

5-25-1992

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Diaz, Arturo; Reginato, Anthony M.; and Jimenez, Sergio A., "Alternative splicing of human prostaglandin G/H synthase mRNA and evidence of differential regulation of the resulting transcripts by transforming growth factor beta 1, interleukin 1 beta, and tumor necrosis factor alpha." (1992). *Department of Medicine Faculty Papers*. Paper 185.
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Alternative Splicing of Human Prostaglandin G/H Synthase mRNA and Evidence of Differential Regulation of the Resulting Transcripts by Transforming Growth Factor β_1 , Interleukin 1β , and Tumor Necrosis Factor α^*

(Received for publication, September 11, 1991, and in revised form, February 3, 1992)

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Prostaglandin G/H synthase (PGG/HS) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins and thromboxanes. We screened a human lung fibroblast cDNA library with an ovine PGG/HS cDNA and isolated a 2.3-kilobase clone (HCO-T9). Sequence analysis of this clone showed that (a) it contained the entire translated region of PGG/HS and (b) it displayed an in-frame splicing of the last 111 base pairs encoded by exon 9, which resulted in the elimination of the *N*-glycosylation site at residue 409. Polymerase chain reaction amplification with specific oligonucleotides of reverse-transcribed mRNA from diverse human tissues and cultured cells yielded 400- and 300-base pair fragments that corresponded, respectively, to the intact and spliced transcripts. The expression of these two transcripts in cultured human lung fibroblasts was differentially regulated by serum, transforming growth factor β_1 , interleukin 1β , tumor necrosis factor α , and phorbol 12-myristate 13-acetate, as each of these conditions stimulated preferentially the expression of the unspliced transcripts. The elimination of one of the four *N*-glycosylation sites by the alternative splicing of exon 9 and the differential regulation of this process by relevant cytokines and growth factors may represent a mechanism for the regulation of PGG/HS enzymatic activity under physiological or pathological conditions.

Prostaglandins (PG)¹ are important mediators involved in diverse biologic processes such as cell proliferation, inflammatory and immune responses, smooth muscle contraction, maintenance of fluid and electrolyte balance, platelet aggregation, and the production of extracellular matrix proteins (1-3). The metabolism of free arachidonic acid to PG and thromboxanes is dependent on the availability of the enzyme

PG G/H synthase (PGG/HS) (EC 1.14.99.1), a membrane-bound homodimer of two 70-kDa polypeptides. PGG/HS has a bisdioxygenase (cyclo-oxygenase) activity mediating the bisoxygenation of arachidonic acid to the hydroperoxide PGG₂, and a hydroperoxidase activity mediating the reduction of PGG₂ to the endoperoxide PGH₂, which is the precursor of PG and thromboxanes (4, 5). PGG/HS is a hemoprotein with a protoporphyrin IX prosthetic group which is required for both cyclo-oxygenase and hydroperoxidase activities. The enzyme contains four oligosaccharides bound to asparagine residues (6). At least one of these residues appears to play a crucial role in the activity of the enzyme.

The primary structure of PGG/HS from ovine vesicular glands (7-9) and murine 3T3 cells (10) has been deduced from full-length cDNA sequences. Similarly, the primary structure of the human enzyme has been deduced from sequences of a genomic clone (11). Northern hybridizations employing ovine or murine cDNAs have identified either 2.7-kb transcripts that correspond to the size of the cDNA (12, 13) or transcripts of 2.7 and 5.5 kb (14-16) in a variety of cells and tissues from different species, including humans. In addition, hybridizations under low stringency conditions showed the presence of 4.0-kb transcripts in murine and ovine tissues, suggesting the existence of a related gene (17, 18). More recently, the avian equivalent of this gene has been cloned (19).

PG have been shown to down-regulate the production of extracellular matrix proteins in fibroblasts by diverse mechanisms (3). PG production is stimulated by various cytokines, such as interleukin 1β (IL- 1β), tumor necrosis factor α (TNF- α), transforming growth factor β_1 (TGF- β_1), epidermal growth factor, and platelet-derived growth factor (20-24). Consequently, PG may modulate the effects that these cytokines have on extracellular matrix protein production (22). The availability of a cDNA for the human PGG/HS would allow a better understanding of the complex regulatory processes involved in PG production in various tissues under diverse conditions.

Here, we report the cloning of a cDNA for the human PGG/HS and present evidence of an alternative splicing of the corresponding mRNA that eliminates 111 bp encoded by exon 9 of the gene. The alternative splicing would result in the elimination of one of the four functionally required *N*-glycosylation sites in the enzyme. Furthermore, we demonstrate the differential regulation of PGG/HS mRNA splicing by serum, TGF- β_1 , IL- 1β , TNF- α , and phorbol esters. These observations suggest that the alternative splicing we identified may play an important role in the regulation of the enzymatic activity of PGG/HS under normal or pathologic conditions.

* This work was supported by National Institutes of Health Grant HL41214. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: PG, prostaglandin(s); PGG/HS, prostaglandin G/H synthase; bp, base pair(s); kb, kilobase(s); IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; MEM, Eagle's minimal essential medium; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction.

MATERIALS AND METHODS

Cell Culture—Primary fibroblast cultures were established from human lung, dermis, and synovium and were cultured as described (25). Confluent fibroblasts were incubated for 24 h in Eagle's minimal essential medium (MEM) supplemented with 1, 5, or 10% FCS, 1% (v/v) vitamins, and 2 mM L-glutamine. After this period, the cells were incubated for different intervals in media containing either no serum or serum at the same concentrations as those received during the previous 24 h. The cultures were incubated under control conditions or with one of the following additives: 0.1 μ M phorbol 12-myristate 13-acetate (PMA), 200 units/ml TNF- α (Boehringer Mannheim), 50 units/ml IL-1 β (Boehringer Mannheim) or various concentrations of TGF- β 1 (10–500 pM) (Collaborative Research Inc., Bedford, MA).

