The CCAAT-box binding factor NF-Y is required for the expression of phospholipid hydroperoxide glutathione peroxidase in human epidermoid carcinoma A431 cells

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Abstract Promoter activation in the expression of phospholipid hydroperoxide glutathione peroxidase (PHGPx) gene in human epidermoid carcinoma A431 cells was studied in the present investigation. Luciferase reporter assays with plasmids carrying a 400 bp of the promoter DNA were performed to analyze the regulatory element in the proximal promoter of human PHGPx gene. Transient transfection with a series of 5′-deletion and internal truncation mutants showed that the 5′-flanking region spanning from −212 to −121 bp was important for the basal expression of PHGPx gene in A431 cells. A region from −170 to −140 bp was protected in DNase I footprinting assays and bound the nuclear proteins in electrophoretic mobility shift assays. This region, denoted FP3, contains the consensus recognition sites for AP-2, CCAAT-box and CRE. The oligonucleotide competitor with the mutation at CCAAT-box could not eliminate the nuclear protein binding in gel-shift assay and the site-directed mutagenesis at the CCAAT-box decreased the luciferase activity of PHGPx promoter for approximate 50% in reporter gene assays. Competition experiments indicate that the binding of nuclear factor to the FP3 region was abolished by oligodeoxyribonucleotide corresponding to NF-Y/CP1 binding site to a greater extent than by those corresponding to sites for CTF/NFI and C/EBP. Taken together, the CCAAT-box in the promoter ranging from −156 to −151 bp, bound to NF-Y/CP1, was essential for the basal expression of human PHGPx gene in A431 cells.

Key words: PHGPx; NF-Y; A431 cell

1. Introduction

Oxidative stress is associated with a disturbance in the reactive oxygen species-antioxidant balance in favor of the former [1,2]. Many of the reactive oxygen species are involved in the oxidative damage in a number of physiological and pathophysiological phenomena and processes as diverse as inflammation, aging, carcinogenesis, drug action and drug toxicity. The reactive oxygen species may be formed from polyunsaturated fatty acids either by an enzymatic or by a non-enzymatic oxygenation [3]. Reduction of the reactive oxygen species can be achieved either by the inhibition of lipid oxygenation enzymes or by the interception of free radicals by antioxidants and some antioxidation enzymes such as superoxide dismutase, catalase and glutathione peroxidase.

Lipoxygenases and fatty acid cyclooxygenases are the major enzymes involved in the metabolism of polyunsaturated fatty acids including arachidonic acid [4]. In studying the regulation of arachidonate metabolism in human epidermoid carcinoma A431 cells, we previously reported a putative inhibitor of 12-lipoxygenase, which masked the biosynthesis of 12(*S*)-hydroxyeicosatetraenoic acid in intact cells [5]. The putative inhibitor in A431 cells was purified to a 22 kDa protein [6] whose sequence data matched the cDNA sequence of human phospholipid hydroperoxide glutathione peroxidase (PHGPx) [7]. So far, five selenium-dependent glutathione peroxidases have been identified [8–12]. Among these isozymes, PHGPx is unique in its substrate specificity because it can interact with lipophilic substrates including the peroxidized phospholipids and cholesterol and reduce these hydroperoxides to hydroxide compounds [13,14]. PHGPx exists ubiquitously in all tissues, but highly expresses in testis [15]. However, the high expression of PHGPx in testis is not directly regulated by testosterone [16].

In the present study, the functional promoter sequence in human PHGPx gene for the basal expression in A431 cells was analyzed. With the aid of in vitro DNase I footprinting study, electrophoretic mobility shift assay and site-directed mutagenesis, the CCAAT-box residing at −155 to −151 bp in the promoter region, bound by the nuclear factor NF-Y/CP1, was demonstrated to be essential for the transcriptional activation of human PHGPx gene in A431 cells. This is the first demonstration of CCAAT-box mediating the expression of human PHGPx gene in cancer cells.

