INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Université d'Ottawa · University of Ottawa

Characterization of a Novel Prostaglandin Endoperoxide H Synthase-1 Transcript and Examination of Prostaglandin Endoperoxide H Synthase-1 Expression

by

Matthew Hilton Plant

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Biochemistry, Microbiology and Immunology
University of Ottawa

ABSTRACT

Characterization of a Novel Prostaglandin Endoperoxide H Synthase-1 Transcript and Examination of Prostaglandin Endoperoxide H Synthase-1 Expression

Matthew Hilton Plant

Graduate Department Biochemistry, Microbiology and Immunology, University of Ottawa

The prostaglandin endoperoxide H synthase (PGHS) enzyme plays a pivotal role in the prostanoid biosynthetic pathway because it catalyzes the formation of prostaglandin H_2 (PGH_2), the common precursor for prostanoids. The object of my research is to characterize a novel PGHS transcript and to examine the mechanisms that regulate PGHS-1 gene expression.

By Northern blotting with the entire hPGHS-1 coding region, 2.8 kb 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 and defined it as a PGHS-1 product. In order to determine the origin of the 4.5 kb PGHS-1 transcript, the remaining 947 bp of the 5.1 kb PGHS-1 transcript was cloned and sequenced. A non-canonical polyadenylation signal (AAGAAA) and a cleavage site (CA) were found and could generate the 4.5 kb PGHS-1 transcript. A commercial RNA dot blot with 50 different human tissues, showed a strong signal for the 4.5 kb PGHS-1 transcript in the bladder and appendix. Subsequent hybridization of a multiple tissue Northern blot detected 2.8, 4.5 and 5.1 kb transcripts in the bladder, indicating the 4.5 kb PGHS-1 transcript is not an artifact of the MEG-01 cell line.

Treatment of MEG-01 cells with the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) elevated PGHS-1 gene expression. The pattern of PGHS-1 mRNA and protein expression in MEG-01 cells after TPA treatment did not match. After a single treatment with TPA, PGHS-1 mRNA levels peaked after 24 hrs and remained elevated for 8 days, whereas PGHS-1 protein which was capable of converting arachidonic acid into thromboxane A_2, did not reach peak levels until after 6 to 8 days. Nuclear run-on experiments provided no interpretable results because of low levels of message. From our results, increased mRNA stability could not be determined because control and TPA treated PGHS-1 mRNA levels remained elevated for at least 8 hrs after treatment with inhibitors of transcription (Actinomycin-D and 5,6-Dichlorobenzimidazole Riboside). The effect of TPA treatment on PGHS-1 mRNA levels disappeared after treatment with the protein synthesis inhibitor cycloheximide, indicating the increases in PGHS-1 mRNA levels involves *de novo* protein synthesis.

ACKNOWLEDGMENTS

First and foremost I would like to thank my wife and best friend Violeta for her guidance, support and love for the past two years. Without her encouragement I was able to keep focused and complete my studies. My parents have supported and stood up for me all my life, therefore, I would like to say thanks and this degree is for you. Three years ago I received a second set of parents and from the start that is what they have been. I would like to thank them for opening their home to me for the past two years and treating me like a son. Finally I would like to thank my two brothers and sister for always being there.

Thanks Everyone: Violeta, David and Sharon Plant, David and Rose Yu,
Andrew and Christopher Plant and Vivian Yu.

ACKNOWLEDGMENTS

I would like to thank Dr. Odette Laneuville for allowing me to be part of her research group for the past two years. Odette's supervision and friendship were an integral part of the completion of this work. This work was made possible by financial support provided to Dr. Laneuville, by the Medical Research Council (Grant # MT-14131).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

15-HPETE - 15 (*R*)-hydroperoxy-eicosatetraenoic acid

3'UTR - 3'-untranslated region

5'UTR - 5'-untranslated region

AA - arachidonic acid

AAGAAA - non-canonical polyadenylation signal

AAUAAA - canonical polyadenylation signal

Act-D - actinomycin-D

AMP - adenosine monophosphate

ATCC - american type culture collection

AUBPs - AURE-binding proteins

AUG - initiator codon

AUREs - Adenoine-Uridine rich elements

AUUUA - Shaw-Kamen instability motifs

BFU-Mk - megakaryocyte burst forming cell

BRI - University of Ottawa Biological Research Institute

cAMP - cyclic adenosine monophosphate

cDNA - complementary deoxyribonucleic acid

CFC-Mk - megakaryocyte colony-forming-cell

CFC-Mk-HPP - colony-forming cell-megakaryocyte-high-proliferative-potential

CHX - cycloheximide

CIAP - calf intestinal alkaline phosphatase

COX - cyclooxygenase

CPM - counts per minute

CPSF - cleavage and polyadenylation specificity factor

CSF - colony stimulating factor

CstF - cleavage stimulation factor

DAG - diacylglycerol

dATP - deoxy adenosine triphosphate

dCTP - deoxy cytidine triphosphate

DEPC - diethylpyrocarbonate

dGTP - deoxy guanosine triphosphate

DMSO - dimethylsulfoxide

DNA - deoxyribonucleic acid

DRB - 5,6-dichloro-1-B-D-ribofuranosylbenzimidazole

DSE - downstream elements

dTTP - deoxy thymidine triphosphate

dUTP - deoxy uridine triphosphate

ECL - enhanced chemiluminescence

ER - endoplasmic reticulum

FBS - fetal bovine serum

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GM-CSF - granulocyte macrophage-colony stimulating factor

GPIIb/IIIa - glycoprotein complex IIb/IIIa

hCG - human chorionic gonadotrophin

IE - immediate early gene

IL-11 - interleukin-11

IL-1α - interleukin-1α

IL-1β - interleukin-1β

IL-3 - interleukin-3

IL-6 - interleukin-6

IPTG - isopropylthiogalactoside

IRE - iron-responsive element

IRP - iron-regulatory protein

kb - kilo base pair

kDa - kilodalton

LB - Luria-Bertani broth

LOX - lipoxygenase

LPS - lipopolyssacharide

MEG-01 - human megakaryoblastic cell line

Mk-CSF - megakaryoctye-colony stimulating factor

MMLV-RT - Moloney Murine Leukemia Reverse Transcriptase

mRNA - messenger ribonucleic acid

NE - nuclear envelope

NSAIDs - non-steroidal anti-inflammatory drugs

OD - optical density

PAB II - poly (A)-binding protein II

PAGE - polyacrylamide gel electrophoresis

PAP - poly (A) Polymerase

pBS - pBluescript vector

PCR - polymerase chain reaction

PGD_2 - prostaglandin D_2

PGE_2 - prostaglandin E_2

$PGF_{2\alpha}$- prostaglandin $F_{2\alpha}$

PGG_2 - prostaglandin G_2

PGH_2 - prostaglandin H_2

PGHS - prostaglandin Endoperoxide H Synthase

PGHS-1 - prostaglandin endoperoxide H synthase-1

PGHS-2 - prostaglandin endoperoxide H synthase-2

PGI_2 – prostacyclin

PLA_2 - phospholipase A_2

PLC - phospholipase C

PmkB - promegakaryoblasts

PPAR - peroxisome proliferator activated receptor

RACE - rapid amplification of cDNA ends

RE - restriction endonuclease

RNA - ribonucleic acid

RT-PCR - reverse transcriptase-polymerase chain reaction

THP-1 - human monocyte cell line

T_m - DNA melting temperature

TPA - 12-0-tetradecanoylphorbol-13-acetate

TXA_2 – thromboxane A_2

TXB_2 - Thromboxane B_2

U937 - human monocytic cell line

UCDEN - ubiquitin-conjugate degrading enzyme

SECTION ONE: INTRODUCTION

1.1 RATIONALE, OVERALL GOAL AND EXPERIMENTAL OBJECTIVES

Prostaglandins, prostacyclins, thromboxanes and leukotrienes (collectively known as the eicosanoids) are oxygenated fatty acids produced enzymatically from the 20 carbon fatty acid, arachidonic acid. Prostanoids (prostaglandins and thromboxanes) are produced by most mammalian tissues from arachidonic acid by the enzyme Prostaglandin Endoperoxide H Synthase (PGHS), also known as cyclooxygenase (COX). Prostanoids have profound physiological effects at extremely low concentrations. For example, they mediate: (1) The inflammatory response; (2) The regulation of renal blood flow; (3) Cyto-protection of the gastric mucosa and (4) Platelet aggregation.

Studies have shown that there are two types of PGHS, denoted PGHS-1 and PGHS-2. Each isoform plays a different role by the type of prostanoids they produce. PGHS-1, the constitutive form is present in most tissues and produces prostanoids for normal body functions, such as stomach mucus production and platelet formation. In contrast, PGHS-2, is induced for its most important role of producing prostaglandins during an inflammatory response.

Recent data indicates that low-dose aspirin diminishes the incidence of cardiovascular disease. The basis for the anti-thrombogenic activity of aspirin is the irreversible inhibition of platelet PGHS-1, suggesting that this enzyme plays a central role in the pathology of cardiovascular diseases. Platelets represent an ideal system to study PGHS-1 expression because they are cells that represent one of the richest sources of PGHS-1 enzyme in the body. The regulation of PGHS-1 expression in platelets is not fully understood at the moment. Progress in our understanding of PGHS-1 regulation in platelets has been hampered because of the lack of a good model to study PGHS-1 expression. As

a model to investigate the regulation of PGHS-1 gene expression, the immortalized human megakaryoblastic cell line MEG-01, a platelet precursor was studied. Therefore, our objective was to determine the mechanism by which PGHS-1 expression is regulated in platelets. To accomplish this, we measured both transcriptional and post-transcriptional activation of the PGHS-1 gene in MEG-01 cells induced to differentiate into megakaryocytes and platelet-like structures after TPA addition. As well, we characterized a novel 4.5 kb PGHS transcript, detected in MEG-01 cells.

1.2 IMPORTANCE OF PROSTANOIDS IN PHYSIOLOGICAL FUNCTIONS

Most mammalian tissues produce prostaglandins, prostacyclins, thromboxanes and leukotrienes. The eicosanoids have important local mediator effects at low concentrations. For example, they regulate: (1) The inflammatory response, in the joints, skin and eyes; (2) The production of pain and fever; (3) The regulation of blood flow ; (4) The induction of platelet aggregation and (5) The control of reproductive functions such as the induction of labor (32). In humans, the precursor to eicosanoids is arachidonic acid, a C_{20} polyunsaturated fatty acid that has four unsaturated double bonds.

1.2.1 Arachidonic acid (5,8,11,14 Eicosatetraenoic acid)

The substrate for eicosanoid production, arachidonic acid (5,8,11,14 eicosatetraenoic acid) is found esterfied in membrane phospholipids and triglycerides in all mammalian tissues. In this form, arachidonic acid serves to sustain membrane fluidity and to act as a substrate storage for eicosanoid

production (1). Because there are no stores of eicosanoids, metabolism of arachidonic acid to eicosanoids depends on the release of free arachidonic acid from cell membranes.

1.2.2 Arachidonic acid release from membranes

The interaction of a hormone with its specific receptor can act as an important step in the release of arachidonic acid from cell membranes, which is followed by the production of eicosanoids (32). For example, thrombin acting on blood platelets can stimulate the release of arachidonic acid from their cellular membranes (2). The production of arachidonic acid metabolites is controlled by the release of arachidonic acid from the sn-2 position of membrane phospholipids by two alternative receptor mediated pathways.

The first pathway is the direct action of Phospholipase A_2 (PLA_2), which hydrolyzes the ester linkage at the sn-2 position of the phospholipid (3,4). Multiple isoforms of PLA_2 have been identified in human tissues, the low-molecular weight (14 kDa) types I and II referred to as secretory or $sPLA_2$ and the high-molecular weight (85 kDa) cytosolic or $cPLA_2$ (39,211). One of the most likely forms of PLA_2 involved in the production of eicosanoids is $cPLA_2$ because it becomes reversibly associated with the cell membrane fraction after Ca^{+2} -dependent activation (5,6). As well, many of the membrane associated forms appear to be the type II $sPLA_2$, such as the liver mitochondrial PLA_2 (7).

The second pathway is the sequential action of Phospholipase C (PLC), which hydrolyzes the phosphatidylinositol head group of phospholipids to produce diacylglycerol (DAG)(8,9). A diacylglycerol lipase can then hydrolyze the DAG to release arachidonic acid (7). Phospholipase C appears to be activated following treatment with irritating agents such as tumor promoters (10).

The levels of free arachidonic acid are normally very low since the liberated fatty acid is rapidly metabolized. Oxygenation of free arachidonic acid can proceed along one of three distinctively different pathways: (1) The Prostaglandin Endoperoxide H Synthase pathway which leads to the production of prostaglandins, prostacyclins and thromboxanes; (2) The Lipoxygenase pathway which leads to the production of leukotrienes, lipoxilins and hepoxilins and (3) The Cytochrome P-450 expoxygenase pathway which produces epoxides and diols (11).

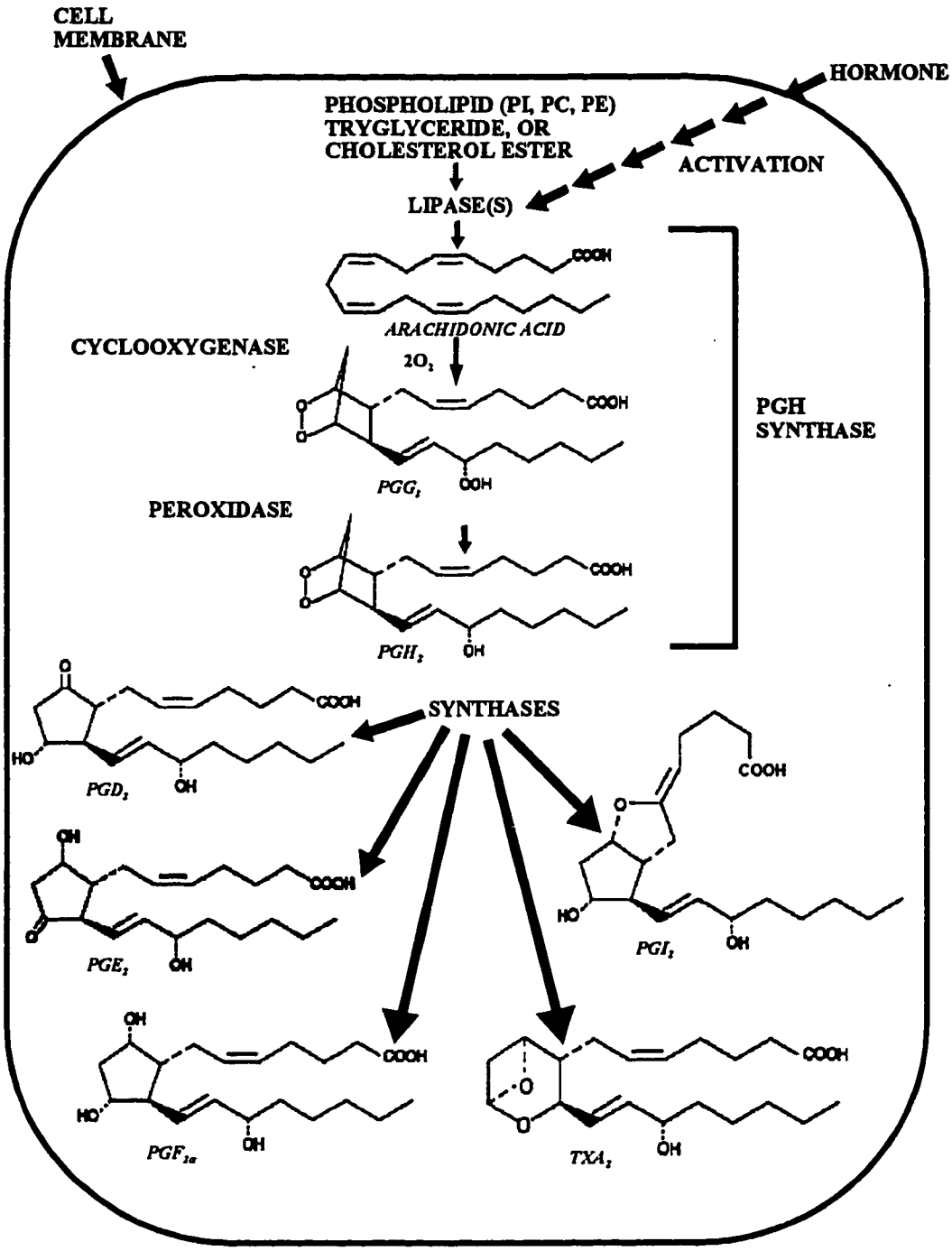
1.2.3 Prostaglandin endoperoxide H synthase pathway

The production of prostanoids such as thromboxane A_2, which is involved in platelet aggregation are produced from arachidonic acid via the Prostaglandin Endoperoxide H Synthase (PGHS) pathway (32). The biosynthetic pathway of prostanoid synthesis is illustrated in Figure 1.1. The PGHS enzyme contains two active sites, a cyclooxygenase moiety and a endoperoxide moiety. The cyclooxygenase moiety catalyzes a *bis*-oxygenation (incorporation of two atoms of oxygen) of the arachidonic acid chain to form the hydroperoxy endoperoxide Prostaglandin G_2 (PGG_2) and the endoperoxide moiety reduces PGG_2 to the hydroxy endoperoxide Prostaglandin H_2 (PGH_2) (32).

1.2.4 Metabolism of prostaglandin H_2

The intermediate PGH_2 generated by PGHS is converted to either prostaglandins (PGD_2, PGE_2 and $PGF_{2\alpha}$), prostacyclin (PGI_2) or thromboxane (TXA_2) by the action of a set of isomerases and synthases called PGH-PGD isomerase, PGH-PGE isomerase, PGF synthase, PGI synthase and

Figure 1.1 **Pathways for the cellular biosynthesis of prostanoids.** (Adapted from Smith, W.L., *Am. J. Physiol.*, 263:F181-F191, 1992.)


CELL MEMBRANE
HORMONE
PHOSPHOLIPID (PI, PC, PE) TRYGLYCERIDE, OR CHOLESTEROL ESTER
ACTIVATION
LIPASE(S)
ARACHIDONIC ACID
CYCLOOXYGENASE
$2O_2$
PGH SYNTHASE
PGG_2
PEROXIDASE
PGH_2
SYNTHASES
PGD_2
PGE_2
PGI_2
$PGF_{2\alpha}$
TXA_2

TX synthase (12). Differentiated cells tend to produce only one of the prostanoid products because cells usually contain a single PGH_2 metabolizing enzyme. In the following paragraphs, information regarding the synthesis and action of each of these prostanoid compounds will be briefly discussed.

Prostaglandin D_2 (PGD_2) can be formed by a disproportionation of PGH_2 by both reduced glutathione (GSH)-dependent and GSH-independent PGD synthase mechanisms (13,14). The major prostaglandin that participates in the inflammatory response in the skin is PGD_2 (215). Although platelets produce only small amounts of PGD_2 when compared to Thromboxane A_2 (TXA_2), PGD_2 is a potent inhibitor of platelet aggregation (15,16,17). PGD_2 is also shown to be involved in bronchoconstriction (18) and is the major prostaglandin produced in mast cells (19).

Prostaglandin E synthase (also known as PGH-PGE isomerase or PGE synthase) catalyzes Prostaglandin E_2 (PGE_2) formation from PGH_2. PGE synthesis appears to involve a GSH-dependent mechanism of catalysis (20,21). Although GSH is required for PGE_2 synthesis, it is not consumed during the synthesis reaction (22). PGE_2 increases vascular permeability which leads to swelling after injection into the skin (23). It is not clear whether this increased permeability is due to direct action on the vasculature or release of vasoactive substances (23).

Two biological forms of the prostaglandin F series ($PGF_{2\alpha}$ and 9α, 11β-PGF_2) have been shown in biological systems (24,25,26,27). The PGF analogs can be produced in three separate ways: (1) PGH_2 can be reduced by an endoperoxide reductase to form $PGF_{2\alpha}$; (2) PGD_2 can be reduced by a 11-keto-reductase to form 9α, 11β-PGF_2 and (3) PGE_2 can be reduced by a 9-keto-reductase to form $PGF_{2\alpha}$ (28,29). $PGF_{2\alpha}$ is one of the major prostanoids formed in vascular endothelial cells (30) and has been shown to enhance the effects of colony stimulating factor (CSF) on bone marrow colony growth (31).

The formation of Prostaglandin I_2 (PGI_2) (also known as prostacyclin) by PGI synthase, involves the acid-catalyzed heterocyclic cleavage of the endoperoxide group of PGH_2 followed by reaction with a transiently positive oxygen at C-6 (32). PGI_2 synthesis occurs in all smooth muscles and virtually all vascular endothelial cells (2,33). Platelet-derived PGH_2 can result in the formation of PGI_2 in vascular endothelial cells, where it is a potent platelet aggregation inhibitor and vasodilator (33). The platelet/vascular wall interaction involving PGI_2 and TXA_2 is one of the best defined roles of prostanoid function (33).

During the study of arachidonic acid metabolism by human platelets, thromboxanes were discovered (34). Thromboxane A synthase catalyzes the conversion of PGH_2 to Thromboxane A_2 (TXA_2) by the transient formation of an electropositive oxygen at C-11 and subsequent cleavage of the 9,11-peroxido group (35). TXA_2 is a potent platelet aggregatory substance, vasoconstrictor and thrombogenic agent (33). Because TXA_2 is exceedingly unstable its presence is usually detected by its biologically inactive hydrolysis product Thromboxane B_2 (TXB_2) (32).

1.3 PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE

The transient metabolites of arachidonic acid metabolism (eicosanoids) are of great importance in the modulation of numerous biological responses, such as vascular homeostasis, induction of labor and inflammatory responses (36). The major rate limiting enzyme in prostanoid formation is prostaglandin endoperoxide H synthase (PGHS). The reaction catalyzed by PGHS includes the *bis*-oxygenation of arachidonic acid to form prostaglandin G_2 (PGG_2) and the reduction of PGG_2 into prostaglandin H_2 (PGH_2). The two enzymatic activities of PGHS; the

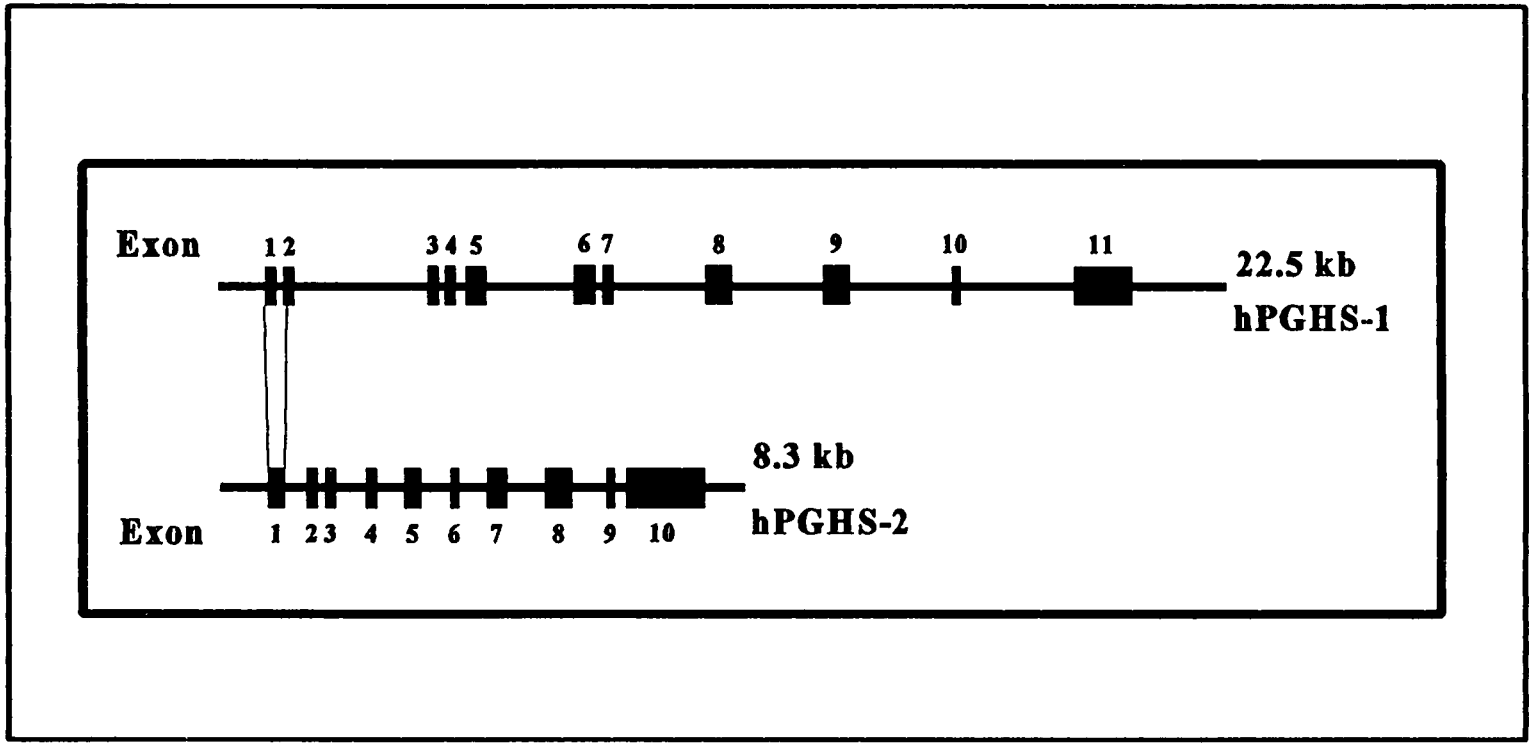
cyclooxygenase activity and the peroxidase activity, have been mapped to distinct sites on the protein (37).

Research prior to 1991 in the arachidonic acid metabolism field described only one PGHS enzyme, the isoform now referred to as prostaglandin endoperoxide H synthase-1 (PGHS-1) or the constitutive form of the enzyme. In 1989 mRNAs were reported whose expression was activated in both mouse and chicken fibroblasts after treatment with either phorbol ester or *src* (38). The deduced amino acid sequence of this mRNA contained 60% amino acid sequence identity with the PGHS enzyme and was called prostaglandin endoperoxide H synthase-2 (PGHS-2) or the inducible form of the enzyme. Recent studies have shown that although the PGHS-2 enzyme is very similar in structure to the PGHS-1 form, they are produced from two separate genes and are very different in their expression patterns (39,40,41,42).

1.3.1 Regulation of PGHS-1 and PGHS-2 gene expression

To fully understand the differences in expression patterns of PGHS-1 and PGHS-2 enzymes, it is important to review the characteristic differences of the two genes. The genomic structures for both PGHS-1 and PGHS-2 are illustrated in Figure 1.2. The gene for PGHS-1 is located on chromosome 9 (9q32-q33.3), spans 22.5kb and contains 11 exons and 10 introns (43). Whereas, the gene for PGHS-2 is located on chromosome 1 (1q25.5-q25.3), spans 8.3 kb and contains 10 exons and 9 introns (44). The intron/exon boundaries are identical except exons 1 and 2 from the PGHS-1 gene (which contains the translational start site and signal peptide) are integrated into one exon in the PGHS-2 gene (43,44). Although the intron/exon boundaries are virtually identical between PGHS-1

Figure 1.2 **Human Prostaglandin Endoperoxide H Synthase 1 and 2 genomic structure.** Adapted from Williams, C.S., and Dubois, R.N. 1996. *Am. J. Physiol.* 270:G393-400. (Map not drawn to scale).


Exon
1 2
3 4 5
6 7
8
9
10
11
22.5 kb
hPGHS-1
8.3 kb
hPGHS-2
Exon
1 2 3
4
5
6
7
8
9
10

and PGHS-2, there is a large difference in the size of the two genes. The decreased size of PGHS-2 compared to PGHS-1 is essentially related to the reduction in the size of the introns of PGHS-2 (45).

The major differences between the two PGHS isoforms is their dissimilar regulation of expression. PGHS-1 appears to be an important housekeeping enzyme since it is constitutively expressed in many tissues and performs various functions, such as cyto-protection of the gastric mucosa, regulation of renal blood flow and platelet aggregation (46). However, higher levels of PGHS-1 expression are detected in specialized cells such as platelets and endothelial cells. As well, studies have shown systems that model developmental events have demonstrated changes in PGHS-1 expression, with the most notable example being phorbol ester TPA induced differentiation of monocytes (THP-1 cells) to a macrophage phenotype (47). This induction of PGHS-1 appears to be associated with differentiation, rather than activation of cell growth. These observations suggest that basal levels of PGHS-1 expression may differ as cells move through differentiation pathways, and may be altered as a consequence of the molecular response to agents that modulate cellular phenotypes (such as TPA in THP-1 cells). Although, studies have shown that reporter constructs containing regions of the PGHS-1 5'UTR can stimulate conservative amounts of transcription (78,79). In contrast, PGHS-2 protein and mRNA are not present in resting unstimulated tissues, but can be rapidly induced in many cell types (macrophages, monocytes, fibroblasts) by tumor promoters and cytokines because PGHS-2 is associated with the inflammatory process (36). Nuclear run-on experiments demonstrate that induction of PGHS-2 mRNA accumulation occurs, at least in part, as a consequence of increased transcription of the PGHS-2 gene (48,49).

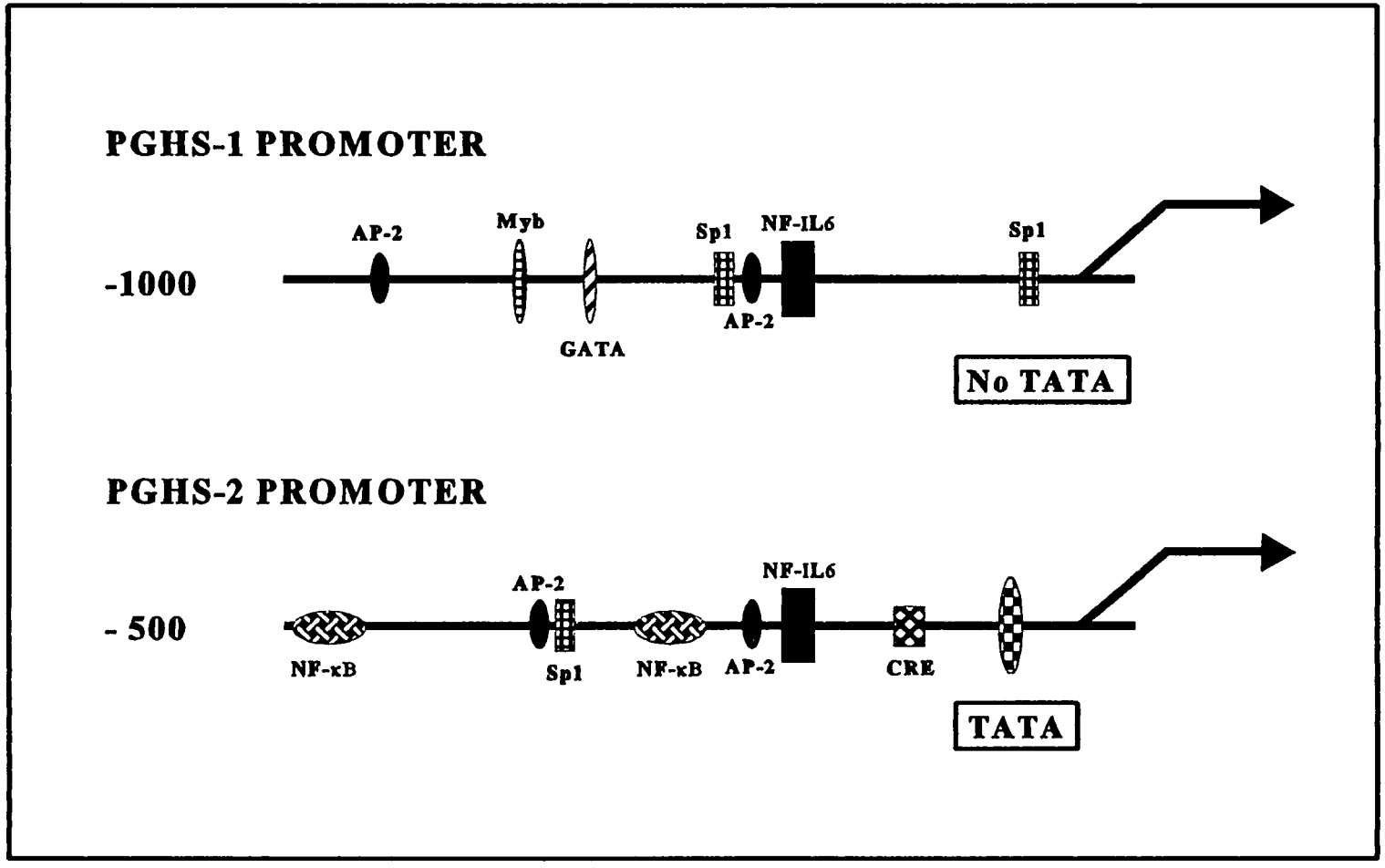
The promoters of the two PGHS genes are indicative of their mode of regulation. The structures of the PGHS-1 and PGHS-2 promoters are illustrated in Figure 1.3. PGHS-1 has a TATA-

less promoter and is GC rich, a feature common to many housekeeping genes. Although the PGHS-1 promoter contains putative binding sites for AP2, SP1, NF-IL6 and GATA-1enhancer elements, the promoter has not shown any significant inducible transcription (43,50). Whereas, the PGHS-2 promoter contains a TATA box and is highly regulatable, which is demonstrated by the number of transcription factor binding sites that are common to highly regulated sequences, such AP-2, CEBP/NFIL-6 and NFκB (36). Because the PGHS-2 gene is upregulated during an inflammatory response and after cytokine, phorbol ester, hormone and bacterial endotoxin treatment, it is not surprising that the enhancer elements contained in the PGHS-2 promoter are known to be upregulated during an inflammatory response (213).

1.3.2 PGHS-1 and PGHS-2 transcripts

The hPGHS-1 and hPGHS-2 open reading frames are both 1.8 kb in length and are 73% homologous at the nucleotide level. PGHS-1 is encoded by transcripts of 2.8 and 5.1 kb (51). The 2.8 kb transcript appears to be derived from the 5.1 kb transcript by alternative polyadenylation at a second canonical polyadenylation site (51). Whereas, PGHS-2 is encoded by transcripts of 2.8 and 4.5 kb, which have 3'UTRs that are AU rich (indicative of inducible genes) (51). The 2.8 kb PGHS-2 mRNA is also derived by alternative polyadenylation from the longer PGHS-2 4.5 kb mRNA (52). Studies have shown that the alternative polyadenylated PGHS-2 transcripts show differential stability in response to agents such as dexamethasone and interleukin-1β (200,201). It has been shown that

Figure 1.3 **Representation of PGHS-1 and PGHS-2 Promoters with the transcription factor binding sites.** Adapted from Crofford, L.J. 1997. *The Journal of Rheumatology.* Vol 24, Sup 49. (Map not drawn to scale).


PGHS-1 PROMOTER
-1000
AP-2
Myb
GATA
Sp1
NF-IL6
AP-2
Sp1
No TATA
PGHS-2 PROMOTER
- 500
NF-κB
AP-2
Sp1
NF-κB
NF-IL6
AP-2
CRE
TATA

the differential stabilities displayed by the two PGHS-2 transcripts are related the number of the Shaw-Kamen instability motifs (AUUUA) contained in their 3'UTRs (53). The full length 4.5 kb PGHS-2 mRNA contains 22 copies and the 2.8 kb transcript contains 7 copies of the AUUUA instability motif (53). The PGHS-2 gene is classified as an immediate early gene, which means that alteration of its gene transcription rate is key to its expression pattern, as well, the differential stabilities displayed by the two transcripts indicates that mRNA stability may also play a potential role in its expression. Therefore, it appears that there is a functional role for both transcriptional and post-transcriptional mechanisms in PGHS-2 expression. The PGHS-1 gene contains only one AUUUA instability motif in the known 3'UTR sequence of both transcripts, therefore, the PGHS-1 transcripts are known to be much more stable than the PGHS-2 mRNA in cultured cells (51). It has not been shown if regulation of mRNA stability is important for PGHS-1 gene expression. Because of the low levels of transcriptional activation reported for the PGHS-1 gene, it has been suggested that post-transcriptional regulation in the 3'UTR may play an important role in PGHS-1 expression (54,55). Therefore, it appears that sequences contained in the 3'UTRs of the PGHS-1 and PGHS-2 transcripts may contribute significantly to the different regulation patterns between the two genes.

