Involvement of Two Sp1 Elements in Basal Endothelial Prostaglandin H Synthase-1 Promoter Activity*

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Prostaglandin H synthase (PGHS,1 EC 1.14.99.1) is a bifunctional enzyme containing a cyclooxygenase activity that catalyzes the biosynthesis of prostaglandins and a peroxidase activity that catalyzes the reduction of prostaglandins to prostacyclins (5). The PGHS-1 is the common precursor of biologically active prostacyclins, while prostacyclin is the downstream product of the PGHS-1 enzyme. The human PGHS-1 gene spans about 22 kb on chromosome 9 containing 2075-base pair fragment, which was transfected in HUVEC. Two important regions were identified. DNase I footprinting identified a protected segment, which contains an Sp1 binding site proximal to the transcription start sites. Band shift assays confirmed specific binding of Sp1 to this segment. Band shift assays further revealed specific binding of Sp1 to a distal region containing a canonical Sp1 site. Mutation of either Sp1 binding site significantly reduced the promoter activity. When both sites were mutated, the activity was reduced to 29% of that of the wild type. Mutation of Sp1 sites did not abrogate promoter activity stimulated by phorbol ester. These results indicate that binding of Sp1 or its related proteins to two widely separated Sp1 sites on the promoter region activates the basal PGHS-1 gene transcription.

Prostaglandin H synthase (PGHS,1 EC 1.14.99.1) is a bifunctional enzyme containing a cyclooxygenase activity that catalyzes the biosynthesis of prostaglandins and a peroxidase activity that catalyzes the reduction of prostaglandins to prostacyclins (5). The PGHS-1 is the common precursor of biologically active prostacyclins, while prostacyclin is the downstream product of the PGHS-1 enzyme. The human PGHS-1 gene spans about 22 kb on chromosome 9 containing 2075-base pair fragment, which was transfected in HUVEC. Two important regions were identified. DNase I footprinting identified a protected segment, which contains an Sp1 binding site proximal to the transcription start sites. Band shift assays confirmed specific binding of Sp1 to this segment. Band shift assays further revealed specific binding of Sp1 to a distal region containing a canonical Sp1 site. Mutation of either Sp1 binding site significantly reduced the promoter activity. When both sites were mutated, the activity was reduced to 29% of that of the wild type. Mutation of Sp1 sites did not abrogate promoter activity stimulated by phorbol ester. These results indicate that binding of Sp1 or its related proteins to two widely separated Sp1 sites on the promoter region activates the basal PGHS-1 gene transcription.

Experimental Procedures

Materials—Reagents for cell cultures were obtained from Sigma. Lipofectin, Optimem 1, and kits for the β-galactosidase assay were obtained from Life Technologies, Inc. Restriction enzymes and Klenow polymerase were obtained from New England Biolabs. pSV-β-gal plasmids, DNase I, Taq polymerase, purified Sp1, and kits for the luciferase assay and DNase I protection assay were obtained from Promega. Radiolabeled nucleotides were obtained from Amersham Corp. Consensus oligonucleotides containing binding sites for Sp1 (ATTGAGGCCCCGGCGCCGAGC), AP-2 (GATCGAACTGACCGCCCGCGG), and NF-κB (AGTGTAGGGACTTCCAGG) were obtained from Promega.

Isolation and Sequencing of PGHS-1 5′-Flanking Region—A bacterio- phage EMBL-3 human genomic library constructed from placental DNA (Clontech) was screened with a 5′-labeled 0.7-kb fragment at the 5′-flanking region of human PGHS-1 genomic DNA, the sequence of which had been previously reported (6). One positive clone containing a 16-kb insert was isolated from 5 × 10⁶ plaques. The positive clone was plaque-purified and mapped. This clone included the first eight exons and part of the first intron. The first intron was subcloned into pGEM7-Zf(Promega) for sequencing. Nucleotide sequences were determined by the chain termination method using specific primers of PGHS-1 genomic DNA.

