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The cyclic AMP response element plays an essential role in the expression of the human prostaglandin-endoperoxide synthase 2 gene in differentiated U937 monocytic cells

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Abstract

The promoter activity of 1432 bp upstream of the human prostaglandin-endoperoxide synthase 2 gene (*PTGS2*) was examined in differentiated U937 monocytic cells expressing prostaglandin-endoperoxide synthase 2 mRNA. Transient transfection experiments were performed using these cells and reporter vectors containing the upstream region of the gene with deletions or site-specific mutations and the luciferase gene. The deletion or destruction of the cyclic AMP response element (nucleotides −59 to −53) markedly reduced the promoter activity of this gene. Electrophoretic mobility shift assays showed that a nuclear protein(s) binding to the cyclic AMP response element was induced during monocytic differentiation of U937 cells. These results indicate that expression of the human prostaglandin-endoperoxide synthase 2 gene in differentiated U937 monocytic cells is regulated by the cyclic AMP response element.

Key words: Prostaglandin-endoperoxide synthase; Cyclooxygenase; Gene expression; Cyclic AMP response element; U937 cell

1. Introduction

Prostaglandin-endoperoxide synthase (PES) catalyzes the first committed step of the biosynthesis of prostaglandins, prostacyclin and thromboxane which are potent biological mediators [1,2]. It is now evident that there exist two distinct isozymes for PES: a well-characterized constitutive isozyme, designated PES-1 or cyclooxygenase-1; and a newly discovered inducible isozyme, PES-2 or cyclooxygenase-2. The human [3] and mouse [4] PES-2 genes spanning 8.0–8.3 kb are smaller than the human [5,6] and mouse [7] PES-1 genes being 22 kb in size and consisting of 10 exons. The exon structures of the PES-2 and -1 genes are similar except for the first and last exons [3,5,7]. The human PES-2 gene was mapped to chromosome 1q25.2-q25.3 whereas the PES-1 gene was mapped to chromosome 9q32-q33.3 [3], indicating that the two genes are not linked. It is noteworthy that PES-2 mRNA is rapidly induced by a variety of factors, i.e. inflammatory mediators, growth factors, mitogens and gonadotropin [8–11].

The nucleotide sequence of the 5′-flanking region of the human PES-2 gene contains a canonical TATA box and various transcriptional regulatory elements [3] but does not show significant similarity to the 5′-flanking regions of human and mouse PES-1 genes which lack a TATA box [6,7]. The nucleotide sequences of the human [3], mouse [4] and rat [11] PES-2 genes in their 5′-flanking regions share 60–63% similarity among the 275-bp nucleotide residues upstream of the transcriptional start site. It is noteworthy that in all three genes the sequences homologous to the consensus NF-IL6 site and the cyclic AMP response element (CRE) are conserved. From this study using human differentiated U937 monocytic cells we report that the conserved CRE in the human PES-2 gene plays an essential role in the efficient promoter activity of the gene.

2. Materials and methods

2.1. Cell culture

U937 cells were supplied by the Japanese Cancer Research Resources Bank and maintained in suspension in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) ('complete culture medium') at 37°C in a humidified atmosphere of 5% CO_2 in air. For differentiation, U937 cells were plated at 1.2×10^7 cells/150 mm dish in the medium containing 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) and allowed to adhere for 48 h.

2.2. RNA blot analysis

RNA blot analysis was performed as described previously [3]. Total RNA was isolated according to the acid guanidinium thiocyanate procedure [12] and 20 μg/lane of the total RNA was used for the electrophoresis. The probe used was the entire 1.5-kb insert of the recombinant plasmid pHPESII17 encoding the human PES-2 cDNA [3].

2.3. Plasmids

pGV-B, the promoterless luciferase reporter vector, and pGV-C, the luciferase reporter vector under the control of the SV40 promoter/enhancer were purchased from Toyo Inc., Tokyo. pCMV-βgal, the

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Abbreviations: PES, prostaglandin-endoperoxide synthase; CRE, cyclic AMP response element; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; NF-IL6, nuclear factor for interleukin-6 expression; NF-κB, nuclear factor κB.

