that constitutive expression of the human antithrombin III gene is regulated by flanking exon I [23] and the expression of plasminogen activator inhibitor I gene by transforming growth factor β [24]. In the present study we have examined the *cis* elements of the human PCI gene required for its constitutive expression in HepG2 cells by the luciferase transient expression assay. We also evaluated the binding of *trans* elements to the promoter, enhancer and silencer regions of the PCI gene by gel mobility-shift assay.

MATERIALS AND METHODS

Materials

Restriction endonucleases were purchased from Toyobo (Osaka, Japan). *Taq* polymerase, T4 polynucleotide kinase and T4 DNA ligase were from Boehringer Mannheim (Mannheim, Germany). Klenow fragment, a PicaGene basic vector, PGV-B, containing a luciferase gene and a luciferase assay system were from Nihon Gene (Tokyo, Japan). A site-directed mutagenesis kit, Transformer, was from Clontech Laboratories (Palo Alto, CA, U.S.A.). Plasmid pCH110, an expression vector of β-galactosidase in mammalian cells, was from Pharmacia Biotech (Uppsala, Sweden). Plasmid Bluescript SKII(+) and SKII(−), exonuclease III and mung bean nuclease were from Stratagene (La Jolla, CA, U.S.A.). Radionucleotides ([α-^{32}P]dCTP and [γ-^{32}P]ATP) were from Amersham (Little Chalfont, Bucks., U.K.). A 7-deaza sequenase sequencing kit and T7 DNA polymerase were from United States Biochemical (Cleveland, OH, U.S.A.). Rabbit

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antiserum against residues 520–538 of human Sp1 and double-stranded oligonucleotides containing the Sp1-binding site (5′-ATTCGATCGGGGCGGGGCGAG-3′), a mutant Sp1-binding site (5′-ATTCGATCGG$\underline{TT}$CGGGGCGAG-3′), the AP2-binding site (5′-GATCGAACTGACCGCCCGCGGCCCGT-3′), and a mutant AP2-binding site (5′-GATCGAACTGACCGC-$\underline{TT}$GCGGCCCGT-3′) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other chemicals and reagents used were of the highest grade commercially available.

Preparation of plasmids for deletion mutants

A 1.6 kb SalI–BglII fragment containing the 5′ flanking region of human PCI gene and a transcription start site was prepared as described previously [12]. To prepare deletion constructs, the SalI–BglII fragment in pBluescript SKII(+) was used as a template. The plasmid was digested with SalI and filled with deoxythio derivates (thio-dNTP) using the Klenow fragment, then this plasmid was digested by BamHI and deleted using exonuclease III and mung bean nuclease. After self-ligation with T4 DNA ligase, each plasmid was transformed into Escherichia coli. XLI-Blue. The KpnI and BglII sites in PGV-B vector containing a luciferase reporter gene were used for the construction of various deletion mutants of the PCI gene. The PGV-B plasmids containing various segments of the 5′ flanking region of the PCI gene were purified by CsCl ultracentrifugation [25]. The nucleotide sequence of each DNA fragment was determined by the dideoxy chain termination method [26]. Plasmids designated pPCI-1587, pPCI-1401, pPCI-1201, pPCI-980, pPCI-789, pPCI-577, pPCI-452, pPCI-329, pPCI-208, pPCI-93 and pPCI-39, shown in Figure 2, contained the nucleotide sequence residues -1587 to $+2$, -1401 to $+2$, -1201 to $+2$, -980 to $+2$, -789 to $+2$, -577 to $+2$, -452 to $+2$, -329 to $+2$, -208 to $+2$, -93 to $+2$ and -39 to $+2$ of the 5′ flanking region of the PCI gene, including a transcription initiation site in PGV-B, respectively.

As pPCI-452 showed the maximum promoter activity (see Figure 2), cis elements located in a region between residues -452 and $+2$ were characterized. Several DNA fragments containing the Sp1-binding site, the AP2-binding site and/or the inverted AP2-binding site were prepared by PCR [27]. A fragment (residues -452 to -209) containing the inverted AP2-binding site (residues -413 to -404), the upstream AP2-binding site (residues -350 to -343) and the Sp1-binding site (residues -302 to -294) of the PCI gene was prepared with two primers (5′-CC$\underline{GGTACC}$TGCCATTCCAGGAAAATGAT-3′ and 5′-CC$\underline{GGTACC}$GGGCTCAGAAAGGGCAGAGG-3′; the inserted KpnI site is underlined). A fragment (residues -452 to -330) containing the inverted AP2-binding site and the upstream AP2-binding site of the PCI gene was prepared with two primers (5′-CC$\underline{GGTACC}$TGCCATTCCAGGAAAATGAT-3′ and 5′-CC$\underline{GGTACC}$TTTCCGGCCAGCAGGCCTGC-3′; the inserted KpnI site is underlined). A fragment (residues -329 to -209) containing the Sp1-binding site of the PCI gene was prepared with two primers (5′-CC$\underline{GGTACC}$CCTGCCAGGA-AAGGAAGGGG-3′ and 5′-CC$\underline{GGTACC}$GGGCTCAGAAA-GGGCAGAGG-3′; the inserted KpnI site is underlined). After PCR amplification and sequence analysis, the fragments were inserted into the KpnI site of pPCI-93. To prepare a fragment containing the inverted AP2-binding site and both the upstream and the downstream AP2-binding sites (residues -136 to -127), the fragment (residues -452 to -330) containing the inverted AP2-binding site and the upstream AP2-binding site was inserted into the KpnI site of pPCI-208, which contains the downstream AP2-binding site.

Mutant fragments with various deletions in the region between residues -203 and -94 containing the downstream AP2-binding site were also prepared by PCR with two primers [5′-CC$\underline{GGTACC}$TGCCATTCCAGGAAAATGAT-3′ (the inserted KpnI site is underlined) and 5′-GGC$\underline{TCTAGA}$ATGCCCTCA-AGCCCTGGGGA-3′, 5′-GGC$\underline{TCTAGA}$CGGCATCGGTAC-TTACTGTG-3′, 5′-GGC$\underline{TCTAGA}$GAGTGTGCACACAGT-GGAGG-3′ or 5′-GGC$\underline{TCTAGA}$GGGCTCAGGAAGGGCA-GAGG-3′ (the inserted XbaI site is underlined) and pPCI-452 as a template. After PCR amplification and sequence analysis, the fragments were inserted into the KpnI and XbaI sites of the pPCI-93 plasmid. No promoter or enhancer sequence was produced by these insertions as shown by homology search analysis with DNASIS (TaKaRa Shuzo, Kyoto, Japan). In plasmids originating from the pPCI-452 and designated pPCI-452 (del$-116/-94$), pPCI-452(del$-146/-94$), pPCI-452(del$-176/-94$) and pPCI-452(del$-203/-94$), the residues -116 to -94, -146 to -94, -176 to -94 and -203 to -94 respectively were deleted (Figure 5).

