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An intronic enhancer regulates cyclooxygenase-1 gene expression

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Abstract

To identify *cis*-elements regulating PMA-induced prostaglandin H synthase-1 (PGHS-1) gene expression in the human megakaryoblast cell line, MEG-01, we performed promoter reporter assays with a luciferase reporter vector containing the −2030/−22 region of the human PGHS-1 gene. PMA treatment for 24 h increased PGHS-1 promoter activity by twofold. Mutagenesis studies of the promoter revealed a single Sp1 site essential for PMA-inducible transcription. Insertion of a highly conserved 100 bp sequence cloned from intron 8 into the −2030/−22 reporter plasmid enhanced PMA-dependent transcription 10-fold. Mutation of either a consensus AP-1 site within intron 8 or the Sp1 site in the promoter reduced PMA-induced activity by 80–100%. Gel shift assays using the intron 8 AP-1 sequence demonstrated the formation of an AP-1-specific DNA–protein complex. Our results suggest that inducible PGHS-1 gene expression involves the coordinate functioning of a Sp1 site in the promoter and an AP-1 site in intron 8.

Keywords: Cyclooxygenase; Megakaryocyte; MEG-01 cells; SP1; AP-1; Transcription; Gene expression

Prostaglandin endoperoxide H synthases (PGHS-1 and PGHS-2) catalyze the synthesis of prostaglandin H_2 (PGH_2) from arachidonic acid [1,2]. PGH_2 formation is the committed step in the production of prostanoids, potent signaling molecules that regulate a number of physiological processes [3]. PGHS-1 and -2 are very similar in structure and catalytic mechanism. Both isoforms are important pharmacological targets of non-steroidal anti-inflammatory drugs (NSAIDs). The most notable difference between the isozymes occurs at the level of gene expression. While PGHS-2 is an inducible enzyme, PGHS-1 is constitutively expressed in most tissues. However, a number of cell types show PGHS-1 upregulation when induced to undergo cellular differentiation by tumor-promoting phorbol ester, including primary megakaryocytes and megakaryoblast cell lines [4–6], human umbilical vein endothelial cells (HUVEC) [7], and a monocyte cell line [8]. PGHS-1 is overexpressed in ovarian cancer and has been proposed as a therapeutic target [9,10]. Despite its importance as a pharmacological target in cardiovascular therapy, and the indications that inducible PGHS-1 expression is involved in developmental processes and pathological conditions, very few studies have addressed the transcriptional mechanism behind PGHS-1 upregulation.

The human PGHS-1 gene is approximately 22 kDa and contains 11 exons [11,12]. The PGHS-1 promoter is typical of "housekeeping" genes in that it lacks a TATA-box, has several transcriptional start sites, and has a high G + C content [13,14]. Within the 5′ flanking region, there are two Sp1 consensus-binding sites at −610 and −111 relative to the ATG start codon that appear to be involved in regulation of basal, but not PMA-induced, expression in human umbilical vein endothelial cells [7]. Another Sp1-binding motif at −89 has been implicated in estradiol-mediated PGHS-1 upregulation

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in primary ovine endothelial cells [15]. No other *cis*-elements have been identified that affect PGHS-1 gene transcription. A post-transcriptional mechanism of regulation has been investigated by Duquette et al. [16], who found a conserved 3′ *cis*-element that may be involved in controlling translation of PGHS-1 mRNA.

We have examined PGHS-1 gene regulation during megakaryocyte differentiation in MEG-01 cells. Megakaryocytes are the precursors of platelets, in which PGH_2 is converted to the potent aggregation mediator thromboxane A_2 by thromboxane synthase. Upregulation of PGHS-1 has been demonstrated in primary megakaryocytes isolated from $CD34^+$ cells treated to differentiate with the primary cytokine thrombopoietin [6]. Using PMA-treated MEG-01 cells, Mroske et al. [17] showed a correlation between the expression of megakaryocyte differentiation markers and PGHS-1. Thus, the MEG-01 cell line provides a model system for studies of inducible PGHS-1 gene regulation.

Materials and methods

Cell culture. MEG-01 cells (American Type Culture Collection) were cultured in RPMI (Invitrogen) containing 10% fetal bovine serum at 37 °C and in a 5% CO_2 atmosphere. For all experiments, the cells were plated at 40% confluency the day before the experiment was initiated.

Reverse transcriptase-PCR. MEG-01 cells were treated with 25 nM PMA for various times and total RNA was isolated using TRIZOL reagent (Invitrogen). DNase I-treated RNA (2 μg) was reverse-transcribed using Superscript II RT (Invitrogen) with random primers according to the protocol of the manufacturer. For PCR amplification, 2 μl of each RT sample was mixed with 1 U of High Fidelity Platinum *Taq*, 1× PCR buffer, 2 mM $MgSO_4$, 0.8 mM dNTPs, and 0.4 μM primer mix. To determine the exponential phase of PCR amplification for semi-quantitation of amplicons, the cycle parameters were 94 °C for 2 min, followed by 15–31 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s. The optimum cycle number for amplification was determined to be 26 cycles for all primer sets. The primers used for the PGHS-1 primary transcript amplified a 397 bp fragment within intron 2 (forward primer ACTTGGGGAGAAGGGACAGT; reverse primer GGACGCAGACAGACCTGAGT). The primers used for PGHS-1 mRNA and 18S rRNA amplicons were from Ambion COX-1 Gene Specific Relative PCR kit. PCRs were electrophoresed in 2% agarose gel stained with SYBR green I (Molecular Probes).