2. Materials and methods

2.1. Reagents

[α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Polyvinyl alcohol, HEPES and *o*-nitrophenyl β-galactopyranoside were from Sigma (St. Louis, MO, USA). Wizard *Plus* MiniPreps DNA purification system, *pfu* polymerase, DNase I, luciferase plasmid pGL-3 and assay system were from Promega (Madison, WI, USA). GENECLEAN III kit was from BIO 101 (La Jolla, CA, USA). β-Galactosidase plasmid (CMVβ) was from Clontech Laboratories (Palo Alto, CA, USA). *TransIT* transfection reagents were from Pan-Vera (Madison, WI, USA). Dulbecco's modified Eagle's medium, ribonuclease inhibitor and Opti-MEM medium were from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was from Hyclone Laboratories (Logan, UT, USA). Restriction enzymes and T_4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA, USA) or TaKaRa (Kyoto, Japan). Antibodies against Sp1, Sp3

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Abbreviations: PHGPx, phospholipid hydroperoxide glutathione peroxidase; CBFs, CCAAT-box binding factors; SECIS, selenocysteine insertion sequence; Sec, selenocysteine; Se-S, selenenyl sulfide; ORE, oxygen responsive element; PdBu, phorbol 12,13-dibutyrate

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and NF-1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and that against NF-YA was from Pharmingen (San Diego, CA, USA). Rapid DNA ligation kit was from Boehringer Mannheim (Mannheim, Germany).

2.2. Cell culture

Human epidermoid carcinoma A431 cells were grown at 37°C under air/CO_2 (19:1) in 10-cm plastic dishes containing 10 ml of culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin.

2.3. Construction of PHGPx gene promoter/luciferase reporter vector

The pGL3-basic vector, which contains a luciferase reporter gene for the analysis of promoter activity of cloned fragments located immediately downstream of a polylinker, was obtained from Promega. The 0.4-kb promoter of PHGPx gene was amplified by PCR using a modified promoter sense primer with an additional *Bgl*II restriction site (5′-GAAGATCTTGGGGACACTTTTCTGCGAGTT-3′) and a modified promoter antisense primer with an additional *Nco*I restriction site (5′-CATGCCATGGCGCGGCGGGGCTGGGA-3′) using genomic DNA of A431 cells as a template. The PCR fragments were purified from 1% agarose gel using the GENECLEAN III kit. Subsequently, the fragments were cleaved with *Bgl*II and *Nco*I and cloned into pGL-3. Identification of isolated clones was confirmed by DNA sequencing. One clone (pGP4-2), which contained the entire 0.4-kb promoter fragment, was chosen for further studies.

2.4. Preparation of promoter-deletion constructs

Human PHGPx gene promoter regions of various lengths were prepared either by restriction enzyme digestion of pGP4-2 for the preparation of pGP4-7, or by the PCR amplification method for the preparation of pGP4-3, 4-4, 4-5, 4-8, 4-11, dFP3, dFP4 and dFP34. The mutants at the sites of CCAAT (pGPdNFY) and AP-2 (pGPdAP2) were constructed by the site-directed mutagenesis method as described by Higuchi et al. [17], and confirmed by DNA sequencing.

2.5. Transfection and reporter gene assays

Cells were replated 18 h before transfection at a density of 3×10^5 cells in 2 ml of fresh culture medium in a 3.5-cm plastic dish. For use in transfection, 1 μl of *TransIT* transfection reagent was mixed with 1 μg of pGP luciferase plasmid and 0.5 μg of β-galactosidase plasmid in 1 ml of Opti-MEM medium and incubated for 30 min at room temperature. Cells were transfected by changing the medium to 1 ml of Opti-MEM medium containing the plasmids and *TransIT* transfection reagent, followed by incubation at 37°C in a humidified atmosphere of air/CO_2 (19:1) for 4 h. After the change of Opti-MEM medium to 2 ml of fresh culture medium, cells were incubated for an additional 24 h and then the luciferase and galactosidase activities in cell lysates were determined as described previously [18].