Preparation of mutant plasmids

Several point mutants of the Sp1-binding site, the inverted AP2-binding site and the upstream or the downstream AP2-binding site were prepared with pPCI-452 as a template and oligonucleotides containing two point mutations, as follows: 5′-TGTCCCGG$\underline{TT}$CGGGGCCAGGG-3′ for the Sp1-binding site, 5′-GTGTAAGGCTT$\underline{TT}$GGGGCAGC-3′ for the inverted AP2-binding site, 5′-TCTGCAGGCA$\underline{TT}$CCTGCTGGC-3′ for the upstream AP2-binding site and 5′-GGGAGGTCC$\underline{TT}$AGGG-CTTGAG-3′ for the downstream AP2-binding site (underlining indicates mutated bases). Site-directed mutagenesis was performed with a Transformer site-directed mutagenesis kit in accordance with the manufacturer's instructions. The mutants inserted into PGV-B were purified by CsCl ultracentrifugation.

Cell culturing and DNA transfection

The HepG2 cells were grown in RPMI1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY, U.S.A.), 0.01% penicillin and 0.01% streptomycin (Sanko Junyaku, Tokyo, Japan) in an air/CO_2 (19:1) atmosphere at 37 °C, with tissue culture dishes 60 mm in diameter (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

Transfection of plasmid DNA into HepG2 cells was performed by the calcium phosphate precipitation method [28]. In brief, cultured cells were co-transfected with 3 μg of plasmid DNA and 1 μg of pCH110 as an internal control. The calcium phosphate/DNA precipitates were removed 4 h later, and the cells were treated with 15% (v/v) glycerol in Hepes/phosphate saline [25 mM Hepes/0.75 mM NaH_2PO_4/140 mM NaCl (pH 7.1)] (2 ml per dish) for 1 min. The cells were then washed with serum-containing RPMI1640 medium and cultured in the same fresh medium in an air/CO_2 (19:1) atmosphere at 37 °C for 48 h. The cells were then washed three times with PBS [10 mM sodium phosphate/140 mM NaCl (pH 7.5)] and incubated in 250 μl of cell lysis buffer [25 mM Tris/phosphate, pH 7.8, containing 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetra-acetic acid, 10% (v/v) glycerol and 1% (v/v) Triton X-100]. After incubation for 15 min at room temperature, the supernatant was collected after centrifugation at 20000 g for 10 min.
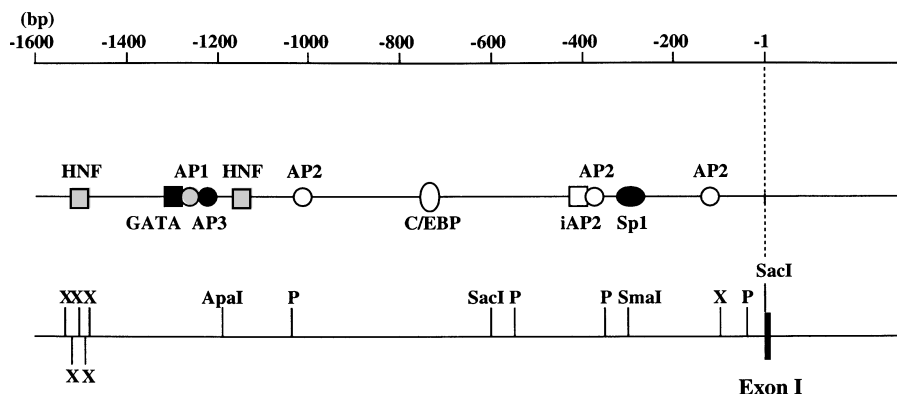
Figure 1   Restriction map and location of the binding site of transcription factors in the 5′ flanking region of the human PCI gene

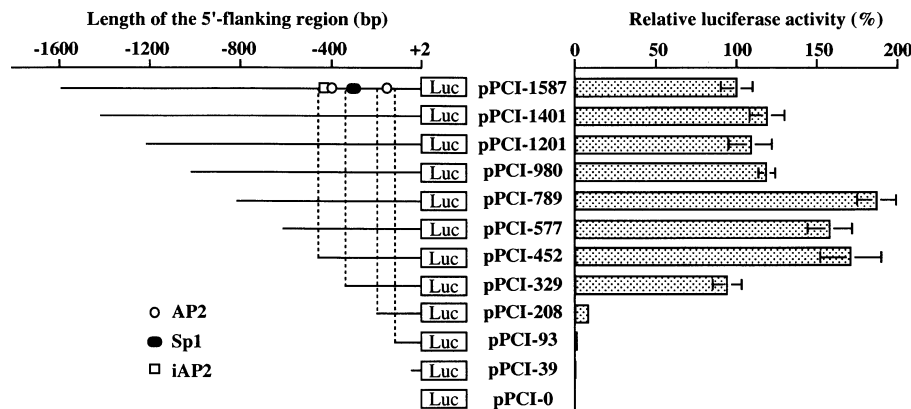Abbreviations: X, *Xba*I; P, *Pst*I.

Figure 2   Transcriptional activity of various deletion constructs of the 5′ flanking region of the human PCI gene in HepG2 cells

Deletion constructs of the 5′ flanking region of the PCI gene fused to the luciferase reporter gene (3 μg per dish) were co-transfected with pCH110 (1 μg per dish), containing the β-galactosidase gene, into HepG2 cells by using the calcium phosphate method. After 48 h of incubation the cells were harvested for assay of luciferase and β-galactosidase activities. Results are means ± S.D. for four independent transfections. Each plasmid construct contains the 5′ flanking region of the human PCI gene as demonstrated by the length (bp) of the gene that was fused to each PGV-B luciferase reporter gene. Abbreviation: Luc, luciferase gene.

Luciferase and β-galactosidase assays

The luciferase assay was performed in accordance with the manufacturer's instruction, with a Lumat LB 9501 instrument (Berthold, Wildbad, Germany). The β-galactosidase assay was performed as described previously [12]. The protein concentration of each extract was determined with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, U.S.A.), with BSA as standard. The luciferase activity obtained from cell extracts was normalized to β-galactosidase activity to correct for differences in transfection efficiency.

Preparation of nuclear extract

Nuclear extract was prepared from HepG2 cells by the method of Dignam et al. [29]. The nuclear extract was dialysed twice into a sterile Hepes buffer [20 mM Hepes, pH 7.9, containing 50 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% (v/v) glycerol and 0.5 mM PMSF] at 4 °C for 12 h. The extract was separated in aliquots, frozen in solid CO_2/ethanol and stored at −80 °C until use. Protein concentration was determined with a bicinchoninic acid protein assay kit with BSA as standard.