Luciferase reporter constructs. A fragment of the human (h) PGHS-1 promoter region (−2095 to −22 relative to the ATG start site) was a generous gift from Dr. Kenneth Wu. This fragment was used as a template to amplify various lengths that differed at their 5′ end. The sequence for the hPGHS-1 gene was obtained from Accession No. AF440204 and data from the Human Genome Project on the UCSC Genome Browser. The primers used for amplification contained a 5′ *Xho*I and *Bgl*II or a *Kpn*I site (forward primer) or a 5′ *Hin*dIII site (reverse primer) for ligation of the amplicon into the pGL3 Basic luciferase reporter vector (Promega). Forward primers: −2030, AAGGTACCACCTGGCACTTCTGCC; −1311, AAGGTACCACC CTGGCTTGCTCTG; −950, CCGCTCGAGATCTGGTGGATGT GAGTCTAGC; −722, CCGCTCGAGATCTACCTTGTCCAAGG TGACACG; −542, CCGCTCGAGATCTTCATCTAGGAAACCAT CAGC; −381, AAGGTACCACTGCGGGGCAGGGTA; −210, AA GGTACCAGCTTTAGCGGCGAGCA; −71, AAGGTACCAAGCT CCGGGCAGTGTG; and reverse primer: −22, GGAAAGCTTGAC GCAGAGTGCAGG. A 152 bp fragment from intron 8 region (+13,071 to +13,222), which contains the 95 bp conserved region plus flanking sequence and is designated Ir-8, was cloned from MEG-01 genomic DNA using primers containing a 5′ *Bam*HI site (forward primer) or a 5′ *Sal*I site (reverse primer) for ligation into −2030/−22 pGL3 after the luciferase gene at the *Bam*HI/*Sal*I site (forward primer: +13,071, AAGCTTGGTACCGCCTTCAGGAATCTTCATGTTC, reverse primer: +13,222, AGCTCCCAGGCAGGAAACTCGAG. The intronic regions from +13,123 to +13,185 (Ir-8a) and +13,186 to +13,217 (Ir-8b) fragments were made by annealing synthesized oligonucleotides containing 5′ *Bam*HI and 3′ *Sal*I overhangs, and ligating them into −2030/−22 pGL3, −380/−22 pGL3, −209/−22 pGL3, or −70/−22 pGL3 at the *Bam*HI/*Sal*I site. Mutations of the various consensus *cis*-elements in −2030/−22 pGL3, −2030/−22 Ir-8 pGL3, or −2030/−22 Ir-8a pGL3 were made according to the protocol for the Stratagene Site-directed Mutagenesis kit. Mutagenesis primers are listed in Table 1.

Luciferase reporter assays. Cells (5×10^5 per well) were plated in six-well plates, transfected with 0.4 μg of promoter-luciferase plasmid using Effectene transfection reagent (Qiagen) according to the instructions of the manufacturer, and incubated at 37 °C for 18 h. PMA (25 nM) was added and cells were harvested after further incubation for 24 h. Cell lysates and samples were prepared using the Luciferase Assay Kit from Invitrogen. Luciferase activity was measured on a Tecan GeniosPro multidetector plate reader. Total protein was measured using the BCA protein assay kit from Pierce. Luciferase activity was normalized to total protein.

Electrophoretic mobility shift assay. Nuclear extracts from untreated or PMA-treated MEG-01 cells were isolated using NE-PER? Nuclear and Cytoplasmic Extraction kit (Pierce). A ^{32}P-labeled oligonucleotide (20,000 cpm) corresponding to 20 nucleotides of the PGHS-1 Ir-8 region surrounding the AP-1 site was mixed with 20 mM Hepes–KOH, pH 7.9, 100 mM KCl, 2 mM EDTA, 1 μg poly(dI–dC), 300 μM dithiothreitol, and the protease inhibitors Pefabloc (2 mM), and Complete EDTA-free (1×) (Roche) in 20 μl total volume. Included in some of the reactions was a 30- or 300-fold molar excess of non-radioactive native or mutant competitor oligonucleotide. Nuclear protein (5 μg) was added to the mixture and incubated at room temperature for 15 min. For antibody supershift assays, 1 μl of antibody was added to the protein extract, and the mixture was incubated on ice overnight prior to addition of the probe mixture. Glycerol was added to reaction tubes to yield a final concentration of 7% prior to loading samples onto a 1× TAE non-denaturing 5% polyacrylamide gel. Samples were subjected to electrophoresis at 150 V for 1.5 h in cold 1× TAE. The gel was dried and autoradiography was performed.

Results

PGHS-1 is transcriptionally upregulated during PMA-induced differentiation of MEG-01 cells

Reverse transcriptase-PCR revealed that untreated MEG-01 cells had no detectable PGHS-1 primary transcripts (Fig. 1); transcripts were detected after 3 h of PMA treatment and continued to increase for at least 24 h. There was a detectable level of PGHS-1 mRNA in untreated cells, which increased in PMA-treated cells over the 24 h treatment period. These results indicate that PMA-dependent PGHS-1 upregulation in MEG-01 cells occurs at the transcriptional level.

Table 1
Oligonucleotide primers used for site-directed mutagenesis of putative *cis*-elements

Mutation	Primer sequence
	−148 GGATTC −108
mAP-4 (−129/−124)	AAGCGGGCAGCCGAGGT**GACAGCTG**GAGGGAGGAGCGGGGG
	−139 GGATTC −99
mSP1 (−122/−113)	GCCGAGGTGACAGCTGG**AGGGAGG**AGCGGGGGTGGAGCCGG
	−127 GGATTC −88
mSP1 (−111/−102)	GCTGGAGGGAGGAGCG**GGGGTGG**AGCCGGGGGAAGGGTGG
	−109 GGATTC −70
mRREB (−99/−86)	GGTGGAGCCG**GGGGAAGGGTGGGG**AGGGGATGGGCTGGAG
	−106 GGATTC −67
mSP1 (−89/−80)	GGAGCCGGGGGAAGGGT**GGGGAGGGGA**TGGGCTGGAGCTC
	+13,127 GAC +13,173
mAP-1 (13,138/13,145)	GTAGCTTCTAGG**TGACTCA**GGGACAGGATATTTTTGTGTTCCCTATG
	+13,127 TTC +13,173
mEvi-1 (13,146/13,156)	GTAGCTTCTAGGTGACTCAGG**GACAGGATAT**TTTTGTGTTCCCTATG
	+13,127 GAC TTC +13,173
mAP-1 + mEvi-1	GTAGCTTCTAGG**TGACTCA**GG**GACAGGATAT**TTTTGTGTTCCCTATG
	+13,122 CTCGAG +13,148
m1 (13,125/13,131)	CCTTCCTGCAGTTT**GGTAGC**TTCTAGGTGACTCAGGG
	+13,130 CTCGAG +13,174
m2 (13,146/13,151)	GCTTCTAGGTGACTCA**GGGACA**GGATATTTTTGTGTTCCCTATGG
	+13,140 CTCGAG +13,175
m3 (13,156/13,161)	GACTCAGGGACAGGAT**ATTTTT**GTGTTCCCTATGGG
	+13,140 CTCGAG +13,181
m4 (13,163/13,168)	GACTCAGGGACAGGATATTTTTG**TGTTCC**CTATGGGGGCGAG
	+13,146 CTCGAG +13,189
m5 (13,170/13,175)	GGGACAGGATATTTTTGTGTTCCC**TATGGG**GGCGAGTCTGCAAC