Northern Hybridizations—Poly(A⁺) mRNA was prepared on oligo(dT)₁₀-cellulose columns using the Fast Track mRNA Isolation Kit (Invitrogen; San Diego, CA). Total RNA from human cartilage was obtained as described (26). Total RNA from confluent lung, dermal, and synovial fibroblasts was prepared by the guanidium isothiocyanate/CsCl₂ discontinuous gradient method as described (27). Total RNA or poly(A⁺) mRNA was electrophoresed on formaldehyde-agarose gels, transferred to nylon-supported nitrocellulose (Schleicher & Schuell), UV cross-linked, and hybridized with either a gel-purified 1.6-kb cDNA for ovine PGG/HS (7), the corresponding human HCO-T9 cDNA (see below), or a murine glyceraldehyde-3-phosphate dehydrogenase cDNA (28). Probes were labeled with [α -³²P]dCTP by nick translation (29), and the filters were hybridized for 24 h at 42 °C in 4 \times SSC, 50% formamide, 0.1% sodium dodecyl sulfate, 2 \times Denhardt's solution, and 200 μ g/ml salmon sperm DNA. Autoradiographs were developed and then scanned in a laser densitometer (UltraScan XL, Pharmacia LKB Biotechnology Inc.).

Isolation and Characterization of the cDNA—Single-stranded cDNA was prepared by reverse transcription of 5 μ g of human lung fibroblast poly(A⁺) mRNA with Moloney leukemia virus reverse transcriptase (GIBCO-BRL) and oligo(dT)₁₀ primers (30), and the second strand was synthesized using RNase H and DNA polymerase I (Boehringer Mannheim). EcoRI linkers (Promega Biotec, Madison, WI) were attached to the double-stranded cDNA, and it was ligated to an EcoRI-predigested λ vector (λ -ZAP II; Stratagene, La Jolla, CA), and packaged *in vitro* using a commercial packaging extract (Giga Pack II Plus; Stratagene, La Jolla, CA). A bacteriophage cDNA library was obtained containing approximately 4.5 \times 10⁸ colonies and was screened by plaque hybridization with the gel-purified 1.6-kb ovine PGG/HS cDNA previously radiolabeled with [α -³²P]dCTP by nick translation to a specific activity of 1.5 \times 10⁸ cpm/ μ g DNA. Hybridizations were carried out for 48 h at 42 °C in a buffer containing 6 \times SSC, 5 \times Denhardt's solution, 1% sodium dodecyl sulfate, and 100 μ g/ml salmon sperm DNA. A positive clone was identified that was plaque-purified, and the Bluescript phagemide containing the cDNA was excised from the λ vector with the helper virus R408. Progressive bidirectional deletions of the double-stranded cDNA were prepared with exonuclease III (Erase-a-Base System, Promega Biotec) and sequenced by the dideoxy chain termination method (31) using modified T7 polymerase (Sequenase, U. S. Biochemical Corp.).

Polymerase Chain Reaction (PCR) Amplification of PGG/HS Transcripts—Total RNA (5–10 μ g) or poly(A⁺) mRNA (2 μ g) from various human tissues and cultured cells was employed to synthesize single-stranded cDNA with Moloney leukemia virus reverse transcriptase and oligo(dT)₁₀ primers. Amplification of the cDNA was performed by 40 cycles of PCR (32) in 50 μ l of Taq DNA polymerase mixture (Perkin-Elmer Cetus Instruments) with 25 pM of two primers flanking a region from nucleotides 1011 to 1415. The reaction cycles were 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min. The 5'-primer (5'-GGAGACCATCAAGATTGT) is complementary to the antisense strand, and the 3'-primer (5'-GTTCTCCAAACCGTACTT) is complementary to the sense strand. The PCR-amplified cDNAs were directly cloned into the pCR-1000 vector (Invitrogen, San Diego, CA) and sequenced by the dideoxy chain termination method.

In other experiments, 4 μ g of total RNA from lung fibroblasts incubated under various conditions were reverse-transcribed using random hexamers and amplified by PCR in the presence of 25 μ Ci of [α -³²P]dCTP for 20 cycles. The reactions were chloroform-extracted and precipitated, resuspended in H₂O, and divided into two aliquots and analyzed by electrophoresis in 3% agarose and 5% acrylamide, respectively. In other reactions, 3 or 8 μ g of total RNA were reverse-transcribed and amplified by PCR in the presence of 50 μ Ci of [α -³²P]dCTP in a reaction volume of 200 μ l, and 38- μ l aliquots were

taken at cycles 15, 18, 21, 24, 27. Each aliquot was divided and analyzed by electrophoresis in agarose and acrylamide gels as described above. Acrylamide gels were fixed in 7% acetic acid, dried, and subjected to autoradiography. The segments of the gels corresponding to the bands detected by autoradiography were excised, and the radioactivity was determined by scintillation counting. The counts/min obtained were divided by the cytidine content of both strands of the cDNA corresponding to the intact and spliced transcripts flanked by the oligonucleotide primers used for amplifications; that is cpm in 400 bp/210 and cpm in 300 bp/144. The resulting values were used for further calculations.

RESULTS

Cloning and Sequencing of a Full-length Human PGG/HS cDNA and Evidence of Alternative Splicing—Northern hybridizations of poly(A⁺) mRNA from normal human lung fibroblasts with the 1.6-kb ovine PGG/HS cDNA showed hybridization to 2.7-kb and 5.5-kb transcripts (Fig. 1, lane 1). A bacteriophage cDNA library was prepared from this mRNA and a positive clone containing a 2.3-kb cDNA, referred to as HCO-T9, was obtained. Northern hybridization analysis of normal human lung fibroblast poly(A⁺) mRNA with the HCO-T9 clone showed transcripts of 2.7 and 5.5 kb (Fig. 1, lane 2). A partial restriction map and the sequencing strategy for the HCO-T9 clone are shown in Fig. 2.

