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Transcriptional Regulation of the Human PNRC Promoter by NFY in HepG2 Cells

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PNRC (proline-rich nuclear receptor co-activator) was previously identified using bovine SF-1 (steroidogenic factor 1) as the bait in a yeast two-hybrid screening of a human mammary gland cDNA expression library. PNRC has been demonstrated to be a novel co-activator for multiple nuclear receptors. To understand the molecular mechanisms that regulate the expression of human PNRC gene, in this study, potential transcriptional start site was determined by 5′ RACE analysis. Functional analysis of the 5′ flanking region of the human PNRC gene by deletion mutagenesis, transient transfection and luciferase assays revealed that the −123/+27 region is the minimal promoter of the human PNRC gene. Within this promoter region, there is one putative binding site for the transcription factor NFY (nuclear factor Y). EMSA and ChIP analyses demonstrated the specific binding of NFY protein to the human PNRC promoter. Transient transfection and luciferase assays further revealed that over-expression of NFY represses promoter activity of PNRC gene in a dose-dependent manner. These results indicate that the transcription factor NFY specifically binds to promoter region of PNRC and negatively regulates the transcription of the human PNRC gene.

Key words: nuclear receptor co-activator, NFY, PNRC, promoter analysis, transcriptional regulation.

Abbreviations: ChIP, chromatin immunoprecipitaion; EMSA, electrophoresis mobility shift and super-shift assay; NFY, nuclear factor Y; PNRC, proline-rich nuclear receptor co-activator; RACE, rapid amplification of cDNA ends; SF1, steroidogenic factor 1; SH3, Src homology domain-3.

The nuclear receptor superfamily comprises a large group of transcription factors that bind to the specific hormone-responsive elements and regulate the target gene expression in response to their cognate ligands (*1*, *2*). Transcription controlled by steroid hormone receptor plays a key role in many important physiological processes include cell growth, development, differentiation and metabolism. Besides binding to hormone-responsive elements and their ligands, the subsequent recruitment of co-regulator proteins is required for nuclear receptor to initiate the transcription of their target genes. These regulatory co-factors include co-activators and co-repressors that enhance or inhibit transactivation activity of nuclear receptor, respectively (*3*, *4*). The best-studied co-activators are of the steroid receptor co-activator (SRC) family. Most of these co-activators of nuclear receptors have molecular weights of ∼160 kDa and interact with the liganded nuclear receptors using the NR-box or LXXLL-motif (*5*).

PNRC (proline-rich nuclear receptor co-regulatory protein) (*6*), and PNRC2 (*7*) were identified as SF-1 (steroidogenic factor 1)-interacting proteins in a yeast two-hybrid screening of a human mammary gland cDNA expression library. These proteins were found to interact with the ligand-binding domains of all the nuclear receptors tested, including ERα, ERβ, PR, GR, TR, RAR and RXR, in a ligand-dependent manner. They were also found to interact in a ligand-independent manner with orphan receptors such as SF1 and ERR. Furthermore, PNRC2 and PNRC were also repeatedly isolated as multiple nuclear receptor-interacting proteins in a large interaction-screening project using an automated version of the yeast two-hybrid system (*8*). In their screening, PNRC was isolated as the interacting partner of RARβ, RARγ, RORα, RORβ, HNF4α, HNF4γ, ERβ, ERRγ and LRH1. In the same study, PNRC2 was isolated as a prey with the ligand-binding domains of 10 different nuclear receptors baits, including ERα, ERβ, ERRγ, HNF4α, HNF4γ, LRH1, PXR, RARγ, RORα and RORβ. Thus, PNRC and PNRC2 appear to be more general co-factors for nuclear receptors than previously thought. These two co-activators are unique in that they are significantly smaller than most of the co-regulatory proteins previously identified and are proline rich. Unlike most of the co-activators that interact with nuclear receptors through its LXXLL motif, these new co-activators interact with nuclear receptors through a proline-rich Src homology domain-3 (SH3)-binding motif, S-D (E)-P-P-S-P-S (*9*).

In addition to functioning as a nuclear receptor co-activator, PNRC also plays a role in the growth factor/Ras-signalling pathway through its interaction with Grb2, a central adaptor protein in Ras pathway (*10*). MCF-7 cells that over-express PNRC protein were found to grow slower than control cells, and the activation of Ras and

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MAP kinase in these PNRC over-expressing cells were found to be down-regulated. Furthermore, PNRC was recently demonstrated to stimulate RNA pol III transcription through its interaction with the subunit RPC39 of RNA pol III (*11*). PNRC is a unique co-activator that has profound effects on many aspects of cellular function by directly influencing both RNA pol II- and RNA pol III-dependent transcription.

Significantly reduced PNRC mRNA expression in stomach, colorectal and hepatocellular carcinomas (*12*) and in breast cancer (*10*) was observed in comparison with the corresponding non-cancerous tissues. Furthermore, the reduced expression of PNRC was observed in association with hepatitis B virus infection of HCC patients (*12*). In addition, time-dependent induction of mRNA expression of PNRC was observed during 5-azacytidine treatment. These data suggest a possibility of PNRC as a tumour-related gene and that the regulation of PNRC expression plays a role in human multi-stage carcinogenesis. However, very little is known about the regulation of the expression of PNRC genes itself. To better understand the molecular mechanisms that regulate the expression of PNRC gene, in this study, we have cloned and characterized the 5′ flanking region of the human PNRC gene. Potential transcriptional start site was determined by 5′ RACE analysis. The minimal promoter region of the human PNRC gene was mapped in the region from residue −123 to +27. The transcriptional activity of human PNRC promoter was found to be regulated negatively by transcription factor NFY through binding NFY putative binding site within PNRC promoter region.

