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Identification of Multiple Cis-Acting Elements Mediating the Induction of Prostaglandin G/H Synthase-2 by Phorbol Ester in Murine Osteoblastic Cells

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Abstract The tumor promoter phorbol 13-myristate 12-acetate (PMA), the best characterized protein kinase C agonist, frequently regulates gene expression via activation of Fos/Jun (AP-1) complexes. PMA rapidly and transiently induces prostaglandin G/H synthase-2 (PGHS-2) expression in murine osteoblastic MC3T3-E1 cells, but no functional AP-1 binding motifs in the 5′-flanking region have been identified. In MC3T3-E1 cells transfected with −371/+70 bp of the PGHS-2 gene fused to a luciferase reporter gene (Pluc), PMA stimulates luciferase activity up to eightfold. Computer analysis of the sequence of the PGHS-2 promoter region identified three potential AP-1 elements in the −371/+70 bp region, and deletion analysis suggested that the sequence 5′-aGAGTCA-3′ at −69/−63 bp was most likely to mediate stimulation by PMA. Mutation of the putative AP-1 sequence reduces the ability of PMA to stimulate Pluc activity by 65%. On electrophoretic mobility shift analysis (EMSA), PMA induces binding to a PGHS-2 probe spanning this sequence, binding is blocked by an unlabeled AP-1 canonical sequence, and antibodies specific for c-Jun and c-Fos inhibit binding. Mutation of this AP-1 site also causes a small (22%) but significant reduction in the serum stimulation of Pluc activity in transiently transfected MC3T3-E1 cells. On EMSA, serum induces binding to a PGHS-2 probe spanning the AP-1 site, binding is blocked by an unlabeled AP-1 canonical sequence, and antibodies specific for c-Jun and c-Fos inhibit binding. Joint mutation of this AP-1 site and the nearby CRE site at −56/−52 bp, previously shown to mediate serum, v-src and PDGF induction of PGHS-2 in NIH-3T3 cells, blocks both PMA and serum induction of Pluc activity in MC3T3-E1 cells. Hence, the AP-1 and CRE binding sites are jointly but differentially involved in both the PMA and serum stimulation of PGHS-2 promoter activity. J. Cell. Biochem. 78: 197–209, 2000.

Key words: cyclooxygenase; prostaglandin; activator protein-1; cAMP response element; serum

Prostaglandins (PGs) are produced by osteoblasts; these complex regulators of bone remodeling both stimulate and inhibit bone resorption and bone formation [Pilbeam et al., 1995]. The major enzyme regulating the conversion of arachidonic acid released from membrane phospholipids to PGs is prostaglandin G/H synthase (PGHS), also called cyclooxygenase. PGHS oxidizes arachidonic acid to PGG_2 and reduces PGG_2 to PGH_2 [Smith et al., 1996]. There are two forms of PGHS, PGHS-1, and PGHS-2, which are products of distinct genes. PGHS-1 is usually constitutively expressed, whereas PGHS-2 is rapidly and transiently induced [Smith et al., 1996; Xie and Herschman, 1996]. PGHS-2 is the enzyme responsible for PG elevation in bone cells in response to hormones, cytokines, and growth factors [Pilbeam et al., 1993; Kawaguchi et al., 1994; Pilbeam et al., 1994; Harrison et al., 1994; Kawaguchi et al., 1995; Klein-Nulend et al., 1997; Pilbeam et al., 1997; Tai et al., 1997; Tetradis et al., 1997; Chen et al., 1997; Min et al., 1998].

Murine PGHS-2 was originally identified as a PMA-inducible gene in fibroblasts [Kujubu et al., 1991, 1993]. PMA is a tumor promoter and the prototypic protein kinase C (PKC) activating agent. PMA regulates expression of many genes by stimulating the binding of activating protein-1 (AP-1) transcription factor com-

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plexes, dimers of basic region leucine zipper (bZIP) proteins, at specific DNA sites. The AP-1 transcription factor family consists of Jun-related proteins (v-Jun, c-Jun, Jun B, Jun D), Fos-related proteins (v-Fos, c-Fos, Fos B, Fra1, Fra2) and activating transcription factor-related proteins (ATF2, ATF3/LRF1, B-ATF) [Karin et al., 1997]. Homodimers of Jun or heterodimers of Fos-Jun preferentially bind to the AP-1 sequence TGA(G/C)TCA, while homodimers of ATF or heterodimers of ATF-Jun preferentially bind to the cAMP-response element (CRE), TGACGTCA [Karin et al., 1997].

The AP-1 complex is involved in cellular proliferation, tumorigenesis, and inflammation in multiple tissues. In bone, Fos and Jun are important in the regulation of growth and development, with selective expression of family members at different stages of osteoblast differentiation [McCabe et al., 1996]. Disruption of the c-fos protooncogene leads to osteopetrosis in mice, characterized by a complete absence of osteoclasts [Grigoriadis et al., 1994]. In mice overexpressing c-Fos, osteoblasts are the targets for transformation, and these mice subsequently develop osteosarcomas [Grigoriadis et al., 1993; Wang et al., 1995]. AP-1 transacting factors are also implicated in inflammatory processes. In mice with collagen-induced arthritis, bone resorption and synovial overgrowth at joints can be inhibited by administering short double-stranded AP-1 DNA oligonucleotides to compete for the binding of AP-1 in vivo [Shiozawa et al., 1997]. The anti-inflammatory actions of glucocorticoids may be mediated in part by interactions of glucocorticoid receptors with AP-1 proteins [Krane, 1993].

We identified three sequences in the −371/+70 bp 5′-flanking region of the murine PGHS-2 gene, which differ by only 1 bp from the AP-1 consensus sequence. Using MC3T3-E1 cells transfected with varying lengths of PGHS-2 5′-flanking DNA fused to a luciferase reporter gene (Pluc), we determined the site most likely to mediate the PMA induction of luciferase expression. Mutation of this site leads to a marked reduction of PMA-stimulated activity. Mutation of a nearby CRE results in an additional reduction in PMA stimulated luciferase activity, and joint mutation of both sites blocks PMA induction of luciferase activity.