Numbers indicate the nucleotide sequence location within the PGHS-1 gene relative to the ATG start site. Underlined nucleotides indicate the consensus sequence. The native sequence containing the core-binding site within each *cis*-element is indicated in bold, with the mutant sequence shown above.

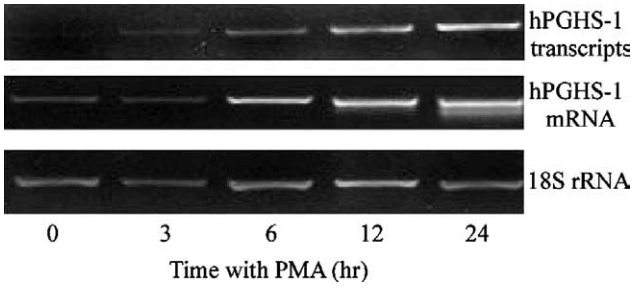

Fig. 1. PGHS-1 is transcriptionally upregulated during PMA-induced differentiation of MEG-01 cells. Cells were treated with 25 nM PMA for the indicated times. Total RNA was reverse-transcribed and PCR amplification was performed using primers specific for PGHS-1 primary transcripts (a 397 bp amplicon within intron 2), PGHS-1 mRNA (a 401 bp amplicon of exons 6, 7, and 8), or 18S ribosomal RNA as a control. Amplicons were visualized on an agarose gel stained with SYBR green I.

Promoter reporter assays reveal a small PMA-dependent induction with 2 kb of the 5′ untranslated region of the human PGHS-1 gene

A 2008 bp region 5′ to the PGHS-1 translational start site (−2030 to −22) was fused to the promoter-less luciferase reporter vector pGL3. The PGHS-1 promoter reporter vector (−2030/−22-pGL3) or the empty pGL3 vector was transiently transfected into MEG-01 cells. The cells were treated with or without PMA for 24 h, and the luciferase activity in cell lysates was measured (Fig. 2A). Basal activity of each of the constructs tested in MEG-01 was typically very low, which made it difficult to interpret the measurements of basal activity. PMA significantly induced promoter activity, even in the empty pGL3 vector. After factoring in the fivefold increase for the empty vector, there was a consistent PMA-dependent induction of two- to threefold in −2030/−22-pGL3.

A 5′ deletion analysis of the 2 kb promoter region was performed to identify regions containing regulatory elements (Fig. 2A). There were no significant changes in basal or PMA-inducible promoter activity until the region between −210 and −71 was deleted, which abrogated all PMA-inducible activity. Because the region between −160 to −80 is highly conserved between human and murine genes (Fig. 3), the region was examined for consensus transcription factor-binding sites using MatInspector (www.Genomatix.de). Five candidate *cis*-elements were found: an activator protein 4 (AP-4) site, three Sp1 sites, and a ras-responsive element-binding (RREB) site. Site-directed mutagenesis of each of the five *cis*-elements was performed. Mutation of the −111 Sp1 site completely abrogated the modest PMA-inducible promoter activity in MEG-01 cells (Fig. 2B). None of the other mutations reproducibly affected PMA-inducible promoter activity.