2.6. Gel-shift assay

Nuclear protein extracts were prepared from A431 cells by homogenization with a Dounce homogenizer, and double-stranded oligonucleotides were prepared by the standard annealing method and labeled with 0.2 μM [γ-^{32}P]ATP using T_4 polynucleotide kinase as probes [18]. The mixture of 2–3 μg of the cell nuclear extracts and the radiolabeled probe (2×10^4 c.p.m.) was incubated at room temperature for 30 min and loaded on a 5% (w/v) polyacrylamide gel. Electrophoresis was performed at a constant 200 V for 2 h. Gel was dried and autoradiographed.

2.7. In vitro DNase I footprinting assay

In vitro DNase I footprinting experiments were mainly performed according to the method of Lemaigre et al. [19]. Labeled probe (2–3×10^4 c.p.m.) was incubated with 25–50 μg of nuclear extracts for 30 min on ice. After digestion with 1–2 U of DNase I for 6 min on ice, the degradation products were separated onto a 8% polyacrylamide, 7 M urea sequencing gel. The G and (G+A) chemical cleavage reactions on the labeled probes were performed according to the standard procedure [20].

Fig. 1. Regulatory elements in human PHGPx gene. Each putative consensus sequence in the 5′-flanking region of human PHGPx gene is enclosed in a box. Abbreviations: GR, glucocorticoid responsive element; AP-2, binding site of AP-2; Sp1, Sp1 binding site; CCAAT, CCAAT-box; CRE, cAMP responsive element.

3. Results

3.1. Expression of DNA constructs with 5′-deletion of human PHGPx gene promoter in A431 cells

The promoter of human PHGPx gene (AC004151) lacks a TATA-box. Each putative consensus sequences in the 5′-flanking region of human PHGPx gene is enclosed in a box as shown in Fig. 1. There are three putative Sp1 binding sites, three AP-2 binding sites, two CCAAT-boxes, one glucocorticoid responsive element, and one cAMP responsive element in the first 400 bp before the ATG start codon. To study the transcriptional regulation of human PHGPx gene, the luciferase reporter vectors bearing various lengths of 5′-flanking regions of human PHGPx were constructed as shown in Fig. 2. Among these DNA constructs, pGP4-1 is a vector bearing the luciferase reporter gene only. Cells were transfected with various DNA constructs, and the basal expression of luciferase activity in these vectors was studied. The results are summarized in Fig. 2. No obvious expression was observed in cells transfected with pGP4-8 (−82 bp), and pGP4-11 (−46 bp), and there was very little expression in pGP4-7 (−121 bp). However, a significant expression was observed in luciferase vectors bearing a promoter longer than 212 bp. The luciferase activity in 5′-deletion vectors apparently is restricted in three regions covering from −305 (pGP4-3) to −237 bp (pGP4-4), from −237 (pGP4-4) to −212 bp (pGP4-5), and from −212 (pGP4-5) to −121 bp (pGP4-7). The results indicated that the two putative binding sites for GR and AP-2 residing in the region from −400 to −305 bp do not play any significant role since no difference of the luciferase activity between pGP4-2 and pGP4-3 was observed. Although a significant decrease in luciferase activity from pGP4-3 (−305 bp) to pGP4-5 (−212 bp) was found, no well-defined consensus binding sequence of transcription factor is present in the region from −305 to −212 bp. However, presence of cis-elements for the binding of some unknown transcription factors in this region is possible. In contrast, multi-binding sites: two potential binding sites for Sp1, one for AP-2, one for CCAAT-box (inverted sequence 'ATTGG'), and one for CRE binding site are present in the region from −212 to −121 bp. This region may be responsible for the decrease in luciferase activity from pGP4-5 (−212 bp) to pGP4-7 (−121 bp).

