Characterization of a novel transcript of prostaglandin endoperoxide H synthase 1 with a tissue-specific profile of expression

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The enzyme prostaglandin endoperoxide H synthase (PGHS) has a pivotal role in the prostanoid biosynthetic pathway because it catalyses the formation of prostaglandin H_2 (PGH_2), the common precursor of prostanoids. Two PGHS isoforms have been reported, PGHS-1 and PGHS-2, which have 61 % identity (at the amino acid level) and 73 % similarity (at the nucleotide level) between the two human enzymes. Transcription of the PGHS-1 gene leads to the formation of two transcripts (2.8 and 5.1 kb); two transcripts of 2.8 and 4.5 kb are produced from the PGHS-2 gene. By Northern blot analysis with the entire coding region of human PGHS-1, 2.8 and 5.1 kb transcripts as well as a novel 4.5 kb transcript were detected in the human megakaryoblastic cell line MEG-01. We designed a strategy to characterize the 4.5 kb PGHS transcript. Probes specific for each PGHS-1 and PGHS-2 were designed on the basis of the 3′ untranslated region (3′ UTR), where no similarity is present. The 4.5 kb transcript was detected only with the PGHS-1-specific 3′ UTR probes and not with the PGHS-2-specific 3′ UTR probe. To investigate the origin of the 4.5 kb PGHS-1 transcript, the remaining 947 bp of the 5.1 kb PGHS-1 transcript was generated by 3′ rapid amplification of cDNA ends (3′ RACE) and sequenced. A non-canonical polyadenylation signal (AAGAAA) located upstream of a potential cleavage site (CA) was found and could generate the 4.5 kb PGHS-1 transcript. Analysis of the sequence also produced several possible G/U-rich elements downstream of the potential cleavage site. An RNA dot-blot with 50 different human tissues was probed with the 4.5 and 5.1 kb PGHS-1-specific probes. A signal for the 4.5 kb PGHS-1 transcript was detected in the bladder and appendix. Signals of lower intensity were detected in the colon, bone marrow, small intestine, uterus, prostate, peripheral leucocyte, lymph node and stomach. In conclusion, our results suggest that the cell line MEG-01, the bladder and the appendix contain a new PGHS-1 transcript of 4.5 kb that can be produced from the PGHS-1 gene and we provide a better strategy for distinguishing PGHS-1 transcripts from PGHS-2.

Key words: cyclo-oxygenase, bladder, 4.5 kb transcript, MEG-01 cells.

INTRODUCTION

Thromboxane A_2 (TXA_2) is a potent pro-aggregatory and vasoconstrictive prostanoid (a group consisting of prostaglandins and thromboxanes) [1]. A major source of TXA_2 is platelets and its synthesis requires the multi-step prostanoid biosynthetic pathway that includes the following series of enzymes [2]: (1) phospholipase A_2 (PLA_2), (2) prostaglandin endoperoxide H synthase (PGHS) and (3) thromboxane A synthase. The first step of the prostanoid biosynthetic pathway is the release of arachidonic acid from membrane phospholipid by PLA_2. After its release from phospholipids, free arachidonic acid is converted to prostaglandin H_2 (PGH_2) by PGHS. Thromboxane A synthase catalyses the final conversion of PGH_2 to TXA_2. PGHS has a pivotal role in the prostanoid biosynthetic pathway because it catalyses the formation of PGH_2, the common precursor for prostanoids. PGHS exhibits both a cyclo-oxygenase activity [which involves the formation of prostaglandin G_2 (PGG_2) from arachidonic acid] and peroxidase activity (which catalyses the reduction of PGG_2 to PGH_2). There are two PGHS isoforms called PGHS-1 and PGHS-2, both of which catalyse the formation of PGH_2 [3]. Each isoform is encoded by a different gene: PGHS-1 is located on human chromosome 9 and contains eleven exons spanning approx. 22 kb [4], whereas the gene for PGHS-2 is located on human chromosome 1 and is considerably smaller (approx. 8 kb), owing to smaller introns [5]. Most of the intron/exon boundaries are conserved except the first two exons of PGHS-1, which are condensed into a single exon in PGHS-2. PGHS-1 is encoded by transcripts of 2.8 and 5.1 kb. The 2.8 kb transcript seems to be derived from alternative polyadenylation at a second canonical polyadenylation site [6]. PGHS-2 is encoded by transcripts of 2.8 and 4.5 kb, with the 2.8 kb also being derived from alternative polyadenylation [7]. The human PGHS-1 (hPGHS-1) and hPGHS-2 open reading frames are 73 % similar at the nucleotide level. The deduced amino acid sequences of PGHS-1 and 2 are 61 % identical for the human enzymes; the residues required for catalysis by PGHS-1 are conserved in PGHS-2 [8].

The major differences between the two PGHS isoforms are their dissimilar regulations of expression. PGHS-1 seems to be an important housekeeping enzyme because it is expressed constitutively in many tissues and performs various functions such as cytoprotection of the gastric mucosa, regulation of renal blood flow and platelet aggregation [3]. However, higher levels of PGHS-1 expression are detected in specialized cells such as platelets and endothelial cells. In addition, systems modelling

Abbreviations used: hPGHS, human PGHS; LPS, bacterial lipopolysaccharide; MEG-01, human megakaryoblastic cell line; PG, prostaglandin; PGHS, prostaglandin endoperoxide H synthase; PLA_2, phospholipase A_2; RACE, rapid amplification of cDNA ends; TXA_2, thromboxane A_2; UTR, untranslated region.

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The nucleotide sequence data reported in this paper will appear in the EMBL and GenBank nucleotide sequence databases under accession number 254414.

developmental events have demonstrated changes in PGHS-1 expression, the most notable example being the differentiation of THP-1 cells (a human monocytic cell line) to a macrophage phenotype induced by the phorbol ester PMA [9]. This induction of PGHS-1 seems to be associated with differentiation rather than the activation of cell growth. In contrast with PGHS-1, PGHS-2 protein and mRNA are not present in resting unstimulated tissues but can be rapidly induced in many cell types (macrophages, monocytes and fibroblasts) by cytokines and tumour promoters, because PGHS-2 is associated with the inflammatory process [8]. The promoters of the two PGHS genes are indicative of their modes of regulation: PGHS-1 has a TATA-less promoter, a feature common to many housekeeping genes [4], whereas the PGHS-2 promoter contains a TATA box and is highly regulatable [8]. The PGHS-2 promoter has been characterized and seems to contain several inducible enhancer elements such as activator protein 1 ('AP-1'), CCAAT-enhancer-binding protein ('C/EBP')/nuclear factor interleukin-6 ('NFIL-6') and the nuclear factor NF-κB [8].