MATERIALS AND METHODS

Database Searches—The cDNA sequence of the human PNRC (NM 006813) was obtained from the National Center for Biotechnology Information (NCBI) (website: www.ncbi.nih.gov). The genomic organization of the human PNRC and the 5′-flanking region sequence of PNRC were obtained from the UCSC human gene browser at: http://genome.ucsc.edu/cgi-bin/hgBlat by Blat research using the PNRC cDNA sequence (NM 006813) against the human genome database. The transcription factor-binding sites in the promoter region were analysed using the TRANSFAC Professional software in the Biobase Biological Database (website: www. biobase. de/ pages / products/ databases.html # t ransfac).

Plasmids Construction—The human PNRC 5′ flanking fragments of different lengths were amplified by PCR using HepG2 cell genomic DNA as template with the oligonucleotide primers, which were designed according to the sequences from human genome database and contained *Kpn*I and *Bgl*II restriction site at 5′ and 3′ ends, respectively. The PCR products were digested with *Kpn*I and *Bgl*II and cloned into the *Kpn*I and *Bgl*II site of the luciferase reporter plasmid, pGL3-Basic (Promega). The orientations and the sequences of the inserts were verified by restriction digestion and sequencing.

RNA Ligase-Mediated Rapid Amplification of cDNA Ends—Total RNA was isolated from HepG2 cells using TRIzol reagent (Invitrogen). The transcriptional start point was determined using RLM-RACE kit (Ambion) according to manufacturer's instructions. Briefly, in order to select full-length mRNAs, 10 μg total human RNA was treated with calf intestine alkaline phosphatase (CIP) to remove free 5′-phosphates from molecules such as ribosomal RNA, fragmented mRNA, tRNA and contaminated genomic DNA. Then, the RNA was treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure from the capped mRNAs and leave a terminal 5′ -monophosphate. A 45-base RNA adapter oligonucleotide was ligated to the RNA population using T4 RNA ligase. During the ligation reaction, the majority of the full length decapped mRNA acquires the adapter sequence as its 5′ end. After random-primed reverse transcription reaction, the cDNA was amplified by nested PCR using two forward primers provided by RLM-RACE kit, 5′ -RACE outer primer and 5′ -inner primer, corresponding to the 5′ RACE adapter sequence and the following two specific reverse primers for human PNRC gene, outer primer, 5′-ACC TTC TTC TTT CGC CGC TTC TT - 3′ and inner primer, 5′ -GTC CGG GAT CCG GGG TAA AG -3′, respectively. After the PCR was complete, run 5–10 μl of each sample in a 2% high-resolution agarose gel containing 1 μg/ml EtBr and visualize on a UV transilluminator. The PCR products then were cloned into pCRIITOPO TA cloning vector (Invitrogen) and were sequenced.

Cell Culture, Transient Transfections and Luciferase Assays—HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics including 100 U of penicillin/ml, 100 mg of streptomycin sulphate per millilitre. Twenty-four hours before transfection, cells were seeded in 12-well plates at 1×10^5/per well. Transfect cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Each well of cells was transfected with 2 μg Lipofectamine 2000 and 1 μg of either pGL3-Basic (as no promoter control) or luciferase reporter containing the 5′ flanking fragments of human PNRC gene. The cells were also transfected with 0.2 μg pSV-β-galactosidase (Promega) to correct variation in transfection efficiency. In co-transfection experiments, 1 μg of NFY expression vector was used.

Luciferase activity was assayed 24 h after transfection. Cells were washed twice with PBS. Then the cells were lysed with 200 μl of 1×reporter lysis buffer (Promega). The lysate was centrifuged at 13,000 r.p.m. for 20 s to pellet the cell debris. The supernatants were transferred to a fresh tube and their β-galactosidase activities were determined using the β-galactosidase enzyme assay reagent according to the manufacturer's protocol (Promega). The luciferase activities were measured using Luciferase assay substrate (Promega). All transfection data were expressed as the ratios of luciferase activities/ β-galactosidase activities. Each transfection was performed in triplicate.

Site-Directed Mutagenesis—Point mutations of CCAAT box (NFY-binding site) was generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutant constructs were generated by mutating two or five bases indicated by the lower case letters in primers. For mutagenesis of CCAAT box (change ATTGG to AAAGG, CA-Mut), primers were 5′ -GCA CTC TAA **aa**G GGC TTT CGC AGG-3′ and 5′ -CCT GCG AAA GCC C**tt** TTA GAG

TGC - 3′; for inverse CCAAT box (change ATTGG to CCAAT, CA-Inverse), primers were 5′ -GCA CTC T**ac caa t**GC TTT CGC AGG - 3′ and 5′-CCT GCG AAA G**ca ttg g**TA GAG TGC- 3′.

Electrophoresis Mobility Shift and Super-Shift Assay (EMSA)—Nuclear extracts were prepared from HepG2 cells as previously described (*13*). Protein concentration was determined using Dye Reagent Concentrate (Bio-Rad). A fragment of 5′ flanking region of the PNRC gene, −123/+27, that contains the NFY-binding site, was amplified by PCR and used as a probe in the gel-shift and super-shift assays. This probe was labelled with [γ-^{32}P-ATP] in the presence of T4 polynucleotide kinase (New England Biolabs) and purified using a ProbeQuant G-50 column (Amersham).