MATERIALS AND METHODS

Materials

Murine PGHS-2 cDNA was described previously [Kujubu et al., 1991]. DNA constructs consisting of −371 to +70 bp of the PGHS-2 gene, or 5′ deletions of this region, fused to a luciferase reporter gene in pXp-2 vector have been described previously [Fletcher et al., 1992; Bazan et al., 1994]. Murine PGHS-1 cDNA was the gift of Drs. David DeWitt and William Smith (Michigan State University, East Lansing MI). Murine cDNA for glyceraldehyde phosphate dehydrogenase (GAPDH) was amplified by polymerase chain reaction (PCR) with a control amplimer set from Clontech (Palo Alto, CA). PMA, cycloheximide (CHX), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

MC3T3-E1 Cell Culture

MC3T3-E1 cells were the kind gift of Dr. Yoshiyuki Hakeda (Meikai University School of Dentistry, Sakado, Saitama, Japan). Cells were plated in six-well dishes at a density of 5,000 cells/cm^2 and grown for 6 days in Dulbecco's modified Eagle's medium (DMEM) without phenol red (Sigma) containing 10% heat-inactivated fetal calf serum (FCS) (Gibco-BRL, Grand Island, NY), penicillin (100 U/ml), and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. On the sixth day of culture, cells reached full confluence (about 200,000 cells/cm^2). At this time, cells were changed to serum free medium with 0.1% BSA for 24 h before treatment with agonists. All treatment groups were controlled for test material vehicle, and the concentration of alcohol was 0.01% or less.

Stable Transfection

Constructs were purified by CsCl banding and co-transfected with pSV2-neo into MC3T3-E1 cells, cultured as described above to 50–80% confluency in six-well dishes. Cells in each well were incubated with 1 μg of promoter-reporter DNA, 0.067 μg of pSV2-neo DNA, and 8 μl Lipofectamine reagent (Gibco-BRL) in 1 ml of serum-free medium without antibiotics. After 5 h of incubation, an additional 1 ml of medium with 20% FCS was added; 19 h later, the medium was replaced with fresh complete medium; 72 h after transfection, cells were split 1:10 into 100-mm dishes and placed under selection with 400 μg/ml of

G418 for 2 weeks. Stable colonies (>200) were pooled to minimize effects secondary to variable integration sites. After selection, cells were grown in medium containing 200 μg/ml G418. To maintain uniform cell phenotype, the −371/+70 bp construct, and all shorter constructs were transfected at the same time in the same passage cells.

Transient Transfection

DNA constructs were purified by CsCl or Qiagen columns (Valencia, CA). Cells were plated at 5,000/cm^2 24 h before transfection and serum deprived 1 h before transfection. For each DNA construct, three wells of a six-well dish were transfected. Each well received 1 μg DNA (in 10 μl of 20 mM Hepes buffer, pH 7.4), 8 μl of DOTAP Liposomal Transfection Reagent (Boehringer-Mannheim, Indianapolis, IN) (in 50 μl Hepes buffer), and 940 μl DMEM. The medium was replaced 5–6 h later with 2 ml/well of 2%-FCS in DMEM. Cells were treated 72 h after transfection. All experiments contained the wild type (−371/+70 bp construct) and were repeated at least three times.

Luciferase Activity

Luciferase activity was measured in soluble cell extracts prepared with a kit from Promega (Madison, WI), using an automatic injection luminometer (Berthold Lumat; Wallac, Gaithersburg, MD). For each experiment, three wells of a six-well dish of cells were analyzed per treatment group. Stably transfected cells and transiently transfected cells were scraped in 200 and 100 μl of lysis buffer, respectively. Activity was normalized to total proteins measured with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

Northern Blot Analysis

Three wells of cells in six-well dishes were pooled for RNA extraction, using the method of Chomczynski and Sacchi [1987]. Briefly, cells were homogenized in 4 M guanidinium thiocyanate, extracted with phenol/chloroform-isoamyl (24:1) and RNA precipitated with isopropanol and washed with 80% ethanol. After quantitation at 260 nm, 20 μg of total RNA was run on a 1% agarose-2.2 M formaldehyde gel and transferred to a nylon membrane (Genescreen; New England Nuclear Research, Boston, MA) by capillary pressure and fixed to the membrane by ultraviolet irradiation. After 3 h of prehybridization in 50% formamide solution in rotating cylinders at 42°C with a random primer [^{32}P]dCTP (New England Nuclear Research)-labeled cDNA probe for PGHS-2, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Filters were washed in a 1× sodium saline citrate (SSC), 1% sodium dodecyl sulfate (SDS) solution, 0.1% ×SSC, at room temperature, then washed three times at 65°C with 0.1% (SDS). Filters were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −70°C. Signals were quantitated by densitometry (Bio-Rad Laboratories, Richmond, CA) or by analysis of scanned in images with Scan Analysis (Biosoft, Ferguson, MO). Filters were stripped with boiling 0.1% SDS-0.1× SSC between hybridizations.

Electrophoretic Mobility Shift Assay (EMSA)

To prepare nuclear extracts, cells were washed with phosphate-buffered saline (PBS), scraped in PBS, collected by centrifugation, suspended in solution A (10 mM Hepes-KCl, pH 7.9, 0.1 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine, 0.5 mM DTT, 0.5 mM PMSF, 1 μg leupeptin, and 1 μg pepstatin A) and allowed to swell for 5 min. Cells were centrifuged again, resuspended in solution A, and homogenized with a Dounce homogenizer. After another centrifugation, the cell pellet was suspended in a solution of 20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. After further homogenization, homogenates were centrifuged and supernatants were dialyzed against a solution of 20 mM Hepes, pH 7.9, 20% glycerol, 0.1 M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 1 μg/ml leupeptin, and 1 μg of pepstatin A.

For gel electrophoresis, single-stranded oligonucleotides (Oligos Etc., Wilsonville, OR) spanning −80 to −57 bp of the 5′-flanking region of the PGHS-2 gene were end-labeled with [γ^{32}P]-ATP (New England Nuclear) using T4 kinase (Gibco). Complementary oligonucleotides were annealed, and the resulting double-stranded DNA purified; 6 μg of nuclear extract was incubated in 20 μl binding reaction mixture (10 mM Tris HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 5% glycerol, and 2 μg poly dI-dC (Pharmacia and Upjohn, Bridgewater, NJ)) with 50,000 cpm of labeled probe. Five percent nondenaturing acrylamide gel electrophoresis was performed for 1.5–2 h and gels fixed for 30

min in 10% methanol and 10% acetic acid. Dried gels were exposed to X-ray film. Competitors (10–30 molar excess) or supershifting antibodies (2 μl of 2 μg/μl solution) were added to the binding mixture 15–30 min before or after addition of the probe, and incubation was continued for 30 min. An oligonucleotide containing the consensus binding sequence for AP-1 complexes (5′-TGACTCA-3′) and antibodies designed to supershift c-Fos or c-Jun binding complexes were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Deletions and Site-Directed Mutations