Recent results indicate that, in low doses, aspirin diminishes the incidence of cardiovascular disease [10,11]. The basis for the anti-thrombogenic activity of aspirin is the irreversible inhibition of platelet PGHS-1, suggesting that this enzyme has a central role in the pathology of cardiovascular diseases. The regulation of PGHS-1 expression in platelets is not yet fully understood. Progress in our understanding of PGHS-1 regulation in platelets has been hampered because of the lack of a good model for studying PGHS-1 expression. As a model for investigating the regulation of PGHS-1 gene expression, the immortalized human megakaryoblastic cell line MEG-01, a platelet precursor, was studied. In this model, we show PMA-induced expression of PGHS-1 protein and mRNA; results indicate the presence of a new 4.5 kb PGHS transcript, which we characterize as a new PGHS-1 message that shows high levels of expression in the human bladder and appendix.

EXPERIMENTAL

Cell culture

MEG-01 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Cells were cultured in RPMI 1640 supplemented with 10 % (v/v) fetal bovine serum without antibiotics and maintained at 37 °C in air/CO_2 (19:1) in a humid atmosphere. Cultures were seeded at 2×10^5 cells/ml (100 ml) and allowed to grow for 4 days, after which the floating cells were re-adjusted to 2×10^5 cells/ml. After 3 days, the floating fraction was again adjusted to 2×10^5 cells/ml; 20 h later, cells were stimulated with PMA (Sigma) and incubated [37 °C, air/CO_2 (19:1)] for various durations.

The human monocytic cell line U937 was obtained from the American Type Culture Collection. Cells were passaged twice a week in RPMI 1640 supplemented with 10 % (v/v) fetal bovine serum and maintained at 37 °C in air/CO_2 (19:1) in the absence of antibiotics. Cells were adjusted to 10^6 cells/ml in the presence of 10 nM PMA and incubated for 3 days to differentiate into macrophages. Non-adherent cells were removed and the remaining adherent cells were made quiescent in fresh complete RPMI 1640 medium in the absence of PMA for 24 h, before stimulation with bacterial lipopolysaccharide (LPS). Differentiated macrophages were stimulated with 50 ng/ml LPS for 4 h, after which they were harvested for RNA isolation. To demonstrate that LPS-induced 4.5 kb PGHS-2 mRNA is sensitive to glucocorticoid, differentiated macrophages were incubated with 1 μM dexamethasone for 4 h before stimulation with LPS.

Table 1 Sequences of primers used for reverse-transcriptase-mediated PCR of specific segments of the genes for PGHS-1 and PGHS-2

Primer	Sequence
PGS15	5′-GGGCAGGAAAGCAGCATTCTGGAG-3′
PGS13	5′-AACAAGGAGTTCAGCATTCTGGAAG-3′
PGS25	5′-AAGTCTAATGATCATATTTATTTAT-3′
PGS23	5′-AACATCTTTACTTTCGTCCTTATAA-3′
PGS1UTR	5′-CCATTTGTTCCTGCTTCCGAGATCC-3′

Treatment with PMA

PMA was dissolved in DMSO at 1 mg/ml and stored at −20 °C. Before use, PMA was diluted in RPMI 1640 and added to each culture. Control cells were treated with the same DMSO concentration that PMA-treated cells received. Cells were then incubated for various durations, counted for viability and harvested for RNA or protein analysis.

Isolation of total and poly(A)$^+$ RNA

Total RNA was extracted from the cell pellets of various treated cell cultures, by using Trizol reagent (Gibco–BRL) and the protocol provided by the supplier. The poly(A)$^+$ RNA was purified with a poly(A)$^+$ extraction kit (Qiagen) in accordance with the instructions provided by the supplier.

Northern-blot hybridization

Samples of poly(A)$^+$ RNA from various cell samples were subjected to electrophoresis on a denaturing 2.2 M formaldehyde/agarose (1 %, w/v) gel. The RNA was transferred by capillary action to a Biodyne nylon membrane (Gibco–BRL) and baked at 80 °C for 2 h. The membranes were prehybridized in 5 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate)/5 × Denhardt's solution/50 % formamide/1 % (w/v) SDS at 42 °C for 1 h. Complementary DNA probes were radiolabelled with [^{32}P]dCTP (Amersham) by random-primer extension (Prime-it II kit; Stratagene) and hybridized overnight in 5 × SSC/5 × Denhardt's solution/50 % formamide/1 % (w/v) SDS at 42 °C. The membranes were washed three times under low stringency (2 × SSC/0.1 % SDS) at room temperature, once under high stringency (0.1 × SSC/0.1 % SDS) at 65 °C for 30 min and then analysed by autoradiography or phosphorimaging. Band intensity was quantified by scanning densitometry with a Hewlett Packard ScanJet II cx and Kodak Digital Science ds 1D analysis software. The PGHS-1 cDNA probe was then removed by washing (95 °C for 3 min in diethyl pyrocarbonate-treated water) to allow rehybridization of the membranes with the human α-tubulin cDNA , to control for the quantity of mRNA loaded in each lane. Results are expressed as the ratio of the PGHS-1 (2.8 kb) band to the corresponding α-tubulin (1.6 kb) band.

RNA master blot and multiple-tissue Northern blot

The human RNA master blot and multiple-tissue Northern blot were obtained from Clontech and hybridized in accordance with the protocols provided by the supplier with various complementary DNA probes radiolabelled with [^{32}P]dCTP by random-primer extension. Dot or band intensities were quantified by scanning densitometry with a Hewlett Packard ScanJet II cx and Kodak Digital Science ds 1D analysis software.

Protein isolation and quantification

Pelleted cells were washed three times in PBS and resuspended in 100–200 μl of 0.1 M Tris/HCl, pH 7.4, and kept on ice. Cells were lysed by sonication for 5 s at a power setting of 80% (ultrasonic cell disruptor; Microson). Protein concentration was determined with the Bio-Rad protein assay, based on the Bradford method [12], with BSA as a standard.

SDS/PAGE and Western blotting

A sample (50 μg), previously denatured in 2.3% (w/v) SDS/5% (v/v) 2-mercaptoethanol at 100 °C for 5 min, was separated electrophoretically by SDS/PAGE by the method of Laemmli [13]. Separated samples were transferred to nitrocellulose membrane by Western blotting (150 V, 30 min). Blots were blocked overnight at 4 °C [3% (v/v) milk/TBST (24.8 mM Tris/HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20)]. Blots were then washed with 1% (v/v) milk/TBST (three changes over 30 min) and then probed for 1.5 h with primary anti-PGHS antibody (directed against residues Leu^{272}–Gln^{283} for hPGHS-1 and against Ser^{584}–Lys^{598} for hPGHS-2) (diluted 1:1900) in 1% (v/v) milk/TBST. After being washed with 1% (v/v) milk/TBST (three changes over 30 min), the blots were probed for 1 h with horseradish-peroxidase-conjugated rabbit anti-IgG secondary antibody (diluted 1:2000). Blots were washed again with TBST (three changes over 30 min) and bound secondary antibody was detected with enhanced chemiluminescence (ECL kit; Boehringer Mannheim).