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β-galactosidase reporter vector under the control of the human cytomegalovirus promoter, was obtained from Karl Normington (University of Texas Southwestern Medical Center at Dallas). These expression vectors were used as controls in the transfection experiments. The *Hae*II-digested fragment, corresponding to nucleotide positions −327 to +59 from the transcription start site of the human PES-2 gene, was isolated from the recombinant phage containing the human PES-2 genomic sequence (λhPESII95) [3], blunt-ended and then subcloned into the *Sma*I site of pBluescript II SK(+) (Stratagene). Then, the inserted fragment digested with *Bam*HI and *Hin*dIII from the subcloned plasmid DNA was ligated into the *Bgl*II and *Hin*dIII sites of pGV-B DNA and this construct was designated phPES2(−327/+59) (Fig. 2A). The construct phPES2(−1432/+59) was prepared using phPES2(−327/+59) and the *Kpn*I–*Hin*dIII restriction fragment of the human genomic PES-2 clone. Fragments coding for the nucleotide residues −52/+59, −124/+59 and −220/+59 were generated by the polymerase chain reaction (PCR) using respective primer sets P6 (5′-TTgagCtCATGGGCTTGGTTTTCAGT-3′)/P7 (5′-CCTCTAGAG-GATAGAATGGG-3′, originated from the sequence of the pGV-B vector), P4 (5′-TTggtacc-TTTTTTTAAGGGGAGAGGA-3′)/P7 and P2 (5′-AGAGTGcac-ACTACCCCCTCTGCTCCCAA-3′)/P7 and phPES2(−327/+59) as a template (Fig. 2A). Lower case letters in the nucleotide sequences described above represent the nucleotide residues which have been changed to introduce new restriction enzyme sites. The mutant constructs were prepared by insertion of PCR-amplified nucleotides into pGV-B. Namely, the amplified DNA fragment using the primer set P6/P7 or P4/P7 was digested by *Hin*dIII/*Sac*I or *Hin*dIII/*Kpn*I and inserted into the *Hin*dIII/*Sac*I or *Hin*dIII/*Kpn*I sites of pGV-B, respectively (Fig. 2A). In addition, the amplified DNA fragment using the primer set P2/P7 was digested by *Alw*44I, blunt-ended by T4 DNA polymerase, digested by *Hin*dIII, and then inserted into the *Hin*dIII/*Sma*I sites of pGV-B (Fig. 2A). These deletion constructs thus prepared were designated phPES2(−52/+59), phPES2(−124/+59) and phPES2(−220/+59). Mutant constructs, phPES2(CRM) with a mutation at CRE and phPES2(ILM) with a mutation at NF-IL6 site were prepared by ligation of each PCR product using primer set P1 (5′-TTGTCC AAACTCATCAATG-3′, originating from the sequence of pGV-B)/P5 (5′-atGaGctc AAATGACTGTTTCTTTCCG-3′) or P1/P3 (5′-AAggtaccAAGCCCGGTGGGGG CAGGG-3′) and phPES2(−52/+59) or phPES2(−124/+59) as shown in Fig. 2A. The oligonucleotide primers were synthesized on an Applied Biosystems 391 DNA synthesizer. Each construct was verified by DNA sequence analysis as described in [3].

2.4. DNA transfections

A total of 1.4×10^7 U937 cells differentiated by TPA were transfected with 30 μg of each reporter plasmid, 10 μg of pCMV-βgal and 100 μg of sonicated salmon sperm DNA by electroporation at 300 V and 960 μF in 700 μl of Dulbecco's phosphate-buffered saline, using a Bio-Rad Gene Pulser. Expression of β-galactosidase derived from pCMV-βgal was used as an internal control of the transfections. Cells were resuspended in the complete culture medium and cultivated for 20 h. Cells were harvested, and luciferase and β-galactosidase activities were determined by a luminometer (Berthold) [13] and a method using chlorophenol red β-D-galactopyranoside as substrate [14], respectively. Experiments were repeated at least three times. The promoter activity in undifferentiated U937 cells could not be evaluated because of the very low transfection efficiency.