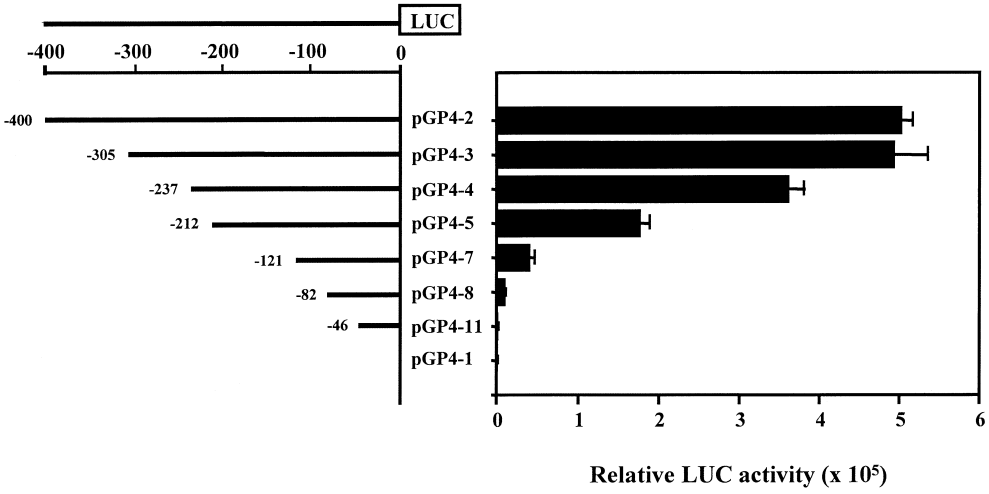

Fig. 2. Transcriptional analysis of luciferase expression vectors bearing various lengths of human PHGPx promoter. Truncated promoter fragment was ligated into a luciferase plasmid as described in Section 2. Numbers indicate the distance in base pairs from the translation start site. A mixture of each pGP luciferase plasmid (1 μg) and β-galactosidase plasmid (0.5 μg) was transfected into A431 cells by the *TransIT* transfection method. The luciferase activity was normalized with the β-galactosidase activity. Values are means ± S.E.M. for three determinations.

3.2. *Identification of the nuclear factors involved in the expression of human PHGPx gene*

The in vitro DNase I footprinting studies were performed with a restriction fragment extending from −400 to −1 bp. Four protected areas on the fragment, designated FP1 (−246 to −212 bp), FP2 (−202 to −172 bp), FP3 (−170 to −140 bp), and FP4 (−106 to −77 bp), were observed. FP3 region covering from −170 to −140 bp played an essential role in the regulation of PHGPx expression (Fig. 2). Three potential binding sites for AP-2, CCAAT-box, and CRE are present in this region. To further examine the protein binding properties to the FP3 region of promoter, DNA mobility shift and super-shift assays were performed with an oligonucleotide extending from −170 to −140 bp (FP3). Synthetic oligonucleotide FP3 was labeled with [γ-^{32}P]ATP by T_4 polynucleotide kinase. When nuclear extracts from A431 cells were allowed to react with FP3 probe, four retarded bands (a, b, c, and d) were observed (Fig. 3B, lane 2). Among them, band b was the

