NOTE TO USERS

PROSTAGLANDIN E_2: A CENTRAL MEDIATOR OF CYTOKINE CELL SIGNALING THROUGH POST-TRANSCRIPTIONAL AND TRANSLATIONAL REGULATORY MECHANISMS IN HUMAN SYNOVIAL FIBROBLASTS

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PART A:
List of Abbreviations

AMPK:	AMP-activated protein kinase
APRIL:	Acidic protein rich in leucine
ARE:	Adenylate/uridylate-rich element
ASK1:	Apoptosis signal-regulating kinase 1
ATF-2:	Activating transcription factor-2
AUBP:	AU-binding protein
AUF1:	ARE/poly(U)-binding factor 1
β-gal:	Beta-galactosidase
BRF:	Butyrate-response factor
CARM1:	Coactivator-associated arginine methyltransferase 1
COX:	Cyclooxygenase
CRD:	Coding region determinant
CREB:	Cyclic AMP response element binding protein
CRM1:	Chromosome maintenance region 1
DNA:	Deoxyribonucleic acid
ELAV:	Embryonic lethal abnormal vision
ERF-1:	Epidermal growth factor response factor
ERK:	Extracellular signal-regulated kinase
FGF-9:	Fibroblast growth factor-9
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF:	Granulocyte/macrophage colony stimulating factor
GRO:	Growth-related oncogene
HNS:	HuR nucleocytoplasmic shuttling sequence
HSF:	Human synovial fibroblast
HuR:	Hu-antigen R
IL:	Interleukin
Impα1:	Importin alpha 1
IRF-1:	Intereferon-regulatory factor-1
JNK:	c-Jun N-terminal kinase
JRE:	JNK-response element
LC/MS/MS:	Liquid chromatograph/mass spectrometry/mass spectrometry
LPS:	Lipopolysaccharide
M-CSF:	Macrophage colony stimulating factor
MALDI:	Matrix-assisted laser desorption/ionization
MAPK:	Mitogen-activated protein kinase
MAPKAPK-2:	MAPK-activated protein kinase-2
MAPKKK:	MAPK kinase kinase
MCP-1:	Monocyte chemotactic protein-1
MEKK:	Mitogen-activated protein/ERK kinase kinase
MIP-1β:	Macrophage inflammatory protein-1beta
MKK:	MAPK kinase
MLK:	Mixed lineage kinase
MMP:	Matrix metalloproteinase
mRNA:	Messenger ribonucleic acid
mRNP:	Messenger ribonucleoprotein particle
NES:	Nuclear export signal
NF-κβ:	Nuclear factor-kappa beta

NF-IL-6:	Nuclear factor interleukin-6
NLS:	Nuclear localization signal
NSAID:	Non-steroidal anti-inflammatory drug
OD:	Optical density
PABP:	Poly(A)-binding protein
PAI-2:	Plasminogen activator inhibitor type 2
PARN:	Poly(A) ribonuclease
PG:	Prostaglandin
PI3-K:	Phosphatidylinositol 3-kinase
PLA_2:	Phospholipase A_2
Poly(A):	Polyadenylate
RA:	Rheumatoid arthritis
RACE:	Rapid amplification of cDNA ends
rhIL-1β:	Recombinant human interleukin-1 beta
RLU:	Reactive light unit
RNA:	Ribonucleic acid
RRM:	RNA recognition motif
rRNA:	Ribosomal RNA
snRNAs:	Small nuclear RNAs
TAK1:	TGF-beta-activated kinase 1
TGF-β:	Transforming growth factor-beta
TIA-1:	T-cell-restricted intracellular antigen-1
TIAR:	TIA-related protein
TNF-α:	Tumor necrosis factor alpha
TPA:	12-O-tetradecanoylphorbol-13-acetate
Trn:	Transportin
TTP:	Tristetraprolin
TZF:	Tandem zinc finger
UTR:	Untranslated region
VEGF:	Vascular endothelial growth factor

PART B:
Acknowledgements

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PART C:
M.Sc. Research Project Abstract

Prostaglandin E_2 (PGE_2), a cyclooxygenase (COX) pathway product, increases COX-2 mRNA stability and translation in human synovial fibroblasts (HSFs) via a positive-feedback loop. We hypothesized that this effect is a result of PGE_2-mediated regulation of mRNA-binding (possibly AU-binding) protein synthesis and/or activity and wished to characterize the factors and mechanisms involved in this process.

When transiently cotransfected with a COX-2 3 UTR-fused luciferase reporter, the AU-binding proteins (AUBPs) TTP and p37AUF1 markedly reduced and mildly increased reporter activity in HSFs, respectively. Western blot analysis of PGE_2's effects on AUF1 and TTP protein synthesis showed that PGE_2 suppressed HSF p37AUF1 expression while regulating the formation of a heterocomplex of which TTP is a subunit. PGE_2 also induced rapid nuclear export of eGFP-fused TTP in HeLa cells. Lastly, PGE_2 induced changes in the protein binding profile of a COX-2 distal 3 UTR probe as analyzed by RNA gel shift analysis (REMSA) and subtractive affinity chromatography.

In summary, the results of this study establish that PGE_2 may influence COX-2 post-transcriptional and translational regulation through a variety of mechanisms involving modulation of AUBP expression, subcellular localization and binding of uncharacterized COX-2 distal 3 UTR binding factors.

La prostaglandine E_2 (PGE_2), synthétisée par la voie de la cyclooxygénase (COX), stabilise et favorise la traduction du messager de la COX-2 via une boucle autocrine dans les synoviocytes humains (SH). Le but de cette étude était de caractériser les facteurs (i.e., protéines liant l'ARNm, surtout les "AU-binding proteins", AUBPs) dont leur synthèse et/ou activité est modulé par la PGE_2 et de préciser les mécanismes effecteurs à cet égard.

Des essais de co-transfection transitoire ont montré que l'AUBP TTP réduit drastiquement l'activité d'un ARNm rapporteur (COX-2 3 UTR) qui consiste de la gène luciférase couplé à la région 3 UTR COX-2, alors que l'AUBP p37AUF1 cause une augmentation mineur à cet effet. L'analyse de p37AUF1 et TTP par Western blot dans des SH traités avec la PGE_2 a révélé que cette prostaglandine supprime l'expression

protéique de p37AUF1 alors qu'elle contrôle l'assemblage d'un complexe covalent dont TTP est une des sous-unités. La PGE_2 a aussi induit l'export nucléaire de la protéine fusion TTP-eGFP dans les cellules HeLa. Finalement, une évaluation du répertoire de protéines liant la région 3 UTR distal COX-2 par technique de retard sur gel (i.e., "gel shift") et chromatographie compétitif a permis l'identification de 3 protéines liant l'ARNm qui sont régulées d'une façon PGE_2-dépendante.

En conclusion, cette étude démontre que les mécanismes de régulation post-transcriptionnelles et traductionnelles PGE_2-dépendants de la COX-2 sont diverses. La PGE_2 module l'expression et localisation subcellulaire d'AUBPs connus, ainsi que l'interaction de facteurs inconnus avec la région COX-2 3 UTR distal.

PART D:
Bibliographic Study

I) INTRODUCTION

Eukaryotic gene expression is a process that begins in a cell's nucleus with the transcription of deoxyribonucleic acid (DNA) into mRNA, which is then translated into protein in the cytoplasm. Eukaryotic cells possess an impressive repertoire of mechanisms for the regulation of this fundamental pathway. For decades, transcriptional regulation has been considered as the major control point for gene expression. This view, however, has been challenged by the growing knowledge of numerous regulatory mechanisms that take place after mRNA synthesis (i.e., post-transcriptionally).

The importance of post-transcriptional regulation of gene expression has long been recognized, most notably in the forms of mRNA capping, polyadenylation, splicing and nucleocytoplasmic export. However, it is becoming increasingly clear that there exists other levels to this form of gene regulation, namely in the form of differential mRNA stabilization/decay and translational efficiency. Global analysis of mRNA turnover has demonstrated that the half-lives of different mRNAs present within a cell vary drastically, ranging from a few minutes to more than 24 hours (Kiledjian *et al.* 1997). Moreover, the half-life of an individual mRNA can vary at different stages of the cell cycle and with changes in cellular state and environment. Given that the differences and changes in mRNA stability are eventually reflected in the amount of protein produced, this form of gene regulation cannot be overlooked.

Recent genome-wide microarray analyses (Raghavan and Bohjanen 2004) have shown that the levels of a significant number of mRNAs are controlled via *regulated* mRNA decay mechanisms implicating specific *cis*-acting elements (i.e., AREs) and their binding proteins. Such messages are transcribed from genes belonging to wide variety key gene families (i.e., cytokines, growth factors, proto-oncogenes, transcription factors, kinases, etc) involved in many crucial cellular processes (i.e., cell growth and proliferation, apoptosis, metabolism, signal transduction and inflammation), thus highlighting the importance of this so-called "ARE-dependent" mechanism of mRNA decay. Yet, despite the importance of regulated mRNA stability in the control of mammalian gene expression, the precise factors and mechanisms that mediate individual mRNA decay rates have only just begun to be elucidated. It is now well established that mRNA decay is not a default process in which an array of general, non-specific nucleases

indiscriminately degrades the substrate, but instead a tightly regulated event central to cell function and viability (Bevilacqua 2003).

The focus of the following bibliographic review will be post-transcriptional gene regulation through mRNA stability and decay. As an introduction to the topic of mRNA metabolism, a general overview of mRNA synthesis will be presented. Subsequently, the discussion will shift to regulated mRNA turnover with particular emphasis on mechanisms of ARE-mediated decay. The final section will serve to establish the importance of this field to my area of research.

II) mRNA Decay And Its Relevance To Gene Regulation: More Than Just a Disappearing Act

In many instances, the level of protein produced in eukaryotes is regulated by the amount of cytoplasmic mRNA available for translation. Intracellular mRNA levels, however, do not only depend on the rate of gene transcription. In effect, they represent a balance between the rates of a series of nuclear events, including mRNA transcription, processing and export, and the rate of cytoplasmic mRNA decay. Although the central focus of this thesis is on mRNA stability, it is nonetheless essential to have a general understanding of the transcriptional and processing events that lead to the synthesis of mRNA. To begin, a general overview of mRNA synthesis and maturation will be provided. A more in depth discussion of regulated mRNA decay will then follow.

II.1) Overview of Transcription and mRNA Maturation

Briefly, the process of transcription **(summarized in Figure 1)** is catalyzed by a large multi-subunit protein complex named RNA polymerase II. This process starts after the recognition of specific DNA sequences called promoters usually (but not always) located upstream (i.e., on the 5 side) of a gene's transcription start site. Promoters contain binding sites for transcription factors; these factors enhance binding of the RNA polymerase II pre-initiation complex to DNA and lead to the start of ribonucleotide polymerization (i.e., elongation) in a 5 -to-3 direction. Polymerization continues until the transcription of a particular sequence (i.e., 5 -AAUAAA-3) that signals for cleavage

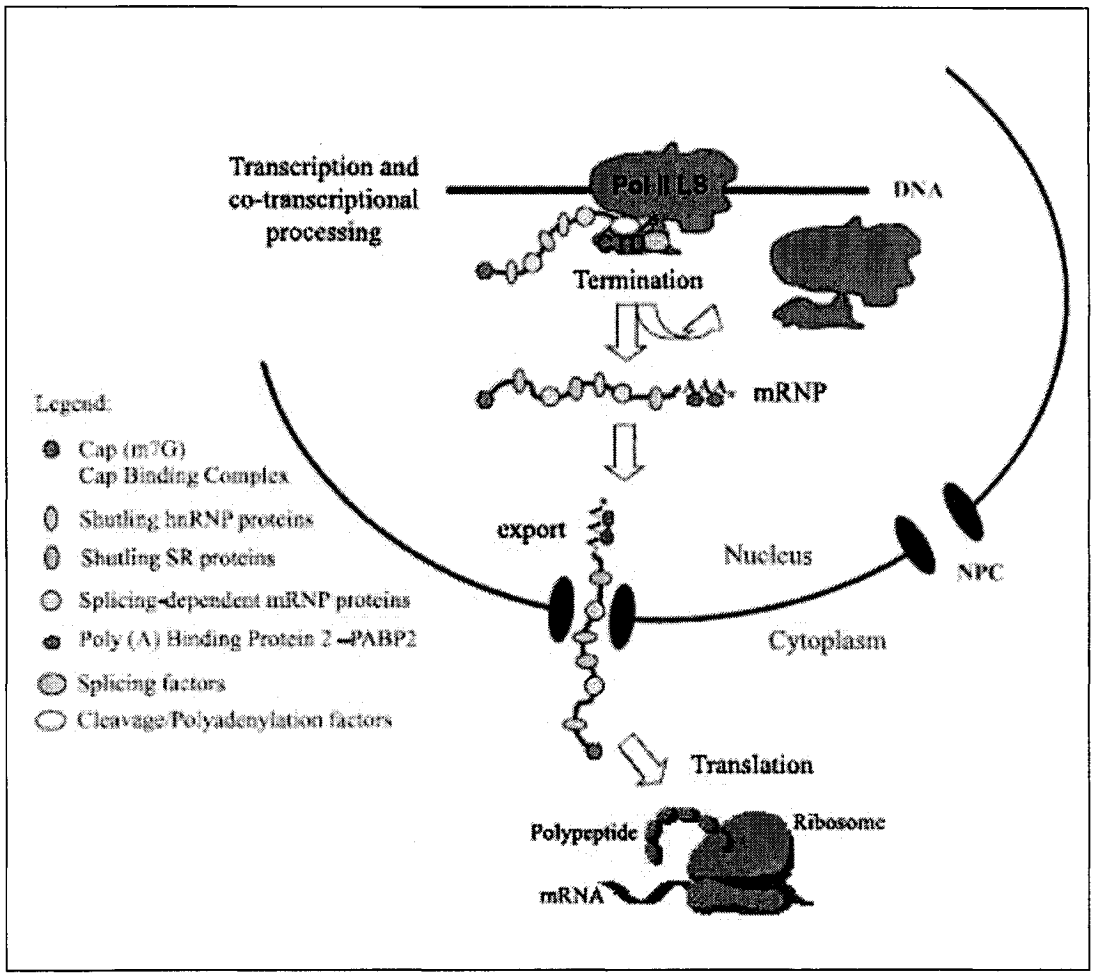


Figure 1: Summary of the eukaryotic gene expression process (refer to text for details). **Pol II LS**: RNA polymerase II large subunit; **CTD**: C-terminal domain; **mRNP**: messenger ribonucleoprotein particle; **NPC**: nuclear pore complex.
(Adapted from Custodio and Carmo-Fonseca 2001)

of the nascent RNA molecule. A specific endonuclease then cleaves the RNA approximately 10 to 30 nucleotides downstream of an AAUAAA sequence, giving rise to a primary transcript (i.e., pre-mRNA or heterogeneous nuclear RNA) (Berg *et al.* 2003).

Virtually all eukaryotic pre-mRNA molecules are subjected to various processing and modification events in the nucleus, many of which occur co-transcriptionally (i.e., while the nascent RNA chain is being synthesized) and continue post-transcriptionally **(Figure 2)** (Neugebauer 2002). It is now known that various pre-mRNA processing factors associate with the C-terminal domain of elongating RNA polymerase II to form a complex (i.e., "mRNA factory") involved in the co-transcriptional processing and packaging of nascent transcripts (Zorio and Bentley 2004). These steps are required for the formation of functional mRNA molecules. Early during on-going transcription, pre-mRNA molecules are first capped at their 5 end with an N-7 methylated GTP molecule (i.e., 7-methylguanosine cap) through an uncommon 5 -5 triphosphate linkage (Shuman 1995). Subsequent to capping, pre-mRNAs are subjected to one or more RNA splicing events. Most eukaryotic protein-coding genes contain segments called introns, which break up the amino acid coding sequence into segments called exons. During splicing, intron sequences are removed from the middle of the RNA molecule and exons are joined by a large ribonucleoprotein complex known as the spliceosome. Finally, these capped and spliced RNA molecules are processed at their 3 ends by cleavage and the addition of 100 to 250 residues of adenine nucleotides (i.e., polyadenylation). Polyadenylation is accomplished by a separate polymerase called poly(A) polymerase. Following these processing steps, certain pre-mRNA molecules are further chemically modified/edited (i.e., human apolipoprotein B) (Smith and Sowden 1996). In the end, the fully processed pre-mRNAs are considered fully functional mRNA molecules fit for nuclear export and translation in the cytoplasm. Note that throughout the process of transcription and mRNA maturation, the mRNA molecule is constantly bound by proteins; this dynamic mRNA-protein complex is named the messenger ribonucleoprotein particle (mRNP).

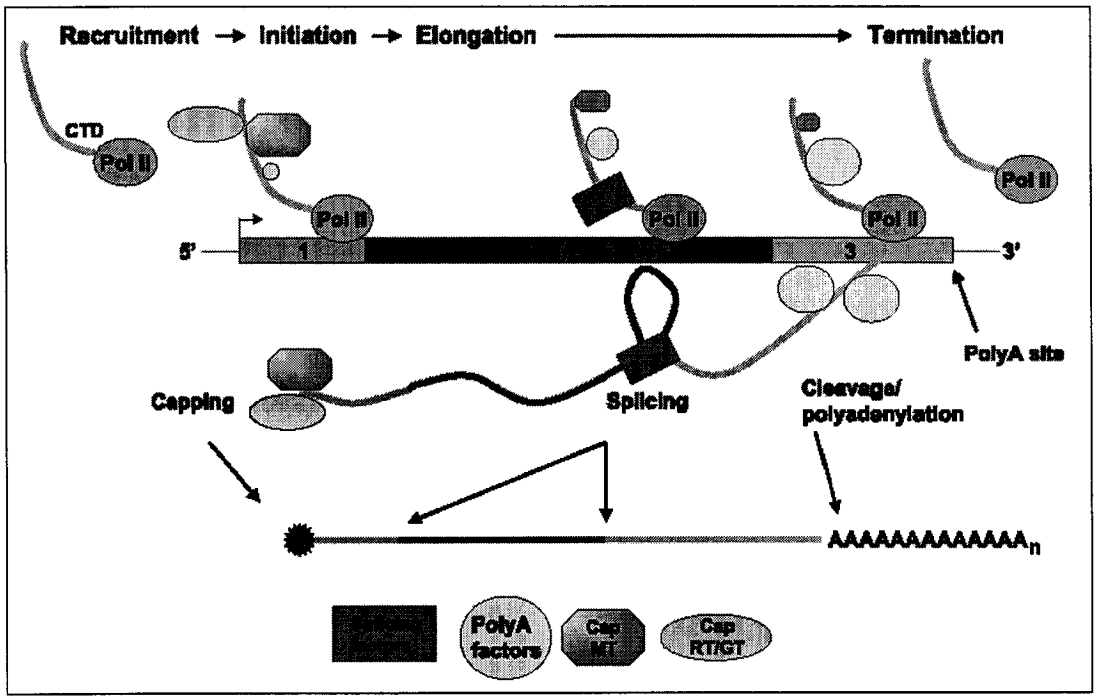


Figure 2: A closer look at eukaryotic mRNA co-transcriptional processing. Processing factors involved in capping and 3 end cleavage/polyadenylation interact with the C-terminal domain (CTD) of RNA polymerase II at the gene's 5 end. Capping of the nascent RNA molecule is conducted co-transcriptionally as the polymerase traverses the gene. At the same time, splicing factors associate with the transcription complex and subsequently perform co-transcriptional splicing of the nascent transcript. Finally, the newly synthesized RNA molecule is cleaved and polyadenylated at its 3 end by RNA polymerase II-associated cleavage/polyadenylation factors. Changes in symbol sizes represent quantitative differences in binding of the corresponding factors to RNA polymerase II at different stages of the transcriptional process. Exons are numbered 1, 2 and 3. Introns are shown in black boxes. The star represents the cap structure.
(Zorio and Bentley 2004)

II.2) Fundamental Mechanisms of Eukaryotic mRNA Decay

II.2.1) Revelations from the Yeast Saccharomyces cerevisiae

As already mentioned, the levels of mRNA in a cell are determined by both the rate of synthesis (described above) and the rate of decay. The most relevant findings in eukaryotic mRNA decay mechanisms have resulted from studies in the yeast *Saccharomyces cerevisiae*. In yeast, the two general decay pathways commence with progressive deadenylation followed by degradation of the mRNA body in either a 5 -to-3 (major pathway) or 3 -to-5 (minor, exosome-mediated pathway) direction. Deadenylation is a critical event in mRNA degradation; it is the rate-limiting step in the turnover of numerous mRNAs. The poly(A) tail can interfere with mRNA decay through the actions of the poly(A) binding protein (PABP) Pab1p (Caponigro and Parker 1995; Coller *et al.* 1998). As well as binding the poly(A) tail, Pab1p interacts with a specific region of the translation-initiation factor eIF4G, which in turn forms a ternary complex with the cap-binding protein eIF4E **(Figure 3)**. The formation of this complex *in vitro* has been shown to circularize the mRNA molecule (Wells *et al.* 1998), which subsequently enhances translation and likely simultaneously stabilizes mRNAs by preventing access of decapping and deadenylating enzymes to their targets (i.e., 5 terminus cap and 3 poly(A) tail, respectively). The importance of this "closed loop" mRNA structure to mRNA decay will be discussed later.