Gel mobility-shift assay

To determine DNA–nuclear protein complex formation, a gel mobility-shift assay was performed with several DNA fragments, as described [30]. The DNA fragment (residues −329 to −209) containing the Sp1-binding site was prepared by PCR as described above. A DNA fragment containing residues −452 to −414, and including residues −355 to −330 (this containing the upstream AP2-binding site) or residues −176 to −147, were prepared by annealing the two synthesized oligonucleotides (5′-TGCCATTCCAGGAAAATGATGGCCCAGCTGGTGGTGTAA-3′ and 5′-TTACACCACCAGCTGGGCCATCATTTTCCTGGAATGGCA-3′), (5′-CTGCAGGCAGGCCTGCTGGCCGGAAA-3′ and 5′-TTTCCGGCCAGCAGGCCTGCCTGCAG-3′) or (5′-AGCTACGGGACACAGTAAGTACCGATGCCG-3′ and 5′-CGGCATCGGTACTTACTGTGTCCCGTAGCT-3′) respectively. HepG2 cell extract was incubated in 20 μl (final volume) of the binding buffer [20 mM Hepes, pH 7.9, containing 50 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol and 10% (v/v) glycerol] containing approx. 0.2 ng of the radiolabelled DNA fragment and 2 μg of poly(dI-dC) (Pharmacia Biotech) as a non-specific competitor, at 4 °C for 1 h. After

incubation the samples were subjected to electrophoresis on a 4 % (w/v) polyacrylamide gel with 0.5 × TBE (TBE being 90 mM Tris/90 mM H_3BO_4/1 mM EDTA) at 4 °C, and the gel was then dried and autoradiographed with Kodak XAR film at −80 °C with an intensifying screen.

For competitive assays the radiolabelled DNA probe was mixed with various molar excesses of unlabelled DNA fragments or double-stranded synthetic oligonucleotides for 30 min before the addition of nuclear extracts. In gel mobility-shift assays with antibodies for nucleic factors, the nuclear extract was pre-incubated on ice with antibody or with normal rabbit IgG for 1 h before addition of the DNA probe.

RESULTS

Nucleotide sequence of the 5′ flanking region of human PCI gene

Figure 1 shows the restriction enzyme map and location of several putative binding sites of transcription factors in the 5′

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CTTTGGTCTT  GTCTCTCAGG  AGTGCCCATG  CCATTCCAGG  AAAATGATGG  CCCAGCTGGT  -421

GGTGTAAGGC TTGGGGGGCA GCGAGTGGGC  ATCGTGGTGA AAGCCTCGGG  ATCAGGGAGC  -361
        iAP2

TGCGTCTGCA GGCAGGCCTG CTGGCCGGAA ACCTGCCAGG AAAGGAAGGG GCTGTCCCGG  -301
          uAP2

GGCGGGGCCA GGGAGGGGTG GAGACAGGGC CGGCTGTGGT CAGTGACAAA TGCTGGCTGC  -241
Sp1

AATCCAGCCA GCCCTCTGCC CTTTCTGAGC CCGAGGGACT GCCACCTCCA CTGTGTGCAC  -181

ACTCAGCTAC GGGACACAGT AAGTACCGAT GCCGCAAAGG GAGGTCCCCA GGGCTTGAGG  -121
                                        dAP2

GCATGTGAGG CGAGGAGAGG ATGGACTCTA GAGTTTTGGG GTTTGGGGTC TGCAAAGCTC  -61

TGAAGGAGTC TCATCTCTGC AGTTTCAGGT ATCCAAGGCA GCAGAGGTGA GTGGGTCCCC  -1
      +1    Exon I
CGAGCTCTGT GACCTTATGC TCCACACTAA CTCTGGCAGA GCCTCCGTTT CCTCATAG   58
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Figure 3   Complete nucleotide sequence of the 452 bp 5′ flanking region of the human PCI gene

The transcription start site is marked by an arrow; the beginning of the transcription start site is numbered +1. Boxes are putative *trans*-acting factor-binding sites. Abbreviations: Sp1, Sp1-binding site; uAP2, upstream AP2-binding site; dAP2, downstream AP2-binding site; iAP2, inverted AP2-binding site.

flanking region of human PCI gene. A consensus sequence of the Sp1-binding site was present in residues −302 to −294, which is currently considered to be the promoter of the housekeeping gene. Sequences corresponding to CCAAT and TATAA were not found in this region. There were also several consensus sequences of the nuclear protein-binding site: three AP2-binding sites, an inverted AP2-binding site, an AP1-binding site, an AP3-binding site and a C/EBP-binding site. Moreover, two binding sites of HNF, a liver-specific factor, and a binding site of GATA-1, an erythroid specific factor, were also present in this region.

Transcriptional activity of the 5′ flanking region of human PCI gene

Figure 2 shows that a region in the pPCI-452 construct is required for the full expression of promoter activity of the PCI gene. Moreover, a region in the pPCI-329 construct seems to be necessary to express the basic promoter activity, because the promoter activity of pPCI-208 construct was markedly decreased. This suggests that the region containing residues −329 to −209 is the promoter of the PCI gene. The pPCI-789 construct expressed the maximum promoter activity, but this very high activity was not observed in the pPCI-980 construct (Figure 2). These findings suggest that the region between residues −980 and −790 functions as the silencer of PCI gene expression.

Because the pPCI-452 construct showed full promoter activity of the PCI gene, some binding sites of this region that bind to nuclear factors might have crucial roles in the regulation of the PCI gene expression in HepG2 cells. Figure 3 shows the nucleotide sequence between residue −452 and the initiation site of the 5′ flanking region of the PCI gene containing an Sp1-binding site, two AP2-binding sites and an inverted AP2-binding site. To elucidate the role of each *cis* element, several fragments containing the Sp1-binding site, the AP2-binding site and/or the inverted AP2-binding site were prepared, and the transcriptional activities of these constructs were determined by luciferase assay after transfection into HepG2 cells. Figure 4 shows that all constructs involving residues −329 to −209 (namely control, Sp1 plus dAP2, iAP2 plus uAP2 plus Sp1, and Sp1) that contain the Sp1-binding site have potent transcriptional activity. Four of the constructs without this region (namely dAP2, iAP2 plus
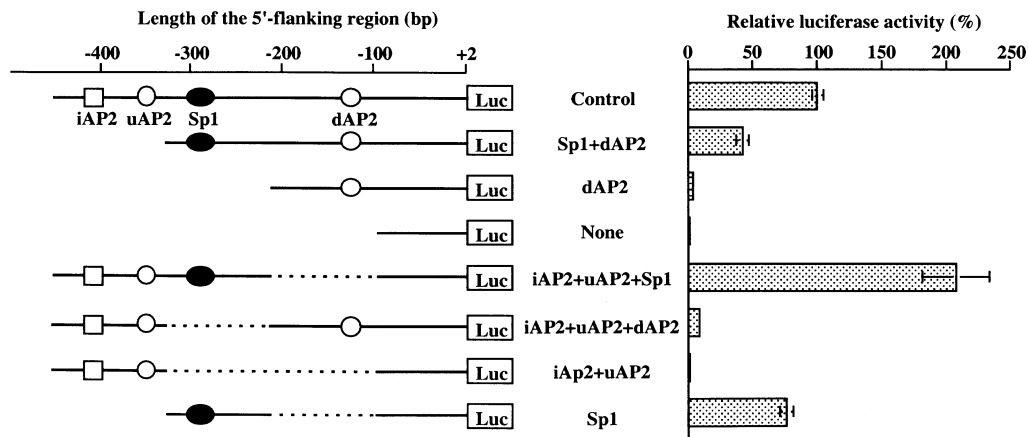