1.3.3 PGHS-1 and PGHS-2 proteins and reactions

Despite the obvious differences in PGHS-1 and PGHS-2 gene structure and regulation, the two distinct PGHS isoforms share 60 % identity at the amino acid level and both catalyze the same enzymatic function.(36). Both PGHS enzymes catalyze a cyclooxygenase reaction (conversion of arachidonic acid to PGG_2) and a peroxidase reaction (conversion of PGG_2 to PGH_2). PGHS-1 and

PGHS-2 are able to perform the same enzymatic reactions because the key residues involved in catalysis are conserved between the two isozymes and the crystalized structures of the two isoforms are remarkably similar (56,57,58,75). The PGHS-1 mRNA encodes a 599-residue, 70 kDa protein and the PGHS-2 mRNA encodes a 604-residue, 70 kDa protein. Both of the PGHS isozymes form tyrosyl radicals (59), undergo suicide inactivation (57,58,60) and are inhibited by Non-steroidal anti-inflammatory drugs (NSAIDs). Although they differ in their sensitivities towards NSAIDs (60). Two distinct differences between the PGHS-1 and PGHS-2 peptide sequences have been reported. The PGHS-2 enzyme contains a cleaved signal sequence (61,212), instead of the large hydrophobic signal peptide of the PGHS-1 enzyme (43,53,63,64,65,66,67,68). The second major difference between the two peptides is PGHS-2 contains an 18 amino acid cassette near its carboxy terminus that is absent in the PGHS-1 enzyme.

After the amino acid sequence of ovine PGHS-1 was determined, four consensus sequences for N-glycosylation (Asn-X-Ser/Thr) were located at Asn^{68}, Asn^{104}, Asn^{144} and Asn^{410} (63,64,65), which are conserved in the human and murine PGHS-1 enzyme sequences (43,68). From the results obtained, it appears that N-glycosylation of the PGHS-1 enzyme at Asn^{68}, Asn^{144} and Asn^{410} is required for the enzyme to attain its native conformation and confer enzyme activity (69). It has been reported that three (Asn^{68}, Asn^{144} and Asn^{410}) of the four N-glycosylation sites of PGHS-1 are conserved and glycosylated in PGHS-2 (Asn^{53}, Asn^{130} and Asn^{396} in the murine PGHS-2 molecule) (69). Two additional N-glycosylation sites in the PGHS-2 sequence (contained within the carboxy 18 amino acid cassette) at Asn^{580} and Asn^{592} have been found and it was determined that the site at Asn^{580} is used (69). The N-glycosylation site at Asn^{580} is utilized in about 50 % of the PGHS-2 molecules, which results in a doublet molecule (72 and 74 kDa) on SDS-PAGE. Enzyme activity

assays proved that glycosylation of both PGHS-1 and PGHS-2 are required for catalysis, but glycosylation of Asn^{580} in the PGHS-2 molecule is not required for activity (69).

1.3.4 PGHS-1 and PGHS-2 intracellular locations

Although the clear conservation of structure and function between PGHS-1 and PGHS-2 has been observed, evidence indicates that PGHS-1 and PGHS-2 may function as separate intracellular enzyme systems. Studies using immunocytochemical and histochemical techniques have shown that PGHS-1 and PGHS-2 isozymes are both present on the lumenal surface of the endoplasmic reticulum and the outer membrane of the nuclear envelope (NE) (70). Despite the fact that both PGHS-1 and PGHS-2 are located in the same areas, evidence indicates that the PGHS-2 enzyme is more concentrated than PGHS-1 in the nuclear envelope (70). Because the different isoforms are contained in the ER and NE at different concentrations, this may lead to separate enzyme systems within the cell that produce prostanoids for paracrine and autocrine functions. Prostanoids produced at the ER by PGHS-1 can act as paracrine mediators, for example, PGI_2 produced by vascular endothelial cells can act on platelets as a platelet aggregation inhibitor (33). Whereas, prostanoids produced by PGHS-2 at the NR can act as autocrine mediators by transducing signals through nuclear prostaglandin receptors, such as the peroxisome proliferator activated receptor (PPAR) class (220). More evidence for the two separate systems is that PGHS-1 and PGHS-2 isoforms in murine 3T3 cells and macrophages utilize separate arachidonic acid pools for prostanoid formation (71). Studies have shown that PGHS-1 in RAW.264.7 macrophage cells was reported to be unable to convert endogenous arachidonic acid that was released in response to LPS stimulation, but was able to

convert exogenously supplied arachidonic acid to PGE_2. As well, mast cells derived from murine bone marrow are linked to different stimulus-initiated pathways (72). The consequences of the separate PGHS-1 and PGHS-2 systems, could occur by directly affecting ongoing processes located at the two separate intracellular locations. For example, recent evidence for eicosanoid nuclear receptors that can act as transcription factors means that the greater concentration of the highly regulatable PGHS-2 at the nuclear membrane can provide prostaglandins that interact with the nuclear receptors and possibly alter related gene expression (73,74).

1.3.5 Action of NSAIDs on PGHS-1 and PGHS-2 enzymes

A number of amino acids have been identified in the catalysis reaction of PGHS-1; Tyr^{385} is located in the cyclooxygenase active site and is oxidized to produce a tyrosyl radical that abstracts the (13*S*)-hydrogen from arachidonic acid, Arg^{120} is in the active site and serves as a counterion for the carboxylate groups of arachidonic acid and common NSAIDs. Finally Tyr^{355} lies on the opposite side of the active site from Arg^{120} and administers the stereospecificity of PGHS-1 to NSAIDs (75). Each of the important amino acids in the active site of PGHS-1 are conserved in the active site of PGHS-2 (39). The conservation of important amino acids in the active sites of PGHS-1 and PGHS-2 implies that the enzyme reactions catalyzed by the two isozymes should be fundamentally the same. There are several reports that indicate there are variations in the pharmacological profiles of many NSAIDs when tested on the PGHS-1 and PGHS-2 purified enzymes (58). For example, acetylation of Ser^{530} of the ovine PGHS-1 protein by aspirin leads to irreversible inhibition of PGHS-1 by the actyl group protruding into the hydrophobic channel, which consequently blocks arachidonic acid

access to the cyclooxygenase active site and subsequent prostanoid formation (68). Whereas, acetylation of PGHS-2 (Ser^{516}) by aspirin leads to a modified protein which can lead to production of 15 (*R*)-hydroperoxy-eicosatetraenoic acid (15-HPETE) and subsequent production of lipoxylins by the 15-lipoxygenase pathway (60,76). The functional role of NSAIDs is to effectively inhibit inflammatory prostanoids produced mainly by the PGHS-2 pathway. Current NSAIDs effectively inhibit both PGHS-1 and PGHS-2 by competing with arachidonic acid for the cyclooxygenase active site (77). The inhibition of the constitutive housekeeping PGHS-1 pathway causes serious side effects from current NSAIDs. For example, PGHS-1 inhibition limits the production of the cyto protective PGE_2 in the stomach, which leads to ulceration and in some cases serious bleeding of the gastrointestinal tract (62). The knowledge of the needed PGHS-2 specificity of NSAIDs has recently lead many pharmaceutical firms to develop PGHS-2 specific inhibitors (216).

1.3.6 Phenotypes of PGHS-1 and PGHS-2 deficient mice

Recently the characteristics of mice deficient in PGHS-1 and PGHS-2 were reported (217, 218). Observations in survival ability, gastric cyto-protection, inflammatory response, reproductive capability and histological changes were recorded. No significant gross or microscopic changes were observed in PGHS-1 deficient mice, which developed normally and were in good physical condition (217). Whereas, the survival and development of PGHS-2 deficient mice was notably altered (218). Gross changes were detected in the kidneys and microscopic renal abnormalities were observed in the PGHS-2 deficient mice (218). PGHS deficient mice were expected to be less responsive to inflammatory agents than wild type animals when inflammatory response was measured with standard

ear and paw inflammation assays. However, there was no difference between PGHS-2 deficient mice and wild type mice in response to the phorbol ester TPA and the PGHS substrate arachidonic acid (218). Whereas, the PGHS-1 deficient mice were less responsive to arachidonic acid but not to TPA (217). PGE_2 produced in the gastrointestinal tract by PGHS-1 is thought to be essential for gastric cyto-protection and inhibition of PGHS-1 by NSAIDs is the presumed mechanism of gastric ulcerogenesis. PGHS-1 deficient mice had no increase in spontaneous gastric ulcerations and were less sensitive to induction of gastric ulcers by NSAIDs (217). No gastric intestinal pathology was detected in the PGHS-2 deficient mice (218). PGHS-1 is the key enzyme in the production of prostanoids involved in platelet aggregation. As expected platelets from the PGHS-1 deficient mice aggregate more slowly and to a lesser extent than those from the wild type mice (217). The role of prostanoids in reproductive function is well known and as expected the fecundity and fertility of PGHS deficient mice was modified (217, 218). When PGHS-1 deficient males and females were mated, the number of live pups was decreased. Recent studies suggest that pup death is caused by a lengthening of the gestation period of the females, by 1-2 days which results in pup death due to dystocia (219). PGHS-2 deficient female mice are infertile due to ovulatory failure (218).

1.4 MEGAKARYOCYTOPOIESIS

Platelets perform two major related functions in hemostasis. When linked by fibrinogen, they form large multicellular aggregates to limit blood loss by creating a physical barrier at the site of vascular injury. The second role of platelets is to moderate the speed at which proteins (thrombin and fibrin) that are involved in coagulation become activated (80,81).

Platelets are produced by the shedding or fragmentation of the cytoplasm of mature megakaryocytes (80,81). Mature megakaryocytes are large polyploid cells that contain highly invaginated membranes (also known as the demarcation membrane), that contain molecules necessary for platelet function (82,83,84,85). Megakaryocytopoiesis is the cellular maturation process of immature megakaryocyte progenitor cells prior to the release of platelets into the circulation.

1.4.1 Megakaryocyte differentiation

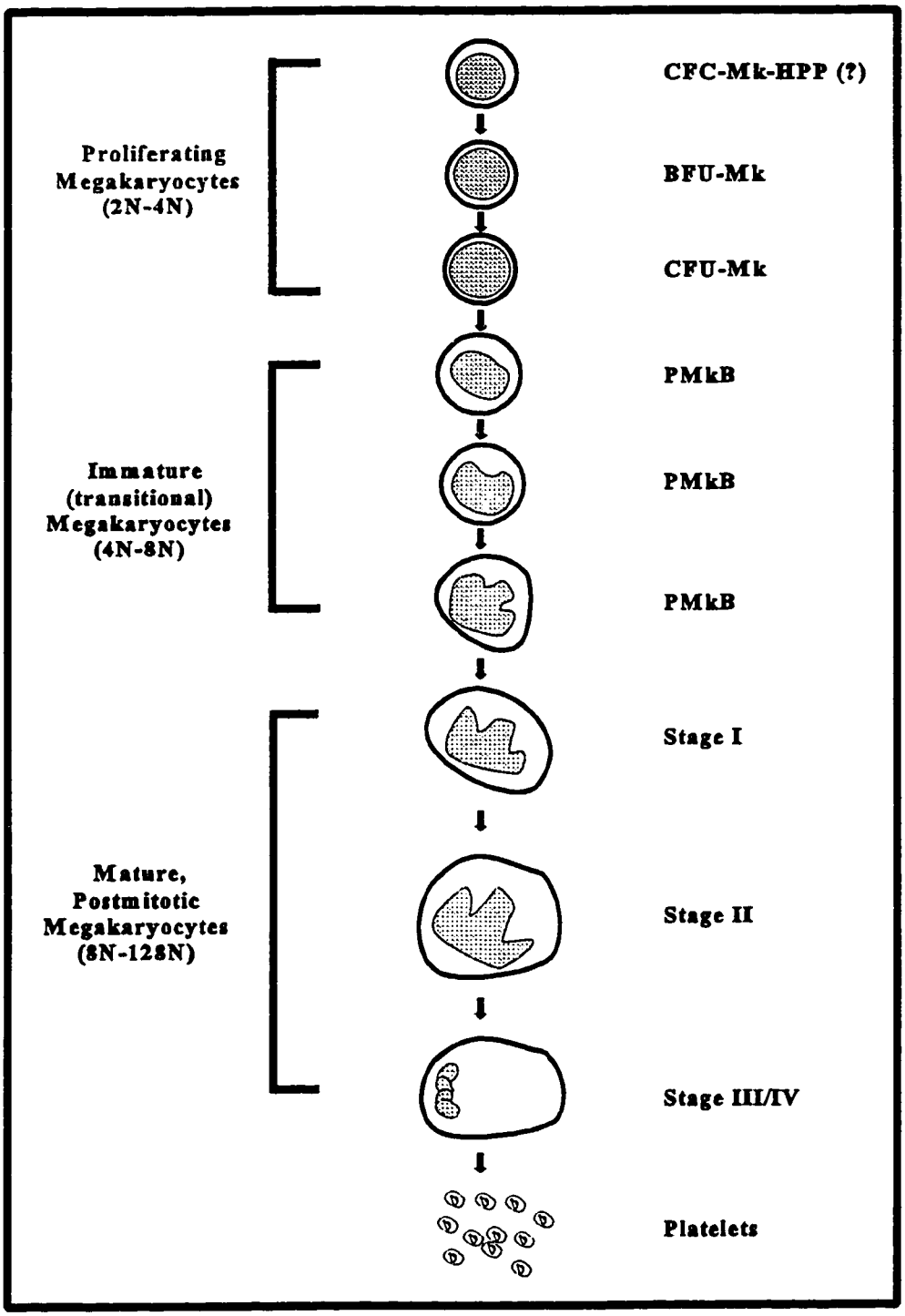
Platelet release from mature megakaryocytes is an elaborate process that starts with the committed maturation of hematopoietic stem cells into the replicative megakaryocyte progenitor cells, which continue on through cellular endomitosis and megakaryocyte differentiation (86,87,88,89). Megakaryocytopoiesis can be divided into three separate stages of development: (1) progenitor cells; (2) promegakaryoblasts (PmkB) or immature megakaryocytes and (3) mature megakaryocytes. This hierarchy of megakaryocyte development is illustrated in Figure 1.4.

In vitro studies have shown as in other cell lineages, the megakaryocyte progenitor cells lose their proliferative potential as they mature. Several different classes of megakaryocyte progenitor cells have been identified (Figure 1.4). The most primitive form is the highly proliferative cell called the colony-forming cell-megakaryocyte-high-proliferative-potential (CFC-Mk-HPP), which was first described in murine bone marrow (90,91). The existence of these cells in human circulation has not been documented and is therefore still just speculation. The CFC-Mk-HPP can generate a few thousand megakaryoctyes per cell, demonstrating around 8-10 replicative cycles (92). It has been reported that proliferation of this primitive progenitor cell requires different mechanisms for

proliferation, which are IL-3 stimulation and co-activation of the protein kinase C and cAMP mediated cell signaling pathways (90). The next progenitor cell type of the megakaryocytic cell lineage is the megakaryocyte burst forming cell (BFU-Mk), which also has a high proliferative potential. Each BFU-Mk cell has the potential to form a large number of megakaryocytes (50 -500), which represents 5 to 7 replicative cycles (92). Serum free cultures of these cells have lead to the discovery of the growth factors required for their development. Investigators have demonstrated that IL-3, GM-CSF and Mk-CSF can stimulate proliferation of human BFU-Mk cells (93). BFU-Mk cell development can be augmented by stimulation with IL-3 in conjunction with c-kit ligand, IL-11 or IL-1α (93,94,95,96,97). The final megakaryocyte progenitor cell is the megakaryocyte colony-forming-cell (CFC-Mk), which is the most differentiated and easily recognized megakaryocyte progenitor cell (94,98). Each CFC-Mk cell has a limited proliferative potential and can only produce a moderate number of megakaryocytes (3-50), which represents 2 to 5 replicative cycles. IL-3 and GM-CSF, as well as crude preparations of thrombopoietin have been shown to promote growth and survival of the CFC-Mk cell type (94,97,99,100).

The cells that bridge the gap between the proliferative progenitor cells and the fully developed megakaryocytes are known as the Immature megakaryocytes or Promegakaryoblast (PMkB). These cells which are not yet identifiable as megakaryocyte, express platelet specific markers (GPIIb/IIIa, platelet peroxidase activity and von Willebrand factor) (101,102,103,104,105,106,107). The PMkB

Figure 1.4 **Cellular hierarchy of megakaryocyte maturation.**
(Adapted from Avraham, H., *Stem Cells*, 11:499-510, 1993.)


Proliferating Megakaryocytes (2N-4N)
Immature (transitional) Megakaryocytes (4N-8N)
Mature, Postmitotic Megakaryocytes (8N-128N)
CFC-Mk-HPP (?)
BFU-Mk
CFU-Mk
PMkB
PMkB
PMkB
Stage I
Stage II
Stage III/IV
Platelets

can be identified by their smaller size, round indented nucleus and their restricted or loss of proliferative potential. Most PMkB cells are endomitotic and are intermediate in ploidy number for mature megakaryocytes. Reports have demonstrated that several factors (IL-3, c-kit ligand and IL-6) can cause PMkB cells to mature into single large megakaryocytes (108,109,110). Three distinct types of PMkBs have been observed and vary based on their nuclear and cytoplasmic complexity, antigen expression and enzymatic content (101,102,106,109,111,112).

The fully developed megakaryocyte which has lost is proliferative potential and sheds platelets is the final cell type produced in megakaryocytopoiesis. These large polyploid cells contain lobulated nuclei and are divided into four detectable stages of maturation, based on their morphology. The stage I megakaryoblast has a high nucleus to cytoplasm ratio and a granular like cytoplasm, which reflects the amount of protein synthesis occurring in these cells (106,107,113,114,115,). The stage II promegakaryocyte is similar in appearance to the stage I megakaryoblast, but the cytoplasmic volume and granular particles is increased (106,107,113,114,115,). The final cell type of megakaryocyte development are stage III and IV megakaryocytes (granular or platelet shedding cells) (106,107,113,114,115,).

1.4.2 PGHS expression during megakaryocytopoiesis

As described earlier, thromboxane A_2 produced by platelets is a potent inducer of platelet aggregation and acts as a vasoconstrictor, whereas Prostaglandin I_2 produced by vascular endothelial cells has an opposite physiological effect of thromboxane A_2 (116,117,118,119,120,121,123). These two prostanoids are thought to influence platelet function by regulating cAMP levels through

activation (PGI_2) or inhibition (TXA_2) adenylate cyclase (116,117,118,119,120,121,123). Platelet activity has shown to play a role in cardiovascular disease (atherosclerosis) and control of the platelet/vascular wall interaction through treatment with NSAIDs has been effective in reducing the incidence of cardiovascular disease (124,125). The source of PGI_2 in vascular endothelial cells and TXA_2 in platelets is the prostaglandin endoperoxide H synthase pathway. The basis for the activity of aspirin is the irreversible inhibition of platelet PGHS-1, suggesting that this enzyme plays a central role in the pathology of cardiovascular diseases. Because platelets are anucleated cells, aspirin-inhibited PGHS-1 can not be regenerated, therefore, PGHS-1 activity can only be recovered when platelets are replaced (every 10 days). Although at the moment, the regulation of PGHS-1 expression in platelets is not fully understood.

1.4.3 Model for studying PGHS-1 regulation in platelets

Because platelets are anucleated cells and the collection of sufficient numbers of human megakaryocytes (0.01% of bone marrow cells) from bone marrow is difficult, progress in the understanding of PGHS-1 regulation in platelets has been hampered by the lack of a good model. As a potential model to investigate megakaryocyte differentiation, the cell line MEG-01, a platelet precursor has been studied (126). MEG-01 is a human megakaryoblastic leukemia cell line which retains the morphological and functional properties of bone marrow megakaryocyte progenitor cell, for example, a high proliferative potential and expression of platelet lineage markers such as GPIIb/IIIa (127). MEG-01 cells exist as three populations: nucleated cells that float in culture, nucleated cells that adhere to the bottom of the culture flask, and anucleated platelet-like structures.

It has been suggested that floating MEG-01 are cells at the early stage of megakaryopoiesis and that adherent MEG-01 are cells that have differentiated into a slightly later stage (130). Also, MEG-01 cells contain no markers for T and B lymphocytes and myloid cells (127). Tumor promoting phorbol esters can alter many cellular differentiation related events and studies with murine megakaryocyte progenitor cells indicates that phorbol esters can induce megakaryocyte differentiation (128). Therefore, studies were undertaken to determine if phorbol esters can cause MEG-01 cells to differentiate into mature megakaryocytes with platelet like structures. These studies indicate that treatment of the megakaryoblastic MEG-01 cells with phorbol esters cause the arrest of cell growth and expression of platelet lineage markers (GPIIb/IIIa) (129). As well, maturation of immature progenitor like cells into mature megakaryocyte like cells was observed by the appearance of prominent cytoplasmic blebs, budding of blebs (platelet release) and multiplication of nuclei (129,130). Also, studies have also shown an apparent induction of PGHS-1 expression in MEG-01 cells after phorbol ester treatment (131). Therefore, MEG-01 cells are considered the best cell line for the analysis of megakaryocytic maturation and PGHS-1 gene expression in platelets (127).

1.5 EFFECT OF POST-TRANSCRIPTIONAL REGULATION ON GENE EXPRESSION

Until recently, it was thought that mRNA processing was not important in the regulation of gene expression and that the 3'UTR had no real significant function. The majority of studies have focused on the effect of transcriptional control mechanisms on gene regulation. Studies are now indicating an increasing role for post-transcriptional control mechanisms in the regulation of gene expression. In the following sections, examples of the already identified post-transcriptional gene

regulatory mechanisms will be discussed.

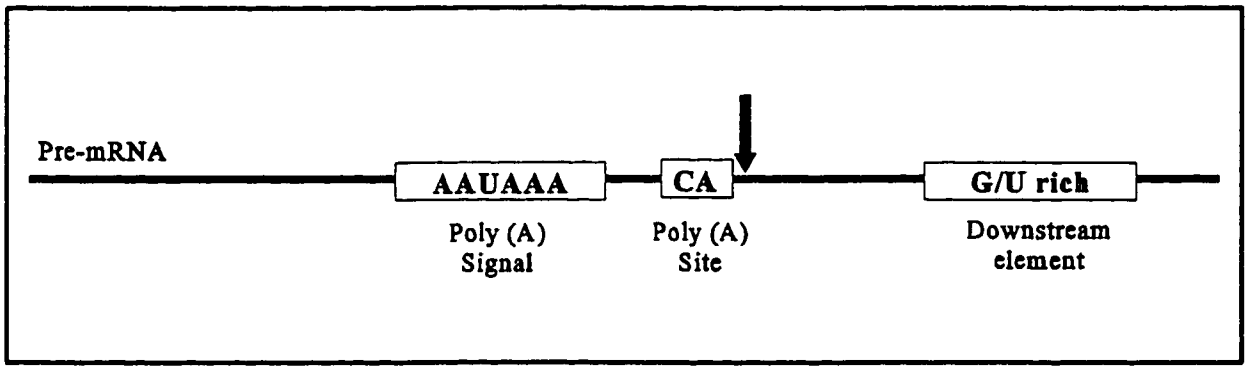
1.5.1 Polyadenylation of mRNA precursors

Most precursor RNAs are not processed efficiently and as a consequence are rapidly degraded in the nucleus and never reach the cytoplasm. Polyadenylation is one of the mechanisms that can affect the efficiency at which RNA is processed. For example polyadenylation site efficiency can directly impact on the amount of cytoplasmic mRNA derived from a primary transcript (132). Once in the cytoplasm, the strength of a poly (A) tail can affect the stability, intracellular location and translatability of a message (133,134,135,136). Therefore, the efficient use and strength of a polyadenylation site can affect gene expression in a tissue or developmental specific manner (137,138,139,140).

1.5.1.1 Genetic signals that influence polyadenylation

Efficient pre mRNA processing requires genetic elements that are located upstream and downstream of the polyadenylation cleavage site (Figure 1.5), which is CA in 70% of vertebrate mRNAs (221). Studies have shown that mutation at and near the cleavage site (CA), alters the accuracy but not the efficiency of the poly (A) tail formation (221). By comparison of 3'UTR sequences of several different mRNA species, the upstream element was first found to play a role in the proper formation of the polyadenylated mRNA (141). From sequence analysis, 80-90 % of

Figure 1.5 ***cis*-acting elements required for proper 3'-processing of a mammalian pre-mRNA**. (Arrow represents the site of pre-mRNA cleavage).

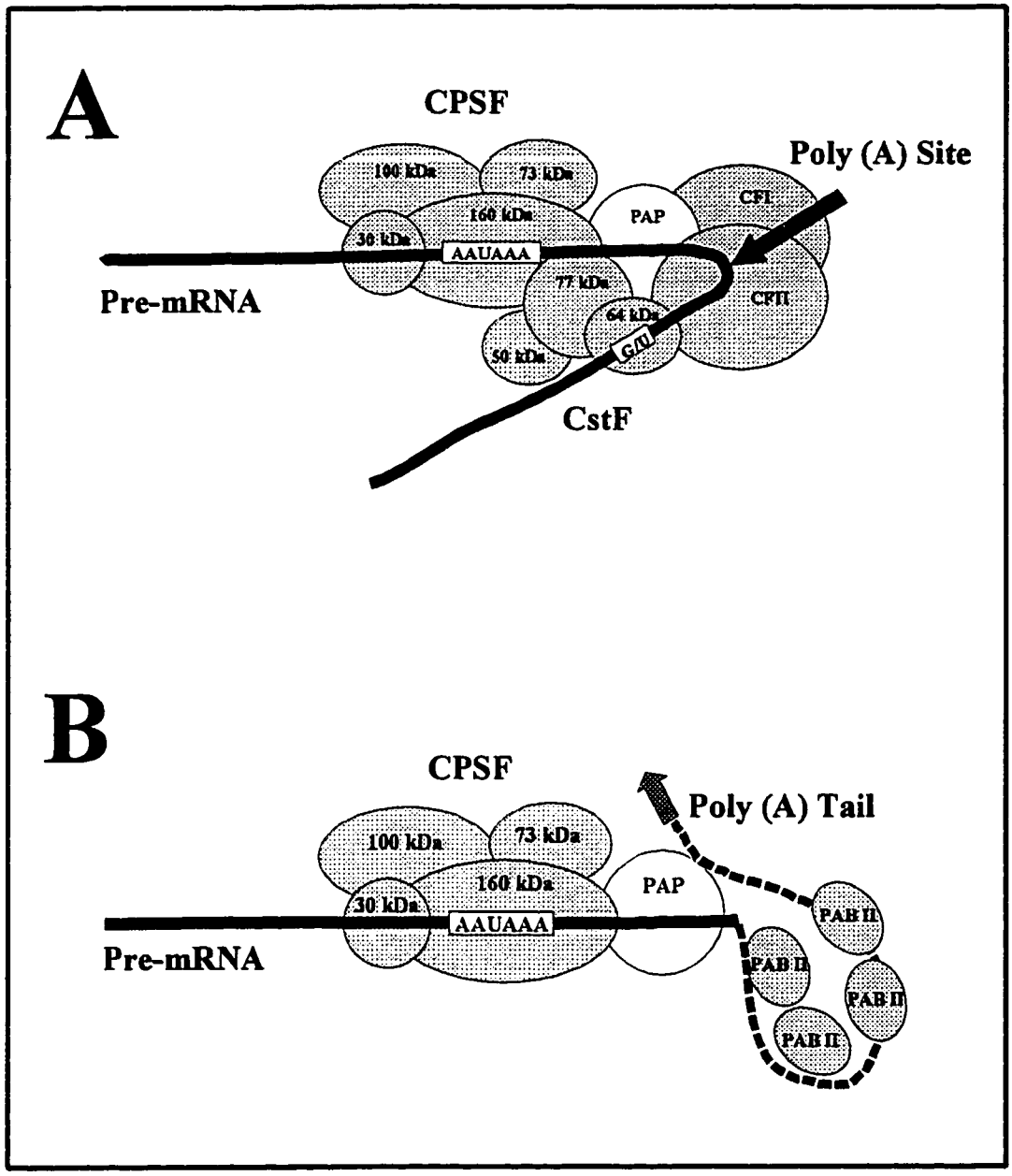

Pre-mRNA
AAUAAA
CA
G/U rich
Poly (A)
Signal
Poly (A)
Site
Downstream
element

mRNAs examined contained the hexanucleotide sequence AAUAAA located 10 to 30 bases upstream of the site at which the poly (A) tail is added (137,142). Proper polyadenylation of mRNA precursors is highly dependent on the AAUAAA sequence, although studies have shown that the mutant hexanucleotide sequence AUUAAA arises at a high frequency and results in efficient polyadenylation (143). Studies have documented that other point mutations of the AAUAAA sequence also occur in nature, for example, reduced the levels of α_2-globin mRNA from a patient (AAUAAG) with α-thalassaemia were found when compared with patients that contain the consensus AAUAAA sequence in their gene (144,145). It has been determined that the presence of the AAUAAA sequence is not sufficient for efficient polyadenylation of precursor RNAs. Further examination of the sequences surrounding cleavage sites revealed the presence of elements located 20-50 bases downstream of the cleavage site, which are rich in U and G residues (146,147). Although there is no consensus sequence for G/U rich elements, they have been shown to be integral in the pre mRNA processing events (147). The G\U rich elements act as a binding site for the cleavage stimulation factor (CstF), which is part of the multi-subunit mRNA processing complex (147).

1.5.1.2 Multiple proteins mediate specific cleavage and polyadenylation of precursor RNA

A proper polyadenylation cleavage reaction proceeds in two distinct stages, which are the endonucleotide cleavage of the precursor RNA and the subsequent addition of a poly (A) tract to the upstream cleavage product. Along with the discussed *cis*-acting elements, there are several *trans*-acting factors that direct the cleavage and polyadenylation of precursor RNA through the formation

Figure 1.6 **Trans-acting factors that direct the proper cleavage and polyadenylation of precursor RNA.** The multi-subunit protein complexes that directs proper cleavage of pre-mRNA is represented in panel **A**. The trans-acting factors required for the polyadenylation of cleaved pre-mRNA is shown in panel **B**. (Adapted from Manley, J.L., and Takagaki, Y. 1996. *Science.* 274:1481-1482).


A
CPSF
Poly (A) Site
100 kDa
73 kDa
160 kDa
PAP
CFI
30 kDa
AAUAAA
77 kDa
CFII
64 kDa
50 kDa
GU
Pre-mRNA
CstF
B
CPSF
Poly (A) Tail
100 kDa
73 kDa
160 kDa
PAP
30 kDa
AAUAAA
PAB II
PAB II
PAB II
PAB II
Pre-mRNA

of a multi-subunit protein complex (figure 1.6A) (148).

Two key subunits of the multi-subunit protein complex responsible for processing pre mRNA are the cleavage and polyadenylation specificity factor (CPSF) and the cleavage stimulatory factor (CstF). CPSF itself is a multi-protein complex, which is comprised of three polypeptide subunits that are 160 kDa, 100 kDa and 73 kDa (149,150). CPSF is the *trans*-acting factor that specifically binds to the AAUAAA upstream element, through a RNA-protein interaction involving the transcript and the 160 kDa subunit (149). CstF, the other major subunit is comprised of three separate polypeptides with molecular weights of 77 kDa, 64 kDa and 50 kDa and it binds to the RNA molecule through an interaction of its 64 kDa subunit with the GU rich downstream element (151,152,153).

For efficient RNA processing, both subunits are required and complement each others interaction with the transcript, by interaction with each other. CPSF is required for tight binding of CstF to the RNA molecule and the presence of CstF stabilizes the CPSF RNA-protein complex (151,152,153). The proteins complexes are thought to interact with each other through the CstF 50 kDa subunit, which is linked to the CstF 64 kDa RNA binding subunit by the CstF 77 kDa protein (151,152).

The other proteins shown to be involved with the pre mRNA processing complex, are the machinery responsible for the actual cleavage and polyadenylation of the precursor RNA molecule. Two proteins which interact with the CPSF and CstF complexes have been identified and have been shown to contribute to the recognition and cleavage of a poly (A) site. These two proteins are termed cleavage factors and are known as CFI and CFII (150). The final part of the processing complex is poly (A) polymerase (PAP) and is responsible for polyadenylation of the cleaved transcript.

Once the cleavage reaction has occurred, it is assumed that the cleavage factors, the downstream cleavage fragment and the CstF complex separate from the large processing unit. Whereas, the CPSF and PAP remain bound to the upstream cleavage fragment. The first reaction that occurs after cleavage of the primary transcript is the slow addition of ten adenosine residues by PAP, which is facilitated by the interaction with CPSF (154). Once the short ten adenosine poly (A) tract has been added, the 3'-processing complex becomes associated with a 50 kDa regulatory protein named the poly (A)-binding protein II (PAB II), which facilitates a rapid addition of approximately 250 adenosine residues to the poly (A) tail. During the addition of the elongated poly (A) tail, it becomes covered with additional PAB II molecules (150,155). The rapid addition of adenosine residues, is dependent on the stable complex (Figure 1.6B) comprised of a pre-mRNA which has a AAUAAA signal, CPSF, PAP and PAB II (154). The molecular mechanisms that regulate the termination of the addition of adenosine residues to the poly (A) tail is not known. Although, it has been postulated that the 3'-processing complex has a way of sensing the number of PAB II molecules attached to the poly (A) tail (150).

1.5.2 mRNA stability

The steady-state level of mRNA is regulated at two steps: (1) rate of synthesis, i.e. transcription and (2) rate of degradation, i.e. stability. Little has been elucidated about the regulation of mRNA stability. Changes in the half-life of a particular mRNA represent a fast and economic way of increasing the amount of mRNA available immediately for protein synthesis in response to environmental stimuli .

1.5.2.1 Mechanisms of mRNA decay

There are several different mechanisms that facilitate the decay of mRNA, but little known about the enzymes that degrade mRNAs in mammalian cells. To date, no mammalian mRNase has been clearly identified. Although isolation and characterization of intermediates of mRNA decay have proven useful in identifying the nature of mRNA decay mechanisms. Two types of enzymes involved in mRNA degradation have been identified as either exoribonucleases or endoribonucleases. It has been shown that the poly (A) tail of several mRNAs plays an important role in mRNA decay, by protecting the molecule from nuclease attack (156,157,158).

Exoribonucleases have been shown to act via several different mechanisms when they are involved in mRNA degradation. The major mRNA decay pathway is initiated by shortening of the poly (A) tail, which is followed by removal of the 7-methylguanosine cap structure and then rapid 5' to 3' exonucleolytic degradation of the mRNA. As well, exonuclease decay of transcripts in the 3' to 5' direction after shortening of the poly (A) tail has been demonstrated. Although poly (A) shortening appears to play a major role in exoribonuclease decay, mRNA degradation independent of poly (A) tail shortening has been demonstrated by removal of the 7-methylguanosine cap and rapid 5' to 3' exonucleolytic activity (159,160,161).