The PGHS-2 promoter-luciferase fusion gene (Pluc), containing 371 bp of the 5′-flanking sequence immediately proximal to the transcription start site and 70 bp of downstream untranslated DNA (−371/+70), and the series of shorter deletion constructs based on the −371/+70 bp construct have been described previously [Fletcher et al., 1992; Bazan et al., 1994]. The promoterless luciferase vector was made by cutting the −371/+70 bp promoter-luciferase construct with *Bam*HI and *Bgl*II, followed by ligation with T4 ligase (Gibco-BRL). The 3′-deletion of −371/+70 to −371/+6 bp was made by PCR, adding a convenient restriction sequence to the 3′ primers to match a cloning site in the pXp2 linker region. PCR products were subcloned using the TA Cloning Kit (Invitrogen, San Diego, CA). The CRE mutation, in which the sequence 5′-CGTCA-3′ at −56 to −52 bp in the PGHS-2 5′-flanking region was changed to 5′-ATTCA-3′ (muCRE), has been discussed previously [Xie et al., 1994]. The putative AP-1 sequence 5′-AGAGTCA-3′ at −69/−63 bp was changed to 5′-AGAGTTG-3′ (muAP-1), using the QuikChange™ Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). In this system, the −371/+70 bp PGHS-2-luciferase plasmid was denatured and annealed to oligonucleotide primers containing the desired mutation. The mutagenic primers were extended with Pfu DNA polymerase, resulting in nicked circular strands. The methylated, nonmutated parental DNA template was digested away with *Dpn*1. XL1-Blue supercompetent *Escherichia coli* were transformed with the circular dsDNA and repaired the nicks. For the joint AP-1, CRE mutation, the plasmid carrying the muCRE DNA construct was used as template. After introduction of deletions or mutations, the PGHS-2 5′-flanking region was sequenced (Automated DNA Sequence Facility, University of Conn. Health Ctr.). To avoid the accidental introduction of mutations into regions outside the PGHS-2 5′-flanking region, the 5′-flanking region was released from the pXp2 plasmid and subcloned into a fresh pXp2 vector. Mutated oligonucleotides were used as unlabeled competitors on EMSA to affirm that binding to the mutated sequence did not occur.

Statistics

Means of groups were compared by analysis of variance (ANOVA). The significance of differences was determined by post hoc testing, using Bonferroni's method [Godfrey, 1985]. Differences were considered significant at $P < 0.05$.

RESULTS

PMA Induction of PGHS-2 mRNA and Pluc Activity

PGHS-2 mRNA is generally undetectable by Northern blotting in unstimulated MC3T3-E1 cells. PMA (1 μM) induces PGHS-2 mRNA expression within 30 min, with peak effects at 1–2 h (Fig. 1A). Treatment with cycloheximide (CHX; 10 μg/ml) for 1 h induces PGHS-2 mRNA expression and enhances the induction of PGHS-2 mRNA by PMA (Fig. 1B), suggesting that the PMA induction of PGHS-2 mRNA does not require de novo protein synthesis. In MC3T3-E1 cells stably transfected with −371/+70 bp of PGHS-2 DNA fused to a luciferase reporter gene (Pluc371), PMA stimulates luciferase activity up to eightfold at 2 h (Fig. 2). The stimulation is very transient and luciferase levels return to control values by 6 h.

Deletion Mapping of the PGHS-2 Promoter

To begin the mapping of the PGHS-2 5′-flanking region for DNA elements mediating the induction by PMA, MC3T3-E1 cells were transiently transfected with either −371/+70 or −963/+70 bp Pluc constructs. There is no difference in PMA stimulated luciferase activities in cells carrying either construct after treatment with PMA (1 μM) for 3 h (Fig. 3). A longer promoter fragment containing approximately 4 kb of 5′-flanking sequences also confers five- to sixfold inducibility on a luciferase promoter (data not shown). These results suggest that the regions that mediate the effects of PMA lie within the −371/+70-bp fragment.

MC3T3-E1 cells were stably transfected with a series of 5′-deletion constructs: −371/+70 bp

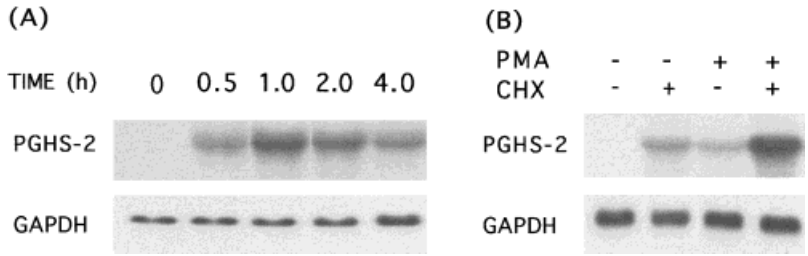

Fig. 1. Phorbol myristate acetate (PMA) induction of PGHS-2 mRNA expression in MC3T3-E1 cells: time course and lack of inhibition by cycloheximide (CHX). Cells were treated with PMA (1 μM) for the indicated times (**A**) or for 1 h (**B**). CHX (5 μg/ml) was added 45 min before PMA. Total RNA was extracted and Northern blot analysis performed as described in Methods and Materials. mRNA levels for the housekeeping gene GAPDH are shown to assess RNA loading.

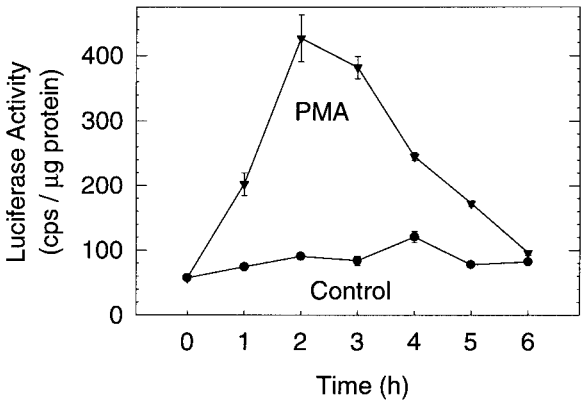

Fig. 2. Time course for stimulation of luciferase activity by phorbol myristate acetate (PMA) in MC3T3-E1 cells stably transfected with −371/+70 bp of the PGHS-2 5′-flanking DNA fused to a luciferase reporter gene. Cells were treated with vehicle (control) or PMA (1 μM) for the indicated times. Luciferase activity, measured as described in Materials and Methods, is expressed as counts per second (cps) per μg total protein. Symbols are means ±SEM for n = 3 samples.