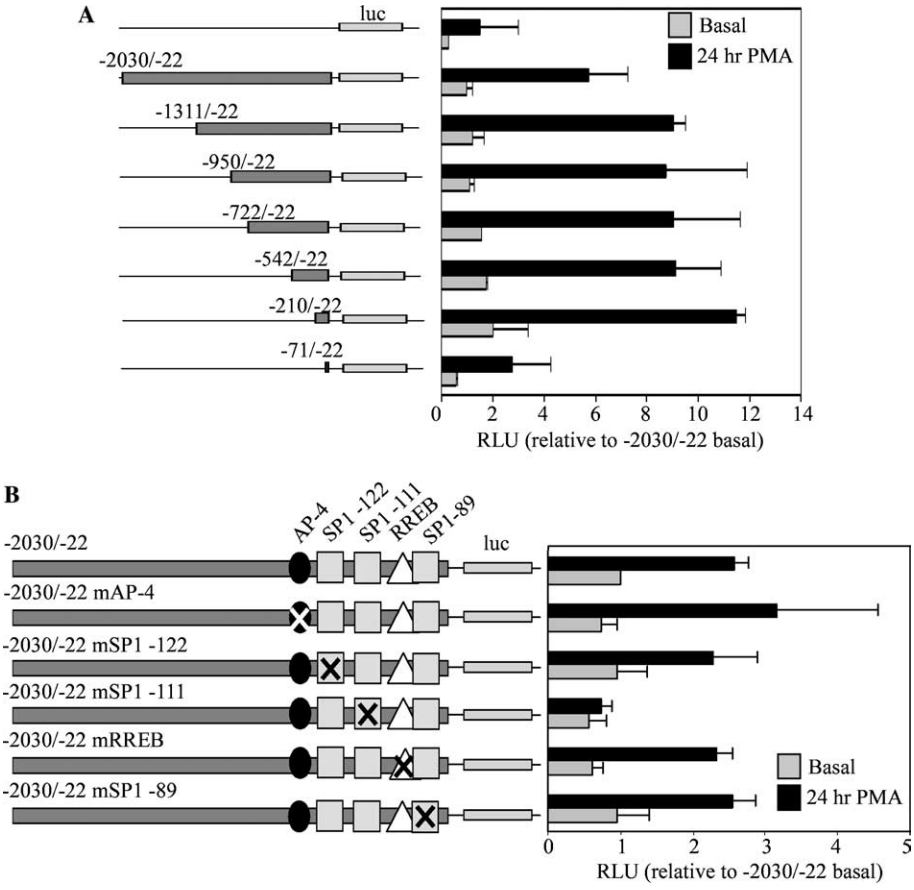

Fig. 2. Analysis of the 2 kb PGHS-1 promoter region. The indicated PGHS-1 promoter constructs in the pGL3 luciferase reporter vector were transiently transfected into MEG-01 cells and incubated for 18 h. PMA (25 nM) was added and cells were incubated for an additional 24 h before being harvested and assayed for luciferase activity and total protein content. Results of promoter assays are normalized to the basal activity of the full-length native construct and are an average of two experiments in duplicate ±SD. (A) Deletion analysis of the 5′ end of the PGHS-1 promoter. (B) Mutagenesis of conserved consensus-binding sequences in the PGHS-1 proximal promoter. Specific mutated sequences are shown in Table 1.

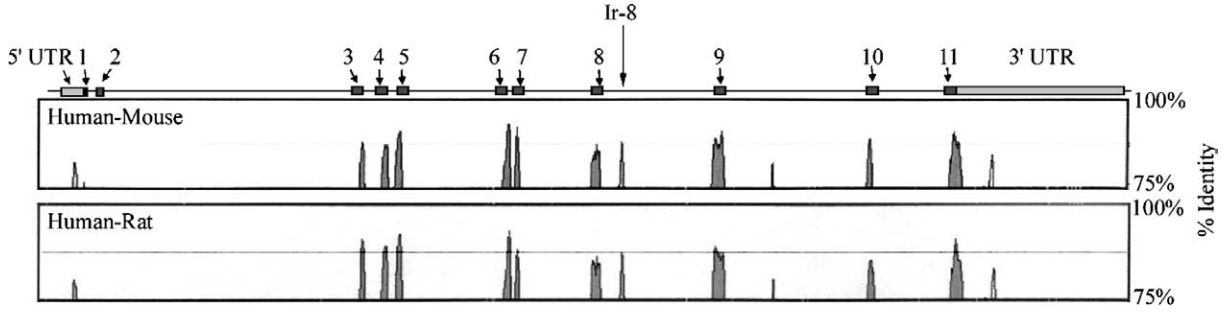

Fig. 3. VISTA gene alignment of human and murine PGHS-1 genes. The human, mouse, and rat genome sequences were aligned using VISTA browser (available through http://gsd.lbl.gov/vista/index.shtml). Peaks within the histogram indicate highly conserved regions between species. The *Y*-axis shows those regions with at least 75% identity. Numbers 1–11 denote exons. Ir-8 denotes the conserved 100 nucleotides in intron 8.

Gene alignments and promoter reporter assays reveal a highly conserved transcriptional enhancer element within intron 8

The results of promoter analyses raised the possibility that distal elements are required for enhancer activity. Gene alignments of human and mouse or human and rat PGHS-1 genes using the VISTA browser [18] (http://gsd.lbl.gov/vista/index.shtml) revealed several highly conserved (at least 75% identity) regions in addition to exons 3–11: (a) the proximal promoter from −160 to −80; (b) about 100 bp in intron 8; (c) about 40 bp in intron 9; (d) about 180 bp in the 3′UTR region that contains the post-transcriptional control element investigated by Duquette and Laneuville [16]. A second method of gene alignment was performed using BLAST

[15] (www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.ht), which revealed the same conserved regions as the VISTA alignment except for the intron 9 region. The intron 8 region (Ir-8) from +13,071 to +13,222 which includes 95 bp of 90% conserved sequence plus flanking sequence was cloned as a candidate regulatory transcriptional region and was inserted downstream of the luciferase gene in −2030/−22 pGL3 to make −2030/−22 Ir-8 pGL3 (Fig. 4). Results shown in Fig. 4A indicate that Ir-8 conferred an approximately 10-fold increase in PMA-inducible transcription from the PGHS-1 promoter. Ir-8 had no effect on PMA-inducible transcription from the SV40 promoter when inserted into SV40-pGL3 (Fig. 4A), suggesting that its regulatory effect has at least some specificity toward the PGHS-1 promoter.

An AP-1 site is involved in the enhancer activity of Ir-8

In order to define the area within Ir-8 that confers the enhancer activity, the 95 bp highly conserved sequence was divided into two fragments and analyzed. A double-stranded fragment corresponding to nucleotides +13,123 through +13,185 (Ir-8a) or nucleotides +13,186 through +13,217 (Ir-8b) was inserted into −2030/−22 pGL3 after the luciferase gene. The enhancer activity was retained only in the Ir-8a fragment (Fig. 4B). In the human, mouse, and rat genes, Ir-8a contains conserved canonical-binding sequences for activating protein-1 (AP-1) and ecotropic viral integration site-1 encoded factor (Evi-1). AP-1 is a protein complex composed of Jun homodimers or Jun/Fos heterodimers that regulates the expression of genes involved in many aspects of hematapoietic function [25]. Evi-1 is a zinc-finger DNA-binding protein that is expressed in normal megakaryocytes and is highly expressed in acute myeloid leukemia cells [19,20]. Promoter assays revealed that mutation of the AP-1 site, but not the Evi-1 element, decreased the response of the −2030/−22 Ir-8a pGL3 luciferase reporter to PMA by 80% (Fig. 4B), suggesting that this AP-1 site in intron 8 is an important element for PMA-induced PGHS-1 gene expression.