In addition to its indirect role in mRNA stabilization (i.e., via formation of the closed-loop mRNA), the 5 cap structure also has a direct function in this process. Its special 5 -5 triphosphate linkage renders mRNAs resistant to general exoribonucleases that would normally cleave a standard 3 -5 phosphodiester bond at an mRNA's 5 terminus. Following Dcp1/Dcp2-catalyzed decapping, mRNA degradation can occur uninhibited in a 5 -to-3 fashion by the exoribonuclease Xrn1 (Tucker and Parker 2000). In both the 5 -to-3' and the 3 -to-5 decay pathways, the residual cap is further hydrolyzed to N-7 methyl guanosine monophosphate (van Dijk *et al.* 2003) by the scavenger decapping enzyme DcpS, which specifically recognizes only free cap structures (Liu *et al.* 2002).

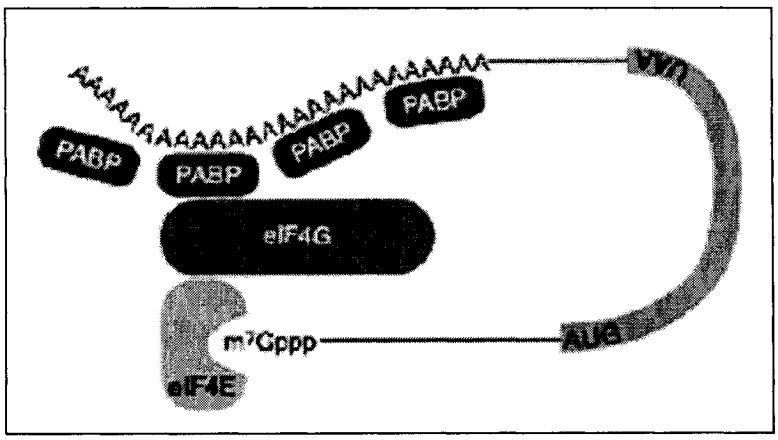


Figure 3: Protein associations involved in the formation of the "closed loop" mRNA structure.
(Adapted from Preiss 2003)

II.2.2) Conservation of Yeast mRNA Decay Pathways in Mammals

Relative to yeast, much less is known about mRNA decay mechanisms in multicellular organisms, especially mammals. Substantial genetic and biochemical evidence now suggests that the above-described *S. cerevisiae* deadenylation-dependent mRNA decay pathways also constitute the major mammalian mRNA degradation mechanisms **(Figure 4)**.

As in yeast, deadenylation appears to be the first step in the major mammalian decay pathways. It has been shown that deadenylation precedes the decay of many mammalian mRNAs *in vivo* and particular elements that stimulate the decay of mammalian mRNAs promote rapid deadenylation (Wilson and Treisman 1988; Shyu *et al.* 1991; Couttet *et al.* 1997). In addition, the interaction between the mammalian PABP and the mRNA poly(A) tail hinders rapid mRNA decay *in vitro* (Guhaniyogi and Brewer 2001). Two deadenylase complexes (i.e., the Ccr4p/Pop2p/Not1p-Not5p protein complex (Dupressoir *et al.* 1999, 2001) and the Pan2p/Pan3p complex (Zuo and Deutscher 2001)) are conserved in eukaryotic genomes and show common structural/functional properties (Chen *et al.* 2002). In addition, a vertebrate-specific deadenylase named PARN (poly(A) ribonuclease) has been purified and shown to mediate poly(A) shortening in mammalian cells (Korner and Wahle 1997; Martinez *et al.* 2000).

Subsequent to deadenylation, mRNA decay in mammals can also occur in either a 3 -to-5 or 5 -to-3 direction. The existence of a mammalian 3 -to-5 degradation mechanism is implied by the high conservation of exosome subunits. This conserved complex comprises 10 core proteins, nine of which have proven, or predicted, 3 -to-5 exoribonuclease activity (Butler 2002). The human exosome was isolated from HeLa cell extracts and displays extensive structural and functional homologies to the yeast complex. Importantly, depletion of the human exosome from cell extracts increased the half-life of unstable mRNAs (Chen *et al.* 2001). The exosome will be further discussed in a later section. Conservation of deadenylation-dependent 5 -to-3 mRNA decay in mammals is suggested by the identification of mRNA decay intermediates that are trimmed from the 5′ end or lack the 5 cap. Analysis of these intermediates showed that they contained short poly(A) tails, thus suggesting that decapping and 5 -to-3 degradation are present and likely preceded by deadenylation (as in yeast) (Couttet *et al.*

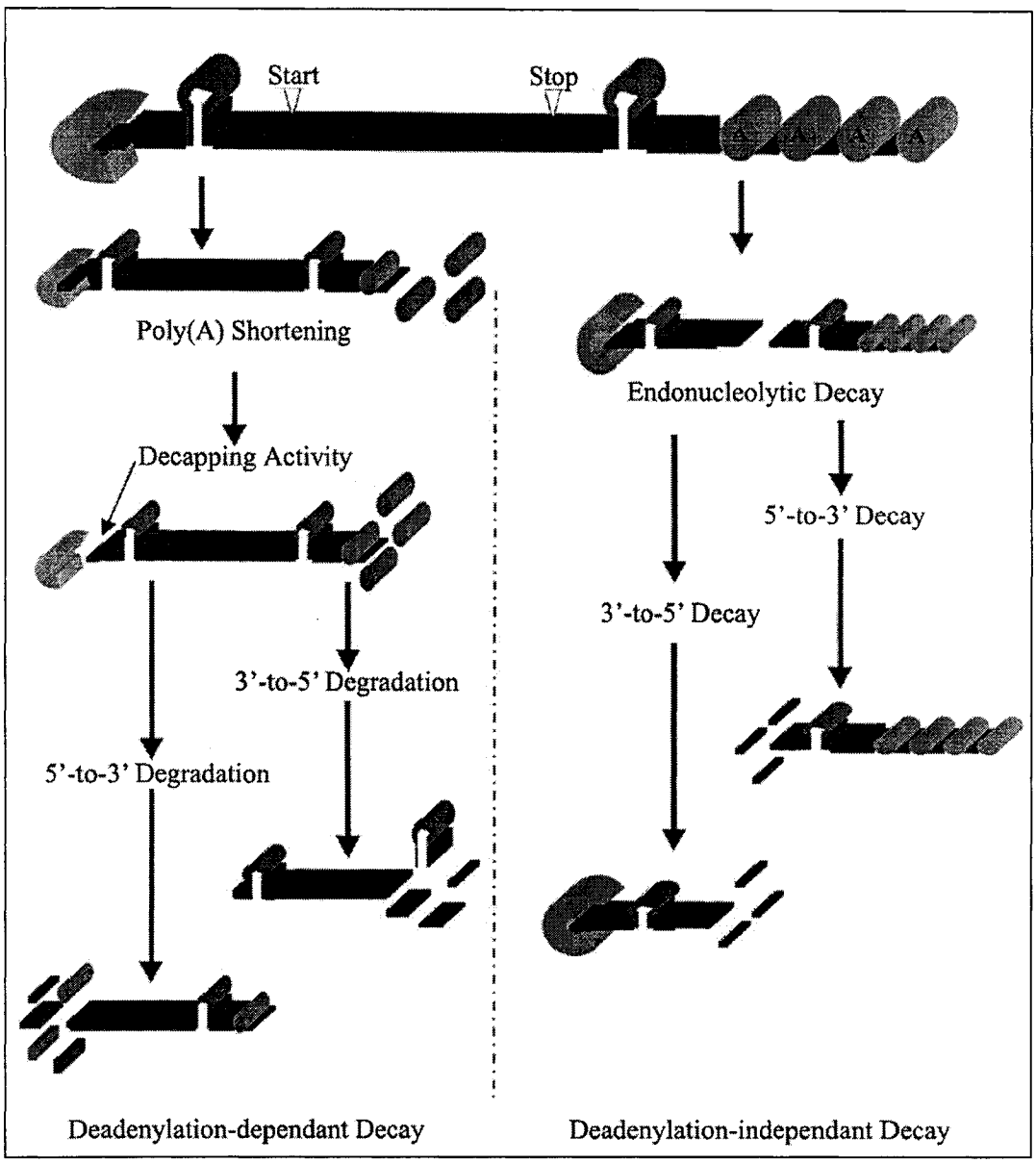


Figure 4: Deadenylation-dependent and -independent pathways of mammalian mRNA decay. In the major mammalian mRNA decay pathways (left), deadenylation is followed by decapping and either 3 -to-5 degradation (exosome-mediated) or 5 -to-3 decay (5 -to-3 exonuclease-mediated). In the less prominent deadenylation-independent decay pathway (right), mammalian mRNAs are degraded by endonucleolytic cleavage and the resulting cleavage fragments are degraded by exonucleolytic decay.
(Guhaniyogi and Brewer 2001)

1997). Furthermore, 5 -to-3 exonuclease activity was displayed in cytosolic HeLa extracts (Mukherjee *et al.* 2002). Several mammalian homologs to yeast decapping enzymes have also been identified. These include human Dcp2 (homolog of yeast Dcp2) (Lykke-Andersen 2002; van Dijk *et al.* 2002), Dcp1A and Dcp1B (homologs to yeast Dcp1p), Xrn1 (Bashkirov *et al.* 1997) and Xrn2 (Zhang *et al.* 1999) (homologs to the yeast XRN-family of 5 -to-3 exonucleases) and DcpS (scavenger decapping enzyme homologous to yeast DcpS) (Liu *et al.* 2002; van Dijk *et al.* 2003). In addition, interactions between certain decapping enzymes in the formation of decapping complexes are also conserved. A human Dcp1/Dcp2 complex, as found in yeast, was isolated by co-immunoprecipitation. Although the precise role played by each enzyme in the decapping process is still unclear, evidence suggests that both are required for decapping *in vivo* in both mammals and yeast. Moreover, aggregation of various decapping enzymes and 5 -to-3 exonucleases into distinct cytoplasmic aggregates (i.e., P-bodies) (Sheth and Parker 2003; Cougot e*t al.* 2004) is also conserved, thus suggesting a structural/functional conservation of these enzymes in the formation of 5 -to-3 decay complexes.

Despite the evidence that deadenylation-dependent mRNA decay is the predominant mRNA decay mechanism in mammals, the relative contribution of the 3 -to-5 and the 5 -to-3 pathways to mRNA degradation *in vivo* is unclear. Furthermore, there exists a less prominent deadenylation-independent decay pathway in which mammalian mRNAs such as 9E3, insulin-like growth factor 2, serum albumin, c-myc and transferrin receptor are degraded by endonucleolytic cleavage (Beelman and Parker 1995) **(Figure 4)**. The fragments resulting from the endonucleolytic cleavage(s) are subsequently targeted for exonucleolytic decay by either 3 -to-5 (for the 5 fragment) and 5 -to-3 (for the 3 fragment) exonucleases. Wang and Kiledjian (2000a) have recently identified a sequence-specific endoribonuclease involved in erythrocyte α-globin mRNA degradation. The same group has also demonstrated that endonuclease-directed α-globin mRNA decay is inhibited by an interaction between PABP and the α-globin-specific stabilizing protein, α-CP (Wang and Kiledjian 2000b).

II.3) Functional Link Between mRNA Decay and Translation in Mammals

As previously discussed, the 5 cap and poly(A) tail are fundamental determinants of mRNA stability. There are good indications that the deadenylation-dependent mRNA turnover pathway is regulated by the status of the translation initiation machinery on the mRNA, which, as suggested by the "closed-loop mRNA" model of translational initiation, is directly dependent on the 5 cap and poly(A) tail. Thus, it is not surprising that the processes of mRNA translation and decay have been closely linked functionally. Further support for this functional association came from the observation that whereas inhibition of translation initiation destabilizes mRNAs (Day and Tuite 1998; Schwartz and Parker 1999), inhibiting the elongation step of translation slows down deadenylation and decapping and subsequently promotes mRNA stabilization (Schwartz and Parker 2000a).

A putative model linking the processes of mRNA translation and turnover suggests that mRNA turnover is influenced by the association of proteins involved in mRNA decay (i.e., PARN, Dcp1p) and translation initiation (i.e., eIF4G, cap-binding protein eIF4E) with the 5 cap through bridging of the mRNA ends **(Figure 5)**. In addition to promoting the protection of the mRNA ends from degradation (as discussed in section II.2), this protein interaction also influences the activities of the decay enzymes. For example, biochemical evidence suggests that direct binding of the PARN deadenylase to the 5 cap structure on a substrate RNA stimulates its deadenylase activity both *in vitro* and *in vivo* (Dehlin *et al.* 2000). Disruption of this interaction *in vitro* by use of either cap analogs or free eIF4E inhibits PARN activity. Conversely, cap analogues enhance decapping of substrate mRNAs by preventing the association of both PARN and eIF4E to the mRNA 5 cap and thus making it accessible to Dcp1p (Gao *et al.* 2000). Hence, disruption of either Dcp1p or PARN interaction with the 5 cap will disfavor decapping or deadenylation, respectively. It must be noted, though, that there exists a competition between eIF4E, PARN and Dcp1p for cap binding. However, eIF4E has a much lower intrinsic affinity for the 5 cap than Dcp1p (Schwartz and Parker 2000b). Implicated in this complicated protein interaction network is the poly(A) binding protein Pab1p. This protein also strengthens the closed-loop mRNA structure by associating with eIF4G. Moreover, Pab1p can directly recruit Dcp1p to the 5 cap, although it has also

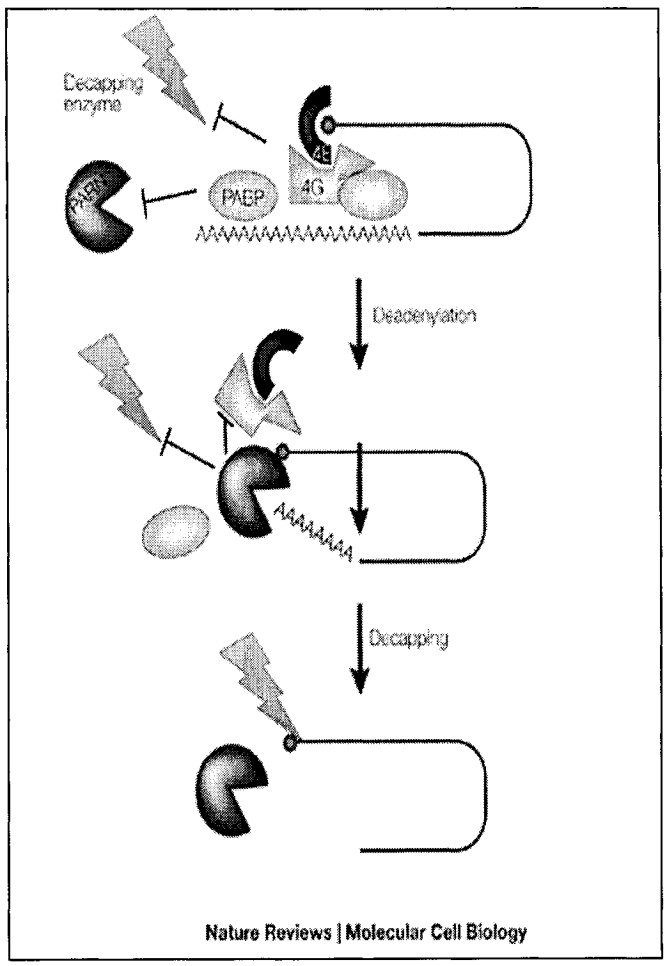


Figure 5: The closed-loop mRNA model as a link between the processes of mRNA translation and decay. Simultaneous interaction of the cap-binding protein eIF4E and poly(A)-associated PABP with the bridging factor eIF4G leads to the formation of a closed-loop mRNA molecule that is resistant to decapping and deadenylation (top). Transient destabilization of the [poly(A)-PABP] and [5 cap-eIF4E] complexes allows PARN to bind simultaneously to both the 5 cap and the 3 poly(A) tail, leading to deadenylation and subsequent disruption of the [PABP-eIF4G-eIF4E] interaction (middle). After deadenylation is complete, PARN dissociates from the now linear mRNA molecule and decapping ensues (bottom), leaving the mRNA molecule susceptible to 3 -to-5 or 5 -to-3degradation.
(Wilusz *et al.* 2001)

been shown that Pab1p acts as an inhibitor of decapping activity (Coller *et al.* 1998).

Thus, the emerging picture is one of a dynamic complex that bridges the mRNA ends and is involved in the switch from translation initiation to mRNA degradation. In this model, an initial event causes a transient destabilization of the [poly(A)-PABP] and [5' cap-eIF4E] complexes, allowing PARN to bind simultaneously to both the 5 cap and the 3 poly(A) tail. It is possible that the [PARN-5 cap-poly(A) tail] interaction can inhibit decapping, even if the ternary [eIF4E-eIF4G-PABP] complex is disrupted. Consequently, the poly(A) tail is progressively shortened to a length too short to allow binding of PABP (i.e., less than 10 adenosines), and thereby completely inhibiting the [PABP-eIF4G-eIF4E] interaction. After deadenylation is complete, PARN dissociates from the now linear mRNA molecule. The decapping enzyme Dcp1p can now recognize the exposed 5 cap and proceeds to decapping the mRNA, which is subsequently susceptible to 3 -to-5 degradation by the exosome or in 5 -to-3 fashion (van Hoof and Parker 1999; Wilusz *et al.* 2001).

III) Regulated mRNA Turnover: Mechanisms and Regulatory Determinants of mRNA Stability

The mechanisms of mRNA decay presented above define the *general* elements and processes involved in all mRNA degradation pathways. However, regulated mRNA decay implicates an added level of determinants and specific processes that either enhance or prevent elimination of the mRNA through the previously-mentioned mechanisms. The remainder of this review will consist of a thorough discussion of regulated mRNA decay.

III.1) *Cis*-Acting Regulatory Elements

Regulated messenger RNA degradation is dependent upon both *cis*-elements in the RNA molecule and *trans*-acting factors. The *cis*-elements that affect mRNA stability are numerous and can be found at various positions along the mRNA molecule. Some elements are ubiquitous, whereas others signal the degradation of specific messages. The stabilizing roles of two ubiquitous elements, namely the 7-methylguanosine cap and the poly(A) tail, have been mentioned above.

Other *cis*-elements that influence mRNA stability can be found in the 5 UTR, protein coding region and/or the 3 UTR **(Figure 6)**. Examples of such elements will be given below.

III.1.1) Elements in the 5' UTR

The 5 UTR corresponds to the non-coding region situated between the 5 cap and the protein-coding sequence of a messenger RNA. This region is a critical regulator of translation through its influence on translation initiation. Modification of the 5 UTR sequence altered mRNA half-lives, thus supporting the idea that the processes of mRNA translation and decay are closely functionally linked. As previously mentioned, translation initiation stabilizes mRNAs (Day and Tuite 1998; Schwartz and Parker 1999); thus, stable secondary structures within the 5 UTR that perturb this step (e.g., stem-loops) by sterically inhibiting binding and scanning of the 43S pre-initiation complex, may lead to mRNA destabilization. Moreover, binding of *trans*-acting factors to structural elements in the 5 UTR may also impede translation initiation and thus contribute to mRNA decay. Conversely, sequence elements in the 5 UTR may enhance translation by favoring the binding of translation initiation-enhancing factors (Millard *et al.* 2000) and thus, by the same reasoning, may increase the mRNA's half-life.