The nucleotide sequence of the HCO-T9 clone was determined and compared with the sequence of the human genomic clone for PGG/HS (11), which encodes for the 2.7-kb transcript (Fig. 3). This analysis showed that the HCO-T9 clone has the following features. (a) It contains the entire coding region plus 638 bp from the 3'-nontranslated region. (b) It

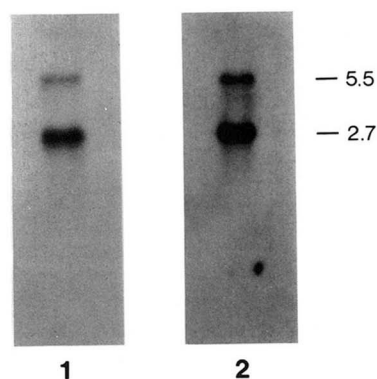


FIG. 1. Northern hybridizations of normal human lung fibroblast mRNA with ovine and human PGG/HS cDNAs. Two μ g of poly(A⁺) mRNA isolated from normal human lung fibroblasts was electrophoresed and blotted onto nitrocellulose as described under "Materials and Methods." Lane 1, mRNA hybridized with the 1.6-kb ovine cDNA for PGG/HS labeled by nick translation with a [α -³²P]dCTP (specific activity, 5.9 \times 10⁸ cpm/ μ g). Lane 2, mRNA hybridized with the 2.3-kb human HCO-T9 clone labeled by nick translation with [α -³²P]dCTP (specific activity, 1.29 \times 10⁸ cpm/ μ g). Sizes in kb were determined by comparison with transcripts for fibronectin and glyceraldehyde-3-phosphate dehydrogenase.

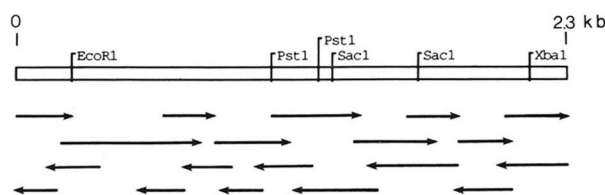


FIG. 2. Partial restriction map and sequencing strategy of the human PGG/HS cDNA. The 2.3-kb HCO-T9 clone was digested with exonuclease III to obtain bidirectional deletions. The arrows indicate the direction and lengths of the sequencing reactions.

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ATGACCGGAGTCTCTTCCCTCCGGTCTTCTGCTTCTGCTCTCTCCGCGCCCTCCCGCTCTCTC 69
M S R S L L L L L L L L L L L L L L L L L L P P L P V L L 23
CGGACCCAGGGGCCCCACCCAGTGAATCCCTGTGTTACTATCCATGCCAGCACCAGGCACTCTGT 138
A D P G A P T P V N P N C C Y Y P C Q H Q G I C 46
GTCCGCTTGGCGTTGACCGCTAGCTAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 207
V R F G L D R Y Q C D C T R T G Y S G P [N] C T 69
ATCCCGGCGCTCTGACCTGACTCGGAGTCTGACTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 276
I P G L W T W L R N S L R P S P S F T H F L L 92
ACTCAGGGGCGCTGTTCTCGAGGTTTGTCTCATGGCCACTCTCATCCGAGAGTCTCATCGCCCTGGTA 345
T H G R W F W E F V [N] A T F I R E M L M R L V 115
CTCAGAGTGGCTCCCAACCTTATCCCGAGTCCCGCCAGCTACCAACTCAGCAGCTGACTACATCAGCTG 414
L T V R S N L I P S P P T Y N S A H D Y I S W 138
GAGCTTCTCCAGGCTGAGCTATTACACTGTGATTCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 483
E S F S [N] V S Y Y T R I L P S V P K D C P T P 161
ATGGGACCAAGGGGAGAGAGAGAGTCCAGGATGCCAGCTCCAGCTCTGCGCGCGCGCTTCTGCTCAGG 552
M G T K G K K Q L P D A Q L L L L L L L R R F L L R R 184
AGTITCATCTAGCCCGCCAGCCAGCCAGCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 621
K F I P D P Q G T N L M F A F F A Q H F T H Q 207
TCTTCAGAACTTCTGCAAGATGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 690
F F K T S G K M G P P G T F K A L G H G V D L G 230
CACATTTATGGAGCAATCTGGAGGCTGAGTATCAACTGGGCTCTTTAGAGTGGGAATCCAGCTAGC 759
H I Y G D N L E R Q Y Q L R L F K D G K L K Y 253
CAGTCTCAGTGGAGAAATGTACCCCGCTCCGAGAGAGAGCGGCTGTTGTGATGCACTACCCCGA 828
Q V L D G E M Y P P S V E E A P V L M H Y P R 276
GCGATCCCGCAGGAGCCAGATGGCTGTTGGCGAGGAGTGTGGCTGCTGCTGCTGCTGCTGCTGCTGCT 897
G I P P Q S Q M A V G Q E V F G L L P G L M L 299
TATGCACTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 966
Y A T L W L R E H N R V C D L L K A E H P T W 322
GGGATGAGCAGCTTTTCCAGGAGCCCGCCCTCATCTCATAGGGGAGCCAGCTGCTGCTGCTGCTGCTG 1035
G D E Q L F Q T T R L I L I G E T I K I V I E 345
GAGTCTGCTGAGAGCTGAGTGTATTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 1104
E Y V Q Q L S G Y F L Q L K F D P E L L F V G 368
CAGTTCAGTCCGCAAGCCAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 1173
Q F Q Y R N R I A M E F N H L Y H W H P L M P 391
GACTCTTCAAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1242
D S F K V G S Q E Y S Y E Q F L F [N] T S M L V 414
GACTTGGGTTGAGGCGCTTGGTGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1311
D Y G V E A L V D A F S R Q T I A G R I G G G R 437
AAGCTGAGCAGCCACTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 1380
N M D H H I L H V A V D V I R E S R E M L Q 460
CCCTTCAGTGGTCCGCAAGGCTTTCATGAAAGCCCTCAGGCTCTGCTGCTGCTGCTGCTGCTGCTGCT 1449
P F N E Y R K R F G M K P Y T S F Q E L V G E 483
AAGCAGTGGCAGAGTGGAGATTTGAGGAGCATGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1518
K E M A A E L E E L Y G D I D A L E F Y P G L 506
CTTCTGAAAGGCTCCCAACTTCATCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1587
L L E K C H P N S I F G E S M I E I G A P P S 529
CTCAGGCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1656
L K G L L G N P I C S P E Y W K P S T F G G E 552
GTGGCTTTAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 1725
V G F N I V K T A T L K K L V C L N T K T C P 575
TAGCTTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 1794
Y V S F R V P D A S Q D D G P A V E R P S T E 598
CTCTGAGGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG 1863
L * 599
CAGGCTGTGAGTCTTAAATGCTCATTTCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 1932
TTTAACTTCCACCATTAATCTGGAATATTGAGTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2001
AGCCCTCAGATTGTAGGAGGTTCTCAATTTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 2070
AGATGCTCAGATTTCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2139
TAGAAGTTAGGGGCTTCTTATTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2208
TGTTCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 2277
GTGCAATTGATTCATTTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2346
GCATTCCTGATTCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2415
AGACCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG 2435