The second type of enzyme shown to be involved in mRNA decay are endoribonucleases. Several examples of mRNA decay that function via endonucleolytic cleavage before shortening of the poly (A) tail have been identified (162,163,164). The majority of endonucleolytic cleavage sites have been identified in the coding region and 3'UTR of several mRNAs (162,163,164). Several mRNAs that are susceptible to endonucleolytic decay have been shown to be regulated by RNA-binding

proteins. These RNA-binding proteins have been shown to bind to the RNA and block the cleavage site, rendering the mRNAs stable (163)

1.5.2.2 *cis* determinants of mRNA stability

1.5.2.2.1 Poly (A) tail

Several results have indicated that the poly (A) tail of several mRNAs protect them from mRNA decay, for example; (1) the decay of most mRNAs is initiated by the deadenylation of the poly (A) tail and (2) the poly (A) binding protein (PAB II) - poly (A) tail complex protects many mRNAs from rapid degradation (158,165,166). Studies show that polyadenylated mRNAs are rapidly degraded when incubated with PAB II depleted extracts (158,167). As well, deadenylated mRNAs incubated in the presence or absence of PAB II are also rapidly degraded (158,167). In most mammalian cells, the amount of PAB II is three times greater than necessary to bind up all of the mRNA within a cell (168). Therefore, these results indicate that the poly (A) tail - PAB II complex is present on all mRNAs and gene regulation through mRNA stability occurs by affecting the strength of this complex (169). The consensus is that *cis*-elements and *trans*-acting factors function by affecting the association of PAB II with poly (A) tail and limit PAB II's ability to protect the poly (A) tail.

1.5.2.2.2 3' untranslated region

Most work completed on *cis* determinants of mRNA stability have been identified in the 3'UTRs of several mRNAs, which suggests that mRNA stability is influenced by this region. Several elements have been well characterized and appear to function through a RNA-protein interaction (170,171,172,173). One of the most well characterized 3'UTR stability elements are regions rich in adenosine and uridine, and are termed AU rich elements or AUREs (174). The 3'UTRs of many unstable mRNAs contain AUREs (174). Many AUREs contain two domains (175). Domain I consists of an AU rich element, which includes multiple copies of the pentamer AUUUA and the adjacent domain II contains a 20 nucleotide U rich region (176). The observation that linked AUREs to mRNA instability was when the AUREs from the 3'UTR of an unstable mRNA (GM-CSF) was placed within the 3'UTR of a stable mRNA (β-globin), the chimeric RNA was degraded within 30 minutes (177). Whereas, the wild type β-globin RNA was stable for over 2 hours (177).

1.5.2.3 *trans*-acting factors affecting mRNA stability

Most mRNAs do not have a fixed half-life. Instead the half-lives of many mRNAs actually fluctuate in response to environmental stimuli such as nutrient levels, cell growth rates, viral infection and exposure to toxins or carcinogens (134). Therefore, due to environmental factors some mRNAs might be more susceptible to degradation than others. The control of intracelllular mRNA half-lives of many mRNAs has been linked to their interaction with RNA binding proteins (170,171,172,173,178,179). Several of these *trans*-acting factors have been identified and

characterized, which lead to the proposal of three general features of these factors; (1) many factors probably have other regulatory roles and do not only act as constitutive stabilizers or destabilizers, (2) some factors (RNA binding proteins) can either stabilize or destabilize the mRNAs to which they bind and (3) several factors can act in conjunction to stabilize or destabilize mRNAs.

A well characterized family of binding proteins have the capacity to bind with high affinity to the previously mentioned AURE sequence elements (159,180). This family of proteins named AURE-binding proteins (AUBPs) are primarily cytoplasmic, but some shuttle between the nucleus and cytoplasm (180,181,182). The influence of AUBPs on mRNA stability has been demonstrated by several observations; (1) changes in intracellular concentrations of AUBPs have been shown to correlate with changes in mRNA half-lives and (2) purified AUBPs can affect mRNA stabilities in cell free extracts (183,184).

1.6 SUMMARY AND SPECIFIC AIMS

One of the most cost-effective drugs in preventing cardiovascular disease is aspirin. The basis for the anti-thrombogenic activity of aspirin is the irreversible inhibition of platelet PGHS-1, suggesting that this enzyme plays a key role in the pathology of cardiovascular diseases. There is evidence that prostanoid biosynthesis is altered in cardiovascular diseases such as atherosclerosis. For example, atherosclerotic platelets form increased amounts of the prostanoid TxA_2, which is a potent mediator of platelet aggregation and increases vasculature constriction. To understand the molecular mechanisms facilitating this increase in thromboxane synthesis, the regulation of PGHS-1 expression in platelets and their precursor cells must be studied.

Therefore, the overall goal of this research is to further clarify the regulation of PGHS-1 expression in platelets. The ability to study gene expression in platelets is limited by the fact that there is no source of DNA to study gene regulation because platelets are anucleated cells. After TPA treatment, the immortalized human megakaryoblastic cell line MEG-01 has been shown to differentiate into megakaryocytes with platelet-like structures (191, 237, 238). Therefore, MEG-01 cells represent an ideal source of DNA of the platelet lineage to study PGHS-1 expression.

The specific aims of the present study are divided into two objectives. The first objective is to characterize the 4.5 kb PGHS transcript detected in MEG-01 cells by Northern blotting with the entire coding region of PGHS-1. To accomplish this, the specific aims are:

- to design a strategy to determine the identity of the 4.5 kb PGHS transcript using PGHS-1 and PGHS-2 specific probes;
- clarify the mechanism for generation of the new 4.5 kb PGHS transcript; and
- to define the tissue expression pattern of the new 4.5 kb PGHS transcript.

The second objective is to examine the regulation of expression of the PGHS-1 gene in MEG-01 cells. To accomplish this, the specific aims are:

- to characterize the profiles of PGHS-1 mRNA and protein expression in MEG-01 cells after TPA treatment;
- to determine if increases in PGHS-1 mRNA after TPA treatment are facilitated through transcriptional or post-transcriptional regulation.

SECTION TWO: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Human PGHS-1 3'UTR cloning and production of various PGHS-1 constructs

The vector pBluescript (pBS) KS(-) was obtained from Stratagene (LaJolla, CA). DNA cloning was performed using the *E. coli* strains DH5α from GibcoBRL, Life Technologies Inc. (Burlington, Ont). Media for the growth of *E.coli* included: LB agar, LB broth base from GibcoBRL, and tryptone from DIFCO-Bacto Labs (Detroit, MI) and yeast extract and agar from Sigma Chemical Co. (St. Louis, MO). Vector bearing bacterial cells were selected with ampicillin from GibcoBRL. All restriction enzymes as well as the DNA modifying enzymes Calf Intestinal Alkaline Phosphatase (CIAP) and Taq DNA polymerase were purchased from either Pharmacia (Baie d'Urfe, Que), Promega (Madison, WI) or GibcoBRL. λ DNA/*Hind* III fragments and the 0.24-9.5 kb RNA ladders were obtained from GibcoBRL and the pBR322 DNA - *Msp* I digest ladder was purchased from New England Biolabs (Beverly, MA) Rapid DNA Ligation Kits, Expand™ High Fidelity PCR System Kits and 5'/3' RACE Kits were obtained from Boehringer Mannheim (Laval, Que). Size-separated DNA fragments were recovered from agarose gel slices using the QIAEX DNA purification kit from Qiagen (Chatsworth, CA). The RT-PCR kit was obtained from Stratagene. Oligonucleotide primers for Polymerase Chain Reaction (PCR) amplification were obtained from Oligos Etc. Inc. (Ottawa, Ont) and the University of Ottawa Biotechnology Research Institute (Ottawa, Ont). PCR reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 (Applied Biosystems Canada, Inc,

Mississauga, Ont).

2.1.2 Western blotting and protein purification

Expressed protein was collected by lysing the MEG-01 cells with a Microson, Ultrasonic cell disrupter obtained from Heat Systems - Ultrasonic Inc.(Farmingdale, NY). Protein concentrations were determined using a dye binding reagent from Bio-Rad Laboratories Inc. that is based on the Bradford method (Mississauga, Ont). Protein samples were electrophoretically separated using the Bio-Rad Mini-Protean II system and transferred to nitrocellulose membrane obtained from Bio-Rad. PGHS-1 protein samples were dectected using enhanced chemiluminescence with the ECL kit from Boehringer Mannheim.

2.1.2 Northern blotting, total RNA and POLY $(A)^+$ mRNA purification

Total RNA was isolated from cells using the TriZol reagent from GibcoBRL. mRNA was purified from total RNA using the Oligotex mRNA purification kit from Qiagen. Electrophoretically separated mRNA samples were transferred to a nylon membrane from GibcoBRL. DNA probes were labelled using the Prime-It II Random Primer Labeling Kit from Stratagene. [α-^{32}P] dCTP and [α-^{32}P]dUTP were obtained from Amersham Life Science Inc. (Oakville, Ont). Band intensities were quantified by scanning densitometry using a Hewlett Packard ScanJet II cx and and Kodak Digital Science ds 1D analysis software. Commercial multiple tissue dot and Northern blots were obtained from Clontech Inc. (Palo Alto, CA).

2.1.3 MEG-01 and U937 cell culture

The MEG-01 and U937 cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Complete MEG-01 and U937 culture medium contained: RPMI-1640 Medium with L-glutamine and 10% Fetal Bovine Serum (FBS), which was obtained from Wisent Inc. (St. Bruno, Que).

All other chemicals were of analytical grade and were obtained from local suppliers.

2.2 METHODS

2.2.1 Standard methods

2.2.1.1 Bacterial growth conditions

Bacterial cultures were used for the isolation of plasmid DNA. Typically, 3 - 5 ml of Luria-Bertani (LB) was seeded with a single vector-bearing colony using a sterile disposable inoculum loop. Vectors used encoded the ampicillin or kanamycin resistance gene, therefore the LB broth was supplemented with 50 μg/ml ampicillin or 30 μg/ml kanamycin. Cultures were grown at 37°C for 18 h in a shaker-incubator at 225 rpm.

2.2.1.2 Preparation of competent cells

For standard competent *E. coli* cells, an overnight culture of DH5α was diluted 1:100 in 60 ml of SOB medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$). Cells were grown at 37°C with shaking until the culture reached an optical density (OD) of 0.3 using a wavelength of 600 nm, which corresponds to the log phase of growth. The culture was transferred into two new 50 ml tubes (30 ml/tube) and centrifuged at 1000 x g at 4°C for 15 min. The cells were resuspended in 20 ml (10 ml/tube) of RF1 medium (100 mM RbCl, 30 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% (w/v) glycerol) and placed on ice for 30 min. Next, the cells were collected by centrifugation again at 1000 x g at 4°C for 15 min. The pellets were resuspended in 5 ml (2.5 ml/tube) of RF2 medium (10 mM MOPS pH 6.8, 10 mM RbCl, 75 mM $CaCl_2$-$2H_2O$, 15% (w/v) glycerol) and incubated on ice for 15 min and divided into 200 µl aliquots. The competent cells were shock frozen in liquid nitrogen and stored at -80°C for later use. The transformation efficiency of these cells was usually about 1 X 10^6 colonies/µg of plasmid DNA (the test plasmid was 2.96 kb).

2.2.1.3 Transformation

Competent cells were thawed on ice. A 200 µl aliquot of cells was then mixed with either 10 µl of ligation mix or 1 µl (1 ng) of plasmid, left on ice for 45 min, heat-shocked at 42°C for 1.5 min and placed on ice again for 2 min. Next, 800 µl of SOC medium (SOB supplemented with 12 mM glucose) was added and the cells were incubated for 45 min at 37°C with shaking to allow for the

expression of plasmid encoded antibiotic resistance genes.

For plasmids, a 50 - 100 µl aliquot of transformed cells was directly plated on LB agar supplemented with 50 µg/ml ampicillin. For ligations, samples were concentrated prior to plating by spinning for 1 min at 7500 x g at room temperature and resuspending in 200 µl of fresh SOC.

2.2.1.4 Small scale isolation of plasmid DNA

The procedure for the small scale isolation of plasmid DNA from bacterial cultures (referred to as a miniprep) was based on the alkaline lysis method (Sambrook et al; 1989). A 1.5 ml aliquot of fresh overnight bacterial culture was centrifuged at 10000 x g for 1 min at room temperature to pellet the cells. The supernatant was aspirated and the pellet was resuspended in 100 µl of ice cold P1 solution (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, 0.4 mg/ml RNase I, pH 8.0) by vortexing. 100 µl of P2 solution (0.2 N NaOH, 1 % SDS) was added to lyse the cells and denature the proteins and chromosomal DNA. Tubes were gently mixed by inversion and incubated on ice for 5 min. Next, 100 µl of ice-cold P3 (3M potassium acetate) was added and tubes were mixed by inversion to precipitate proteins, chromosomal DNA and cellular debris. The mixture was centrifuged at 8500 x g for 10 min at 4°C and the supernatant was transferred to a fresh tube. 900 µl of 100 % ethanol was added and the mixture was vortexed. The samples were centrifuged at 8500 x g for 20 min at 4°C to pellet the plasmid DNA. The ethanol was aspirated and the pellets were washed with 500 µl of ice-cold 70 % ethanol and centrifuged at 8500 x g at 4°C for an additional 5 min to remove excess salt. The supernatant was aspirated and the pellets were air dried in a 37°C heating block for 5 min. The DNA was resuspended in 30 - 100 µl of sterile dd$H_2$0 (depending on the size of the

pellet).

2.2.1.5 Genomic DNA isolation

To isolate genomic DNA, cells were collected by centrifugation (1000 x g at 4°C for 5 min) and the pellet was resuspended in 500 µl PK buffer (100 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 400 µg/ml proteinase K) and incubated overnight at 42°C in a shaker-incubator at 225 rpm. The next day, 1 ml of 100 % ethanol was added to each tube. The tubes were mixed by inversion until the DNA precipitated. The DNA was collected by centrifuging for 10 min at 7500 x g. The ethanol was aspirated and the DNA was allowed to air-dry by placing the open tubes in a heating block at 37°C for 5 min. The pellets were then dissolved in 2 -4 mls (depending on pellet size) of ddH_2O and stored at 4°C.

2.2.1.6 Estimation of DNA concentrations

A 500 µl aliquot of diluted DNA (100X in ddH_20) was transferred to a 1.0 ml quartz cuvette and the OD was measured at 260 nm and 280 nm. The OD_{260}/OD_{280} ratio was used to estimate purity of the DNA (ratios were at least 1.8). The OD_{260} was used to calculate the concentration of DNA given that 1 OD_{260} = 50 µg/ml of double stranded DNA and 1 OD_{260} = 33.3 µg/ml single stranded DNA.

2.2.1.7 Restriction digestions, electrophoresis, dephosphorylation, DNA fragment isolation and ligation reactions

Restriction digests

Each restriction endonuclease (RE) was used under temperature and salt conditions producing optimal activity. The amount of enzyme used was typically 3-5 units per μg of DNA, where 1 unit is defined as the amount of enzyme required to cleave 1 μg of lambda bacteriophage DNA in 1 h under optimal conditions. Restriction was allowed to proceed for 1 - 24 h. When non-specific cutting activity was a concern, incubations were kept to the minimum time required. Restriction enzymes were inactivated by either heating at 65°C for 20 min or with the addition of loading buffer dye (0.25 % bromophenol blue, 0.25 % xylene cyanol, 30 % glycerol in 1 X Tris-acetate-EDTA).

Electrophoresis

Following the addition of loading buffer dye, DNA samples were size-separated on 1 X Tris-acetate-EDTA (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) agarose gels containing ethidium bromide (0.5 μg/ml). Gels were prepared based on the size of the DNA fragments to be separated (*e.g.* 0.8% agarose for fragments greater than 5 kb, 1.2 % for 0.5 - 5 kb fragments, 2 % for fragments less than 0.5 kb). The DNA was visualized under ultraviolet light.

When required, desired bands were cut and the DNA was extracted using the QIAEX gel purification kit (Qiagen) as follows. 300 μl of solubilization buffer (3 M NaI, 4 M $NaClO_4$, 10 mM Tris-HCl pH 7.0 and 10 mM sodium thiosulfite) and 10 μl of QIAEX slurry were added to the gel slice and the tube was heated at 50 °C for 10 min with occasional mixing to dissolve the agarose and enable the QIAEX beads to bind DNA. The mixture was spun at 7500 x g for 30 sec and the

supernatant was aspirated. The pellet was then washed once with 500 µl of 8 M $NaClO_4$, 10 mM Tris-HCl pH 7.0 and once with 70 % EtOH, 100 mM NaCl, 10 mM Tris-HCl pH 7.5. To elute the DNA, the pellet was briefly dried by aspiration and then resuspended in 20 µl Tris-EDTA (pH 8.0) or sterile ddH_2O and incubated at 37-50°C for 5 min. The QIAEX beads were then pelleted by spinning at 7500 x g for 30 sec and the DNA was collected by transferring the supernatant to a fresh microfuge tube.

Dephosphorylation

5'-phosphate groups were removed from blunt and cohesive DNA ends to prevent vector re-ligation. Typically, 0.1 units of calf intestinal phosphatase (CIP) was added to a reaction volume of 50 µl containing 1 X One Phor All (OPA) buffer (Pharmacia - 10 mM Tris-acetate pH 7.5, 10 mM Magnesium acetate, 50 mM Potassium acetate). Reactions were incubated either at 37°C for 15 min or overnight, after which another 0.1 units of CIP was added and incubated for a further 15 min. Following dephosphorylation, the enzyme was then heat-inactivated at 65°C for 20 min. The vectors were then gel-purified by electrophoresing on an agarose gel and extracting as previously described.

Ligations

A 3:1 insert to vector molar ratio and 1 µl of ligase (7.5 Weiss units/µl) was used for cohesive ends. Each reaction was performed in 1X Ligation buffer (supplied in the Boehringer Mannheim Rapid DNA Ligation Kit) in a volume of 20 µl. Ligations were incubated at room temperature for 5 minutes. Typically, half of the ligation mixture was used for transformation.

TABLE 2.1

Sequences of primers used for RT-PCR and 3'RACE of specific segments of the PGHS-1 and PGHS-2 genes

Primer		Sequence
T3	(forward)	5' - ATTAACCCTCACTAAAG - 3'
T7	(reverse)	5' - TTAATACGACTCACTAT - 3'
PGS15	(forward)	5' - GGGCAGGAAAGCAGCATTCTGGAG - 3'
PGS13	(reverse)	5' - AACAAGGAGTTCAGCATTCTGGAAG - 3'
PGS25	(forward)	5' - AAGTCTAATGATCATATTTATTTAT - 3'
PGS23	(reverse)	5' - AACATCTTTACTTTCGTCCTTATAA - 3'
PGS1UTR	(forward)	5' - CCATTTGTTCCTGCTTCCGAGATCC - 3'

2.2.1.8 Polymerase chain reaction (PCR) amplification

PCR was used to generate inserts for cloning, and to determine the presence and orientation of DNA sequences of interest. All of the primers used for PCR are listed in Table 2.1. For standard PCR, each reaction contained: 1X PCR buffer with 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH8.3), and 0.001 % (w/v) gelatin), 200 µM dNTPs, 300 nM of each primer, 2.6 U of Expand™ High Fidelity PCR System enzyme mix (Boehringer Mannheim) and 0.1 - 0.75 µg template cDNA in a volume of 20 µl. Next, tubes were heated at 94°C for 1 min to denature the DNA. Mixtures were then subjected to sequential cycles of denaturation, hybridization and primer elongation. Hybridization temperatures were calculated based on the lower melting temperature (T_m) of each primer pair (see Table 2.1). T_m , defined as the temperature at which 50 % of the primer-template DNA duplex is single stranded, was estimated by the equation, 2(A + T) + 4(G + C). The cycle parameters were as follows: denaturation at 94°C for 10 sec, hybridization at T_m- 5°C for 10 sec, and primer elongation at 72°C for 1 sec per 40 nucleotides of expected PCR product. Typically 25 -32 cycles were used. For the last cycle, a final extension of 7 min at 72°C was performed.

To identify bacterial colonies harboring desired DNA constructs, colony PCR was performed. For each reaction, an isolated colony was picked with a sterile toothpick and seeded into 30 - 50 µl of sterile ddH_2O. The toothpick was then used to streak a labeled section on an LB agar plate (supplemented with 50 µg/ml ampicillin). The solution containing the bacteria was boiled at 100°C for 10 min to lyse the cells and the cellular debris was subsequently pelleted by centrifuging at 10000 x g for 2 min. 4 µl of supernatant was used for PCR using standard conditions.

2.2.1.9 Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification

The RT-PCR kit (Stratagene) was used to generate DNA templates for PCR, from either total or poly $(A)^+$ mRNA. Typically, total RNA (5 - 10 µg) dissolved in 33 µl DEPC-treated water was combined with 3 µl of random primers (100 ng/ml), mixed gently and incubated at 65°C for 5 min and then cooled to room temperature. The following reagents were added in the stated order: 10 µl of 5 X first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 1 µl of RNase Block Ribonuclease Inhibitor (40 U/µl), 2 µl of 100 mM dNTPs and 1 µl of MMLV-RT (Moloney Murine Leukemia Reverse Transcriptase) (50 U/µl). All components were mixed and incubated at 37°C for 1 h. Next, the reactions were stopped by incubation at 95°C for 5 min and then cooled on ice. Typically, 1 -5 µl of this reaction was used as a template for PCR using standard conditions previously described.

2.2.1.10 3'- rapid amplification of cDNA ends (3'-RACE)

3' RACE was used to generate cDNA templates for PCR, by taking advantage of the natural poly $(A)^+$ tail of mRNA using an oligo dT-anchor primer (Table 2.1). Typically, total RNA (0.5 - 2 µg) dissolved in 35.5 µl DEPC-treated water was combined with 1 µl of oligo dT-anchor primer (37.5 µM), mixed gently and incubated at 65°C for 5 min and then cooled at room temperature for 10 min. The following reagents were added in the stated order: 10 µl of 5 X first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 1 µl of RNase Block Ribonuclease Inibitor

(40 U/µl), 2 µl of 100 mM dNTPs and 0.5 µl of MMLV-RT (Moloney Murine Leukemia Reverse Transcriptase) (50 U/µl). All components were mixed and incubated at 37°C for 1 h. Next, the reactions were stopped by incubation at 95°C for 5 min and then cooled on ice. Typically, 1 -5 µl of this reaction was used as a template for PCR using standard conditions previously described and a PCR anchor primer (Table 2.1).

2.2.1.11 DNA probe labeling

DNA probes were labeled with [α-^{32}P] dCTP by random priming using the Stratagene Prime-It II Random Primer Labeling Kit. 25 ng of gel purified unlabelled probe DNA in a total of 25 µl of ddH_2O was mixed with 10 µl of random oligonucleotide primers and denatured by heating at 100°C for 5 min. The denatured DNA was then cooled briefly at room temperature and mixed with 10 µl of 5 X primer buffer, 5 µl of [α-^{32}P] dCTP (50 µCi) and 1 µl of Exo (-) klenow enzyme (5 U/µl). After incubating at 37°C for 10 min, the reaction was stopped with 2 µl of 0.5 M EDTA. 170 µl Tris-HCl-EDTA (TE) pH 8.0 (10 mM Tris-HCl pH 8.0, 1 mM EDTA) was then added. 1 µl was removed for Trichloroacetic acid (TCA) precipitation and the remaining mixture was combined with 5 µl salmon sperm DNA (10 mg/ml) and 5 µl 0.1 M spermine and incubated on ice for 10 min. The DNA was pelleted by centrifugation at 8500 x g at 4°C, after which it was resuspended in 135 µl TE, 15 µl 4 N NaOH, 15 µl 5 M NaCl. Before use, probes were denatured by heating at 95°C for 5 min.

The 1 µl removed for TCA precipitation was mixed with 2 mls 5 % TCA and 100 ml 0.2 % BSA and incubated on ice for 10 min, after which the solution was filtered under suction through a Whatman GF/C microfiber glass filter. To determine the amount of radiolabeled probe, the filter was

counted by liquid scintillation using a Beckman LS5000CE scintillation counter.

2.2.1.12 Total RNA isolation

Cells were collected by centrifugation (1000 x g at 4°C for 5 min) and the pellet was resuspended in 1 ml of TriZol reagent (GibcoBRL) and incubated at room temperature for 5 min. 200 µl of chloroform was added and the tubes were mixed vigorously by hand and incubated at room temperature for 2 to 3 minutes. The tubes were centrifuged for 15 min at 7500 x g at 4°C. The clear upper aqueous phase was removed and combined with 500 µl of isopropyl alcohol, mixed by hand and incubated at room temperature for 10 minutes. The samples were then centrifuged at 7500 x g for 10 min at 4°C to pellet the total RNA. The ethanol was aspirated and the pellets were washed with 1 ml of ice-cold 70 % ethanol and centrifuged at 3000 x g at 4°C for an additional 5 min to remove excess salt. The supernatant was aspirated and the pellets were air dried at room temperature for 5 to 10 min. The RNA was resuspended in 100 - 200 µl of sterile diethyl pyrocarbonate (DEPC) treated ddH_20 (depending on the size of the pellet). The total RNA was stored at - 80°C.

2.2.1.13 Purification of poly $(A)^+$ mRNA from total RNA

The poly $(A)^+$ RNA was purified from total RNA using a poly $(A)^+$ extraction kit (Qiagen). Total RNA (up to 250 µg) in 250 µl DEPC ddH_20 was combined with 250 µl 2 X binding buffer (20 mM Tris-HCl (pH 7.5), 1000 mM NaCl, 2 mM EDTA, 0.2% SDS), 15 µl Oligotex suspension (10 % (w/v) Oligotex particles, 10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, 0.1% SDS and

0.1% NaN_3) and incubated for 3 min at 65°C to disrupt any secondary structure in the RNA. The samples were incubated at room temperature for 10 min to allow hybridization between the oligotex particles and the poly $(A)^+$ tail of the mRNA. Samples were centrifuged at 10000 x g for 2 min at room temperature to pellet the oligotex particles. The supernatant was aspirated and the pellets were resuspended in 400 µl of OW2 wash buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA) and pipetted onto a oligotex spin column and centrifuged at 10000 x g for 30 sec. The spin column was transferred to a new RNase-free tube and washed with 400 µl of OW2 wash buffer and centrifuged again at 10000 x g for 30 sec. The spin column was transferred to a new RNase-free tube and the mRNA was eluted with 20 µl of preheated (70°C) elution buffer (5 mM Tris-HCl (pH 7.5)) by centrifugation at 10000 x g for 30 sec. The purified poly A^+ mRNA was stored at - 80°C.

2.2.1.14 Northern blotting

Northern blotting was performed based on the capillary transfer method of Southern (185). Samples of poly $(A)^+$ RNA from various cell samples were subjected to electrophoresis on a denaturing formaldehyde (2.2 M)- agarose (1%) gel. Prior to setting up the transfer apparatus, the gel was soaked in transfer buffer (10 X SSC: 1.5 M NaCl, 0.15 M tri-sodium citrate). To set up the capillary transfer apparatus, a sponge was placed in a glass dish filled with transfer buffer so that the level of liquid almost reached the top of the sponge. Next a piece of 3MM Whatman filter paper (10 cm larger than the gel on all sides) soaked in 10 X SSC was placed on top of the sponge and the gel was centered on the filter paper. A piece of biodyne (GibcoBRL) nylon membrane that had been soaked in 10 X SSC and cut to the size of gel was placed on top of the gel, followed by a piece of

3 MM Whatman filter paper also soaked in 10 X SSC and cut to the size of gel. To complete the capillary apparatus, 3 inches of blotting paper cut to the size of the gel was placed on top of the apparatus, followed by a glass plate and finally a 500 g weight. The transfer was allowed to proceed for 18 - 24 h. After blotting, the stack was disassembled. The gel was discarded and the nylon membrane was baked in an oven at 80°C for 2 - 2.5 h to immobilize the RNA on the membrane.

For hybridization, the blot was transferred to a hybridization tube and 15 mls of pre-hybridization solution (5 X SSC, 5 X Denhardt's, 50 % Formamide, 1 % SDS). Blots were prehybridized at 42°C in a hybridization oven for 2 h. Denatured labeled probe was subsequently added to the pre-hybridization solution and the blots were hybridized overnight at 42°C.

The next day, the hybridization solution was discarded and the blot was sequentially washed 3 times at room temperature for 15 min with 2 X SSC/0.1% SDS and 1 time at 65°C for 45 min with 0.1 X SSC/0.1% SDS. Blots were then analyzed by autoradiography. Autoradiographs were quantified by scanning densitometry. The cDNA probes were removed by washing (95 °C for 3 min in DEPC H_2O), to allow reprobing of the membranes with the human alpha-tubulin cDNA to control for equal loading between lanes. Band intensities for PGHS-1 were divided with the corresponding α-tubulin intensity.

2.2.1.15 Protein isolation and estimation of concentration

Pelleted cells were washed 3 times (0.1 M Tris pH 7.4) and resuspended in 100 to 200 µl of 0.1 M Tris pH 7.4 and kept on ice. Cells were lysed by sonication for 5 s at a power setting of 80 % (Microson, Ultrasonic cell disrupter). Protein concentrations were determined using the Bio-Rad

protein assay kit based on the method described by Bradford (214). Standards were prepared by diluting a known concentration of BSA. 1 ml of diluted dye reagent (diluted 5 fold with ddH_2O) was added to 20 µl of diluted standard or sample (diluted 10 - 50 fold) and incubated at room temperature for 10 min. The absorbance was measured at 595 nm with a spectrophotometer. A standard curve was constructed by plotting the known standard concentrations against their absorbance values. This standard curve was used to interpolate sample protein concentration values.

2.2.1.16 SDS-PAGE and Western blotting

Lysate protein (50 µg) in 30 µl of ddH_2O was diluted in 4 X protein sample loading buffer (125 mM Tris-HCl (pH 6.8), 2% SDS, 4% β-mercaptoethanol, 40% glycerol, 0.02% bromophenol blue) and boiled for 3 min. Protein samples were size-separated on 10% SDS-PAGE gels by the method of Laemmli (186) using a Bio-Rad mini gel apparatus. A High Molecular Weight size marker (containing - rabbit muscle myosin (205 kDa), *E.coli* β-galactosidase (116 kDa), rabbit muscle phoshorylase β (97 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and bovine erythrocyte carbonic anhydrase (29 kDa)), as well as purified PGHS-1 protein (70 kDa) was run with each gel. Separated samples were electrophorectically transferred to nitrocellulose membrane (Bio-Rad) at 150 Volts for 30 min.

Following transfer, each blot was blocked overnight at 4 °C (3 % Milk/TBST). Blots were washed with 1% Milk/TBST (3 changes over 30 min) and probed for 1.5 h with a primary antibody specific for amino acids L272-Q283 of the PGHS-1 enzyme (1:1900 in 1% Milk/TBST). After washing with 1% Milk/TBST (3 changes over 30 min), the blots were probed with horseradish

peroxidase-conjugated secondary antibody (1:2000 in 1% Milk/TBST) for 1 h. Blots were then washed with 1% TBST (3 changes over 30 min) and bound secondary antibody was detected with enhanced chemiluminescence (ECL kit from Boehringer Mannheim).

2.2.2 Specific methods

2.2.2.1 MEG-01 and U937 cell culture standard growth conditions

The human megakaryoblastic cell line MEG-01 was cultured in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS) and in the absence of antibiotics. Cultures were maintained at 37 °C in 5% CO_2. Cultures were seeded at $2X10^5$ cells/ml (100 mls) and allowed to grow for 4 days, after which the floating cells were readjusted to $2X10^5$ cells/ml. 3 days later, the floating fraction was again adjusted to $2X10^5$ cells/ml. 24 hours later, flasks were stimulated with 12-O-tetradecanoylphorbol 13-acetate (TPA) and incubated (37 °C, 5% CO_2) for various time intervals The human monocytic cell line U937 was obtained from the American Type Culture Collection (Rockville, MD). Cells were passaged twice a week in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS) and maintained at 37 °C in 5% CO_2 in the absence of antibiotics. Cells were adjusted to $1X10^6$ cells/ml in the presence of 10 nM TPA and incubated for 3 days to differentiate into macrophages. Non-adherent cells were removed and the remaining adherent cells were quiesced in fresh complete RPMI 1640 medium in the absence of TPA for 24 hours before stimulation with LPS. Differentiated macrophages were stimulated with 50 ng/ml lipopolyssacharide (LPS) for 4 hours, after which they were harvested for RNA isolation. To demonstrate that LPS-induced 4.5 kb

PGHS-2 mRNA is glucocorticoid sensitive, differentiated macrophages were incubated with 1 μM Dexamethasone for 4 hours prior to LPS stimulation..

2.2.2.2 TPA stimulation of cell cultures

TPA was dissolved in dimethylsulfoxide (DMSO) at 1 mg/ml and stored at - 20 °C. Prior to use, TPA was diluted in RPMI 1640 and added to each culture. Control cells were treated with the same corresponding DMSO concentration that TPA treated cells received. Cells were incubated for various time intervals, counted for viability and harvested for RNA or protein.

2.2.2.3 Poly $(A)^+$ mRNA half-life studies

Cells were grown and treated with TPA (1.6×10^{-8} M) as previously described. After two days of TPA stimulation, the transcriptional inhibitors actinomycin-D (7.97×10^{-6} M) or 5,6-dichloro-1-B-D-ribofuranosylbenzimidazole (DRB) (40 mM) were added to the TPA stimulated cultures. Cells were processed for total and poly $(A)^+$ mRNA isolation 0, 4, and 8 h after addition of the transcriptional inhibitors.

2.2.2.4 Membrane preparation for nuclear run-on transcription assays

Plasmid DNA (100 - 200 μg) was linearized with *Xho* I and purified by adding an equal

volume of phenol:chloroform (50% Tris-HCl (pH 8.0) equilibrated phenol and 50% chloroform), mixed well by inversion, and spun at 8500 x g for 10 minutes at room temperature. The aqueous phase was extracted again with an equal volume of chloroform. To precipitate the DNA, the aqueous phase from the second extraction was mixed with 0.1 volumes of 3 M NaOAc and 2.5 volumes of 100 % ethanol and incubated at -80°C for 30 min. The samples were centrifuged at 8500 x g for 20 min at 4°C to pellet the plasmid DNA. The ethanol was aspirated and the pellets were washed with 500 µl of ice-cold 70 % ethanol and centrifuged at 8500 x g at 4°C for an additional 5 min to remove excess salt. The supernatant was aspirated and the pellets were air dried in a 37°C heating block for 5 min. The DNA was resuspended in 100 µl of sterile dd$H_2$0. Next, an equal volume of 1 N NaOH was added and the samples were incubated at 65°C for 60 min, after which the tubes were cooled to room temperature. 1.6 volumes of 2 M ammonium acetate (pH 7.0) was added and the tubes were then cooled on ice. Precut biodyne (GibcoBRL) nylon membrane that had been pre-soaked in 2 M ammonium acetate (pH 7.0) was placed in the assembled slot blot apparatus (GibcoBRL) and 5 µg of plasmid DNA or genomic DNA was added to each slot. The slots were washed with 500 µl of 6 X SSC and the membranes were baked in an oven at 80°C for 2 - 2.5 h to immobilize the DNA on the membrane.

Prior to hybridization with the labeled run-on transcripts, membranes were pre-hybridized overnight in hybridization buffer (50 % formamide, 5 X SSC, 1 X Denhardt's, 0.2 % SDS, 0.4 mg/ml salmon sperm DNA) at 42°C.

2.2.2.5 Nuclear run-on transcription assays

Cells were grown and treated with TPA (10 ng/ml) as previously described. Cells were harvested under the following conditions 0, 4, 8 and 16 h after TPA stimulation. Cells were collected by centrifugation (1000 x g at 4°C for 5 min) and washed twice with ice cold Phosphate Buffered Saline (PBS) (137 mM NaCl, 3 mM $MgCl_2$, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.3). The pelleted cells were resuspended in 10 ml of pre-chilled NP-40 buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and 0.2% NP-40) and kept on ice for 10 min. The cells were centrifuged at 750 x g at 4°C for 5 min to collect the nuclei after which they were washed once in pre-chilled NP-40 buffer. The washed nuclei were resuspended in 200 µl of nuclei freezing buffer (50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 40% glycerol, 0.1 mM EDTA (pH 8.0), 1 mM PMSF) split into two aliquots and stored in small sterile, pre-chilled, siliconized eppendorf tubes at -80°C.