The 5 UTR may modulate mRNA stability in a translation-independent manner. Such a mechanism is seen in lymphoma and plasmacytoma cells harboring reciprocal translocations of immunoglobulin introns in the 5 UTR of the c-myc mRNA. Despite similar translation efficiencies, the chimeric c-myc mRNA is significantly more stable relative to the wild-type transcript (Johnston and Carroll 1992). Binding of *trans*-acting factors to 5' UTR structural elements may also play a role in translation-independent mechanisms of mRNA decay. Examples of the latter include regulated 5 UTR-dependent stabilization of HeLa IL-2 (Chen *et al.* 1998) and the mouse KC chemokine (Tebo *et al.* 2000). The 5 element leading to the stabilization of IL-2 mRNA is named the JNK-response element (JRE) given its ability to specifically stabilize IL-2 mRNA following activation of the JNK MAPK pathway through a mechanism likely involving the binding of YB-1 and nucleolin (Chen *et al.* 2000). A 68-nucleotide region in the KC 5 UTR was also shown to impart stability to the mRNA. More precise mechanisms behind this

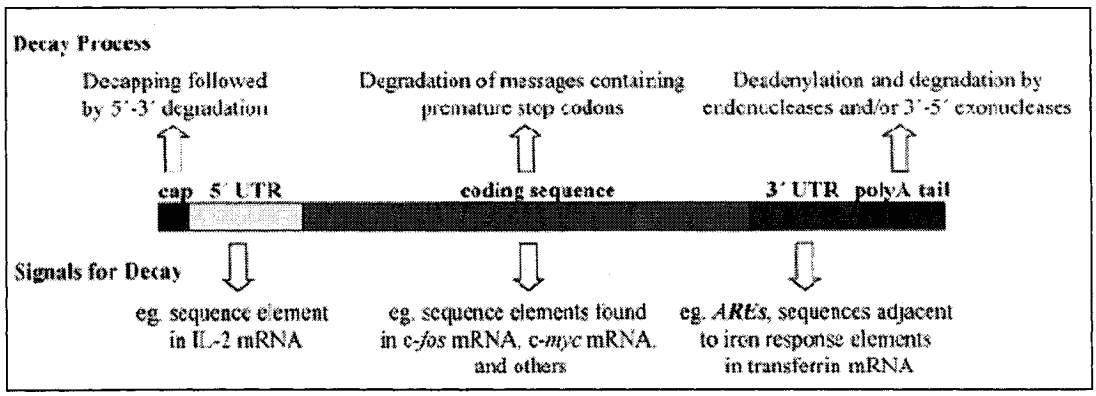


Figure 6: Location of various *cis*-acting elements affecting mRNA stability (with specific examples listed below) and their associated decay processes (listed above). (Brennan and Steitz 2001)

stabilization are still unclear, although it involves a cooperative interaction of elements in the 3 UTR and is IL-1α-dependent.

III.1.2) Elements in the Coding Region

mRNA instability elements are also present within the coding region of certain transcripts and are known collectively as coding region determinants (CRDs). Like the 5 UTR stability elements, the effects of CRDs on mRNA stability are closely linked to translation. CRD-containing mRNAs include c-fos (Schiavi *et al.* 1994), β-tubulin (Gay *et al.* 1987) and c-myc (Wisdom and Lee 1991).

c-fos contains two CRDs denoted CRD-1 and CRD-2. When the 320-nucleotide c-fos major CRD (mCRD, CRD-1) is placed in frame with the β-globin coding region, the resulting [5'-globin-fos mCRD-globin-3] chimeric transcript displays a fourfold decrease in stability compared to wild type β-globin mRNA (Shyu *et al.* 1991). This mCRD-imparted instability is dependent on its primary mRNA sequence and is independent of the encoded protein (Wellington *et al.* 1993). In contrast, the instability imparted on β-tubulin mRNA by its CRD is dependent on the encoded protein sequence. The β-tubulin CRD comprises the first 13 translated nucleotides, which give rise to the first 4 amino acids (i.e., MREI, single letter amino acid code) (Pittenger and Cleveland 1985). β-tubulin mRNA stability is inversely correlated to intracellular tubulin monomer concentrations and likely involves co-translational degradation. Given the inability of the β-tubulin protein to bind the polypeptide encoded by its CRD, it is believed that β-tubulin monomers destabilize β-tubulin mRNA by favoring an interaction between the nascent peptide and a ribosome-associated mRNase (Theodorakis and Cleveland 1992; Bachurski *et al.* 1994). The c-myc CRD encodes the C-terminal 60 amino acids and dictates c-myc mRNA destabilization during the differentiation of myoblasts to myotubes (Yeilding and Lee 1997). In both intracellular and *in vitro* mRNA decay assays, an in-frame insertion of the c-myc CRD into β-globin mRNA resulted in increased destabilization compared to the same transcript harboring an insert from the stable glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Herrick and Ross 1994). Interestingly, this determinant has been found to specifically associate with a 70 kDa protein that prevents

endonucleolytic cleavage of c-myc mRNA in vitro (Prokipcak *et al.* 1994), suggesting a bi-functional role of the CRD in mRNA decay.

III.1.3) Elements in the 3' UTR

The best-characterized *cis*-acting mRNA stability determinants are located in the 3 UTR. Although various elements have been found in this region (i.e., iron-response element in transferrin receptor mRNA (Addess *et al.* 1997), cytidine-rich element in α-globin mRNA (Weiss and Liebhaber 1995), stem-loop in histone mRNA (Pandey and Marzluff 1987)), the most prominent and ubiquitous 3 UTR stability determinant is referred to as the ARE. These adenylate/uridylate-rich elements are found in a variety of short-lived mRNAs including those coding for cytokines, growth factors, transcription factors and proto-oncogenes. Shaw and Kamen were the first to display that these AREs promote rapid mRNA turnover in a manner independent from the remainder of the mRNA. Their seminal study demonstrated that the introduction of a 51-nucleotide ARE-coding segment from the human GM-CSF gene into the 3 UTR of the normally stable (i.e., $t_{1/2}$ > 2h) rabbit β-globin gene resulted in its rapid (i.e., $t_{1/2}$ < 30 min) turnover (Shaw and Kamen 1986).

There is no defined consensus sequence that defines an entire ARE, although they often, but not always, consist of one or several copies of the AUUUA pentamer or UUAUUUA(U/A)(U/A) nonamer usually within a U-rich sequence. Based on general sequence features (i.e., presence/absence and number of pentamer and/or nonamer repeats) and mRNA decay and deadenylation kinetics, AREs are classified into 3 classes. Characteristic features of each ARE class are summarized in **Table 1**. It is important to note that the mere presence of an AU-rich sequence within the 3 UTR of an mRNA does not necessarily influence its stability and that such sequences are not necessarily required to transmit mRNA instability (i.e., c-jun).

Individual AREs are structurally and functionally distinct elements, each possessing different properties that, as a whole, determine the half-life of an mRNA molecule. An elaborate genome-wide study on such structural and functional properties has been conducted by Pesole *et al.* (2001). Moreover, a non-redundant ARE-database (i.e., ARED-mRNA version 2.0) in which AREs are grouped into clusters based on ARE

Table 1: Sequence features and functional properties of the three classes of AREs. *Below:* nucleotide sequence of human c-fos and GM-CSF AREs.
(Chen and Shyu 1995)

ARE	Example	Sequence features	Decay kinetics	Deadenylation
AUUUA-containing				
class I	*c-fos*	Has 1–3 copies of scattered AUUUA motifs coupled with a nearby U-rich region or U stretch	Biphasic, deadenylation precedes decay of the RNA body	Synchronous, results in decay intermediates with poly(A) tails of 30–60 nucleotides
class II	GM-CSF	Has at least two overlapping copies of the nonamer UUAUUUA(U/A)(U/A) in a U-rich region	Biphasic, deadenylation precedes decay of the RNA body	Asynchronous, results in poly(A)$^+$ decay intermediates
Non-AUUUA	*c-jun*	Has a U-rich region and other features (?)	Biphasic, deadenylation precedes decay of the RNA body	Synchronous, results in decay intermediates with poly(A) tails of 30–60 nucleotides

h*c-fos*: GUUUUUA**AUUUAUUUA**UUAAGAUGGAUUCUCAGAU**AUUUA**UAUUUUUAUUUUAUUU

h*GM-CSF*: UAAU**AUUUA**UAU**AUUUA**UAUUUUUAAAAU**AUUUAUUUAUUUAUUUA**UUUAA

length has been created (accessible via the web at http://rc.kfshrc.edu.sa/ared). Analyses employed in the creation of this database have shown that ARE-mRNAs constitute up to 5 8% of human genes and encode functionally diverse proteins that are important in many biological processes including cell growth and differentiation, signal transduction, transcriptional and translational control, hematopoiesis, apoptosis, nutrient transport and metabolism (Bakheet *et al.* 2003).

Despite the original findings by Shaw and Kamen showing that AREs promote mRNA decay, it is now clear that these sequences may promote mRNA stabilization under certain conditions (i.e., cellular stimulation by inflammatory cytokines and growth factors, stress, oncogenic transformation) (Gallouzi *et al.* 2000). The reason for this apparent ambiguity is that the effects of an ARE on the mRNA decay process is not an intrinsic property of the sequence element. Instead, an ARE's regulatory function is mediated by selective interactions with a family of regulated RNA binding proteins known as AUBPs. In the next section, specific examples of AUBP-mediated mRNA decay mechanisms will be provided, followed by a more in-depth characterization of three notable AUBPs.

III.2) *Trans*-Acting Regulatory Factors and ARE-mRNA Decay

The half-life of an ARE-containing mRNA molecule is ultimately determined by the binding and specific activity of one or more AUBPs, which either promote mRNA stabilization or destabilization **(Table 2)**. The binding of such factors is dependent on both primary (i.e., ribonucleotide sequence, ARE) and secondary (i.e., conformation, stem-loops) structures present in the ARE-mRNA 3 UTR

Evidence for the involvement of AUBPs in ARE-mediated decay is plentiful and incontestable: (*i*) Specific AUBP-ARE interactions have been identified for various mRNAs (i.e., TTP-TNF-α (Lai *et al.* 1999); AUF1-bcl-2 (Lapucci *et al.* 2002)); (*ii*) the abundance and/or localization of some AUBPs is altered concurrently with the stabilization or destabilization of target mRNAs in response to a stimulus (Doninni *et al.* 2001; Kloss *et al.* 2004); (*iii*) genetic deletion or overexpression of certain AUBPs directly modulates the half-lives of target ARE-containing mRNAs (Carballo *et al.* 2000; Lai *et al.* 2000; Rodriguez-Pascual *et al.* 2000).

Table 2: Identified AUBPs with demonstrated mRNA stability-modifying effects.
(Zhang *et al.* 2002; Bevilacqua *et al.* 2003)

Protein	Cellular Localization	Examples of AREs bound	Role in mRNA decay
AUBF	Cytoplasmic	c-fos, IL-3	Stabilizing
AUF-1/hnRNP D 4 isoforms	nuclear, cytoplasmic	c-myc, c-fos, GM-CSF, bcl-2	Destabilizing, stabilizing
AU-A, AU-B, AU-C	Nuclear (AU-A) and cytoplasmic (AU-A, AU-B , AU-C)	Various lymphokine mRNAs, c-myc	Destabilizing
hnRNPA1	Nuclear	GM-CSF, IL-2, c-myc	Destabilizing
hnRNP C	Nuclear	APP, IL-3	Destabilizing
HuR/HuA, HuB/Hel-N1, HuC HuD	Nuclear (HuA/HuR, HuB/Hel-N1, HuC HuD); cytosolic (HuR/HuA)	EGF, c-myc, c-fos, TNF-α, GM-CSF	Stabilizing
KSRP	Nuclear, cytoplasmic	c-fos	Destabilizing
TIA-1, TIAR	Nuclear, cytoplasmic	TNF-α, GM-CSF	Destabilizing, translation
TTP	Nuclear, cytoplasmic	TNF-α, IL-3, GM-CSF	Destabilizing

As a consequence of being a protein-mediated process, ARE-dependent mRNA decay is subject to fine regulation. Many AUBPs are responsive to various kinase signaling cascades that have also been shown to modulate mRNA stability. These include the JNK (Ming *et al.* 1998), PI3-K (Ming *et al.* 2001) and p38 MAPK/MAPKAPK-2 cascades (Carballo *et al.* 2001) (see section IV for further details about the p38 MAPK). As will be more thoroughly discussed in the following sections, kinase-mediated phosphorylation of AUBPs alters their RNA binding affinity (Wilson, Lu, Sutphen, Suarez *et al.* 2003), activity and/or interaction with other factors (Chrestensen *et al.* 2004). Moreover, numerous AUBPs associate with nucleocytoplasmic shuttling factors (which are themselves regulated) via nuclear shuttling signals and translocate upon cellular stimulation. Hence, regulation of ARE-mediated decay is an extremely complex process that can occur at multiple levels and by various mechanisms.

The exact mechanisms by which AUBPs modulate mRNA stability are still unclear, although various reports have shown that AUBPs may affect mRNA decapping, deadenylation and 3 -to-5 degradation. As already mentioned, the mRNA 5 cap and poly(A) tail are critical determinants of mRNA translation and stability; thus, it is not surprising that AREs (and associated AUBPs) have been shown to influence the 5 cap and poly(A) tail structure either directly (by recruiting and/or competing with the binding of factors that control decapping and/or deadenylation) or indirectly (by modifying the mRNA 5 cap-poly(A) tail interaction). AREs have long been shown to increase deadenylation *in vivo* (Shyu *et al.* 1991), but the exact mechanisms were not known. Later, Lai *et al.* (1999) provided strong evidence for the involvement of AUBPs in this mechanism by showing that TTP (an mRNA destabilizing AUBP) promotes the deadenylation and subsequent decay of TNF-α and other class-II ARE-containing mRNAs. A link between AUBPs and mRNA decapping was made by Gao *et al.* (2001), who demonstrated that sequence-specific destabilizing AUBPs are required for decapping activity in HeLa. Note that these observations would suggest that the mechanisms underlying AUBP-regulated mRNA decay are in some manner linked to the translation process. In fact, AREs have been shown to influence the translational efficiency of a variety of mRNAs (Kruys and Huez 1994). The best documented example is the strong translational blockade imposed by the TNF-α ARE in both macrophage (Han *et al.* 1990)

and non-macrophage (Kruys *et al.* 1992) cells. In further support of a functional link between translation and mRNA stability, it was reported that blockage of translation results in increased ARE-mRNA stability (Curatola *et al.* 1995).

Recently, studies by Chen *et al.* (2001) and Mukherjee *et al.* (2002) have demonstrated a link between AUBPs and the human exosome, thus defining another mechanism by which these factors affect mRNA decay. As already briefly mentioned, the exosome consists of a complex of 3 -to-5 exoribonucleases and RNA-binding proteins that together, play a key role in the processing and degradation of several RNA species **(Figure 7)**. The human exosome was identified and characterized based on (*i*) the homology of its components to that of the yeast exosome and to known *E. coli* exoribonucleases **(Table 3)** and (*ii*) the observation that human components can restore exosome activity in yeast lost after mutations in yeast homologues. Initial studies in yeast demonstrated that the exosome is involved in the processing of the large 35S precursor ribosomal RNA (rRNA) into the functional 5.8S, 18S and 25S products required for proper ribosomal assembly (Zanchin and Goldfarb 1999; Allmang *et al.* 2000). A mutation in any of the yeast exosome component proteins resulted in various defects in rRNA maturation, including blocked early endonucleolytic 35S rRNA cleavages, stabilization of aberrant rRNAs resulting from these missed cleavages and ineffective 3 end processing of the 5.8S rRNA. The exosome also plays a role in the 3 end processing of small nuclear RNAs (snRNAs) involved in precursor mRNA splicing (i.e., U1, U2, U4 and U5 snRNAs) or in the processing and modification of rRNAs (i.e., small nucleolar RNAs U3, U14, U18 and U24) (van Hoof *et al.* 2000). Subsequently, Mukherjee *et al.* (2002) demonstrated that depletion of HeLa cytoplasmic (S100) extracts of key exosome components (i.e., PM/Scl75, hRrp40 or hRrp46) resulted in reduced efficiency of deadenylated mRNA decay *in vitro*, thus demonstrating the involvement of the exosome in 3 -to-5 cytoplasmic mRNA decay.

Interestingly, nuclear and cytoplasmic forms of the exosome complex have been characterized. These complexes were shown to consist of the same 10 core components while differing by one constituent: the nuclear complex contained the RNase Rrp6p (yeast homolog of human PM/Scl100), while the cytoplasmic exosome contained the

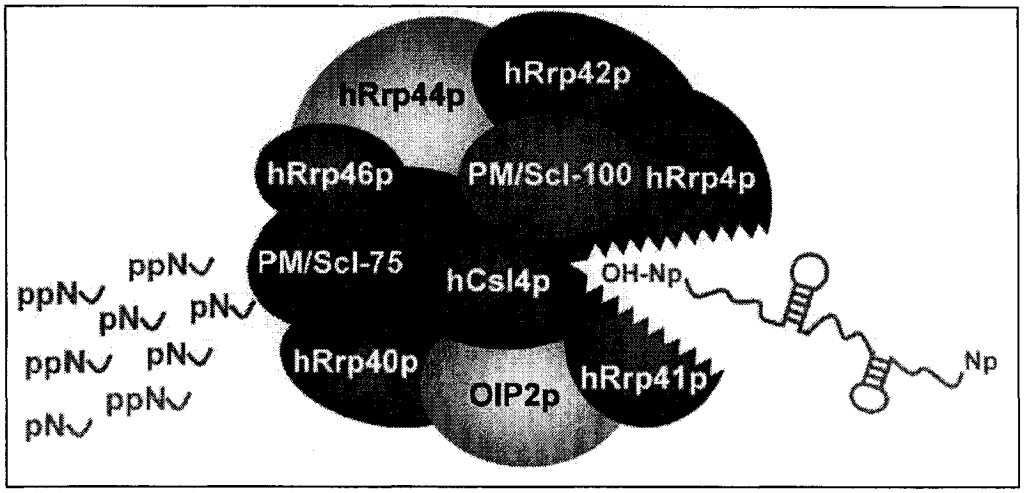


Figure 7: The composition of the human exosome complex. Functional characterization of each subunit is provided in **Table 3**.
(Brouwer *et al.* 2001)

Table 3: Functional characterization of human exosome components as predicted by sequence homology to *S. cerevisiae* and *E. coli* proteins.
(Brouwer *et al.* 2001; Chen *et al.* 2001)

Human exosome component	Homologous *S. cerevisiae* exosome subunit	Homologous *E.coli* protein or protein domain	Function
hRrp4p	Rrp4p	S1 RNA-binding domain	RNA binding
hRrp40p	Rrp40p	S1 RNA-binding domain	RNA binding
hRrp41p	Rrp41p/Ski6p	RNase PH	3 -to-5 Exoribonuclease
hRrp42p	Rrp42p	RNase PH	3 -to-5 Exoribonuclease
OIP2p	Rrp43p	RNase PH	3 -to-5 Exoribonuclease
PM/Scl-75	Rrp45p	RNase PH	3 -to-5 Exoribonuclease
hRrp44p/hDis3p	Rrp44p/Dis3p	RNase R	3 -to-5 Exoribonuclease
hRrp46p	Rrp46p	RNase PH	3 -to-5 Exoribonuclease
PM/Scl-100	Rrp6p	RNase D	3 -to-5 Exoribonuclease
hCsl4p	Csl4p/Ski4p	S1 RNA-binding domain	RNA binding

putative GTPase Ski7p (Allmang *et al.* 1999). Recently, another component, Rrp47p (homologous to human protein C1D), was identified as a substrate-specific nuclear cofactor for exosome activity in the processing of stable RNAs (Mitchell *et al.* 2003). Genetic studies have demonstrated that the nuclear and cytoplasmic functions of the exosome are separable; all characterized *in vivo* nuclear functions of the exosome require the putative RNA helicase Mtr4p/Dob1p (de la Cruz *et al.* 1998), whereas exosome-mediated cytoplasmic mRNA turnover pathways are dependent upon Ski7p and the Ski complex (i.e., Ski2p, Ski3p and Ski8p) (Brown *et al.* 2000; van Hoof *et al.* 2000).

Of greatest relevance to this discussion is the observation that AREs and destabilizing AUBPs regulate exosome activity by recruiting this complex to mRNA. Using a cell-free system containing purified exosome and recombinant AUBPs, Chen *et al.* (2001) recently showed that the destabilizing AUBPs TTP, AUF1 and KSRP (KH-type splicing regulatory protein) can directly interact with the exosome and enhance decay of an mRNA containing the c-fos ARE. Interestingly, the stabilizing AUBP HuR did not associate with the exosome. In support of this data, Ford *et al.* (1999) had already shown that HuR can oppose exosome-mediated ARE-mRNA decay and stabilize deadenylated mRNA intermediates in a cell free system. In a separate *in vitro* decay study, Mukherjee *et al.* (2002) showed that several AREs are able to bind directly to the PmScl75 subunit of the exosome and thereby enhance decay. However, this result was not confirmed by Chen *et al.* (2001) whose studies displayed that AUBPs are required for recruitment of the exosome to ARE-mRNAs.

Hence, all the above-mentioned data suggests that *trans*-acting factors involved in ARE-mRNA decay act by altering mRNA access to and/or activity of degradative enzymes. In the following section, a comprehensive discussion of three prominent AUBPs with proven *in vivo* functions will be provided.