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FIG. 3. Nucleotide and predicted amino acid sequences of the HCO-T9 cDNA. Nucleotides and amino acids are numbered from the ATG initiation codon. The nucleotides corresponding to exon 9 that are absent from clone HCO-T9 are underlined with a solid line. Binding sites for forward and reverse oligonucleotide primers for PCR analysis are underlined with a discontinuous line. The 5'- and 3'-ends of exon 9 are marked with arrowheads. The four N-glycosylation sites (residues 67, 103, 143, and 409) are boxed. The serine that is acetylated by aspirin (residue 529) is marked with a dot.

has a complete homology with the genomic sequence, except for the nucleotides at positions 36, 338, 393, and 1133. (c) It shows the absence of 111 bp (from nucleotides 1186 to 1296) corresponding to the last 111 bp of exon 9, coding for 37 amino acids (amino acids 396–432). The 3'-acceptor site of exon 10 is conserved, thereby maintaining the PGG/HS single open reading frame. The presence of the consensus sequence C/AAG:GTG/A of 5'-splice donor sites (33) from nucleotides 1183 to 1188 of exon 9 strongly suggested an alternative splicing of the PGG/HS transcripts.

Expression of Intact and Spliced PGG/HS Transcripts in Various Tissues and Cultured Cells—To investigate whether transcripts corresponding to the intact and alternatively spliced forms of the PGG/HS gene were expressed in different human cells, PCR amplification of cDNA prepared from fetal and adult cartilage and from dermal, lung, and synovial fibroblast mRNA was performed. The ovine 1.6-kb (intact) and

the human HCO-T9 (spliced) cDNAs were also amplified as controls. The 18-mer primers used were designed to bind regions of perfect homology between the human and ovine cDNAs. PCR amplification of the reverse transcribed mRNA from the five different human sources resulted in two fragments of approximately 400 and 300 bp in every case (Fig. 4). PCR amplification of mRNAs extracted from synovial tissue produced fragments of the same sizes (data not shown). PCR amplification of the ovine 1.6-kb cDNA yielded a single fragment of the expected 400 bp, whereas amplification of the HCO-T9 yielded a single fragment of 300 bp (Fig. 4). The 300-bp and 400-bp fragments amplified from the human mRNA and the 400- and 300-bp fragments amplified from the 1.6-kb ovine and the HCO-T9 cDNAs, respectively, were cloned in the pCR-1000 vector and sequenced. Analysis of the sequences showed that (a) the 400-bp fragments from both species were derived from transcripts corresponding to an intact exon 9 and (b) the human 300-bp fragment lacked the last 111 bp encoded by exon 9. We conclude that there was indeed an alternative splicing of the human PGG/HS mRNA at a position corresponding to nucleotide 1185 of exon 9 of the gene.

Regulation of the Expression of Intact and Spliced PGG/HS Transcripts by Growth Factors and Cytokines—The possibility that there may be differences in the enzymatic activity of the proteins translated from the intact and alternatively spliced transcripts led us to investigate the relative expression of these transcripts in normal human lung fibroblasts, under *in vitro* conditions known to up-regulate the expression of the gene (12, 14, 17, 34–39). Fibroblasts incubated with increasing concentrations of TGF- β 1 showed a dose-dependent increase (up to 17-fold at 500 pM TGF- β 1) in the steady-state levels of the 2.7-kb PGG/HS transcript (Fig. 5A). It was also noted that the 5.5-kb transcript described above was coordinately expressed with the 2.7-kb PGG/HS transcript. To determine the effects of the higher concentrations of TGF- β 1 on the relative proportions of intact and spliced PGG/HS transcripts, total RNA from control and TGF- β 1-treated cells was reverse-transcribed and amplified by PCR. Aliquots taken at different cycles were electrophoresed in agarose and acryl-

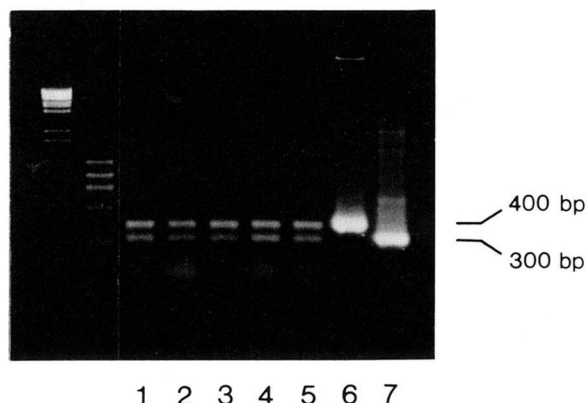


FIG. 4. Analysis of mRNA from various human tissues and cells by PCR amplification with PGG/HS-specific oligonucleotide primers. Ten μ g of total RNA (cartilage, dermal, and lung fibroblasts), or 2 μ g of poly(A⁺) mRNA (synovial fibroblasts), was reverse-transcribed, and equal aliquots were amplified for 40 cycles by PCR as described under "Materials and Methods." The PCR products were analyzed in a 1.5% agarose gel stained with ethidium bromide. Lane 1, fetal tibial cartilage; lane 2, adult tibial cartilage; lane 3, dermal fibroblasts; lane 4, lung fibroblasts; lane 5, synovial fibroblasts; lane 6, ovine 1.6-kb cDNA; lane 7, HCO-T9 clone. The DNA standard ladders (left) are λ DNA-BstEII and Phix174 RF DNA-HaeIII.