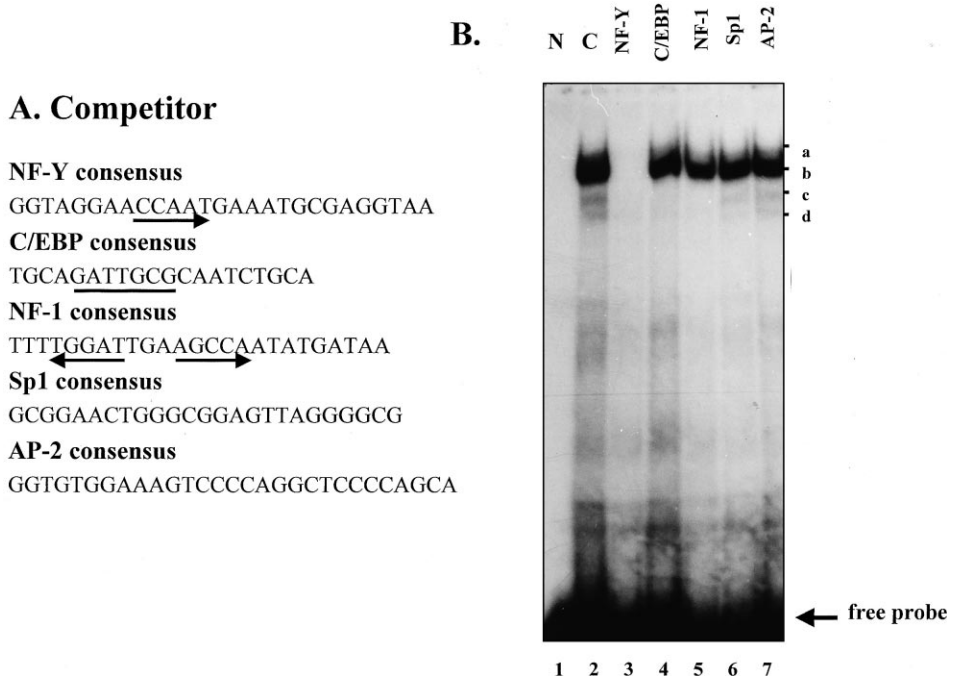

Fig. 3. Determination of the binding of nuclear factors to FP3 (−170/−140) probe by gel-shift assay. DNA probe, FP3 (−170/−140), was labeled with [γ-^{32}P]ATP as described in Section 2. A: DNA sequences of NF-Y, C/EBP, NF-1, Sp1 and AP2 consensus are indicated. The CCAAT-box consensus sequences are denoted by arrows and thick lines. B: The radiolabeled FP3 probe was incubated with the nuclear extracts of A431 cells. Lane 1 is a free probe; lane 2, four retarded bands (a, b, c and d) are indicated; lanes 3–7, competitors NF-Y, C/EBP, NF-1, Sp1 and AP-2 consensus were preincubated with nuclear extracts with a 50-fold molar excess of radiolabeled FP3 probe for 10 min at room temperature, and then the radiolabeled FP3 probe was added. After 30 min at room temperature, the preparation was electrophoretically separated.