Construction of 5′-Deletion and Site-directed Mutants—5′-Deletion constructs of the PGHS-1 promoter were generated by polymerase chain reaction (PCR), using the 5′-kb XhoI/EcoRI fragment of this clone containing the 5′-flanking region, the first two exons, and part of the second intron was subcloned into pGEM7-Zf(Promega) for sequencing. Nucleotide sequences were determined by the chain termination method using specific primers of PGHS-1 genomic DNA.

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-761 to -744. The PCR products were purified from agarose gel, digested, and cloned into a promoterless luciferase expression vector, pXP1 (7). The constructs were designated according to their positions relative to the ATG codon as shown in Fig. 2. To generate the constructs of -2916/-221, where the two Sp1 sites were changed, a PCR-mediated site-directed mutagenesis was employed. The primers used for mutations were as follows (from 5' to 3', with mutated bases underlined):

- primer A, GGGCTGGCTCTGAAACCTGAAGCCA;
- primer A', TGGCT-TTCAGGTTTCAGAGCCAGCCC;
- primer B, GGAGGAGCGGTTTTAGAGCCCGGG;
- primer B', CCCCCGGGCTCTAAAACCGCTCCTCC.

The mutant -2610/-2604 (see Fig. 5, construct b) was obtained by first amplifying the template -2916/-221 with the primers A and 241/-221 to generate a 0.6-kb fragment. In a separate tube, a 0.3-kb fragment was generated by 30 cycles of PCR using primers A' and 2916/2899. Both 0.6- and 0.3-kb fragments were gel-purified and combined for one cycle of PCR. Subsequently, primers 2916/2899 and 241/-221 were added for another 30 cycles of PCR. The amplified fragment was digested and subcloned into pXP1 vector.

The double mutant (construct d) was obtained using the mutant -2610/-2604 as the template and primers B and B' as mutagenized primers. The mutants were confirmed by nucleotide sequencing.

Cell Culture, Transient Transfection, and Luciferase Assays—Human umbilical vein endothelial cells (HUVECs) were cultured as described previously (8). To ensure consistent results, passage 1 cultured cells were used throughout the studies unless otherwise indicated. One day before transfection, cells were seeded at 30–40% confluence in a six-well dish. Liposome-mediated transient transfection was performed as described (9). Briefly, HUVECs were transfected with a Lipofectin/DNA mixture containing 12 μg of Lipofectin (Life Technologies, Inc.) and 2 μg of the promoter construct with or without 0.3 μg of pSV-β-gal (Promega) in 1.2 ml of OptiMEM I for 4 h. Lipofectin and DNA plasmids were subsequently removed and replaced with complete medium. Cells were harvested and lysed with 200 μl of reporter lysis buffer (Promega). Cell extracts were centrifuged in a microcentrifuge for 5 s to remove debris. 50 μl of the supernatant was removed for luciferase assay in a luminometer (Monolight, model 2010) according to the manufacturer’s procedures. The protein content was determined by the BCA protein assay kit (Pierce) using bovine serum albumin as a standard. β-Galactosidase activity was assayed by chemiluminescence (Clontech) as described (10).

Preparation of Nuclear Extracts—HUVEC nuclear extracts were prepared as described previously (11) with the following modifications. HUVECs were harvested by scraping, washed in cold phosphate-buffered saline, and incubated in two packed cell volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 300 mM sucrose, 0.5 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 mM dithiothreitol, and 1.0 mg/ml each leupeptin and aprotinin). Nuclei were disrupted by passing through a 23-gauge syringe 10 times. The homogenate was gently stirred on ice for 30 min, and the debris was removed by microcentrifugation for 2 min. The resulting supernatant was removed for luciferase assay in a luminometer (Monolight, model 2010) according to the manufacturer’s procedures. The protein content was determined by the BCA protein assay kit (Pierce) using bovine serum albumin as a standard. β-Galactosidase activity was assayed by chemiluminescence (Clontech) as described (10).
 extracts were frozen on dry ice and stored at −80 °C. The final protein concentration of the nuclear extracts ranged from 5 to 8 mg/ml.