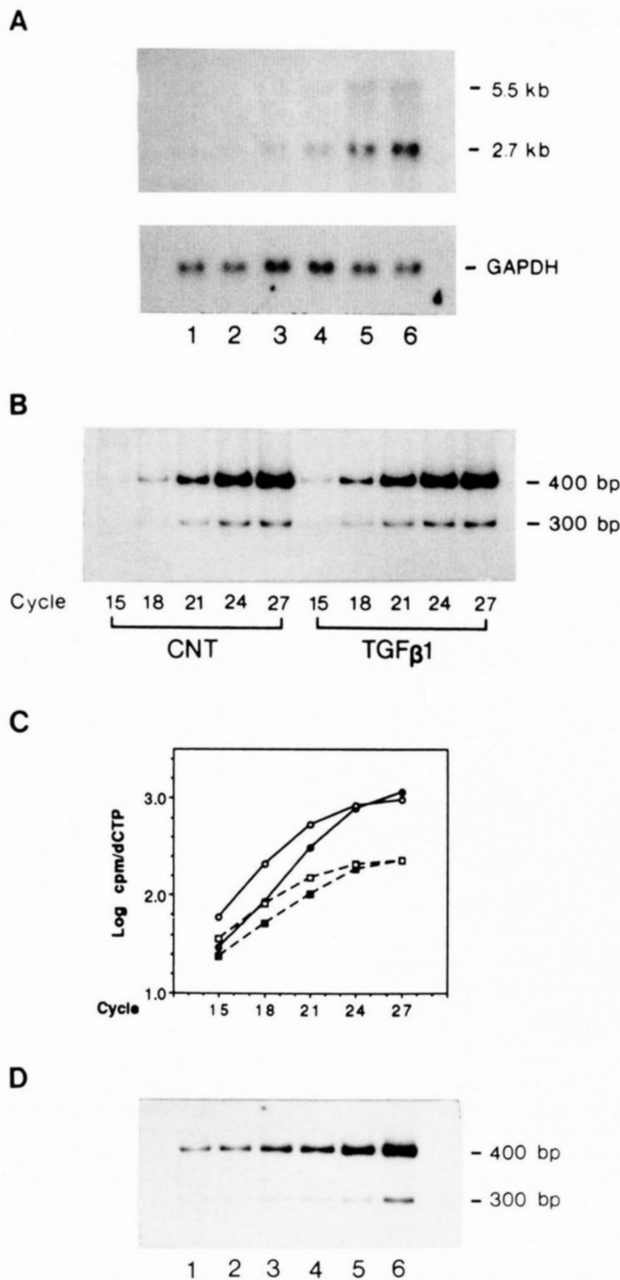


FIG. 5. Northern hybridizations and PCR analysis of mRNA from normal human lung fibroblasts stimulated with TGF- β ₁. A, confluent normal human lung fibroblasts were incubated in MEM (1% FCS) with various concentrations of TGF- β ₁ for 24 h. Total RNA was isolated as described under "Materials and Methods" and 10- μ g aliquots were analyzed by Northern hybridizations with the HCO-T9 clone (specific activity, 1×10^8 cpm/ μ g) and a murine glyceraldehyde-3-phosphate dehydrogenase cDNA (specific activity, 1×10^8 cpm/ μ g). Lane 1, control; lanes 2-6, TGF- β ₁, 10, 50, 100, 250, and 500 pM, respectively. B, 8 μ g of total RNA from control and 500 pM TGF- β ₁-treated cells was reverse-transcribed and amplified by PCR. Equal aliquots taken at different cycles were electrophoresed in 5% acrylamide and processed as described under "Materials and Methods." An autoradiograph of a 15-min exposure of the gel is shown. C, the radioactivity from gel slices corresponding to the autoradiographic bands was corrected for the dCTP content of the templates, as described under "Materials and Methods," and their logarithms were plotted against the number of cycles. The *r* values were calculated from cycles 15-21. ●, 400-bp control (*r* = 0.9984); ■, 300-bp control (*r* = 0.9990); ○, 400-bp TGF- β ₁ (*r* = 0.9965); □, 300-bp TGF- β ₁ (*r* = 0.9533). D, 4 μ g of total RNA were reverse-transcribed and PCR-amplified for 20 cycles, and the samples were processed as described under "Materials and Methods." An autoradiograph of a 15-min exposure of the gel is shown. Lane 1, control; lanes 2-6, TGF- β ₁, 10, 50, 100, 250, and 500 pM, respectively.

amide as described under "Materials and Methods." The autoradiographs of the acrylamide gel showed a progressive amplification of the intact (400 bp) and spliced (300 bp) PGG/HS transcripts, with higher levels of both transcripts in TGF- β ₁-treated cells at all cycles of amplification examined (Fig. 5B). The relative extent of amplification of the intact and spliced transcripts showed a linear increase from cycles 15 to 21 (Fig. 5C). It was, therefore, possible to obtain an accurate estimate of the relative ratios of the 400-bp/300-bp PCR-amplified products at cycle 18, the midpoint of the linear phase of the reaction. This ratio increased from 1.67 in control cells to 2.58 in TGF- β ₁-treated cells. A similar analysis of cells treated with increasing concentrations of TGF- β ₁ showed a dose-dependent increase in the levels of the intact and spliced transcripts (Fig. 5D), with a more pronounced increase in the levels of the intact transcripts relative to those of the spliced transcripts (Table I).