The transcription reactions were prepared by combining 100 µl of transcription reaction (10 mM Tris-HCl (pH 8.0), 2.5 mM Dithiothreitol, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM $MnCl_2$, 1 mM GTP, 1 mM ATP, 1 mM CTP, 250 µCi [α-P^{32}] UTP, 1 mM PMSF and 25 Units of RNase inhibitor) with a 100 µl thawed nuclei aliquot, which were then incubated at 27°C for 45 min. Next, 20 units of RNase free DNase I and 110 ng tRNA were added and the reactions were incubated for an additional 30 min at 37°C. The reaction mixture was then transferred to a new sterile, siliconized, screw cap tube and an equal volume of TRIzol reagent (GibcoBRL) and 0.1 volumes of chloroform. Next, the reaction was vortexed and incubated on ice for 15 min, followed by centrifugation at 8500 x g at 4°C for 10 min. The supernatant was transferred to a new tube and combined with 500 µl of isopropanol and incubated at -20°C for overnight. The RNA is pelleted by centrifugation at 8500 x

g at 4°C for 10 min and washed once with 70 % ethanol to remove any excess salts. The pelleted RNA is finally resuspended in 500 µl of hybridization solution (50 % formamide, 5 X SSC, 1 X Denhardt's, 0.2 % SDS, o.4 mg/ml salmon sperm DNA) and heated at 65°C for 15 min. A 10 µl aliquot is removed for Cerenkov counting and the remaining reaction is denatured at 85°C for 10 min and then hybridized with the prepared nylon membranes for 48 h at 42°C. Following the 48 h incubation, the hybridization solution was discarded and the membranes were sequentially washed 3 times at room temperature for 15 min with 2 X SSC/0.1% SDS and 1 time at 65°C for 45 min with 0.1 X SSC/0.1% SDS. Membranes were then analyzed by autoradiography. Autoradiographs were quantified by scanning densitometry.

2.2.2.6 Assay of PGHS activity

Following treatment, MEG-01 cells were harvested in PBS using a rubber policeman, collected by centrifugation at 1200 x *g* for 5 minutes and resuspended in RPMI 1640 without serum before incubation with a final concentration of 10 µM 1-[^{14}C]arachidonic acid (42 µCi/mmol) for 15 minutes at 37°C. After incubation, cells were removed by centrifugation (1200 x *g* for 5 minutes), and the radioactive products present in the supernatant were extracted with ethyl acetate/methanol/citric acid 0.2M; (30:4:1, v:v:v) and analyzed by thin layer chromatography (TLC) and autoradiography. Proteins in the cell supernatant were precipitated by adding 1.5 ml of ice-cold acetone. After centrifugation (2000 x *g*, 10 minutes) to remove precipitated proteins, the resulting supernatant was acidified with 0.2 ml of 0.2 M citric acid and the aqueous phase extracted with 1.5 ml of of the following mixture: ethyl acetate/methanol/citric acid 0.2M; (30:4:1, v:v:v) . The organic

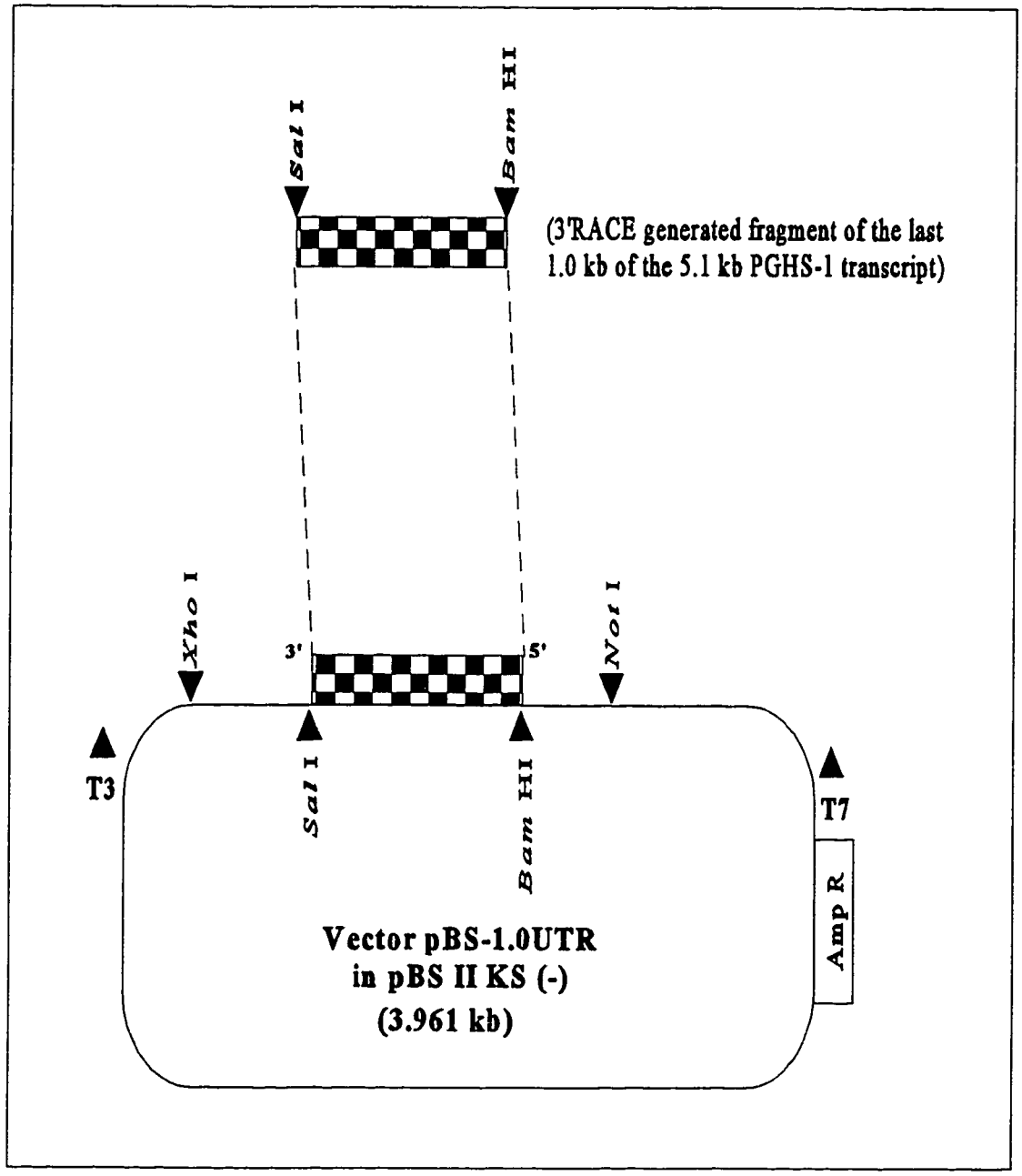
phase was evaporated under nitrogen and the residue dissolved in 50 µl of the TLC solvent before spotting on a Silica Gel 60 thin layer chromatography plate. The lipid products were chromatographed twice for 1 hour in benzene/dioxane/formic acid/acetic acid (82:14:1:1, v:v:v:v). The thin layer chromatography plate was then air-dried and subjected to autoradiography for 40 hours using Kodak XAR-5 film. Cold TxB_2 standard (Cayman Chemical Co.) visualized with iodine vapor was used to confirm the identity fo the major prostanoid produced by the MEG-01 cells; TxB_2. Autoradiographic bands corresponding to TxB_2 were collected and quantified by measuring radioactivity in counts per minute (CPM). Signal density was normalized to total protein.

2.2.2.7 Vector design and construction

2.2.2.7.1 Cloning of the last 1.0 kb of the PGHS-1 3'UTR

3'-RACE was used to amplify the last 1.0 kb of the 5.1 kb PGHS-1transcript, using the primer PGS1UTR (Table 2.1). In the original reverse transcriptase reaction, the oligo dT anchor primer produced a *Mlu* I, *Cla* I and a *Sal* I site in the 3' end and a single *Bam* HI site is present in the 5' end of the cDNA fragment. The 1.0 kb PCR fragment was cohesive end ligated into the *Bam* HI - *Sal* I site of pBS II KS (-) (Figure 2.1). The presence and orientation of the insert was tested by restriction digests with various enzymes. The sequence of the fragment was determined using the primers T3, T7 and INT-1 (Table 2.1). Sequencing of the fragment was contracted out to the University of Ottawa Biological Research Institute (BRI).

Figure 2.1 **Design and production of Vector pBS-1.0UTR for cloning (by 3'RACE) the last 1.0 kb of the 5.1 kb PGHS-1 message into the *Bam* HI - *Sal* I site of pBS II KS (-).**


Sal I
Bam HI
(3'RACE generated fragment of the last
1.0 kb of the 5.1 kb PGHS-1 transcript)
Xho I
3'
5'
Not I
T3
Sal I
Bam HI
T7
Amp R
Vector pBS-1.0UTR
in pBS II KS (-)
(3.961 kb)

SECTION THREE: RESULTS

3.1 CHARACTERIZATION OF A NOVEL PGHS-1 TRANSCRIPT WITH A TISSUE SPECIFIC PROFILE OF EXPRESSION

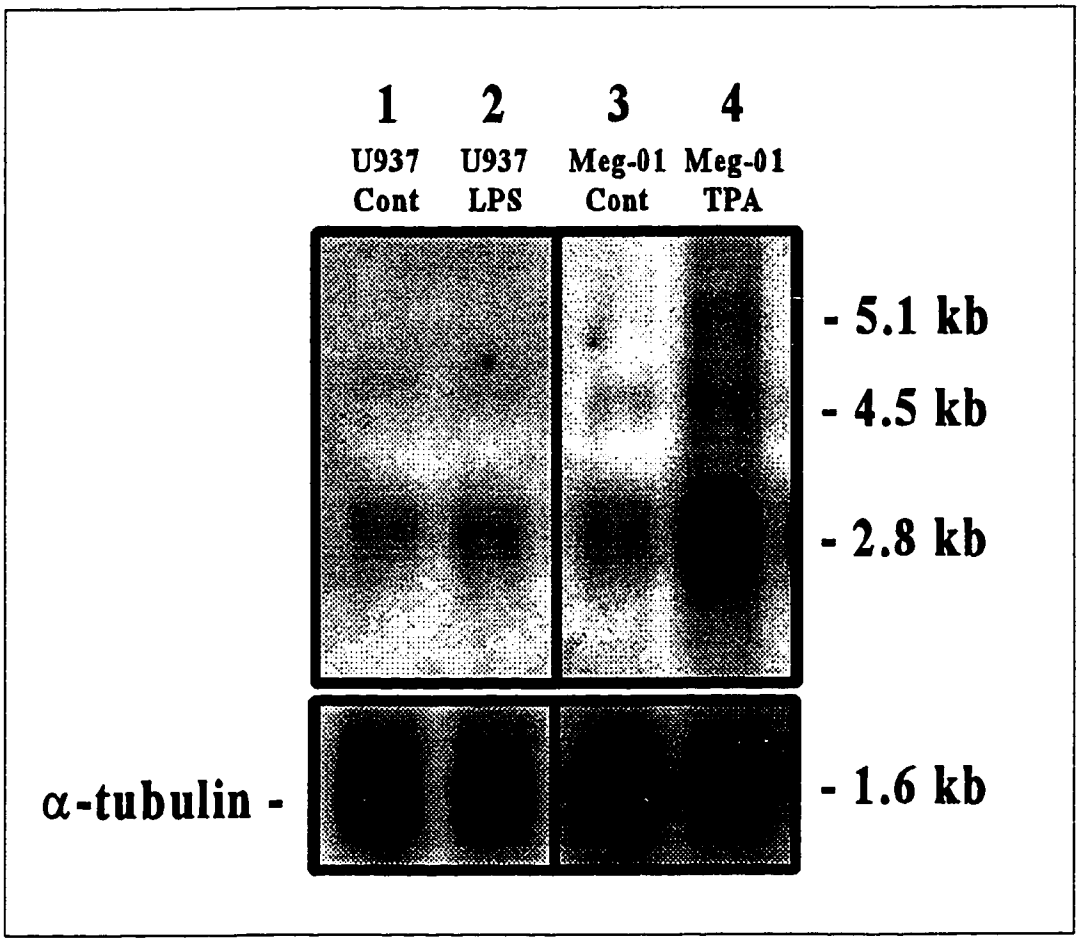
3.1.1 PGHS transcripts present in TPA treated MEG-01 cells

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. Using this approach with the entire coding region of the human PGHS-1 (hPGHS-1) gene, we detected transcripts of three different sizes in TPA treated MEG-01 cells: 5.1, 4.5 and 2.8 kilobases (kb) (Fig 3.1). In agreement with the literature, the most abundant PGHS-1 transcript is the 2.8 kb message (187). The 5.1 kb message is much less abundant since it contains a possible polyadenylation site used to produce the PGHS-1 2.8 kb message (51). Since 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 based on size and the fact that there is 73% homology between the coding regions of the two human PGHSs.

3.1.2 The 4.5 kb transcript is not a PGHS-2 transcript

The most obvious characteristic of the 4.5 kb PGHS-2 transcript is the transient expression pattern that is characterized by a maximal increase observed within two hours and a return to basal

Figure. 3.1 . **PGHS-1 transcripts detected in TPA treated MEG-01 cells and LPS treated U937 cells.** Representative autoradiograph of a Northern blot of poly $(A)^+$ RNA extracted from MEG-01 cells and U937 cells. MEG-01 cells were treated with 1.6×10^{-8} M TPA for 24 hours and U937 cells were treated with 50 ng/ml LPS for 4 hours, total RNA was then extracted and poly $(A)^+$ RNA was prepared from 150 μg of total RNA, resolved by gel electrophoresis, transferred onto nylon membrane and hybridized with the entire coding region of hPGHS-1 under high stringency conditions as described in the experimental methods section.


1
2
3
4
U937 Cont
U937 LPS
Meg-01 Cont
Meg-01 TPA
- 5.1 kb
- 4.5 kb
- 2.8 kb
α-tubulin -
- 1.6 kb

levels within 4 to 8 hours after stimulation (53). To investigate the expression pattern of the 4.5 kb transcript detected with the hPGHS-1 coding region, mRNA was measured at various time intervals after stimulation with TPA. The changes in mRNA levels during an eight 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 TPA treated MEG-01 cells. As shown in Figure 3.2A, the 4.5 kb message was observed in control cells at every time point over the 8 day period of study. The level of PGHS-1 2.8 and 4.5 kb transcripts increased in a time dependent fashion upon TPA (Fig 3.2B) stimulation. The 2.8 and 4.5 kb messages (Fig 3.2B) peaked after 24 hours of stimulation and remained essentially at that level over the remaining period of study. The profile of expression of the 4.5 kb transcript (Fig 3.2C) is therefore more similar to the profile of PGHS-1 transcript than to the profile of PGHS-2 mRNA expression, which is transient upon TPA stimulation (53).

Western blot analysis was conducted using antibodies specific for each hPGHS enzyme. For PHGS-2 analysis, hour time points were used since TPA induced PGHS-2 in NIH-3T3 cells occurs within hours. As shown in Figure 3.3, no detectable PGHS-2 protein was present in either control or TPA treated cells at any time over the period of study. Whereas, PGHS-1 protein was induced upon TPA treatment. The PGHS-1 standard in Figure 3.3A appears as a doublet. This is not an unexpected results as the PGHS-1 and -2 proteins are known to exist in various glycosylation states (69).

Figure. 3.2 **Time course of PGHS transcripts expressed in MEG-01 cells.** **A** and **B** are representative autoradiographs of Northern blots of poly $(A)^+$ RNA extracted from control (**A**) and TPA (**B**) treated MEG-01 cells. Panel **C** represents the quantification of three separate experiments and the values represent the ratio (+/- s.d.) of the intensity of the 2.8 or 4.5 kb PGHS-1 band over the α-tubulin intensity. Cells were treated with or without 1.6×10^{-8} M TPA for periods of time varying between 2 hours and 8 days as indicated.

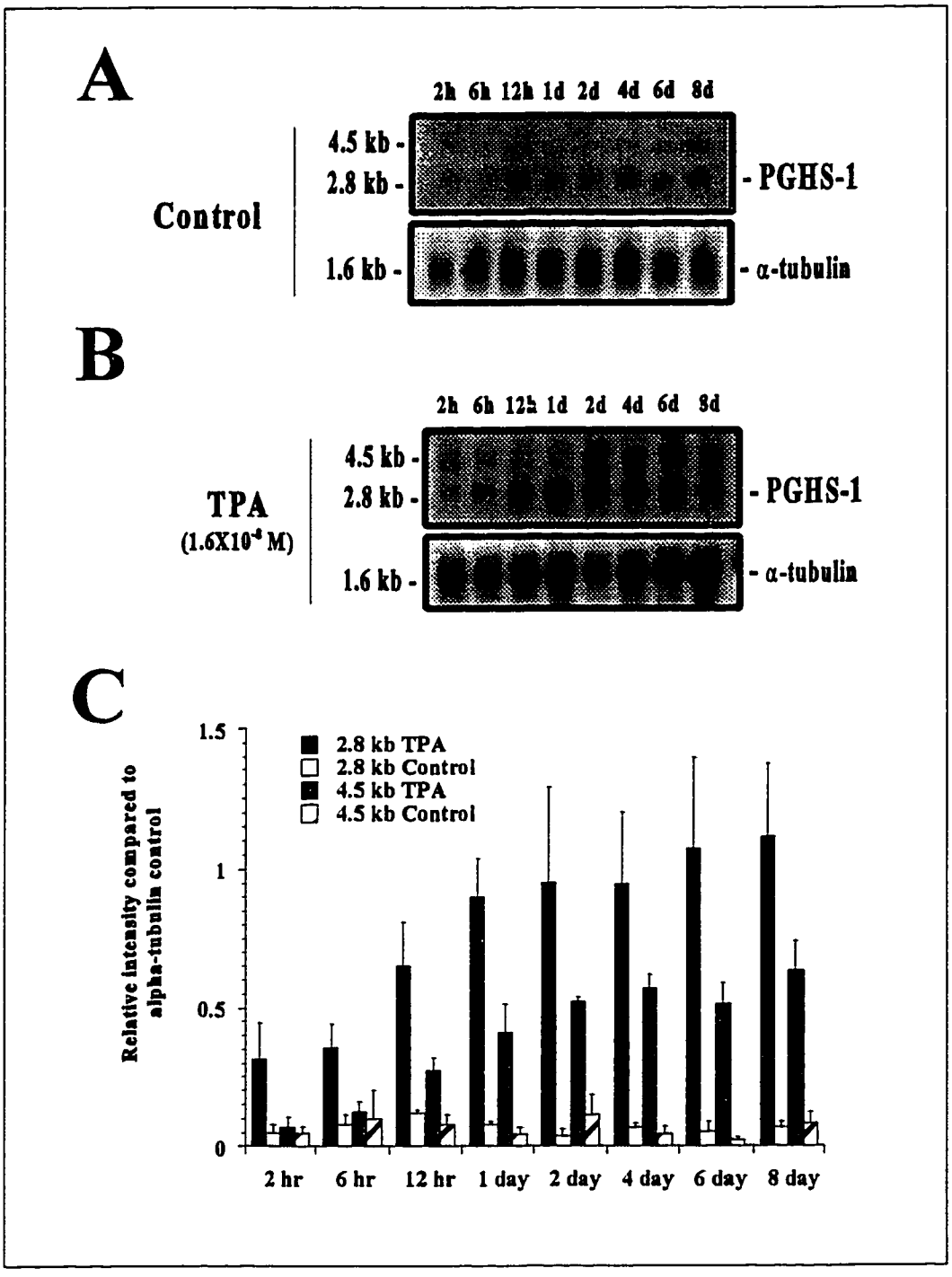
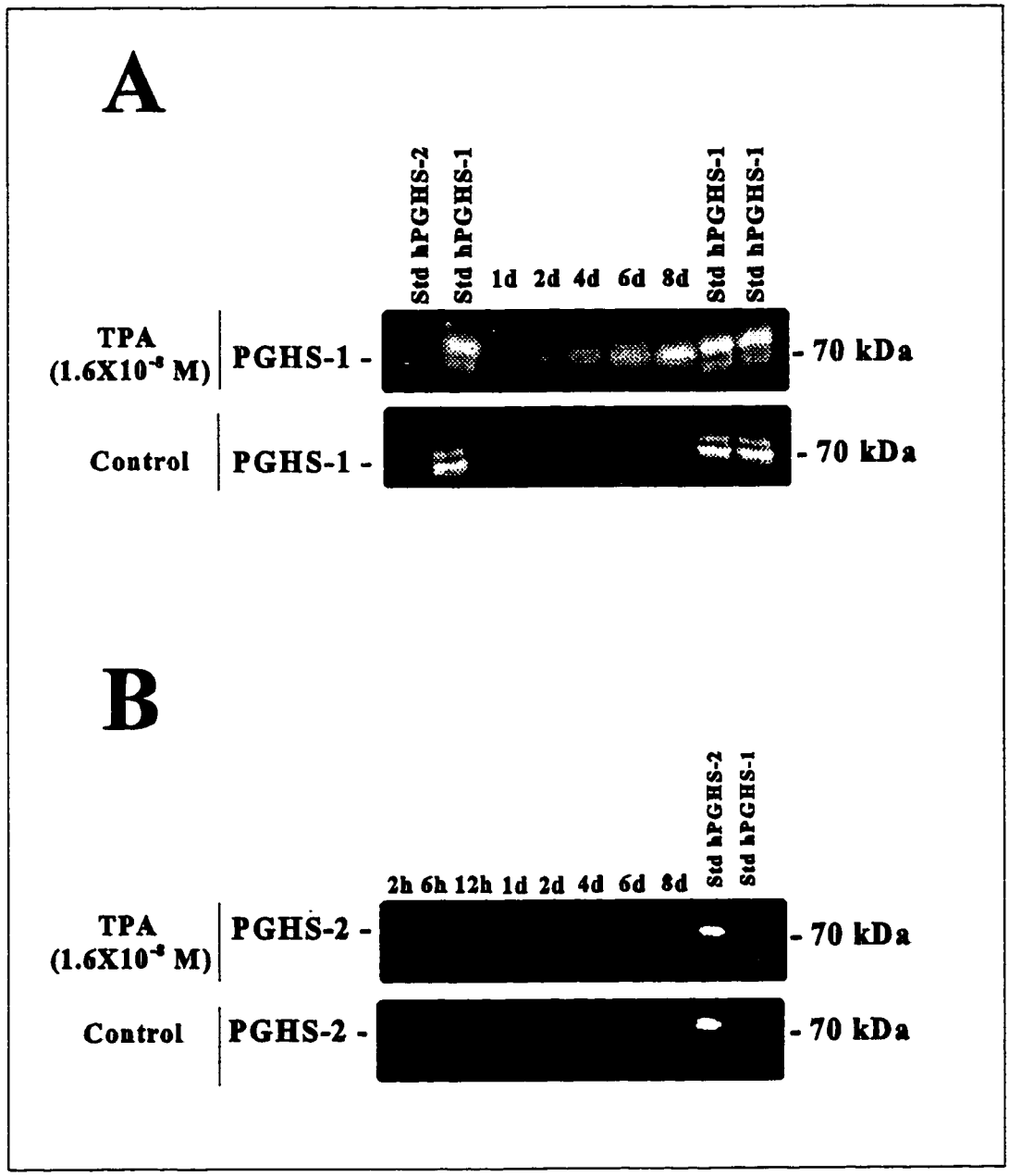

A
2h 6h 12h 1d 2d 4d 6d 8d
Control
4.5 kb -
2.8 kb -
- PGHS-1
1.6 kb -
- α-tubulin
B
2h 6h 12h 1d 2d 4d 6d 8d
TPA
(1.6X10⁻⁶ M)
4.5 kb -
2.8 kb -
- PGHS-1
1.6 kb -
- α-tubulin
C
Relative intensity compared to alpha-tubulin control
1.5
1
0.5
0
2.8 kb TPA
2.8 kb Control
4.5 kb TPA
4.5 kb Control
2 hr
6 hr
12 hr
1 day
2 day
4 day
6 day
8 day

Figure. 3.3 **Time course of PGHS-1 and PGHS-2 protein expression in MEG-01 cells.** Representative photographs of Western blots from MEG-01 cells. Cells were treated with or without 1.6×10^{-8} M TPA for various times, harvested and sonicated. Fifty micrograms of total protein was resolved by SDS-PAGE, transferred onto nitrocellulose membranes and probed with specific PGHS-1 (directed against amino acids L272-Q283 of the human PGHS-1 human enzyme) or PGHS-2 (directed against amino acids S584-K598 of the human PGHS-2 enzyme) antibodies. The specificity of the PGHSs antibodies used is shown.


A
Std hPGHS-2
Std hPGHS-1
1d 2d 4d 6d 8d
Std hPGHS-1
Std hPGHS-1
TPA
(1.6X10⁻⁸ M)
PGHS-1 -
- 70 kDa
Control
PGHS-1 -
- 70 kDa
B
Std hPGHS-2
Std hPGHS-1
2h 6h 12h 1d 2d 4d 6d 8d
TPA
(1.6X10⁻⁸ M)
PGHS-2 -
- 70 kDa
Control
PGHS-2 -
- 70 kDa

3.1.3 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. Since the coding region of the human PGHS-1 and PGHS-2 gene are 73% homologous, the specificity of the coding regions used as probes to detect PGHS-1 or PGHS-2 sequences is limited even when high stringency conditions are used. Therefore, we designed probes specific for each of the PGHS-1 and PGHS-2 sequences in the 3'UTR where no homology is found between the two genes. The localization of the probes on the PGHS-1 and PGHS-2 transcripts are indicated in Figure 3.4. Using the coding region of hPGHS-1 as a probe, transcripts of 4.5 and 2.8 kb were detected in MEG-01 cells (Fig 3.5A). A monocytic cell line, U937 cells, which constitutively express PGHS-1 and can be induced to produce PGHS-2 transcript in abundance (188) was used as a control to determine the hybridization specificity of the PGHS-2 probes. When the hPGHS-2 coding region was used as a probe (Fig 3.5B), as expected, the U937 cells treated with LPS gave a major signal at 4.5 kb. The 4.5 kb signal was faint but detectable in U937 control and LPS + Dexamethasone treated cells suggesting that the 4.5 kb signal in U937 cells is a PGHS-2 message (Fig 3.5B). On the same blot, the coding region of hPGHS-2 recognized a faint signal at 4.5 kb in TPA treated MEG-01 cells but not in control cells. Because the 4.5 kb transcript in TPA 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 no homology is found (Fig 3.4). When Northern blots were hybridized with Probe 3 (PGHS-1 specific) (Fig 3.5C) 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)

Figure. 3.4 **A 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 A and B were used generate the specific PGHS-1 probe and Primers C and D were used generate the specific PGHS-2 probe. Digestion of the cloned PGHS-1 3'UTR with *Apa* I/*Xho* I produced Probe 5 and digestion with *Hinc* II produced Probe 6. Closed arrows indicate canonical polyadenlyation signals and open arrows indicate non-canonical polyadenylation signals.

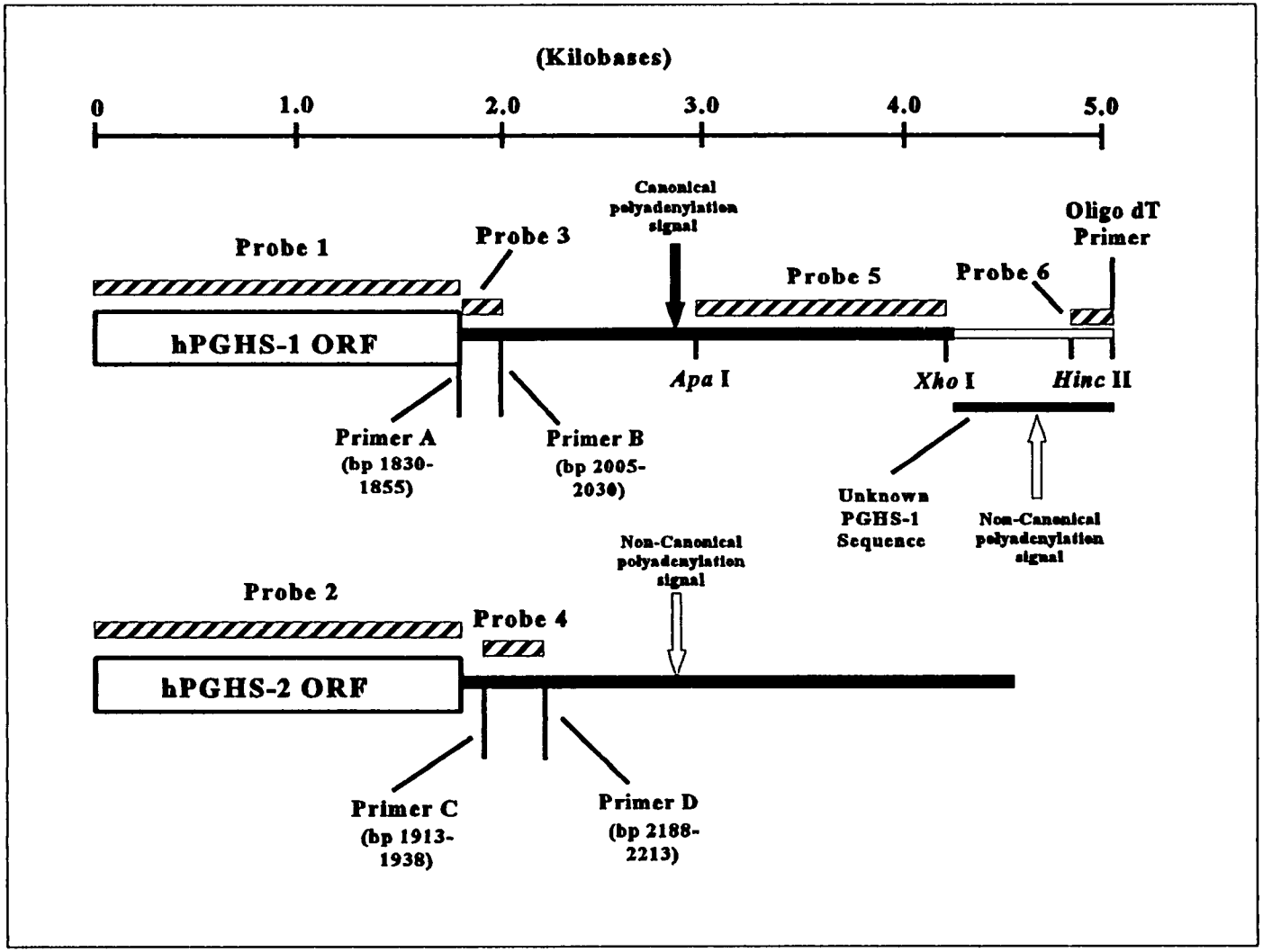
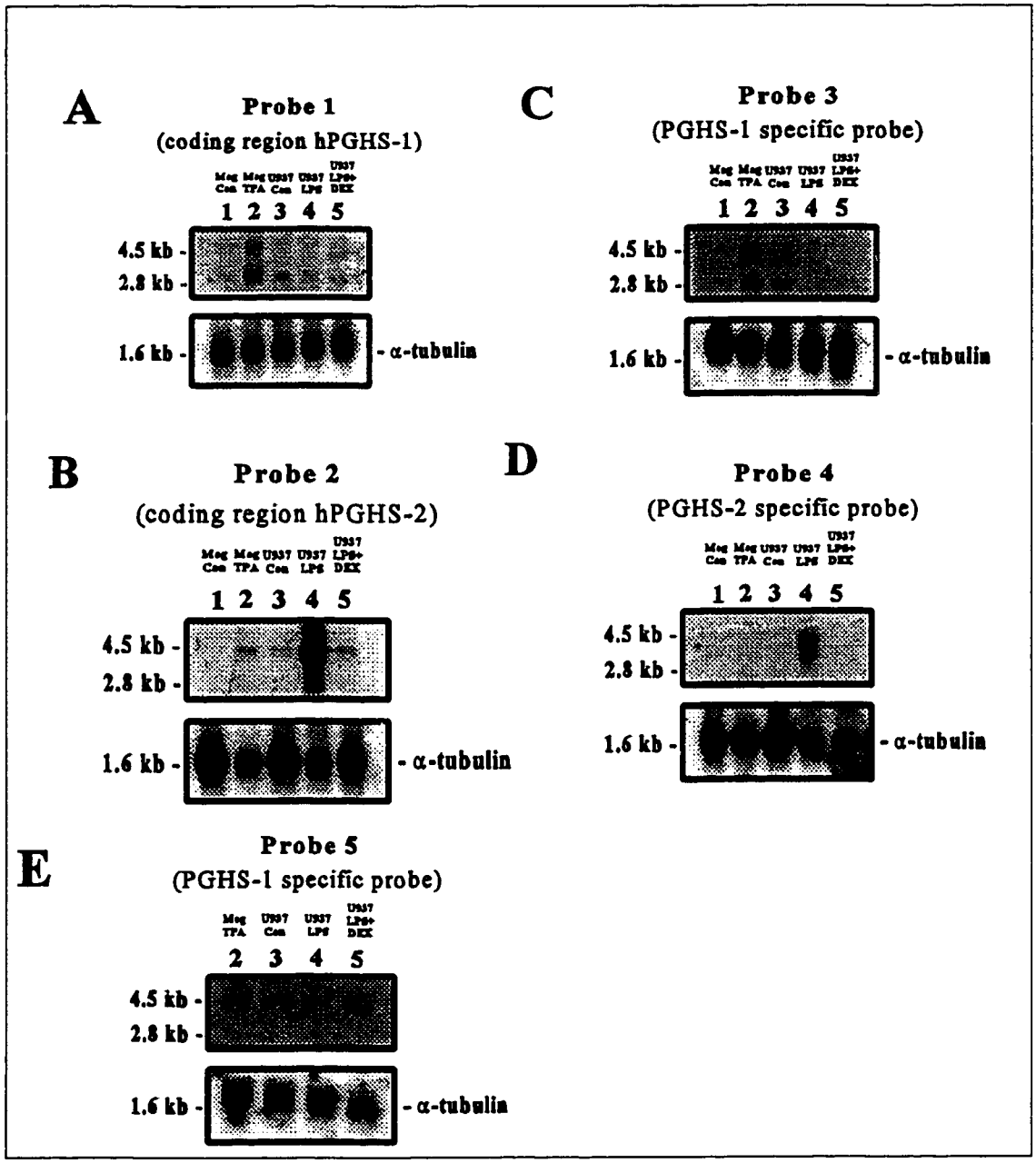

(Kilobases)
0
1.0
2.0
3.0
4.0
5.0
Canonical polyadenylation signal
Oligo dT Primer
Probe 1
Probe 3
Probe 5
Probe 6
hPGHS-1 ORF
Apa I
Xho I
Hinc II
Primer A (bp 1830-1855)
Primer B (bp 2005-2030)
Unknown PGHS-1 Sequence
Non-Canonical polyadenylation signal
Non-Canonical polyadenylation signal
Probe 2
Probe 4
hPGHS-2 ORF
Primer C (bp 1913-1938)
Primer D (bp 2188-2213)

Figure. 3.5 **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 Experimental Procedures. Probes used to analyze the resolved RNA are described in Figure 3.4 were as follows: (**A**) entire coding region of hPGHS-1; (**B**) entire coding region of hPGHS-2; (**C**) hPGHS-1 specific (3'UTR segment of hPHGS-1); (**D**) hPGHS-2 specific (3'UTR segment of hPGHS-2) and (**E**) hPGHS-1 specific (3'UTR segment of hPGHS-1). Each blots lanes correspond to the following: 1-Control MEG-01 cells, 2-TPA treated MEG-01cells, 3-Control U937 cells, 4-LPS treated U937 cells and 5-LPS + Dexamethasone treated U937 cells. Each was hybridized with α-tubulin to control for equal loading between lanes.