Figure 4   Identification of the regions functioning as promoter, enhancer or silencer in human PCI gene expression

Various deletion constructs of the 5′ flanking region of the human PCI gene (residues −452 to −94) fused to the luciferase reporter gene (3 μg per dish) were co-transfected with pCH110 (1 μg per dish), containing the β-galactosidase gene, into HepG2 cells, by using the calcium phosphate method. After 48 h of incubation the cells were harvested for assay of luciferase and β-galactosidase activities. Results are mean ± S.D. for four independent transfections. Abbreviations: Sp1, Sp1-binding site; uAP2, upstream AP2-binding site; dAP2, downstream AP2-binding site; iAP2, inverted AP2-binding site; Luc, luciferase gene. Dotted lines indicate regions deleted from the control construct.
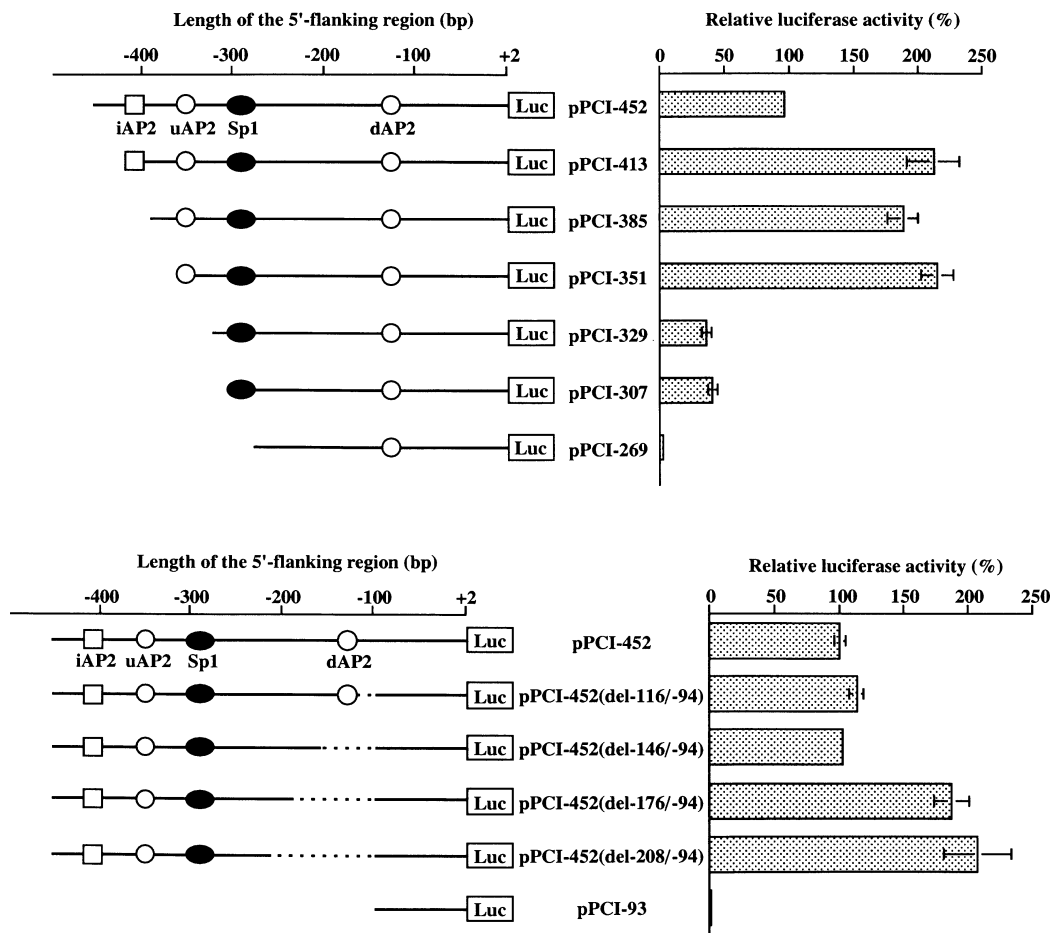
Length of the 5'-flanking region (bp)   **Relative luciferase activity (%)**

Length of the 5'-flanking region (bp)   **Relative luciferase activity (%)**

Figure 5   Promoter activity of various deletion constructs of the human PCI gene 5′ flanking region

Upper panel: various deletion constructs of the region containing the inverted AP2-binding site, the upstream AP2-binding site and the Sp1-binding site of the PCI gene 5′ flanking region (residues −452 to −269) fused to the luciferase reporter gene (3 µg per dish) were co-transfected with pCH110 (1 µg per dish), containing the β-galactosidase gene, into HepG2 cells, by using the calcium phosphate method. The construct name of each plasmid indicates the length (bp) of the 5′ flanking region of human PCI gene contained in each PGV-B luciferase reporter gene. Lower panel: various deletion constructs of the region containing the downstream AP2-binding site of the human PCI gene (residues −203 to −94) fused to the luciferase reporter gene (3 µg per dish) were co-transfected with pCH110 (1 µg per dish), containing the β-galactosidase gene, into HepG2 cells, by using the calcium phosphate method. Abbreviations: Sp1, Sp1-binding site; uAP2, upstream AP2-binding site; dAP2, downstream AP2-binding site; iAP2, inverted AP2-binding site; Luc, luciferase gene. Dotted lines indicate regions deleted from the pPCI-452.

uAP2 plus dAP2, iAP2 plus uAP2, and none) showed almost no transcriptional activity. The promoter activity of the region containing the Sp1-binding site depended on the direction of the sequence of this site; the activity of the fragment containing the reverse-oriented Sp1-binding site decreased to 30 % of that of the fragment containing the forward-oriented Sp1-binding site (results not shown). These findings indicate that the region containing the Sp1-binding site is the major promoter for the expression of the PCI gene in HepG2 cells.

Figure 4 also shows that the promoter activity of the fragment containing all four *cis* elements (control) was significantly higher than that of the fragment containing the Sp1-binding site and the downstream AP2-binding site (namely Sp1 plus dAP2). Moreover, the promoter activity of the fragment containing the inverted and upstream AP2-binding sites and the Sp1-binding site (namely iAP2 plus uAP2 plus Sp1) was significantly higher than that of the fragment containing the Sp1-binding site alone (namely Sp1). These findings strongly suggest that the region containing the inverted AP2-binding site and the upstream AP2-binding site is the enhancer of the promoter activity of the Sp1-

binding site. In contrast, the promoter activity of the fragment containing all *cis* elements (control) was significantly lower than that of the fragment containing the inverted and upstream AP2-binding sites and the Sp1-binding site (namely iAP2 plus uAP2 plus Sp1). In addition, the promoter activity of the fragment containing the Sp1-binding site and the downstream AP2-binding site (namely Sp1 plus dAP2) was lower than that of the fragment containing the Sp1-binding site alone (control). These findings suggest that the region containing the downstream AP2-binding site is the silencer of the promoter activity of the Sp1-binding site.