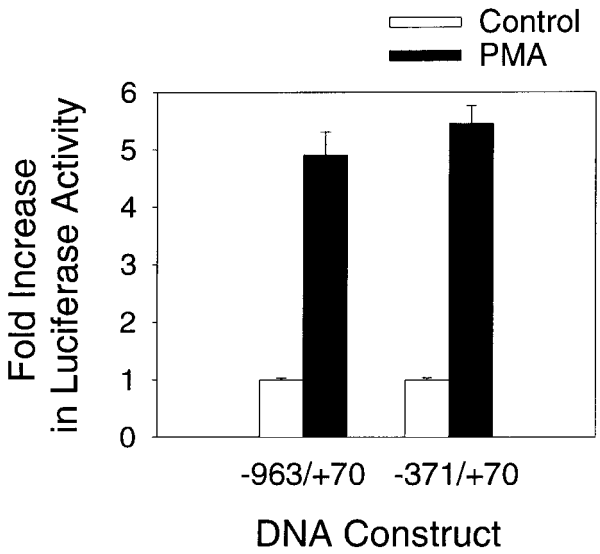

Fig. 3. Comparison of phorbol myristate acetate (PMA)-stimulated luciferase activity in MC3T3-E1 cells transiently transfected with either -973/+70 bp or −371/+70 bp of the PGHS-2 5′-flanking DNA fused to a luciferase reporter. Cells were treated with vehicle (control) or PMA (1 μM) for 3 h. Luciferase activity was normalized to total protein and the fold increase in activity calculated as treated/control ratios. Bars = ±SEM for n = 3 replicates.

(Pluc371), −150/+70 bp (Pluc150), −40/+70 bp (Pluc40), and the luciferase vector without any PGHS-2 5′-flanking region (Fig. 4). Basal luciferase activity is significantly less ($P < 0.01$) in Pluc150 cells than in Pluc371 cells in this series of experiments, but this decrease has not been a consistent finding. The fold increase in luciferase activity with PMA is the same in both Pluc371 and Pluc150 cells (Fig. 4A). As seen in studies with other agents [Pilbeam et al., 1997], basal and stimulated luciferase activities are reduced 96–98% by deletion to −40/+70 bp. The fold increase in luciferase activity with PMA is reduced 83% by deletion to −40/+70 bp, and the small increase seen in PMA-treated Pluc40 cells is not statistically significant.

Mutation of a Putative AP-1 Binding Sequence

Computer analysis identified three potential AP-1 binding sites within the −371/+70 bp region with the sequences shown in Figure 5. All these potential binding sites differ from the canonical AP-1 binding site by 1 bp, as indicated by the lowercase letters. The deletion analyses above suggest that the potential AP-1 site at −69/−63 bp is the most likely AP-1 site to mediate PMA induction of luciferase activity.

To examine the role of the −69/−63 bp sequence, we made a 2-bp mutation, changing

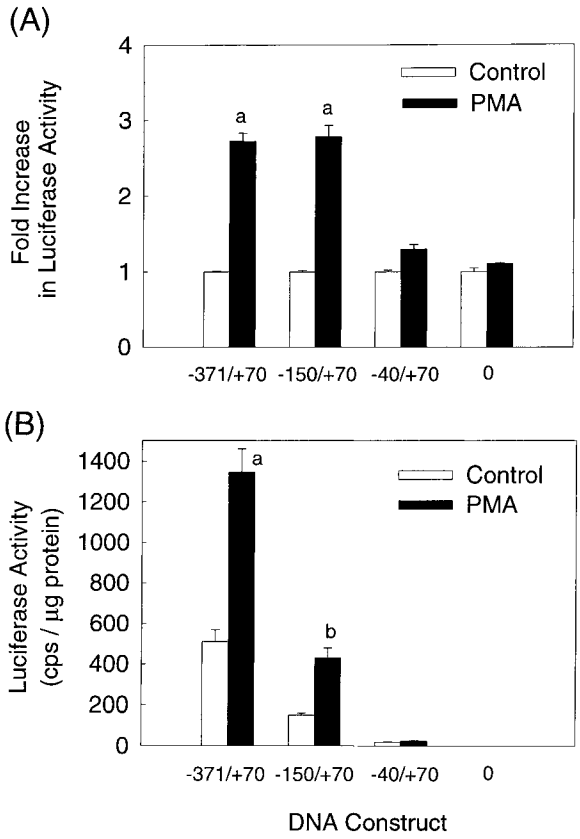

Fig. 4. Comparison of phorbol myristate acetate (PMA)-stimulated luciferase activity in MC3T3-E1 cells stably transfected with either −371/+70, −150/+70, −40/+70, or 0 bp of the PGHS-2 5′-flanking PGHS-2 DNA fused to a luciferase reporter. Cells were treated with vehicle (control) or PMA (1 μM) for 3 h. **A:** Luciferase activity, normalized to total protein, is expressed as the fold increase in luciferase activity, calculated as treated/control ratios. **B:** Absolute luciferase activity is expressed as counts per second (cpm) per μg protein. Bars = means ±SEM for three experiments, each with n = 3 replicates. [a] Significantly different from control, $P < 0.01$. [b]Significantly different from control, $P < 0.05$.

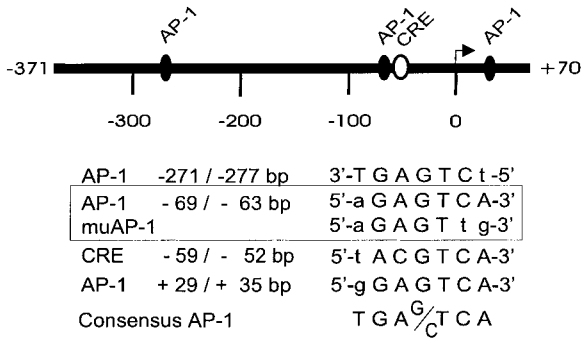

Fig. 5. Potential AP-1 transactivating factor binding sites in the 5′-flanking DNA of the murine PGHS-2 gene. A 2-bp mutation (muAP-1) was made in the −69/−63 bp putative AP-1 site as described in Materials and Methods.