2.5. Nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared as described by Andrews and Faller [15]. The following oligonucleotides were made by a DNA synthesizer: 5′-AAACAGTCA TTTCGTCACATGGGCTTG-3′, containing the consensus CRE sequence of the human PES-2 gene (indicated by underline; PES-2CRE); 5′-AAACAGTCA TTTgagCtCATGGGCTTG-3′, containing a four-point mutation (indicated by lower case letters) within the consensus CRE sequence by introducing the *Sac*I site (PES-2CRM). Complementary oligonucleotides synthesized separately were annealed in 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol. The annealed oligonucleotide PES-2CRE was phosphorylated at the 5′-end with [γ-^{32}P]ATP (110 TBq/mmol, Amersham) and T4 polynucleotide kinase. Electrophoretic mobility shift assays using synthesized oligonucleotides were carried out as described [13].

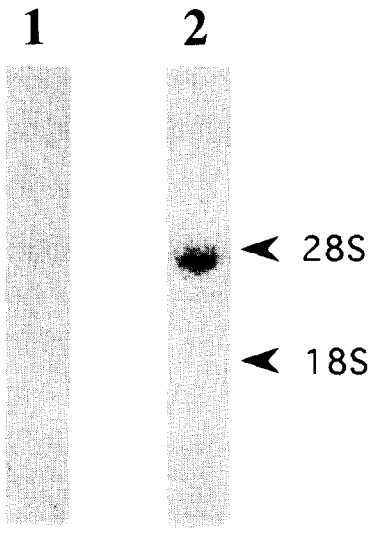

Fig. 1. Expression of the human PES-2 gene. RNA blot analysis of total RNAs (20 μg/lane) from undifferentiated U937 cells (lane 1) and from the monocytic differentiated U937 cells treated with TPA for 48 h and cultured overnight in TPA-free medium (lane 2). The RNA markers are 18 S and 28 S ribosomal RNAs.

3. Results and discussion

3.1. Expression of PES-2 mRNA in differentiated U937 human monocytic cells

Human monoblastoid U937 cells differentiate into macrophage-like cells following incubation with TPA for 48 h [16]. TPA-induced differentiation results in the cessation of proliferation of cells and alteration in their morphology from non-adherent to adherent cells. Previously the rapid and transient induction of PES-2 mRNA by TPA was reported with HEL cells [17], Swiss 3T3 cells [8] and human umbilical vein endothelial cells [9]; the mRNA levels increased maximally within 2–6 h after the stimulation and decreased rapidly. The human monoblastoid U937 cells, however, did not show such a rapid and transient increase of PES-2 mRNA by TPA treatment, and a gradual and stable increase in mRNA was observed after the treatment of the cells for 24 h (data not shown). Therefore, we treated the cells with TPA for 48 h and cultured them overnight in TPA-free medium. RNA blot analysis showed that these cells expressed detectable levels of PES-2 mRNA with an expected size of about 4 kb [9] as shown in Fig. 1. The PES-2 mRNA was undetectable in undifferentiated U937 cells. Thus, the transfection experiments were carried out using U937 cells treated with TPA for 48 h, and after transfection the cells were maintained in TPA-free medium for 20 h prior to harvest.

3.2. Functional activity of the human PES-2 promoter in differentiated U937 monocytic cells

To determine if the 5′-flanking region of the human PES-2 gene contains functional domains for its pro-

moter, transient DNA transfection experiments using luciferase as a reporter gene in differentiated U937 cells were performed using deletion and site-specific mutants of the human PES-2 promoter (Fig. 2). As shown in Fig. 2B, the luciferase activity observed with the reporter vector phPES2(−1432/+59) or phPES2(−327/+59) in the transfected cells was about 10-times higher than that with pGV-C. The luciferase activity derived from the pGV-C expression vector with the SV40 promoter/enhancer was about 5-times higher than that derived from a promoterless expression vector pGV-B (data not shown). These results show that the functional promoter region is located within the 5′-flanking sequence −327/+59 from the transcription start site which contains the consensus sequences for NF-κB, NF-IL6 and CRE.