Binding reactions were performed in a 15 μl reaction mixture containing gel shift binding 5× buffer [5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl (pH7.5), 25 mg/ml poly(dI–dC), 20% glycerol] and 5 μg of nuclear extract. In the competition experiments, unlabelled competitors as indicated in Fig. 4 were added to the reaction mixtures. The mixtures were incubated at room temperature for 10 min. In the super-shift experiments, 4 μg of a polyclonal antibody against NFYA (Santa Cruz Biotechnology, CA, USA) or 4 μg of control normal IgG was added to the reaction mixtures and incubated on ice for 30 min. After binding reactions, the 6,000 c.p.m. of ^{32}P-labeled probe was added to the mixture and the mixtures were incubated at 25°C for another 20 min. After addition of 1 μl of gel loading 10× buffer per reaction, the reaction products were analysed on a 4% non-denaturing polyacrylamide (59:1 acrylamide:bisacrylamide) gel. At the end of electrophrosis, the gel was dried in gel dryer and the dried gel was exposed to X-ray film (Kodak) overnight at −70°C with intensifying screen.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP assay was performed using ChIP Assay kit (Upstate) according to the manufacturer's instructions. Briefly, ~10^6 cells were treated with 1% formaldehyde (final concentration, v/v) in cell culture medium for 10 min at 37°C to cross-link proteins to DNA. The medium was aspirated and the cells were washed twice with ice-cold PBS containing protease inhibitor cocktail (Roche Molecular Biochemicals). The cells were harvested by centrifugate for 4 min at 2,000 r.p.m. at 4°C, and resuspended in 200 μl SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1) and protease inhibitor cocktail] and incubated on ice for 10 min. To shear DNA to lengths between 200 and 1,000 bp, the cell lysate was sonicated with a Digital Sonifier (Dranson Ultrasonics Corporation, CT, USA) at 30% of maximum power 10 times for 15 s each, with 2 min of cooling on ice in between sonications.

The sonicated DNA was diluted 10-fold in ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl (pH 8.1), 167 mM NaCl and protease inhibitor cocktail]. A portion of the diluted cell supernatant 1% (~20 μl) was kept as input for PCR. The rest of the supernatant was pre-cleared by incubation with protein A Sepharose beads for 30 min at 4°C with agitation to reduce non-specific background. Agarose was pelleted by brief centrifugation and the supernatant fraction was transferred into a fresh tube. The antibodies against NFYA (2 μg each) was added to the supernatant fraction and incubated overnight at 4°C with rotation. The negative controls, with no antibody immunoprecipitation or normal rabbit IgG immunoprecipitation, were included. Immunocomplexes were precipitated for 2 h with protein A Sepharose beads. Then, the beads containing specific precipitates were collocated and washed once with 1 ml of low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 150 mM NaCl], once with 1 ml of high-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), and 500 mM NaCl], once with LiCl wash buffer [0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1] and twice with TE buffer. All washes were for 5 min, rotating at 4°C. The protein–DNA complexes were eluted from beads with 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) at room temperature for 15 min with rotation. Formaldehyde cross-links were reversed by overnight incubation at 65°C and the proteins subsequently degraded by proteinase K. DNA was recovered by phenol–chloroform extraction and ethanol precipitation in the presence of 0.3 M sodium acetate and 20 μg glycogen. PCR amplification of a 150 bp (−123/+27) of the human PNRC promoter region, which harbours the NFY-binding site, was performed using a sense primer, 5′ -CGGAGGCGGAGCGAGG-3′, and an anti-sense primer, 5′ - TGACTCGGGCGGTGGC - 3′.

For additional control of non-specific binding, another PCR amplification reaction of the anti-NFY precipitated chromatin DNA was also performed to detect a 150-bp fragment that is corresponding to exon II of the human PNRC gene and locates about 2.6 kb downstream from the −123/+27 promoter region. This control PCR reaction was performed using a sense primer, 5′ -TAG TCA CTG GAT GGG AAG CAC-3′, and an anti-sense primer, 5′ -AAG AGT CCA GGG ATA TGG GAA- 3′. PCR was performed using Hot StarTaq DNA polymerase (Qiagen) as following conditions: Initial denaturation for 15 min at 95°C followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 30 s, ending with a final 7 min extension at 72°C. The PCR products were resolved electrophoretically on a 2% agarose gel and visualized with EtBr staining.

Western Blot Analysis—Fifty micrograms of nuclear extract was analysed by 12% SDS–PAGE. Proteins were transferred to PVDF Membrane and the membrane was blocked 1 h with 5% milk in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.5% Tween-20). The membrane was then incubated with the affinity purified rabbit anti-NFY (1:100) IgGs or anti-β-actin antibody (1:2,000) at 4°C overnight, and HRP-conjugated goat anti-rabbit antibodies for an additional 1 h at room temperature. After washing three times in TBST, enhanced chemiluminescence reagents (ECL) were used for the final detection.

RESULTS

Genomic Organization of the Human PNRC Gene—Human PNRC mRNA sequence (NM 006813) which was obtained by searching GenBank was used to perform human genome Blat searching by using UCSC human

genome browser to obtain information regarding the genomic organization of the human PNRC gene. Through alignment of human genomic sequence with human cDNA sequence (NM 006813), the human PNRC gene is defined to be located on chromosome 6 and comprised of 2 exons and 1 intron: 657-bp exon I, 1,048-bp exon II and 2,319-bp intron I. The translational start site is located in exon I. The 984-bp coding sequence in exon I and exon II encode 327 amino acids (Fig. 1A). Each intron begins with a GT and ends with an AG, confirming to the GT/AG rule.