To characterize the role of these *cis* elements further, several deletion mutants were prepared and their promoter activities were determined. As illustrated in Figure 5 (upper panel), the promoter activities of fragments pPCI-413, pPCI-385 and pPCI-351 were approx. double that of pPCI-452, whereas the promoter activities of pPCI-329 and pPCI-307 were less than 50 % of the promoter activity of pPCI-452. These results indicate that the region containing residues −452 to −414 has a strong silencer activity and that the region containing residues −413 to −352 involving the inverted AP2-binding site has no enhancer activity.
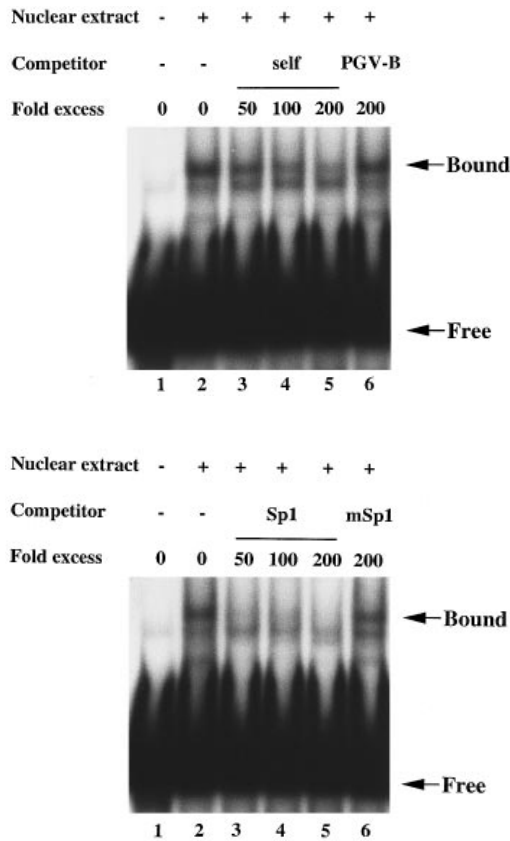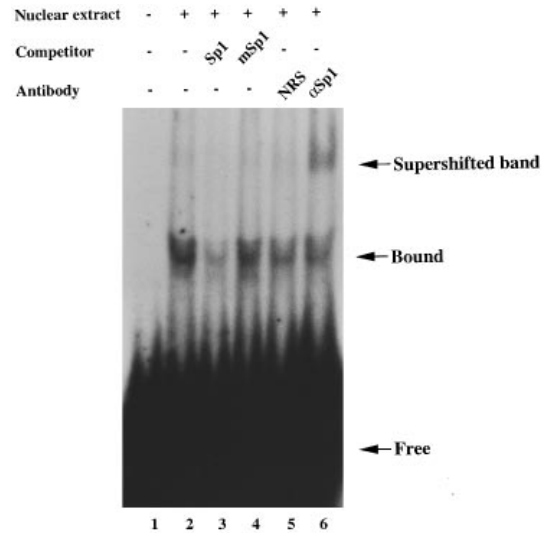
Figure 7   Gel mobility-shift assay performed with nuclear factors of HepG2 cells and a double-stranded oligonucleotide containing the consensus Sp1-binding site sequence

HepG2 nuclear extract was preincubated with an oligonucleotide containing the consensus Sp1-binding site sequence, a mutant Sp1-binding site sequence, with normal rabbit serum, or with anti-Sp1 antibody for 1 h on ice; the end-labelled Sp1 oligonucleotide probe was added to the incubation mixture, which was then analysed by gel mobility-shift assay. Lane 1, no extract; lane 2, nuclear extract; lane 3, nuclear extract and 100-fold excess of oligonucleotide with consensus sequence of Sp1-binding site (Sp1); lane 4, nuclear extract and 100-fold excess of oligonucleotide with mutant Sp1-binding site (mSp1); lane 5, nuclear extract preincubated with normal rabbit serum (NRS); lane 6, nuclear extract preincubated with anti-Sp1 antibody (αSp1).

Figure 6   Gel mobility-shift assay with nuclear extracts of HepG2 cells and a fragment containing the Sp1-binding site (residues −329 to −209) of the 5′ flanking region of the human PCI gene

Upper panel: competitive assay in the presence of a fragment containing the Sp1-binding site or the heterologous DNA fragment PGV-B. The end-labelled Sp1-binding site fragment was preincubated with a 50-fold, 100-fold or 200-fold molar excess of unlabelled fragment containing the Sp1-binding site or PGV-B, before the addition of 5 μg of nuclear extract of HepG2 cells. The DNA–protein complexes were subjected to electrophoresis on a 4% (w/v) gel, followed by autoradiography. Lane 1, no nuclear extract; lane 2, no competitor; lanes 3–5, 50-fold, 100-fold and 200-fold excess amounts of unlabelled fragment containing the Sp1-binding site respectively; lane 6, 200-fold excess of PGV-B. Lower panel: competitive assay with double-stranded synthetic oligonucleotide containing the consensus sequence of the Sp1-binding site. The end-labelled Sp1-binding site fragment was preincubated with different amounts of oligonucleotide with the consensus sequence of the Sp1-binding site (50-fold, 100-fold or 200-fold excess of the Sp1-binding site fragment) or a 200-fold excess of oligonucleotide with the mutant Sp1-binding site. Nuclear extract of HepG2 cells (5 μg) was added to the incubation mixture; the mixture was then analysed as described above. Lane 1, no extracts; lane 2, HepG2 nuclear extracts; lanes 3–5, 50-fold, 100-fold and 200-fold excesses of oligonucleotide with consensus sequence of the Sp1-binding site respectively; lane 6, 200-fold excess of oligonucleotide with the mutant Sp1-binding site.

The results also suggest that the region containing residues −351 to −330 involving the upstream AP2-binding site has a strong enhancer activity. Moreover, the promoter activity of the fragment without the Sp1-binding site (pPCI-269) was almost absent (Figure 4). In separate examinations with the nucleotide sequence mutants of the *cis* elements of the PCI gene, the promoter activities of a two-point mutant of the upstream AP2-binding site and that of the Sp1-binding site decreased to 75% and 40% respectively of that of the control construct (results not shown). These results support the idea that the Sp1-binding site and the upstream AP2-binding site are the promoter and enhancer regions respectively.