The DNA sequence flanking the AP-1 site was subjected to scanning mutagenesis to find *cis*-elements that may be coordinately involved in the enhancer activity of Ir-8. Five more separate mutations were made within the Ir-8a region with the aim of obtaining six-point

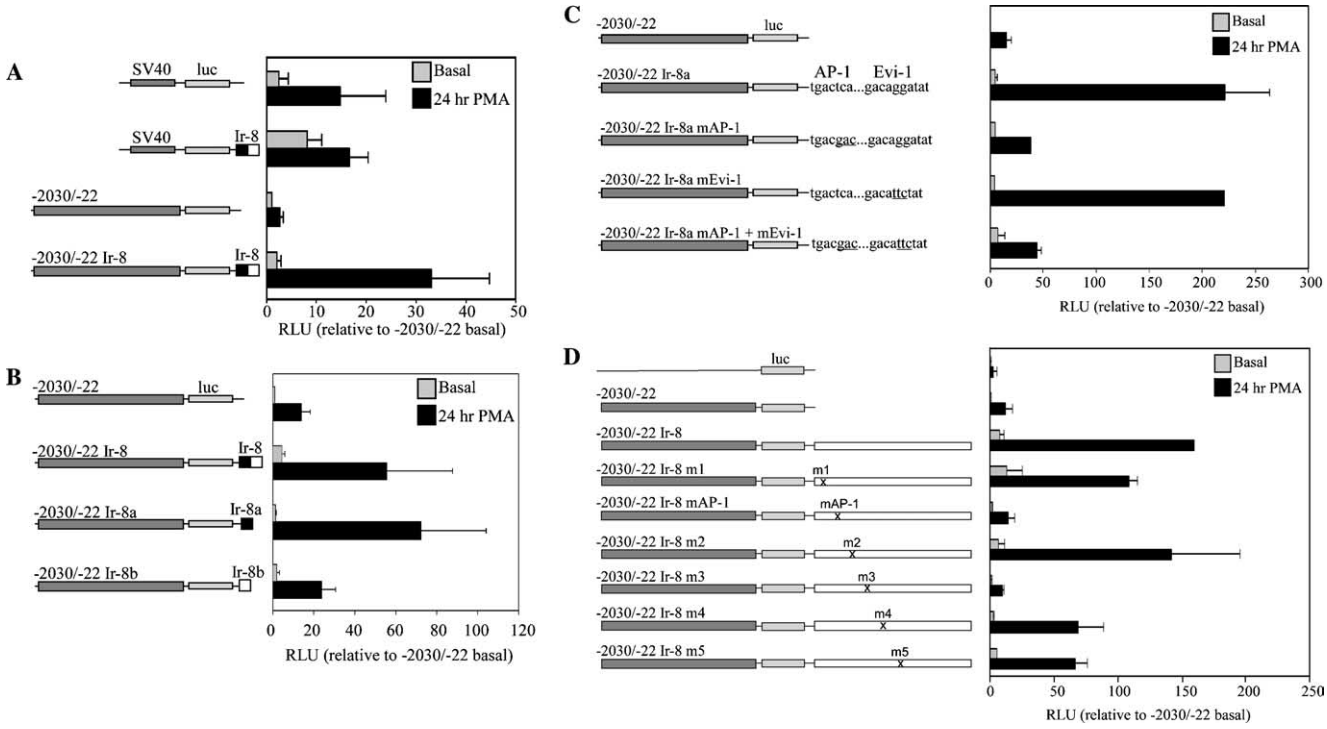

Fig. 4. Analysis of the effect of the intronic region Ir-8 on hPGHS-1 promoter activity. Promoter assays were performed as described in Fig. 2. (A) Effect of Ir-8 on SV40 minimal promoter vs. hPGHS-1 promoter. The conserved intron 8 region, designated Ir-8, was inserted downstream of the luciferase coding region in the SV40 pGL3-promoter vector or the −2030/−22 hPGHS-1 pGL3 vector. (B) Deletion analysis of Ir-8. The enhancer effect of Ir-8 on −2030/−22 hPGHS-1 pGL3 was tested in its entirety or as two separate component constructs. The black box represents base pairs +13,123 to +13,185 of the Ir-8 region (Ir-8a) and the white box represents base pairs +13,186 to 13,217 (Ir-8b). (C) Mutation of the AP-1 and Evi-1 sites in Ir-8a. Canonical DNA-binding sequences for the AP-1 and Evi-1 transcription factors are shown in the Ir-8a wild-type sequence of −2030/−22 Ir-8a pGL3. Site-directed mutagenesis of the −2030/−22 Ir-8a pGL3 plasmid was performed to alter the core sequences of either the AP-1 site or the Evi-1 site or both. Mutated nucleotides are underlined. (D) Scanning mutagenesis of Ir-8. Site-directed mutagenesis of the −2030/−22 Ir-8a pGL3 plasmid was performed to replace 6 bp of the native sequence with a *Xho*I site, at an average of every 10 bp between +13,123 and +13,175 on the Ir-8 sequence.

mutations every 10 bp. Fig. 4D shows that one mutation (m3), which is 11 bp downstream of the AP-1 mutation, decreases promoter activity to control (promoter-only) levels. Additionally, mutations of two other downstream sites (m4 and m5) each reduced Ir-8 promoter activity by approximately 60%.

Ir-8a interacts with sites within the PGHS-1 proximal promoter

Region Ir-8a was inserted into deletion constructs −380/−22, −209/−22, and −70/−22 pGL3 after the luciferase gene to assess the requirement for proximal promoter elements for interaction (Fig. 5A). The results suggested an interaction between the Ir-8a AP-1 enhancer and site(s) within the region from −209 and −70. Mutations were made in the consensus *cis*-elements in this region (Fig. 5B). Mutation of the Sp1 site at −111 abrogated all activity, whereas mutation of RREB decreased PMA-dependent activity by 85%. Mutations in the other two Sp1 sites decreased PMA-dependent activity by about 35%. Mutation of the most upstream element, AP-4, had no effect.

The intronic AP-1 site forms a PMA-dependent protein–DNA complex with MEG-01 nuclear extracts

The AP-1 site in Ir-8 was able to bind MEG-01 nuclear proteins to form a protein–DNA complex in a time-dependent manner with PMA treatment (Fig. 6). The protein–DNA complex was AP-1 sequence specific, since formation of the complex was inhibited by co-incubation with unlabeled wild-type probe but not with an unlabeled probe containing a three-nucleotide mutation of the AP-1 sequence. Antibody supershifts were performed with antibodies directed against Jun, Fos, or individual Jun and Fos protein family members (Fig. 7). The antibodies directed against the Jun conserved DNA-binding region and the Fos conserved DNA-binding region completely prevented formation of the AP-1 complex. To further characterize the AP-1 components, assays with antibodies directed against c-Jun, phospho-c-Jun, JunB, JunD, c-Fos, FosB, fra-2, and IgG as negative control were performed. Although none of the antibodies supershifted the complex, JunD and FosB antibodies prevented formation of the AP-1 complex.