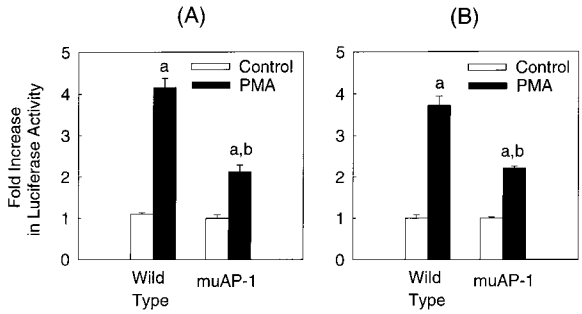

Fig. 6. Effect of mutating the −69/−63 bp AP-1 site (muAP-1) in the −371/+70 bp reporter construct (Wild Type) on phorbol myristate acetate (PMA)-stimulated luciferase activity in MC3T3-E1 cells. Cells were treated for 3 h with vehicle (control) or phorbol myristate acetate (PMA) (1 μM). **A:** Data are means ±SEM from 7 experiments, each with n = 3 replicates per treatment group, on transiently transfected cells. **B:** Data are means ±SEM from 2 experiments, each with n = 3 replicates per treatment group, on stably transfected cells. [a]Significantly different from control, $P < 0.01$. [b]Significantly different from PMA-treated wild type, $P < 0.01$.

the sequence from 5′-AGAGTCA-3′ to 5′-AGAGTTG-3′. MC3T3-E1 cells were transiently transfected in 7 separate experiments, each with three replicates, with either the unmutated Pluc371 construct (wild-type) or the Pluc371 construct carrying the 2-bp mutation (muAP-1). The mutation reduced PMA (1 μM)-stimulated luciferase activity at 3 h by 65% (Fig. 6A). For comparison, we examined the effect of this mutation on PMA-stimulated luciferase activity in MC3T3-E1 cells stably transfected with wild-type or mutated Pluc constructs. The data presented in Figure 6B come from two separate experiments, each with three replicates per treatment group, and show a 55% decrease in fold increase in luciferase activity with the mutation. There is no consistent difference in basal luciferase activities between cells carrying the wild-type Pluc and cells carrying the mutated Pluc (data not shown).

Because the potential AP-1 site at +29/+35 bp is very similar to the site at −69/+70 bp and because the marked decrease of basal luciferase activity on deletion to −40/+70 bp might obscure a role for this site, we also examined cells transfected with −371/+6 bp of the PGHS-2 promoter fused to luciferase. Comparison with cells transfected with the −371/+70 bp construct shows no difference in the fold stimulation by PMA of luciferase activity (data not shown).

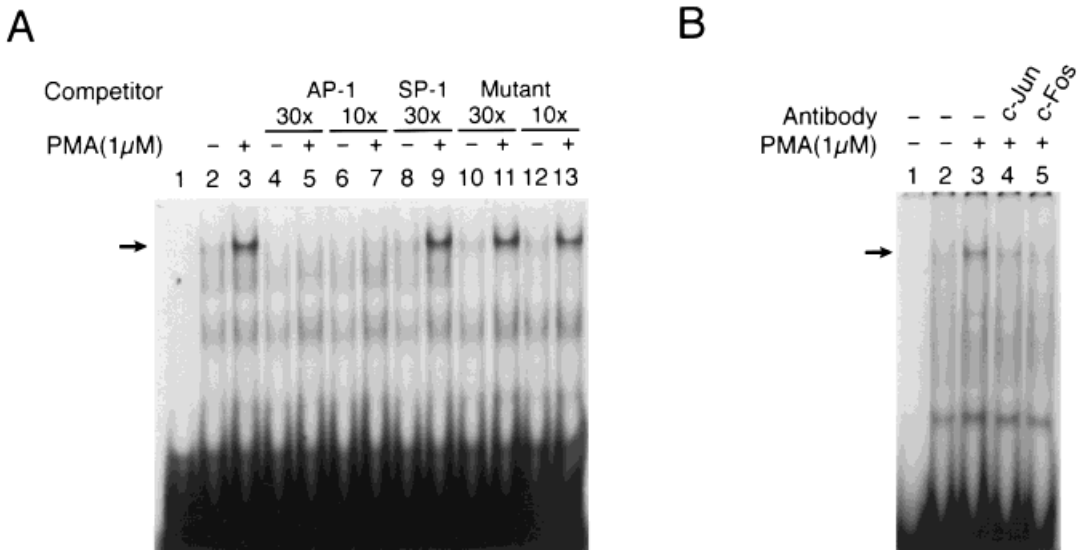

Fig. 7. Electrophoretic mobility shift assay (EMSA) analysis of phorbol myristate acetate (PMA)-induced nuclear binding to a ^{32}P-labeled PGHS-2 oligonucleotide (−80/−57 bp) spanning the −69/−63 bp AP-1 site. MC3T3-E1 cells were treated with vehicle or PMA for 1 h and nuclear proteins extracted as described in Materials and Methods. **Lane 1**, the free probe. **A:** PMA induces binding (**lane 3**) that is blocked by addition of 30-fold molar excess (**lane 5**) or 10-fold excess (**lane 7**) of an unlabeled oligonucleotide with the consensus AP-1 sequence. Binding is not blocked by an unlabeled SP-1 consensus oligonucleotide (**lane 9**) or the unlabeled −80/−57 bp PGHS-2 probe with the AP-1 site mutated (mutant; **lanes 11, 13**). **B:** phorbol myristate acetate (PMA)-induced binding (**lane 3**) is inhibited by antibodies specific for c-Jun (**lane 4**) or c-Fos (**lane 5**).

PMA-Induces Binding to the Putative AP-1 Sequence

To examine binding to the −69/−63 bp PGHS-2 sequence, we prepared a double-stranded oligonucleotide ^{32}P-labeled probe spanning −80 to −57 bp of the PGHS-2 5′-flanking sequence. The probe did not overlap the CRE site (Fig. 5). MC3T3-E1 cells were treated for 1 h with or without PMA (1 μM). Electrophoretic mobility shift assay (EMSA) shows increased binding to the PGHS-2 probe after treatment with PMA (Fig. 7A, lane 3). This binding is competed by the unlabeled PGHS-2 probe (data not shown) and by an oligonucleotide containing the AP-1 canonical sequence (Fig. 7A, lanes 5, 7), suggesting that this is indeed a binding site for AP-1 complexes. Binding is not competed by an SP-1 consensus sequence (Fig. 7A, lane 9), confirming specificity, or by the unlabeled −80/−57 bp PGHS-2 oligonucleotide with the 2-bp AP-1 mutation used in the transfection studies above (Fig. 7A, lanes 11, 13), confirming that the mutation disrupts binding to the PGHS-2 AP-1 sequence. A light band is seen in untreated cells (Fig. 7A, lane 2), which is also competed by the AP-1 consensus sequence (Fig. 7A, lanes 4, 6) and not competed by the SP-1 or mutated AP-1 oligonucleotide (Fig. 7A, lanes 10, 12). Antibodies, specific for c-Jun and c-Fos, do not supershift the PMA-induced binding but do inhibit binding (Fig. 7B, lanes 4, 5). Similar inhibition was seen when these antibodies were added before or after the PGHS-2 probe, as well as, with antibodies that bind to multiple members of the c-Fos and c-Jun families (data not shown).