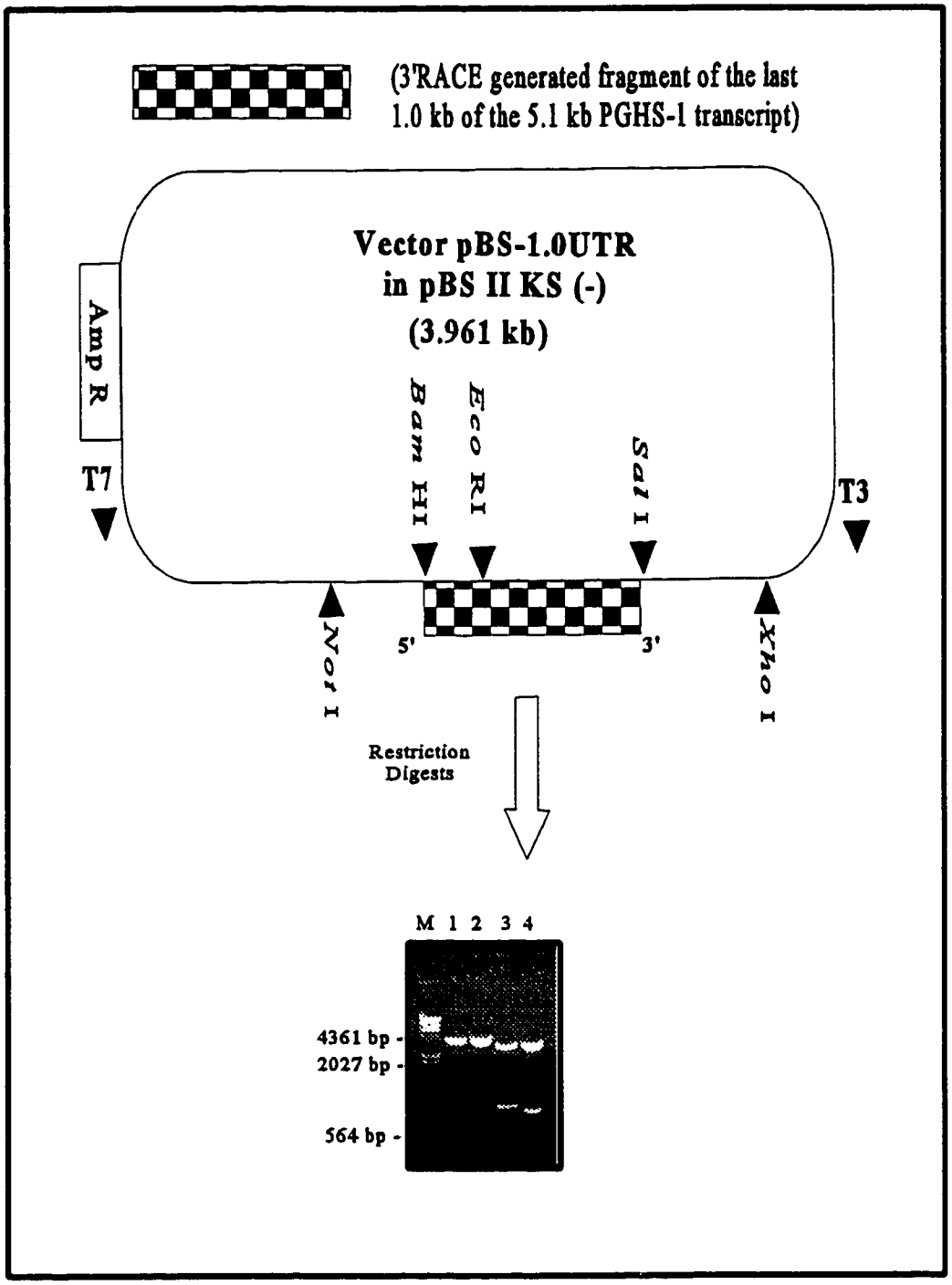

A
Probe 1
(coding region hPGHS-1)
Meg Con
Meg TPA
U937 Con
U937 LPS
U937 LPS+ DEX
1 2 3 4 5
4.5 kb -
2.8 kb -
1.6 kb -
- α-tubulin
B
Probe 2
(coding region hPGHS-2)
Meg Con
Meg TPA
U937 Con
U937 LPS
U937 LPS+ DEX
1 2 3 4 5
4.5 kb -
2.8 kb -
1.6 kb -
- α-tubulin
C
Probe 3
(PGHS-1 specific probe)
Meg Con
Meg TPA
U937 Con
U937 LPS
U937 LPS+ DEX
1 2 3 4 5
4.5 kb -
2.8 kb -
1.6 kb -
- α-tubulin
D
Probe 4
(PGHS-2 specific probe)
Meg Con
Meg TPA
U937 Con
U937 LPS
U937 LPS+ DEX
1 2 3 4 5
4.5 kb -
2.8 kb -
1.6 kb -
- α-tubulin
E
Probe 5
(PGHS-1 specific probe)
Meg TPA
U937 Con
U937 LPS
U937 LPS+ DEX
2 3 4 5
4.5 kb -
2.8 kb -
1.6 kb -
- α-tubulin

shows a single message at 4.5 kb only in LPS treated U937 cells and not in control or TPA treated MEG-01 cells (Fig 3.5D). This suggests that the PGHS-2 gene is not expressed in MEG-01 cells either before or after TPA stimulation. 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 (Fig 3.4). As shown in Figure 3.5E, only a 4.5 kb transcript was detected in TPA treated MEG-01 cells. Probe 5 did not detect the PGHS-1 2.8 kb message in TPA treated cells since the Probe 5 sequence is not present in the 2.8 kb transcript. Having established that the 4.5 kb transcript is indeed a PGHS-1 and not a PGHS-2, we investigated how the 4.5 kb PGHS-1 message could be produced.

3.1.4 Mechanism for the generation of the 4.5 kb PGHS-1 transcript

To determine if the 4.5 kb PGHS-1 message is 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. To construct the vector pBS-1.0KBUTR, we derived a specific hPGHS-1 oligonucleotide (Primer PGHS1UTR) upstream of a unique *Bam* HI site at position 4103 of the 5.1 kb cDNA. This oligonucleotide together with degenerated oligo dT primers were used to amplify cDNA from TPA treated MEG-01 cells. The generated 3'-RACE product was confirmed by gel electrophoresis. The gel purified PCR fragment was then ligated into the *Bam* HI - *Sal* I site of pBS II KS (-) to produce the vector pBS-1.0KBUTR, as illustrated in Figure 3.6. Following transformation, the individual colonies bearing the vector pBS-1.0KBUTR were identified by digestion with various restriction enzymes (*Bam* HI, *Eco* RI, *Bam* HI/Sal I and *Eco* RI/ *Sal* I) to see

Figure 3.6 **Conformation of Vector pBS-1.0UTR.** The results from the diagnostic restriction digests of Vector pBS-1.0UTR are shown. **M** represents the marker lane, **1** represents a *Bam* HI digest, **2** represents a *Eco* RI digest, **3** represents a *Bam* HI/*Sal* I digest and **4** represents a *Eco* RI/*Sal* I digest.


(3'RACE generated fragment of the last 1.0 kb of the 5.1 kb PGHS-1 transcript)
Vector pBS-1.0UTR
in pBS II KS (-)
(3.961 kb)
Amp R
T7
T3
Bam HI
Eco RI
Sal I
Not I
Xho I
5'
3'
Restriction Digests
M 1 2 3 4
4361 bp -
2027 bp -
564 bp -

if fragments of predicted sizes could be released. As expected, *Bam* HI produced a 3.8 kb fragment, *Eco* RI produced a 3.8 kb fragment, *Bam* HI/Sal I produced 2.9 and 0.95 kb fragments and *Eco* RI/ *Sal* I produced fragments of 2.9 kb and 0.9 kb, as illustrated in Figure 3.6.

3.1.4.1 Analysis of the cloned PGHS-1 3'UTR sequence

Shown in Figure 3.7 is the 907 bp consensus sequence produced by 3'-RACE experiments. The consensus sequence was obtained by comparing sequences of 3 separate clones 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. In the cloned sequence, we identified a non-canonical polyadenylation signal (AAGAAA) located 12 bp upstream of a cleavage site (CA) that generates the 4.5 kb PGHS-1 transcript detected in MEG-01 cells. Two G/U-rich or U-rich downstream elements (DSE) located 34 and 46 bp downstream of the cleavage site were also identified in the generated novel sequence.

3.1.5 Tissue expression profile of the 4.5 kb PGHS-1 transcript

Using a commercial mRNA dot blot containing poly $(A)^+$ RNA from 50 different human tissues (obtained from several different patients and pooled), we observed a tissue specific expression pattern of the 4.5 kb PGHS-1 transcript. Comparison between the different tissues was performed after normalization for variations in loading using the ubiquitin control cDNA probe provided (Fig 3.8C). As shown in figure 3.8A, using Probe 5 (Fig 3.4) (which detects the PGHS-1 4.5 and 5.1 kb

Figure. 3.7 **Cloned sequence of the last 1.0 kb of the hPGHS-1 3'UTR.** The unknown 1.0 kb sequence on the 3'UTR end of the hPGHS-1 5.1 kb transcript was generated by 3'-RACE, cloned into the multiple cloning site of pBS II KS (-) and sequenced using the T3 and T7 primers. The identity of the clone was obtained by an overlapping 57 bp stretch of the reported PGHS-1 3'UTR sequence (highlighted 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 by a solid box) and (3) a GT rich elements downstream (underlined by a dashed boxes) of the cleavage site.

1 GGATCCCTGCCCTTCTCAAGCACTTTAGCTTTTCCTTCCATCCGGTGGC

51 TATTCCAGGAATTCCTCTTTTGCTTAAATCAGTTGGAGTTTGTGTCTGT

101 GCTTGTAATCAAGCCTTTATGGCTGCTGGGCTGAGTGACACAAGCACTT

151 AATGGCCTGGAGGGACTTTTAATCAGTGAAGATGCAATCAGACAAGTGT

201 TTGGAAAGAGCACCCTCGAGAAGGGTGGATGACAGGGCAGAGCAGGAAGG

251 ACAGGAAGCTGGCAGAACGGAGGAGGCTGCAGCCGTGGTCCAACCAGGAG

301 CTGATGGCAGCTGGGGCTAGGGGAAGGGCTTTGAGGGTGGAAGGATGGG

351 TGGGTCCAGAGGTATTCCTCTCTTAAATGCAAGTGCCTAGATTAGGTAG

401 CTTTGCTTAGTATTGACAACTGCACATGAAAGTTTTGCAAAGGGAAACAG

451 GCTAAATGCACCAAGAAAGCTTCTTCAGAGTGAAGAATCTTTAATGCTTG

501 TAATTTAAACATTTGTTCCTGGAGTTTTGATTTGGTGGATGTGATGGTTG

551 GTTTTATTTGTCAGTTTGGTTGGGCTATAGCACACAGTTATTTAATCAA

601 CAGTAATCTAGGTGTGGCTGTGAAGGTATTTTGTAGATGTGATTAACAT

651 TACAATCAGTTGACTTTAAGTGAAAGAGATTACTTAAATAATTTGGGTG

701 GCTGCACCTGATTAGTTGAAAGGCCTCAAGAACAAACACTGCAGTTTCC

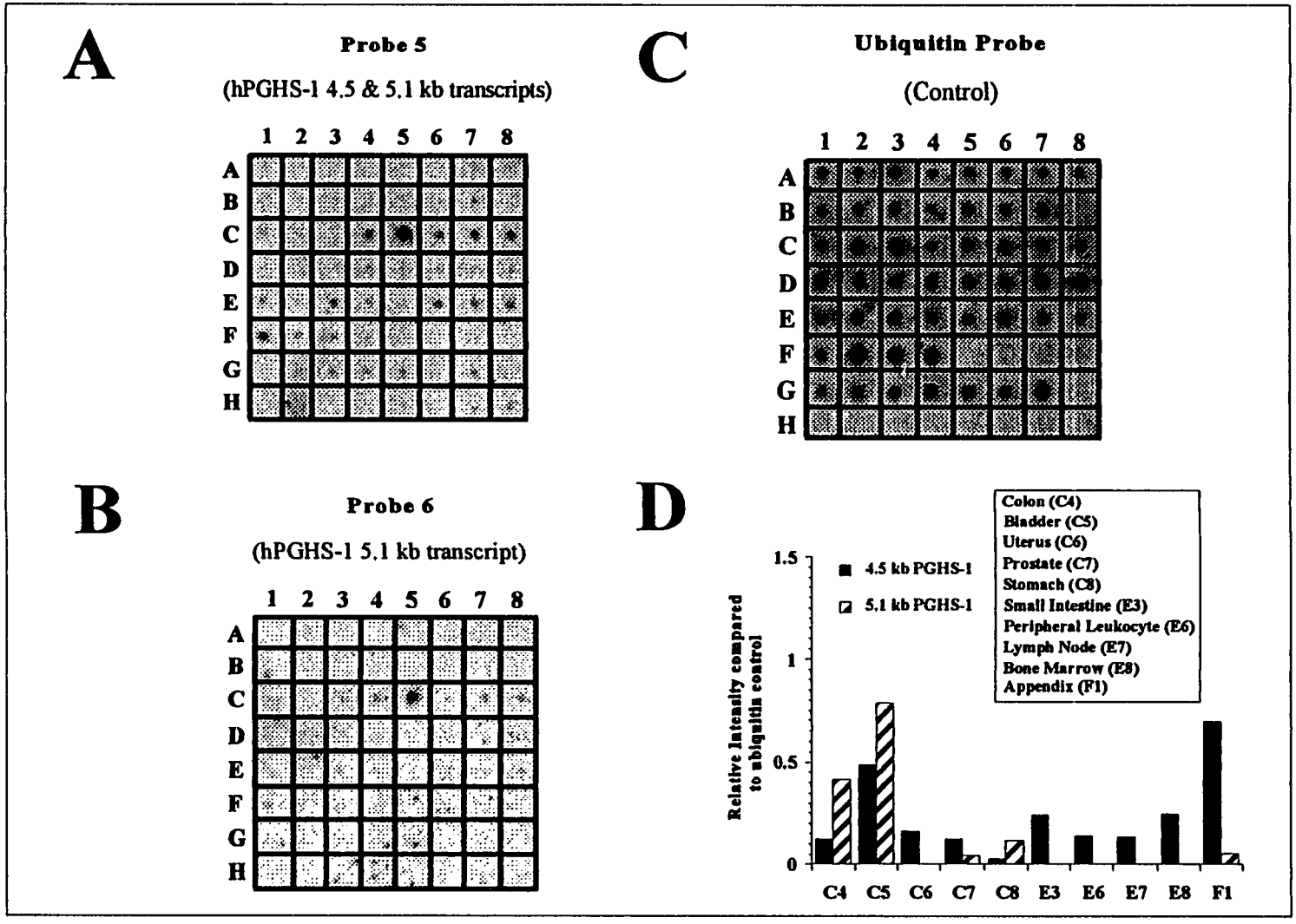
751 GGAAAAGAAGAAACTTTGCCTCAAGACTATAGCCATCGACTCCTGCCTG

801 GTTTCCAGCCTGCTAGTCTGCCCTATGGATTTGAAGTTTGCCAACCCCAA

851 CAATTGTGTGAATTAATTTCTAAAAATAAAGCTATATACAGCCAAAAAA

901 AAAAAAA

Figure. 3.8 **Tissue specific expression of the hPGHS-1 4.5 and 5.1 kb transcripts.** Autoradiogram of a RNA dot blot containing poly $(A)^+$RNA from 50 different human tissues. The membrane was incubated with Probe 5 (**A**) which detects both the 4.5 and 5.1 kb PGHS-1 transcripts and with Probe 6 (**B**) which detects 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.

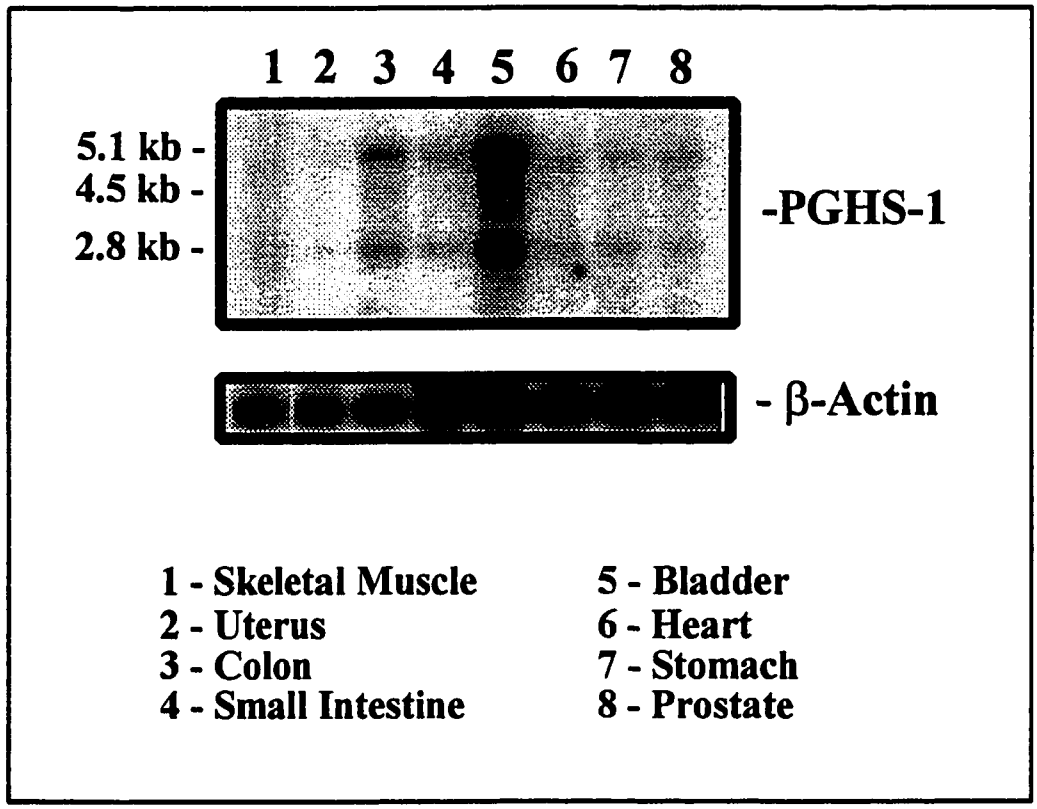

A
Probe 5
(hPGHS-1 4.5 & 5.1 kb transcripts)
1 2 3 4 5 6 7 8
A
B
C
D
E
F
G
H
B
Probe 6
(hPGHS-1 5.1 kb transcript)
1 2 3 4 5 6 7 8
A
B
C
D
E
F
G
H
C
Ubiquitin Probe
(Control)
1 2 3 4 5 6 7 8
A
B
C
D
E
F
G
H
D
Relative Intensity compared to ubiquitin control
1.5
1
0.5
0
4.5 kb PGHS-1
5.1 kb PGHS-1
Colon (C4)
Bladder (C5)
Uterus (C6)
Prostate (C7)
Stomach (C8)
Small Intestine (E3)
Peripheral Leukocyte (E6)
Lymph Node (E7)
Bone Marrow (E8)
Appendix (F1)
C4 C5 C6 C7 C8 E3 E6 E7 E8 F1

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 smaller intensity were also detected in the colon, small intestine, bone marrow, uterus, prostate, peripheral leukocyte, lymph node and stomach. When the membrane was hybridized with Probe 6 (Fig 3.4) 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 (Fig 3.8B). The signal obtained using Probe 6 was subtracted from the signal obtained with Probe 5 in order to determine the level of 4.5 kb PGHS-1 transcript, as demonstrated in Figure 3.8D. Although all probes are of different sizes and their affinites will differ, this was corrected for by probing with equal amounts of radioactively labelled probes.

3.1.6 Detection of the 4.5 kb PGHS-1 transcript using a multiple tissue Northern

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 is indeed generated in a tissue specific fashion. Figure 3.9 shows that the bladder contains detectable levels of the 4.5 kb PGHS-1 transcript along with the 5.1 and 2.8 kb transcripts. Whereas, the other tissues contain no detectable levels of the 4.5 kb PGHS-1 transcript. These results confirm 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. 3.9 **Tissue specific expression of the hPGHS-1 4.5 kb transcript.** Autoradiogram of a multiple tissue Northern blot containing 2 μg of poly $(A)^+$ RNA. The membrane was incubated with Probe 3 which detects the 2.8, 4.5 and 5.1 kb PGHS-1 transcripts. The β-Actin probe was used as a control for equal loading between lanes.


1 2 3 4 5 6 7 8
5.1 kb -
4.5 kb -
2.8 kb -
-PGHS-1
- β-Actin
1 - Skeletal Muscle
2 - Uterus
3 - Colon
4 - Small Intestine
5 - Bladder
6 - Heart
7 - Stomach
8 - Prostate

3.2 Regulation of PGHS-1 gene expression in MEG-01 cells

The following studies were performed before the characterization of the 4.5 kb PGHS-1 transcript was completed. Therefore, the PGHS-1 and PGHS-2 specific probes were not available, so all Northern blots were probed with the entire PGHS-1 coding region.

3.2.1 TPA stimulation markedly increases PGHS-1 mRNA and protein levels in MEG-01 cells

TPA stimulation increased PGHS-1 mRNA levels (2.8 and 4.5 kb) in a concentration-dependent fashion (Fig 3.10 A and B). For maximum PGHS-1 mRNA expression (2.8 and 4.5 kb), cultures were incubated with TPA for 1 day after which they were harvested for Northern analysis. PGHS-1 mRNA production (2.8 and 4.5 kb) was greatest at 0.16 X 10^{-8} M and remained elevated at the higher concentrations, as illustrated in Figure 3.10B. A concentration-dependent increase in PGHS-1 protein expression was also observed after TPA stimulation (Fig 3.11 A and B). For maximum protein expression, cultures were incubated with TPA for 8 days after which the cells were harvested for Western analysis. Protein induction appears to be less sensitive to TPA concentrations than mRNA, Figure 3.11B shows that PGHS-1 protein production was greatest at 1.6 X 10^{-8} M and remained elevated at the higher concentrations. Therefore, to obtain both maximal PGHS-1 mRNA and protein expression, a common TPA concentration of 1.6 X 10^{-8} M was selected. To confirm that the increased PGHS-1 protein is biologically active, TPA and control treated MEG-01 cell lysates were incubated with [1-^{14}C] arachidonic acid. Figure 3.12 demonstrates that the

Figure. 3.10 **Concentration-response relation of PGHS-1 mRNA accumulation in MEG-01 cells following TPA stimulation.** **A** (autoradiograph) shows a representative Northern blot of poly $(A)^+$ RNA extracted from 150 μg of total RNA from MEG-01 cells. Cells were treated with the indicated concentrations of TPA for 24 hours, RNA was extracted and hybridized with the ^{32}P labeled entire coding region of hPGHS-1 (1.8 kb), followed by a ^{32}P labeled DNA specific for α-tubulin to control for equal loading between lanes. RNA bands were quantified by Densitometric analysis arbitrary units of PGHS-1/α-tubulin mRNA are presented in **B** (graph) as the average +/- s.d. of three independent experiments.

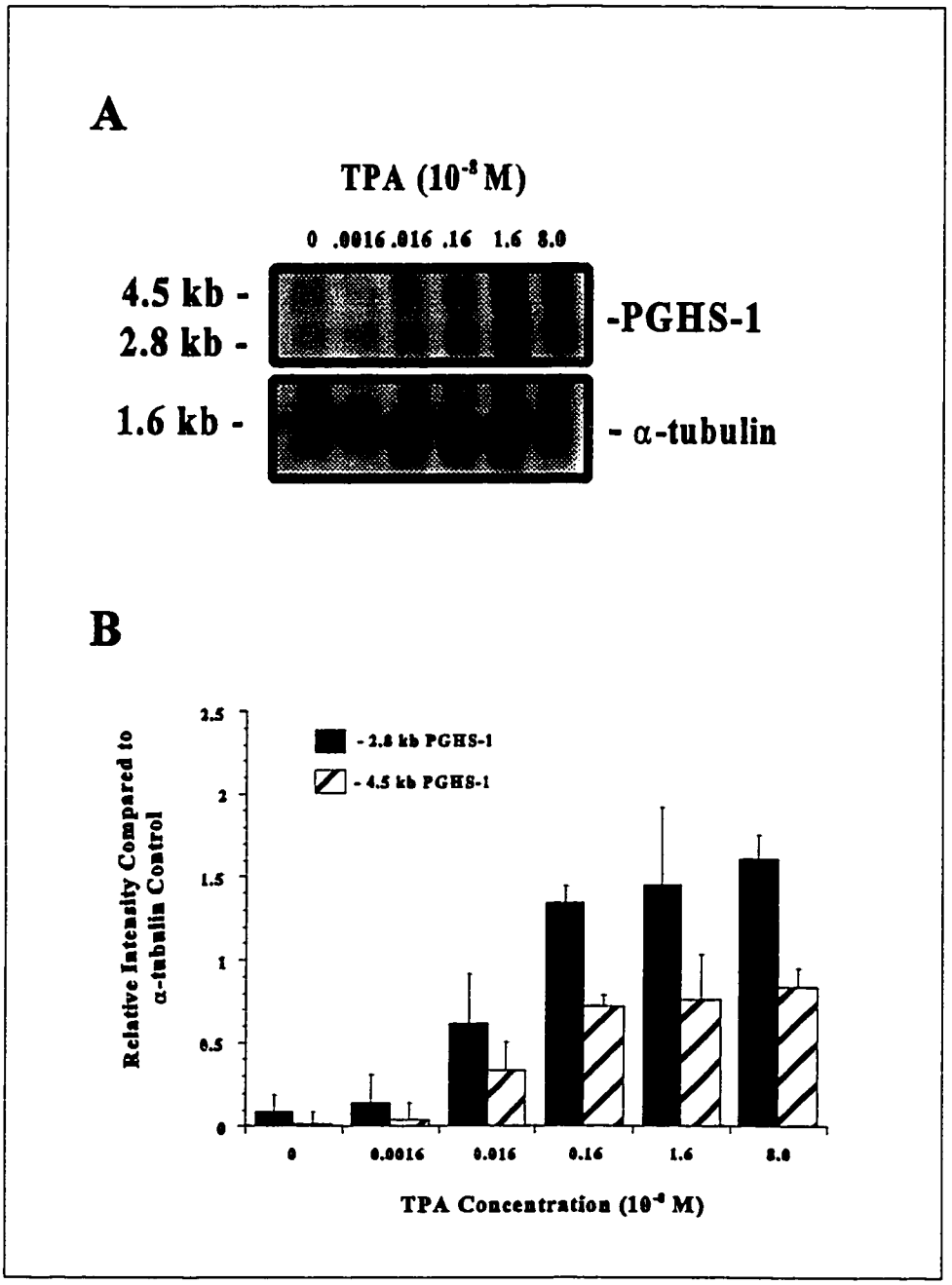
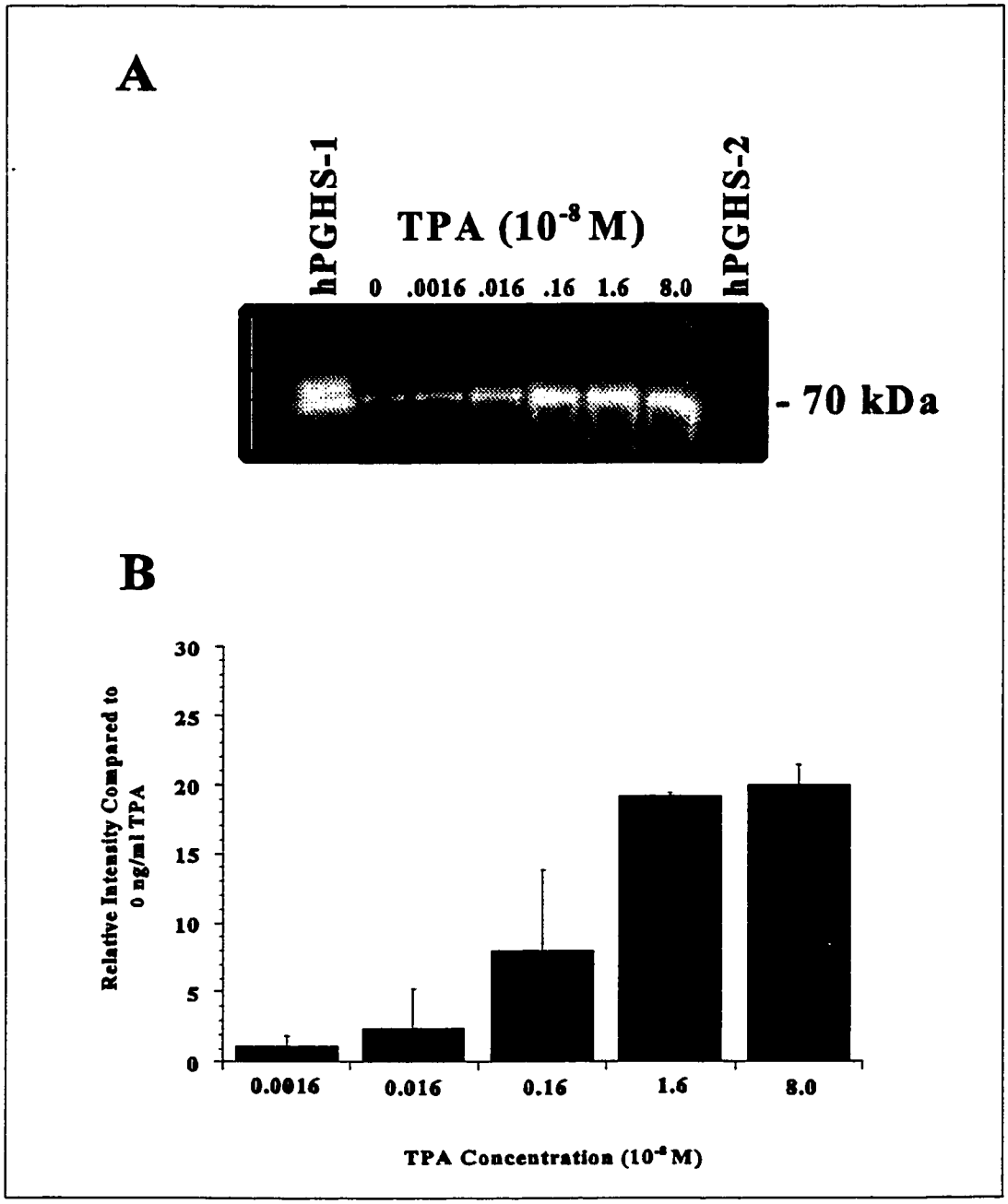

A
TPA (10^{-8} M)
0 .0016 .016 .16 1.6 8.0
4.5 kb -
2.8 kb -
-PGHS-1
1.6 kb -
- α-tubulin
B
Relative Intensity Compared to α-tubulin Control
- 2.8 kb PGHS-1
- 4.5 kb PGHS-1
2.5
2
1.5
1
0.5
0
0
0.0016
0.016
0.16
1.6
8.0
TPA Concentration (10^{-8} M)

Figure. 3.11 **Concentration-response relation of PGHS-1 protein accumulation in MEG-01 cells following TPA stimulation.** **A** (Photograph) shows a representative Western blot of SDS-PAGE separated proteins (from MEG-01 cells treated with the indicated TPA concentrations for 8 days) that were transferred onto nitrocellulose membranes. Membranes were probed with a PGHS-1 specific antibody directed against amino acids L272 to Q283 inclusively of the human enzyme. Protein bands were quantified by densitometric analysis and **B** (graph) presents the average of three separate experiments +/- s.d., with data represented as fold induction over the control sample.


A
hPGHS-1
TPA (10^{-8} M)
0 .0016 .016 .16 1.6 8.0
hPGHS-2
- 70 kDa
B
Relative Intensity Compared to 0 ng/ml TPA
30
25
20
15
10
5
0
0.0016
0.016
0.16
1.6
8.0
TPA Concentration (10^{-8} M)

increase in PGHS-1 protein correlates with an increase in thromboxane B_2 from the exogenous arachidonic acid, which is maximal eight days after TPA stimulation. This data suggests that TPA is able to induce a functionally active PGHS-1 enzyme in MEG-01 cells.

3.2.2 Pattern of PGHS-1 mRNA and protein expression in MEG-01 cells after TPA stimulation

To investigate the temporal expression of PGHS-1 in control and TPA treated MEG-01 cells, protein and mRNA was measured at various time intervals after stimulation. The changes in mRNA levels during an 8 day period were measured by Northern blot analysis. As shown in Figure 3.2A and C, the level of PGHS-1 mRNA (2.8 and 4.5 kb) in control cells remained at a constant constitutive level over the period of study. Whereas, the levels of PGHS-1 transcripts (2.8 and 4.5 kb) increased in a time dependent fashion upon TPA stimulation (Fig 3.2B and C). Both PGHS-1 messages (Fig 3.2B and C) peaked after 24 hours of stimulation and remained essentially at that level over the remaining period of study. To examine PGHS-1 protein expression the changes in protein levels during an 8 day period were measured in control and TPA (1.6×10^{-8} M) treated cells by Western blot analysis. In contrast to Northern analysis, Western blot analysis showed little PGHS-1 protein production in control cell. Whereas, after TPA treatment a time-dependant increase in PGHS-1 protein was observed with a maximum expression after 6-8 days (Fig 3.13A and B). Figure 3.14 is a schematic representation illustrating the pattern of PGHS-1 mRNA and protein expression in

Figure. 3.12 **PGHS-1 activity in TPA treated MEG-01 cells increased [^{14}C]Thromboxane B_2 formation from [^{14}C] arachidonic acid.** After incubation with or without 1.6 X 10^{-8} M TPA for 1 to 8 days as indicated, MEG-01 cells were harvested and washed before incubation with [^{14}C] arachidonic acid for 15 minutes at 37°C, as described in "Materials and Methods". Radioactive products in the supernatant were extracted and separated by thin layer chromatography as described in "Materials and Methods". This figure is representative of the average of 3 independent experiments +/- s.d.