**DNase I Footprint Assay**—DNase I footprinting was performed according to a method previously described (11). Labeled probe (−476 to −21) was prepared by *Pst*I digestion of construct pXP1 (−744 to −21), and the digested probe was isolated and labeled with [α-32P]dATP by Klenow DNA polymerase. The labeled probe (2.5 × 10⁶ cpm) was incubated with either purified Sp1 (Promega) or HUVEC nuclear extracts (30 μg) at room temperature for 15 min in 50 μl of binding buffer containing 50 μg/ml bovine serum albumin, 10 μg/ml poly(dI·dC), and 0.03% Nonidet P-40 to allow binding and then digested with 0.15 μg/ml DNase I at room temperature for 1 min. The samples were analyzed on a sequencing gel.

**Electrophoretic Mobility Shift Assay**—The shift assay was performed by a previously described procedure (12). The binding mixture (20 μl) contained 5 × 10⁶ cpm of 32P-labeled Sp1 consensus oligonucleotide or DNA probe, 10 μg of HUVEC nuclear extracts, or 5 ng of purified Sp1 and 2.5 μg of poly(dI·dC) in a binding buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml each of leupeptin and aprotinin). After a 15-min incubation on ice, the samples were incubated at room temperature for 20 min. Band shift patterns were resolved by electrophoresis. In competition experiments, nuclear extracts or purified Sp1 were incubated for 5 min with the unlabeled oligonucleotide or DNA fragment in a 50–150-fold molar excess prior to the addition of the labeled probe. The gel supershift assay was performed by adding 2 μg of rabbit polyclonal anti-Sp1 antibody (PEP2, Santa Cruz Biotechnology) to the DNA/protein mixture for 30 min on ice, and the band formation was analyzed in gel electrophoresis as described above. An unrelated rabbit polyclonal anti-PGHS-1 antibody was included as negative control.

**Primer Extension Analysis**—Primer extension using HUVEC PGHS-1 mRNA as the template was performed by a procedure previously described (6). Primer extension using the PGHS-1 promoter/luciferase construct was done similarly. The primer used in the extension analysis is shown in Fig. 6. The extension products were analyzed on a 6% polyacrylamide sequencing gel.

**Rapid Amplification of cDNA Ends (RACE)**—The 5′-end RACE for determining the 5′-end of the PGHS-1 transcript was based on the procedure previously described (13). 3 μg of cellular RNA prepared from HUVECs was reverse transcribed to cDNA using an antisense PGHS-1 oligonucleotide (+63/+107 relative to the translation start site). The aliquoted cDNA products were used for amplification by PCR using the following primers: primer a. 5′-GACTGCAGTCGACATCATCGTTTTTTTTTTTTTTTT-3′, which contains an XhoI digestion site as underlined, and primer b, 5′-CCCTGACCGAGGGACGGG-3′, which also contains an XhoI digestion site. This primer contains an antisense sequence corresponding to PGHS-1 nucleotides +70 to +51. RACE reaction products were digested with XhoI and cloned into the XhoI sites of pGEM7. The transformed cells were screened with PGHS-1 genomic probe (−744/+183).