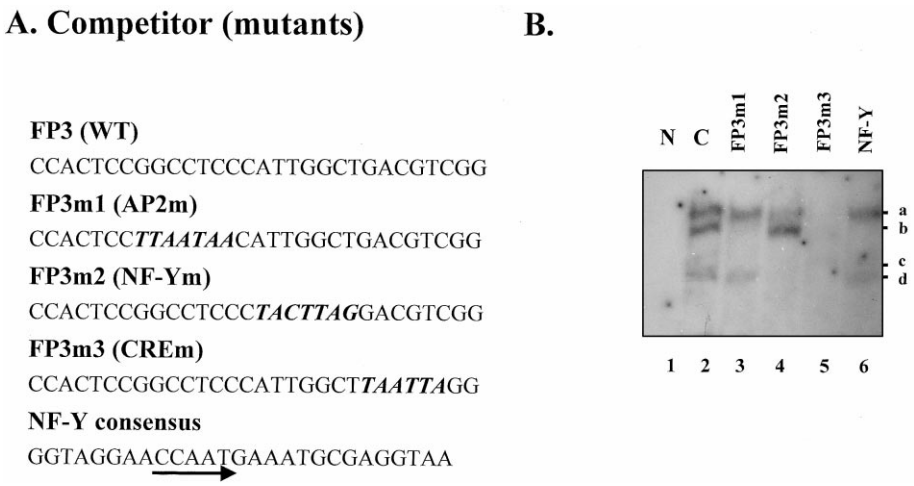

Fig. 4. Effect of mutated competitors on the nuclear factors interacting with the radiolabeled FP3 probe. A: Wild-type and mutated FP3 sequence were used in competitive gel-shift analysis. B: The radiolabeled FP3 oligonucleotide was used as a probe and incubated with the nuclear extracts of A431 cells. Lane 1 is a free probe; lane 2, four retarded bands (a, b, c and d) are indicated; lanes 3–6, competitors FP3m1, FP3m2, FP3m3 and NF-Y consensus were preincubated with nuclear extracts with a 50-fold molar excess of radiolabeled FP3 probe for 10 min at room temperature, and then the radiolabeled FP3 probe was added. After 30 min at room temperature, the preparation was electrophoretically separated.

most significant. As there is a CCAAT-box in the oligonucleotide FP3, study on the binding of CCAAT-box binding factors (CBFs) to the FP3 region was then followed. CBFs include a family of proteins including NF-Y/CP1, C/EBP, and NF-1 [21,22]. To determine the specificity of protein binding, the oligonucleotides that selectively bind CBFs, Sp1, and AP-2 were used respectively in the competition experiments (Fig. 3A). Formation of band b was abolished by an oligonucleotide with an NF-Y consensus, but not by C/EBP, NF-1 and other competitors used (Fig. 3B). There are three putative binding sites for AP-2, CCAAT-box, and CRE in the FP3 region. Three mutated oligonucleotides designated FP3m1, FP3m2, and FP3m3 for AP-2, CCAAT-box, and CRE mutants were synthesized and used as competitors, respectively (Fig. 4A). Among these three mutants, mutant FP3m2 was the only one that could not abolish the formation of band b (Fig. 4B), indicating that band b is the only retarded band formed by NF-Y protein interacting with the sequence of CCAAT-box in FP3 probe. To further identify whether NF-Y binds the FP3 probe, monoclonal antibody specifically recognizing human NF-YA protein was used in the gel-shift assay. The retarded band b was completely abolished by antibody against NF-YA but not by antibodies against Sp1, Sp3, and NF-1, as determined with oligonucleotide FP3 as a probe (Fig. 5). These results indicate that NF-Y was the major nuclear factor that bound the sequence of PHGPx gene promoter from −170 to −140 bp.

3.3. Luciferase activity of mutants with internal truncation and site-directed mutagenesis

To determine which of these putative binding sites in the promoter region from −164 to −75 bp is functionally relevant to the expression of human PHGPx gene, effect of the internal truncation and site-directed mutagenesis was studied. Mutants with the regions truncated from −164 to −115 bp (pGPdFP3), from −117 to −75 bp (pGPdFP4), and from −164 to −75 bp (pGPdFP34) were prepared. Vectors with mutations at the CCAAT-box (pGPdNFY), and at the AP-2 site (pGPAP2) were obtained by site-directed mutagenesis (Fig. 6A). Plasmids with internal truncation at the FP3 region (pGPdFP3), FP4 region (pGPdFP4) and both FP3 and FP4 regions (pGPdFP34) eliminated the luciferase activity by 50%, 25%, and 70%, respectively (Fig. 6B). Plasmid with CCAAT-box site-directed mutation (pGPdNFY) eliminated the luciferase activity by 50%, similar to the effect induced by pGPdFP3. However, no significant effect was observed in pGPdAP2 with a mutation at the putative AP-2 binding site in the FP4 region. These results indicate that the CCAAT-box in the FP3 region was essential for the basal expression of human PHGPx gene.