TNF- α and IL-1 β caused a 1.6- and 2.3-fold increase in the steady-state levels of the 2.7-kb PGG/HS transcript, respectively, and a coordinated increase on the 5.5 kb band described above (Figs. 6A and 7A). The kinetics of PCR amplification of the reverse-transcribed mRNA showed a progressive increment of the amplified products of the intact and spliced transcripts as the number of cycles increased, with higher levels of both in TNF- α and IL-1 β -treated cells (Figs. 6B and 7B). The relative extent of amplification of the intact and the spliced transcripts showed linear increases from cycles 15 to 21 (Figs. 6C and 7C). The ratios of the 400-bp/300-bp PCR-amplified products at cycle 18 increased from 2.19 in control cells to 3.18 in TNF- α -treated cells and from 2.32 to 3.75 in IL-1 β -treated cells (Table I).

Treatment of fibroblasts with serum or PMA showed a time-dependent increase in the steady-state levels of the 2.7-kb PGG/HS transcript that reached 18- and 10-fold higher levels over control cells, respectively, (Fig. 8A). In addition, there was a coordinate increase in the expression of the 5.5-kb transcript and of a minor transcript of 4.4 kb. PCR analysis

TABLE I

Relative expression of intact and spliced PGG/HS transcripts in normal human lung fibroblasts under various treatments

Total RNA from normal human lung fibroblasts cultured under control conditions or under various treatments was reverse-transcribed and amplified by PCR in the presence of [α -³²P]dCTP, and the products were resolved in 5% acrylamide gels as described under "Materials and Methods." The radioactivity of the gel slices corresponding to the autoradiographic bands was divided by the cytidine content of the templates, and the ratios of the amplified cDNAs were calculated with the following equation. Ratio = (cpm in 400 bp/210)/(cpm in 300 bp/144).

Experiment	Condition	400-bp	300-bp	Ratio 400/300
1 ^a	Control	87.0	52.0	1.67
	TGF- β ₁ , 500 pM	211.1	81.6	2.58
2 ^b	Control	114.5	48.0	2.38
	TGF- β ₁ , 10 pM	150.7	59.2	2.54
	TGF- β ₁ , 50 pM	248.6	89.2	2.78
	TGF- β ₁ , 100 pM	274.8	89.0	3.08
	TGF- β ₁ , 250 pM	434.0	131.4	3.30
	TGF- β ₁ , 500 pM	834.9	266.3	3.13
3 ^a	Control	18.2	8.3	2.19
	TNF- α , 200 units/ml	83.8	26.3	3.18
4 ^a	Control	13.0	5.6	2.32
	IL-1 β , 50 units/ml	81.1	21.6	3.75
5 ^b	Control	29.2	24.8	1.17
	10% FCS, 2 h	41.5	27.5	1.50
	10% FCS, 4 h	96.3	50.3	1.91
	10% FCS, 6 h	145.7	70.2	2.07
	PMA, 0.1 μ M	129.1	58.8	2.19

^a Amplified for 18 cycles.

^b Amplified for 20 cycles.

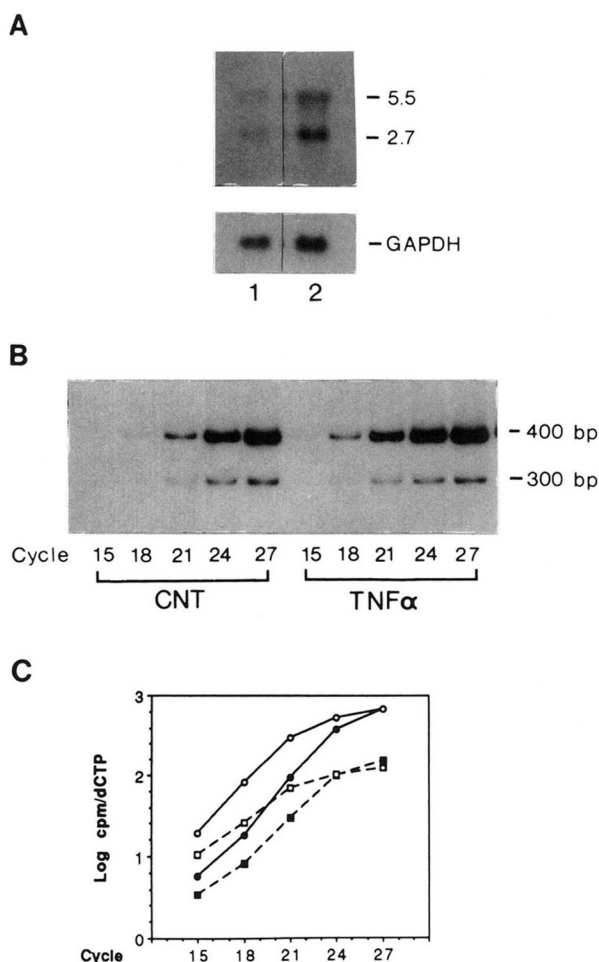


FIG. 6. Northern hybridizations and PCR analysis of mRNA from normal human lung fibroblasts stimulated with TNF- α . A, confluent normal human lung fibroblasts were incubated in MEM (5% FCS) with or without 200 units/ml TNF- α for 24 h. Total RNA was isolated as described under "Materials and Methods", and 5- μ g aliquots were analyzed by Northern hybridizations with the HCO-T9 clone (specific activity, 4.5×10^8 cpm/ μ g) and a murine glyceraldehyde-3-phosphate dehydrogenase cDNA (specific activity, 2.6×10^8 cpm/ μ g). Lane 1, control; lane 2, TNF- α . B, three μ g of total RNA were reverse-transcribed and PCR-amplified. Equal aliquot taken at a different cycle was electrophoresed on a 5% acrylamide gel and processed as described under "Materials and Methods." An autoradiograph of a 30-min exposure of the gel is shown. C, the radioactivity of gel slices corresponding to the autoradiographic bands was corrected for the dCTP content of the templates as described under "Materials and Methods", and their logarithms were plotted against the number of cycles. The r values were calculated from cycles 15–21. ●—●, 400-bp control ($r = 0.9940$); ■—■, 300-bp control ($r = 0.9930$); ○—○, 400-bp TNF- α ($r = 0.9930$); □—□, 300-bp TNF- α ($r = 9996$).