Generation of probes specific for PGHS-1 and PGHS-2 for Northern-blot analysis

The known human sequences of the 3′ untranslated regions (3′ UTRs) of PGHS-1 and PGHS-2 were used to design oligonucleotide primers [6,15]. The first 200 bp after the stop codon in exon 11 of the 3′ UTR of PGHS-1 and the first 300 bp after the stop codon in exon 10 of the 3′ UTR of PGHS-2 were generated by PCR with cDNA templates made from RNA of PMA-stimulated MEG-01 cells for 24 h. The products of these reactions were sequenced to confirm identity and used as specific PGHS-1 and PGHS-2 probes for Northern-blot analysis.

For a PGHS-1-specific probe, primers PGS15 and PGS13 (Table 1) were designed. Primer PGHS15 is a 24-mer sense oligonucleotide at position 1 after the stop codon of the 3′ UTR of PGHS-1; primer PGS13 is a 25-mer anti-sense oligonucleotide at position 200 after the stop codon of the 3′ UTR of PGHS-1. For a PGHS-2-specific probe, primers PGS25 and PGS23 were designed (Table 1). Primer PGS25 is a 25-mer sense oligonucleotide at position 1 after the stop codon of the 3′ UTR of PGHS-2; primer PGS23 is a 25-mer anti-sense oligonucleotide at position 300 after the stop codon of the 3′ UTR of PGHS-2.

3′ Rapid amplification of cDNA ends (3′ RACE)

The 5′/3′ RACE kit (Boehringer Mannheim) was used to generate the remaining sequence at the 3′ end of the 5.1 kb transcript of PGHS-1. 3′ RACE was used to generate cDNA templates for PCR , by taking advantage of the natural poly(A)$^+$ tail of mRNA, with an oligo(dT) anchor primer. The appropriate sequence was generated by PCR with the PGHS-1 primer PGS1UTR (Table 1) a 25-mer sense oligonucleotide at position 4103 of the 3′ UTR of PGHS-1 and a PCR anchor primer supplied with the kit. The sequence generated was cloned into the *Bam*HI–*Sal*I site of pBS II KS (−) and three separate clones were sequenced with the T3 and T7 primers. A consensus sequence was obtained by using the sequence analyser Gene Jockey II (Biosoft).

RESULTS

PGHS transcripts present in PMA-treated MEG-01 cells

The conventional approach used to detect messages produced from expressed genes is Northern blotting with probes corresponding to part of or the entire coding region of the gene of interest. By using this approach with the entire coding region of the hPGHS-1 gene, we detected transcripts of three different sizes in PMA-treated MEG-01 cells: 5.1, 4.5 and 2.8 kb (Figure 1). In agreement with published results, the most abundant PGHS-1 transcript was the 2.8 kb message [16]. The 5.1 kb message is much less abundant because it contains a possible polyadenylation site used to produce the PGHS-1 2.8 kb message [6]. Because the PGHS-2 message is 4.5 kb in size, our first assumption was that the 4.5 kb transcript detected with the PGHS-1 coding region was a PGHS-2 transcript; this was based on its size and the fact that there is 73% similarity between the coding regions of the two human PGHSs.

The 4.5 kb transcript is not a PGHS-2 transcript

The most obvious characteristic of the 4.5 kb PGHS-2 transcript is its transient expression: the maximal increase is observed within 2 h and returns to basal levels within 4–8 h of stimulation [17]. To investigate the expression pattern of the 4.5 kb transcript detected with the hPGHS-1-coding region, mRNA was measured at various durations after stimulation with PMA. The changes in mRNA levels during an 8-day period were measured by Northern-blot analysis by hybridization with the entire coding region of hPGHS-1 (1.8 kb). We observed the 4.5 kb transcript in both control and PMA-treated MEG-01 cells. As shown in Figure 2(A), the 4.5 kb message was observed in control cells at every time point over the 8 days of study. The levels of 2.8 and

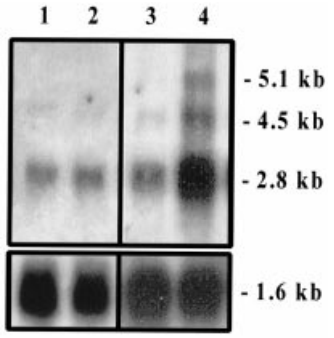
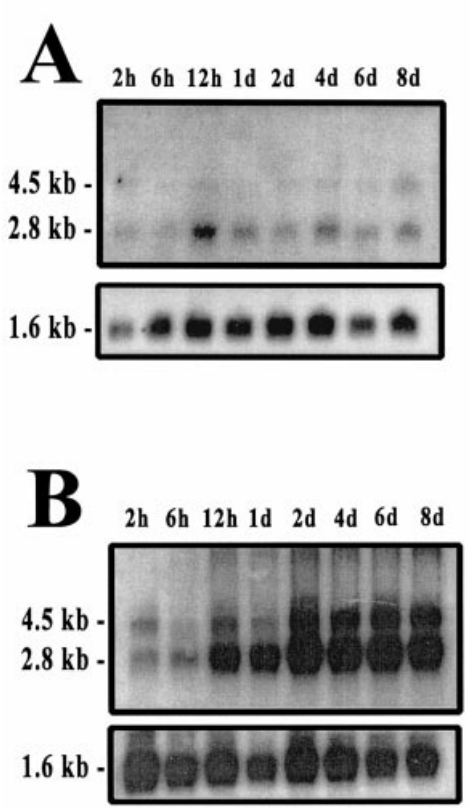


Figure 1 PGHS-1 transcripts detected in PMA-treated MEG-01 cells and LPS treated U937 cells

Top panel: representative autoradiograph of a Northern blot of poly(A)$^+$ RNA extracted from U937 cells (lanes 1 and 2) and MEG-01 cells (lanes 3 and 4). MEG-01 cells were treated with 16 nM PMA (lane 4) for 24 h and U937 cells were treated with 50 ng/ml LPS for 4 h (lane 2), total RNA was then extracted and poly(A)$^+$ RNA was prepared from 150 μg of total RNA, resolved by gel electrophoresis, transferred to nylon membrane and hybridized with the entire coding region of hPGHS-1 under of high stringency as described in the Experimental section. Lanes 1 and 3 are controls (no addition of PMA or LPS) for lanes 2 and 4 respectively. Bottom panel: α-tubulin cDNA was used as a control for equal loading between lanes.