Determination of the Transcription Start Site of Human PNRC Gene—Ambion's First Choice 5′ RLM-RACE kit was used to determine the transcription initiation site of human PNRC gene of in HepG2 cells (Fig. 2). The relative positions of all the primers used for 5′ RACE analysis are shown in Fig. 2A. The results of the analysis of 5′ RACE are shown in Fig. 2B. A 300-bp fragment was obtained from nested PCR reactions using the 5′ RACE adapter inner primer and PNRC reverse inner primer pair and cDNA template derived from tobacco acid pyrophosphatase (TAP)-treated HepG2 cells RNA (Fig. 2B, lane 2). No product was obtained when RNA was not treated with TAP (lane 3), indicating that the 300-bp product was derived from full-length mRNA. As a control to validate the assay, the expected 248-bp (lane 4) and 412-bp product (lane 5) were obtained from cDNA template that derived from TAP-treated RNA using human PNRC primers, control forward primer 5′ -TCT TCT CAG GCT CTC CTA GCA GCA T- 3′ and reverse inner primer 5′ -ACC TTC TTC TTT CGC CGC TTC TT- 3′ or reverse outer primer 5′ -ACC TTC TTC TTT CGC CGC TTC TT- 3′, respectively. A sample of the outer PCR using 5′ RACE outer primer and PNRC outer primer (lane 1) was also run for evaluation. The 300-bp PCR product was subcloned into pCRIII-TOPO TA cloning vector (Invitrogen) and the resulting clones containing the PCR inserts were sequenced. The result showed that the major human PNRC transcription initiation site was mapped at the nucleotide G, 27 nt more 5′ upstream from the 5′ end of

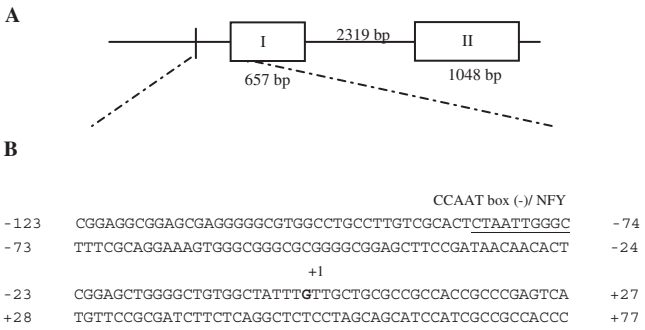

Fig. 1. **Genomic organization of human PNRC gene and its 5′ flanking sequence.** (A) A schematic representation of exon–intron structure of the human PNRC gene. A human PNRC mRNA (GenBank Accession No. NM 006813) was used for the determination of genomic structure of PNRC gene by Blat search using UCSC human genome browser. The human PNRC gene, located on human chromosome 6, consists of two exons, indicated by the boxes. Sizes of exons I, exons II and intron I are given in base pairs. (B) Nucleotide sequences of the 5′ flanking region of the human PNRC gene. The sequence shown contains 123 nt 5′to the major transcription start site, nucleotide G (+1) determined in this study. The major transcription initiation site (identified by our 5′ RACE analysis) is Guanine nucleotide and is designed +1 and in bold. Putative binding site for transcription factor is underlined and identified above the sequence.

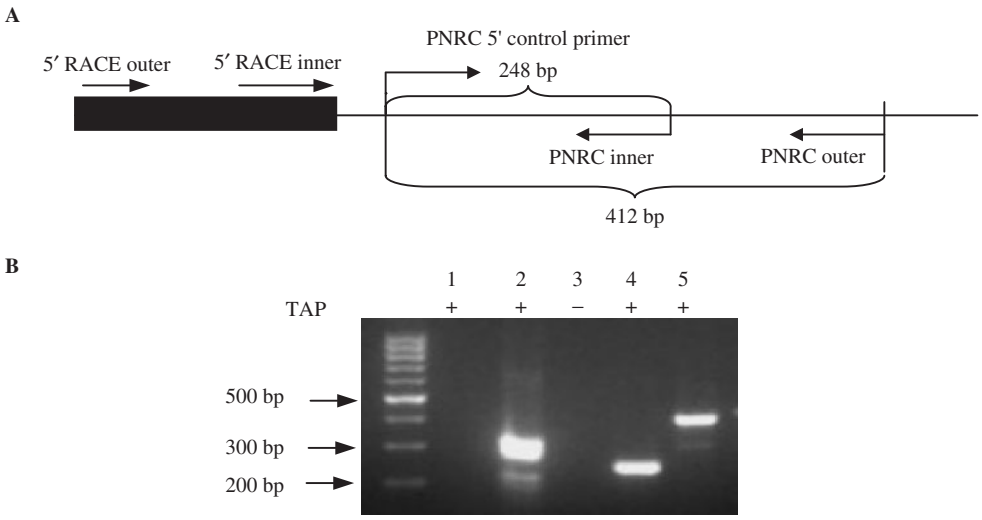

Fig. 2. **Identification of transcription initiation site by 5′ RACE analysis.** (A) The relative positions of all the primers used for 5′ RACE analysis and the expected distances between primers are shown. (B) The results of the 5′ RACE of total RNA isolated from HepG2 cells. Ten-microgram total RNA was sequentially treated with calf intestinal alkaline phosphatase, tobacco acid pyrophosphatase and then ligated to a designated RNA adapter provided in Ambion 5′ RLM-RACE kit. Following nested PCR reactions, a 300-bp PCR product was detected by gel electrophoresis in 2% agarose (lane 2, primers 5′ RACE inner and PNRC inner). No product was obtained when RNA was not treated with TAP (lane 3). As control, an expected 412-bp product (lane 5) was obtained when the primers PNRC 5′ control and PNRC outer were used. An expected 248-bp product (lane 4) was also obtained from the PCR reaction using the primers PNRC 5′ control and PNRC inner. A sample of the outer PCR using 5′ RACE outer primer and PNRC outer primer (lane 1) was also included in the gel.

the longest PNRC EST in available databases. This major transcription initiation site is indicated in bold letter and is designed +1 (Fig. 1B).