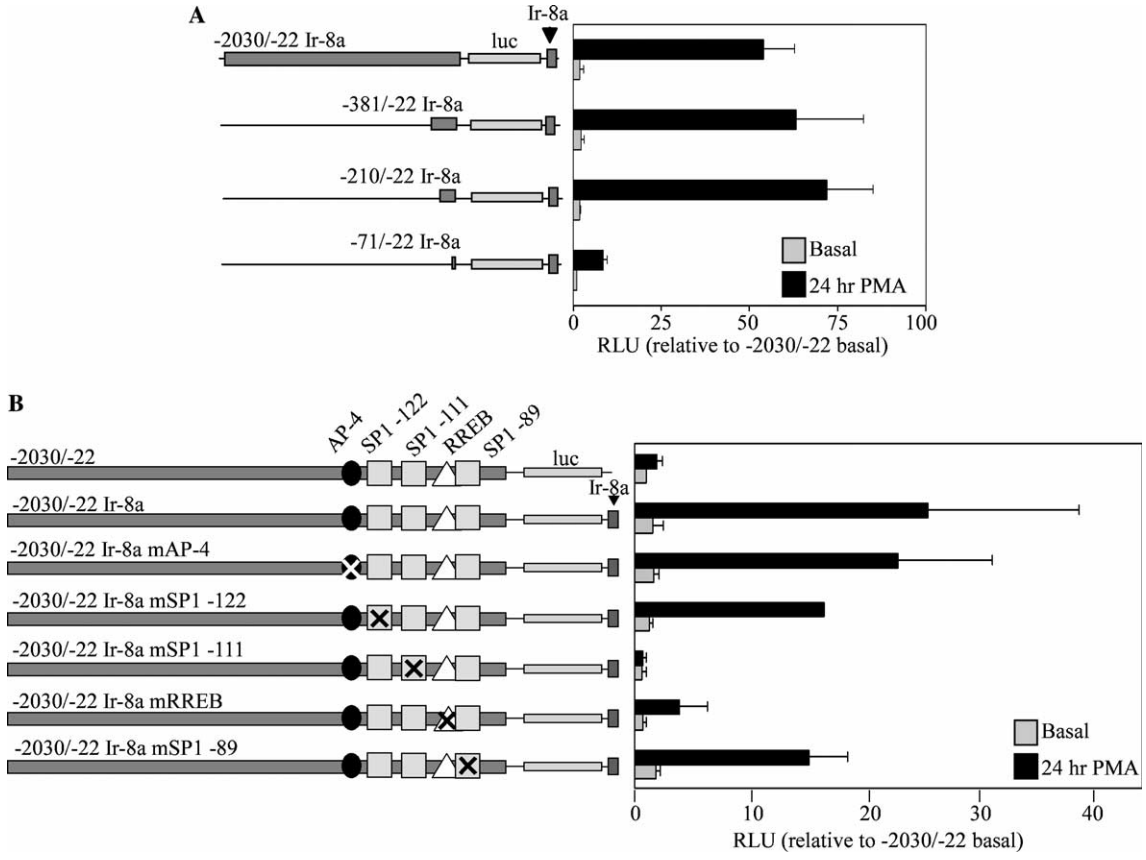

Fig. 5. Analysis of interaction between Ir-8a and the proximal promoter region of the hPGHS-1 gene. Promoter assays were performed as described in Fig. 2. (A). Deletion analysis of −2030/−22 Ir-8a pGL3 reporter plasmid. (B) Mutagenesis of conserved consensus-binding sequences in the PGHS-1 proximal promoter of −2030/−22 Ir-8a pGL3. Specific mutated sequences are shown in Table 1.

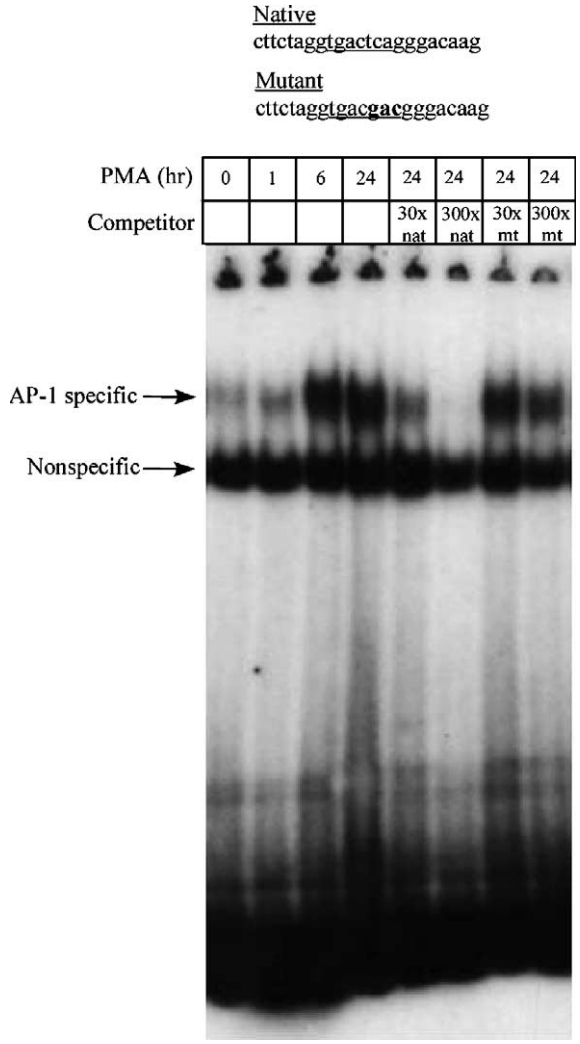

Fig. 6. MEG-01 nuclear extracts form a protein–DNA complex with Ir-8. Nuclear protein (5 μg) from cells treated without or with 25 nM PMA for the indicated times was incubated with a ^{32}P-labeled probe corresponding to 20 nucleotides of the Ir-8 region surrounding the AP-1 site. In the last four samples, unlabeled probe having the native sequence (nat) or a mutation (mt) in the AP-1 site was added to the reaction mixture at 30- or 300-fold molar excess over the ^{32}P-labeled probe. The native and mutant probe sequences are shown, with the AP-1 consensus sequence underlined and the specific mutated nucleotides in bold.