The role of the downstream AP2-binding site was also evaluated. As shown in Figure 5 (lower panel), the promoter activities of pPCI-452(del−176/−94) and pPCI-452(del−208/−94) were twice that of the control constructs, pPCI-452(del−116/−94) and pPCI-452(del−146/−94), suggesting that the region containing residues −176 to −147 functions as a silencer. Moreover, the promoter activity of a two-point mutant construct of the downstream AP2-binding site was not different from that of the control construct (results not shown). These findings suggest that the downstream AP2-binding site does not have any role in PCI gene expression. However, the region between residues −176 and −147 was found to be a potent silencer of the Sp1-mediated PCI gene expression in HepG2 cells.

Binding of nuclear proteins to oligonucleotides

A gel mobility-shift assay was performed to elucidate whether the fragment containing the Sp1-binding site, which seems to be the major promoter of the PCI gene, is able to interact with nuclear proteins of HepG2 cells, and whether this interaction is competitively inhibited by a double-stranded oligonucleotide containing the consensus sequence of the Sp1-binding site. A gel mobility-shift assay was performed with the ^{32}P-labelled Sp1-binding site fragment (residues −329 to −209) of the PCI gene as a probe and nuclear extract of HepG2 cells. As shown in Figure 6 (upper panel), a nuclear extract of HepG2 cells formed two distinct complexes with the Sp1-binding site fragment; of the two complexes, that with a high molecular mass was dose-dependently decreased by the addition of a 50–200-fold molar
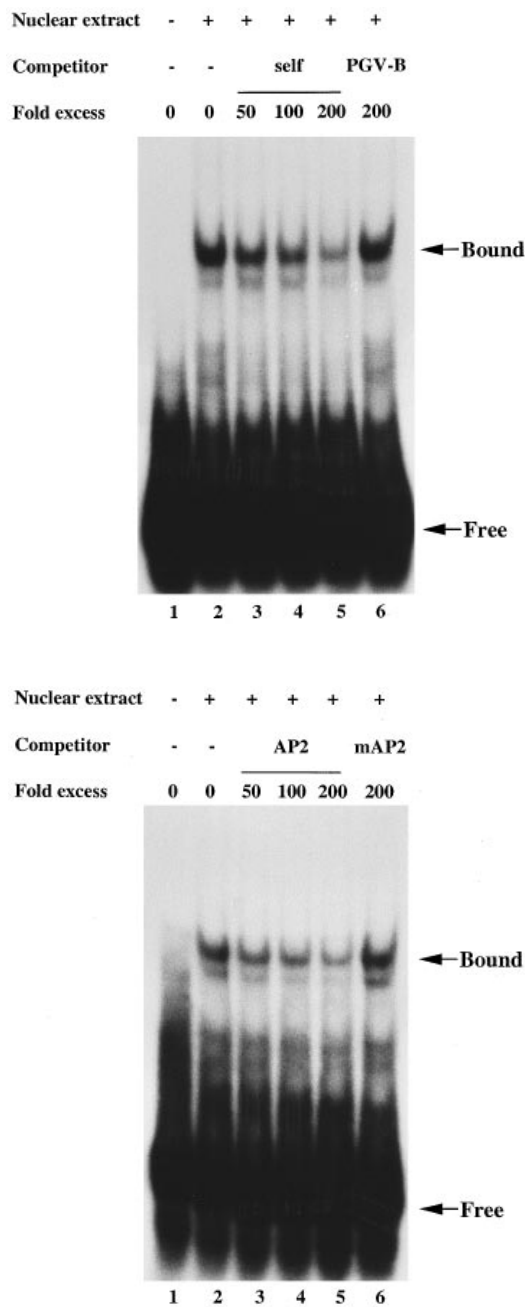
Figure 8   Gel mobility-shift assay performed with nuclear extract of HepG2 cells and oligonucleotides containing the upstream AP2-binding site of the 5′ flanking region of the human PCI gene

Upper panel: the end-labelled upstream AP2-binding site fragment (residues −355 to −330) was preincubated with a 50-fold, 100-fold or 200-fold molar excess of unlabelled fragment containing the upstream AP2-binding site or PGV-B with 5 μg of nuclear extract of HepG2 cells. The DNA–protein complexes were analysed by gel mobility-shift assay. Lane 1, no nuclear extract; lane 2, no competitor; lanes 3–5, 50-fold, 100-fold and 200-fold excesses of unlabelled fragment containing the upstream AP2-binding site respectively; lane 6, addition of 200-fold excess of PGV-B. Lower panel: competitive assay with a double-stranded synthetic oligonucleotide containing the consensus sequence of the AP2-binding site. The end-labelled upstream AP2-binding site oligonucleotide was preincubated with different amounts of oligonucleotide with the consensus sequence of the AP2-binding site (50-fold, 100-fold or 200-fold excess of the upstream AP2-binding site fragment) or a 200-fold excess of oligonucleotide with the mutant AP2-binding site. Nuclear extract of HepG2 cells (5 μg) was added to the incubation mixture; the mixture was then analysed as described in the Materials and methods section. Lane 1, no extract; lane 2, HepG2 nuclear extract; lanes 3, 4 and 5, 50-, 100- or 200-fold excess of oligonucleotide with consensus sequence of the AP2-binding site; lane 6, 200-fold excess of oligonucleotide with the mutant AP2-binding site.

excess of the unlabelled Sp1-binding site fragment. However, the complex with a low molecular mass was not changed by the addition of a 200-fold molar excess of heterologous DNA, PGV-B (luciferase reporter plasmid). These findings indicate that the complex with a high molecular mass in Figure 6 (upper panel) does not result simply from non-specific interaction or from protein–protein aggregation but rather from the binding between a nuclear protein and the Sp1-binding site fragment of the PCI gene. The inhibition of complex formation between nuclear protein of HepG2 cells and the Sp1-binding site fragment by a double-stranded 22 bp oligonucleotide containing the consensus sequence of the Sp1-binding site was even more significant. As shown in Figure 6 (lower panel), a 200-fold molar excess of the oligonucleotide containing the consensus sequence of the Sp1-binding site almost completely abolished complex formation, whereas the oligonucleotide containing the mutant Sp1-binding site sequence did not. In addition, the nuclear protein from HepG2 cells formed a complex with the ^{32}P-labelled 22 bp oligonucleotide containing the consensus Sp1-binding site sequence (Figure 7). This complex band was decreased by a 100-fold molar excess of unlabelled 22 bp oligonucleotide containing the consensus Sp1-binding site sequence, but not by the mutant Sp1-binding site sequence. Furthermore this complex band was supershifted by pretreatment with anti-Sp1 antibody but not with a non-specific rabbit serum antibody. These findings indicate that Sp1 of the nuclear extract of HepG2 cells specifically recognizes the Sp1-binding site of the human PCI gene promoter.