Identification and Characterization of the Human PNRC Gene Promoter—To map the minimal promoter activity and the regulatory sequences, a series of luciferase reporter constructs containing various 5′ flanking region deletions of PNRC gene were generated (Fig. 3, left panel) by PCR, based on the information of both transcription factor-binding site and the PNRC gene transcription start site. Promoter activity for each PNRC gene fragment is shown in the right panel of Fig. 3. Transfection of the longest PNRC fragment in pGL3h PNRC (−1,373/+727) reporter construct into HepG2 cells resulted in a luciferase activity near to that of empty pGL3 Basic reporter vector (no promoter, no enhancer). Deleting the region from −1,373 to −724, −723 to −324 and −323 to −124, [construct pGL3hPNRC (−723/+727), pGL3hPNRC (−323/+727), pGL3hPNRC (−123/+727)] resulted in 2-fold, 4-fold and 6.3-fold increase in the promoter activity, suggesting that the region from −1,373 to −123 harbours negative-response elements. Further deletion of the sequence from −123 to −24 [construct pGL3hPNRC2 (−23/+727)] resulted in the complete loss of the promoter activity, suggesting that the region of −123 to −23 contains the core elements necessary for the basal promoter function of the human PNRC gene. Several constructs with the deletion from 3′end were also generated. Deletion of the region +727 to +327 [construct pGL3hPNRC (−123/+327)] resulted in 16.6-fold increase in the promoter activity. Deletion of the region +327 to +57 [construct pGL3hPNRC (−123/+57)] resulted in 120-fold increase in the promoter activity, respectively, further deletion of the sequence from +57 to +27 [construct pGL3hPNRC (−123/+27)] resulted in 86-fold increase in the promoter activity, respectively. These results suggest that the region from +727 to +27 harbours negative-response elements. When further deletion of +27 to −23 [construct pGL3hPNRC (−123/−23)], this deletion almost completely abolished the promoter activity. Taken together, our deletion and transfection results demonstrated that the minimal human promoter activity is located in the region of −123/+27.

Specific Binding of NFY Protein to the Human PNRC Promoter in vitro and in vivo—As shown in Fig. 1B, the region spanning −123/+27 harbours one CCAAT box (NFY-binding site). To identify protein interacting with the −123/+27 sequences, EMSA was performed by using the nuclear extract prepared from HepG2 cells and a probe spanning −123/+27 that contains the NFY-binding site. As shown in Fig. 4, at least five DNA–protein complexes, marked as A, B, C, D and E were formed between the probe and the nuclear proteins in HepG2 cells (lane 2). All five of the complexes were competed by the addition of a 50-fold molar excess of unlabelled probe DNA (lane 3), but not competed by non-specific competitor DNA, poly(dI–dC) (lane 6), demonstrating that all the complexes are the sequence-specific complexes between −123/+27 DNA and the nuclear proteins. These complexes could also be competed by the oligonucleotide that contains wild-type inverted CCAAT box sequence (*i.e.* NFY-binding site, lane 5),

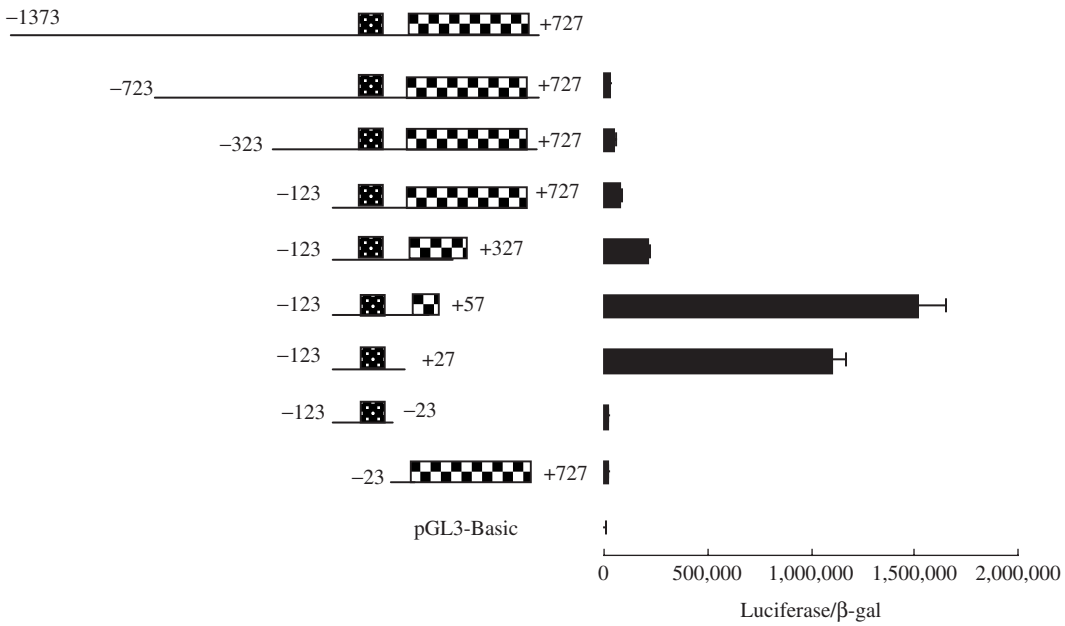

Fig. 3. **Identification of the minimal promoter region of the human PNRC gene.** Left panel, schematic representation of 5′- or 3′-deleted fragments of the human PNRC promoter in conjunction with the luciferase gene in pGL3 basic vector. The nucleotides are numbered from the transcriptional starting site that was assigned +1. Right panel, the activities of deleted promoter constructs were determined by luciferase assays. HepG2 cells were transiently transfected with 1 μg DNA in 12-well dish by lipofectiamine 2000 (2 μg) (Invitrogen). Twenty-four hours after transfection, luciferase activity was measured using luciferase assay substrate. The relative luciferase activities were calculated as luciferase activities/β-galactosidase activities. Results represent three independent experiments performed in triplicates.