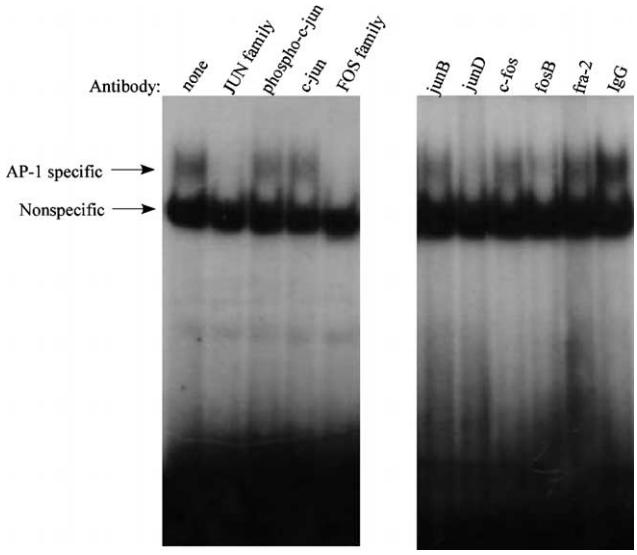

Fig. 7. Characterization of the intron 8 AP-1 enhancer implicates a JunD/FosB complex. EMSAs were performed as described for Fig. 6, except that the nuclear extract was incubated with 1 μl of antibody on ice overnight prior to incubation with the ^{32}P-labeled native probe.

Discussion

Our studies of PMA-induced PGHS-1 transcription in MEG-01 cells revealed a requirement for a Sp1-binding site at −111 relative to the ATG codon. No other regulatory *cis*-elements were found within 2 kb upstream of the start codon. Although densitometric analysis (not shown) of the autoradiogram shown in Fig. 1 revealed that the total PGHS-1 transcript level was induced at least 20-fold by treating MEG-01 cells for 24 h with PMA, there was only a two- to threefold increase in luciferase activity using reporter constructs containing only the PGHS-1 promoter. This modest level of PGHS-1 transcriptional activity is similar to those seen in HUVEC [7] and ovine endothelial cells [15] induced by PMA and estradiol, respectively, in promoter assays with 2 kb or less of 5′-sequence. Vista gene alignment revealed that within the PGHS-1 sequence from 10 kb upstream of the ATG codon to 10 kb downstream of the 3′UTR (not shown), the 100 bp Ir-8 region of intron 8 was the only sequence that was as highly conserved as exons 3–11. This intronic region enhanced PMA-inducible PGHS-1 promoter activity by 10-fold. Ir-8 contained a fully conserved AP-1 site; mutation of this site reduced PMA-dependent promoter activity by at least 80%. The AP-1 site and its flanking DNA sequence when mixed with nuclear proteins formed a PMA-dependent protein–DNA complex in gel shift assays. Antibody supershift assays revealed the formation of a Jun/Fos heterodimer.

There are several indications in our report that there is coordinate functioning between elements within the Ir-8 region and the promoter: (a) the insertion of the Ir-8 region immediately downstream of the luciferase gene in the promoter reporter plasmid significantly enhanced the PMA-induced luciferase activity from the PGHS-1 promoter, but not from the SV40 promoter; an important difference between these two promoters is that the SV40 promoter contains a TATA-box, while the PGHS-1 promoter is TATA-less but contains several consensus Sp1-binding sites; (b) deletion of the conserved proximal promoter region completely abolished the enhancer activity of Ir-8, and mutations within the proximal promoter identified a requirement of the −111 Sp1-binding site and, to a lesser degree, its flanking sequence, for Ir-8-mediated activity; (c) scanning mutagenesis of Ir-8 revealed a 6 bp sequence starting 11 bp downstream of the AP-1 site that is also required

for Ir-8 enhancer activity; this result suggests that Ir-8 is composed of a complex of DNA-binding factors rather than a single transcription factor. Analysis of Ir-8 using MatInspector did not reveal any consensus transcription factor-binding sites for this 6 bp sequence, suggesting that a non-consensus-binding sequence and perhaps an unknown transcription factor is involved.

Sp1 proteins are ubiquitous transcription factors that bind to GC-rich DNA core sequences and activate transcription by recruiting the basal transcription apparatus. In addition to its role in recruiting basal transcriptional machinery, Sp1 can also interact with other transcription factors to allow for context-specific gene regulation. Sp1 and AP-1 interactions have been found within adjacent regions of the promoters for keratin 16 [21], vimentin [22], and transglutaminase 1 [23]. There is a precedent for long-range interplay between a distal promoter AP-1 site and a proximal promoter Sp1 site [24]. However, in our PGHS-1 promoter system in MEG-01 cells, we did not investigate the effects of *trans*-acting factors on the promoter, and therefore we cannot rule out the possibility that other members of the Sp family of transcription factors or other transcription factors that recognize the Sp-binding sequence may be bound to the −111 G/C element. In HUVEC, the −111 consensus Sp1 site is required for basal transcription but antibody supershift assays did not demonstrate binding of Sp1 or Sp3 to the oligonucleotide probe [7]. Likewise, the −89 and −111 consensus Sp1 sites are involved in estradiol-induced PGHS-1 promoter activity in ovine endothelial cells and produce a protein–DNA complex in gel shifts, but antibody supershifts only detected the binding of the transcription factor AP-2, and not Sp1 or Sp3, to the −89 site [15]. Thus, further studies are required to identify the *trans*-acting factors that bind the Sp1-binding site and the Ir-8 sequence. Although our results do not provide a detailed mechanism for interaction of Ir-8 with the promoter, they do indicate that Ir-8 interacts specifically with the PGHS-1 promoter to act as a transcriptional enhancer in promoter reporter assays.