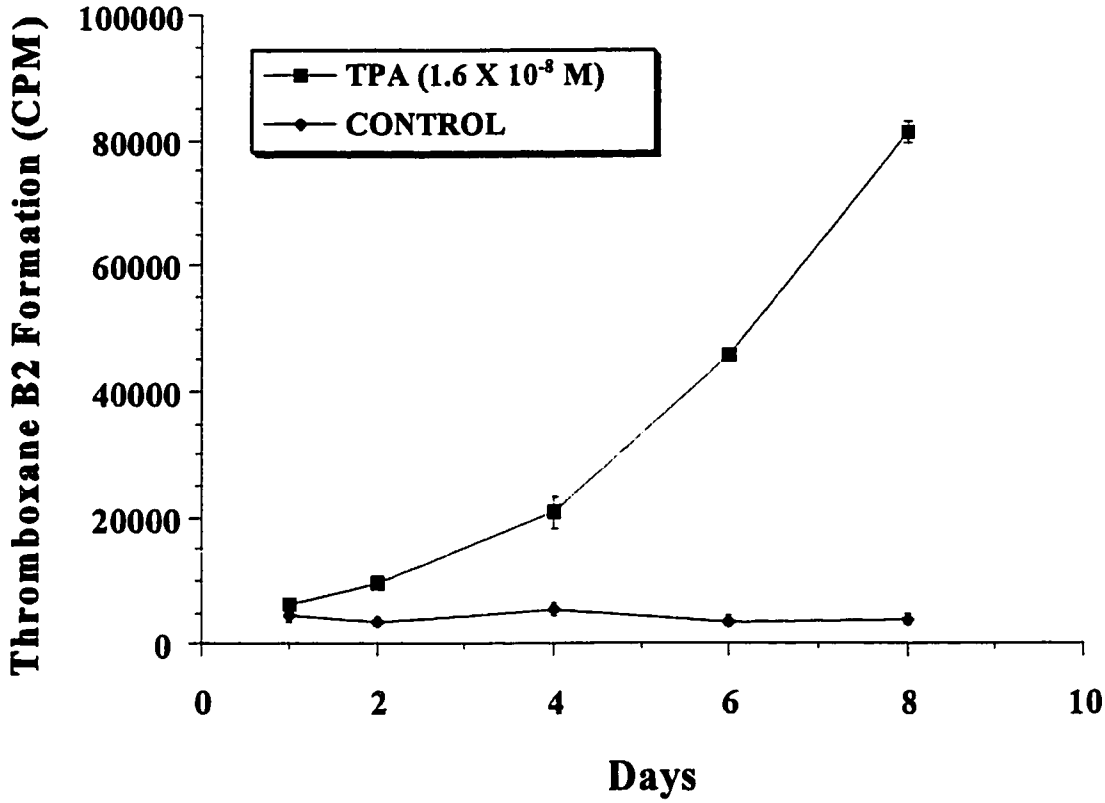

Thromboxane B2 Formation (CPM)
100000
80000
60000
40000
20000
0
TPA (1.6 X 10-8 M)
CONTROL
0
2
4
6
8
10
Days

Figure. 3.13 **Time course of PGHS-1 protein accumulation in MEG-01 cells after TPA stimulation.** **A** (Photograph) shows a representative Western blot of SDS-PAGE separated proteins (from MEG-01 cells treated with or without 1.6 X 10^{-8} M TPA for 1 day to 8 days) that were transferred onto nitrocellulose membranes. Membranes were probed with a PGHS-1 specific antibody directed against amino acids L272 to Q283 of the human enzyme followed by an antibody specific for β-actin, to ensure equal loading between lanes. Protein bands were quantified by densitometric analysis and arbitrary units of PGHS-1/β-actin protein are presented in **B** (graph) as the average of three separate experiments +/- s.d.

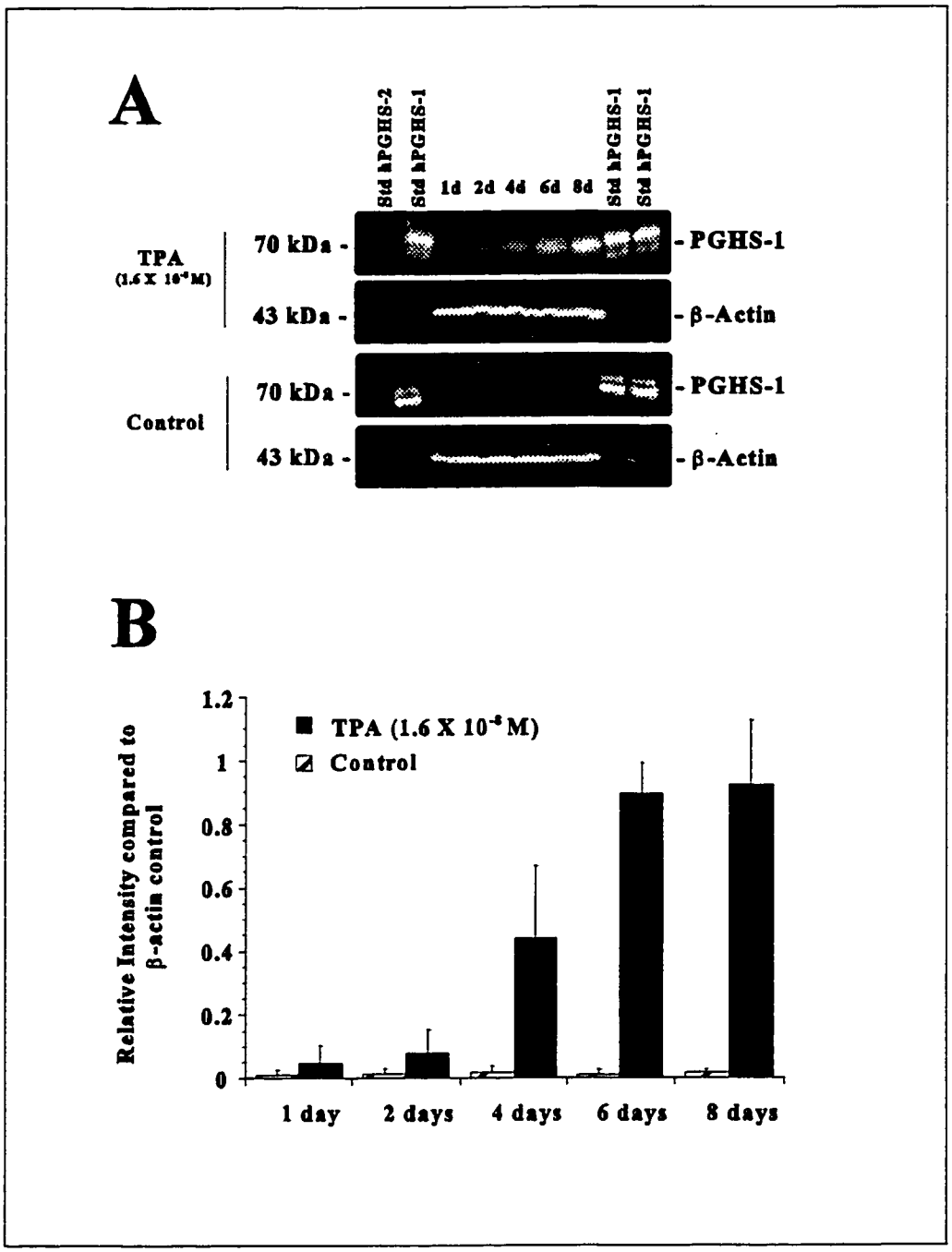
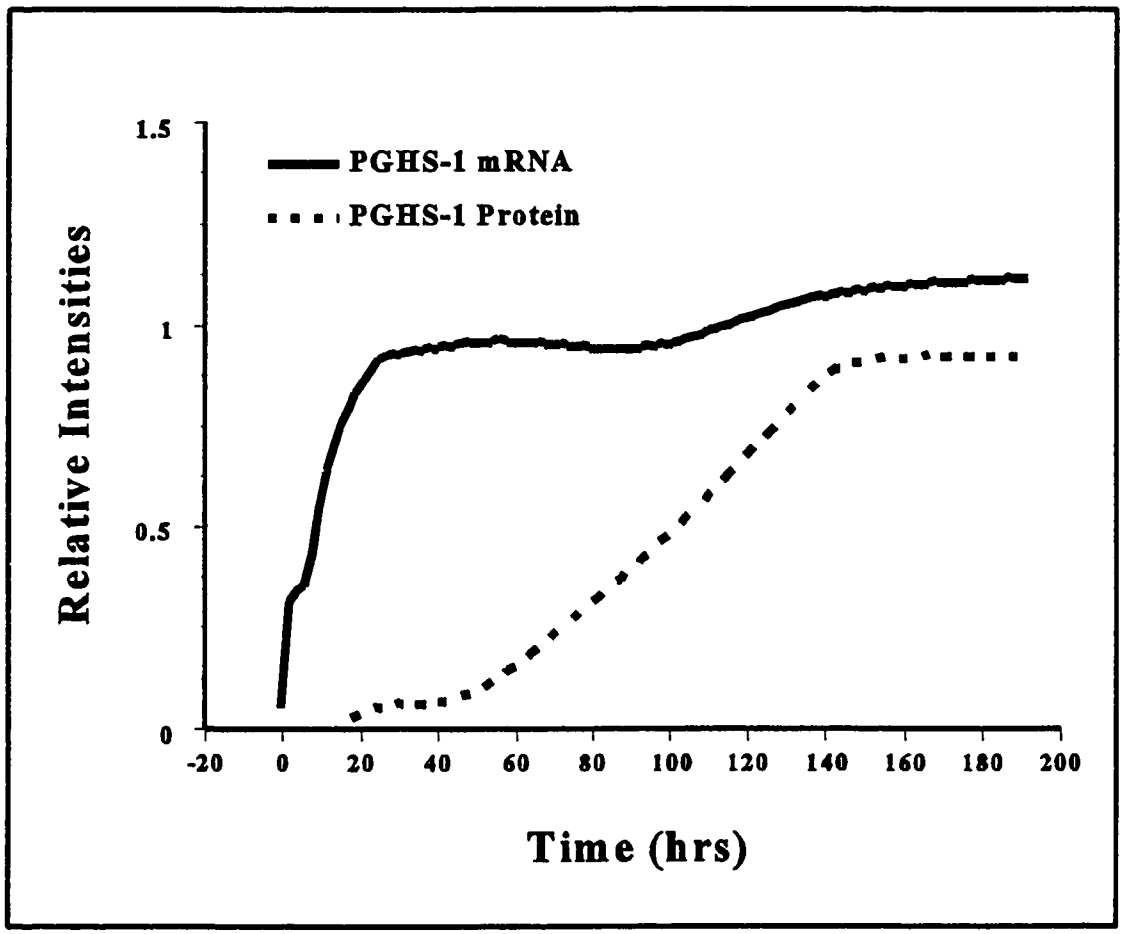

A
Std hPGHS-2
Std hPGHS-1
1d
2d
4d
6d
8d
Std hPGHS-1
Std hPGHS-1
TPA
(1.6 X 10⁻⁶M)
70 kDa -
- PGHS-1
43 kDa -
- β-Actin
Control
70 kDa -
- PGHS-1
43 kDa -
- β-Actin
B
Relative Intensity compared to β-actin control
1.2
1
0.8
0.6
0.4
0.2
0
TPA (1.6 X 10⁻⁶ M)
Control
1 day
2 days
4 days
6 days
8 days

Figure 3.14 **A schematic representation of the pattern of PGHS-1 mRNA and protein expression in TPA treated MEG-01 cells.** The solid line represents the pattern of PGHS-1 mRNA from 2 hours to 8 days of TPA stimulation. The dashed line represents the pattern of PGHS-1 protein expression from 1 day to 8 days of TPA stimulation.


PGHS-1 mRNA
PGHS-1 Protein
Relative Intensities
1.5
1
0.5
0
-20
0
20
40
60
80
100
120
140
160
180
200
Time (hrs)

MEG-01 cells after TPA stimulation. The dissimilar pattern of PGHS-1 mRNA and protein expression suggests there is transcriptional and/or post-transcriptional regulation of the PGHS-1 gene occurring in TPA treated MEG-01 cells.

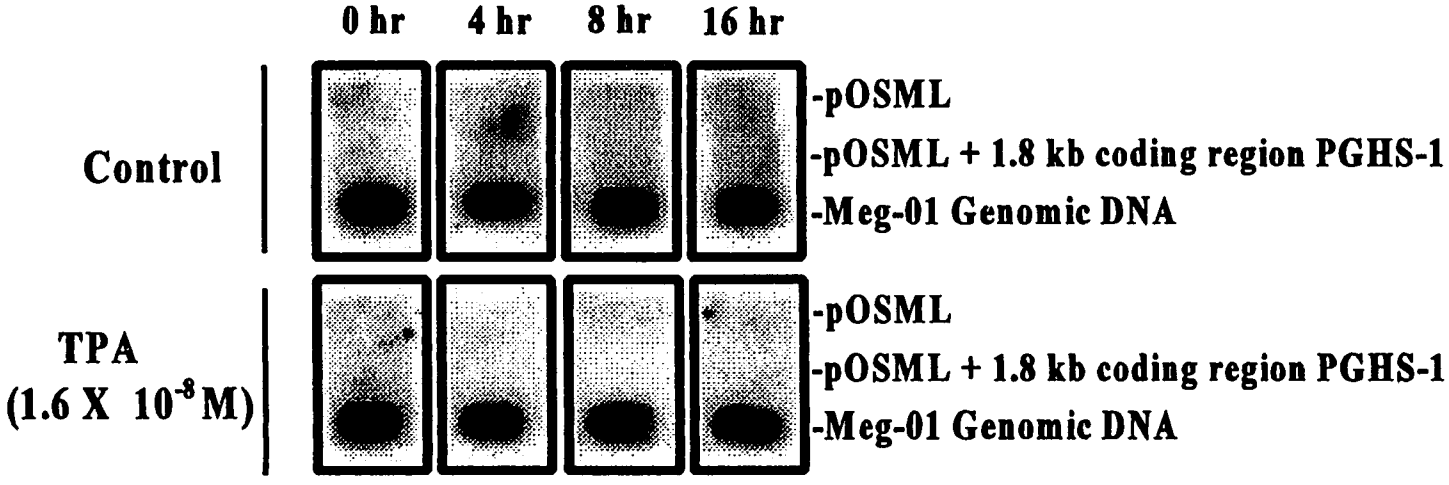
3.2.3 Effect of TPA stimulation on the transcriptional rate of the PGHS-1 gene

To assess whether the observed elevation in steady-state PGHS-1 mRNA was due to a concomitant increase in the transcription rate, nuclear run-on assays were performed in the presence and absence of TPA. The PGHS-1 basal transcription rate in MEG-01 cells was compared to that obtained following TPA treatment for 0, 4, 8 and 16 hours. A signal was detected with the genomic DNA control, indicating that the isolated nuclei were intact and actively labeling nascent RNA with radioactivity (Fig 3.15). There was no detectable difference between control and TPA treated samples suggesting TPA may not increase the rate of PGHS-1 transcription. Nonetheless, it is important to note, that the sensitivity of the assay did not allow for the detection of basal PGHS-1 gene transcription. Therefore, no solid conclusions can be made from these experiments about the effect of TPA on the PGHS-1 transcription rate.

3.2.4 Stability of PGHS-1 mRNA in control and TPA stimulated MEG-01 cells

Elevated steady state levels of mRNA can also result from a decreased rate of message degradation. Since the amount of any mRNA measured results from both synthesis and degradation, the synthesis process must be blocked first, so the rate of decay can be measured. To determine the

Figure. 3.15 **Detection of PGHS-1 gene induction after TPA stimulation by nuclear run-on analysis.** MEG-01 cells were treated with or without 1.6 X 10^{-8} M TPA for 0 to 16 hours as indicated. Nuclear RNA samples labeled with [^{32}P] UTP were hybridized to PGHS-1 coding region, MEG-01 cell genomic DNA (positive control) and the vector pOSML (to detect background). The results are illustrated by autoradiography and represent one of two separate experiments with similar results.


0 hr
4 hr
8 hr
16 hr
Control
-pOSML
-pOSML + 1.8 kb coding region PGHS-1
-Meg-01 Genomic DNA
TPA
(1.6 X 10-8 M)
-pOSML
-pOSML + 1.8 kb coding region PGHS-1
-Meg-01 Genomic DNA

effect of TPA on PGHS-1 mRNA stability, we examined the effects of two mechanistically distinct inhibitors of transcription - DRB and Actinomycin-D. DRB specifically interferes with the RNA polymerase II transcription unit whereas Actinomycin-D acts by intercalating between nucleic acids. After the addition of the RNA synthesis block, the time dependant decay of the mRNAs were analyzed by Northern blotting. As illustrated in Figures 3.16, neither the 2.8 or 4.5 kb PGHS-1 transcripts decayed appreciably in either control or TPA treated cells during the course of the study.

3.2.5 Effect of cycloheximide on PGHS-1 mRNA induction in MEG-01 cells

Although some mRNAs might be inherently more susceptible than others to RNase attack (e.g. by virtue of their primary structure), the half-lives of most mRNAs are probably determined by other factors, including their affinity for proteins (222). To determine whether induction of PGHS-1 mRNA by TPA requires ongoing protein synthesis, cycloheximide (CHX) was used to inhibit translation. Cells were incubated for 24 hours with cycloheximide (35μM) in the presence and absence of TPA after which they were harvested for Northern analysis. As shown in Figure 3.17 PGHS-1 mRNA was detected in both control and TPA treated MEG-01 cells, with the appropriate increases in PGHS-1 mRNA after TPA treatment. The treatment of MEG-01 cells with cycloheximide had a stimulatory effect on basal PGHS-1 mRNA levels. However, cycloheximide completely blocked the stimulatory effect of TPA on PGHS-1 mRNA production.

Figure. 3.16 **Transcriptional inhibitors were used to investigate PGHS-1 mRNA stability.** MEG-01 cells were treated with or without 1.6 X 10^{-8} M TPA for 48 hours, which was followed by either the transcriptional inhibitor DRB (40 μM) or Actinomycin-D (7.97 X 10^{-6} M) for 0 to 8 hours as indicated. Results are shown as a representative autoradiograph (**A**) and as arbitrary densitometric units of PGHS-1/α–tubulin in (**B**), a graph of the average of three independent experiments +/- s.d.

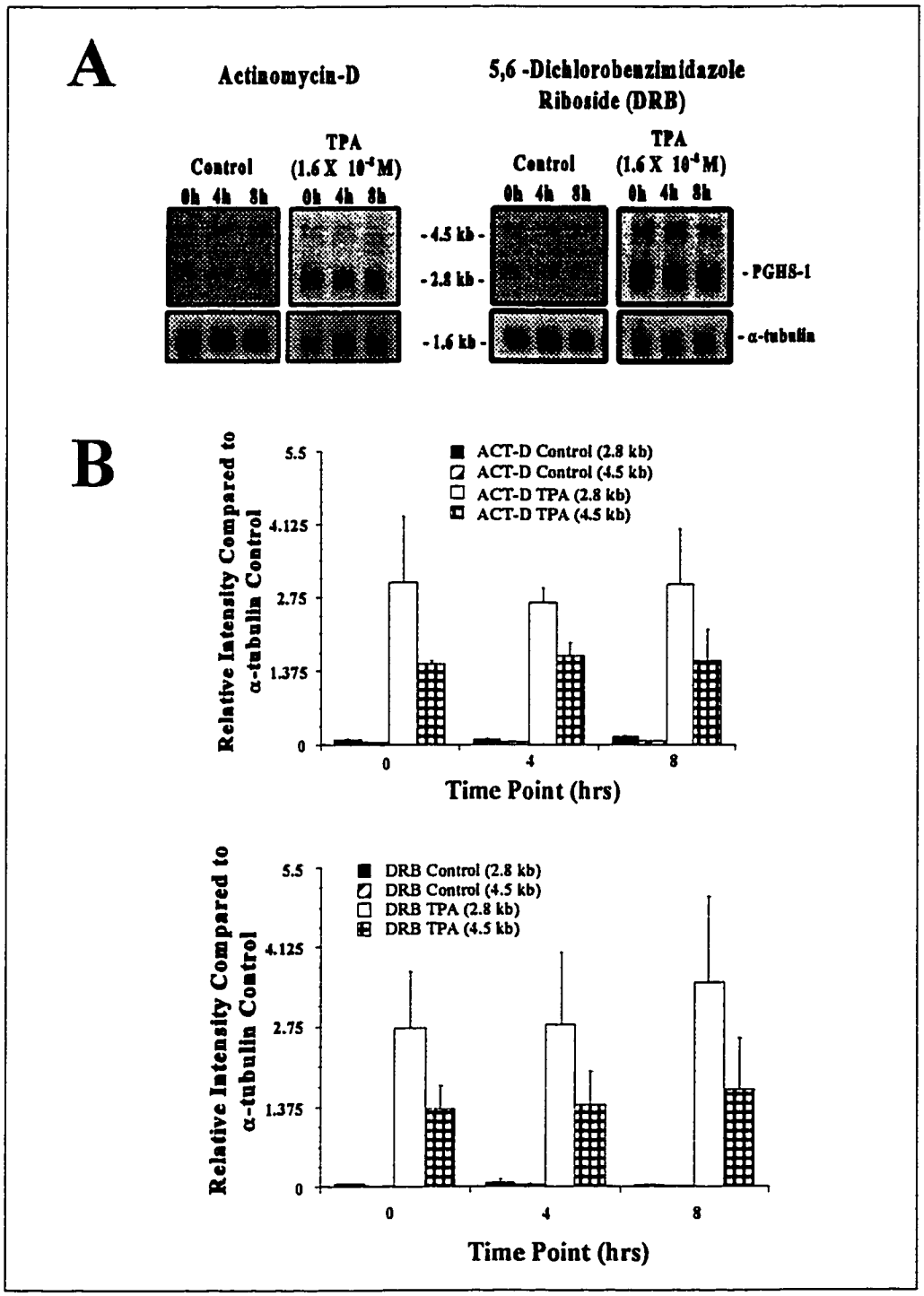
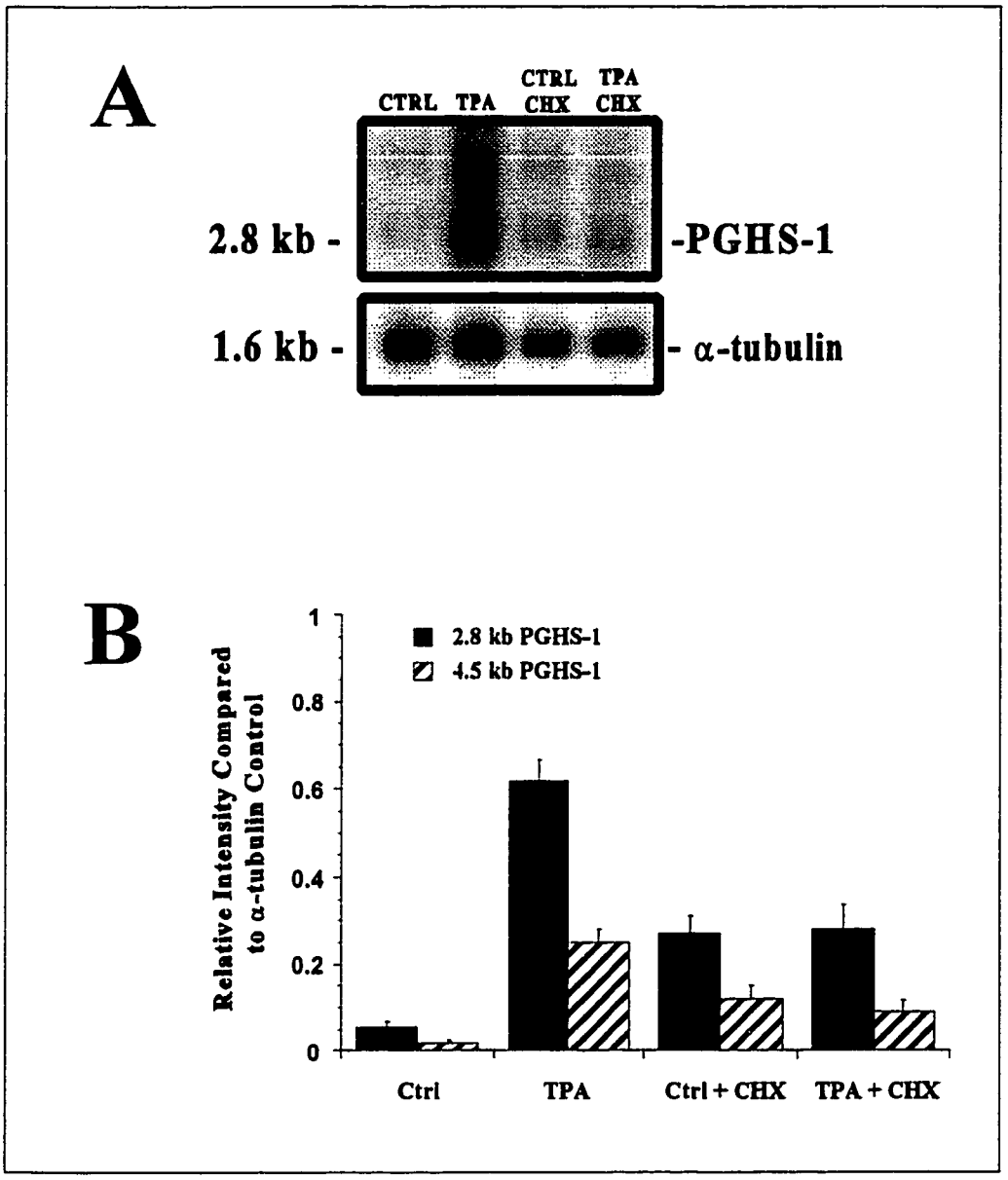

A
Actinomycin-D
5,6 -Dichlorobenzimidazole Riboside (DRB)
Control
TPA (1.6 X 10⁻⁴M)
0h 4h 8h
-4.5 kb-
-2.8 kb-
-1.6 kb-
-PGHS-1
-α-tubulin
B
Relative Intensity Compared to α-tubulin Control
ACT-D Control (2.8 kb)
ACT-D Control (4.5 kb)
ACT-D TPA (2.8 kb)
ACT-D TPA (4.5 kb)
Time Point (hrs)
DRB Control (2.8 kb)
DRB Control (4.5 kb)
DRB TPA (2.8 kb)
DRB TPA (4.5 kb)
Time Point (hrs)

Figure 3.17 **Effect of cycloheximide on the accumulation of PGHS-1 mRNA in MEG-01 cells after TPA stimulation.** MEG-01 cells were treated with cyclohexamide (35 μM) for 24 hours in the presence and absence of TPA (1.6×10^{-8} M). As well, cells were treated with or without TPA (1.6×10^{-8} M) for 24 hours in the absence of cyclohexamide. Results are shown as a representative autoradiograph (**A**) and as arbitrary densitometric units of PGHS-1/α–tubulin in (**B**), a graph of the average of three independent experiments +/- s.d.


A
CTRL
TPA
CTRL CHX
TPA CHX
2.8 kb -
-PGHS-1
1.6 kb -
- α-tubulin
B
Relative Intensity Compared to α-tubulin Control
2.8 kb PGHS-1
4.5 kb PGHS-1
1
0.8
0.6
0.4
0.2
0
Ctrl
TPA
Ctrl + CHX
TPA + CHX

SECTION FOUR: DISCUSSION

4.1 IMPORTANCE OF PGHS-1 EXPRESSION IN PLATELETS

Blood platelets are a rich source of PGHS-1 (54), the enzyme targeted by aspirin (189). Inhibition of platelet PGHS-1 by aspirin prevents the formation of the aggregatory agent TXA_2 and reduces the incidence of cardiovascular disease associated with platelet hyperfunction (190). Because of the importance of PGHS-1 in regulating cardiovascular homeostasis, it is crucial to elucidate the molecular mechanisms regulating the expression of PGHS-1 enzyme in platelets. Platelets are anucleated cells, therefore, to study the mechanism regulating PGHS-1 expression we used the human nucleated platelet precursor cell line MEG-01. MEG-01 cells are relatively undifferentiated megakaryoblastic cells committed to the platelet pathway (130). Studies have shown that *in vitro* differentiation of megakaryoblastic MEG-01 cells into mature megakaryocytes and platelet-like structures can be achieved upon addition of the phorbol ester TPA (127,191).An induction of PGHS-1 expression in MEG-01 cells after phorbol ester treatment has also been documented(129). Therefore, MEG-01 cells are considered an ideal model for the analysis of megakaryocytic maturation and PGHS-1 gene expression in platelets.

4.2 MOLECULAR CHARACTERIZATION OF THE NOVEL 4.5 KB PGHS-1 TRANSCRIPT

4.2.1 PGHS transcripts present in TPA treated MEG-01 cells

We observed that untreated MEG-01 cells do not express detectable levels of the enzyme PGHS-1 when analyzed by Western blotting, but after a single addition of TPA (1.6×10^{-8} M) an increase in the levels of PGHS-1 mRNA and protein was detected. Using the entire coding region of PGHS-1 as a probe under high stringency conditions, transcripts of 2.8, 4.5 and 5.1 kb were detected in the Poly $(A)^+$ RNA fraction of MEG-01 cells analyzed by Northern blotting. As previously reported, the 2.8 kb transcript is the most abundant PGHS-1 transcript (129,131) and is postulated to be generated from the 5.1 kb transcript through a canonical polyadenylation signal. Previous reports studying 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, using the hPGHS-1 cDNA as a probe (192). No work has been done on the possible sources of this new 4.5 kb PGHS transcript detected with the hPGHS-1 coding region. Therefore, we further characterized the 4.5 kb transcript detected in MEG-01 cell Poly $(A)^+$ RNA fraction and identified it as a PGHS-1, using a probe specific to the PGHS-1 3'UTR that contains no homology to the PGHS-2 sequence.

4.2.2 Mechanism for the generation of the 4.5 kb PGHS-1 transcript

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 and found by computer analysis to contain elements that define a potential polyadenylation site. When used, this site provides a source of the novel 4.5 kb PGHS-1 transcript detected in MEG-01 cells. The most critical sequence of a polyadenylation site is the canonical polyadenylation signal (AAUAAA) found almost invariably 10 to 30 bases upstream of a cleavage site (193). The polyadenylation signal found in our sequence is a non-canonical signal (AAGAAA). *In vitro* studies indicate that mutation of the AAUAAA hexanucleotide to AAGAAA dramatically reduces the efficiency of polyadenylation (194). This reduction 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). Upon TPA treatment both the 2.8 and 4.5 kb transcripts are increased, with their ratios remaining the same; the 2.8 kb transcript being the most abundant. Altogether, the polyadenylation signal (AAGAAA), the G/U rich element located downstream of the site of cleavage and the CA sequence immediately 5' to the site of cleavage, correspond to the consensus elements which define a poly $(A)^+$ cleavage site (193).

4.2.3 Tissue expression profile of the 4.5 kb PGHS-1 transcript

To our surprise, 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 express 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 leukocyte, lymph node and bone marrow, suggesting that tissues with an endodermal origin preferentially express the PGHS-1 4.5 kb transcript. To confirm that expression of the 4.5 kb transcript is limited to tissues with endodermal origin, *in situ* hybridization will have to be performed to confirm that expression is limited to the epithelial region of these organs.

4.2.4 Possible role of the 4.5 KB PGHS-1 transcript

Although the majority of 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 (193). The hPGHS-1 gene can now be added to that list with 3 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 or used in a tissue specific fashion. At least two poly $(A)^+$ signals have been reported in the amphiglycan gene, with the longer message being ubiquitous while the shorter message seems to be tissue-specific (195). As well, a switch in poly $(A)^+$ site usage during chondrocyte differentiation has been reported (196). The human activin βA subunit gene contains a tandem of 8 possible poly $(A)^+$ sites and TPA treatment of HT1080 fibrosarcoma cells which stimulates erythroid differentiation causes a shift over time to use of proximal poly $(A)^+$ sites (197). Also, the murine tissue inhibitor of the metalloproteinase-3 gene in pre-neoplastic JB 6 cells treated with TPA produces three transcripts of 2.3, 2.8 and 4.6 kb, with the 4.6 kb being the most abundant (198).

Since the multiple forms of PGHS-1 mRNA differ only by the selection of their polyadenylation signal sites in the 3' end, presently it is not obvious how differential poly $(A)^+$ site usage could influence protein expression. However, if the different forms of mRNAs have different stabilities, then use of alternative poly $(A)^+$ sites can positively or negatively impact the final amount of protein product per unit 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 (52). Recently, post-transcriptional mechanisms that stabilize the mRNA have been reported to increase PGHS-2 mRNA levels (199). 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 PGHS-2 4.5 kb transcript stability (53). Since the 2.8 and 4.5 kb messages of PGHS-2 have 7 and 22 copies respectively of the instability motif, AUUUA, they would be expected to show different stabilities, with the 4.5 kb fragment being the most unstable. Studies have shown that both PGHS-2 transcripts displayed half-lives in excess of 2 hours (36). Increases in stability for both transcripts were reported following IL-1β treatment, with stabilization of the 4.5 kb transcript occurring earlier than for the 2.8 kb transcript (199,200). In response to dexamethasone, the 2.8 kb transcript became more stable than the 4.5 kb transcript (201). Because IL-1β tends to favor stabilization of the 4.5 kb transcript and dexamethasone treatment favored stabilization of the 2.8 kb message, mechanisms other than the number of AUUUA instability motifs play a role in the post-transcriptional regulation of PGHS-2 gene. Stabilization of the PGHS-2 mRNA has also been reported in HUVEC cells, suggesting a functional post-transcriptional role of the 3' UTR (199). The molecular mechanisms responsible for the increase in PGHS-2 mRNA stability remains to be determined.

The results of our studies show that the novel 4.5 kb and the major 2.8 kb PGHS-1 transcripts are regulated in the same manner. Both transcripts are increased in the same concentration and time dependant fashion. As well, the 4.5 and 2.8 kb transcripts show the same profile of mRNA stability (stable over the 8 hours of study). This is to no surprise because the full length PGHS-1 3'UTR contains only 4 copies of the AUUUA instability motif. Therefore, the biological significance of the three different PGHS-1 transcripts is at present unknown. Since TPA predominantly induced the formation of the shortest version; the 2.8 kb message, this mRNA isoform may be primarily involved in the housekeeping role of constitutive PGHS-1 expression. However, the longer transcripts, 5.1 and 4.5 kb, which are also inducible but at much lower levels, may therefore contribute to the induction of PGHS-1 during differentiation. Different functions from the different sized transcripts could be achieved by mRNA intracellular localization. Since mRNAs are the templates for translation, their localization allows for proteins to be produced in the subcellular regions where they are required. mRNA localization is a very efficient way to target proteins to their correct sites, as more energy would be needed to localize many protein molecules, instead of a single mRNA that can be translated many times. Studies using immunocytochemical and histochemical techniques have shown that PGHS-1 is present on the lumenal surface of the endoplasmic reticulum and the outer membrane of the nuclear envelope (NE) (70). Because the PGHS-1 protein is located at the ER and NE, this may lead to separate enzyme systems within the cell that could possibly produce prostanoids for paracrine and autocrine functions. Prostanoids produced at the ER by PGHS-1 can act as paracrine mediators. For example, PGI_2 produced by vascular endothelial cells can act on platelets as a platelet aggregation inhibitor (33). Whereas, prostanoids produced by PGHS-2 at the NR have been shown to act as autocrine mediators by transducing signals through nuclear prostaglandin

receptors, such as the peroxisome proliferator activated receptor (PPAR) class (220). A common feature of most localized mRNAs is that the *cis*-acting sequences required for localization reside in the 3'UTR of the transcript (223). An example of localized mRNA occurs in differentiating myoblasts, the mRNAs encoding two actin isoforms, α-cardiac and β-cytoplasmic can occupy different cytoplasmic compartments (224). Reporter constructs demonstrated that isoform-specific 3'UTR sequences play a key role in modulating the distribution of actin mRNAs (224). It is possible that the extra 3'UTR sequence contained on the 4.5 kb message may direct the mRNA to a different intracellular location than the 2.8 kb transcript (NE or ER). Therefore, two separate enzyme systems could produce prostanoids for different functions (constitutive or differentiation roles). To confirm that expression of the 4.5 kb transcript is limited to specific regions within the cell, *in situ* hybridization will have to be performed to confirm the localization of the various PGHS-1 transcripts.

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 TPA 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 human 4.5 kb PGHS-1 and 4.6 kb PGHS-2 transcripts share 73% sequence homology, the standard probes used (entire coding regions) for hybridization may have often misidentified the 4.5 kb PGHS-1 as a PGHS-2. Therefore, we provide a strategy to detect specifically PGHS-1 and PGHS-2 transcripts by using probes in the 3'UTR regions of these genes where no homology is found.