**RESULTS**

**Functional Analysis of the 5′-Untranslated Region of the Human PGHS-1 Gene**—A genomic clone containing a 2.5-kb fragment of the 5′-flanking region of the human PGHS-1 gene was isolated and sequenced. The sequence reveals that this region bears several putative binding sites for transcriptional activators (Fig. 1). The adenine residue of the ATG is designated as +1. A GC box containing a canonical Sp1 binding site (GGGCGCG) is located at nucleotides −610 to −604. Three shear stress-responsive elements (SSRE) are localized near nucleotides −395, −625, and −1810, respectively. In addition, two Sp1 sites are located at nucleotides −83 to −89 and −105 to −111 proximal to the ATG site. To characterize cis-acting elements in the human PGHS-1 promoter region, we constructed a 2075-bp fragment (−2095/−21) and a series of 5′-deletion mutants into a promoterless luciferase expression vector, pXP1. These constructs were transiently transfected into the cultured HUVECs by lipofection according to the methods described under "Experimental Procedures." The parental construct containing nucleotides from −2095 to −21 of the PGHS-1 gene conferred strong constitutive luciferase expression. The promoter activity of this parental construct was about 22% of that of the pSV2-luc construct, which utilizes the SV40 early promoter and enhancer to drive luciferase expression (Fig. 2). The promoter activity remained unchanged by progressive 5′-deletion until reaching nucleotide −565, where the activity of construct −565/−21 dropped to about 50% of the parental construct activity (Fig. 2). There was a small gradual decline in the promoter activity with further progressive deletion. However, even a short fragment −137/−21 still conferred about 100-fold higher activity than the promoterless vector, pXP1. To demonstrate that the basal promoter activity resides between −137 and −21, we constructed two 3′-deletion mutants, −916/−126 and −257/−126, into the luciferase reporter gene and carried out transient transfection experiments. As shown in Fig. 2, both constructs almost entirely lost the basal promoter activity (Fig. 2). The promoter activity remained unchanged by progressive 5′-deletion until reaching nucleotide −565, where the activity of construct −565/−21 dropped to about 50% of the parental construct activity (Fig. 2). There was a small gradual decline in the promoter activity with further progressive deletion. However, even a short fragment −137/−21 still conferred about 100-fold higher activity than the promoterless vector, pXP1. To demonstrate that the basal promoter activity resides between −137 and −21, we constructed two 3′-deletion mutants, −916/−126 and −257/−126, into the luciferase reporter gene and carried out transient transfection experiments. 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with those of the experiments with luciferase alone (Fig. 2). These results strongly suggest that two regions located between nucleotides 2744 and 2565 and between 2137 and 221 are critical for PGHS-1 promoter activity.

Binding of HUVEC nuclear proteins to these two regions were investigated. Fig. 3A shows band shift when nuclear extracts of HUVECs were incubated with a 744/569 probe containing the distal activator region. Two bands were noted with this probe. Since this region comprises putative binding sites for Sp1 and AP-2, a 150-fold molar excess of AP-2 consensus oligonucleotides; Sp1, a 150-fold excess of Sp1 oligonucleotides; mut, a 50-fold molar excess of four-base Sp1 mutated unlabeled probe; cold, a 50-fold molar excess of unlabeled wild-type probe. B, binding of HUVEC nuclear extracts to a 22-mer consensus Sp1 recognition oligonucleotide. Lane 1, formation of two bands between nuclear extract and Sp1 probe in the absence of competitors; lane 2, competitive inhibition of both bands by a 150-fold molar excess of unlabeled Sp1 probe; lanes 3 and 4, lack of inhibition by a 150-fold molar excess of AP-2 or NF-kB oligonucleotide; lane 5, competitive inhibition by a 75-fold molar excess of unlabeled DNA fragment 2744/2569.

FIG. 3. Analysis by band shift assays of Sp1 binding to the distal enhancer elements of the PGHS-1 gene. A, binding of HUVEC nuclear extracts (NE) to the 32P-labeled fragment, 744/569 (50 × 10^6 cpn). –, absence of any competitor; AP-2, a 150-fold molar excess of AP-2 consensus oligonucleotides; Sp1, a 150-fold excess of Sp1 oligonucleotides; mut, a 50-fold molar excess of four-base Sp1 mutated unlabeled probe; cold, a 50-fold molar excess of unlabeled wild-type probe. B, binding of HUVEC nuclear extracts to a 22-mer consensus Sp1 recognition oligonucleotide. Lane 1, formation of two bands between nuclear extract and Sp1 probe in the absence of competitors; lane 2, competitive inhibition of both bands by a 150-fold molar excess of unlabeled Sp1 probe; lanes 3 and 4, lack of inhibition by a 150-fold molar excess of AP-2 or NF-kB oligonucleotide; lane 5, competitive inhibition by a 75-fold molar excess of unlabeled DNA fragment 2744/2569.