at 20 cycles showed a parallel increase in the intact and spliced transcripts (Fig. 8B). Estimation of the 400-bp/300-bp ratios in serum-deprived cells showed an increase from 1.17 to 2.07 and 2.1 following 6 h of serum stimulation and 4 h of PMA stimulation, respectively (Table I).

DISCUSSION

We have been studying the role of PG as regulators of various human fibroblast functions and their participation in the modulation of the effects of several cytokines on collagen production by these cells (2, 22). Cytokines such as IL-1 β and TNF- α cause inhibition of collagen production, whereas TGF- β_1 is a potent stimulant. All three cytokines also stimulate

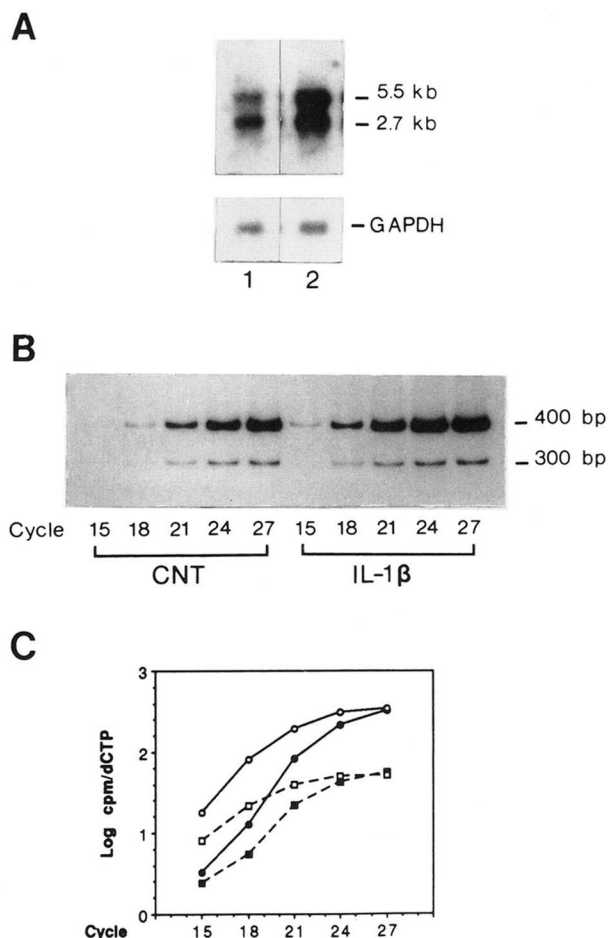


FIG. 7. Northern hybridizations and PCR analysis of mRNA from normal human lung fibroblasts stimulated with IL-1 β . A, confluent normal human lung fibroblasts were incubated in MEM (5% FCS) with and without 50 units/ml IL-1 β for 24 h. Total RNA was isolated as described under "Materials and Methods", and 5- μ g aliquots were analyzed by Northern hybridizations with the HCO-T9 clone (specific activity, 4.8×10^8 cpm/ μ g) and the murine glyceraldehyde-3-phosphate dehydrogenase cDNA (specific activity, 2.6×10^8 cpm/ μ g). Lane 1, control; lane 2, IL-1 β . B, three μ g of total RNA were reverse-transcribed and PCR-amplified. Equal aliquots taken at different cycles were electrophoresed on a 5% acrylamide gel and processed as described under "Materials and Methods." An autoradiograph of a 30-min exposure of the gel is shown. C, the radioactivity of gel slices corresponding to the autoradiographic bands was corrected for the dCTP content of the templates as described under "Materials and Methods", and their logarithms were plotted against the number of cycles. The r values were calculated from cycles 15 to 21. ●—●, 400-bp control ($r = 0.9963$); ■—■, 300-bp control ($r = 0.9891$); ○—○, 400-bp IL-1 β ($r = 0.9887$); □—□, 300-bp IL-1 β ($r = 9909$).

production of PG, which are known inhibitors of collagen production. These interactions suggest that PG play a central role in the modulation of extracellular matrix protein production, as well as in other biological processes. To explore the mechanisms responsible for the regulation of PG production in human cells under diverse conditions, we obtained a cDNA for the human PGG/HS, the rate-limiting enzyme in the metabolism of arachidonic acid to PG. The screening of a normal human lung fibroblast cDNA library with an ovine cDNA for PGG/HS resulted in the isolation of a human PGG/HS cDNA. Northern hybridizations of lung fibroblast mRNA with this cDNA showed strong hybridization to 2.7-kb PGG/HS transcripts and to additional transcripts of 5.5 and 4.4 kb that were coordinately expressed in culture cells