Therefore, the consensus sequences for NF-κB (−233/−214), NF-IL6 (−132/−124) and CRE (−59/−53) in the proximal 5′-flanking region of the human PES-2 gene [3] were deleted singly or in combinations in order to analyze their roles in controlling the expression of the human PES-2 gene (Fig. 2A). The luciferase activity using a deletion mutant reporter vector phPES2(−52/+59) with no consensus sequence for NF-κB, NF-IL6 and CRE was only about 5% of that using a wild-type vector phPES2(−327/+59), whereas luciferase activities using phPES2(−124/+59) with only the CRE sequence and phPES2(−220/+59) with both NF-IL6 site and CRE sequences were about 77% and 70% of that using phPES2(−327/+59), respectively.

These results suggested that the CRE is essential for efficient transcription of the human PES-2 gene in TPA-differentiated U937 cells. To confirm this possibility, a reporter vector phPES2(CRM) with a mutation at CRE was transfected into TPA-differentiated U937 cells. The luciferase activity derived from this construct was only about 20% of that using phPES2(−327/+59), whereas the luciferase activity using a reporter vector phPES2(ILM) with a mutation at the NF-IL6 site was about 85% of that using phPES2(−327/+59). These results clearly indicate that the CRE appears to be more important than the NF-IL6 site for expression of the human PES-2 gene in differentiated U937 human monocytic cells.

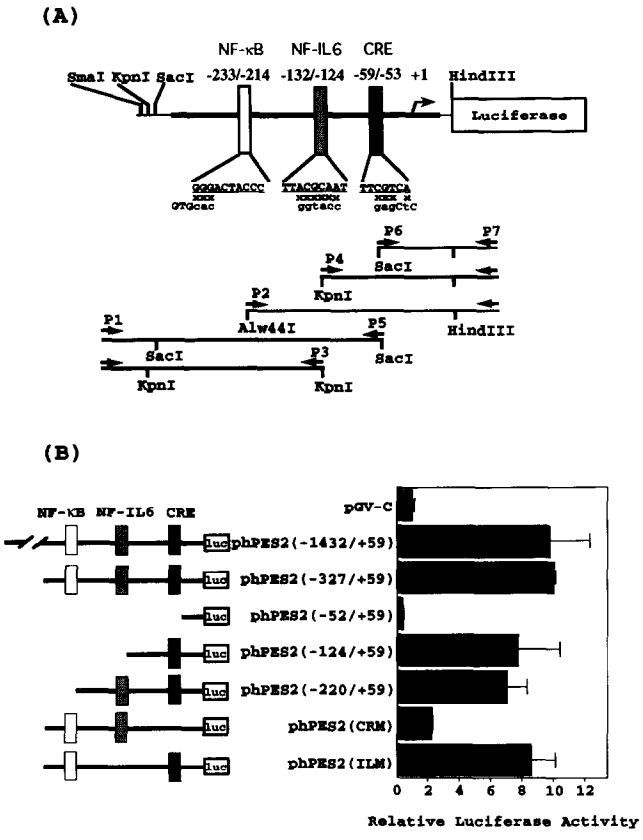

Fig. 2. (A) Schematic representation of the 5′-flanking region of the human PES-2 gene used in the transfection experiments. The 5′-flanking region (thick line) and multi-cloning sites derived from pBluescript II (thin line) in the luciferase reporter vector phPES2(−327/+59) (see section 2) are shown. The NF-κB site (open box), NF-IL6 site (shaded box) and CRE (closed box) are indicated with their sequences, together with the sequences introduced with the restriction enzymes *Alw*44I, *Sac*I and *Kpn*I (lower case letters and 'x' represent the changed nucleotide and its positions, respectively). PCR products are indicated as *lines* with the restriction enzyme sites and the relative positions of their primers (P1–P7) shown as *arrows*. (B) Promoter activity of the 5′-flanking region of the human PES-2 gene. Constructs were transiently transfected into the differentiated U937 cells, together with pCMV-βgal used as an internal control of the transfections. Results are represented as relative luciferase activities obtained by dividing the normalized luciferase activity from the reporter vector pGV-C under the control of the SV40 promoter/enhancer. Experiments were carried out in triplicate. The data are presented as means ± S.D.