4. Discussion

Several pieces of evidence were provided in this study to indicate that the CCAAT-box in the promoter region from

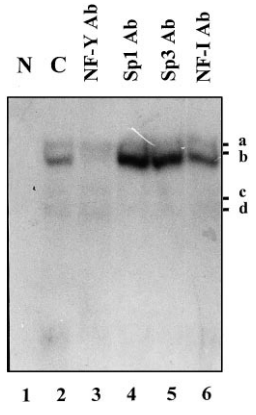

Fig. 5. Effect of antibodies to NF-Y, Sp1, Sp3 and NF-1 on the nuclear factors interacting with the radiolabeled FP3 probe. Radiolabeled FP3 oligonucleotide was used as a probe and incubated with the nuclear extracts of A431 cells. Lane 1, free probe; lane 2, nuclear extracts only; lanes 3–6, 0.2 μg of each NF-Y, Sp1, Sp3 and NF-1 antibodies was preincubated with nuclear extracts (2 μg) for 2 h on ice, and then the radiolabeled FP3 probe was added. After 30 min at room temperature, the preparation was electrophoretically separated.

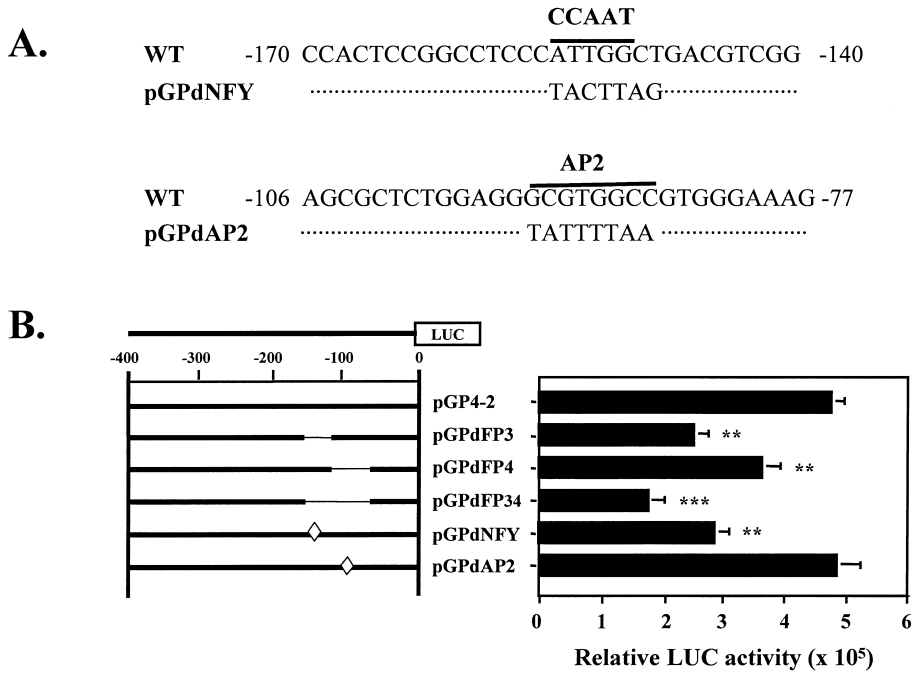

Fig. 6. Effect of internal truncation and site-directed mutation of CCAAT-box on the promoter activity. A: The sequence of wild-type (WT) promoter and the mutated nucleotides in mutant constructs are indicated. B: A mixture of each pGP luciferase plasmid (1 μg) and β-galactosidase plasmid (0.5 μg) was transfected into A431 cells by the *TransIT* transfection method. The luciferase activity was normalized with the β-galactosidase activity. Values are means ± S.E.M. for three determinations. (**$P < 0.01$; ***$P < 0.001$ compared with pGP4-2.)