III.2.1) AUF1 (hnRNP D)

AUF1 is a stabilizing and destabilizing AUBP that exists as four different isoforms generated by alternative splicing of the same gene (Wagner *et al.* 1998). Each isoform is denoted by its apparent molecular weight as p37AUF1, p40AUF1, p42AUF1 and p45AUF1, with p37AUF1 considered as the most active and "core" isoform. As

shown in **Figure 8,** all the AUF1 isoforms contain two nonidentical RNA recognition motifs (labeled as RRM1 or RRM2) and an 8-amino acid glutamine-rich motif C-terminal to RRM2 (labeled as Q). p40AUF1 and p45AUF1 contain a 19-amino acid insert (HSNSSPRHSEAATAQREEW, single-letter amino acid code) generated by alternative splicing of exon 2. p42AUF1 and p45AUF1 share a 49-amino acid insert (PSQNWNQGYSNYWNQGYGNYGYNSQGY GGYGGYDYTGYNNYYGYGDYSN, single-letter amino acid code) obtained by alternative splicing of exon 7. AUF1 isoforms are mostly nuclear, but may undergo quick nucleocytoplasmic shuttling. Nuclear import (or retention) is facilitated by sequences in the C-terminal domain of the two smaller isoforms; nuclear export is facilitated by the exon 7-interrupted C-terminal domain of p42AUF1 and p45AUF1, which also binds the transport receptor transportin 1 (Trn1) *in vitro* (Siomi *et al.* 1997). The special distribution of shuttling signals seen among AUF1 isoforms might represent a mechanism to assure co-shuttling of a subset of isoforms that interact in a heterocomplex (Sarkar, Lu *et al.* 2003). Each isoform also displays a different binding affinity, as shown by EMSA with a c-fos ARE probe (relative binding affinities: p37AUF1 > p42AUF1 > p45AUF1 >> p40AUF1) (Wagner *et al.* 1998).

AUF1 protein tissue distribution has been analyzed in mice (Lu and Schneider 2004); AUF1 displayed strong expression in the thymus and within lymphocytic cells of the spleen and moderate expression in gonadal tissues, epithelial linings of lungs and nuclei of most neurons in the brain. Little expression was observed in other tissues. Interestingly, the expression pattern of the four AUF1 isoforms differed among these organs. In the brain, testis and uterus, p40, p42 and p45 proteins were predominant with low levels of p37 evident. The reverse expression pattern was observed in the lung and ovary, with undetectable p45 and p37, p40 and p42 constituting the major isoforms. Given that AUF1 mRNA expression is strong in all adult mouse organs except the brain (Gouble and Morello 2000), differential expression of murine AUF1 protein isoforms is related to different patterns of alternate exon 2 and 7 splicing or differential protein stabilities (Lu and Schneider 2004). This isoform-specific expression of AUF1 in different organs is therefore suggestive of differential AUF1 regulation adapted to the function of a specific organ or tissue.

AUF1 has been shown to posses both ARE-mRNA stabilizing and destabilizing

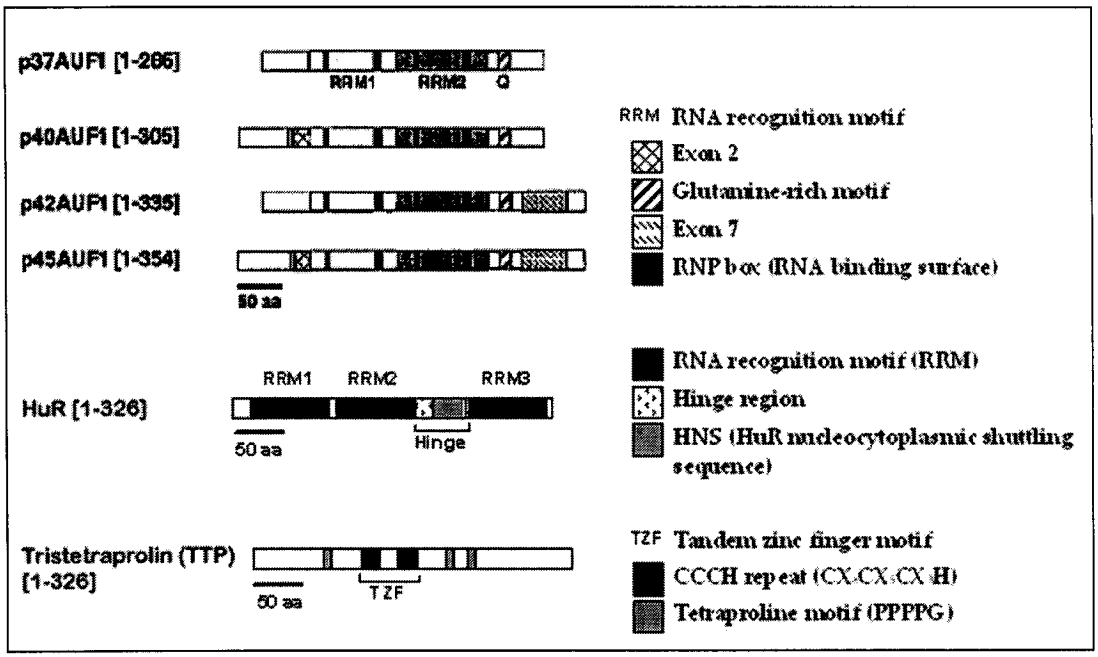


Figure 8: Domain structures of AUBPs shown to alter mRNA stability *in vivo* (refer to text for details about specific domains).
(Compiled from Fan and Steitz 1998a and Wagner *et al.* 1998)

properties in different contexts. Human mononuclear cells with low levels of endogenous p37AUF1 and p40AUF1 (Buzby *et al.* 1996) and erythroleukemic K562 cells in which AUF1 is sequestered into a hemin-induced protein complex (Loflin e*t al.* 1999) displayed compromised ARE-mRNA degradation. Sarkar, Xi *et al.* (2003) recently showed that p37AUF1 and, to a lesser extent, p40AUF1 when overexpressed in the context of all endogenous AUF1 isoforms in various cell lines promoted destabilization of a GM-CSF ARE reporter transcript. A putative role for AUF1 in mRNA stabilization was initially suggested by Kiledjian *et al.* (1997), who demonstrated that AUF1 was a component of the alpha-globin mRNA stability complex. More recently, Xu *et al.* (2001) showed that p37AUF1 overexpression in NIH 3T3 cells resulted in increased levels of the reporter mRNAs, especially those harboring class II AREs. Given the diverse nature of the experimental results on AUF1, it is difficult to come to one conclusion about its effects on ARE-mRNA stability. AUF1 s functions apparently vary depending on cell type, cellular environment and ARE class. However, it is important to consider that a majority of AUF1 information has been derived from overexpression assays, which are extremely artificial and may greatly disrupt cell function and the *in vivo* process being studied.

AUF1 activity is subject to regulation via various mechanisms. Polysome-associated p40AUF1 is serine-phosphorylated in untreated cells from the monocytic leukemia cell line THP-1. Treatment of these cells with phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) resulted in the loss of p40AUF1 phosphorylation with a concomitant reduction in its ability to bind a TNF-α ARE probe *in vitro* and to stabilize IL-1β and TNF-α ARE-mRNAs *in vivo* (Wilson, Lu, Sutphen, Sun *et al.* 2003). Furthermore, inhibition of the p38 MAPK pathway in human monocytes has been reported to reduce AUF1 ARE binding (Sirenko *et al.* 1997); this result, however, is debatable given the use of highly nonspecific concentrations of p38 inhibitor (i.e., SKF 86002 at doses of 20 μM and above). Hence, although no kinase or phosphatase have been conclusively linked to AUF1 phosphorylation or dephosphorylation *in vivo*, it is evident that phosphorylation is a key mechanism in the regulation of AUF1 activity.

AUF1 activity is also regulated by ubiquitination. As reported by Laroia and Schneider (2002), AUF1 isoforms are differentially ubiquitinated and degraded in a manner dependent on the presence of exon 7, which blocks ubiquitination. AUF1

isoforms have been shown to form multiprotein complexes on AREs; such complexes comprise ubiquitin-proteasome pathway-related (heat shock proteins Hsc-70 and Hsp70, ubiquitin conjugating enzyme E1) and non-related (eIF4G, PABP, RNA binding proteins NSEP-1, NSAP-1 and IMP-2) proteins (Laroia *et al.* 1999; Moraes *et al.* 2003). A specific complex comprising AUF1, Hsc70, Hsp70, PABP and eIF4G has been associated to the stabilization of ARE-mRNAs during heat shock and cell stress (Laroia *et al.* 1999). Hsp70, which itself can directly bind AREs (Henics *et al.* 1999) is induced during heat shock and sequesters AUF1 to the nucleus as part of a stabilizing ARE-bound Hsp70-Hsc70-eIF4G-PABP complex. Dissociation of this complex, which involves displacement of eIF4G from AUF1, ubiquitination of AUF1 and degradation of AUF1 by proteasomes, leads to ARE-mRNA destabilization.

Recently, Dean *et al.* (2002) identified a protein showing high sequence homology to AUF1 and named it AUF2. This protein is expressed as two isoforms, namely p37 and p42. Its role in ARE-dependent mRNA decay is still unclear given conflicting experimental data. AUF2, along with AUF1, were identified as components of an intense complex associated with a TNF-α ARE probe in RNA electromobility shift assays of RAW264.7 chromatographic fractions. However, these two factors were not identified in any of the TNF-α ARE-probe-associated complexes resolved by electromobility shift assays of crude cell extracts, although they were part of a complex specific for the COX-2 ARE. Moreover, overexpressed AUF2 co-immunoprecipitated with endogenous HeLa cell COX-2 mRNA and led to a slight, but noticeable stabilization of a COX-2 ARE reporter.

III.2.2) HuR

HuR is a member of the ELAV-like family of RNA binding proteins. These proteins have proven essential for neural development in Drosophila (Campos *et al.* 1985) and been shown to stabilize and/or to activate translation of target mRNAs. Unlike other members of the Hu family proteins (i.e., HuB/Hel-N1, HuC and HuD), whose expression is developmentally regulated and restricted to the brain, HuR (also called HuA) has a wider tissue distribution and is expressed in the human brain, heart, kidney, liver, lungs, spleen, testes and to a lower degree in skeletal muscle (Gallouzi *et al.* 2000).

In adult mice, HuR expression parallels that seen in humans and also detected in the thymus, stomach and intestine. In addition, HuR mRNA and protein expression levels display disparities, thereby implicating translational control or differential mRNA versus protein stabilities in HuR regulation (Gouble and Morello 2000; Lu and Schneider 2004).

HuR is predominantly (~ 90%) nuclear in unstimulated cells, although it may undergo nucleocytoplasmic shuttling upon cellular stimulation. HuR contains a unique shuttling signal termed HNS (HuR nucleocytoplasmic shuttling sequence) that contains both nuclear localization and nuclear export activity (Fan and Steitz 1998a). Rebane *et al.* (2004) demonstrated that HNS is sufficient for interactions with nuclear import receptors Trn1 and two alternatively spliced isoforms of Trn2 (i.e., Trn2a and Trn2b). A recent study by Wang *et al.* (2004) identified the adapter importin α 1 (Impα1) as another HuR cytoplasmic ligand involved in nuclear import, albeit following AMP-activated protein kinase (AMPK) activation and subsequent Impα1 phosphorylation and acetylation. Nuclear export was shown to involve the association of HuR with two nuclear ligands, pp32 and acidic protein rich in leucine (APRIL), which contain leucine-rich nuclear export signals (NES) that are recognized by the export receptor chromosome maintenance region 1 (CRM1) (Gallouzi and Steitz 2001).

HuR contains three classical RRMs; RRM 2 and RRM3 are separated by a hinge region that harbors the HNS (**Figure 8**). *In vitro* studies using HuR mutants have demonstrated that RRM1 and RRM2 are required for ARE binding while RRM3 simultaneously associates with the poly(A) tail (Ma *et al.* 1997). Further studies by Fan and Steitz (1998b) demonstrated that RRM3 is indispensable for HuR's stabilizing effects *in vivo*.

In overexpression experiments, HuR displays high affinity and stabilizes ARE-mRNAs containing class I and II ARE-mRNAs (i.e., GM-CSF (Fan and Steitz 1998b), TNF-α (Dean *et al.* 2001), IL-3 (Ming *et al.* 2001), COX-2 (Sully *et al.* 2004)), and to a lesser extent, class III ARE-containing mRNAs (i.e., β-adrenergic receptor (Blaxall *et al.* 2000), GAP-43 (Chung *et al.* 1997)). In support of the overexpression data, depletion of HuR by RNA interference or antisense cDNA lead to the destabilization of ARE-mRNAs for vascular endothelial growth factor (VEGF) (Levy *et al.* 1998), Cyclins A and B1

(Wang, Caldwell *et al.* 2000) and cyclin-dependent kinase inhibitor p21 (Wang, Furneaux *et al.* 2000).

Both *in vitro* (Ford *et al.* 1999) and overexpression (Peng *et al.* 1998) assays have demonstrated that HuR acts by stabilizing the body of deadenylated mRNA intermediates and has no effect on the deadenylation process. Although unproven, multiple studies have suggested that HuR's functions are linked to its presence in the cytoplasm (Atasoy *et al.* 1998; Wang, Caldwell *et al.* 2000). Notably, increases in cytoplasmic endogenous HuR levels in RKO colorectal carcinoma cells following UV treatment is associated with an increase in stability of the ARE-containing cyclin-dependent kinase inhibitor p21 mRNA (Wang, Furneaux *et al.* 2000). Similarly, the increased stability of ARE-mRNAs for proteins involved in myocyte differentiation (i.e., myogenin, MyoD, and p21) coincided with elevated cytoplasmic levels of HuR at the onset of myogenesis; these mRNAs were subsequently destabilized at the end of myogeneis, at which time HuR displayed a predominantly nuclear presence (Figueroa *et al.* 2003). Similarly, the cytoplasmic localization of other Hu-family members appears to be crucial for their activity (Antic and Keene 1997). Given the above mentioned data, it is suggested that HuR may bind the ARE-containing mRNAs in the nucleus and accompany them to the cytoplasm, protecting them from degradation and possibly delivering them to the translational apparatus.

HuR's subcellular localization and activity is modulated by binding partners. Four abundant HeLa cell proteins, namely SETα, SETβ, pp32, and APRIL, specifically and directly interact with HuR by recognizing its hinge region and RRM3 (Brennan *et al.* 2000). As previously mentioned, pp32 and APRIL influence HuR nucleocytoplasmic shuttling via their NESs. Furthermore, HuR's association with pp32 and APRIL increased its affinity for ARE-mRNAs in the nucleus of CRM1 export pathway-arrested cells (Brennan *et al.* 2000). SETα, SETβ, and pp32 also function as inhibitors of protein phosphatase 2A (Li *et al.* 1996; Saito *et al.* 1999), thus raising the possibility that protein phosphatase 2A and/or phosphorylation/dephosphorylation are implicated (*i*) in the signaling cascades that regulate the stability of ARE-containing mRNAs or (*ii*) directly in the mechanism of ARE-mediated mRNA decay.

HuR is specifically methylated *in vivo* by the coactivator-associated arginine

methyltransferase 1 (CARM1) in both lipopolysaccharide (LPS) -stimulated RAW264.7 macrophages and CARM1/HuR co-transfected COS-7 cells (Li *et al.* 2002). Methylation occurs on Arginine 217, which is located within HuR's hinge region. The exact role of this methylation is unclear, although it is believed to affect HuR's association with its nuclear ligands pp32 and APRIL. Given the roles of these proteins in nucleocytoplasmic shuttling and ARE-mRNA binding affinity, methylation may thus enhance or diminish any of these processes.

In addition to all the above mentioned regulatory mechanisms, HuR's activity is further regulated by the previously discussed AUBP AUF1 (Gouble and Morello 2000; Lu and Schneider 2004). Such mutual regulation is a result of their significantly overlapping substrate specificities and tissue distribution, as well as their opposing influence on target mRNA stability. Using p21 and cyclin D1 transcripts as targets, Lal *et al.* (2004) reported that HuR and AUF1 can concurrently bind specific mRNA transcripts *in vivo* on both separate, nonoverlapping sites and on identical sites in a competitive fashion. Such simultaneous binding was evident in the nucleus, whereas in the cytoplasm, HuR and AUF1 appeared to bind target mRNAs individually. Hence, a model was presented whereby HuR and AUF1 both interact with a subset of ARE-mRNAs in the nucleus. Upon exiting this compartment, either AUF1 is released and HuR remains bound, followed by the recruitment of the mRNA-protein complex to polysomes, or HuR is released and AUF1 remains bound, leading to exosome-mediated decay of the mRNA.

III.2.3) TTP

TTP (TIS11 in mouse), is a product of the human immediate early gene *ZFP36* and the prototype of a family of known zinc finger proteins containing two CCCH zinc fingers spaced 18 amino acids apart (i.e., CCCH tandem zinc finger (TZF) proteins) (Taylor *et al.* 1991; Kaneda *et al.* 1992). The TZF family includes two other members in mammals, namely human butyrate-response factor (BRF)-1 (also known as epidermal growth factor response factor, ERF-1; TIS11b in mouse) and human BRF-2 (TIS11d in mouse) (Lai *et al.* 2000). In addition to the TZF motif, TTP contains three tetraproline

motifs consisting of the amino acid sequence PPPPG (single letter amino acid code) **(Figure 8)**.

TTP's involvement in ARE-mRNA decay has been highlighted by the severe phenotypes displayed in TTP knockout mice. Although normal at birth, these mice develop a systemic syndrome characterized by severe polyarticular erosive arthritis and myeloid hyperplasia, both within and outside the bone marrow, as well as alopecia, cachexia, dermatitis, conjunctivitis and autoimmunity (Taylor, Carballo *et al.* 1996). These effects were later attributed to the overexpression of TNF-α (Carballo *et al.* 1997) and TTP is now recognized as a major, negative-feedback regulator of TNF-α expression that acts by destabilizing TNF-α mRNA (Lai *et al.* 1999). TTP has also been shown to associate with and destabilize the following ARE-mRNAs: GM-CSF, IL-2, IL-3 (Raghavan *et al.* 2001), plasminogen activator inhibitor type 2 (PAI-2) (Yu *et al.* 2003), COX-2 (Sully *et al.* 2004) and TTP itself (Tchen *et al.* 2004). Given its effect on a variety of inflammatory mediators, it is clear that TTP is a critical component of inflammatory processes and as such, is usually studied within this context.

In mice, TTP mRNA is expressed in developing oocytes and regenerating liver, intestine, lung, spleen, thymus and macrophages (Taylor, Carballo *et al.* 1996). However, TTP mRNA translation and protein expression is tissue-restricted; analysis of TTP protein in 6-week-old male and female mice showed expression only in the livers of male and female mice and in the testes of male mice, with weaker expression in ovaries of female mice (Lu and Schneider 2004). In humans, TTP protein has been localized immunohistochemically to synovial-lining leukocytes of rheumatoid synovium (Brooks *et al.* 2002).

TTP promotes mRNA decay by two independent mechanisms. In both cases, the destabilizing effect on target ARE-mRNAs requires both zinc-fingers (Lai *et al.* 2000). In one mechanism, TTP enhances the deadenylation of target transcripts. The expression of exogenous TTP in TTP-null HEK 293 cells promotes deadenylation of reporter mRNAs bearing the TNF-α ARE (Lai *et al.* 1999). GM-CSF mRNA is also more stable and has a longer poly(A) tail in TTP-null macrophages relative to wild-type cells (Carballo *et al.* 2000) *In vitro*, TTP promotes the deadenylation of RNAs containing the TNF-α ARE, but not a mutated version (Lai *et al.* 2003). TTP also promotes deadenylation and poly(A)

tail-independent decay of target mRNAs, as shown by transfection assays wherein transfected TTP destabilized reporter mRNAs in which the poly(A) tail was replaced with a histone mRNA stem loop structure (Lai and Blackshear 2001). The latter mechanism likely involves the recruitment of the exosome subsequent to ARE binding (Chen *et al.* 2001).

TTP, along with the other mammalian CCCH TZF protein family members, are nucleocytoplasmic shuttling proteins. TTP contains both a nuclear localization (NLS) and nuclear export signal (NES) in its TZF region and N-terminal leucine-rich region, respectively (Murata *et al.* 2002). TTP's nuclear export is mediated by the nuclear export receptor CRM1, which binds directly to TTP's NES (Phillips *et al.* 2002). Another mechanism by which TTP is targeted to the cytoplasm is through binding 14-3-3 proteins. This highly conserved class of proteins is known for associating with various ligand proteins (i.e., glucocorticoid receptor (Kino *et al.* 2003), histone deacetylase-4 (Wang and Yang 2001) and histone deacetylase-7 (Kao *et al.* 2001)) and directing their cytoplasmic localization and/or sequestration. 14-3-3 binding to TTP is a regulated process and is further discussed below.