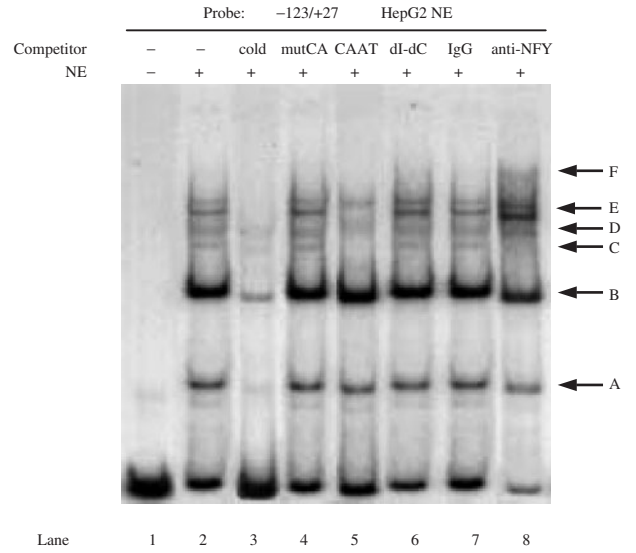

Fig. 4. **Electrophoretic mobility shift and super-shift assays of NFY binding to hPNRC gene promoter.** The DNA probe, corresponding to nucleotides −123/+27, was amplified by PCR using pGL3-hPNRC (−123/+27) Luc reporter plasmid as template, CIP treated and end-labelled with γ-^{32}P-ATP and T4 kinase. The labelled probe was incubated with or without 5 μg of nuclear extract prepared from HepG2 cells in the absence or presence of various competitor DNA or antibodies, as indicated on the top of the gel image. Separation of the free and bound probes was done by electorphoresis on a 4% 59:1 polyacrylamide gel. The specific DNA–protein complexes and super-shift probe band are indicated by arrows.

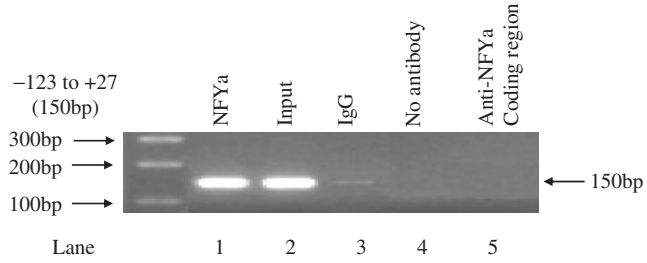

Fig. 5. **ChIP analysis to detect *in vivo* association of nuclear transcriptional factors NFY to the hPNRC promoter.** HepG2 cells were treated with formaldehyde to cross-link endogenous proteins and DNA. Samples of sonicated chromatin were immunoprecipitated with no antibody, preimmuno IgG (IgG), anti-NFYA antibody as indicated. DNA isolated from immunoprecipitated material was amplified by PCR with primers to amplify the 150-bp human PNRC promoter sequences corresponding to the −123 to +27 region or the 150-bp exon II region (coding region). The amplified PCR fragments were analysed on 1.5% agarose gel.

but not competed by the mutated CCAAT oligonucleotide (mutCA: 5′- TCG CAC TCT AAAAGG GCT TTC GCA G-3′) (lane 4), indicating that NFY protein present in HepG2 cells binds to the probe used in the gel shift assay. The binding of NFY protein was further confirmed in a super-shift reaction. The addition of anti-NFY antibody to the binding reaction furthermore caused a loss of complex C, a density increase of complexes D and E, and a super-shift band F (lane 8). As expected, normal mouse IgG did not compete with any of the complexes (lane 7).

To determine whether NFY interact with the human PNRC promoter *in vivo*, we performed ChIP assay (Fig. 5). Proteins were cross-linked to genomic DNA in HepG2 cells using 1% formaldehyde, then the chromatin was sonicated and immunoprecipitated with normal rabbit IgG, or polyclonal antibody NFYA. Before the immunoprecipitations, a small aliquot of chromatin was saved and used as an input control. The precipitated DNA was subjected to PCR utilizing primers designed to amplify a 150-bp fragment (−123/+27) of the 5′ flanking region of the human PNRC gene. As shown in Fig. 5, the 150-bp DNA fragment was only amplified from the precipitates by anti-NFYA (lane 1) and the input chromatin DNA (positive control) (lane 2). In contrast, the PNRC promoter fragment was not amplified from negative control immunoprecipitations using no antibody (lane 3) or normal rabbit IgG (lane 4, very weak band). To further rule out the possibility that the PNRC promoter region precipitated by anti-NFY antibody is due to a non-specific binding of NFY antibody, an additional PCR amplification of a distinct genomic region 2.6-kb downstream of PNRC promoter was performed on the anti-NFY precipitated chromatin DNA. As shown in Fig. 5, lane 5, this region was not precipitated by anti-NFY antibody, demonstrating that NFY specifically bind to the PNRC promoter region. To validate that the DNA fragment amplified was the expected 150-bp area of the human PNRC promoter region, this fragment was subcloned into TA cloning vector and then sequenced. Sequence data confirmed that the PCR product has the exact sequence of PNRC promoter-containing fragment, −123/+27 (data not shown). These results demonstrated the recruitment of NFY transcriptional factor to the human PNRC promoter *in vivo*.

Regulation of PNRC Transcriptional Activity by NFY— After demonstration of the specific binding *in vitro* and *in vivo* of NFY to the promoter region of the human PNRC gene, the function of the binding by this transcription factor was also checked. The NFYs expression vector was co-transfected along with the PNRC promoter luciferase reporter plasmids into HepG2 cells. As shown in Fig. 6A, co-transfection of pGL3hPNRC (−123/+57) reporter plasmid and NFYA expression plasmid decreased the activity of PNRC promoter by 2-fold, while NFYB or NFYC did not affect, or only slightly decreased, the promoter activity. When HepG2 cells were co-transfected with the expression plasmids for all three NFY subunits along with the pGL3hPNRC (−123/+57) reporter plasmid, the luciferase activity in these transfected cells was 3.9-fold lower than that in the cells transfected with human PNRC promoter alone. Furthermore, NFY inhibited the luciferase activity driven by the human PNRC promoter in a dose-dependent manner (Fig. 6B). However, co-transfection of the NFY expression plasmids along with reporter plasmid that contains the mutated NFY-binding site (CA-Mut and CA-Inverse) did not inhibit, rather slightly stimulated, the luciferase activity (Fig. 6C). These results demonstrated that NFY functions as a transcriptional repressor in PNRC transcription through the binding to the CCAAT sequences within PNRC promoter region.