Gel mobility-shift assays were also performed with the ^{32}P-labelled upstream AP2-binding site oligonucleotide (residues −355 to −330) of the PCI gene as probes and a nuclear extract of HepG2 cells to elucidate whether the upstream AP2-binding site is actually the enhancer of the Sp1-mediated PCI gene expression. Figure 8 (upper panel) shows that the nuclear extract formed a distinct complex with the upstream AP2-binding site oligonucleotide, and that this complex was dose-dependently decreased by a 50–200-fold molar excess of the unlabelled upstream AP2-binding site oligonucleotide but not by a 200-fold molar excess of the heterologous DNA, PGV-B. Furthermore the oligonucleotide containing the consensus sequence of the AP2-binding site inhibited complex formation, whereas the oligonucleotide containing a two-point mutant AP2-binding site sequence did not (Figure 8, lower panel). In addition, the nuclear protein of HepG2 cells formed a complex with the ^{32}P-labelled 22 bp oligonucleotide containing the consensus AP2-binding site sequence (results not shown). These findings suggest that AP2 of the nuclear extract of HepG2 cells recognizes the upstream AP2-binding site of the PCI gene promoter.

As shown in Figure 5, the regions containing residues −452 to −414 and residues −176 to −147 showed a potent silencer activity; we therefore searched for a sequence similar to that of the nuclear protein-binding site in these regions. This evaluation disclosed the presence of a similar sequence to that of the STATx-binding site (residues −447 to −439, 94.3 % similarity), an IFN-α response element (residues −439 to −434, 100 % similarity) and an A-activator (AA)-binding site (residues −422 to −414, 100 % similarity) in residues −452 to −414, and a similar sequence to that of the GATA-1-binding site (residues −169 to −164, 85.3 % similarity) and IFN-γ response element (residues −164 to −157, 100 % similarity) in residues −176 to −147. To determine which of these *cis* elements were functional, gel mobility-shift assays were performed with ^{32}P-labelled oligonucleotides containing residues −452 to −414 or residues −176 to −147, and a nuclear extract of HepG2 cells. In this experiment, various 20 bp double-stranded oligonucleotides were used as competitors. As shown in Figures 9 and 10, the gel mobility-shift
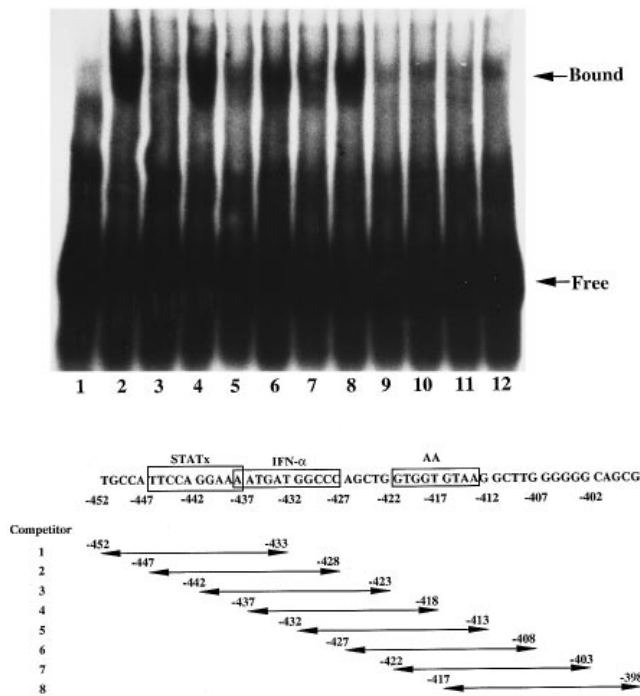
Figure 9   Gel mobility-shift assay performed with nuclear extract of HepG2 cells and oligonucleotides containing residues −452 to −414 of the 5′ flanking region of the human PCI gene, and competitive assay with various double-stranded synthetic oligonucleotides

The end-labelled fragment containing residues −452 to −414 was preincubated with various 20-bp double-stranded synthetic oligonucleotides (200-fold excess of the end-labelled fragment containing residues −452 to −398). The nuclear extract was added to the incubation mixture, and the mixture was then analysed as described in the Materials and methods section. Lane 1, no extract; lane 2, HepG2 nuclear extract; lane 3, 200-fold excess of unlabelled oligonucleotide containing the residues from −452 to −414; lane 4, 200-fold excess of PGV-B; lanes 5–12, 200-fold excess of unlabelled 20 bp oligonucleotides 1–8 as indicated in the lower panel.
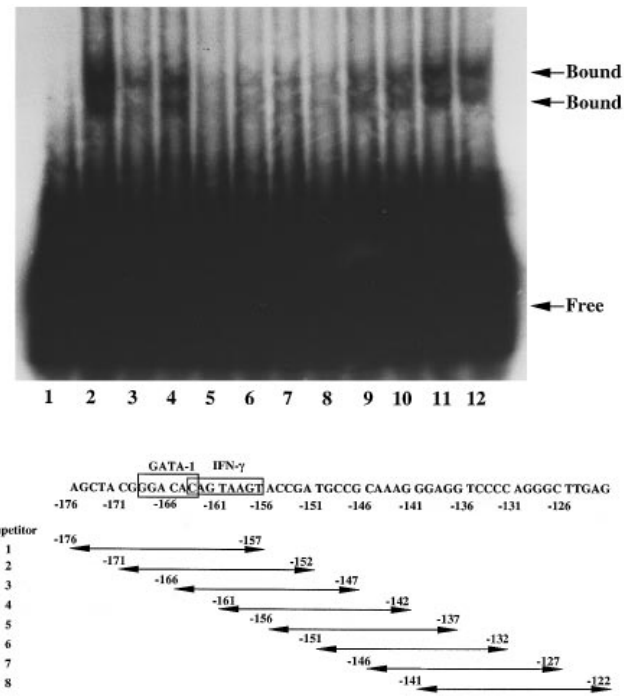
Figure 10   Gel mobility-shift assay performed with nuclear extract of HepG2 cells and oligonucleotides containing residues −176 to −147 of the 5′ flanking region of the human PCI gene, and competitive assay with various double-stranded synthetic oligonucleotides

The end-labelled oligonucleotide containing residues −176 to −147 was preincubated with various 20 bp double-stranded synthetic oligonucleotides (200-fold excess of the end-labelled oligonucleotide containing residues −176 to −122). The nuclear extract was then added to the incubation mixture, which was then analysed as described in the Materials and methods section. Lane 1, no extract; lane 2, HepG2 nuclear extract; lane 3, 200-fold excess of unlabelled oligonucleotide containing residues −176 to −147; lane 4, 200-fold excess of PGV-B; lanes 5–12, 200-fold excess of unlabelled 20 bp oligonucleotides 1–8 as indicated in the lower panel.

assays showed that the HepG2 nuclear extract formed a complex with the fragment containing residues −452 to −414 or residues −176 to −147, and that the addition of a 200-fold molar excess of the same unlabelled fragment decreased complex formation, but not the addition of a 200-fold molar excess of the heterologous DNA, PGV-B. Figure 9 shows that a 200-fold molar excess of four 20 bp oligonucleotides containing residues −432 to −413, −427 to −408, −422 to −403 and −417 to −398 (competitors 5, 6, 7 and 8), inhibited complex formation, whereas other 20 bp oligonucleotides containing residues -452 to −433, −447 to −428, −442 to −423 and −437 to −418 (competitors 1, 2, 3 and 4) did not. These findings suggest that nuclear proteins of HepG2 cells interact with the region containing residues −417 to −413, which is a part of the AA-binding site (residues −422 to −414). Furthermore Figure 10 shows that a 200-fold molar excess of four 20 bp oligonucleotides containing residues −176 to −157, −171 to −152, −166 to −147 and −161 to −142 (competitors 1, 2, 3 and 4) inhibited complex formation, whereas four 20 bp oligonucleotides containing residues −156 to −137, −151 to −132, −146 to −127 and −141 to −122 (competitors 5, 6, 7 and 8) did not. These findings suggest that nuclear proteins of HepG2 cells interact with the region containing residues −161 to −157, which is a part of the IFN-γ response element (residues −164 to −157).