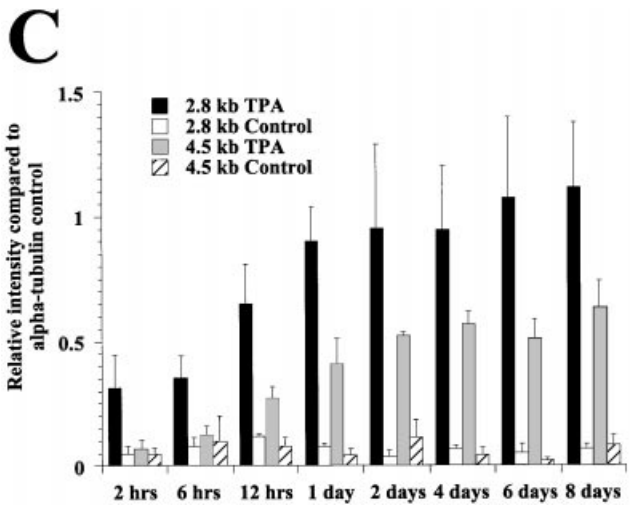


Figure 2 Time course of PGHS transcripts expressed in MEG-01 cells

(**A**, **B**) Representative autoradiographs of Northern blots of poly(A)$^+$ RNA extracted from control (**A**) and PMA-treated (16 mM) (**B**) MEG-01 cells. Upper panels, PGHS-1; lower panels, α-tubulin (loading control). d, days. (**C**) Quantification of three separate experiments; results are the ratios (means $\pm$ S.D.) of the intensity of the 2.8 kb or the 4.5 kb PGHS-1 band to the α-tubulin intensity. Cells were treated with or without 16 nM PMA (TPA) for periods of between 2 h and 8 days as indicated. RNA was extracted and analysed as described in the legend to Figure 1.

4.5 kb transcripts of PGHS-1 increased in a time-dependent manner on stimulation with PMA (Figures 2B and 2C). The 2.8 kb message (Figures 2B and 2C) peaked after 24 h of

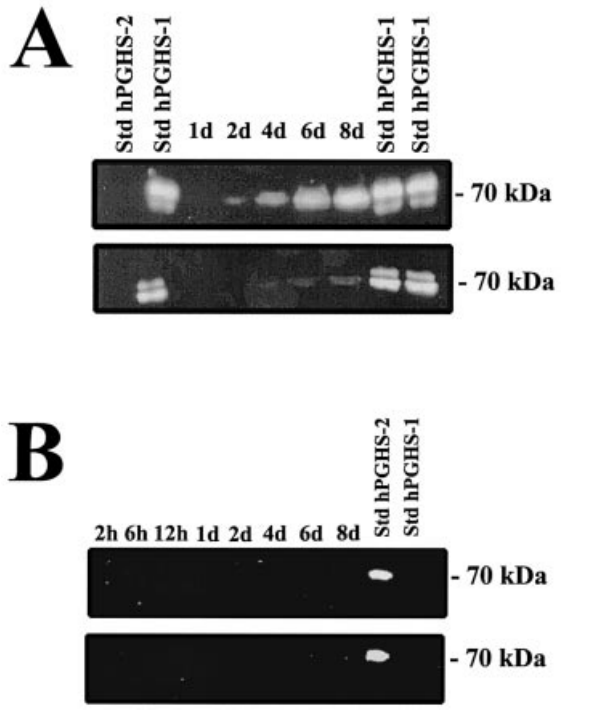


Figure 3 Time course of PGHS-1 and PGHS-2 protein expression in MEG-01 cells

MEG-01 cells (upper panels of **A** and **B**) were treated with a single addition of 16 nM PMA [control cells (lower panels of **A** and **B**) were treated with DMSO (0.0001%, v/v)], the cells were harvested at the indicated times and sonicated. Total protein (50 μg) was resolved by SDS/PAGE (10% gels), transferred on to nitrocellulose membranes and probed with specific antibodies against PGHS-1 (directed against residues Leu^{272}–Gln^{283} of hPGHS-1) or PGHS-2 (directed against residues Ser^{584}–Lys^{598} of hPGHS-2). (**A**) Representative autoradiographs of Western blots detecting PGHS-1; (**B**) representative autoradiographs of Western blots detecting PGHS-2. Abbreviation: Std, standard.

stimulation and remained essentially at that level over the remaining period of study, whereas the 4.5 kb transcript levels (Figures 2B and 2C) peaked later, after 48 h of stimulation, and remained essentially at that level over the remaining period of study. The profile of expression of the 4.5 kb transcript was therefore more similar to the profile of PGHS-1 transcript than to that of PGHS-2 mRNA expression, which was transient on stimulation with PMA.

Western blot analysis was conducted with antibodies specific for each hPGHS enzyme. As shown in Figure 3, no detectable PGHS-2 protein was present in either control or PMA-treated cells at any time over the period of study, whereas PGHS-1 protein was induced on treatment with PMA.

The 4.5 kb transcript is a PGHS-1 transcript

A strategy was designed to determine the identity of the PGHS 4.5 kb transcript detected in MEG-01 cells. Because the coding regions of the human PGHS-1 and PGHS-2 genes are 73% similar, the specificity of the coding regions used as probes to detect PGHS-1 or PGHS-2 sequences is limited even when conditions of high stringency are used. We therefore designed probes specific for each of the PGHS-1 and PGHS-2 sequences in the 3′ UTR, where no similarity is found between the two

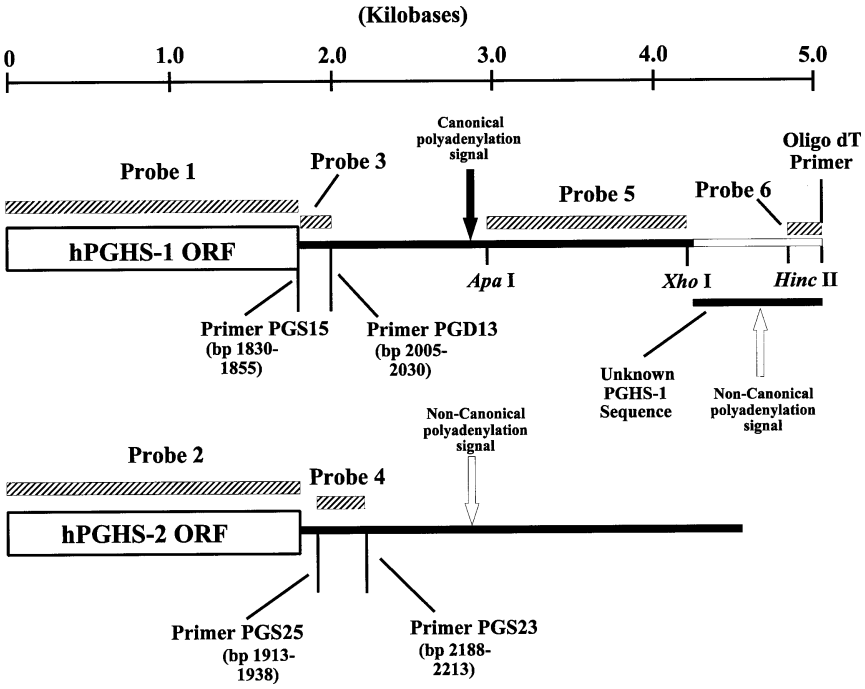


Figure 4 Schematic representation of the hPGHS-1 and hPGHS-2 transcripts and localization of the probes used for Northern-blot analysis