The exact sub-cellular distribution of TTP is specific to both cell-type and cell state (i.e., activated or quiescent). Initial studies by Taylor, Thompson *et al.* (1996) showed that in serum-deprived, quiescent NIH 3T3 cells that constitutively express TTP, 70% of the protein was nuclear. Upon mitogen stimulation, rapid (within 1 minute) cytoplasmic translocation was observed. In transiently transfected COS-7, HeLa and HEK293 cells, rodent TTP is predominantly, but not exclusively, cytoplasmic and is concentrated to the nucleus upon treatment of cells with leptomycin B (a specific inhibitor of CRM1) (Murata *et al.* 2002; Phillips *et al.* 2002). However, endogenous TTP in human neutrophils, lymphocytes and monocytes (Brooks *et al.* 2002; Fairhurst *et al.* 2003) and murine macrophages (Carballo *et al.* 1998) was found to be solely cytoplasmic prior to and following cytokine stimulation. These discrepancies are most likely due to effects of overexpression and/or cell-specific differences.

TTP activity is subject to regulation at multiple levels, most of which are dependent on phosphorylation. TTP exists in different phosphorylated forms; phosphorylation on serines 52 and 178 of murine TTP, along with several other minor

sites, has been detected *in vivo* (Chrestensen *et al.* 2004). It is suspected that TTP is targeted by several signaling pathways, including the p42 MAPK (extracellular signal-regulated kinase-2, ERK-2) and the p38 MAPK/MAPKAPK-2 cascades (Taylor *et al.* 1995; Carballo *et al.* 2001; Mahtani *et al.* 2001; Zhu *et al.* 2001).

The effects of TTP phosphorylation on its ARE binding affinity are not well defined. One study compared ARE binding activity between phosphatase-treated and -untreated crude extracts from TTP-transfected HEK293 cells. The relative ARE binding activity of the phosphatase-treated preparations was higher than that of the untreated samples (Carballo *et al.* 2001). Conversely, a subsequent *in vitro* study by the same group showed that binding of bacterially-expressed recombinant TTP (which is unphosphorylated) to a TNF-α ARE probe was unaffected by p38 phosphorylation (Cao *et al.* 2003). It is important to consider that although TTP is clearly phosphorylated *in vivo*, the exact details and mechanisms behind these phosphorylations are still speculative.

Functional roles of TTP phosphorylation have been demonstrated. *In vivo* phosphorylation of putative MAPKAPK-2 residues serine 52 and serine 178 of murine TTP results in the creation of functional binding sites for the 14-3-3 proteins (Chrestensen *et al.* 2004; Stoecklin *et al.* 2004). This sequence-specific and phosphorylation-dependent interaction promotes localization of TTP to the cytoplasm and may thus serve as a phosphorylation-mediated mechanism to control TTP's subcellular localization. TTP's interaction with 14-3-3 proteins also reverses TTP's ability to bind and destabilize LPS-induced TNF-α mRNA in macrophages and TTP's association with stress granule complexes, which are dynamic cytoplasmic foci consisting of stalled translation initiation complexes formed in cells subjected to environmental stress (Stoecklin *et al.* 2004).

In addition to the above, TTP is involved in other cellular functions besides mRNA stability that may be regulated by phosphorylation. TTP influences cell growth and survival pathways, as suggested by its induction by various mitogens and growth factors and its expression in regenerating murine small intestine, liver, lung and hematopoietic tissues (reviewed in Johnson, Stehn *et al.* 2002). TTP may also induce apoptosis, alone or synergistically with TNF-α, by either mitochondria-dependent or

independent pathways (Johnson *et al.* 2000; Johnson and Blackwell 2002). Hence, TTP may function in the cellular decision between activation and apoptosis.

IV) The p38 MAPK Signaling Cascade and ARE-mediated mRNA Stability

As previously mentioned, ARE-mediated mRNA decay is a tightly regulated process that is sensitive to various extracellular signals. Phosphorylation through activation of kinase signaling cascades has been shown to be a critical event in the regulation of this process in various cells types and for different ARE-mRNAs. Of the various kinase cascades known to control gene expression, the p38 MAPK pathway has proven to be the key player in post-transcriptional regulation. In the upcoming section, a brief introduction to the p38 MAPK cascade will be provided, followed by a discussion about its roles in ARE-mediated post-transcriptional gene regulation.

IV.1) Overview of the p38 MAPK Pathway

The p38 MAPK pathway is activated by a variety of environmental stresses, transforming growth factor-β (TGF-β) and pro-inflammatory stimuli. As with other MAPK cascades (i.e., ERK, JNK/SAPK, BMK1), the p38 MAPK pathway consists of a hierarchy of kinases grouped into three tiers **(Figure 9)**. The first activation tier, known as MAPK kinase kinase (MAPKKK), usually consists of a membrane-proximal mitogen-activated Protein/ERK kinase kinase (MEKK), such as the apoptosis signal-regulating kinase 1 (ASK1), a mixed lineage kinase (MLK) or TGF-β-activated kinase 1 (TAK1). The MAPKKK then phosphorylates and activates the second tier MAPK kinase (MKK)-3 and 6, which in turn phosphorylates and activates the terminal (i.e., third tier) MAP kinase, p38 (Ono and Han 2000). Once activated, the p38 MAPK may influence a variety of cellular functions (Shi and Gaestel 2002). It may induce gene transcription by directly or indirectly (via activation of downstream kinases) phosphorylating various transcription factors, as displayed in **Figure 9.** Activated p38 also influences translation through phosphorylation and activation of MNK1, which phosphorylates and activates eIF4E and allows for the formation of a functional eIF4F complex required for translation (Waskiewicz *et al.* 1999). In addition, p38 MAPK signaling is associated to post-

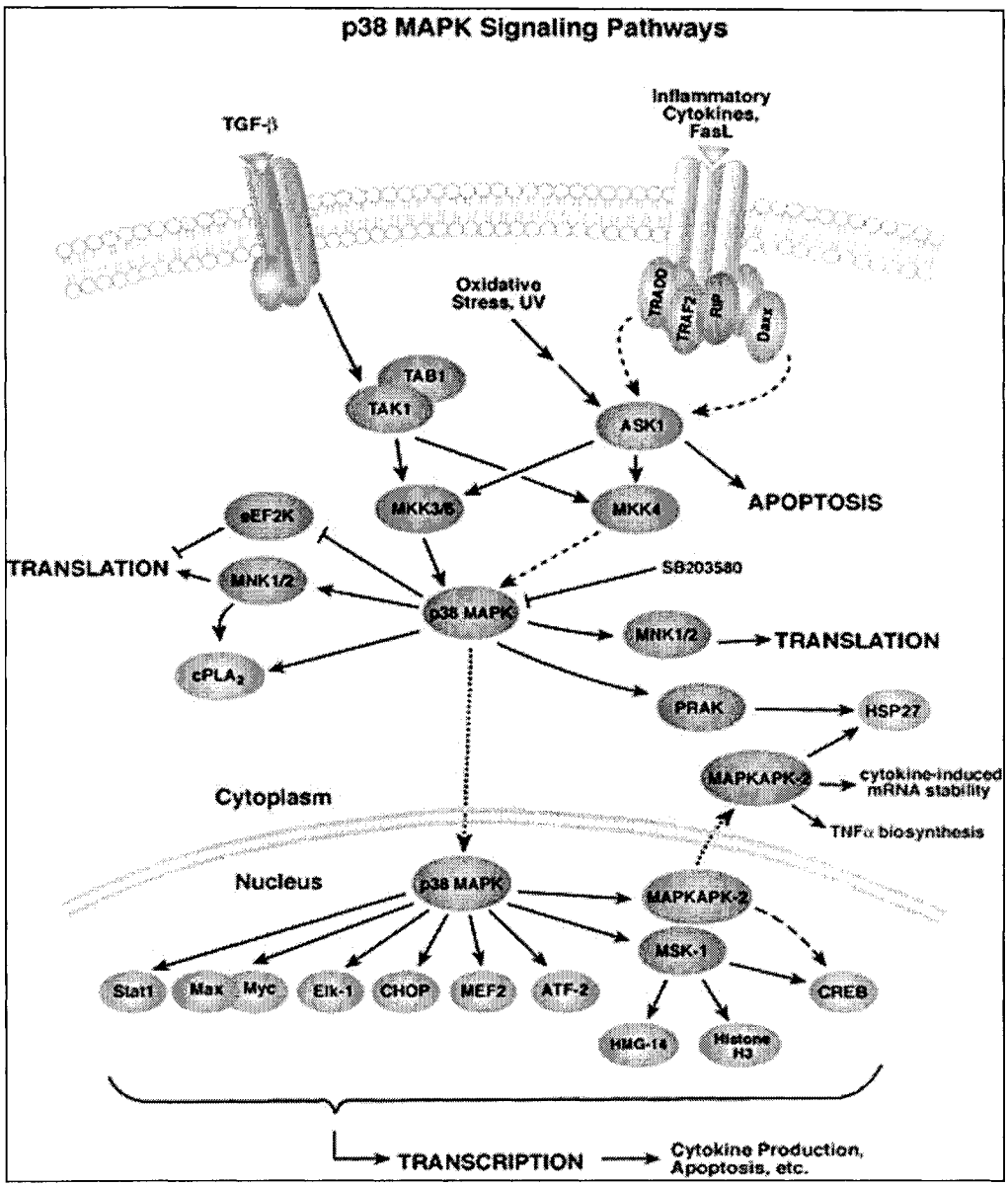


Figure 9: The p38 MAPK signaling cascade.
(Obtained online from Cell Signaling Technology, Inc; "Pathways Activating p38 MAPK." <http://www.cellsignal.com/reference/pathway/p38MAPK.asp>)

transcriptional regulatory mechanisms through the phosphorylation and activation of MAPKAPK-2 (Kotlyarov and Gaestel 2002). This aspect of p38 MAPK signaling will be the focus of the next section.

IV.2) Involvement of the p38 MAPK in ARE-mediated mRNA Stability

Activation of the p38 MAPK cascade has been closely linked to the stabilization of a variety of inflammatory response-related and unrelated ARE-mRNAs (Clark *et al.* 2003) **(Table 4)**. According to recent studies by *Dean et al.* (2003), signaling through p38 MAPK stabilizes target ARE-mRNAs by inhibiting their deadenylation. This effect was ARE-dependent and sequence-specific since deadenylation of reporter mRNAs bearing either the COX-2 or TNF-α ARE were regulated by p38, whereas deadenylation of reporters lacking an ARE or carrying the c-myc ARE (which is not p38-regulated) were unaffected by p38 MAPK activation.

The specificity and precise mechanistic details behind p38-regulated ARE-mRNA stability are unclear, but likely involve regulation of AUBP activity and/or expression through direct or indirect (i.e., via MAPKAPK-2 activation) phosphorylation. Such AUBPs may hypothetically have a direct and/or indirect influence on deadenylase activity. Experimental evidence would suggest that the most likely factor linking the p38 MAPK pathway to stabilization of ARE-mRNAs is TTP. As stated previously, TTP is involved in ARE-mRNA destabilization and has been shown to be a target for MAPKAPK-2 and p38 MAPK itself. This relationship had initially been reported in bone-marrow derived murine macrophages, in which LPS-stimulated TNF-α synthesis is stabilized following p38-induced TTP phosphorylation. This effect was attributed to decreased affinity of phosphorylated TTP for the TNF-α ARE and suggested a possible mechanism by which LPS leads to sustained TNF-α levels (Carballo *et al.* 2001). Paradoxically, Mahtani *et al.* (2001) later demonstrated that the level of TTP protein in mouse RAW264.7 macrophages was itself increased by LPS in a p38 MAPK-dependent fashion. This event can be explained by the stabilization of TTP mRNA by p38 MAPK via an AU-rich region present in the TTP 3 UTR (Tchen *et al.* 2004). Hence, an extremely complex picture is painted in which the signaling pathway involved in TTP's synthesis is the same one that regulates its ability to bind target ARE-mRNAs. Clearly,

Table 4: ARE-mRNAs stabilized by p38 MAPK.

Inflammatory response-related		*Inflammatory response-unrelated*	
Gene	**Reference**	**Gene**	**Reference**
Cyclooxygenase -2 (COX-2)	Ridley *et al.* 1998	**Jun-B, SOX-9, carbonic anhydrase 2, Bcl2, Bcl2-like 2, nuclear factor erythroid 2-like 2, Intereferon-regulatory factor-1 (IRF-1), MKK6, Fibroblast growth factor-9 (FGF-9)**	Frevel *et al.* 2003
GM-CSF	Tebo *et al.* 2003		
Growth-related oncogene (GRO)-2, GRO-3, IL-1α, IL-8, Macrophage colony stimulating factor (M-CSF), Macrophage inflammatory protein-1β (MIP-1β)	Frevel *et al.* 2003		
IL-1β, GRO-α	Sirenko *et al.* 1997		
IL-3	Ming *et al.* 2001		
IL-6	Miyazawa *et al.* 1998		
MIP-1α	Wang *et al.* 1999		
Matrix metalloproteinases (MMP)-1 and -3	Reunanen *et al.* 2002		
Monocyte chemotactic protein-1 (MCP-1)	Waterhouse *et al.* 2001		
TNF-α	Wang *et al.* 1999		
TTP	Tchen *et al.* 2004		
VEGF	Pages *et al.* 2000		

there exist other uncharacterized levels of complexity in the interactions between TTP and the p38 MAPK pathway that would account for the ability of the p38 MAPK to induce the synthesis of this destabilizing factor (through transcriptional and post-transcriptional/stabilization mechanisms) while increasing the stability of its target ARE-mRNAs.

V) Cyclooxygenase-2 as a Model For p38 MAPK-mediated ARE-mRNA Stability

Inflammation is thought to be central to the pathogenesis of a lengthy list of diseases, of which rheumatoid arthritis is one of the most common and physically debilitating. RA is a chronic, systemic, autoimmune disease that affects various joints and surrounding tissues. Although the etiology of RA is still unknown, the pathology associated with the disease is related to the resulting inflammatory response initiated within the synovial membrane, which is subsequently transformed into a proliferating invasive cell mass (i.e., pannus) that erodes the surrounding cartilage and bone. At the center of this inflammatory response is the p38 MAPK pathway, which is involved in the recruitment (T-cells and monocytes only), activation and proliferation of T-cells, monocytes and synovial fibroblasts. p38 MAPK also regulates the expression of the COX-2, which, as will be seen, is involved in the synthesis a crucial inflammatory mediator in RA. In the following section, a comprehensive overview of COX-2 and its isoform, COX-1, will be presented and immediately followed by a discussion of the roles of p38 MAPK in the regulation of COX-2 expression.

V.1) The Cyclooxygenases: Overview of Structure, Function and Molecular Biology

The cyclooxygenases (prostaglandin H synthase) are highly conserved enzymes responsible for catalyzing the rate limiting step in the synthesis of lipid-derived bioactive compounds called prostaglandins (PG) and thromboxanes (known collectively as prostanoids). Two COX isoforms (Vane and Botting 1996) (and a potential third (Chandrasekharan *et al.* 2002)) have been identified. COX-1 is a ubiquitous, constitutively expressed enzyme involved in various aspects of physiological homeostasis, playing key "housekeeping" roles in gastroprotection, platelet aggregation

and kidney function, among other actions (Crofford *et al.* 1997). The second COX isoform, COX-2 (Xie *et al.* 1991), is a highly inducible protein expressed at extremely low levels, although constitutively expressed COX-2 is present in the brain, kidney, ovary, testes and tracheal epithelia. COX-2 is potently induced by pro-inflammatory stimuli (i.e., IL-1, TNF-α, LPS), growth factors (i.e., EGF, FGF, PDGF, TGF-β), hormones (i.e., follicle-stimulating hormone, luteinizing hormone, estrogen) and phorbol esters (*in vitro*). Such factors induce COX-2 transcription through several *cis*-regulatory promoter elements via MAPK signaling pathways (reviewed in Stack and DuBois 2001; Tanabe and Tohnai 2002). Both COXs are homodimers and monotopic membrane proteins (i.e., inserted into only one leaflet of the membrane) that appear to be targeted to the lumenal membrane of the endoplasmic reticulum (where they are *N*-glycosylated) and to the nuclear envelope (Morita *et al.* 1995).

Despite the above mentioned differences, the COX isoenzymes share many common structural and biochemical properties **(summarized in Table 5)**. COX-1 and -2 are encoded from separate genes and share approximately 60-65% amino acid identity (Smith and DeWitt 1996) **(Figure 10)**. In accordance with the high degree of amino acid identity, COX crystal structures confirmed that the isoforms are structurally homologous and fairly superimposable (Luong *et al.* 1996). The COX monomer consists of three structural domains: the N-terminal EGF domain, a membrane binding domain (MBD) of about 48 amino acids in length and a large C-terminal globular catalytic domain containing a heme binding site **(Figure 11)**. The COX catalytic domain harbors two catalytic activities: cyclooxygenase (or *bis*-dioxygenase) activity and peroxidase activity. These two activities occur at distinct sites, but are structurally and functionally connected and give rise to a "branch-chain" reaction mechanism (Dietz *et al.* 1988). The cyclooxygenase activity of COXs oxidizes arachidonic acid (which is initially liberated from cellular membrane phospholipids by phospholipase A_2 (PLA_2)), producing prostaglandin G_2 (PGG_2; a cyclopentane hydroperoxy endoperoxide). Subsequently, the peroxidase activity of COXs reduces PGG_2 to PGH_2 (Needleman *et al.* 1986) **(Figure 12)**. PGH_2 is the root PG from which the major active prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TXA_2 (thromboxane), are made by downstream terminal

Table 5: Properties of human COX-1 and COX-2 genes, mRNAs and proteins.
(Chandrasekharan and Simmons 2004)

Property	COX-1	COX-2
Chromosomal location of gene	9q32-q33.3	1q25.2-25.3
Gene size	About 22 kb	About 8 kb
Number of exons	11	10
Number of introns	10	9
Length of primary mRNA	2.8 kb	4.5 kb
Length of differentially polyadenylated variants	4.5 kb, 5.2 kb	4.0 kb, 2.8 kb
Length of coding region	1,797 nucleotides	1,812 nucleotides
Putative transcription regulatory elements found in:		
- promoter	TATA-less	TATA box
- 5' upstream region	AP-2, GATA-1, NF-IL6, NF-κβ, PEA-3, SP-1, SSRE	AP-2, C/EBP, CRE, GATA-1, GRE, NF-IL6, NF-κβ, PEA-3, SP-1
- 3' untranslated region		**22 AUUUA repeats**
Expression	Constitutive	Inducible (by cytokines, growth factors, phorbol esters); constitutive in selected tissues (see text)
Length of protein (with signal peptide)	599 amino acids	604 amino acids
Length of mature protein (without signal peptide)	576 amino acids	581 amino acids
MW_{app} (SDS PAGE)	~67 kDa (single band)	~68-72 kDa (multiple bands)
Number of glycosylation sites	3	3-4 (variable)
Cofactors	Heme	Heme
Substrates	Arachidonic acid	Arachidonic acid; C18 and C20:3 fatty acids
Quaternary structure	Homodimer	Homodimer
Subcellular location	Endoplasmic reticulum (some in nuclear envelope)	Endoplasmic reticulum and nuclear envelope

Abbreviations: CRE: cyclic AMP response element; **GATA-1**:, binding site containing GATA sequence bound by the GATA 1 transcription factor; **GRE**: glucocorticoid-response element; **SSRE**: shear-stress response element. Other abbreviations denote the transcription factors bound by the regulatory elements shown: **AP-2**: activator protein 2; **C/EBP**: CCAAT/enhancer-binding protein; **NF-IL-6**: nuclear factor IL-6; **NF-κβ**: nuclear factor-kappa beta; **PEA-3**: polyoma enhancer activator; **SP-1**: transcription factor SP-1.