hated with nuclear extracts prior to the addition of the probe. Both bands were specifically competed by Sp1 or unlabeled probes but not by AP-2 oligonucleotide. Furthermore, a 50-fold molar excess of 744/569 in which the canonical Sp1 site,
Sp1 in Prostaglandin H Synthase-1 Gene Expression

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**Fig. 5. Effects of Sp1 mutations on promoter activity.** Mutations of either and both Sp1 sites as shown in this figure were performed by three-step PCR mutagenesis methods as described in detail under “Experimental Procedures.” The hatched boxes denote the two Sp1 binding sites. The bent arrow denotes the TSS. The promoter activity was determined as a ratio of luciferase activity conferred by PGHS-1 promoter to β-galactosidase activity driven by SV40 early promoter. The value of basal promoter activity conferred by (−916−21) was taken as 100%. The S.D. column denotes the promoter activity induced by 50 nM PMA for 4 h. The values shown on the right are percentages relative to the wild-type (−916−21). The values represent the mean ± S.D. of three independent transfection experiments.

−610GGCCGG−604 was mutated to GTTTCTG to inhibit the formation of these two DNA-protein complexes (Fig. 3A, lane 5). These results indicate that Sp1 and/or closely related proteins bind to the canonical Sp1 site. This is further confirmed by the formation of two bands when labeled consensus Sp1 oligonucleotides were incubated with HUVEC nuclear extracts (Fig. 3B). Both bands were competed by unlabeled Sp1 oligonucleotides (lane 2) and −744/−569 fragment (lane 5) but not by AP-2 or NF-κB oligonucleotides (lanes 3 and 4). In all of the experiments, nuclear extracts formed two bands with the canonical Sp1 site. Since both bands are specifically competed by Sp1 sequences but not by mutated Sp1 sequence, both bands are complexes formed between Sp1 and/or Sp1-related protein and the Sp1 sequence. Our results are in keeping with several reports of the formation of two distinct complexes between nuclear extracts and Sp1 binding sites (14–17).

**Identification of the Proximal Sp1 Element—**The proximal activating region (nucleotides −137 to −21) is GC-rich and contains at least two Sp1 sites (Fig. 1). The DNase I footprinting assay revealed a protected area from nucleotide −114 to −98 when labeled probes were incubated with Sp1 (Fig. 4A) or HUVEC nuclear extracts (data not shown). This protected area bears the Sp1 site (GGGTTGG) (Fig. 4A). When labeled probes (−137 to −21) containing the protected region were incubated with HUVEC nuclear extracts, two bands were formed (Fig. 4B, lane 2), and both bands were competitively inhibited by unlabeled probes, Sp1 consensus sequence, and/or an Sp1-containing oligonucleotide (Fig. 4B, lanes 3, 5, and 6, respectively). More importantly, both bands were not competed by a 50-fold excess of the parental probe where only the −111GGGTTGG−105 site had been mutated (Fig. 4B, lane 4). The results clearly demonstrated the binding of Sp1 and/or its related proteins to the −111/−105 site but not to the −89/−83 site. A single band shift was noted when purified Sp1 proteins were incubated with labeled probe containing the protected area (Fig. 4C, lane 2), and this band was competitively inhibited by Sp1 oligonucleotides (lane 5). This band was super-shifted with specific antibody directed against Sp1 (lane 3) but not with unrelated antibody such as anti-PGHS-1 antibody (lane 4). Incubation of HUVEC nuclear extracts with this probe resulted in the formation of two complexes (Fig. 4C, lane 7). Both bands were competitively inhibited by Sp1 oligonucleotides (Fig. 4C, lane 7 versus lane 8) and supershifted with anti-Sp1 antibodies (lane 6). Hence, Sp1 and/or Sp1-related proteins bind to a Sp1 site at −111 to −105.