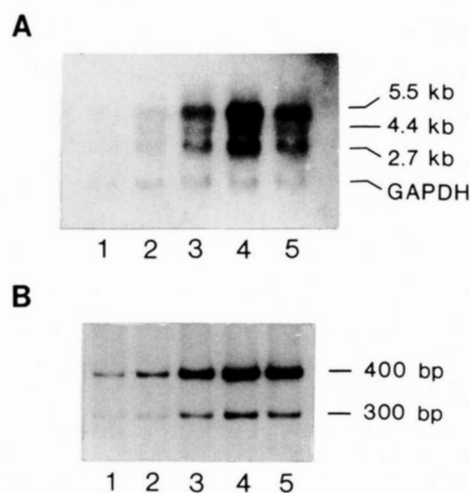


FIG. 8. Northern hybridizations and PCR analysis of mRNA from normal human lung fibroblasts stimulated with serum or PMA. A, confluent normal human lung fibroblasts were incubated for 3 h in serum-free MEM, and then the media were replaced with fresh MEM containing either 10% FCS or 0.1 μ M PMA and incubated for various intervals. Total RNA was isolated as described under "Materials and Methods," and 5- μ g aliquots were analyzed by Northern hybridizations with the HCO-T9 clone (specific activity, 2.6×10^8 cpm/ μ g) and a murine glyceraldehyde-3-phosphate dehydrogenase cDNA (specific activity, 2.5×10^8 cpm/ μ g). Lane 1, control; lane 2, FCS, 2 h; lane 3, FCS, 4 h; lane 4, FCS, 6 h; lane 5, PMA, 4 h. B, four μ g of total RNA were reverse-transcribed and amplified by PCR for 20 cycles. Equal aliquots of each reaction were electrophoresed on a 5% acrylamide gel and processed as described under "Materials and Methods." An autoradiograph of a 2-h exposure of the gel is shown. Lane 1, control; lane 2, FCS, 2 h; lane 3, FCS, 4 h; lane 4, FCS, 6 h; lane 5, PMA, 4 h.

under the treatments explored. Whether the 4.4- and 5.5-kb transcripts are from related genes or represent immature forms of PGG/HS mRNA remains to be determined. The presence of two pools of PGG/HS in human endothelial cells (40) and monocytes (41) and the identification of two antigenically distinct variants of PGG/HS localized to specific cell types in the rat ovary (42) suggest that the 5.5- and 4.4-kb transcripts may correspond to genes coding for different proteins with cyclooxygenase activity. The cloning of a murine PMA-induced 4-kb cDNA corresponding to a protein with a predicted amino acid sequence that is 80% homologous to PGG/HS supports this possibility (43).

The HCO-T9 clone we isolated is a 2.3-kb cDNA containing the entire translated coding region of the human PGG/HS, as compared with an ovine cDNA (7) and a human genomic clone (11), except that it lacked a fragment corresponding to the last 111 bp encoded by exon 9. This observation suggested an alternative splicing of PGG/HS transcripts. PCR amplification of reverse-transcribed mRNA and the presence of a consensus 5'-splice donor site at the area around position 1185 (33) confirmed this notion. Because the alternative splicing occurs in-frame, it is expected that it will be translated into an identical protein, except for the lack of residues 396-432.

The intact and spliced PGG/HS transcripts were found in mRNA extracted directly from tissues (synovium and cartilage) or from cultured cells (dermal, synovial, and lung fibroblasts), indicating that alternative splicing of PGG/HS mRNA is a naturally occurring phenomenon. Treatment of normal human lung fibroblasts with the inducers of PG production, TGF- β_1 , TNF- α , IL-1 β , serum, and PMA, increased the steady-state levels of PGG/HS mRNA. PCR amplification of RNA from stimulated cells showed that each of these

conditions increased the expression of the intact and spliced transcripts. However, all conditions caused a preferential stimulation of the intact transcripts (Table I).

PGG/HS is a membrane-bound glycosylated protein with bisdioxygenase and hydroperoxide activities that requires a heme group for each 70-kDa monomer for the expression of both catalytic activities. The bisdioxygenase and hydroperoxide activities reside on different domains of the protein (44, 45). It has been proposed that the region responsible for the bisdioxygenase activity is located in the stretch encompassing residues 476-546. This region is highly conserved among different species and contains the active site Ser⁵³⁰ (10). It has also been shown that the bisdioxygenase activity of the enzyme requires a tyrosine residue that has been identified by site-directed mutagenesis as Tyr³⁸⁵ (46). The hydroperoxide active site appears to reside around His²⁵⁰-Arg²⁵³ residues (47). It has been postulated that the His³⁰⁹ and His²²⁶ residues are the axial and distal heme ligand sites, respectively (10). Substitution of His³⁰⁹ by site-directed mutagenesis completely abrogated both enzymatic activities of PGG/HS (48). The alternative splicing of the PGG/HS transcripts from exon 9 demonstrated here spares all these critical regions and residues.

The human PGG/HS has four potential glycosylation sites located at residues Asn⁶⁷, Asn¹⁰³, Asn¹⁴³, and Asn⁴⁰⁹. These are considered to be involved in maintaining the conformation of the tertiary structure, as well as in anchoring the enzyme to the cell membrane. Site-directed mutagenesis has shown that substitution of the Asn⁴¹⁰ of the ovine enzyme completely abrogates its cyclooxygenase activity (48). The corresponding residue in the human enzyme (Asn⁴⁰⁹) is eliminated by the alternative splicing of PGG/HS transcripts from exon 9. The elimination of Asn⁴⁰⁹ is the most important alteration of the primary structure of the human enzyme that would result from the alternative splicing of the PGG/HS transcripts described here. The consequences of such alteration on the function of the translated protein are, at the moment, only speculative. However, the findings that the expression of nonspliced transcripts is preferentially stimulated by cytokines and growth factors known to increase PG production suggests that the alternative splicing of transcripts encoded by exon 9 may be a mechanism to limit the conversion of arachidonic acid to PG and thromboxanes at an early step of the biosynthetic pathway. Critical functional studies of the protein coded by the spliced transcripts are in progress.

Acknowledgments—We are indebted to Dr. David L. DeWitt for the kind gift of ovine PGG/HS cDNA. We thank Meredith Billman and Elaine Braddock for excellent assistance in the preparation of this manuscript.

Addendum—While this manuscript was being prepared, the sequence of a human platelet/erythrocyte cell PGG/HS cDNA was published (49).

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