Probes 1–6 were used to characterize the PGHS 4.5 kb transcript by Northern blot analysis. Primers PGS15 and PGS13 were used generate the specific PGHS-1 probe; primers PGS25 and PGS23 were used generate the specific PGHS-2 probe. Digestion of the cloned 3′ UTR of PGHS-1 with *Apa*I/*Xho*I produced probe 5; digestion with *Hinc*II produced probe 6. Filled arrows indicate canonical polyadenylation signals and open arrows indicate non-canonical polyadenylation signals. Abbreviation: ORF, open reading frame.

genes. The localizations of the probes on the PGHS-1 and PGHS-2 transcripts are indicated in Figure 4. By using the coding region of hPGHS-1 as a probe, transcripts of 4.5 and 2.8 kb were detected in MEG-01 cells (Figure 5A). U937 cells, which constitutively express PGHS-1 and can be induced to produce PGHS-2 transcript in abundance [18], were used as a control to determine the specificity of the PGHS-2 probes. When the hPGHS-2 coding region was used as a probe (Figure 5B), the U937 cells treated with LPS gave a major signal at 4.5 kb, as expected. The 4.5 kb signal was faint but detectable in U937 control and LPS plus dexamethasone-treated cells, suggesting that the 4.5 kb signal in U937 cells is a PGHS-2 message (Figure 5B). On the same blot, the coding region of hPGHS-2 recognized a faint signal at 4.5 kb in PMA-treated MEG-01 cells but not in control cells. Because the 4.5 kb transcript in PMA-treated MEG-01 cells was detected with both the coding regions of hPGHS-1 and hPGHS-2, specific probes were designed in the 3′ UTR of these genes, where there is no similarity (Figure 4). When Northern blots were hybridized with probe 3 (PGHS-1-specific) (Figure 5C), both the 2.8 and 4.5 kb messages were detected in MEG-01 cells, with the 2.8 kb transcript being the most abundant. U937 control cells also expressed PGHS-1 messages of 2.8 and 4.5 kb. Hybridization with probe 4 (PGHS-2-specific) showed a single message at 4.5 kb only in LPS-treated U937 cells and not in control or PMA-treated MEG-01 cells (Figure 5D). This suggests that the PGHS-2 gene is not expressed in MEG-01 cells either before or after stimulation with PMA. To confirm the 4.5 kb transcript as a PGHS-1 message in MEG-01 cells, a probe located downstream of the polyadenylation signal that generates the 2.8 kb transcript was designed (Figure 4). As shown in Figure 5(E), only a 4.5 kb transcript was detected in PMA-treated MEG-01 cells. Probe 5 did not detect the 2.8 kb message for PGHS-1 in PMA-treated cells because the probe 5 sequence is not present in the 2.8 kb transcript. Having established that the 4.5 kb transcript was indeed for PGHS-1 and not for PGHS-2, we investigated how the 4.5 kb PGHS-1 message could be produced.

Mechanism for generation of the 4.5 kb PGHS-1 transcript

To determine whether the 4.5 kb PGHS-1 message was generated by alternative polyadenylation in the same manner as the 2.8 kb message, we generated the unknown 3′ sequence of the full-length 5.1 kb PGHS-1 message. We derived a specific hPGHS-1 oligonucleotide upstream of a unique *Bam*HI site at position 4103 of the 5.1 kb PGHS-1 cDNA. This oligonucleotide, together with degenerated oligo(dT) primers, was used to amplify cDNA from PMA-treated MEG-01 cells. Two PCR fragments of 400 and 957 bp were obtained. Shown in Figure 6 is the 937 bp consensus sequence produced by 3′ RACE. The consensus sequence was obtained by comparing the sequences of three separate clones by using the Gene Jockey II program. The sequence was confirmed by a 57 bp overlap with the known hPGHS-1 3′ UTR sequence and the presence of a poly(A)$^+$ tail. We have identified in the cloned sequence a non-canonical polyadenylation signal (AAGAAA) located 12 bp upstream of a possible cleavage site (CA). Two G/U-rich or U-rich downstream elements located 34 and 46 bp downstream of the cleavage site were also identified in the cDNA obtained. If the identified polyadenylation site were used, it would generate a 4.5 kb PGHS-1 transcript, as detected in MEG-01 cells. The 400 bp PCR product obtained with the specific hPGHS-1 oligonucleotide (PGS1UTR) and degenerate oligo(dT) primers was sequenced: it

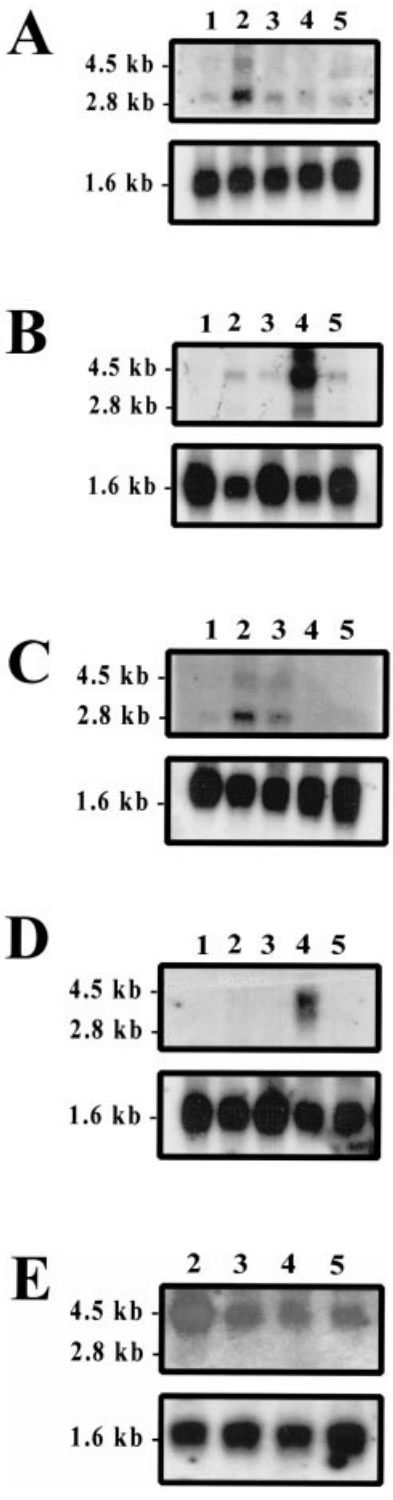


Figure 5 Characterization of the 4.5 kb PGHS transcript

Representative autoradiographs of Northern blots of poly(A)$^+$ RNA extracted from MEG-01 cells or U937 cells. Cells were treated as described in the Experimental section. Upper panels: probes used to analyse the resolved RNA described in Figure 4 were as follows: (**A**) probe 1, entire coding region of hPGHS-1; (**B**) probe 2, entire coding region of hPGHS-2; (**C**) probe 3, hPGHS-1-specific (3′ UTR segment of hPHGS-1); (**D**) probe 4, hPGHS-2-specific (3′ UTR segment of hPGHS-2); (**E**) probe 5, hPGHS-1-specific (3′ UTR segment of hPGHS-1). Lanes 1, control MEG-01 cells; lanes 2, PMA-treated MEG-01 cells; lanes 3, control U937 cells; lanes 4, LPS-treated U937 cells; lanes 5, U937 cells treated with LPS plus dexamethasone. Each sample was hybridized with α-tubulin (lower panels) to control for equal loading between lanes.