−155 to −151 bp played a significant role in the basal expression of the human PHGPx gene in A431 cells. Firstly, the promoter region, which was essential for the expression of luciferase activity, was narrowed down to 91 bp ranging from −212 to −121 bp with the aid of 5′-deletion constructs of human PHGPx gene promoter. Secondly, in vitro DNase I footprinting analysis revealed that there were transcription factors binding on the region from −170 to −140 bp designated FP3 (data not shown). Thirdly, in gel-shift assays, the NF-Y-specific competitor and NF-YA monoclonal antibody abolished the major nuclear protein bound to the FP3 region (Figs. 3 and 5), and the competitor with the mutation at CCAAT-box could not eliminate the binding of NF-Y protein to the promoter DNA (Fig. 4). The results indicate that the major retarded band was due to the binding of NF-YA to its binding element in the promoter. Lastly, mutants with internal truncation and site-directed mutagenesis at the CCAAT-box eliminated the luciferase activity of basal expression by 50%. We conclude that NF-Y is a specific transcription factor that binds the CCAAT-box of human PHGPx gene, and the protein-DNA complex is essential for the basal expression of human PHGPx gene. NF-Y is a heterotrimeric transcription factor composed of three subunits (A, B, and C), and formed a heterotrimeric complex with DNA [23,24]. Mutation at the CCAAT motifs of promoter, including mouse α1 collagen gene, mouse albumin gene, human multidrug resistance gene, and human blood coagulation factor X gene, decreases the basal promoter activity [25]. From the present study, NF-Y specifically bound the CCAAT-box in the promoter of PHGPx gene. But not all the DNA oligonucleotides containing the CCAAT-box interacted with NF-Y. Among those associated with CBFs, only NF-Y/CP1 did, but not C/EBP and NF-1 (Fig. 3), suggesting that in addition to CCAAT other specific nucleotides were also required for NF-Y binding.

Maiorino et al. [26] reported that ^{75}Se-labeled PHGPx existed in the cytosol of several human tumor cell lines. A high expression of PHGPx mRNA and its 12-lipoxygenase inhibitory activity was observed in several human cancer cell lines including cervical carcinoma P26 cell, prostatic carcinoma PC3 cell, breast cancer HTB-20 and lung cancer CL-1 cell in addition to A431 cell [27]. Although the pathophysiological significance of high level expression of PHGPx in cancer cells is not understood, this enzyme as well as GPx-1 may play an important role in protecting cells against oxidative damage. Overexpression of PHGPx in a guinea pig cell line protects the cells from injury induced by lipid hydroperoxides such as dilinoleoyl phosphatidylcholine hydroperoxide and linoleic acid hydroperoxide [28]. Overexpression of PHGPx suppresses cell death due to the oxidative damage in rat basophile leukemia cells [29]. Mice deficient in GPx-1 are healthy and fertile and show no increased sensitivity to hyperoxia [30], but show increased susceptibility to other oxidative stress-inducing agents paraquat and hydrogen peroxide [31]. Asahi et al. [32] used mass spectrometry to demonstrate that nitric oxide and its derivatives directly oxidized Sec of bovine GPx-1 to form a selenenyl sulfide (Se-S) with a free thiol, leading to inactivate the enzyme. On the other hand, the human GPx-1 gene contains two oxygen responsive elements (ORE1 and ORE2) in the 5′-flanking promoter region, which are responsive to the oxygen tension in primary cultured human ventricular cardiomyocytes [33]. In addition, after exposure to phorbol 12,13-dibutyrate (PdBu), GPx-1 in cultured human endothelial cells undergoes transcriptional activation via the AP-1 interaction with the TRE sites in the GPx-1 promoter [34].

In the present study of PHGPx gene, neither ORE nor TRE exists in the 5′-flanking region, but CCAAT-box of the proximal promoter, bound to NF-Y/CP1, is essential for the basal

expression of human PHGPx gene in A431 cells. Recently, Schnurr et al. [35] found that IL-4 and IL-13 can up-regulate 12/15-lipoxygenase and simultaneously down-regulate PHGPx, and then create an oxidative environment inside the human lung carcinoma cells A549. These findings may be of pathophysiological importance for disorders associated with reduction-oxidation reactions such as cancer, atherosclerosis, and inflammation. Although the selenocysteine residue in the active site of selenoproteins is important to catalyze the reduction-oxidation reactions [32], the other significant role of CCAAT-box in the PHGPx gene promoter on the high gene expression in several cancer cells and its role on the protection to oxidative damage should be considered and further elucidated.

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