4.3 REGULATION OF PGHS-1 EXPRESSION IN THE MEGAKARYOCYTIC CELL LINE MEG-01

4.3.1 Profile of PGHS-1 expression in MEG-01 cells

We observed that untreated MEG-01 cells express low levels of the enzyme PGHS-1, but showed a concentration and time dependent increase in functional PGHS-1 enzyme after a single addition of the phorbol ester TPA. Also upon TPA stimulation, PGHS-1 protein was preceded by an increase in PGHS-1 mRNA. As stated earlier, the novel 4.5 kb and the major 2.8 kb PGHS-1 transcripts are regulated in the same manner. Both transcripts are increased in the same concentration and time dependant fashion. Induction of PGHS-1 expression has been reported in systems that model developmental events. The most notable example of PGHS-1 induction is TPA induced differentiation of THP-1 monocytes to a macrophage phenotype (47). Also, a study has reported that PGHS-1 mRNA abundance rises during development of ovine pulmonary artery (202). 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 is reported, it remains to be determined whether the PGHS-1 4.5 kb transcript is also induced in these systems. In MEG-01 cells, maximal PGHS-1 mRNA transcript levels were reached after 24 hours of TPA stimulation, whereas, PGHS-1 enzyme levels did not peak until 6 to 8 days after TPA treatment. Another study has reported increases in PGHS-1 mRNA and protein in MEG-01 cells after TPA treatment (129). Although this study reports only small changes in PGHS-1 levels after TPA stimulation. Methodology differences could explain this discrepancy. Matijevic-Aleksic et al. (129) used RT-PCR analysis and indicated only small increases in PGHS-1

mRNA after TPA treatment. Whereas, our results clearly show by Northern and Western blot analysis an increase in PGHS-1 mRNA transcripts and protein that are separated by a period of 5 to 7 days.

Observation of a 40 hour lag between mRNA maximal levels and the appearance of PGHS-1 protein, indicates a post-transcriptional control mechanism. Possible mechanisms facilitating this lag could be through the control of translation initiation or protein stability. Controlling gene expression by regulation of translation allows a cell to respond to stimuli faster than *de novo* transcription permits. The assembly of a functional ribosome onto an mRNA is required for translation to begin. Briefly, the 7-methyl guanosine cap is bound by a large complex of proteins (including the cap binding protein eIF-4E and the RNA helicase eIF-4A) on to the 5' end of an mRNA. Once binding has taken place, the small ribosomal subunit-initiation factor complex scans the 5'UTR for an initiator codon AUG. After the initiator codon is selected, the large ribosomal subunit joins the complex and protein synthesis begins (225). The overall rates of protein synthesis can be affected by changes in the concentration or activities of components of the translational apparatus, such as initiation factors (226). Work in the recent years has provided a list of examples of mRNAs translationally regulated by sequence motifs contained either in their 5' or 3' UTRs. The sequence motifs that are responsible for translational regulation presumably act by binding specific *trans*-acting factors. An example of a *trans*-acting factor that binds a target mRNA and is able to confer translational control *in vitro* has been demonstrated for the erythroid 15-lipoxygenase (LOX) gene (227). Erythroid 15-LOX participates in the breakdown of internal membranes (such as mitochondrial membranes), during reticulocyte maturation. During the differentiation of red blood cells, the mRNA encoding rabbit erythroid 15-LOX is transcribed during the early stages of erythropoiesis, but is only activated for

translation in peripheral reticulocytes (227). Rabbit erythroid 15-LOX contains 10 tandem repeats of a slightly varied 19 nucleotide motif located in its 3'UTR (227). Ostareck-Lederer *et al.* demonstrated the presence of a novel 48 kDa reticulocyte protein that specifically binds to this 19 nucleotide repeat and specifically represses translation of the 15-LOX mRNA (227). If repression of PGHS-1 mRNA translation is occurring during TPA induced differentiation of MEG-01 cells is a question that still needs to be answered. A possible way to determine this is performing *in vitro* translation assays in the presence of cell lysates from MEG-01 cells treated with TPA for various time intervals. Increases or decreases in protein levels can also be caused by changes in the stability of the protein itself. If a protein were totally stable, it would theoretically accumulate until it approached an infinite concentration. Therefore, differential stability of proteins is a fundamental mechanism that makes biological regulation possible. Eukaryotic cells contain a selective ATP-dependent cytosolic mechanism which specifically marks proteins for degradation. Analysis of a cell-free rabbit reticulocyte system has demonstrated that a protein known as ubiquitin is required for ATP-dependent protein degradation (228). Proteins that are selected for degradation are marked by a covalent linkage to ubiquitin. The marked protein is proteolytically degraded in an ATP-dependant process that is mediated by a large multi-protein complex known as ubiquitin-conjugate degrading enzyme (UCDEN) (228). This multi-protein protease only degrades ubiquitin linked proteins. A correlation exists between a proteins liability and the presence of a structural feature called the PEST element, which represents proteins with segments rich in Pro (P), Glu (E), Ser (S), and Thr (T) residues (229). These elements are found in segments of labile proteins such as oncogene products, transcription factors and regulatable enzymes (229). Pulse-chase experiment need to be performed, to determine if the stability of the PGHS-1 protein changes in TPA treated MEG-01 cells over the

8 day time course.

The second objective of our study is to examine the regulation of expression of the PGHS-1 gene in MEG-01 cells after TPA treatment.

4.3.2 Transcriptional regulation of PGHS-1 expression

Transcriptional activation is the main mechanism for increasing gene expression. To assess whether the observed elevation in steady-state PGHS-1 mRNA was due to a concomitant increase in the transcription rate, nuclear run-on assays were performed in the presence and absence of TPA. A signal was detected with the genomic DNA control, indicating that the isolated nuclei were intact and actively labeling nascent RNA with radioactivity. Nonetheless, it is important to note, that the sensitivity of the assay did not allow for the detection of basal PGHS-1 gene transcription. Therefore, from the reported results no solid conclusions can be made about the effect of TPA on the PGHS-1 transcription rate in MEG-01 cells. A signal was detected with genomic DNA because it would have detected all of the genes within the genome being transcribed at the time of nuclei isolation. As well, repeated sequences (such as Alu repeats) within the genome could have added to the signal detected with the genomic DNA control (231). Since the genome only contains one copy of the PGHS-1 gene the sensitivity of the assay needed to be determined using a control gene that has a similar copy number to PGHS-1 and has shown measurable levels of transcription by nuclear run-on assays. For example, when basal transcription levels were detected for the PGHS-1 gene in human lung fibroblast, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control to measure transcription rates (232). Controls that could have been included were cells pre-treated with transcriptional

inhibitors such as Actinomycin-D, which would determine if a detected signal was a specific measure of transcription rates. To clearly determine if TPA has an effect on the PGHS-1 gene transcription rate, more sensitive methods could have been used to measure increases in gene transcription. For example, nuclear run-on reactions could be performed as usual, but in the presence of unlabeled dUTP and then quantitative RT-PCR could be performed from the isolated run-on RNA. Also, reporter constructs containing the PGHS-1 promoter region linked to a reporter gene could have been used to determine the effects of TPA on the 5'-flanking region. Other studies have used reporter constructs to measure the effect of TPA on PGHS-1 promoter activity because nuclear run-on assays did not produce any detectable results due to low levels of messages (79). This study reported a 1.8 fold increase in promoter activity after TPA treatment of NS-20 cells transfected with a luciferase reporter gene linked to the promoter of the PGHS-1 gene (79). In this study, the magnitude of stimulation of PGHS-1 mRNA and protein levels was consistent with the augmentation of the 5'-promoter activity, indicating that TPA can affect the transcription rate of the PGHS-1 gene (79). TPA has been shown to mediate increases in transcription of several genes (233). For example, TPA treatment allows NFκB to become activated and bind to its DNA recognition sequence and increase the transcription rate of the highly inducible PGHS-2 gene (230). Wu *et al* characterized the promoter of the PGHS-1 gene and identified several transcriptional factor-binding sites (78). Interestingly, one of the identified sites (PEA3) has shown to be activated by phorbol esters (203). Since 1993 many new transcription factors and regulatory elements have been discovered, therefore, re-analysis of the PGHS-1 promoter could provide new information about its regulation.

4.3.3 Post-transcriptional regulation of PGHS-1 expression

mRNA stability, although not as widely and thoroughly studied as transcriptional control, is a regulated property that can determine the level of gene expression. Although, studies have shown stability determinants in mRNA 5'UTRs and coding regions, the majority of papers dealing with mRNA stability determinants have identified mRNA decay signals in the 3'UTR (134). The majority of the characterized 3'UTR elements have been shown to be protein binding sites and are regulated through *trans*-acting factors (134). One of the best reviewed 3'UTR determinants is the Iron-responsive element (IRE) (172). The transferrin receptor plays a key role in the regulation of intracellular iron homeostasis by importing iron into the cell. The mRNA encoding the transferrin receptor is regulated post-transcriptionally by processes dependent on intracellular iron concentrations. Regulation of the mRNA is achieved through the IREs contained in its 3'UTRs, which functions by binding an iron-regulatory protein (IRP). The 3'UTR of the transferrin receptor mRNA contains five IREs, three of which serve to regulate the mRNAs half-life (172). The levels of transferrin receptor mRNA and intracellular iron concentrations are inversely correlated. When iron levels are ample, the IRE-IRP complex does not form and the mRNA has a short half-life (172). However, when intracellular iron levels are depleted the conformation of IRP changes and the IRE-IRP complex forms and the transferrin receptor mRNA is stabilized 20 to 30 fold (172). As a result, the transferrin receptor synthesis increases, as does the amount of iron imported into the cell. TPA has been shown to affect gene regulation post-transcriptionally through altering message stability. For example, TPA treatment has been shown to stabilize mRNAs for transforming growth factor-β1, *c-fms*, IL-3 and IL-1 (204,205,206,207). mRNA stability assays were performed to determine if the

increases in steady state PGHS-1 mRNA levels after TPA treatment was caused by a corresponding increase in PGHS-1 mRNA stability. After addition of two mechanistically distinct transcriptional inhibitors, we found that the PGHS-1 transcripts in both control and TPA treated cells were very stable after 8 hours of treatment. Studies using human lung fibroblasts have also shown that PGHS-1 mRNA from untreated cells did not decay after 10 hours of treatment with transcriptional inhibitors (232). As in our case, this study does not see maximal PGHS-1 mRNA levels until 24 hours of treatment, therefore, it is possible that the control message could begin to decay between 8 and 24 hours (232). To determine if the PGHS-1 mRNA in control MEG-01 cells begins to degrade between 8 and 24 hours of treatment with transcriptional inhibitors, time points in this range need to be included in the study. The half lives of stable mRNAs can not be readily determined since prolonged treatment of cells with transcriptional inhibitors compromises cellular viability by interfering with the expression of housekeeping genes (232). Therefore, if longer time points are to be included in the study, assays such as propidium iodide staining will need to be performed to make sure cell viability is not affected. As well, a labile control mRNA such as interleukin-3 should be included in the study to make sure that the transcriptional inhibitors are indeed working. Interleukin-3 mRNA has been detected in MEG-01 cells and has shown to have a half-life between 45 minutes and 2 hours (234, 235). If prolonged treatment with transcriptional inhibitors does affect cell viability, less toxic methods such as measurement of mRNA degradation *in vitro* or using inducible promoters can be used. Briefly, protein free mRNA produced *in vitro* from expression vectors are incubated with cell extracts for various time intervals and mRNA levels are then measured (134). mRNA stability can also be measured by using short-term activation of an inducible promoter. Prior to induction, the promoter is silent and following induction, the promoter is briefly activated and then shuts down

(134). The mRNA half-life is then simply determined by monitoring the subsequent rate of mRNA loss. Therefore, using either method no transcriptional inhibitors are needed and cell viability will not be affected.

The 24 hour time period required for maximal levels of PGHS-1 2.8 and 4.5 kb messages after TPA administration suggests that the effect of TPA is indirect and requires the biosynthesis or activation of factors mediating the response. Therefore, we studied the possibility that *de novo* protein synthesis was required for the induction of the PGHS-1 mRNA by carrying out TPA treatments in the presence of the protein synthesis inhibitor cycloheximide. We found that PGHS-1 mRNA accumulation was dependent on *de novo* protein synthesis: CHX, when added simultaneously with TPA completely inhibited the accumulation of PGHS-1 mRNA. The effect displayed by CHX treatment could be interpreted in two ways:. (1) TPA treatment requires the synthesis of a transcription factor that binds to a unique or already identified TPA mediated regulatory element in the promoter region of the PGHS-1 gene or (2) TPA treatment requires the synthesis of a *trans*-acting factors that binds to a stabilizing element contained within the PGHS-1 mRNA. TPA treatment has been shown to modulate the synthesis of both transcription factors and mRNA stabilizing proteins (183, 184, 230). Whether the increase in PGHS-1 mRNA levels is dependent on the synthesis of a transcription factor or a stabilizing mRNA binding protein remains to be determined. When MEG-01 cells were treated with CHX alone, an increase in PGHS-1 mRNA levels was observed. This is an interesting observation because it has been well documented that treatment of quiescent cultures with protein synthesis inhibitors can elicit immediate early (IE) gene expression (236). IE genes are a class of genes tightly regulated by a control system that allows the transient induction of their gene products such as the transcription factors *c-Fos* and *c-Jun* (233). IE genes

exhibit a phenomenon known as 'super-induction', which is a massive over-accumulation of their transcripts in the presence of an inducing factor (i.e. Epidermal Growth Factor for *c-Fos* and *c-Jun*) and a protein synthesis inhibitor such as CHX (233). Four contributory mechanisms have been proposed to explain the super-induction phenomenon caused by treatment with protein synthesis inhibitors: (1) Stabilization of the labile mRNA can occur because ongoing translation is required for mRNA degradation; (2) Augmented transcription of the IE gene; (3) Degradation of a labile repressor protein that keeps IE genes inactive in control cells and (4) Protein synthesis inhibitors can independently stimulate the same nuclear signalling pathways as the IE inducer, for example, CHX activates the mitogen activated protein kinase pathway (233). IE genes do not require ongoing protein synthesis to elicit gene expression, therefore, PGHS-1 can not be characterized as a IE gene because TPA stimulation requires *de novo* protein synthesis. Therefore, the increase in PGHS-1 mRNA after CHX treatment can be addressed in three ways: (1) Activation of an intracellular signaling pathway that leads to the activation of a transcription factor; (2) Degradation of a labile repressor protein that keeps PGHS-1 transcription at basal levels and (3) CHX can cause mRNA stabilization by trapping the mRNA on polysomes and shielding the mRNA from cytoplasmic ribonucleases (233).

In summary, from our results no clear conclusions can be made about PGHS-1 transcription rates and mRNA stability after TPA treatment. Although it is apparent that the increase in PGHS-1 mRNA after TPA treatment appears to be regulated by control mechanism involving *de novo* protein synthesis.

In conclusion, we have identified a new PGHS-1 transcript of 4.5 kb that arises through alternative polyadenylation, is induced by TPA in MEG-01 cells and displays a tissue specific

expression pattern. As well, it appears that after TPA treatment, the increases in PGHS-1 transcripts are regulated by control mechanism involving *de novo* protein synthesis.

REFERENCES

1. Ziboh, V.A. 1985. Arachidonic Acid Metabolism in the Skin. In: Fisher, S.M., Slaga, T.J., PB (eds) Arachidonic acid metabolism and Tumor promotion. Martinus Nijhoff Publ., Boston, pp 5-20.

2. Smith, W.L. 1986. Prostaglandin biosynthesis and its compartmentation in vascular smooth muscle and endothelial cells. Ann. Rev. Physiol. 48:251-262.

3. Dennis, E.A. 1987. Regulation of eicosanoid production: role of phospholipases and inhibitors. Biotechnology. 5:1294.

4. Dennis, E.A., Rhee, S.G., Billah, M.M., and Hannun, Y.A. 1991. Role of phospholipase in generating lipid second messengers in signal transduction. FASEB J. 5:2068-2077.

5. Gronich, J.H., Bonventre, J.V., and Nemenoff, R.A. 1990. Purification of a high-molecular-mass form of phospholipase A2 from rat kidney activated at physiological calcium concentrations. Biochem. J. 271:37-43.

6. Gronich, J.H., Bonventre, J.V., and Nemenoff, R.A. 1988. Identification and characterization of a hormonally regulated form of phospholipase A2 in rat renal mesangial cells. J.Biol. Chem. 263:16645-16651.

7. Aarsman, M.J., de Jong, J.G.N., Arnoldussen, E., Neys, F.W., van Wassenaar, P.D., and Van den Bosch, H. 1989. Immunoaffinity purification, partial sequence, and subcellular localization of rat liver phospholipase A2. J. Biol. Chem. 264:10008-10014.

8. Balsine, J., Diez, E., and Mollinedo, F. 1991. Arachidonic acid release from diacylglycerol in human neutrophils. Translocation of diacylglycerol-deacylating enzyme activities from an intracellular pool to plasma membrane upon cell activation. J. Biol.Chem. 266:15638-15643.

9. Neufeld, E.J., and Majerus, P.W. 1983. Arachidonate release and phosphatidic acid turnover in stimulated human platelets. J. Biol. Chem. 258:2461-2467.

10. Galey, C.I., Ziboh, V.A., Marcelo, C.L., and Voorhees, J.J. 1985. Modulation of phospholipid metabolism in murine keratinocytes by tumor promoter, 12-O-tetradecanoylphorbol-13-acetate. J. Invest. Dermatol. 85:319-323.

11. Needleman, P., Turk, J., Jakschik, B.A., Morrsion, A.B., and Lefkowith, J.B. 1986. Arachidonic acid metabolism. Ann. Rev. Biochem. 55:69-102.

12. Smith, W.L., and Marnett, L.J. 1991. Prostaglandin endoperoxide synthase: structure and catalysis. Biochim. Biophys. Acta. 1083:1-17.

13. Ujihara, M., Tsuchida, S., Satoh, K., and Urade, Y. 1988. Biochemical and immunological demonstration of prostaglandin D2, E2, and F2 alpha formation from prostaglandin H2 by various rat glutathione S-transferase isozymes. Arch. Biochem. Biophys. 264:428-437.

14. Urade, Y., Fujimoto, N., and Hayaishi, O. 1985. Purification and characterization of rat brain prostaglandin D synthetase. J. Biol. Chem. 260:12410-12415.

15. Whittle, B.J.R., Moncada, S., and Vane, J.R. 1978. Comparison of the effects of prostacyclin (PGI2), prostaglandin E1 and D2 on platelet aggregation in different species. Prostaglandins 16:373-388.

16. Siegl, A. 1982. Receptors for PGI2 and PGD2 on human platelets. Methods Enzymol. 86:179-192.

17. Thierauch, K.H., Sturzebecher, C.-St., Schillinger, E., Rehwinkel, H., Raduchel, B., Skuballa, W., and Vorbruggen, H. 1989. Stable 9 beta- or 11 alpha-halogen-15-cyclohexyl-prostaglandins with high affinity to the PGD2-receptor. Adv. Prostagl. Thromb. Leuk. Res. 19:655-658.

18. Wasserman, M.A., DuCharme, D.W., Griffin, R.L., DeGraaf, G.L., and Robinson, F.G. 1977. Bronchopulmonary and cardiovascular effects of prostaglandin D2 in the dog. Prostaglandins. 13:255-269.

19. Lewis, R.A., and Austen, K.F. 1981. Mediation of local homeostasis and inflammation by leukotrienes and other mast cell-dependent compounds. Nature. 293:103-108.

20. Nagata, A., Suzuki, Y., Igarashi, M., Eguchi, N., Toh, H., Urade, Y., and Hayaishi, O. 1991. Human brain prostaglandin D synthase has been evolutionarily differentiated from lipophilic-ligand carrier proteins. Proc. Natl. Acad. Sci. U.S.A. 88:4020-4024.

21. Igarashi, M., Nagata, A., Toh, H., Urade, Y., and Hayaishi, O. 1992. Structural organization of the gene for prostaglandin D synthase in the rat brain. Proc. Natl. Acad. Sci. U.S.A. 89:5376-5380.

22. Ogino, N., Miyamoto, T., Yamamoto, S., and Hayaishi, O. 1977. Prostaglandin endoperoxide E isomerase from bovine vesicular gland microsomes, a glutathione-requiring enzyme. J. Biol. Chem. 252:890-895.

23. Zurier, R.B. 1988. Prostaglandins and Inflammation. In:Curtis-Prior, PB (eds) Prostaglandins. Biology and chemistry of prostaglandins and related eicosanoids. Churchill Livingstone, New York, pp 595-607.

24. Bergstrom, S., Ryhage, R., Samuelsson, B., and Sjovall, J. 1963. Prostaglandins and related factors: the structures of prostaglandins E1, F1alpha and F1beta. J. Biol. Chem. 238:355.

25. Dunn, M.J., Liard, J.F., and Dray, F. 1978. Basal and stimulated rates of renal secretion and excretion of prostaglandings E2, Falpha, and 13, 14-dihydro-15-keto Falpha in the dog. Kidney Int. 13:36-43.

26. Liston, T.E., and Roberts, L.J. 1985. Transformation of prostaglandin D2 to 9 alpha, 11beta-(15S)-trihydroxyprosta-(5Z,13E)-dien-1-oic acid (9 alpha, 11 beta-prostaglandin F2): a unique biologically active prostaglandin produced enzymatically in vivo in humans. Proc. Natl. Acad. Sci. U.S.A. 82:6030-6034.

27. Beasly, C.R.W., Robinson, C., Feathersone, R.L., Varley, J.G., Hardy, C.C., Church, M.K., and Holgate, S.T. 1987. 9 alpha,11 beta-prostaglandin F2, a novel metabolite of prostaglandin D2 is a potent contractile agonist of human and guinea pig airways. J. Clin. Invest. 79:978-983.

28. Watanabe, K., Yoshida, R., Shimizu, T., and Hayaishi, O. 1985. Enzymatic formation of prostaglandin F2 alpha from prostaglandin H2 and D2. Purification and properties of prostaglandin F synthetase from bovine lung. J. Biol. Chem. 260:7035-7041.

29. Stone, K.J. and Hart, M. 1975. Prostaglandin-E2-9-ketoreductase in rabbit kidney. Prostaglandins 10:273-288.

30. Weksler, B.B., Marcus, A.J., and Jaffe, E.A. 1977. Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine endothelial cells. Proc. Natl. Acad. Sci. U.S.A. 74:3922-3926.

31. Miller, A.M., Russel, T.R., Gross, M.A., and Yunis, A.A. 1978. Modulation of granulopoiesis: opposing roles of prostaglandins F and E. J. Lab Clin. Med. 92:983-990.

32. Smith, W.L., and Laneuville, O.L. 1994. Cyclooxygenase and Lipoxygenase Pathways of Arachidonic Metabolism. In: Prostaglandin Inhibitors in Tumor Immunology and Immunotherapy, CRC Press, Boca Raton, Chapter 1:1-24.

33. Smith, W.L., DeWitt, D.L., and Allen, M.L. 1983. Bimodal distribution of the prostaglandin I2 synthase antigen in smooth muscle cells. J. Biol. Chem. 258:5922-5926.

34. Hamberg, M., and Samuelsson, B. 1974. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. Proc. Natl. Acad. Sci. U.S.A. 71:3400-3404.

35. Pace-Asciak, C.R. and Smith, W.L. 1983. Enzymes in the Biosynthesis and Catabolism of the Eicosanoids: Prostaglandins, Thromboxanes, Leukotrienes and Hydroxy Fatty Acids. In: The Enzymes. Vol 16, Boyer, P.D. Ed. Academic Press, New York. pp 543-570.

36. Herschman, H.R. 1996. Prostaglandin synthase 2. Biochim. Biophys. Acta. 1299:125-140.

37. Marshall, P.J., and Kumacz, R.J. 1988. Prostaglandin H synthase: distinct binding sites for cyclooxygenase and peroxidase substrates. Arch. Biochem. Biophys. 266:162-170.

38. Simmons, D.L., Levy, D.B., Yannoni, Y., and Erikson, R.L. 1989. Identification of a phorbol ester-repressible v-src-inducible gene. Proc. Natl. Acad. Sci. U.S.A. 86:1178-1182.

39. Smith, W.L. 1992. Prostanoid biosynthesis and mechanisms of action. Am. J. Physiol. 263:F181-F191.

40. Sigal, E. 1991. The molecular biology of mammalian arachidonic acid metabolism. Am. J. Physiol. 260:L13-L28.

41. Simonson, M.S., Wolfe, J.A., and Dunn, M.J. 1990.Regulation of Prostaglandin Synthesis by Differential Expression of the Gene Encoding Prostaglandin Endoperoxide Synthase. In: Advances in Prostaglandin, Thromboxane and Leukotriene Research, Ed: Sammuelsson, B., Ramwell, P., Paoletti, R. Raven Press, New York. pp 21-69.

42. Smith, W.L., DeWitt, D.L., Kraemer, S.A., Andrews, M.J., Hla, T., Maciag, T., and Shimokawa, T. 1990. Structure Function Relationships in Sheep, Mouse and Human Prostaglandin Endoperoxide G/H Synthase. In: Advances in Prostaglandin, Thromboxane and Leukotriene Research, Ed: Sammuelsson, B., Ramwell, P., Paoletti, R. Raven Press, New York. pp 20-24.

43. Yokoyama, C. and Tanabe, T. 1989. Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. Biochem. Biophys. Res. Commun. 165: 888-894.

44. Kosaka, T., Miyata, A., Ihara, H., Hara, S., Sugimoto, T., Takeda, O., Takahashi, E., and Tanabe, T. 1994. Characterization of the human gene (PTGS2) encoding prostaglandin-endoperoxide synthase 2. Eur. J. Biochem. 221:889-897.

45. Appleby, S. B., Ristimaki, A., Neilson, K., Narko, K. and Hla, T. 1994. Structure of the human cyclo-oxygenase-2 gene. Biochem. J. 302: 723-727.

46. Smith, W.L., Garavito, R. and DeWitt, D. 1996. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J. Biol. Chem. 271: 33157-33160.

47. Smith, C. J., Morrow, J. D., Roberts, L. J. I. and Marnett, L. J. 1993. Differentiation of monocytoid THP-1 cells with phorbol ester induces expression of prostaglandin endoperoxide synthase-1 (COX-1). Biochem. Biophys. Res. Commun. 192: 787-793.

48. Herschman, H.R., Kujubu, D.A., Fletcher, G.S., Ma, Q-F., Varnum, D.C., Gilbert, R.S., and Reddy, S.T. 1994. In: Prog. in Nucleic Acids Res. and Mol. Biol. Eds: Cohen, W. and Moldave, K. Academic Press, Orlando. Pp 115-148.

49. DeWitt, D.L., and Meade, E.A. 1993. Serum and glucocorticoid regulation of gene transcription and expression of the prostaglandin H synthase-1 and prostaglandin H synthase-2 isozymes. Arch. Biochem. Biophys. 306:94-102.

50. Smith, W.L., and DeWitt, D.L. 1995. Biochemistry of prostaglandin endoperoxide H synthase-1 and synthase-2 and their differential susceptibility to nonsteroidal anti-inflammatory drugs. Semin. Nephrol. 15:179-94.

51. Hla, T. 1996. Molecular characterization of the 5.2 KB isoform of the human cyclooxygenase-1 transcript. Prostaglandins. 51: 81-85.

52. O'Banion, M. K., Winn, V. D. and Young, D. A. 1992. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. Proc. Natl. Acad. Sci. U.S.A. 89: 4888-4892.

53. Kujubu, D.A., Fletcher, B.S., Varnum, B.C., Lim, R.W., and Herschman, H.R. 1991. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J. Biol. Chem. 266:12866-12872.

54. Funk, C. D., Funk, L. B., Kennedy, M. E., Pong, A. S. and FitzGerald, G. A. 1991. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. FASEB J. 5: 2304-2312.

55. Bailey, J.M., and Verma, M. 1990. Identification of a highly conserved 3' UTR in the translationally regulated mRNA for prostaglandin synthase. Prostaglandins. 40:585-590.

56. Kulmacz, R.J., Pendleton, R.B., and Lands, W.E. 1994. Interaction between peroxidase and cyclooxygenase activities in prostaglandin-endoperoxide synthase. Interpretation of reaction kinetics. J. Biol. Chem. 269:5527-5536.

57. Barnett, J., Chow, J., Ives, D., Chiou, M., Mackenzie, R., Osen, E., Nguyen, B., Tsing, S., Bach, C., Freire, J., Chan, H., Sigal., and Ramesha, C. 1994. Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. Biochem. Biophys. Acta. 1209:130-139.

58. Laneuville, O.L., Breuer, D.K., DeWitt, D.L., Hla, T., Funk, C.D., and Smith, W.L. Differential inhibition of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. 1994. J. Pharmcol. Exp. Ther. 271:927-934.

59. Hsi, L.C., Hoganson, C.W., Babcock, G.T., and Smith, W.L. 1994. Characterization of a tyrosyl radical in prostaglandin endoperoxide synthase-2. Biochem. Biophys. Res. Commun. 202:1592-1598.

60. Meade, E.A., Smith, W.L., and DeWitt, D.L. 1993. Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 268:6610-6614.

61. Simmons, D.L., Xie, W., Chipman, J.G., and Evett, G.E. 1991. Multiple Cyclooxygenases: Cloning of a Mitogen Inducible Form. In: Prostaglandins, Leukotrienes, Lipoxins, and PAF. Eds:Bailey, J.M. Plenum Press, New York. pp 67-78.

62. Isakson, P., Seibert, K., Masferrer, J., Salvemini, D., Lee, L., and Needleman, P. 1995. Discovery of a better aspirin. Adv. Prostaglandin Thromboxane Leukotriene Res. 23:49-54.

63. DeWitt, D.L., and Smith, W.L. 1988. Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. Proc. Natl. Acad. Sci. U.S.A. 85:1412-1216.

64. Merlie, J.P., Fagan, D., Mudd, J., and Needleman, P. 1988. Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). J. Biol.Chem. 263:3550-3553..

65. Yokoyama, C., Takai, T., and Tanabe, R. 1988. Primary structure of sheep prostaglandin endoperoxide synthase deduced from cDNA sequence. FEBS Lett. 231:347-351.

66. Xie, W., Chipman, J.G., Robertson, D.L., Erikson, R.l., and Simmons, D.L. 1991. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc. Natl. Acad. Sci. U.S.A. 88:2692-2696.

67. Hla, T. and Neilson, K. 1992. Human cyclooxygenase-2 cDNA. Proc. Natl. Acad. Sci. U.S.A. 89:7384-7388.

68. DeWitt, D.L., El-Harith, E.A., Kraemer, S.A., Andrews, M.J., Yao, E.F., Armstrong, R.L., and Smith, W.L. 1990. The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. J. Biol. Chem. 265:5192-5198.

69. Otto, J.C., DeWitt, D.L., and Smith, W.L. 1993. N-glycosylation of prostaglandin endoperoxide synthases-1 and -2 and their orientations in the endoplasmic reticulum. J. Biol. Chem. 268:18234-18242.

70. Morita, I., Schindler, M., Regier, M.K., Otto, J.C., Hori, T., and DeWitt, D.L. 1995. Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. J. Biol. Chem. 270:10902-10908.

71. Reddy, S.T., and Herschman, H.R. 1994. Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages. J. Biol. Chem. 269:15473-15480.

72. Murakami, M., Matsumoto, R., Austen, K.F., and Arm, J.P. 1994. Prostaglandin endoperoxide synthase-1 and -2 couple to different transmembrane stimuli to generate prostaglandin D2 in mouse bone marrow-derived mast cells. J. Biol. Chem. 269:22269-22275.

73. Forman, B.M., Tontonoz, P., Chem, J., Brun, R.P., Spiegelman, B.M., and Evans, R.M. 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell. 83:803-812.

74. Devchand, P.R., Keller, H., Peters, J.M., Vazquez, M., Gonzalez, F.J., and Whali, W. 1996. The PPARalpha-leukotriene B4 pathway to inflammation control. Nature. 384:39-43.

75. Picot, D., Loll, P.J., and Garavito, M. 1994. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. Nature. 367:243-249.

76. Holtzman, M.J., Turk, J., and Shornick, L.P. 1992. Identification of a pharmacologically distinct prostaglandin H synthase in cultured epithelial cells. J. Biol. Chem. 267:21438-21445.

77. Smith, W.L., and DeWitt, D.L. 1996. Prostaglandin Endoperoxide H Synthases (Cyclooxygenases) -1 and -2. In:Advances in Immunology. Eds:Dixon, F.J. Academic Press, Orlando. 62:167-215.

78. Wang, L,-H., Hajibeigi, A., Xu, X,-M., Loose-Mitchell, D., and Wu, K.K. 1993. Characterization of the promoter of human prostaglandin H synthase-1 gene. Biochem. Biophys. Res. Comm. 190(2):406-411.

79. Xu, X.-M., Tang, J.-H., Hajibeigi, A., Loose-Mitchell, D., and Wu, K.K. 1996. Transcriptional regulation of endothelial constitutive PGHS-1 expression by phorbol ester. Amer. J. Physiol. 270:C259-C264.

80. Becker, R.P., and DeBruyn, P.P.H. 1976. The transmural passage of blood cells into myeloid sinusoids and the entry of platelets into the sinusoidal circulation; a scanning electron microscopic investigation. Am. J. Anat. 183:206-216.

81. Stenberg, P.E., and Levin, J. 1989. Mechanisms of platelet production. Blood Cells. 15:23-47.

82. Gerwirtz, A.M. 1986. Human megakaryocytopoiesis. Semin. Hematol. 23:27-30.

83. Mazur, E.M. 1987. Megakaryocytopoiesis and platelet production: a review. Exp. Hematol. 15:340-350.

84. Levine, R.F. 1986. Megakaryocyte Development and Function. In: Progress in Clinical and Biological Research. Alan R. Liss Inc. New York. pp 1-20.

85. Breton-Gorius, J., and Vainchenker, W. 1986. Expression of platelet proteins during the in vitro and in vivo differentiation of megakaryocytes and morphological aspects of their maturation. Semin. Hematol. 23:43-67.

86. Hoffman, R. 1989. Regulation of megakaryocytopoiesis. Blood. 74:1196-1212.

87. Long, M.W. 1993. Population heterogeneity among cells of the megakaryocyte lineage. Stem Cells. 11:33-40.

88. Williams, N. 1990. Stimulators of megakaryocyte development and platelet production. Prog. In Growth Fac. Res. 2:81-95.

89. Murphy, M.J. 1989. Megakaryocyte colony-stimulating factor and thrombopoiesis. Hematol./Oncol. Clin. North Am. 3:465-478.

90. Long, M.W., Heffner, C.H., and Gragowski, L.L. 1988. Cholera toxin and phorbol diesters synergistically modulate murine hematopoietic progenitor cell proliferation. Exp. Hematol. 16:195-200.

91. Long, M.W. 1989. Signal transduction events in in vitro megakaryocytopoiesis. Blood Cells. 15:205-229.

92. Long, M.W., Gragowski, L.L. Heffner, C.H., and Boxer, L.A. 1985. Phorbol diesters stimulate the development of an early murine progenitor cell. The burst-forming unit-megakaryocyte. J. Clin. Invest. 67:431-438.

93. Briddell, R.A., and Hoffman, R. 1990. Cytokine regulation of the human burst-forming unit-megakaryocyte. Blood. 76:516-522.

94. Briddell, R.A., Bruno, E., and Cooper, R.J. 1991. Effect of c-kit ligand on in vitro human megakaryocytopoiesis. Blood. 78:2854-2859.

95. Bruno, E., Briddell, R.A., Cooper, R.J., and Hoffman, R. 1991. Effects of recombinant interleukin 11 on human megakaryocyte progenitor cells. Exp. Hematol. 19:378-381.

96. Teramura, M., Kabayashi, S., and Hoshiro, S. 1992. Interleukin-11 enhances human megakaryocytopoiesis in vitro. Blood. 79:327-331.

97. Avraham, H., Scadden, D.T., and Chi, S. 1992. Interaction of human bone marrow fibroblasts with megakaryocytes: role of the c-kit ligand. Blood. 80:1679-1684.

98. Williams, N., and Jackson, H. 1982. Kinetic analysis of megakaryocyte numbers and ploidy levels in developing colonies from mouse bone marrow cells. Cell Tissue Kinet. 15:483-494.

99. Bruno, E., Miller, M.E., and Hoffman, R. 1989. Interacting cytokines regulate in vitro human megakaryocytopoiesis. Blood. 72:671-677.