Involvement of the AP-1 Sequence in the Serum Induction of PGHS-2

Because serum induction of PGHS-2 has been shown to be mediated via c-Jun activation [Xie and Herschman, 1995, 1996] in NIH-3T3 cells, we examined the effect of the AP-1 mutation on serum stimulation. MC3T3-E1 cells were transiently or stably transfected with the wild type Pluc371 or the Pluc371 with the mutated AP-1 site and treated with or without 10% fetal calf serum (FCS) for 3 h (Fig. 8). The results in Figure 8A are from eight independent transient transfections, each with three replicates per treatment group, and show a small (22%), but significant, decrease in FCS-stimulated activity by the mutated AP-1 site. The results in Figure 8B are from three separate experiments, each with three replicates per treatment group, in stably transfected cells and show a larger decrease of 71% in FCS-stimulated Pluc activity.

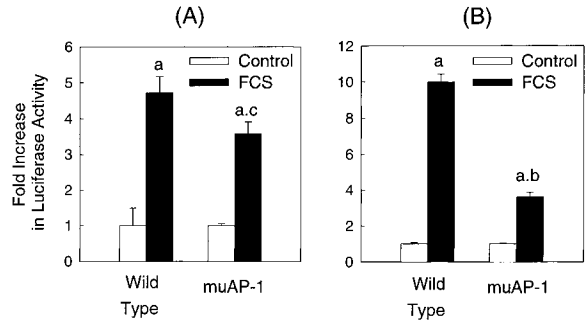

Fig. 8. Fetal calf serum (FCS)-stimulated luciferase activity in MC3T3-E1 cells transiently or stably transfected with the −371/+70-bp PGHS-2 construct (wild-type) or the −371/+70-bp construct with the AP-1 site mutated (muAP-1). Cells were treated with 10% FCS for 3h. (A) Data are means and SEM from 8 experiments, each with n = 3 replicates per treatment group, on transiently transfected cells. **B:** Data are means and SEM from three experiments, each with n = 3 replicates per treatment group, on stably transfected cells. [a]Significantly different from control, $P < 0.01$. [b]Significantly different from FCS-treated wild-type cells, $P < 0.01$. [c]Significantly different from FCS-treated wild-type cells, $P < 0.05$.

On EMSA, treatment of serum-deprived MC3T3-E1 cells with 10% FCS for 2 h induces nuclear factor binding to the ^{32}P-labeled −80/−57 bp PGHS-2 probe (Fig. 9A, lane 3, 9B, lane 2). In addition to the prominent lower band, there are one or more lighter upper bands (top arrow). All FCS-induced bands are competed by an unlabeled oligonucleotide containing the AP-1 canonical sequence (Fig. 9A, lane 5). The mutated AP-1 oligonucleotide does not compete (data not shown). As seen for the PMA study, addition of antibodies specific for c-Jun and/or c-Fos does not supershift the FCS-induced bands but does inhibit all bands (Fig. 9B, lanes 4, 6).

Involvement of the CRE Site in PMA and Serum Induction of Pluc Activity

Mutation of the putative AP-1 site reduces but does not abrogate PMA-stimulated Pluc activity. Moreover, a 2-bp mutation of the CRE site at −56/−52 bp (Fig. 5) in the Pluc371 construct has previously been shown to mediate serum induction of luciferase activity in NIH-3T3 fibroblasts [Xie and Herschman, 1996]. Hence, we examined the effect of this CRE mutation, which replaces 5′-CGTCA-3′ with 5′-ATTCA-3′, on PMA and serum induction of Pluc activity in MC3T3-E1 cells. Cells were transiently transfected with Pluc371, the mutated AP-1 construct, the mutated CRE construct, or a Pluc371 construct with both the AP-1 and the CRE mutations (Fig. 10). Cells were treated with PMA (Fig. 10A) or 10% FCS (Fig. 10B) for 3 h. The results for PMA in Figure 10A are from five separate experiments in which all four DNA constructs were simultaneously transiently transfected. The PMA-stimulated increase in Pluc activity is reduced 66% and 30% by the AP-1 mutation and the CRE mutation, respectively. With the joint mutation, PMA-stimulated activity is reduced 84%, and the PMA treated group is not significantly different from the untreated control. The results for FCS in Figure 10B are from four separate experiments in which all 4 DNA constructs were simultaneously transiently transfected. There is a 22% reduction in FCS-stimulated Pluc activity with the AP-1 mutation, similar to the larger set of experiments, but the reduction is not statistically significant. The CRE mutation and the combined CRE and AP-1 mutations reduce activity 56% and 75%, respectively, and neither are significantly different from their control groups. These results suggest that the AP-1 and CRE elements are jointly involved in mediating PMA and serum induction of PGHS-2 but that the relative importance of the sites is different for the two agonists.

DISCUSSION

No functional AP-1binding motifs in the 5′-flanking region of the PGHS-2 gene have previously been identified. A recent study in human chondrocytes shows that okadaic acid induces PGHS-2 gene expression and stimulates binding to AP-1 and CRE consensus sequences on EMSA, but no AP-1 binding site is identified in the PGHS-2 promoter in this study [Miller et al., 1998]. The tumor promoter PMA, which frequently acts via induction of binding of Fos/Jun complexes to an AP-1 site, rapidly induces PGHS-2 expression in osteoblastic MC3T3-E1 cells. We screened for functional AP-1 motifs in the PGHS-2 promoter region by examining luciferase responses to PMA in MC3T3-E1 cells transfected with PGHS-2 promoter-luciferase reporter constructs. Deletion analyses show that the 5′-flanking region of PGHS-2 from −371 to −40 bp is sufficient to mediate PMA stimulation of Pluc activity. Mutation of the putative AP-1 sequence at −69/−63 bp in the 5′-flanking region of the murine PGHS-2 gene reduces PMA-stimulated luciferase activity, measured as treated/control ratios, in transiently transfected cells by 65%. On EMSA, binding to a PGHS-2