**Effects of Mutation of Sp1 Binding Sites on PGHS-1 Promoter Activity—**To ascertain that these two separate Sp1 binding sites are functionally important in enhancing the PGHS-1 basal promoter activity, one or both Sp1 binding sites in the 5′-flanking promoter (−916−21) of the PGHS-1 gene were altered by site-directed mutagenesis. These mutants were constructed in pXP1 luciferase expression vectors and transfected in HUVECs. Alteration of the distal Sp1 (−610GGCCGG−604 to GTTTCTG) reduced the promoter activity to 53% of that of the wild-type promoter (Fig. 5, a versus b). Alteration of the proximal Sp1 binding site from −111GGGTTGG−105 to GTTTTAG reduced the promoter activity to 38% of the wild-type promoter (Fig. 5, c versus a). When both Sp1 binding sites were simultaneously mutated, the promoter activity was reduced to 29% of that of the wild-type promoter. This reduction is statistically significantly larger (p < 0.05) than the reduction caused by individual mutation of the proximal or distal site.

The extent of promoter activity reduction caused by the distal Sp1 site mutation is comparable with that of the 5′-deletion mutants in which the distal Sp1 site was deleted (−565−21 in Fig. 2 versus Fig. 5b). To determine whether mutation of the Sp1 site in the construct (−257−21) would lead to a comparable reduction in the promoter activity, we mutated the proximal Sp1 site located in the −257−21 fragment and expressed it in HUVECs. The promoter activity expressed by this was only 27% of that of the wild-type promoter (−916−21) (Fig. 5f). This value was essentially identical to that of the double Sp1 site mutations in −916−21, confirming that these two Sp1 binding sites contribute to 73% of the basal
PGHS-1 promoter activity.

Experiments were then carried out to determine the impact of Sp1 site mutations on PMA-stimulated promoter activity conferred by the $-916/-21$ region. PMA treatment (50 nM, 4-h incubation) increased the promoter activity of the wild-type $-916/-21$ by 1.8-fold (Fig. 5a). This result was comparable with that previously reported (18). Mutations of either or both Sp1 sites were accompanied by a marked decrease in the basal promoter activity as described above. However, the level of stimulation by PMA treatment was not significantly altered by the mutations (Fig. 5b–f). These results suggest that PMA stimulation of PGHS-1 promoter activity depends on additional activators.

Mapping the TSS—Multiple TSS for PGHS-1 were identified by primer extension and S1 nuclease mapping in our previous study (6). A major TSS was identified as adenine $-135$, relative to the ATG translation start codon. TSS were situated upstream from the proximal Sp1 cognate site ($-111/-105$). However, primers used in those experiments were corresponding to nucleotide sequence $-22$ to $-48$, which might mask TSS downstream from the proximal Sp1 site. Additional primer extension experiments were, therefore, carried out to reevaluate the TSS. A P2 primer corresponding to nucleotides +115 to +137 (Fig. 6a) was used as an antisense primer in extension experiments. The results from one experiment are shown in Fig. 7A. Multiple bands corresponding to A $-31$, G $-33$, G $-37$, and A $-135$ were noted on the primer extension gel. Of these four TSS, A $-135$ is in accord with that detected by using nucleotides $-22/-48$ as the primer. Results from two other experiments revealed four bands corresponding to A $-31$, G $-33$, G $-37$, and A $-135$ or G $-111$. Hence, TSS at A $-31$, G $-33$, G $-37$, and A $-135$ are consistent in all three experiments. A $-135$ and G $-111$ are, on the other hand, alternative TSS. We observed a similar alternative extension between these two TSS when nucleotides $-22$ to $-48$ were not present in the promoter region relative to the ATG codon. B, use of promoter construct (-816/-21)-luc transcript and PLuc shown in Fig. 6B in primer extension. Lane 1, in the presence of transcript; lane 2, in the absence of transcript. Multiple extension bands were detected in lane 1, whereas no extension bands were noted in control lane 2. The boldface numbers denote the nucleotide positions relative to the 5’-nucleotide of PLuc shown in Fig. 6B. The numbers in italics denote the nucleotide positions relative to the ATG codon of the PGHS-1 gene.