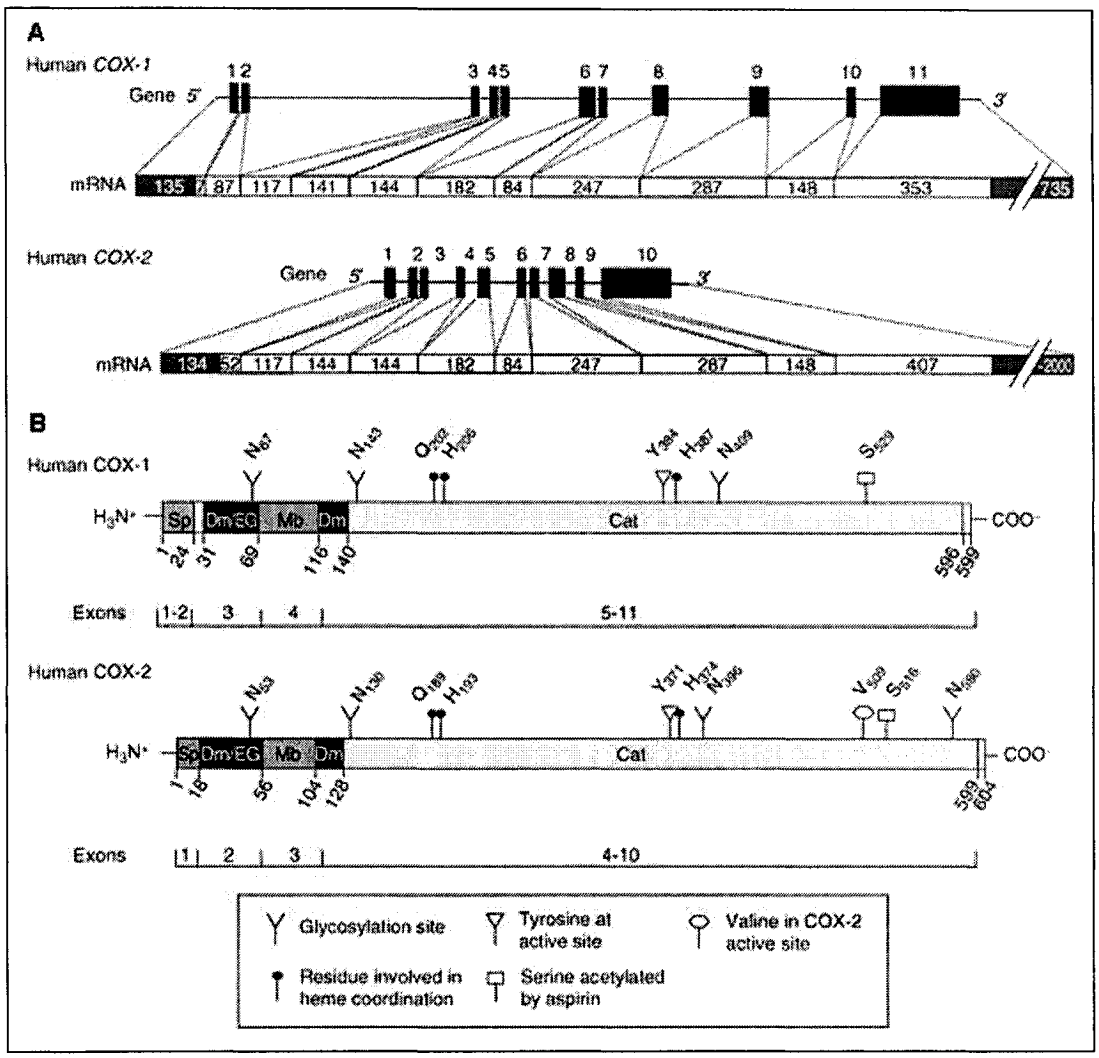


Figure 10: Primary structures of COX genes and COX proteins. (**A**) Schematic of human COX genes and mRNAs. Black boxes in the genes and white boxes in the mRNAs denote exons; numbers above each gene are exon numbers while numbers within the white boxes indicate the size of each exon in nucleotides. (**B**) Schematic of human COX proteins. Numbers denote amino-acid residues; the exons encoding each domain are shown on bars below the proteins; important residues are indicated as shown in the key (and with letters in the single-letter amino-acid code, with a subscript number indicating the residue number). **Sp**: signal peptide; **Dm**: dimerization domain; **EG**: epidermal growth factor domain; **Mb**: membrane-binding domain; **Cat**: catalytic domain.
(Chandrasekharan and Simmons 2004)

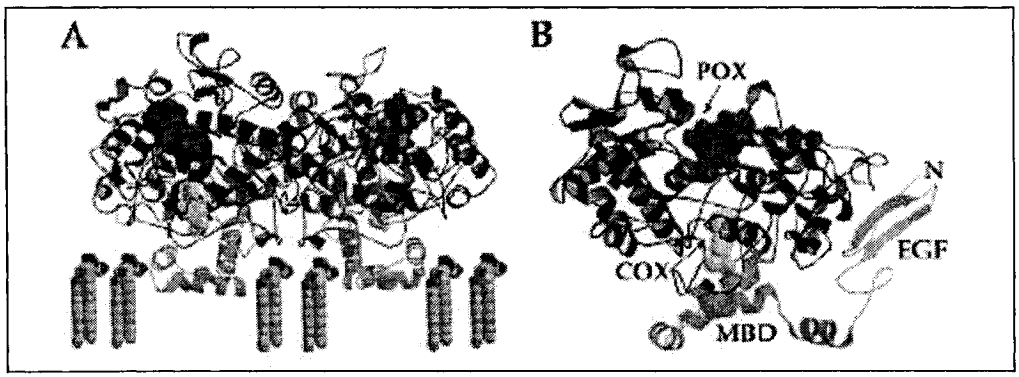


Figure 11: Ribbon diagram of ovine COX-1 displaying structural domains and catalytic sites. (**A**) Lumenal membrane-associated COX-1 homodimer with bound heme (red) and inhibitor (Flurbiprofen, yellow). Blue, orange and green ribbons correspond to the catalytic, membrane binding (MBD) and epidermal growth factor (EGF) domains, respectively. (**B**) Localization of catalytic sites within the ovine COX-1 monomer (POX: peroxidase active site; COX: cyclooxygenase active site). Domains are indicated using the same color scheme as in (**A**).
(Smith *et al.* 2000)

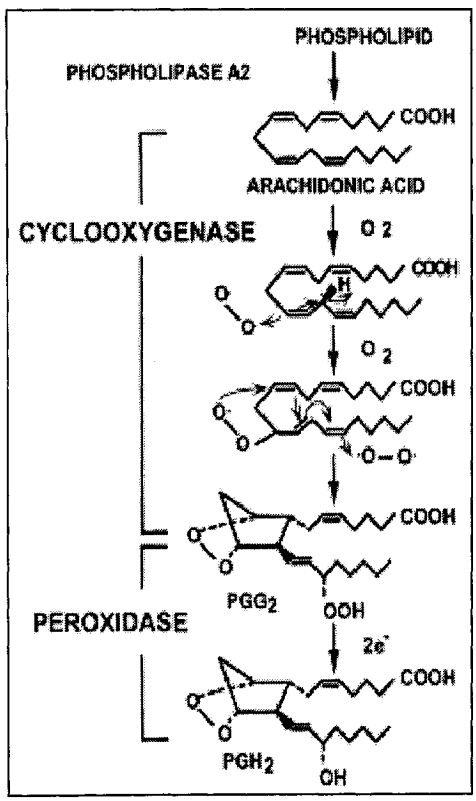


Figure 12: Summary of the two cyclooxygenase catalytic activities: the conversion of arachidonic acid to prostaglandin G_2 (PGG_2) via oxidation of arachidonic acid by the cyclooxygenase activity followed by reduction of PGG_2 to PGH_2 by the peroxidase activity.
(Adapted from Smith *et al.* 2000)

synthases via isomerization and oxidation or reduction reactions **(summarized in Figure 13)**. These synthases may themselves be subject to complex cell-specific regulation (Helliwell *et al.* 2004). Once released from the cell, PGs act as auto- and paracrine physiological and pathophysiological (i.e., inflammatory) mediators. They are synthesized in a variety of tissues and cells and modulate many systems including the cardiovascular, gastrointestinal, genitourinary, endocrine, respiratory, immune and central nervous systems. More specifically, they are involved in the regulation of a variety of processes including cell proliferation and differentiation, fever, algesia, apoptosis, ovulation, gastroprotection, kidney function, platelet aggregation and bone metabolism. In addition, these compounds are likely implicated in disease processes, as implied by various studies linking COX to different cancers (Zha *et al.* 2004) and neurological disorders (reviewed in Minghetti 2004). The vast array of biological activities mediated by PGs is explained by the large repertoire of PG receptors **(Table 6)**. These plasma membrane G-protein-coupled receptors are classified into five basic types, termed DP, EP, FP, IP and TP, on the basis of their sensitivity to the five primary prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 , respectively. Furthermore, EP is subdivided into four subtypes, namely EP1, EP2, EP3 and EP4, on the basis of their responses to various agonists and antagonists (Coleman *et al.* 1994). Further adding to PG receptor diversity is the existence of splice variants for several receptor subtypes. The precise roles these receptors play in physiologic and pathologic settings are unclear and complicated by multiple factors, including cellular context, ligand affinity and differential coupling to downstream signal transduction pathways. Furthermore, these receptors have been shown to be expressed in a cell- and tissue-specific manner with variable expression levels between different tissues **(Table 7)** (reviewed in Sugimoto *et al.* 2000).

V.2) Post-transcriptional Regulation of COX-2 Expression by the p38 MAPK Pathway

Unlike COX-1, which represents a housekeeping gene displaying constitutive expression in selected cells and tissues (i.e., endothelium, monocytes, platelets, renal collecting tubules and seminal vesicles), the COX-2 gene is highly inducible by various factors. As shown in **Figure 14**, the COX-2 promoter contains a TATA box and response elements for several transcription factors commonly associated to inflammatory

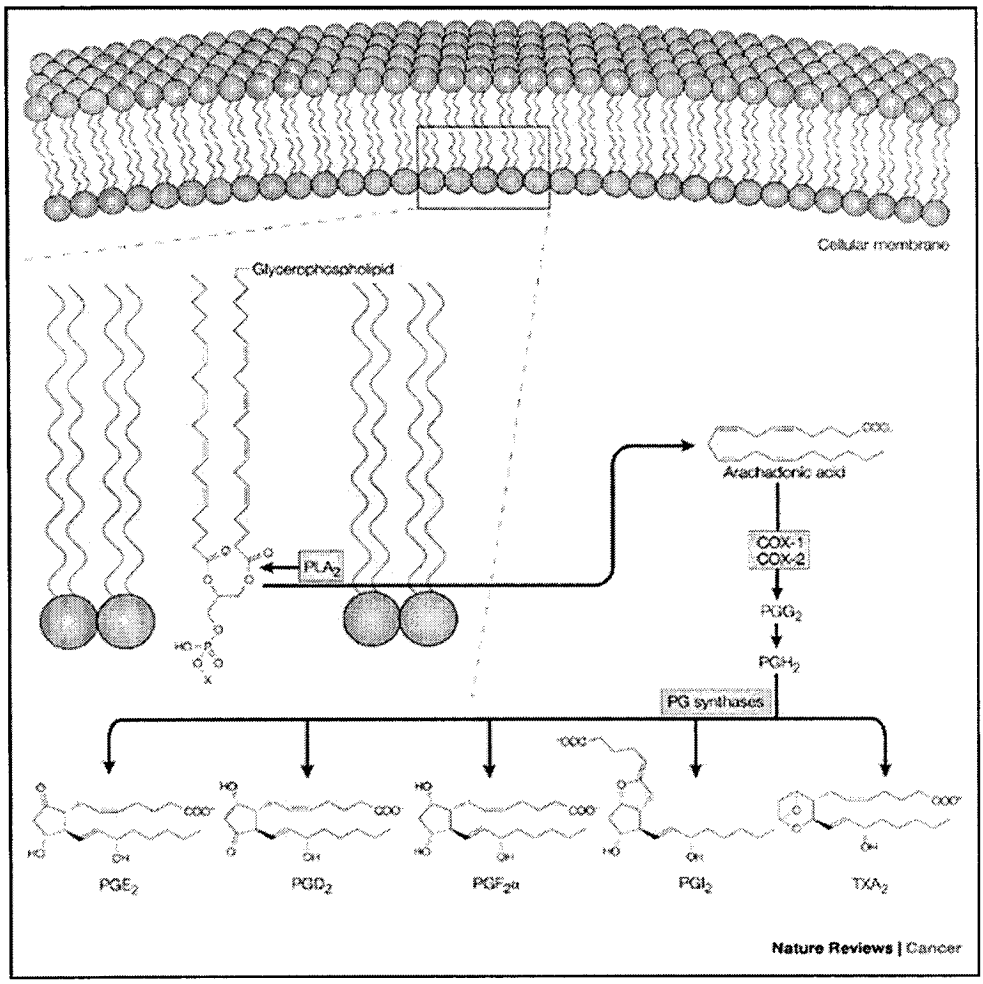


Figure 13: Summary of the prostanoid biosynthetic pathway. PLA_2-catalyzed hydrolysis of the central acyl group from a membrane phospholipid leads to the release of arachidonic acid. Liberated arachidonic acid is then acted on by COX enzymes (COX-1 and -2), which first oxidize arachidonic acid into PGG_2 and then reduce PGG_2 into PGH_2. PGH_2 is finally converted into a variety of prostanoids via cell-specific PG synthases. (Adapted from Gupta and Dubois 2001)

Receptor type		Kd, nM (radio ligand)	Rank order of binding affinity	Signaling	Gene locus mouse / human	Alternatively spliced isoforms
EP	EP1	21 [^{3}H]PGE_2	PGE_2 > iloprost > PGE_1	$[Ca^{2+}]_i\uparrow$	chr 8 / 19p13.1	2 (rat)
	EP2	27 [^{3}H]PGE_2	PGE_2 = PGE_1 > butaprost	cAMP↑	–/ 14q22	None
	EP3	3 [^{3}H]PGE_2	PGE_2 = PGE_1 > iloprost	cAMP↓ $[Ca^{2+}]_i\uparrow$	chr 3 / 1p31.2	3 (mouse) 7 (human) 4 (bovine)
	EP4	11 [^{3}H]PGE_2	PGE_2 = PGE_1	cAMP↑	chr 15 / 5p13.1	None
DP		40 [^{3}H]PGD_2	PGD_2 > BW245C	cAMP↑	chr 14 / –	None
FP		1.3 [^{3}H]$PGF_{2\alpha}$	$PGF2\alpha$ > PGD_2	$[Ca^{2+}]_i\uparrow$	chr 3 / 1p31.1	2 (ovine)
IP		4.5 [^{3}H]iloprost	cicaprost > iloprost > PGE_1	cAMP↑ $[Ca^{2+}]_i\uparrow$	chr 7 / 19q13.3	None
TP		3.3 [^{3}H]S-145	S-145 > STA_2 > U46619	$[Ca^{2+}]_i\uparrow$ cAMP↓	chr 10 / 19p13.3	2 (human)

Table 6: Properties of mouse prostaglandin receptors.
(Sugimoto *et al.* 2000)

Table 7: Tissue distribution of mouse prostaglandin receptors. (Sugimoto *et al.* 2000)

Tissue/Cell	EP1	EP2	EP3	EP4	DP	FP	IP	TP
CNS	Neuron[b]	Neuron Leptomeninges[b][34]	Neuron [40]	Neuron[b] [34]	Leptomeninges[41]	–	–	–
PNS	Neuron (DRG)[31]	–	Neuron (DRG & enteric ganglion) [40]	Neuron (DRG) [31]	–	–	Neuron (DRG) [31]	–
Lung	–	–	+	+	Epithelial cell[f][69]	+	SM (Pulmonary artery)[31]	+
Heart	–	–	+	+	–	+	SM (Aorta & Coronary Artery) [31]	+
Thymus	–	–	+	Thymocyte[b]	–	–	Thymocyte (Medulla) [31]	Thymocyte (Cortex) [45]
Spleen	–	–	+	+	–	–	Lymphocyte & Megakaryocyte [31]	Megakaryocyte [46]
Gastrointestinal tract	SM (Muscularis mucosa) [32]	–	Epithelial cell (Deep layer in stomach SM (Longitudinal layer) [32]	Epithelial cell (Superficial layer) [32]	+(intestine)	Epithelial cell (stomach) [42]	+	–
Kidney	Epithelial cell (Collecting duct) [33]	–	Epithelial cell (Distal tubule) [33]	(Glomerulus) [33]	–	Epithelial cell (Convoluted tubule) [42]	SM (Glomerular arteriole) [31]	(Glomerulus) [47]
Ovary	–	Theca cell[c] & Cumulus cell[d][35]	–	+	–	Luteal cell (Corpus luteum) [43]	–	–
Uterus	SM[g,h]	Luminal epithelial cell[e,g][36,37]	SM (Longitudinal[f,g] & circular layer[e,g]) [36]	Epithelial cell & Stromal cell[e][36]	–	SM[g][44]	–	+
Macrophage	–	+[b][38,39]	–	+ [38,39]	–	–	+ [38]	–

[a] +, significant expression detected by Northern blot; –, below detection levels; CNS, central nervous system; DRG, dorsal root ganglion; PNS, peripheral nervous system; SM, smooth muscle cells.

[b] Expression increased by lipopolysaccharide treatment.

[c] Ovary isolated from mice treated with pregnant mare serum gonadotropin (PMSG).

[d] Ovary isolated from mice treated with PMSG/human chorionic gonadotropin.

[e] Uterus on day 5 of pseudopregnancy.

[f] Uterus on day 0 of pseudopregnancy. The day a vaginal plug was observed was taken as day 0.5.

[g] Uterus at the expected term.

[h] [Sugimoto et al. unpublished data].

[i] Expression is increased by the stimulation of antigen challenge in an asthma model.

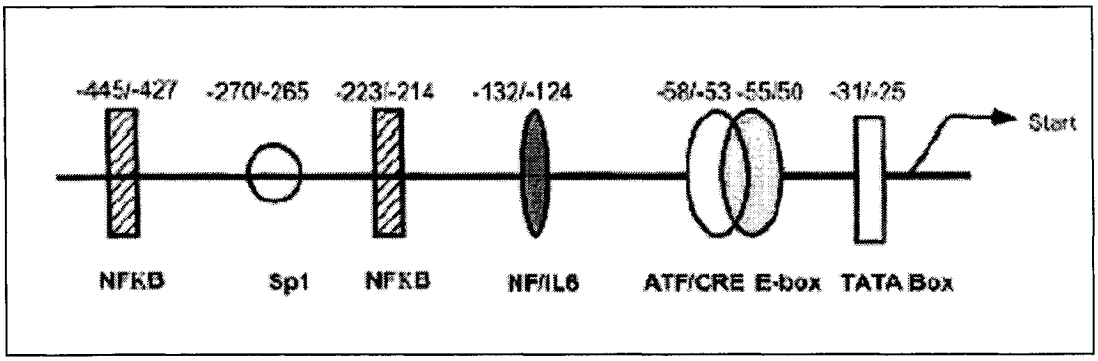


Figure 14: *Cis*-acting elements in the human COX-2 promoter. Numbers above each element indicate its position relative to the transcription start site.
(Smith *et al.* 2000)

processes (i.e., nuclear factor-κB (NF-κB), activating transcription factor-2 (ATF-2), nuclear factor IL-6 (NF-IL-6) and cyclic AMP response element binding protein (CREB)) (Appleby *et al.* 1994). Consequently, the COX-2 gene is subject to strong transcriptional regulation via activation of inflammatory signaling cascades, including the NF-κB and C/EBP signaling cascades (Ghosh *et al.* 1998; Poli 1998), as well as the three MAPK cascades (i.e., ERK1/2, JNK/SAPK and p38) (Su and Karin 1996). Each of these pathways has been shown to contribute to COX-2 transcription concertedly or independently in cell- and context-specific fashion by targeting one or more of the above-listed transcription factors **(Figure 15)**.