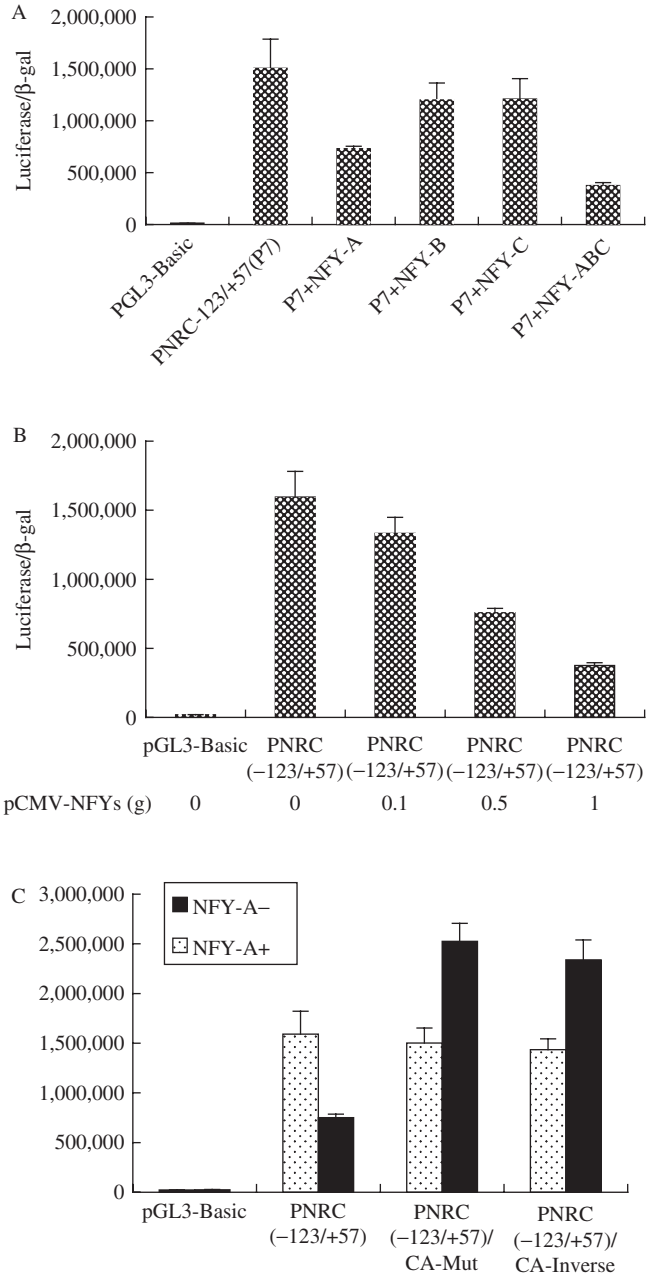

Fig. 6. **Regulation of hPNRC promoter by NFY.** (A) NFY represses the transcription activity of the human PNRC promoter. HepG2 cells were transfected with the pGL3-PNRC (−123/+57) (1.0 μg) alone or together with 1.0 μg of the expression plasmid for NFYA, NFYB or NFYC, separately or combination of three NFY subunits. (B) NFY represses the transcription activity of human PNRC promoter in a dose-dependent manner. HepG2 cells were transfected with pGL3-PNRC (−123/+57) and the increasing amount of expression plasmids for all three NFY subunits (ratio of three subunits was 1 : 1 : 1). (C) The NFY-binding site is responsible for NFY-mediated transcriptional repression of the human PNRC promoter. HepG2 cells were co-transfected with 1.0 μg of either pGL3-PNRC (−123/+57) or the mutant constructs [pGL3-PNRC (−123/+57)/CA-Mut or pGL3-PNRC (−123/+57)/CA-Inverse] and 1.0 μg of pCMV-NFY-A (NFY-A+) or empty pCMV vector (NFY-A-). Luciferase assays were performed in triplicate and the relative luciferase activities (Luciferese/β-gal) are expressed as an average of three independent experiments with the SEM.

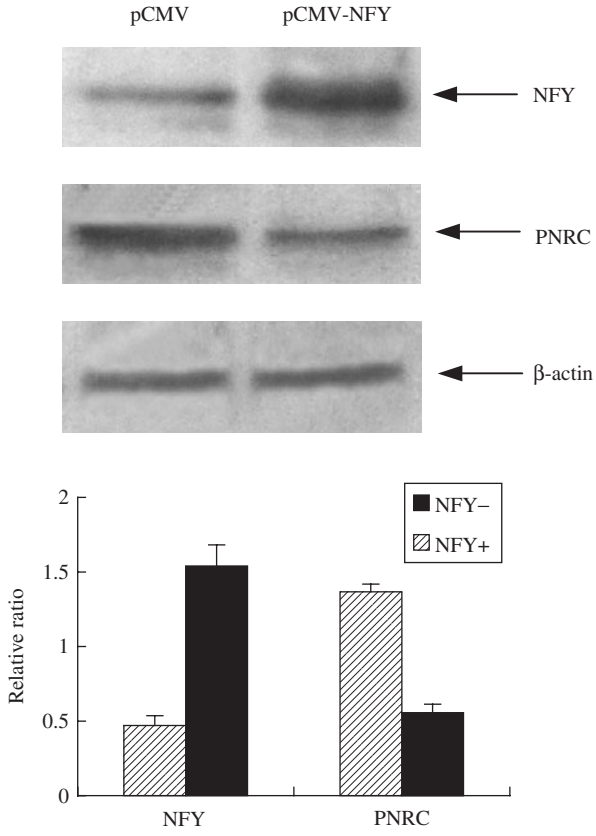

Fig. 7. **Western blot analysis to detect PNRC expression in HepG2 cells.** When HepG2 cells were transfected with pCMV-NFYA, the level of PNRC protein in HepG2 cells showed decrease. Total protein extracts (50 μg) from HepG2 cells transfected with pCMV vector, pCMV-NFYA. Incubated with the anti-NFYA, anti-PNRC and anti-β-actin antibodies. Quantitative densitometry of the immuno-images was performed using Bio-Rad densitometer (Imaging Densitometer, Model GS-700) with specific software (Molecular Analyst) and showed as the ratios to the density of β-actin bands.