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1    GGATCCCTGCCCTTCTCAAGCACTTTAGCTTTTCCTTCCATCCGGTGGCC
51   TATTCCAGGAATTCCTCTTTTGCTTAAATCAGTTGGAGTTTGTGTCTGTT
101  GCTTGTAATCAAGCCTTTATGGCTGCTGGGCTGAGTGACACAAGCACTTT
151  AATGGCCTGGAGGGACTTTTAATCAGTGAAGATGCAATCAGACAAGTGTT
201  TTGGAAAGAGCACCCTCGAGAAGGGTGGATGACAGGGCAGAGCAGGAAGG
251  ACAGGAAGCTGGCAGAACGGAGGAGGCTGCAGCCGTGGTCCAACCAGGAG
301  CTGATGGCAGCTGGGGCTAGGGGAAGGGCTTTGAGGGTGGAAGGATGGGA
351  TGGGTCCAGAGGTATTCCTCTCTTAAATGCAAGTGCCTAGATTAGGTAGA
401  CTTTGCTTAGTATTGACAACTGCACATGAAAGTTTTGCAAAGGGAAACAG
451  GCTAAATGCACCAAGAAAGCTTCTTCAGAGTGAAGAATCTTTAATGCTTG
501  TAATTTAAACATTTGTTCCTGGAGTTTTGATTTGGTGGATGTGATGGTTG
551  GTTTTATTTGTCAGTTTGGTTGGGCTATAGCACACAGTTATTTAATCAAA
601  CAGTAATCTAGGTGTGGCTGTGAAGGTATTTTGTAGATGTGATTAACATC
651  TACAATCAGTTGACTTTAAGTGAAAGAGATTACTTAAATAATTTGGGTGA
701  GCTGCACCTGATTAGTTGAAAGGCCTCAAGAACAAACACTGCAGTTTCCT
751  GGAAAAGAAGAAACTTTGCCTCAAGACTATAGCCATCGACTCCTGCCTGA
801  GTTTCCAGCCTGCTAGTCTGCCCTATGGATTTGAAGTTTGCCAACCCCAA
851  CAATTGTGTGAATTAATTTCTAAAAATAAAGCTATATACAGCCAAAAAAA
901  AAAAAAA
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Figure 6 Cloned sequence of the last 1.0 kb of the 3′ UTR for hPGHS-1

The unknown 1.0 kb sequence on the 3′ UTR end of the 5.1 kb transcript of hPGHS-1 was generated by 3′ RACE , cloned into the multiple cloning site of pBS II KS (—) and sequenced with the T3 and T7 primers. The identity of the clone was obtained by an overlapping 57 bp stretch of the reported 3′ UTR sequence of PGHS-1 (shown in bold). Elements required to generate the 4.5 kb hPGHS-1 transcript were identified as follows: (1) a non-canonical polyadenylation signal (boxed sequence), (2) a cleavage site (underlined with a solid box) and (3) GT-rich elements downstream (underlined by hatched boxes) of the cleavage site.

overlapped with the 957 bp PCR product. This confirms that the 4.5 kb PGHS-1 transcript is produced by alternative polyadenylation in MEG-01 cells.

Profile of 4.5 kb PGHS-1 transcript expression

By using a commercial mRNA dot-blot containing poly(A)$^+$ RNA from 50 different human tissues, we observed a tissue-specific expression pattern of the 4.5 kb PGHS-1 transcript. A comparison between the different tissues was performed after normalization for variations in loading with the use of the ubiquitin control cDNA probe provided (Figure 7C). As shown in Figure 7, by using probe 5 (which detects the PGHS-1 4.5 and 5.1 kb transcripts but not the 2.8 kb transcript) we were able to detect a high level of expression in the bladder (C5) and the appendix (F1). Signals of lower intensity were also detected in the colon, small intestine, bone marrow, uterus, prostate, peripheral leucocyte, lymph node and stomach. When the membrane was hybridized with probe 6 to detect only the 5.1 kb transcript, the bladder and the colon gave maximal signals and weak signals were detected in the appendix, stomach, prostate and uterus. Figures 7(A) and 7(B) represent two different membranes; Figure 7(C) is the from membrane presented in Figure 7(B) after re-probing with ubiquitin. The signal obtained with probe 6 was subtracted from that obtained with probe 5 to determine the level of 4.5 kb PGHS-1 transcript.