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Sp1 in Prostaglandin H Synthase-1 Gene Expression

used as the primer. In all three experiments, A\textsuperscript{−31} and G\textsuperscript{−33} had the highest density and were considered to be major TSS. Transcription start sites for the PGHS-1 promoter-luciferase construct were determined using PLuc primer corresponding to nucleotides +44 to +70 downstream from the luciferase start codon (Fig. 6B). Multiple bands were detected (Fig. 7B). Four bands with higher densities are mapped to G\textsuperscript{−37}, A\textsuperscript{−43}, G\textsuperscript{−78}, and G\textsuperscript{−111} of the PGHS-1 promoter region. Several less dense bands are scattered between A\textsuperscript{−42} and G\textsuperscript{−111} (Fig. 7B). Judging by the density of the primer extension bands, the major TSS for native PGHS-1 transcript resides in the region at nucleotides A\textsuperscript{−31} and G\textsuperscript{−33}, whereas the major TSS for luciferase fusion transcript resides in the region at A\textsuperscript{−43}. Another region that serves as TSS from both transcripts resides at G\textsuperscript{−111}. 5′-End RACE experiments were carried out to further determine TSS. The 3′-primer used in the 5′-end RACE experiments corresponds to the nucleotide sequence from +51 to +70. Two TSS were identified by the RACE procedure, G\textsuperscript{−33} and A\textsuperscript{−17}. Taken together, the results indicate that a promoter region proximal to the Sp1 enhancer contains multiple TSS including A\textsuperscript{−43}, G\textsuperscript{−37}, A\textsuperscript{−31}, and A\textsuperscript{−17}. G\textsuperscript{−111} is a TSS identified by primer extension of native and luciferase fusion transcripts but is not detected as a TSS by 5′-end RACE. A\textsuperscript{−135} was identified as an alternative TSS to G\textsuperscript{−111} only when primer extension was performed on native PGHS-1 transcript. G\textsuperscript{−78} and several less dense bands were noted only in luciferase fusion transcripts. The importance of G\textsuperscript{−111}, A\textsuperscript{−135}, or G\textsuperscript{−78} as TSS for the PGHS-1 gene in vivo is unclear. It is intriguing to note that these nucleotides are localized upstream from the proximal Sp1 recognition site. A similar spatial relationship has been reported for the promoter of the human cyclin-dependent kinase-2 gene (19), in which among multiple TSS identified, one TSS was located upstream from an Sp1 site functionally important in basal transcription for this gene.

DISCUSSION

Results from this study demonstrate that two Sp1 elements are essential for basal transcription of the human PGHS-1 gene. Evidence to support this consists of specific binding of purified Sp1 and nuclear extract proteins to these two regions and a marked loss of promoter activity by Sp1 site mutations. Sp1 is a sequence-specific, ubiquitously expressed nuclear factor essential for basal expression of a variety of eukaryotic genes (for review, see Ref. 20). It confers transcriptional activation by interacting with transcription-associated factors, thereby facilitating the assembly of the basal transcription machinery (21). Reported data suggest that for activation of constitutive transcription of mammalian TATA-less gene, Sp1 is required to bind to a spatially defined region, within 100 base pairs upstream from the TSS (22–26). Hence, Sp1 involvement in basal transcription of the PGHS-1 gene is different from that of other reported mammalian housekeeping genes in that two Sp1 sites required for basal transcription are separated by about 500 bp on the 5′-flanking region. To our knowledge, this is the first instance of critical involvement of two distantly located Sp1 sites in the activation of basal mammalian housekeeping gene transcription.