Despite the importance of transcriptional control of COX-2 gene expression, post-transcriptional regulation is a major element in the regulation of this gene. The full length COX-2 mRNA contains an unprecedented 22 AREs spread throughout its 3 UTR **(Figure 16)** and is extremely unstable in quiescent cells. It is classified as a class II ARE and in addition to being a transcriptional target of the p38 MAPK, it has been shown to be stabilized by p38 MAPK-dependent mechanisms in a variety of cell types and contexts (Alcaide and Fresno 2004; Mifflin *et al.* 2004). In a study in human A549 alveolar type II cells, Newton *et al.* (1997) observed a stabilization of endogenous full-length COX-2 transcripts (as opposed to shorter polyadenylation variants with truncated 3 UTR) following stimulation with IL-1β (a potent activator of the p38 MAPK). Ridley *et al.* (1998) later demonstrated by an actinomycin D chase experiment that in HeLa cells, the reduction in IL-1β-induced COX-2 upon p38 MAPK blockade was due to destabilization of COX-2 mRNA; no effect on COX-2 transcription was observed. These results indicated that in these cells, p38 MAPK controlled the expression of COX-2 predominantly through mRNA stabilization. The p38 MAPK cascade was later shown to stabilize COX-2 mRNA in LPS-treated human monocytes (Dean *et al.* 1999). In an important reporter study performed in COS-7 cells, Dixon *et al.* (2000) identified a conserved 116-nucleotide AU-rich sequence that acted as a potent mRNA-destabilizing and translational blocking element and mediated mRNA stabilization and translation following p38 MAPK pathway activation. This proximal sequence contains 6 closely-spaced AUUUA motifs, 3 of which overlap **(Figure 16)**. In subsequent studies using the same reporter constructs in HSFs, Faour *et al.* (2001, 2003) demonstrated that PGE_2 and

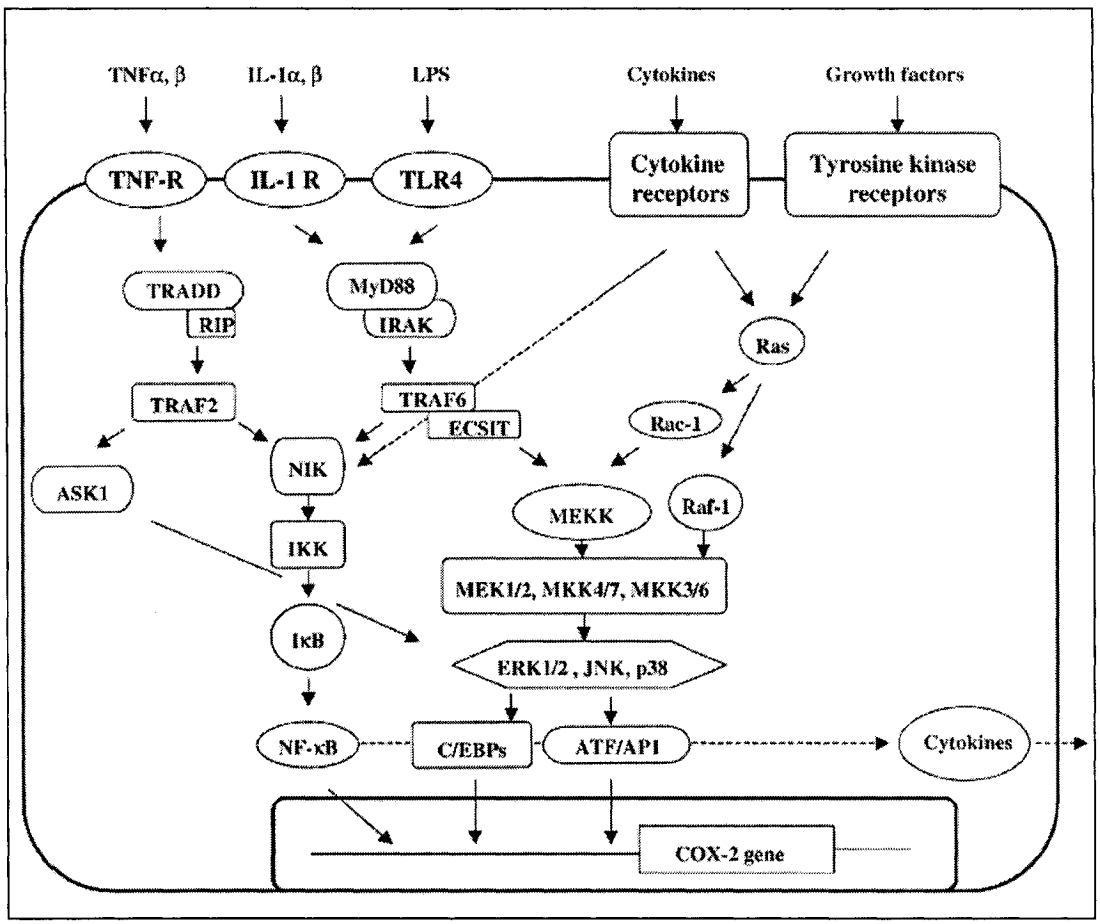


Figure 15: Signaling pathways involved in transcriptional activation of the COX-2 gene.
(Tanabe and Tohnai 2002)

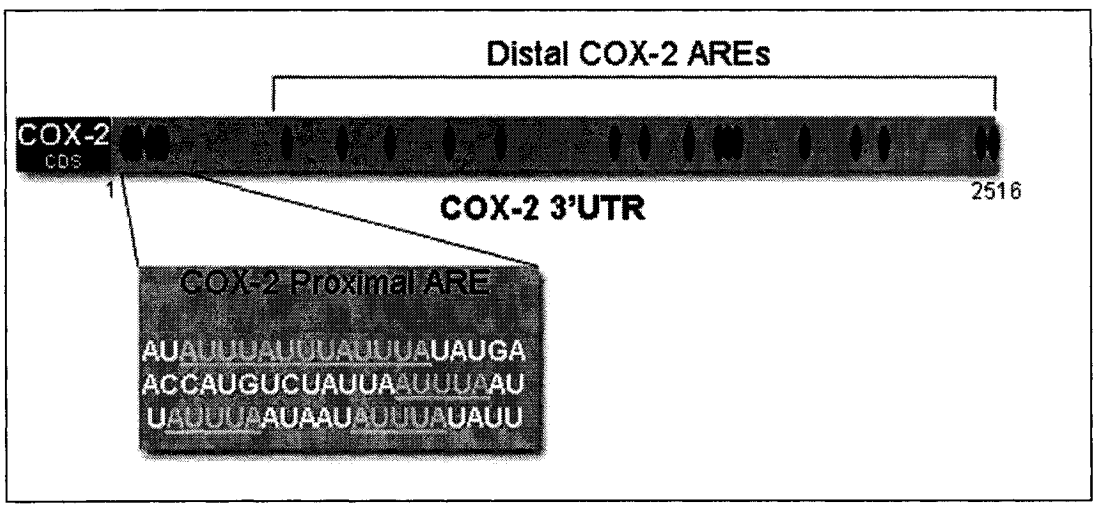


Figure 16: COX-2 3 UTR showing the location of the proximal and distal AREs.
CDS: coding sequence.

IL-17 enhanced COX-2 reporter mRNA stability and translation via distal AREs by a p38 MAPK-dependent mechanism. The former of the two studies highlighted an extremely interesting and biologically relevant signaling paradigm in which the main end-product of the cyclooxygenase pathway (i.e., PGE_2) sustains its own synthesis by a cyclic, self-perpetuating positive feedback loop (i.e., through stabilizing and enhancing translation of COX-2 mRNA).

A number of AUBPs have now been shown to bind to the COX-2 3 UTR. These factors include AUF1 and AUF2 (Lasa *et al.* 2000; Dean *et al.* 2002), CUGBP2 (Mukhopadhyay *et al.* 2003), HuR (Dixon *et al.* 2001; Sengupta *et al.* 2003), T cell-restricted intracellular antigen-1 (TIA-1) and TIA-related protein (TIAR) (Dixon *et al.* 2003; Cok *et al.* 2003, 2004) and TTP (Sully *et al.* 2004). Almost all of the above-cited studies focused on the proximal COX-2 ARE as this element is frequently reported to contain the main control elements responsible for post-transcriptional and translational regulation of COX-2 expression. However, the pertinence of these COX-2 ARE-AUBP interactions to the p38 MAPK-mediated stability of COX-2 mRNA *in vivo* is still unclear. CUGBP2 silenced endogenous COX-2 mRNA translation and stabilized a COX-2-proximal ARE-luciferase reporter mRNA when transfected into a human colon cancer cell line (i.e., HT-29). Yet, no links between these events and the p38 MAPK pathway were reported (Mukhopadhyay *et al.* 2003). Sully *et al.* (2004) showed that overexpression of HuR in Tet-off HeLa cells resulted in the stabilization of a β-globin-COX-2-proximal ARE reporter mRNA. On the other hand, HuR knockdown by RNAi in the same cells had no effect on reporter stability either in the presence or absence of p38 MAPK activation. Transient overexpression of the p42AUF2 isoform stabilized a COX-2 ARE reporter mRNA in HeLa Tet-off cells (Dean *et al.* 2002); however, a mutated COX-2 ARE that had lost destabilizing function and failed to respond to p38 activation still bound AUF1 and AUF2 (Sully *et al.* 2004). In TTP knockout mice, LPS-induced COX-2 protein levels are much higher than in wild-type mice. Yet, the contribution of TTP to mRNA stabilization and its link to the p38 MAPK in this mechanism are not clearly defined.

In brief, p38 MAPK-mediated factors and mechanisms involved in the stabilization of COX-2 mRNA are still elusive. Reasons for the failure to identify these

factors may be related to the inability of the short COX-2 ARE mRNA probes/reporters to form secondary structures required for RNA-AUBP interactions. Furthermore, the lack of the distal AREs, which may be essential to the formation of secondary structures and/or contain sites needed for AUBP (or accessory protein) association, may also be a reason. Whatever the case may be, further research is needed to resolve this issue.

PART E:
M.Sc. Research Project Rationale and Objectives

The rationale for this research project stems from our recent discovery of a molecular paradigm for cytokine signaling in which specific membrane phospholipid-derived metabolites (i.e., prostaglandins), which are released immediately following cytokine cellular activation, modulate cytokine-target gene expression (Faour *et al.* 2001). Notably, our group showed that prostaglandin E_2 (PGE_2) upregulated the level and stability of COX-2 mRNA (a class II ARE-mRNA with 22 AREs) and enhanced COX-2 mRNA translation through a positive feed-back loop mediated by the EP4 receptor and the p38 MAP kinase cascade in IL-1β-treated human synovial fibroblasts (HSFs). The PGE_2-dependent effect appeared to be mediated by distal AREs in the 3 UTR region of COX-2 mRNA as judged by deletion mutant analysis in transiently transfected HSFs. The data support the notion that COX-2 and eicosanoid production are sustained through a self-perpetuating autocrine/paracrine cycle. In a pathological state, such as osteoarthritis (OA) and rheumatoid arthritis (RA), the latter mechanism would serve as an adaptive homeostatic reaction to an inflammatory stimulus(i) whose origin and mode of action are still unknown in terms of COX-2 upregulation. It provides, nevertheless, a plausible explanation for why OA and RA synovial membranes produce abundant quantities of PGE_2 but almost no macrophage-derived cytokines (e.g., IL-1β, tumor necrosis factor alpha (TNF-α)), no T-cell derived cytokines (e.g., IL-17) and no matrix metalloproteinases (MMPs) (He *et al.* 2002). When the cycle is interrupted, such as with non-steroidal anti-inflammatory drug (NSAID) pharmacotherapy, the levels of inflammatory cytokines and MMPs increase dramatically concomitant with the inhibition of COX-2 synthesis, activity and PGE_2 release (He *et al.* 2002). The net clinical effect is the morbidity and matrix erosion observed with long-term users of NSAIDs (Fries 1991).

The central objective of the current study was to test the hypothesis that the physiological role of PGE_2 is as a feed-forward/feed-back signaling intermediate that controls the expression of many cytokine or stress effector target genes (e.g., COX-2, MMPs, IL-6, TNF-α, IL-1β) primarily, but not exclusively, by post-transcriptional mechanisms. More precisely, we sought to use the previously described PGE_2/COX-2 paradigm as a model system to characterize the post-transcriptional and/or translational mechanisms and putative *trans*-acting factors implicated in PGE_2-mediated mRNA stabilization and/or translation in human synovial fibroblasts (i.e., membranes). Our

experimental approach was designed to obtain a global perspective on the various facets to these post-transcriptional/translational regulatory mechanisms.

PART F:
M.Sc. Research Project
Experimental Study

I) MATERIALS AND METHODS

Chemicals: Sodium fluoride (NaF), leupeptin, aprotinin, pepstatin, phenylmethylsulphonylflouride (PMSF), actinomycin D, puromycin, dithiothreitol (DTT), sodium orthovanadate, Ponceau S and bovine serum albumin (BSA) were products of Sigma-Aldrich Canada (Oakville, ON, Canada). Human recombinant IL-1β (rhIL-1β) was purchased from Genzyme Corp. (Cambridge, MA, USA). *N*-[2-(Cyclohexyloxy)-4-nitrophenyl)-methanesulfonamide] (NS-398), prostaglandin E_2 (PGE_2), 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB202190) and NP-40 were products of Calbiochem (La Jolla, CA, USA). Sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED) and Bio-Rad protein reagent originated from Bio-Rad Laboratories (Richmond, CA, USA). Tris-base, EDTA, $MgCl_2$, $CaCl_2$, chloroform, dimethylsulphoxide (DMSO), anhydrous ethanol (95%), methanol (99%), trichloroacetic acid (TCA), sulfosalicylic acid, acetic acid, formaldehyde and formamide were obtained from Fisher Scientific (Nepean, ON, Canada). Dulbecco's Modified Eagle Medium (DMEM), phosphate-free and phenol-red free DMEM, Trizol reagent, heat inactivated fetal bovine serum (FBS) and an antibiotic mixture [10,000 units of penicillin (base), 10,000 μg of streptomycin (base)] were products of Invitrogen Canada Inc. (Burlington, ON, Canada).

Tissue sources/specimen selection/cell culture: A majority of the experiments were conducted in human synovial lining cells (synovial fibroblasts, HSF) since these cells are potent producers of PGE_2 following IL-1β-induced COX-2 synthesis. Consequently, these cells provide an ideal system to study PGE_2-regulated post-transcriptional factors and mechanisms involved in gene expression. Furthermore, the existence of the PGE_2/COX-2 autoregulatory feedback loop (Faour *et al.* 2001) serves as an extremely sensitive readout for the accomplishment of this study's objective. In addition to the aforementioned technical and experimental advantages, the decision to use these cells was also based on their central role in RA pathogenesis following their activation and adoption of an aggressive, transformed phenotype. Hence, understanding the molecular mechanisms behind HSF gene expression in RA is essential to help

rationalize the use of some currently used therapeutic modalities and to offer hope for more evidence-based anti-cytokine and anti-inflammatory drug development

HSFs were derived at necrospy from the synovial membranes (synovia) of donors with no history of arthritic disease (mean age 30±27 years). Details of HSF isolation (by sequential enzymatic digestion) and enrichment have been previously described (Di Battista, Zhang *et al.* 1999). A large bank of these cells has been established in our laboratory and cell lines have been screened for optimal transfection efficiency (Di Battista, Zhang *et al.* 1999; Faour *et al.* 2001). HSFs were seeded in tissue culture dishes and cultured until confluence in DMEM supplemented with 10% FBS and antibiotics at 37°C in a humidified atmosphere of 5% CO_2, 95% air. The cells were incubated in fresh medium containing 0.5-1% FBS for a minimum of 3 hours before the experiments. Where specified, HeLa cells (from ATCC, Rockville, MD, USA) were used and cultured like synoviocytes. Seven to 11 passage cells were used for all applications.

Preparation of cellular protein extracts: Cell extracts were prepared using different techniques as required for each experiment. Separate nuclear and cytosolic fractions from adherent control and treated cells grown to confluence in 15 cm culture dishes were obtained by pelleting washed (in PBS) cells and resuspending in 100 µl of hypotonic buffer (Buffer A: 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1% NP-40, 0.5 mM DTT, 1 mM each of PMSF, Na_3VO_4, NaF and βGPO_4 and a cocktail of protease inhibitors (COMPLETE^{TM}, Roche Diagnostics, Laval, QC, Canada)). Cell lysis was achieved by vortexing intermittently on ice for 30 minutes. Lysates were then centrifuged at 4°C for 10 minutes at 3 000 RPM; supernatants were considered cytosolic extracts. The remaining pellet (containing intact nuclei) was washed in PBS and resuspended in 40 µl of high salt buffer (Buffer C: 20 mM HEPES pH 7.9, 25% v/v glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 mM each of Na_3VO_4, NaF and βGPO_4 and protease inhibitor cocktail). Nuclei were lysed by vortexing intermittently on ice for 1 hour. Following centrifugation at 4°C for 30 minutes at 14 000 RPM, the remaining supernatant was isolated and considered as the nuclear extract.

Extracts for immunoprecipitation were prepared by resuspending washed intact-cell pellets in cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 µg/ml each of aprotinin, leupeptin and pepstatin, 1% NP-40, 1 mM sodium orthovanadate and 1 mM NaF). Cells were kept on ice for 30 minutes with occasional vortexing and then subjected to one freeze-thaw cycle. Following a 4°C centrifugation at 14 000 RPM for 10 minutes, the supernatant containing the soluble proteins was collected. All protein extracts were quantified by BCA assay (Pierce Biotechnology, Inc., Rockford, IL, USA) using a BSA (diluted in sample buffer) standard curve and then stored at -80°C. For denatured total cell extracts, cells were lysed in hot SDS-PAGE loading buffer.

mRNA quantitation: Total cellular RNA was isolated from control and treated cells (1 x 10^6 cells = 15-20 µg RNA) using the Trizol (Invitrogen Canada Inc.) reagent and Northern blot was performed as previously described (Di Battista, Martel-Pelletier *et al.* 1999; Di Battista, Zhang *et al.* 1999; Faour *et al.* 2001). The following probes, labeled with digoxigenin (DIG)-dUTP (Roche Diagnostics Canada) by random priming were used for hybridization: The TTP probe was generated by RT-PCR amplification of a 513 bp fragment (sense primer: 5 -GAT CTG ACT GCC ATC TAC GAG AGC C-3 and antisense primer: 5 -CAG GTC TTC GCT AGG GTT GTG G-3) from human genomic DNA and cloned into an EcoR1 site of the pCRII-TA TOPO vector (Invitrogen Canada Inc.). A 780 bp PstI/XbaI fragment from GAPDH cDNA (1.2 kb; American Type Culture Collection, Rockville, MD, USA) was subcloned into pGEM-3Z vector (Promega Biosciences, Inc., Madison WI, USA) and a cRNA probe was synthesized after linearization with PstI. This latter probe served as a control of RNA loading as GAPDH is constitutively expressed. All blots were subjected to autoradiography and densitometric analysis for semi-quantitative measurements (1Dscan EX, Scanalytics, Inc., Fairfax, VA, USA).

Western immunoblotting: Thirty-50 µg of cellular protein extracts were subjected to SDS-PAGE through 10% or 12% gels (final concentration of acrylamide) under reducing conditions, transferred onto nitrocellulose membranes (Amersham

Pharmacia Biotech Inc, Baie d'Urfé, QC, Canada) and stained with Ponceau-S Red (2% Ponceau S (w/v) in 30% TCA, 30% sulfosalicylic acid diluted 1:10 in 1% (v/v) acetic acid) in order to verify transfer efficiency and ensure equal loading of samples. Following destaining in ddH_2O, membranes were immunoblotted as previously described (Di Battista, Zhang *et al.* 1999). The following antibodies and blocking peptide were used: goat polyclonal anti-human TTP H-120 and N-18, mouse monoclonal anti-human HuR 3A2 and TTP blocking peptide (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:1 000 dilution for both); rabbit polyclonal anti-AUF1 (Upstate Inc., Waltham, MA, USA; 1:1 000 dilution); rabbit polyclonal anti-phosposerine (Zymed Laboratories, Inc., San Francisco, CA, USA; 1:125 dilution).

TTP antibody specificity was assayed by preabsorbing the antibody with TTP blocking peptide (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (1:10 and 1:50 m:m) overnight at 4°C. Western blotting was then performed as previously described using both unabsorbed and preabsorbed antibody.

Probe preparation, plasmid construction and *in vitro* RNA transcription: The COX-2 3 UTR-luciferase chimeric plasmid was a kind gift of Dr. Steven Prescott (Dixon *et al.* 2000) and was used in our previous publication (Faour *et al.* 2001). Briefly, the luciferase reporter expression construct was prepared in the pcDNA3.1/Zeo(+) containing the luciferase cDNA from pGL3-basic (Promega Biosciences, Inc.) cloned into the HindIII and XbaI sites to yield pLucΔ3 UTR (Dixon *et al.* 2000). Addition of the human COX-2 3 UTR was accomplished by PCR amplification of the COX-2 3 UTR using XbaI–tailed primers and inserting it adjacent to the luciferase coding region to yield pLuc+3 UTR (designated pCOX 3 UTR-Luc). The plasmid was analyzed by sequencing and restriction mapping and purified by endo-free plasmid maxi kit column purification (Qiagen Inc., Canada) (Dixon *et al.* 2000; Faour *et al.* 2001). The TTP expression construct was kindly donated by Dr. Seth A. Brooks and constructed as previously described (Brooks *et al.* 2002).