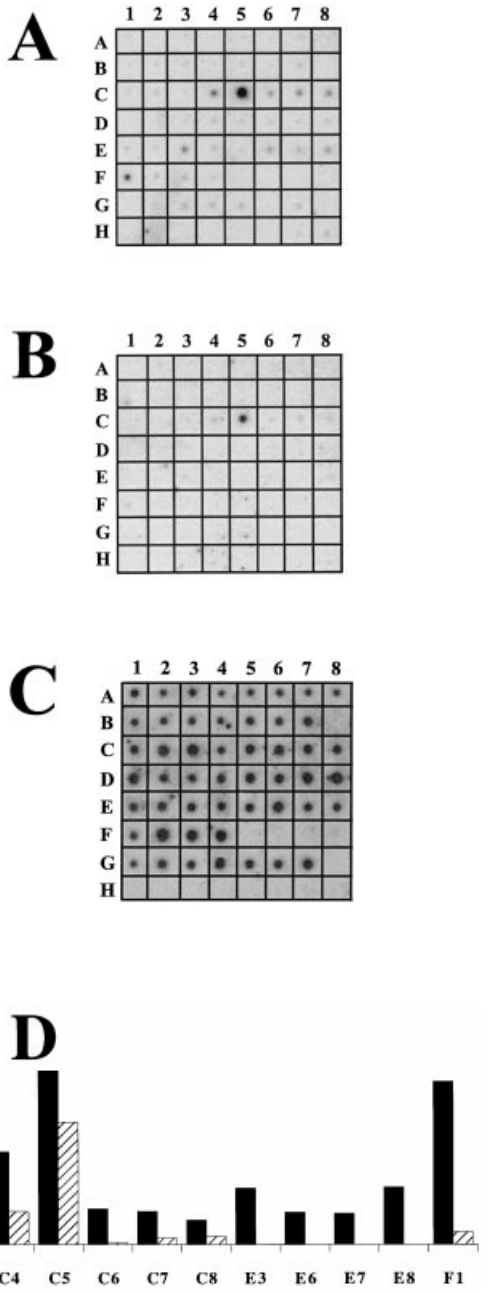

Figure 7 Tissue-specific expression of the 4.5 and 5.1 kb transcripts of hPGHS-1

Autoradiogram of a RNA dot-blot containing poly(A)$^+$ RNA from 50 different human tissues. The membrane was incubated with probe 5 (**A**), which detected both the 4.5 and 5.1 kb PGHS-1 transcripts, and with probe 6 (**B**), which detected only the 5.1 kb transcript. The ubiquitin probe (**C**) was used as a control for equal loading between dots. The intensity of each PGHS-1 transcript signal was divided by the corresponding ubiquitin signal. Levels of the 4.5 kb transcript (**D**) were calculated by subtracting the probe 6 signals from the probe 5 signals for each tissue; filled columns, 4.5 kb PGHS-1; hatched columns, 5.1 kb PGHS-1. C4, colon; C5, bladder; C6, uterus; C7, prostate; C8, stomach; E3, small intestine; E6, peripheral leucocyte; E7, lymph node; E8, bone marrow; F1, appendix.

Detection of the 4.5 kb PGHS-1 transcript with a multiple-tissue Northern blot

By using a commercial mRNA Northern blot containing poly(A)$^+$ RNA from the skeletal muscle, uterus, colon, small intestine, bladder, heart, stomach and prostate (obtained from several different patients and pooled), we observed that the 4.5 kb transcript of PGHS-1 is indeed generated in a tissue-specific fashion. Figure 8 shows that only the bladder contains detectable levels of the 4.5 kb PGHS-1 transcript. Both the 2.8 and 5.1 kb PGHS-1 transcripts were expressed at significantly higher levels in the bladder than in the other tissues. Other tissues contained no detectable levels of the 4.5 kb PGHS-1 transcript. These results confirmed that the 4.5 kb PGHS-1 transcript detected in MEG-01 cells is produced in a tissue-specific fashion and is not an artifact of the MEG-01 cell line.

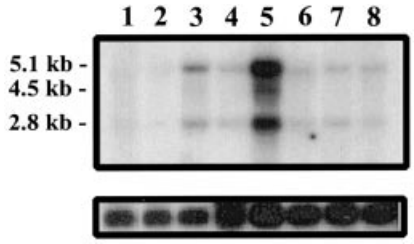

Figure 8 Tissue-specific expression of the 4.5 kb transcript of hPGHS-1

Autoradiogram of a multiple-tissue Northern blot containing 2 μg of poly(A)$^+$ RNA. Lane 1, skeletal muscle; lane 2, uterus; lane 3, colon; lane 4, small intestine; lane 5, bladder; lane 6, heart; lane 7, stomach; lane 8, prostate. Upper panel: the membrane was incubated with probe 3, which detected the 2.8, 4.5 and 5.1 kb PGHS-1 transcripts. Lower panel: a β-actin probe was used as a control for equal loading between lanes.

DISCUSSION

Blood platelets are a rich source of PGHS-1 [19], the enzyme targeted by aspirin [20]. Inhibition of platelet PGHS-1 by aspirin prevents the formation of the aggregatory agent TXA_2, which decreases the incidence of cardiovascular disease associated with platelet hyperfunction [21]. Because of the importance of PGHS-1 in regulating cardiovascular homoeostasis, it is crucial to elucidate the molecular mechanisms regulating the expression of PGHS-1 in platelets. Platelets are anucleate cells; to study the mechanism regulating PGHS-1 expression we therefore used the human nucleated platelet precursor cell line MEG-01. MEG-01 cells are relatively undifferentiated megakaryoblastic cells committed to the platelet pathway [22]. Studies have shown that the differentiation of MEG-01 cells *in vitro* into mature megakaryocytes and platelet-like structures can be achieved by addition of the phorbol ester PMA [23,24].

We observed that untreated MEG-01 cells do not express detectable levels of the enzyme PGHS-1 but after a single addition of PMA (16 nM) a linear increase in PGHS-1 enzyme was detected between days 2 and 6. Also, after stimulation with PMA an increase in PGHS-1 protein was preceded by an increase in PGHS-1 mRNA as early as day 1. Because maximum increases in PGHS-1 mRNA and protein are separated by 4 days, these results together suggest that both transcriptional and post-transcriptional mechanisms of regulation have a role in PGHS-1 expression. To address the post-transcriptional regulation of PGHS-1 expression, we first characterized the PGHS-1 transcripts detected in MEG-01 cells. By using the entire coding region of PGHS-1 as a probe under conditions of high stringency, transcripts of 2.8, 4.5 and 5.1 kb were detected in the poly(A)$^+$ RNA fraction of MEG-01 cells analysed by Northern blotting. As previously reported, the 2.8 kb transcript is the most abundant PGHS-1 transcript [25,26] and is postulated to be generated from

the 5.1 kb transcript through a canonical polyadenylation signal. Previous reports on the effect of human chorionic gonadotrophin ('hCG') on PGHS-1 mRNA levels in fetal membranes have described significant increases in both a 4.1 and 2.8 kb transcript, with hPGHS-1 cDNA as a probe [27]. No work has been done on the possible sources of this new 4.5 kb PGHS transcript detected with the hPGHS-1 coding region. We therefore further characterized the 4.5 kb transcript detected in the poly(A)$^+$ RNA fraction in MEG-01 cells and identified it as PGHS-1, with the use of a probe specific to the PGHS-1 3′ UTR , which contains no similarity to the PGHS-2 sequence.

To examine how the 4.5 kb PGHS-1 transcript could be generated, the last 1.0 kb at the 3′ end of the 5.1 kb PGHS-1 transcript was cloned, sequenced (sequence submitted to Bank it, no. 254414) and found by computer analysis to contain elements that define a potential polyadenylation site. If used, this site would provide a source of the 4.5 kb transcript detected. The most critical sequence of a polyadenylation site is the canonical polyadenylation signal (AAUAAA) found almost invariably 10–30 bases upstream of a cleavage site [28]. The polyadenylation signal found in our sequence is a non-canonical signal (AAGAAA). Studies *in vitro* indicate that mutation of the AAUAAA hexanucleotide to AAGAAA markedly decreases the efficiency of polyadenylation [29]. This decrease in polyadenylation efficiency is consistent with our observation that the PGHS-1 2.8 kb transcript derived by a canonical polyadenylation signal (AAUAAA) is much more abundant than the 4.5 kb transcript produced through the non-canonical polyadenylation signal (AAGAAA). On treatment with PMA, the levels of both the 2.8 and 4.5 kb transcripts are increased but their ratio remains the same; the 2.8 kb transcript is the more abundant. Altogether, the polyadenylation signal (AAGAAA), the G/U-rich element located downstream of the site of cleavage and the CA sequence immediately 5′ of the site of cleavage correspond to the consensus elements that define a poly(A)$^+$ cleavage site [28].