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References

[1] W.L. Smith, D.L. DeWitt, R.M. Garavito, Cyclooxygenases: structural, cellular, and molecular biology, Annu. Rev. Biochem. 69 (2000) 145–182.
[2] L.J. Marnett, R.N. DuBois, COX-2: a target for colon cancer prevention, Annu. Rev. Pharmacol. Toxicol. 42 (2002) 55–80.
[3] W.L. Smith, R.C. Murphy, in: Vance D.E., J.E. Vance (Eds.), fourth ed., Biochemistry of Lipids, Lipoproteins and Membranes, Elsevier Science B.V., Amsterdam, 2002, pp. 341–372.
[4] N. Matijevic-Aleksic, S.K. Sanduja, L.H. Wang, K.K. Wu, Differential expression of thromboxane A synthase and prostaglandin H synthase in megakaryocytic cell line, Biochim. Biophys. Acta 1269 (1995) 167–175.
[5] N. Ueda, R. Yamashita, S. Yamamoto, K. Ishimura, Induction of cyclooxygenase-1 in a human megakaryoblastic cell line (CMK) differentiated by phorbol ester, Biochim. Biophys. Acta 1344 (1997) 103–110.
[6] N. Tanaka, T. Sato, H. Fujita, I. Morita, Constitutive expression and involvement of cyclooxygenase-2 in human megakaryocytopoiesis, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 607–612.
[7] X.M. Xu, J.L. Tang, X. Chen, L.H. Wang, K.K. Wu, Involvement of two Sp1 elements in basal endothelial prostaglandin H synthase-1 promoter activity, J. Biol. Chem. 272 (1997) 6943–6950.
[8] C.J. Smith, J.D. Morrow, L.J. Roberts 2nd, L.J. Marnett, Differentiation of monocytoid THP-1 cells with phorbol ester induces expression of prostaglandin endoperoxide synthase-1 (COX-1), Biochem. Biophys. Res. Commun. 192 (1993) 787–793.
[9] M. Dore, L.C. Cote, A. Mitchell, J. Sirois, Expression of prostaglandin G/H synthase type 1, but not type 2, in human ovarian adenocarcinomas, J. Histochem. Cytochem. 46 (1998) 77–84.
[10] R.A. Gupta, L.V. Tejada, B.J. Tong, S.K. Das, J.D. Morrow, S.K. Dey, R.N. DuBois, Cyclooxygenase-1 is overexpressed and promotes angiogenic growth factor production in ovarian cancer, Cancer Res. 63 (2003) 906–911.
[11] C. Yokoyama, T. Tanabe, Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme, Biochem. Biophys. Res. Commun. 165 (1989) 888–894.
[12] S.A. Kraemer, E.A. Meade, D.L. DeWitt, Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5′-flanking regulatory sequences, Arch. Biochem. Biophys. 293 (1992) 391–400.
[13] L.H. Wang, A. Hajibeigi, X.M. Xu, D. Loose-Mitchell, K.K. Wu, Characterization of the promoter of human prostaglandin H synthase-1 gene, Biochem. Biophys. Res. Commun. 190 (1993) 406–411.
[14] T. Tanabe, N. Tohnai, Cyclooxygenase isozymes and their gene structures and expression, Prostaglandins Other Lipid Mediat. 68–69 (2002) 95–114.
[15] L.L. Gibson, L. Hahner, S. Osborne-Lawrence, Z. German, K.K. Wu, K.L. Chambliss, P.W. Shaul, Molecular basis of estrogen-induced cyclooxygenase type 1 upregulation in endothelial cells, Circ. Res. 96 (2005) 518–525, Epub 2005 Feb, 10.
[16] M. Duquette, O. Laneuville, Translational regulation of prostaglandin endoperoxide H synthase-1 mRNA in megakaryocytic MEG-01 cells. Specific protein binding to a conserved 20-nucleotide *cis* element in the 3′-untranslated region, J. Biol. Chem. 277 (2002) 44631–44637.
[17] C. Mroske, M.H. Plant, D.J. Franks, O. Laneuville, Characterization of prostaglandin endoperoxide H synthase-1 enzyme expression during differentiation of the megakaryocytic cell line MEG-01, Exp. Hematol. 28 (2000) 411–421.
[18] O. Couronne, A. Poliakov, N. Bray, T. Ishkhanov, D. Ryaboy, E. Rubin, L. Pachter, I. Dubchak, Strategies and tools for whole-genome alignments, Genome Res. 13 (2003) 73–80.
[19] J. Jolkowska, M. Witt, The EVI-1 gene—its role in pathogenesis of human leukemias, Leuk. Res. 24 (2000) 553–558.
[20] H. Hirai, The transcription factor Evi1, Int. J. Biochem. Cell Biol. 31 (1999) 1367–1371.
[21] Y.N. Wang, W.C. Chang, Induction of disease-associated keratin 16 gene expression by epidermal growth factor is regulated through cooperation of transcription factors Sp1 and c-Jun, J. Biol. Chem. 278 (2003) 45848–45857.

[22] Y. Wu, X. Zhang, Z.E. Zehner, c-Jun and the dominant-negative mutant, TAM67, induce vimentin gene expression by interacting with the activator Sp1, Oncogene 22 (2003) 8891–8901.
[23] M.A. Phillips, B.A. Jessen, Y. Lu, Q. Qin, M.E. Stevens, R.H. Rice, A distal region of the human TGM1 promoter is required for expression in transgenic mice and cultured keratinocytes, BMC Dermatol. 4 (2004) 2.
[24] S.P. Reddy, H. Vuong, P. Adiseshaiah, Interplay between proximal and distal promoter elements is required for squamous differentiation marker induction in the bronchial epithelium: role for ESE-1 Sp1, and AP-1 proteins, J. Biol. Chem. 278 (2003) 21378–21387.
[25] D.A. Liebermann, B. Gregory, B. Hoffman, AP-1 (Fos/Jun) transcription factors in hematopoietic differentiation and apoptosis, Int. J. Oncol. 12 (1998) 685–700.