100. Long, M.W., Hutchinson, R.J., and Gragowski, L.L. 1988. Synergistic regulation of human megakaryocyte development. J. Clin. Invest. 82:1779-1786.

101. Long, M.W., Williams, N., McDonald, T.P. 1982. Immature megakaryocytes in the mouse: in vitro relationship to megakaryocyte progenitor cells and mature megakaryocytes. J. Cell Physiol. 112:339-344.

102. Long, M.W., and Williams, N. 1981. Immature Megakaryocytes in the Mouse: Morphology and quantitation by acetylcholinesterase staining. Blood. 58:1032-1039.

103. Jackson, C.W. 1973. Cholinesterase as a possible marker for early cells of the megakaryocytic series. Blood. 42:413-421.

104. Jackson, C.W. 1974. Some Characteristics of Rat Megakaryocyte Precursors Identified Using Cholinesterase as a Marker. In: Platelets: Production, Function, Transfusion and Storage. Eds: Baldini, M.G., and Ebbe, S. Grune and Stratton, New York. pp 33-40.

105. Nakeff, A., and Floeh, D.P. 1976. Separation of megakaryocytes from mouse bone marrow by density gradient centrifugation. Blood. 48:133-138.

106. Rabellino, E.M., Levene, R.B., Leung, L.L.K., and Nachman. R.L. 1981. Human megakaryocytes. II. Expression of platelet proteins in early marrow megakaryocytes. J. Exp. Med. 154:88-100.

107. Rabellino, E.M., Nachman, R.L., Williams, N., Winchester, R.J., and Ross, G.D. 1979. Human megakaryocytes. I. Characterization of the membrane and cytoplasmic components of isolated marrow megakaryocytes. J. Exp. Med. 149:1273-1287.

108. Vainchenker, W., Guichard, J., and Deschamps, J.F. 1982. Megakaryocyte cultures in the chronic phase and in the blast crisis of chronic myeloid leukaemia: studies on the differentiation of the megakaryocyte progenitors and on the maturation of megakaryocytes in vitro. Br. J. Hematol. 51:131-146.

109. Long, M.W., Williams, N., and Ebbe, S. 1982. Immature megakaryocytes in the mouse: physical characteristics, cell cycle status, and in vitro responsiveness to thrombopoietic stimulatory factor. Blood. 59:569-575.

110. Hegyi, E., Navarro, S., and Debili, N. 1990. Regulation of human megakaryocytopoiesis: analysis of proliferation, ploidy and maturation in liquid cultures. Int. J. Cell. Cloning. 8:236-244.

111. Breton-Gorius, J., Vainchenker, W., and Nurden, A. 1981. Defective alpha-granule production in megakaryocytes from gray platelet syndrome: ultrastructural studies of bone marrow cells and megakaryocytes growing in culture from blood precursors. Am. J. Pathol. 102:10-19.

112. Debili, N., Issaad, C., and Masse, J.M. 1992. Expression of CD34 and platelet glycoproteins during human megakaryocytic differentiation. Blood. 80:3022-3035.

113. Vainchenker, W., Deschamps, J.F., Bastin, J.M., Guichard, J., Titeux, M., Breton-Gorius, J., and McMichael, A.J. 1982. Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation: immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in in vivo cells from normal and leukemic patients. Blood. 59:514-521.

114. Worthington, R.E., Nakeff, A., and Micko, S. 1984. Flow cytometric analysis of megakaryocyte differentiation. Cytometry. 5:501-508.

115. Levene, R.B., Lamaziere, J.M.D., Broxmeyer, H.E., Lu, L., and Rabellino, E.M. 1985. Human megakaryocytes. V. Changes in the phenotypic profile of differentiating megakaryocytes. J. Exp. Med. 161:457-474.

116. Gerrard, J.M., and Carroll, R.C. 1981. Stimulation of platelet protein phosphorylation by arachidonic acid and endoperoxide analogs. Prostaglandins. 22:81-94.

117. Pollock, W.K., Armstrong, R.A., and Brydon, L.J. 1984. Thromboxane-induced phosphatidate formation in human platelets. Relationship to receptor occupancy and to changes in cytosolic free calcium. Biochem. J. 219:833-842.

118. Best, L.C., McGuire, M.B., and Martin, T.J. 1979. Effects of epoxymethano analogues of prostaglandin endoperoxides on aggregation, on release of 5-hydroxytryptamine and on the metabolism of 3',5'-cyclic AMP and cyclic GMP in human platelets. Biochim. Biophys. Acta. 583:344-351.

119. Brace, L.D., Venton, D.L., and Le Breton, G.C. 1985. Thromboxane A2/prostaglandin H2 mobilizes calcium in human blood platelets. Am. J. Physiol. 249:H1-H7.

120. Brass, L.F., Woolkalis, M.J., and Manning, D.R. 1988. Interactions in platelets between G proteins and the agonists that stimulate phospholipase C and inhibit adenylyl cyclase. J. Biol. Chem. 263:5348-5355.

121. Mills, D.C.B., and Smith, J.B. 1971. The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3':5'-cyclic monophosphate in platelets. Biochem. J. 121:185-196.

122. Haslam, R.J. 1973. Interactions of the pharmacological receptors of blood platelets with adenylate cyclase. Ser. Haematol. 6:333-350.

123. Steer, M.L., and Wood, A. 1979. Regulation of human platelet adenylate cyclase by epinephrine, prostaglandin E1, and guanine nucleotides. Evidence for separate guanine nucleotide sites mediating stimulation and inhibition. J. Biol. Chem. 254:10791-10797.

124. Goldhaber, S. Z., Manson, J. E., Stampfer, M. J., LaMotte, F., Rosner, B., Buring, J. E. and Hennekens, C. H. 1992. Low-dose aspirin and subsequent peripheral arterial surgery in the Physicians' Health Study. The Lancet. 340: 143-145.

125. Boger, R. H., Bode-Boger, S. M., Gutzki, F. M., Tsikas, D., Weskott, H.-P. and Frolich, J. C. 1993. Rapid and selective inhibition of platelet aggregation and thromboxane formation by intravenous low dose aspirin in man. Clinical Sci. 84: 517-524.

126. Yoshino, T., Sakaguchi, M., Masuda, T., Kawakita, M., and Takatsuki, K. 1996. Two regulation points for differentiation in the cell cycle of human megakaryocytes. Br. J. Haematol. 92:780-787.

127. Ogura, M., Morishima, Y., Okumura, M., Hotta, T., Takamoto, S., Ohno, R., Hirabayashi, N., Nagura, H. and Saito, H. 1988. Functional and morphological differentiation induction of a human megakaryoblastic leukemia cell line (MEG-01s) by phorbol diesters. Blood. 72: 49-60.

128. Long, M.W., Smolen, J.E., Szczepanski, P., and Boxer, L.A. 1984. Role of phorbol diesters in in vitro murine megakaryocyte colony formation. J. Clin. Invest. 74:1686-1692.

129. Matijevic-Aleksic, N., Sudershan, K.S., Wang, L-H., and Wu, K.K. 1995. Differential expression of thromboxane A synthase and prostaglandin H synthase in megakaryocytic cell line. Biochim. Biophys. Acta. 1269:167-175.

130. Ogura, M., Morishima, Y., Ohno, R., Kato, Y., Hirabayashi, N., Nagura, H., and Saito, H. 1985. Establishment of a novel human megakaryoblastic leukemia cell line, MEG-01, with positive Philadelphia chromosome. Blood. 66:1384-1392.

131. Ueda, N., Yamashita, R., Yamamoto, S., and Ishimura, K. 1997. Induction of cyclooxygenase-1 in a human megakaryoblastic cell line (CMK) differentiated by phorbol ester. Biochim. Biophys. Acta. 1344:103-110.

132. Denome, R.M., and Cole, C.N. 1988. Patterns of polyadenylation site selection in gene constructs containing multiple polyadenylation signals. Mol. Cell. Biol. 8:4829-4839.

133. Curtis, D., Lehmann, R., and Zamore, P.D. 1995. Translational regulation in development. Cell. 81:171-178.

134. Ross, J. 1995. mRNA stability in mammalian cells. Microbiol. Rev. 59:423-450.

135. Sachs, A., and Wahle, E. 1993. Poly(A) tail metabolism and function in eucaryotes. J. Biol. Chem. 268:22955-22958.

136. Ford, L.P., Bagga, P.S., and Wilusz, J. 1997. The poly(A) tail inhibits the assembly of a 3'-to-5' exonuclease in an in vitro RNA stability system. Mol. Cell. Biol. 17:398-406.

137. Wickens, M., and Stephenson, P. 1984. Role of the conserved AAUAAA sequence: four AAUAAA point mutants prevent messenger RNA 3' end formation. Science. 226:1045-1051.

138. Eckner, R., Ellmeier, W., and Birnstiel, M.L. 1991. Mature mRNA 3' end formation stimulates RNA export from the nucleus. EMBO J. 10:3513-3522.

139. Huang, Y., and Carmichael, C.G. 1996. A suboptimal 5' splice site is a cis-acting determinant of nuclear export of polyomavirus late mRNAs. Mol. Cell. Biol. 16:6046-6054.

140. Huang, Y., and Carmichael, C.G. 1996. Role of polyadenylation in nucleocytoplasmic transport of mRNA. Mol. Cell. Biol. 16:1534-1542.

141. Proudfoot, N.J. and Brownlee, G.G. 1976. 3' non-coding region sequences in eukaryotic messenger RNA. Nature. 263:211-214.

142. Berget, S.M. 1984. Are U4 small nuclear ribonucleoproteins involved in polyadenylation? Nature. 309:179-182.

143. Swimmer, C., and Shenk, T. 1985. Selection of sequence elements that substitute for the standard AATAAA motif which signals 3' processing and polyadenylation of late simian virus 40 mRNAs. Nucleic Acids Res. 13:8053-8063.

144. Higgs, D.R., Goodburn, S., Lamb, J., Clegg, J.B., Weatherall, D.J., and Proudfoot, N.J. 1983. Alpha-thalassaemia caused by a polyadenylation signal mutation. Nature. 306:398-400.

145. Orkin, S.H., Cheng, T.-C., Autonarakis, S.E., and Kazazian, H.H. 1985. Thalassemia due to a mutation in the cleavage-polyadenylation signal of the human beta-globin gene. EMBO J. 4:453-456.

146. Taya, Y., Devos, R., Tavernier, J., Cherokutre, H., Engler, G., and Fiers, W. 1982. Cloning and structure of the human immune interferon-gamma chromosomal gene. EMBO J. 1:953-958.

147. McLauchlan, J., Gaffney, D., Whitton, J.L., and Clements, J.B. 1985. The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3' termini. Nucleic Acids Res. 13:1347-1368.

148. Manley, J.L. 1995. A complex protein assembly catalyzes polyadenylation of mRNA precursors. Curr. Opin. Genet. Dev. 5:222-228.

149. Chen, J., and Moore, C. 1992. Separation of factors required for cleavage and polyadenylation of yeast pre-mRNA. Mol. Cell. Biol. 12:3470-3481.

150. Wahle, E., and Keller, W. 1992. The biochemistry of 3'-end cleavage and polyadenylation of messenger RNA precursors. Annu. Rev. Biochem. 61:419-440.

151. Takagaki, Y., and Manley, J.L. 1995. A polyadenylation factor subunit is the human homologue of the Drosophila suppressor of forked protein. Nature. 372:471-474.

152. Takagaki, Y., MacDonald, C.C., Shenk, T., and Manley, J.L. 1992. The human 64-kDa polyadenylylation factor contains a ribonucleoprotein-type RNA binding domain and unusual auxiliary motifs. Proc. Natl. Acad. Sci. U.S.A. 89:1403-1407.

153. Takagaki, Y., and Manley, J.L. 1992. A human polyadenylation factor is a G protein beta-subunit homologue. J. Biol. Chem. 267:23471-23474.

154. Bienroth, S., Keller, W., and Wahle, E. 1993. Assembly of a processive messenger RNA polyadenylation complex. EMBO J. 12:585-594.

155. Wahle, E. 1995. Poly(A) tail length control is caused by termination of processive synthesis. J. Biol. Chem. 270:2800-2808.

156. Bernstein, P., and Ross, J. 1989. Poly(A), poly(A) binding protein and the regulation of mRNA stability. Trends Biochem. Sci. 14:373-377.

157. Peltz, S.W., and Jacobson, A. 1992. mRNA stability: in trans-it. Curr. Opin. Cell Biol. 4:979-983.

158. Bernstein, P., Peltz, S.W., and Ross, J. 1989. The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro. Mol. Cell. Biol. 9:659-670.

159. Chen, C.Y.A., Chen, T.M., and Shyu, A.B. 1994. Interplay of two functionally and structurally distinct domains of the c-fos AU-rich element specifies its mRNA-destabilizing function. Mol. Cell. Biol. 14:416-426.

160. Maicas, E., Shago, M., and Freisen, J.D. 1990. Translation of the Saccharomyces cerevisiae tcm1 gene in the absence of a 5'-untranslated leader. Nucleic Acids Res. 18:5823-5828.

161. Muhlrad, D., and Parker, R. 1992. Premature translational termination triggers mRNA decapping. Nature. 340:578-581.

162. Bernstein, P.L., Herrick, D.J., Prokipcak, P.D., and Ross, J. 1992. Control of c-myc mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant. Genes Dev. 6:642-654.

163. Binder, R., Horowitz, J.A., Basilion, J.P., Koeller, D.M., Klausner, R.D., and Harford, J.B. 1994. Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening. EMBO J. 13:1969-1980.

164. Dompenciel, R.E., Garnepudi, V.R., and Schoenberg, D.R. 1995. Purification and characterization of an estrogen-regulated Xenopus liver polysomal nuclease involved in the selective destabilization of albumin mRNA. J. Biol. Chem. 270:6108-6118.

165. Brewer, G., and Ross, J. 1988. Poly(A) shortening and degradation of the 3' A+U-rich sequences of human c-myc mRNA in a cell-free system. Mol. Cell. Biol. 8:1697-1708.

166. Fort, P., Rech, J., Vie, A., Piechaczyk, M., Bonnieu, A., Jeanteur, P., and Blanchard, J.M. 1987. Regulation of c-fos gene expression in hamster fibroblasts: initiation and elongation of transcription and mRNA degradation. Nucleic Acids Res. 15:5657-5667.

167. Ross, J., Kobbs, G., Brewer, G., and Peltz, S.W. 1987. Properties of the exonuclease activity that degrades H4 histone mRNA. J. Biol. Chem. 262:9374-9381.

168. Gorlach, M., Burd, C.G., and Dreyfuss, G. 1994. The mRNA poly(A)-binding protein: localization, abundance, and RNA-binding specificity. Exp. Cell Res. 211:400-407.

169. Sachs, A.B., Bond, M.W., and Kornberg, R.D. 1986. A single gene from yeast for both nuclear and cytoplasmic polyadenylate-binding proteins: domain structure and expression. Cell. 45:827-835.

170. Sheiness, D., and Darnell, J.E. 1973. Polyadenylic acid segment in mRNA becomes shorter with age. Nat. New Biol. 241:265-268.

171. Marzluff, W.F., and Pandey, N.B. 1988. Multiple regulatory steps control histone mRNA concentrations. Trends, Biochem. Sci. 13:49-52.

172. Klausner, R.D., Rouault, T.A., and Harford, J.B. 1993. Regulating the fate of mRNA: the control of cellular iron metabolism. Cell. 72:19-28.

173. Harford, J.B., Rouault, T.A., and Klausner, R.D. 1994. In: Iron metabolism in health and disease. Eds: Brock, J.H., Halliday, J.W., Pippard, M.J., and Powell, L.W. The W.B. Saunders Co., Philadelphia. pp 123-149.

174. Alterman, R.B., Ganguly, S., Schulze, D.H., Marzluff, W.F., Schildkraut, C.L., and Skoultchi, A.I. 1984.. Cell cycle regulation of mouse H3 histone mRNA metabolism. Mol. Cell. Biol. 4:123-132.

175. Altus, M.S., and Nagamine, Y. 1991. Protein synthesis inhibition stabilizes urokinase-type plasminogen activator mRNA. Studies in vivo and in cell-free decay reactions. J. Biol. Chem. 266:21190-21196.

176. Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. 1986. Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. Proc. Natl. Acad. Sci. U.S.A. 83:1670-1674.

177. Shaw, G., and Kamen, R. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell. 46:659-667.

178. Pandey, N.B. 1994. Point mutations in the stem-loop at the 3' end of mouse histone mRNA reduce expression by reducing the efficiency of 3'end formation. Mol. Cell. Biol. 14:1709-1720.

179. Chen, F.Y., Amara, F.M., and Wright, J.A. 1993. Mammalian ribonucleotide reductase R1 mRNA stability under normal and phorbol ester stimulating conditions:involvement of a cis-trans interaction at the 3' untranslated region. EMBO J. 12:3977-3986.

180. Burd, C.G., and Dreyfuss, G. 1994. Conserved structures and diversity of functions of RNA-binding proteins. Science. 265:615-621.

181. Chen, C.Y.A., and Shyu, A.B. 1995. AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem. Sci. 20:465-470.

182. Katz, D.A. 1994. AU-A, an RNA-binding activity distinct from hnRNP A1, is selective for AUUUA repeats and shuttles between the nucleus and the cytoplasm. Nucleic Acids Res. 22:238-246.

183. Malter, J.S., and Hong, Y. 1991. A redox switch and phosphorylation are involved in the post-translational up-regulation of the adenosine-uridine binding factor by phorbol ester and ionophore. J. Biol. Chem. 266:3167-3171.

184. Brewer, G. 1991. An A + U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro. Mol. Cell. Biol. 11:2460-2466.

185. Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

186. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

187. Dewitt, D. L. 1991. Prostaglandin endoperoxide synthase: regulation of enzyme expression. Biochim. Biophys. Acta. 1083:121-134.

188. Hoff, T., DeWitt, D. L., Kaever, V., Resch, K. and Goppelt-Struebe, M. 1993. Differentiation-associated expression of prostaglandin G/H synthase in monocytic cells. FEBS Letters 320: 38-42.

189. Vane, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature New Biol. 231:232-234.

190. Patrono, C. 1994. Aspirin as an antiplatelet drug. The New England J. Med. 330:1287-1294.

191. Takeuchi, I., Ogura, M., Saito, H., Satoh, M. and Takeuchi, M. 1991. Production of platelet-like particles by a human megakaryoblastic leukemia cell line (MEG-01). Exp. Cell Res. 193:223-226.

192. Mijovic, J. E., Zakar, T., Nairn, T. K. and Olson, D. M. 1998. Prostaglandin endoperoxide H synthase (PGHS) activity and PGHS-1 and -2 messenger ribonucleic acid abundance in human chorion throughout gestation and with preterm labor. J. Clin. Endocrinol. Metabol. 83:1358-1367.

193. Edwalds-Gilbert, G., Veraldi, K. L. and Milcarek, C. 1997. Alternative poly(A) site selection in complex transcription units: means to an end? Nucl. Acids Res. 25:2547-2561.

194. Sheets, M. D., Ogg, S. C. and Wickens, M. P. 1990. Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation in vitro. Nucl. Acids Res. 18:5799-5805.

195. Grover, J. and Roughley, P. J. 1995. Expression of cell-surface proteoglycan mRNA by human articular chondrocytes. Biochem. J. 309:963-968.

196. Kojima, T., Inazawa, J., Takamatsu, J., Rosenberg, R. D. and Saito, H. 1993. Human ryudocan core protein: molecular cloning and characterization of the cDNA, and chromosomal localization of the gene. Biochem. Biophys. Res. Commun. 190:814-822.

197. Tanimoto, K., Murakami, K. and Fukamizu, A. 1993. Possible roles of the 3'-flanking sequences of the human activin beta A subunit gene in its expression. Arch. Biochem. Biophys. 302:409-416.

198. Sun, Y., Hegamyer, G., Kim, h., Sithanandam, K., Li, H., Watts, R. and Colburn, N. H. 1995. Molecular cloning of mouse tissue inhibitor of metalloproteinases-3 and its promoter. J. Biol. Chem. 270:19312-19319.

199. Ristimaki, A., Garfinkel, S., Wessendorf, J., Maciag, T. and Hla, T. 1994. Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation. J. Biol. Chem. 269: 11769-11775.

200. Newton, R., Seybold, J., Liu, S. F. and Barnes, P. J. 1997. Alternate COX-2 transcripts are differentially regulated: implications for post-transcriptional control. Biochem. Biophys. Res. Commun. 234: 85-89.

201. Ristimaki, A., Narko, K. and Hla, T. 1996. Down-regulation of cytokine-induced cyclo-oxygenase-2 transcript isoforms by dexamethasone: evidence for post-transcriptional regulation. Biochem. J. 318: 325-331.

202. Brannon, T. S., North, A. J., Wells, L. B. and Shaul, P. W. 1994. Prostacyclin synthesis in ovine pulmonary artery is developmentally regulated by changes in cyclooxygenase-1 gene expression. J. Clin. Invest. 93:2230-2235.

203. Wasylyk, C., Flores, P., Gutman, A., and Wasylyk, B. 1989. PEA3 is a nuclear target for transcription activation by non-nuclear oncogenes. EMBO J. 8:3371-3378.

204. Wager, R.E., and Assosian, R.K. 1990. A phorbol ester-regulated ribonuclease system controlling transforming growth factor beta 1 gene expression in hematopoietic cells. Mol. Cell. Biol. 10:5983-5990.

205. Weber, B., Horiguchi, J., Luebbers, R., Sherman, M., and Kufe, D. 1989. Posttranscriptional stabilization of c-fms mRNA by a labile protein during human monocytic differentiation. Mol. Cell. Biol. 9:769-775.

206. Wodnar-Filipowicz, A., and Moroni, C. 1990. Regulation of interleukin 3 mRNA expression in mast cells occurs at the posttranscriptional level and is mediated by calcium ions. Proc. Natl. Acad. Sci. U.S.A. 87:777-781.

207. Yamoto, K., El-Hajjaoui, Z., and Koeffler, H.P. 1989. Regulation of levels of IL-1 mRNA in human fibroblasts. J. Cell. Physiol. 139:610-616.

208. Jones, T., and Cole, M. 1987. Rapid cytoplasmic turnover of c-myc mRNA: requirement of the 3' untranslated sequences. Mol. Cell. Biol. 7:4513-4521.

209. Amara, F.M., Chen, F.Y., and Wright, J.A. 1994. Phorbol ester modulation of a novel cytoplasmic protein binding activity at the 3'-untranslated region of mammalian ribonucleotide reductase R2 mRNA and role in message stability. J. Biol. Chem. 269:6709-6715.

210. Gillis, P., and Malter, J.S. 1991. The adenosine-uridine binding factor recognizes the AU-rich elements of cytokine, lymphokine, and oncogene mRNAs. J. Biol. Chem. 266:3172-3177.

211. Bereziat, G., Etienne, J., Kokkinidis, M., Oliviera, J.L., and Pernas, P. 1990. New trends in mammalian non-pancreatic phospholipase A2 research. J. Lipid Mediators. 2:159-172.

212. Sirios, J., and Richards, J.S. 1992. Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles. J. Biol. Chem. 267:6382-6388.

213. Williams, C.S., and DuBois, R.N. 1996. Prostaglandin endoperoxide synthase: why two isoforms? Amer. J. Physiol. 270:G393-G400.

214. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

215. Ikai, K., and Imamura, S. 1988. Prostaglandin D2 in the skin. Inter. J. Dermatol. 27:141-149.

216. Hawkey, C.J. 1999. COX-2 inhibitors. The Lancet. 353:307-314.

217. Langenbach, R., Morham, S.G., Tiano, H.F., Loftin, C.D., Ghanayem B.I., Chulada, P.C., Mahler, J.F., Lee, C.A., Goulding, E.H., Kluckman, K.D., Kim, H.S., and Smithies, O. 1995. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. Cell. 83:483-492.

218. Morham, S.G., Langenbach, R., Loftin, C.D., Tiano, H.F., Vouloumanos, N., Jennette, J.C., Mahler, J.F., Kluckman, K.D., Ledford, A., Lee, C.A., and Smithies, O. 1995. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell. 83:473-482.

219. Mahler, J.F., Davis, B.J., Morham, S.G., and Langenbach, R. 1996. Disruption of cyclooxygenase genes in mice. Toxicological Pathology. 24:717-719.

220. DuBois, R.N., Gupta, R., Brockman, J., Reddy, S.B., Krakow, L.K., and Lazar, M.A. 1998. The nuclear eicosanoid receptor, PPAR, is aberrantly expressed in colonic cancers. Carcinogenesis. 19:49-53.

221. Sheets, D.M., Ogg, S.C., and Wickens, M.P. 1990. Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation *in vitro*. Nucleic Acids Research, 18:5799-5805.

222. Bernstein, R., and Ross, J. 1989. Poly (A), poly (A) binding protein and the regulation of mRNA stability. Trends Biochem. Sci. 14:373-377.

223. Johnston, D.S. 1995. The intracellular localization of messenger RNAs. Cell. 81:161-170.

224. Kislauskis, E.D., Li, Z., Singer, R.H., and Taneja, K.L. 1993. Isoform specific 3'-untranslated sequences sort α-cardiac and β-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. The Journal of Cell Biology. 123:165-172.

225. Hershey, J.W.B. 1991. Translational control in mammalian cells. Annu. Rev. Biochem. 60:717-755.

226. Thach, R.E. 1992. Cap recap: the involvement of eIF-4F in regulating gene expression. Cell. 68:177-180.

227. Ostareck-Lederer, A., Ostareck, D.H., Standart, N., and Thiele, B. 1994. Translation of 15-lipoxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3' untranslated region. EMBO J. 13:1476-1481.

228. Bachmair, A., Finley, D., and Varshavsky, A. 1986. *In vivo* half-life of a protein is a function of its amino terminal residue. Science. 234:179-186.

229. Rogers, S., Wells, R., and Rechsteiner, M. 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science. 234:364-368.

230. Xie, W., Chipman, J.G., Robertson, D.L., Erikson, E.L., and Simmons, D.L. 1991. Proc. Natl. Acad. Sci. USA. 88:2692-2696.

231. Jelinek, W.R., and Schmid, C.W. 1982. Repetitive sequences in eukaryotic DNA and their expression. Ann. Rev. Biochem. 51:813-844.

232. Diaz, A., Chepenik, K.P., Korn, J.H., Reginato, A.M., and Jimenez, S.A. 1998. Differential Regulation of Cyclooxygenases 1 and 2 by Interleukin-1β, Tumor Necrosis Factor-α and Transforming Growth Factor-β1 in Human Lung Fibroblasts. Exp. Cell Res. 241:222-229.

233. Edwards, D.R., and Mahadevan, L.C. 1992. Protein synthesis inhibitors differentially superinduce *c-fos* and *c-jun* by three distinct mechanisms: lack of evidence for labile repressors. EMBO. J. 11:2415-2424.

234. Chen, Y.Z., Gu, X.F., Caen, J.P., and Han, Z.C. 1994. Interleukin-3 is an autocrine growth factor of human megakaryoblasts, the DAMI and MEG-01 cells. Br. J. Haematol. 88:481-486.

235. Wodnar-Filipowicz, A., and Moroni, C. 1990. Regulation of interleukin-3 mRNA expression in mast cells occurs at the post-transcriptional level and is mediated by calcium ions. Proc. Natl. Acad. Sci. USA. 87:777-781.

236. Dean, D.M., and Sanders, M.M. 1996. Ten Years After: Reclassification of Steroid-Responsive genes. Mol. Endocrinol. 10:1439-1495.

237. Takeuchi, M., Kuno, H., Satoh, M., Yoshida, T., Ogura, M., Takeuchi, K. 1995. Platelet like particles released by inhibition of DNA synthesis in the human megakaryoblastic cell line MEG-01s. Tissue Culture Research Communications. 14:165-172.

238. Takeuchi, M., Satoh, M., Kuno, H., Yoshida, T., Kondo, H., and Takeuchi, M. 1998. Platelet-like particle formation in the human megakaryoblastic leukaemia cell line MEG-01 and MEG-01s. Br. J. Haematol. 100:436-447.

MATTHEW HILTON PLANT

Apartment #910, 14255 Gouin Blvd, West
Pierrefonds, Québec
H8Z 3C2
(514) 620-5499
email: mplank@uottawa.ca

SPECIAL SKILLS

- Productive and motivated scientist when working independently and in conjunction with others
- Adept with both Macintosh and IBM computer word processing, data analysis and presentation software

EDUCATION

May 1997 - Present — University of Ottawa, Ottawa, Ontario, Canada.
- Candidate for **Master of Science** in Biochemistry
- Defence date: June 11, 1999
- Thesis title: *Importance of Post-transcriptional regulation on Prostaglandin Endoperoxide H Synthase-1 Expression.*

Sept. 1989 - May 1994 — University of Waterloo, Waterloo, Ontario, Canada.
- Honors **Bachelor of Science** in Biology, Co-op program
- Courses included Molecular Biology, Microbiology, Immunology, Biochemistry and Chemistry
- Electives in Statistics, Computer Science, and Economics

WORK EXPERIENCE

May 1997 - Present — **Graduate Student, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ont.**
- Examined whether transcriptional or post-transcriptional regulation accounts for the high levels of Prostaglandin Endoperoxide H Synthase-1 (PGHS-1) enzyme during platelet differentiation
- Characterized a novel PGHS-1 transcript

July. 1996 - May 1997 — **Research Associate, Spectral Diagnostics Inc. , Toronto, Ont.**
- Optimized ELISA conditions for screening of antiserum directed against cardiac specific proteins
- Purified and characterized antibodies

May 1994- July 1996 **Research Assistant, Merck Frosst Centre for Therapeutic Research, Montréal, Qué.**

- Developed whole cell and broken cell based assays for characterization of potential phosphodiesterase inhibitors and adapted these assays to Biomek™ instrumentation
- Developed an animal model used for the quantification of acidic and neutral mucins produced in respiratory airway diseases

Sept. 1992 - Aug. 1993 **Research Assistant, Health Canada, Bureau of Radiation and Medical Devices, Radiobiology Section, Ottawa, Ont.**

- Examined the effect of magnetic fields on the generation of superoxide radicals by stimulated granulocytes
- Studied the effect of magnetic fields on tumor promotion using the SENCAR mouse skin model and planarian flatworms

Jan. 1992- May 1992 **Research Assistant, Canadian Space Agency, Ottawa, Ont.**

- Performed a tumor promotion study using the SENCAR mouse skin model to compare the fluorescent phorbol ester Sapintoxin-D with 12-O-tetradecanoylphorbol 13-acetate (TPA)

May 1991- Aug. 1991 **Research Assistant, Canadian Space Agency, Ottawa, Ont.**

- Explored the possible effects of microgravity environments on the dynamics of cellular interactions (the interest was in the observation of decreased responses in single cells)
- The fluorescent phorbol ester Sapintoxin-D was examined for its ability to accumulate in mouse erythro leukemia (MEL) cells

May 1990- Aug. 1990 **Research Assistant, Environment Canada, Pollution Measurement Division, Ottawa, Ont.**

- Measured C_2 to C_5 compounds in the ambient air from locations throughout Canada by gas and high pressure liquid chromatography

SUMMARY OF RESEARCH TECHNIQUES

- Adept with several molecular biology techniques (*e.g.* Restriction enzyme digests, ligations, transformations, small and large scale preparation of plasmid DNA, transient transfection by electroporation, PCR, RT-PCR, 3'-RACE, nuclear run-on assays, total and messenger RNA isolation and Northern blotting)

- Extensive experience with the development and use of whole cell and broken cell based assays for high throughput screening of novel compounds
- Adept with tissue culture procedures related to the culturing of different mammalian cell types as well as techniques related to the isolation of different cell types from whole blood
- Proficient with the handling, dissection and isolation of organs from mice and guinea pigs
- Skilled with techniques related to protein purification and quantification from cell lysates and tissue samples (*e.g.* western blotting, ELISA and column chromatography)
- Competent with many microscopy techniques used for the analysis of cultured cells (*e.g. in situ* hybridization and immunocytochemistry)

PUBLICATIONS

Manuscripts:

1. **Plant, M.**, and Laneuville, O.L. Characterization of a Novel Prostaglandin Endoperoxide H Synthase Transcript with a Tissue Specific Profile of Expression. This paper is in preparation to be submitted to the *Biochemical Journal.* 1999.

2. **Plant, M.**, Mroske, C., and Laneuville, O.L. Regulation of Prostaglandin Endoperoxide H Synthase-1 Gene Expression in the Megakaryocytic Cell Line MEG-01. This paper is in preparation to be submitted to *Experimental Cell Research.* 1999.

3. Mroske, M., **Plant, M.**, Franks, D.J., and Laneuville, O.L. Characterization of Prostaglandin Endoperoxide H Synthase-1 Enzyme Expression During the Differentiation of the Megakaryocytic Cell Line, MEG-01. This paper is in preparation to be submitted to *Blood.* 1999.

4. Aminian, M., Gagnon, F., **Plant, M.**, and Laneuville, O.L. Thromboxane A_2 Mimetic Increases the Expression of Prostaglandin Endoperoxide H Synthase-1 Enzyme in MEG-01 Cells. This paper has been submitted to the *Journal of Pharmcology and Experimental Therapeutics.* 1999.

5. Pon, D.J., **Plant, M.**, Tkach, J., Boulet, L., Muise, E., Allen, R.A., and Rodger. I.W. Characterization of CHO-K1 Cells Stably Expressing PDE-IV Enzymes (Whole-Cell cAMP Determinations vs Broken-Cell Enzymatic Assays). *Cell Biochemistry and Biophysics,* **29**:159-178, 1998.

6. Gordon, T., Nadziejko, C., **Plant, M.**, Pon, D.J., and Rodger, I.W. One Month Exposure to Inhaled Endotoxin Produces a Dose-dependent Increase in Stored Mucosubstances in Rat Intrapulmonary Airways. *Experimental Lung Research*, **22** : 509-523, 1996.

7. McLean, J.R., **Plant, M.**, Lecuyer, D.W., Davidson, C., and Troung, J. Sapintoxin-D is a Weak Tumor Promoter in SENCAR Mouse Skin. *Journal of Pharmacy and Pharmacology*, **47**, N3, P263, 1995.

8. Savoie, C., **Plant, M.**, Zwikker, M., Van Staden, C.J., Boulet, L., Chan. C.C, Rodger, I.W., and Pon, D.J. Effect of Dexamethasone on Antigen-induced Mucous Glycoprotein Secretion in Allergic Guinea Pigs. *Am. J. Respir. Cell Mol. Biol.*, **13** : 133-143, 1995.

Abstracts:

239. Effect of Dexamethasone on Antigen-induced Secretion of Mucus in Allergic Guinea Pigs. Douglas J. Pon, Chantal Savoie, **Matthew Plant**, Michelle Zwikker, Carlo Van Staden, Louise Boulet, Chi Chung Chan and Ian W, Rodger. *Am. J. Respir. Crit. Care Med.* V151, N4, A226, 1995.

240. A Simplified Immunoradiometric Assay for the Measurement of U937 Whole-Cell Adenosine 3',5'-Cyclic Monophosphate. Douglas J. Pon, Phillip T. Tagari, **Matthew Plant**, Louise Boulet and Ian W. Rodger. This abstract was presented at the 2nd World Congress on Inflammation (Brighton, UK. 1995).

EXTRA-CURRICULAR ACTIVITIES AND INTERESTS

- Sports interests include downhill skiing, golf, hockey and volleyball.
- Musical talents include the drums and guitar
- Refinishing antique furniture

REFERENCES

1. Dr. Odette Laneuville
   Associate Professor
   University of Ottawa
   Faculty of Medicine, Department of Biochemistry, Microbiology and Immunology
   Ottawa, Ontario
   Phone: (613)-562-5800 ext. 8402

2. Dr. Douglas J.Pon
Research Fellow
Merck Frosst Centre for Therapeutic Research
Department of Pharmacology
Montreal, Québec
Phone: (514)-428-8668