Over-expression of NFY Decreases PNRC Expression Levels In Vivo—To investigate whether over-expression of NFY regulates PNRC expression levels *in vivo*, HepG2 cells were transiently transfected with the expression plasmid of NFY. The levels of PNRC protein in transfected HepG2 cells were then analysed by western blot. Contrast to the group that transfected with the empty vector, pCMV, the level of PNRC expression was decreased in the group that transfected with the expression plasmid of NFY (Fig. 7). These results further demonstrated that NFY negatively regulates the expression of PNRC in HepG2 cells.

DISCUSSION

PNRC is a unique nuclear receptor co-activator that interacts with nuclear receptors through its SH3-binding motif. It plays an important role in regulation of gene transcription. Therefore, it is important to understand the mechanisms of PNRC gene expression. In this study, we have identified and characterized the human PNRC

promoter and found that one important transcription factor, NFY, regulated the promoter activity of the human PNRC gene.

After the transcriptional start site was verified, the 5′ flanking region of the human PNRC gene was amplified and analysed in detail for minimal promoter localization utilizing a series of 5′ and 3′ deletions of the PNRC 5′flanking region fused upstream of the firefly luciferase reporter gene. These experiment identified the minimal promoter region, −123/+27, that is necessary for the expression of human PNRC gene. Sequence analysis of −123/+27 region revealed that there is an inverted CCAAT box sequences (NFY-binding site) (Fig. 1B). We therefore determine to examine whether transcriptional factor NFY binds to its consensus sequences in PNRC gene and thereby regulates the transcription of PNRC gene. One technique that is central in studying gene regulation and determining protein–DNA interactions is the EMSA. To verify the specific binding of the NFY transcription factor to the PNRC promoter −123/+27 region, we carried out the EMSA analysis. Our results from EMSA demonstrated that there were five sequence-specific DNA–protein complexes caused by the binding of specific protein to PNRC promoter region as shown in Fig. 4, lane 2, and some of these complexes were probably caused by the binding of NFY as that the oligos carrying the mutated NFY-binding site did not compete the binding. We understand that the change in relative mobility does not identify the bound proteins. Therefore, we have carried out the super-shift assays using NFY antibody to confirm the binding of this protein to the promoter region. In addition to forming a relative less clear, less sharp super-shift band F (Fig. 4), the addition of antibodies repeatedly caused the disappearance of the complex C and density increase of complexes D and E.

ChIP analysis is a powerful assay that defines the interaction or the recruitment of transcription factors with specific chromatin sites in living cells, thereby providing a snapshot of the native chromatin structure and factors bound to genes in different functional stages. In our ChIP assays, NFY was demonstrated to associate with the DNA sequence of −123/+27 of the human PNRC gene in HepG2 cells (Fig. 5). Taken together, the results from gel shift binding and super-shift assay, ChIP analysis and mutagenesis/luciferase assay, transcription factor NFY was demonstrated to bind to their binding site in the promoter region of the human PNRC gene.

To address the function of the NFY binding to PNRC promoter region, we utilized a transient co-transfection assay with PNRC promoter region containing NFY-binding site along with the expression plasmid for NFYs. NFY was found to suppress the promoter activity of the human PNRC gene (Fig. 6). We also found over-expression of NFY in HepG2 cells that decreases the expression of PNRC protein *in vivo* (Fig. 7).

NFY is a ubiquitous transcription factor which binds with high specificity to CCAAT motifs in the promoters of genes. NFY consists of three subunits, NFYA, NFYB and NFYC, all of which are essential for DNA binding. The NFYB and NFYC subunits form a tight heterodimer that offers a complex surface for NFYA association. The N-terminal part of NFYA is required for association with the NFYB/NFYC heterodimer, while the NFYA C-terminus is required for DNA binding (*14*). Therefore, NFYA represents the regulatory subunits of the NFY trimer (*15*). In this study, NFYA was found to be the dominant subunit in repressing the promoter activity of human PNRC gene.

It has been suggested that binding of NFY factor to the promoter alone is not able to exert its effect on transcription (*16*), unless it interacts with relevant protein factors, including Sp1 (*17*), c-myb (*18*), SREBP-1 (*19*) and GATA-1 (*20*). NFY functions as both an activator and a repressor, depending on its interacting co-factors. The activation effect of NFY can be mediated through its direct interaction with histone acetylases (*21*), such as p300 (*22*), or by facilitating the binding of TFIID to the core promoters (*23*, *24*). NFY also recruits transcriptional repressors to negatively regulate gene expression. For example, NFY directly recruits histone deacetylases (HDACs) to repress the expression of the von Willebrand factor in non-endothelial cells (*25*). In our co-transfection experiment, NFY was shown to repress the promoter activity of human PNRC gene.

There is an increasing evidence suggesting a possibility of PNRC as a tumour-related gene and that the regulation of PNRC expression plays a role in human multistage carcinogenesis. In our study, the expression of PNRC was regulated by NFY protein, but the biological significance of this finding is not clear at the present time. Future experiments will be aimed at revealing the mechanism of regulation of PNRC expression, and the possible role of PNRC in the cell cycle progression and tumour suppression.

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