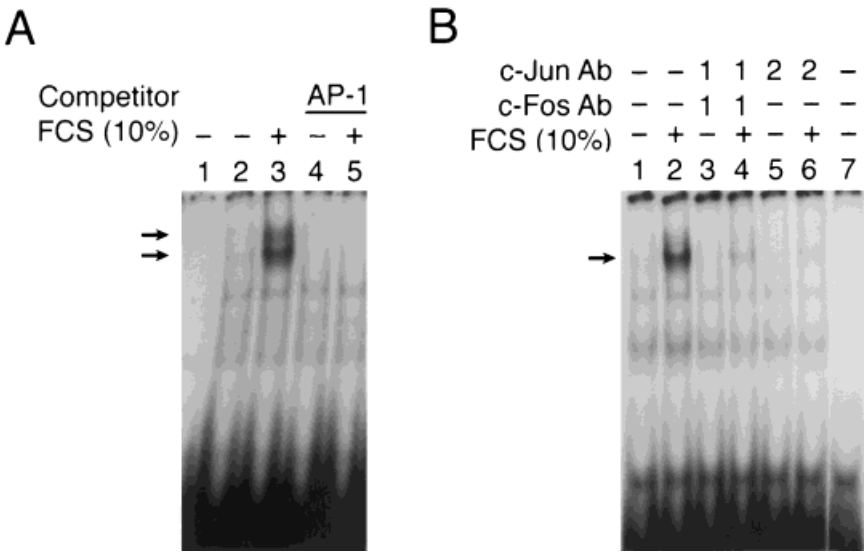

Fig. 9. Electrophoretic mobility shift assay (EMSA) analysis of fetal calf serum (FCS)-induced nuclear binding to a ^{32}P-labeled PGHS-2 oligonucleotide (−80/−57 bp) spanning the −69/−63 bp AP-1 site. MC3T3-E1 cells were treated 10% FCS for 1 h and nuclear proteins extracted as described in Materials and Methods. **A: Lane 1**, free probe; **lanes 2,4**, from cells treated with serum-free media; **lanes 3,5**, from FCS-treated cells. FCS induces binding (**lane 3**) that is blocked by the addition of 30-fold molar excess of an unlabeled oligonucleotide with the consensus AP-1 sequence (**lane 5**). **B: Lanes 1,3,5**, from cells treated with serum-free media; **lanes 2,4,6**, from FCS-treated cells; **lane 7**, free probe. FCS induces binding (**lane 2**) that is inhibited by antibodies specific for c-Fos and c-Jun; **lane 4**, 1 part c-Jun and 1 part c-Fos antibody or **lane 6**), an equal amount of antibody only for c-Jun.

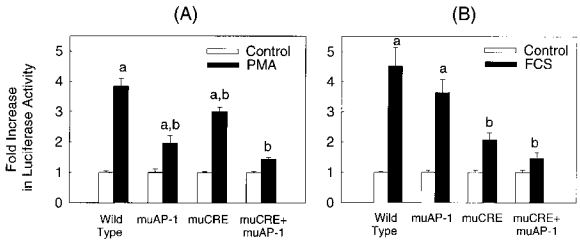

Fig. 10. Combined effects of AP-1 and CRE mutations on the stimulation of luciferase activity by PMA or fetal calf serum (FCS). MC3T3-E1 cells were transiently transfected simultaneously with four DNA reporter constructs: −371/+70 bp (wild type), −371/+70 bp with the AP-1 site mutated (muAP-1), −371/+70 bp with the CRE site mutated (muCRE) or −371/+70 bp with both AP-1 and CRE sites mutated (muCRE + muAP-1). **A:** Cells were treated with vehicle (control) or phorbol myristate acetate (PMA) (1 μM) for 3 h. Data are means ±SEM from five experiments, each with n = 3 replicates per group. **B:** Cells were treated with serum-free media (control) or 10% FCS for 3 h. Data are means ±SEM for four experiments, each with n = 3 replicates per group. [a]Significantly different from control, $P < 0.01$. [b]Significantly different from treated wild-type, $P < 0.01$.

probe spanning the putative AP-1 sequence is competed by the unlabeled canonical AP-1 oligonucleotide and inhibited by antibodies specific for c-Fos and c-Jun. These results suggest that the −69/−63 bp PGHS-2 motif is a functional AP-1 binding site that mediates, in part, the induction of PGHS-2 gene expression by PMA.

To assess the importance of this site for a more physiologic agonist, we examined the effect on serum stimulation because previous studies have shown that serum induction of PGHS-2 in NIH-3T3 cells is mediated via c-Jun activation [Xie and Herschman, 1995, 1996]. Mutation of the AP-1 site causes a small (22%) reduction in the serum stimulated increase in luciferase activity in transiently transfected MC3T3-E1 cells. As seen for PMA, serum induced binding on EMSA to a PGHS-2 probe spanning the PGHS-2 AP-1 sequence, which was competed by the unlabeled canonical AP-1 oligonucleotide and inhibited by antibodies specific for c-Fos and c-Jun. We have found that when MC3T3-E1 cells differentiate into more mature osteoblastic cells, based on their ability to express the osteoblastic marker, osteocalcin, serum stimulation of PGHS-2 expression, Pluc activity, and nuclear protein binding to the AP-1 site on EMSA are lost (data not shown). Hence, the role of this AP-1 site in the serum induction of PGHS-2 may be restricted to early stages of osteoblastic differentiation.

Reporter assays comparing responses of different constructs in transiently transfected cells can be confounded by variation in trans-

fection efficiencies. For this reason, we performed multiple independent experiments, each containing all constructs to be compared; used constructs from more than one plasmid preparation; calculated results for each construct in an experiment as treated/control ratios before comparison of constructs; and performed similar experiments in stably transfected cells. For PMA, mutation of the AP-1 site caused similar reductions in Pluc activity of 65% and 55% in transient and stably transfected cells, respectively. For serum stimulation, however, there was a markedly greater inhibition of treated/control activity in stably transfected cells (71%) than in transiently transfected cells (22%). Regulation of stably transfected transfected DNA constructs may more realistically reflect regulation at the chromosomal level. Although stably integrated constructs are subject to regulation by unrelated elements adjacent to the random integration site, multiple clones were pooled to minimize this effect. The difference in serum-stimulated Pluc activities between transiently and stably transfected cells may simply reflect differences in cell phenotype resulting from different culture histories and confluence states at the time of treatment.

The CRE sequence at −57/−52 bp in the murine PGHS-2 promoter has been shown by site-directed mutation to mediate the induction of promoter activity by v-src [Xie and Herschman, 1995; Xie et al., 1994], serum, and PDGF [Herschman and Xie, 1996] in NIH-3T3 fibroblasts. The CRE site is also involved in mediating PMA induction of PGHS-2. The PMA-stimulated increase in Pluc activity is reduced 30% by the CRE mutation and 84% by the combined AP-1/CRE mutations. Binding to the CRE sequence is constitutive in these cells and was not upregulated by either PMA or serum (data not shown). The single AP-1 mutation has a larger effect on PMA-stimulated activity than the single CRE mutation, whereas the reverse is true for serum-stimulated activity, but induction of Pluc activity by both PMA and serum is blocked by the combination of mutations. Preliminary data suggest that both AP-1 and CRE sites may also be involved in mediating induction of PGHS-2 by fibroblastic growth factor-2 (FGF-2) [Okada et al., 1998] and tumor necrosis factor-α (TNF-α) [Tomita et al., 1998]. Hence, it seems likely that members of the AP-1 and CRE/ATF family may interact to mediate regulation of PGHS-2 by a variety of agonists.