DISCUSSION

We have previously identified the transcription initiation site by primer extension analysis, with human liver mRNA as a template, and showed that, of the five exons involved in the human PCI gene, the exon I has 58 bp [12]. Meijers and Chung [11] reported that exon I is composed of 98 bp, on the basis of an RNase protection assay with HepG2 mRNA as a template. These preliminary studies suggested the presence of multiple transcription initiation sites in the PCI gene. In the present study, using as a basis the numbering of the 5′ flanking region of human PCI gene determined in our previous study [12], we analysed the nucleotide sequence up to −1587 bp of the 5′ flanking region, and found that several consensus binding sites for transcription regulation factors, such as GATA-1 [31], HNF [32], AP2 [33], Sp1 [34] and C/EBP [35], are present in this region. We then performed transient expression analysis of the 5′ flanking region of the PCI gene to characterize which cis elements of the gene participate in the regulation of PCI gene expression in HepG2 cells.

In the preliminary transient expression assay of various deletion constructs of the 5′ flanking region of the PCI gene, we found two positively acting regions at residues −452 to −330 involving an inverted AP2-binding site and an AP2-binding site, at residues −329 to −209 involving an Sp1-binding site, and at

least one negatively acting region at residues −980 to −790. This negatively acting region has no consensus sequence serving the nuclear factor-binding site. The results of this investigation showed that the region containing the Sp1-binding site acts independently as a promoter of PCI gene expression in HepG2 cells, that the region containing the inverted AP2-binding site and the upstream AP2-binding site is the enhancer, and that the region containing the downstream AP2-binding site is the silencer. Moreover, studies with detailed deletion mutants of the region containing the inverted AP2-binding site and upstream AP2-binding site or downstream AP2-binding site, and site-directed mutants of the Sp1-binding site and the upstream AP2-binding site, suggest that the Sp1-binding site is the major promoter for PCI gene expression in HepG2 cells and that the upstream AP2-binding site is a strong enhancer. The inverted AP2-binding site and the downstream AP2-binding site do not have any specific function in Sp1-mediated PCI gene expression. The present study also suggests that the region containing residues −452 to −414 and that containing residues −176 to −147 exert a strong silencer activity on PCI gene expression in HepG2 cells.

Several genes were found to be regulated tissue-specifically by Sp1 and Sp1-related proteins [36,37]. In the present study, a gel mobility-shift assay showed that the region containing the Sp1-binding site interacts with nuclear proteins of HepG2 cells, and that this interaction is inhibited by an unlabelled PCI gene fragment containing the Sp1-binding site and also by the oligonucleotide containing the consensus sequence of the Sp1-binding site, but not by the oligonucleotide containing the mutant Sp1-binding site. Moreover, this oligonucleotide–protein complex was supershifted by pretreatment with anti-Sp1 antibody. These findings suggest that Sp1 regulates the gene expression of PCI in HepG2 cells by binding to the Sp1-binding site.

Sp1 interacts with many cellular and viral promoters and has a role in constitutive transcription [38]. Moreover, Sp1 participates in the regulation of several inducible genes, such as transforming growth factors β1 and β3 [39] and tissue factor [40], and co-operates with other transcription factors such as NFκB [41]. Extracellular signals might modulate *trans*-activation through Sp1 (1) by inducing the expression of Sp1 protein [42], (2) by altering the DNA-binding affinity of Sp1 [43], and/or (3) by altering the *trans*-activation potential of Sp1 [44]. We reported that plasma PCI concentration is markedly decreased in patients with severe liver disease or with disseminated intravascular coagulation, and that it is slightly increased in patients with endotoxin shock or inflammation [45]. These clinical observations can be explained by the lack of cytokine-responsive or lipopolysaccharide-responsive elements in the 5′ flanking region of human PCI gene, as shown in the present study.

The results of our study showed that the AP2-binding site just upstream of the Sp1-binding site enhances the Sp1-mediated expression of the PCI gene. The transcriptional factor, AP2, which can be induced by cAMP or phorbol ester [46], activates the transcription of cAMP- and phorbol ester-inducible genes. In contrast, there are several reports showing that the AP2-binding site acts as an enhancer of constitutively expressed genes [47,48]. For example, it is well known that the AP2-binding site is an enhancer of the gene expression of glutamine synthase in rat liver, and that this enhancer activity is correlated with its constitutive expression [47]. AP2 was found recently to be an important factor in the normal development of the craniofacial region; a mouse knock-out of AP2 was found to be associated with death at birth [49]. The results of these previous studies suggest that AP2 constitutively regulates several genes that have

fundamental roles during development. PCI might constitute one of these genes.

Concerning the region containing residues −452 to −414 and that containing residues −176 to −147, the gel mobility-shift assay showed that the AA-binding site (residues −422 to −414) and the IFN-γ response element (residues −164 to −157) are silencer regions of the PCI gene expression in HepG2 cells. The AA-binding site is the promoter region of the A2 vitellogenin gene. C/EBP or LFB1/HNF-1, which are believed to be determinants of tissue specificity, binds to this AA-binding site [50]. In contrast, the IFN-γ response element is known to be located in the upstream region of IFN-γ-inducible genes [51,52]. The results of our transient-expression and gel mobility-shift assays suggest that some nucleic factors of HepG2 cells bind to the AA-binding site and the IFN-γ response element of the PCI gene and that they constitutively and negatively regulate Sp1-mediated PCI expression in these cells.

As well as in the liver, PCI is also expressed in kidneys, prostate and seminal vesicles [9]. It has been suggested that Sp1 mainly regulates the housekeeping genes and that it is present in various tissue cells. The *cis* elements that modulate PCI gene expression in cells other than hepatocytes are probably regulated by Sp1.

In conclusion, the present study suggests that Sp1 has a crucial role in PCI gene expression in HepG2 cells, possibly through co-operative interaction with AP2, which binds to the AP2-binding site, and/or with proteins that bind to the AA-binding site and to the IFN-γ response element.

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