3.3. *Induction of a protein binding to CRE during monocytic differentiation of U937 cell*

To analyze the expression of nuclear binding protein(s) specific for CRE during monocytic differentiation of U937 cells, electrophoretic mobility shift assays were performed using the oligonucleotide for the CRE of the human PES-2 gene (PES-2CRE), or its mutant (PES-2CRM). As shown in Fig. 3, a band indicated by an arrow was observed in the differentiated U937 cells (lane 2) but not in the undifferentiated U937 cells (lane 1) when PES-2CRE was used as a labeled probe. Moreover, this band was chased by the unlabeled PES-2CRE as a competitor (lanes 3–5) but not by the unlabeled mutant oligonucleotide PES-2CRM (lanes 6–8). These results show that a nuclear binding protein(s) specific for the CRE consensus sequence of the human PES-2 gene is induced during monocytic differentiation of U937 cells. These results are consistent with our previous observation that PES-2 mRNA is rapidly induced by forskolin in differentiated U937 cells but not in undifferentiated cells [3]. Taken together, these results show that CRE plays an essential role in the expression of the human PES-2 gene in U937 cells.

The results of this study clearly demonstrate that the expression of the human PES-2 gene appears to be regu-

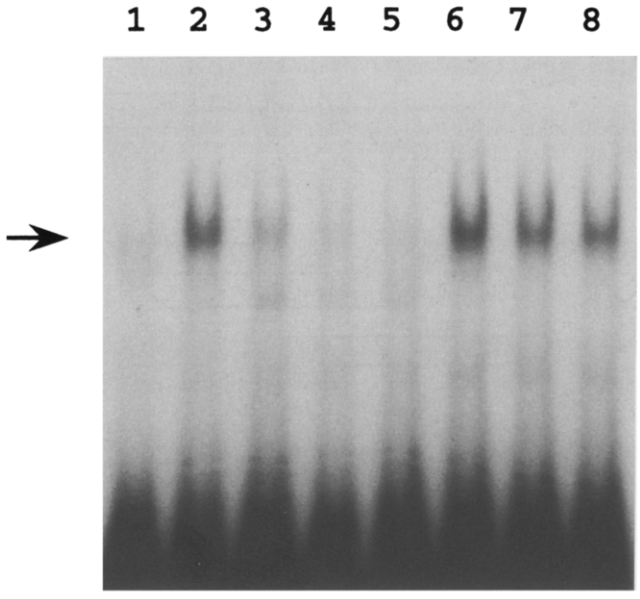

Fig. 3. Electrophoretic mobility shift assays using the labeled oligonucleotide containing the consensus CRE sequence from the human PES-2 gene (PES-2CRE). The nuclear extract (5 μg) from undifferentiated (lane 1) or TPA-differentiated (lanes 2–8) U937 cells was incubated with the ^{32}P-labeled PES-2CRE. 'Cold-chase' experiments were performed using a 10-fold (lanes 3 and 6), 30-fold (lanes 4 and 7) and 50-fold (lanes 5 and 8) molar excess of competitor oligonucleotide PES-2CRE (lanes 3–5) or its mutant PES-2CRM (lanes 6–8). The sequences of these oligonucleotides are shown in section 2.

lated by CRE in differentiated U937 human monocytic cells. Recently, Herschman et al. also showed that CRE is responsible for expression of the murine PES-2 gene (H.R. Herschman, personal communication). On the other hand, the NF-IL6 (C/EBPβ) site has been suggested to play a key role in regulating the induction of the rat PES-2 gene in granulosa cells prior to ovulation [18]. In this context, we note that the luciferase activity of phPES2(CRM), which contains NF-IL6 and NF-κB sites but lacks CRE, was higher than that of phPES2(−52/+59) lacking all these sites (Fig. 2). Interestingly, there is evidence showing that NF-IL6 and NF-κB are involved in the expression of genes by inflammatory stimulants such as lipopolysaccharide and interleukin 1 [19]. Further, the induction of PES-2 mRNA has been reported with lipopolysaccharide in macrophages and monocytes [20]. These facts suggest that the NF-IL6 and/or NF-κB sites in the human PES-2 gene may contribute to the expression of the human PES-2 gene. In addition to CRE, NF-IL6 and NF-κB sites, the 5′-flanking region of the human PES-2 gene contains many other transcriptional regulatory elements [3] and the PES-2 mRNA can be induced by a variety of factors. Thus further studies are necessary for an understanding of the transcriptional regulation of the PES-2 gene.

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