Induction of PGHS-2 promoter activity in NIH-3T3 cells by v-src, serum, and PDGF has been shown to occur through the CRE element via activation of a Ras/mitogen-activated protein kinase kinase/Jun N-terminal kinase (JNK) signal transduction pathway leading to c-Jun transactivation [Xie and Herschman, 1995; Xie et al., 1994; Herschman and Xie, 1996]. In human chondrocytes, the induction of PGHS-2 by okadaic acid is reported to involve interaction of this MAP kinase pathway and the protein kinase A pathway, leading to transactivation by members of the AP-1 and CRE/ATF families [Miller et al., 1998]. Interactions between the AP-1 and CRE/ATF families of transcription factors are common, and it has been suggested that they can be grouped into a superfamily [Hai and Curran, 1991]. AP-1 complexes are dimers of bZip proteins consisting of Jun-related proteins and Fos-related proteins. The CRE/ATF family consists of the bZip proteins CREB, CREM, and ATF. Some members of the ATF family are considered to belong to the AP-1 family [Karin et al., 1997]. Homodimers of Jun or heterodimers of Fos-Jun preferentially bind to the AP-1 sequence, TGA(G/C)TCA, while homodimers of ATF or heterodimers of ATF-Jun preferentially bind to the CRE, TGACGTCA [Karin et al., 1997]. The CRE/ATF family members prefer to bind to the CRE motif as homodimers [Montminy, 1997]. CREM and CREB can also bind to the AP-1 motif [Masquilier and Sassone-Corsi, 1992]. Members of the CRE/ATF family and the AP-1 family can heterodimerize via their basic leucine zipper domains and can bind as heterodimers to both the AP-1 and CRE motifs [Hai and Curran, 1991]. Cooperative interactions of AP-1 have also been reported with C/EBP [Klampfer et al., 1994] and NF-κB [Martin et al., 1997]. CREB-binding protein (CBP), a signal dependent co-activator, may interact with several of these transacting factors and with the basal transcription factors, thereby integrating or cross-coupling signal pathways at this level [Goldman et al., 1997; Arias et al., 1994].

Studies of regulation of PGHS-2 expression by proinflammatory agents, such as IL-1, TNFα, and lipopolysaccharide (LPS), have focused on the roles of NF-κB and C/EBP transacting factors. In rheumatoid synovial fibroblasts and pulmonary cells, the IL-1 in-

duction of PGHS-2 expression may involve NF-κB, as suggested by increased binding on EMSA and by studies with NF-κB antisense oligonucleotides and inhibitors [Roshak et al., 1996; Newton et al., 1997; Crofford et al., 1997]. In MC3T3-E1 cells, IL-1 also stimulates increased binding to the NF-κB site at −402/−393 bp in the murine PGHS-2 promoter, but deletion of this site has no effect on IL-1-stimulated promoter activity (J. Harrison, unpublished data). Similarly, in pancreatic islet cells, IL-1 induces binding of NF-κB to PGHS-2 promoter elements on EMSA, but mutation of the NF-κB motif does not affect promoter activity [Sorli et al., 1998]. In this latter study, mutation of the NFIL-6 binding motif reduces basal promoter activity but does not block IL-1 induction of activity. NF-κB has also been associated with the LPS induction of PGHS-2 promoter activity in macrophages based on such binding studies [D'Acquisto et al., 1997]. Both NF-κB and C/EBP motifs have been implicated in the TNFα induction of promoter activity in MC3T3-E1 cells [Yamamoto et al., 1995], and both C/EBP and CRE motifs have been implicated in the LPS induction of activity in vascular endothelial cells [Inoue et al., 1995]. In both of the latter studies, site-directed mutations of these motifs reduced basal activity but had little effect on specific induction by the agonists.

A previous study of PMA stimulation of human PGHS-2 promoter activity was conducted by Inoue et al. [1995], using bovine arterial endothelial cells transiently transfected with a −327/+59 bp human PGHS-2 promoter-reporter construct. These investigators found a 75% reduction in basal promoter activity with a joint mutation of the C/EBP (NFIL-6) site at −132/−124 bp and the CRE site at −59/−53 bp. The reduction in promoter activity with the individual C/EBP and CRE mutations was 30 and 10%, respectively. The authors reported that the PMA-stimulated fold increase in luciferase activity did not differ in cells carrying the wild type construct and cells carrying constructs with mutations of the CRE site, the C/EBP site, or both.

There is a C/EBP canonical binding sequence in the 5′-flanking region of the murine PGHS-2 gene at −139/−130 bp. We have examined MC3T3-E1 cells transiently and stably transfected with Puc371 carrying a 2-bp mutation of this C/EBP site [Harrison et al., 1994] and found no effect on the specific induction of luciferase activity by PMA (data not shown). Serum treatment of MC3T3-E1 cells induces binding on EMSA to this C/EBP site, but there is no effect of the mutation on the specific stimulation of Pluc activity by serum in MC3T3-E1 cells (data not shown).

Signaling pathways involved in the PMA induction of PGHS-2 may be also implicated in the elevated expression of PGHS-2 that has been associated with tumorigenesis in several tissues [DuBois et al., 1998]. Overexpression of PGHS-2 is implicated as an early abnormality in colon carcinoma, and inhibition of PGHS-2 activity can suppress growth of colon cancer cells in vitro and tumors in animal models [Sheng et al., 1997; Tsujii et al., 1997; Williams et al., 1996; Oshima et al., 1996]. Resistance to apoptosis, delayed progression through G1, and increased adhesion to extracellular matrix proteins are seen in cultured rat intestinal cells programmed to overexpress PGHS-2 and may relate to the tumorigenic potential of PGHS-2 [DuBois et al., 1996; Tsujii and DuBois, 1995]. Programmed overexpression of PGHS-1 has also been associated with tumorigenesis [Narko et al., 1997] and both PGHS-1 and -2 overexpression is found in other cancers [Hwang et al., 1998]. A physiologic role for the AP-1 sequence is suggested by its relative conservation. The aGAGTCA at −69/−63 bp in the murine PGHS-2 5′-flanking region is similar to the sequence acAGTCA found at −68/−62 bp in the rat and at −68/−62 bp in the human PGHS-2 gene [Kosaka et al., 1994; Sirois and Richards, 1993], suggesting that this motif may be important for regulating these genes as well.

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