It has been reported that the activity of the thymidine kinase promoter of the herpes simplex virus and an artificially constructed promoter is enhanced by interaction of two spatially widely separated Sp1 proteins to form Sp1 multimers (27–29). Detailed analysis of the promoter region by electron microscopy revealed DNA looping whereby the separated Sp1 proteins were brought into contact to form a tetramer followed by multiple tetramer formation (30). It is conceivable that basal PGHS-1 transcription regulated by two Sp1 sites may be mediated by a similar mechanism.

It is estimated from the mutation experiments that these two Sp1 enhancer elements contribute to about 70–75% of the promoter activity conferred by the 2.0-kb promoter/enhancer fragment of PGHS-1 gene. Full basal promoter activity may require the involvement of additional enhancer element(s). Comparison of the promoter activity conferred by the construct shown in Fig. 5d versus Fig. 5f suggests that the region between nucleotides −257 and −21 bears additional enhancer elements important in full PGHS-1 gene expression. In this region, besides the functionally active Sp1 site at −105 to −111, there is another Sp1 site located at −83 to −89. The 3′-mutant fragment −257/−126 (Fig. 2) in which both Sp1 sites are removed exhibited almost no promoter activity. This result implies that the Sp1 site at −83 to −89 may be important in PGHS-1 basal transcription in vivo. However, this 3′-deletion mutant is probably devoid of the binding site for the transcription initiation complex and consequently is expected to confer minimal (if any) promoter activity, even when enhancer elements are present distally such as the −916/−126 mutant (Fig. 2). Since Sp1 did not bind to the Sp1 site at −83 to −89 by DNase I footprinting, it would be highly unlikely that this Sp1 site is functionally active. Other sites in this region including a putative PEA3 binding site (−155AGGAAG−156) may be the potential enhancer element for a full PGHS-1 promoter activity. This is now being investigated.

It has recently been reported in different types of cells that PGHS-1 gene expression is stimulated by serum, cytokines, or growth factors (for a review, see Ref. 2). We have shown that endothelial PGHS-1 expression is stimulated approximately 2-fold over the basal level by PMA and interleukin-1β (18). In this study, our results indicate that PMA increased the promoter activity conferred by the 5′-flanking promoter/enhancer of the PGHS-1 gene in HUVECs. Stimulation of the promoter activity by PMA is not entirely dependent on the two Sp1 sites but requires additional elements. Sp1 is involved in stimulation of gene expression by interacting with other transcriptional activators such as GATA, NF-κB, Egr-1, YY1, and Rb (31–37). It is possible that PMA stimulates PGHS-1 transcription by a similar mechanism. Further studies are needed to elucidate the mechanism by which PMA stimulates PGHS-1 promoter activity.

Nuclear extracts of HUVECs form two distinct bands with distal or proximal probes and with consensus Sp1 recognition sequences. Both bands are specifically competed by unlabeled Sp1 cognate oligonucleotides but not by Sp1 mutant, AP-2, or NF-κB sequences. These two bands are complexes of DNA with Sp1 and/or Sp1-related proteins. Purified Sp1, on the other hand, forms only a single DNA-Sp1 complex with fragment −119 to −94 (Fig. 4C). These results are similar to those reported in several recent studies (13–16). The reason for the double band formation with nuclear extracts is unclear. It has been attributed to binding of Sp1 and a closely related protein to Sp1 recognition sites (38). Three Sp1-related proteins, Sp2, Sp3, and Sp4, have been identified (39–42). These isoforms of Sp1 bind to Sp1 recognition sites and are antigenically very close to Sp1. Hence, despite specific inhibition of binding by Sp1 cognate sequences and supershift by specific Sp1 antibodies, the additional band is probably formed as a result of binding of a Sp1-related protein to the Sp1 binding site.

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