The biological significance of the three different PGHS-1 transcripts is at present unknown. We have observed that the expression of the newly identified PGHS-1 4.5 kb transcript is tissue-specific. The bladder expressed high levels of the 5.1 and 2.8 kb PGHS-1 transcripts and moderate levels of the 4.5 kb transcript, whereas MEG-01 cells expressed high levels of the 4.5 and 2.8 kb PGHS-1 transcripts and moderate levels of the 5.1 kb transcript. Therefore these results suggest preferential use of PGHS-1 poly(A)$^+$ sites in a tissue-specific manner. Expression was also detected in the colon, uterus, prostate, stomach, small intestine, peripheral leucocyte, lymph node and bone marrow, suggesting that tissues with an endodermal origin preferentially express the PGHS-1 4.5 kb transcript. To confirm that the expression of the 4.5 kb transcript is limited to tissues with endodermal origin, hybridization *in situ* will have to be performed to confirm that expression is limited to the epithelial region of these organs.

Although most eukaryotic gene transcription units possess a single polyadenylation signal, numerous examples of genes with multiple poly(A)$^+$ sites, all within a single 3′-terminal exon, have been reported [28]. The hPGHS-1 gene can now be added to that list, with three polyadenylation sites spread over the 3.3 kb of 3′ UTR sequence. Changes in the use of the various poly(A)$^+$ sites have been shown to be developmentally regulated. At least two poly(A)$^+$ signals have been reported in the amphiglycan gene: the longer message is ubiquitous, whereas the shorter message seems to be tissue-specific [30]. In addition, a switch in poly(A)$^+$ site use during chondrocyte differentiation has been reported [31]. The human activin βA subunit gene contains a tandem of eight possible poly(A)$^+$ sites; the treatment of HT1080 fibrosarcoma cells with PMA, which stimulates erythroid differentiation, causes a shift over time to the use of proximal poly(A)$^+$ sites [32]. Moreover, the murine tissue inhibitor of the metalloproteinase-3 gene in pre-neoplastic JB 6 cells treated with PMA produces three transcripts of 2.3, 2.8 and 4.6 kb, with the 4.6 kb transcript being the most abundant [33]. All three PGHS-1 transcripts detected are subject to up-regulation in MEG-01 cells by treatment with PMA, which indicates that they are regulated in a similar fashion.

Because the multiple forms of PGHS-1 mRNA differ only by the selection of their polyadenylation signal sites at the 3′ end, at present it is not obvious how differential poly(A)$^+$ site use could influence protein expression. However, if the different forms of mRNA have different stabilities, then the use of alternative poly(A)$^+$ sites can affect (positively or negatively) the final amount of protein product per unit of precursor RNA transcribed. An example of a gene that shows a pattern of differential stability of the various mRNA products is the PGHS-2 gene. The PGHS-2 gene produces a major transcript of 4.5 kb and a minor transcript of 2.8 kb, which are derived by polyadenylation in the 3′ UTR [7]. Post-transcriptional mechanisms that stabilize the mRNA have been reported to increase PGHS-2 mRNA levels [34]. The multiple copies of the AUUUA instability element in the 3′ UTR of the PGHS-2 gene have been shown to be associated with the stability of the 4.5 kb PGHS-2 transcript [17]. Because the 2.8 and 4.5 kb messages have respectively 7 and 22 copies of the AUUUA instability motif, they would be expected to show different stabilities, with the 4.5 kb fragment being the more unstable. Studies have shown that both PGHS-2 transcripts displayed half-lives in excess of 2 h [8]. Increases in stability for both transcripts were reported after treatment with interleukin 1β; stabilization of the 4.5 kb transcript occurred earlier than that for the 2.8 kb transcript [34,35]. In response to dexamethasone, the 2.8 kb transcript became more stable than the 4.5 kb transcript [36]. Because interleukin 1β tends to favour the stabilization of the 4.5 kb transcript and dexamethasone treatment favoured the stabilization of the 2.8 kb message, mechanisms other than the number of AUUUA instability motifs have a role in the post-transcriptional regulation of the PGHS-2 gene. Stabilization of the PGHS-2 mRNA has also been reported in human umbilical-vein endothelial cells, suggesting a functional post-transcriptional role of the 3′ UTR [34]. The molecular mechanisms responsible for the increase in mRNA stability remain to be determined.

Because the full-length human PGHS-1 3′ UTR contains only four copies of the AUUUA instability motif, we propose that expression is influenced instead by the use of the three poly(A)$^+$ polyadenylation sites spread over the 3.3 kb of 3′ UTR sequence that generate the three PGHS-1 messages. The three possible messages produced could have different stabilities or be translated at different rates, possibilities that we are currently investigating. Because PMA predominantly induced the formation of the shortest version, the 2.8 kb message, this mRNA isoform might be involved primarily in the housekeeping role of constitutive PGHS-1 expression. The longer transcripts, 5.1 and 4.5 kb, which are also inducible but at much lower levels, might therefore contribute to the induction of PGHS-1 during differentiation. The induction of PGHS-1 expression was reported in systems that model developmental events. The most notable example of PGHS-1 induction is the PMA-induced differentiation of THP-1 monocytes to a macrophage phenotype [9]. A study has reported that the abundance of PGHS-1 mRNA increases during the development of sheep pulmonary artery [37]. Because probes designed in the coding region of PGHS-1 were used in those studies and only the expression of the 2.8 kb PGHS-1 message

was reported, it remains to be determined whether the PGHS-1 4.5 kb transcript is also induced in these systems.

In conclusion, we have identified a new PGHS-1 transcript of 4.5 kb that arises through alternative polyadenylation. This 4.5 kb message is induced by PMA in MEG-01 cells, together with the previously reported 2.8 and 5.1 kb transcripts. We report tissue-specific expression of this new PGHS-1 transcript, with the bladder and appendix expressing the highest levels. Because the coding regions of the 4.5 kb PGHS-1 and 4.6 kb PGHS-2 transcripts share 73 % sequence similarity, the standard probes used (entire coding regions) for hybridization might have often misidentified the 4.5 kb PGHS-1 as PGHS-2. We therefore provide a strategy to detect PGHS-1 and PGHS-2 transcripts specifically by using probes in the 3′ UTR regions of these genes, where no similarity is found.

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