For *in vitro* transcription, the first 129 nucleotides of the COX-2 3 UTR sequence in pCOX 3 UTR-Luc were deleted by PCR (sense primer: 5 -TGC GGA GAA AGG AGT CAT ACT TGT GA-3 ; antisense primer: 5 -ATA CAC ATT TGT CTG AGG

CAC TG-3). This PCR-amplified fragment, which contained only the distal COX-2 AREs, was subcloned into pCRII-TOPO (Invitrogen Canada Inc.) and used as a template for cRNA probe synthesis. Transcription was performed as per the Promega Biosciences, Inc. Riboprobe® kit protocol with the following modifications: 0.5 mM (final concentration) rATP, rGTP and rUTP and 100 μM (final concentration) rCTP in 20 μL reaction volume. cRNA was purified by overnight NH_4OAc/ethanol precipitation and counted by scintillation spectrophotometry. Conditions were established to obtain about 15 000 cpm/fmol. The labeled cRNA (COX-2_dist3 UTR) was used for UV cross-linking studies. Radioinert probe (used for competition reactions) was synthesized by omitting ^{32}P-rCTP and using 0.5 mM rCTP (final concentration).

Cell transfections and reporter assays: Transient transfection experiments were conducted in HSFs by previously established methods that our routine in our laboratory and described in detail (Di Battista, Martel-Pelletier *et al.* 1999; Di Battista, Zhang *et al.* 1999; Faour *et al.* 2001). Briefly, adherent cells were transfected with FuGENE 6 transfection reagent (Roche Diagnostics Canada) exactly as per the manufacturer's suggestions. Transfection efficiencies were controlled by co-transfection with 0.5 μg of pCMV-β-gal, a β-galactosidase reporter vector under the control of CMV promoter (Invitrogen Canada Inc.). Total amount of plasmid did not exceed 1.5 μg. β-gal activity was measured spectrophotometrically using a colorimetric assay and luciferase activity was assessed with the aid of a luminometer (Lumat LB 9507, EG&G Berthold, Germany). All luciferase enzyme activities (expressed in reactive light units, RLUs) were normalized to β-gal activity and cell protein (determined by BCA assay).

Isolation of RNA binding proteins and analysis of protein-RNA interactions- RNA electrophoretic mobility shift- (REMSA)/UV crosslinking assay and subtractive affinity chromatography with poly-uridylate (poly(U)) RNA: For REMSA/UV cross-linking assays, RNA binding proteins were isolated separately from cytoplasmic and nuclear extracts essentially as described above under "Preparation of Cellular protein extracts". Thirty μg of either nuclear or cytosolic protein extract was incubated with 150 000 cpm of ^{32}P-labeled heat-denatured COX-2_dist3 UTR probe.

After 20 minutes incubation at room temperature, 100 units of RNaseT1 was added to each reaction and incubated for 15 minutes at room temperature. Subsequently, samples were UV cross-linked on ice (254 nm, 300 mJ/cm^2, Stratalinker 2400; Stratagene, La Jolla CA, USA) for 20 minutes and then incubated with 2 µg RNaseA (Invitrogen Canada Inc.) for 30 minutes at 37°C to remove non-interacting RNA. Finally, samples were boiled in 3XSDS sample buffer and resolved by SDS-PAGE (10% gel). RNA-bound proteins were visualized by autoradiography. For competition reactions, 20 µg of both nuclear and cytosolic protein extracts were pre-incubated for 10 minutes with 100-fold molar excess of radioinert heat-denatured COX-2_dist3 UTR probe prior to the addition of the radioactive probe. The subsequent steps were conducted as described above.

For use in subtractive poly(U) affinity chromatography experiments, total protein extracts were obtained by resuspending PBS-washed cell pellets in 200 µl Buffer C (50 mM Tris-HCl, pH 7.4, 150 mM KCl, 0.1 mM EDTA, 1% NP-40, 4 mM DTT, 20 mM NaF, 40 mM sodium β-glycerophosphate and 2 mM Na_3VO_4) on ice for 30 minutes with intermittent vortexing. The cell lysate was cleared at 4°C for 10 minutes at 13 000 RPM and the supernatant quantified by BCA protein assay. For each reaction, 100 µg of protein was diluted in 40 µl binding buffer (25 mM Tris-HCl, pH 7.4, 150 mM KCl, 0.05 mM EDTA, 0.5% NP-40, 2 mM DTT, 10 mM NaF, 20 mM sodium β-glycerophosphate, 1 mM Na_3VO_4, 5 mM $MgCl_2$, 5% glycerol and 1 mg/ml heparin) with either 7.5 nmol of heat-denatured unlabeled COX-2 dist3 UTR probe (for competition reactions) or an equal volume of RNAse-free H_2O. Following a 15-minute pre-incubation at 4°C with agitation, each reaction was supplemented with 75 pmol of a 50% Poly(U) Sepharose™ 4B (Amersham Pharmacia Biotech Inc.) bead slurry (prepared in Buffer C) and shaken for 2 hours at 4°C. Protein-associated poly(U) beads were spun down and washed twice in 1 ml wash buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$ and 5% glycerol) with brief centrifugation and elimination of the supernatant after each wash. At the end of the second wash, the poly(U) bead-protein complexes were boiled in 1XSDS sample buffer and resolved by SDS-PAGE on 10% gels. Isolated proteins were visualized by silver staining of gels using the ProteoSilver™ Plus Silver Stain Kit (Sigma-Aldrich Canada).

Immunoprecipitation: A 50 % slurry of Protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Inc.) was prepared in PBS as per the manufacturer's instructions. Five hundred µg of protein extract in 300-500 µl of RIPA buffer was pre-cleared by mixing with 50 µl of Protein G Sepharose (50% slurry) and 1 µg of normal IgG (from same host species as the antibody being used for immunoprecipitation) for 1 hour at 4°C. Subsequent to centrifugation at 12 000 x *g* for 10 minutes at 4°C, the pre-cleared supernatant was collected and combined with 2 µg of the required immunoprcipitating antibody. This mixture was shaken for 2 hours at 4°C, afterwhich time 20 µl of Protein G Sepharose (50% slurry) was added and mixed overnight at 4°C. The following day, the mixture was centrifuged at 2 500 x *g* for 3 minutes at 4°C; the supernatant was removed and frozen at -80°C and the pellet was washed 3 times in RIPA buffer with centrifugation and discarding of supernatant between each wash. The antigen was eluted by heating the washed pellet at 95°C for 10 minutes in 30 µl 2XSDS sample buffer. Samples were then spun down and the supernatants were analyzed directly by SDS-PAGE.

Real-time TTP-eGFP nuclear / cytoplasmic shuttling studies by confocal microscopy: HeLa cells were seeded at 50 000 cells / chamber on 4-chamber Lab-Tek coverglasses (VWR International, Ville Mont-Royal, QC, Canada) in 900 µl medium (as per the conditions described previously) and cultured overnight. The following day, cells were transiently transfected with FuGENE 6 transfection reagent (using manufacturer's protocol) with 50 ng of TTP-eGFP fusion plasmid and 150 ng of pcDNA3.1 plasmid as carrier DNA. The TTP-eGFP fusion construct was a kind gift from Dr. T. K. Blackwell and its construction is described elsewhere (Johnson, Stehn *et al.* 2002). After 6 hours, the transfection medium was replaced with normal culture medium; cells were allowed to recover and express the transfected protein overnight. The next day, cells were serum-deprived for 3 hours in culture medium containing 1% FBS. The medium was then replaced with fresh serum-deprived medium supplemented with 50 mM HEPES in order to maintain stable medium pH during the transport of cells to the confocal microscopy facility. HeLa cells were stimulated directly on the microscope stage by adding 2 µM of PGE_2. An image of the cells was taken prior to stimulation and served as the control

image (i.e. 0 min); image capture was started immediately after stimulation for a period of 8 minutes. Cell viability and morphology was verified microscopically prior to addition of PGE_2. Stimulation and image capture steps were conducted at slightly higher than ambient temperatures.

II) RESULTS

Quiescent human synovial fibroblasts express AUBPs: Given the large number of AREs present in the COX-2 3 UTR, we thought it logical to begin the study by focusing on the involvement of AUBPs in COX-2 mRNA post-transcriptional regulation. Of the various AUBPs identified to date, only HuR, AUF1 and TTP have been demonstrated to alter the stability of ARE-containing mRNAs *in vivo* (Sela-Brown *et al.* 2000; Wang, Caldwell *et al.* 2000; Brennan and Steitz 2001). Consequently, we decided to focus our work on these three factors. As a first step toward fulfilling the study's objectives, it was crucial to determine whether HSFs are capable of expressing the latter AUBPs. Individual Western blot analyses of nuclear and cytosolic cell extracts from unstimulated, serum-reduced (i.e., 1%) HSFs for HuR, AUF1 and TTP showed that these cells are potent producers of all 3 factors **(Figure 1)**. HuR was found mostly in the nucleus of HSFs, although a significant amount of protein was also present in the cytoplasm **(Figure 1*A*)**. The 4 AUF1 isoforms p37, p40, p42 and p45 were also mostly located in the nucleus (relative nuclear protein levels: p42 ≈ p45 > p37 > p40); p40-, p42- and p45AUF1 were also cytoplasmic (relative cytoplasmic protein levels: p42 >> p45 > p40) **(Figure 1*B*; Figure 3*A*, *bottom*)**. TTP showed an inverse pattern of localization relative to the other 2 AUBPs, displaying a predominantly cytoplasmic presence **(Figure 1*C*)**. Interestingly, a higher molecular weight protein specifically recognized by the anti-TTP antibody was also detected in cytosolic fractions.

Exogenous TTP and p37AUF1 influence the post-transcriptional regulation of a luciferase-COX-2 3 UTR reporter mRNA: The COX-2 3 UTR has been shown to bind various AUBPs in a range of cell lines, although the role(s) of these factors in COX-2 mRNA stability and translation, particularly in primary cells, is not yet fully understood. In order to obtain some insight into the effects of HuR, AUF1 and TTP on

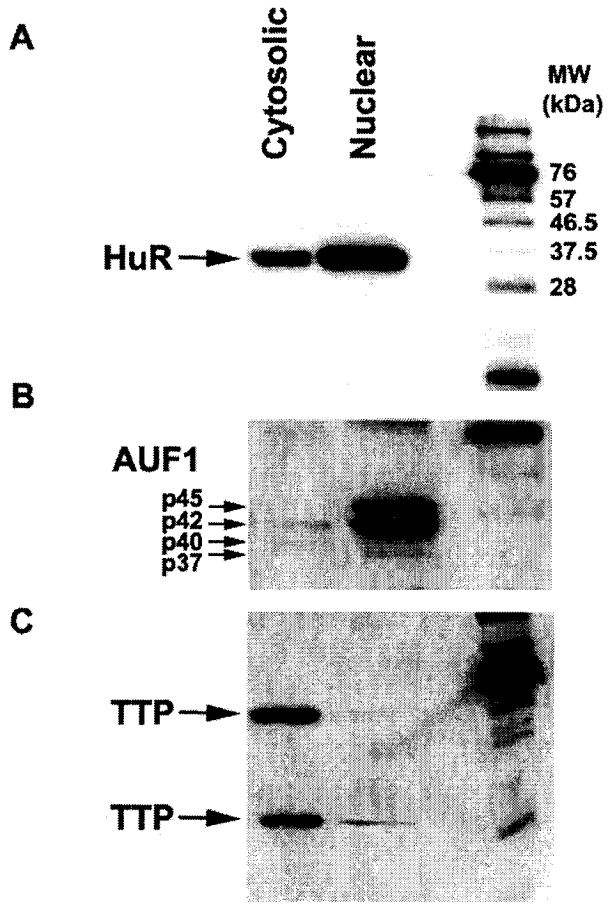
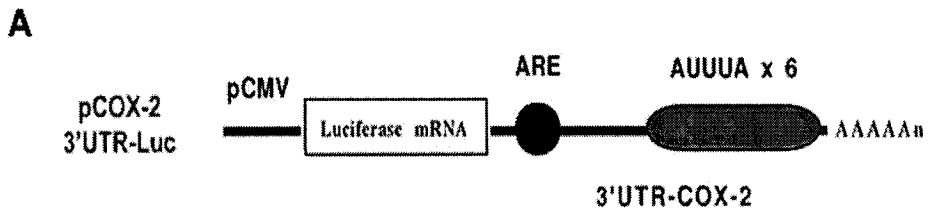
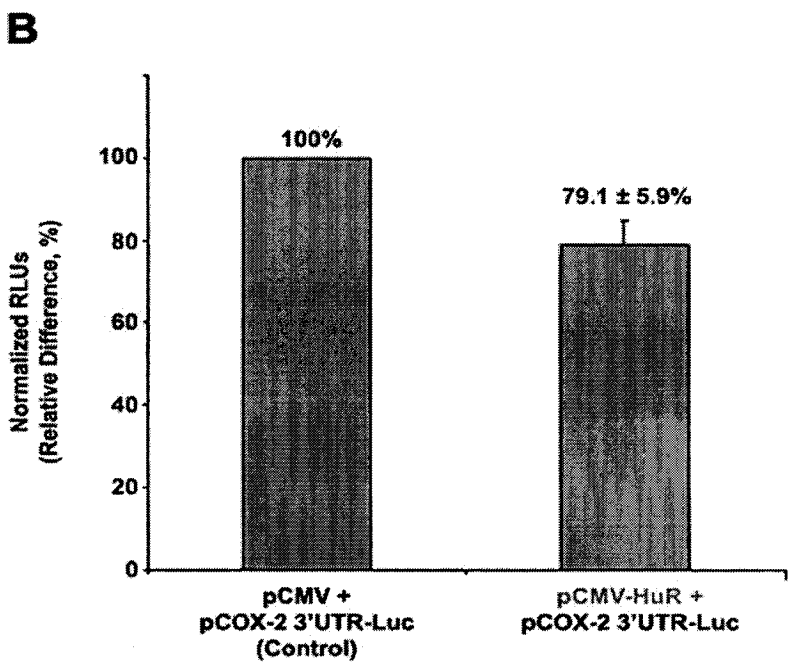


Figure 1: AUBP expression in quiescent untreated HSFs. Nuclear and cytosolic fractions were obtained from unstimulated HSFs rendered quiescent and synchronous by preincubation for 3 h in DMEM supplemented with 1% FBS plus antibiotics at 37°C. Fifty μg of protein from each fraction was analyzed separately for expression of the AUBPs HuR (*A*), AUF1 (*B*) and TTP(*C*) by Western blotting as described under "Material and Methods".

overall COX-2 mRNA post-transcriptional regulation (i.e., mRNA stabilization and translation) in primary HSFs, cells were transiently co-transfected with 300 ng of a β-galactosidase expression plasmid and 500 ng of the pCOX 3 UTR-Luc reporter plasmid **(Figure 2*A*)** with either 500 ng of empty pCMV vector or a vector expressing one of the AUBPs. End-point measurements of reporter activity were obtained by luminometry and results were always compared to those obtained for cells transfected with the empty pCMV expression vector. Transient expression of HuR in HSFs did not significantly affect luciferase activity **(Figure 2*B*)**. Transfections with expression plasmids for the different AUF1 isoform resulted in isoform-specific effects on reporter activity **(Figure 2*C*)**. The most significant effects were seen with p37- and p42AUF1, which increased luciferase activity by 61% and 53%, respectively, compared to empty pCMV vector-transfected HSFs. However, the results for p42AUF1 displayed greater standard deviation and such variability must be taken into account when drawing final conclusions about the effects of this isoform. The other two AUF1 isoforms, p40- and p45AUF1, did not significantly affect reporter activity. The most impressive changes in pCOX 3 UTR-Luc-derived reporter activity were observed with TTP **(Figure 2*D*)**. HSFs transfected with the TTP expression plasmid displayed a 71% decrease in luciferase activity compared to cells transfected with the empty pCMV plasmid.

PGE_2 modulates the expression of p37AUF1 and TTP in human synovial fibroblasts: In light of both the central aim of this study and the results from the AUBP transfection assay, we wished to know if the expression and/or localization of endogenous AUF1 and TTP were regulated by PGE_2. rhIL-1β-treatment of HSFs at the EC_{50} for COX-2 gene induction (i.e., 5.7 pM) (Faour *et al.* 2001) increased the nuclear level of p40AUF1 and cytosolic levels of both p42- and p45AUF1 **(Figure 3*A*, *bottom*)**. In order to determine whether the inductive effect of rhIL-1β was dependent on PGE_2 synthesis and activity, cells were co-incubated with 100 nM of the selective COX-2 inhibitor NS-398 and 5.7 pM rhIL-1β. We discovered that rhIL-1β's effects on p40- and p42AUF1 were PGE_2-independent since no change was observed upon addition of NS-398 **(Figure 3*A*, *top*, *lane 5*)**. Conversely, inhibition of PGE_2 synthesis by NS-398 in rhIL-1β-treated cells resulted in a noticeable increase of nuclear p37AUF1 expression


A
pCOX-2
3'UTR-Luc
pCMV
Luciferase mRNA
ARE
AUUUA x 6
AAAAAn
3'UTR-COX-2


B
Normalized RLUs
(Relative Difference, %)
100
80
60
40
20
0
100%
79.1 ± 5.9%
pCMV +
pCOX-2 3'UTR-Luc
(Control)
pCMV-HuR +
pCOX-2 3'UTR-Luc

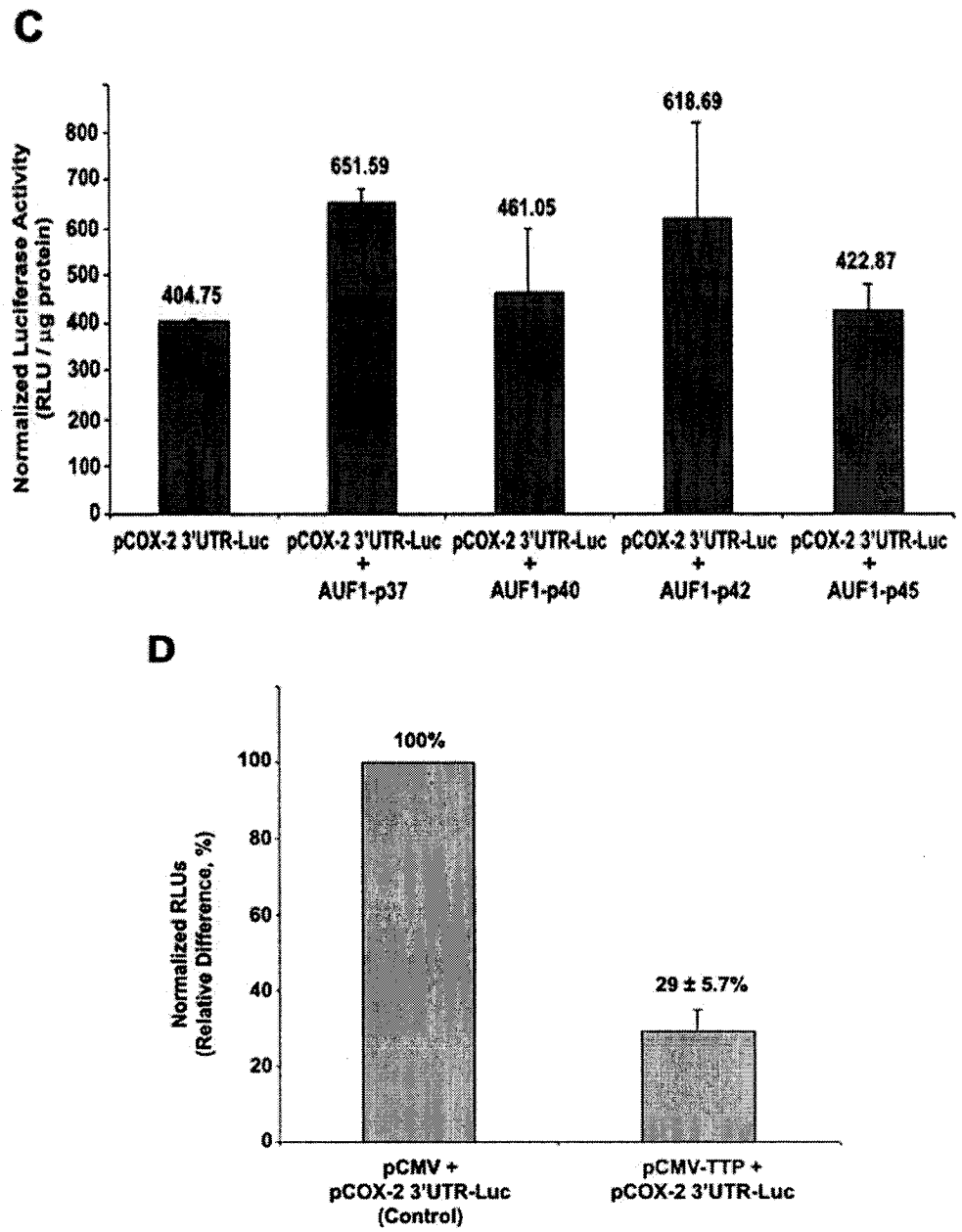
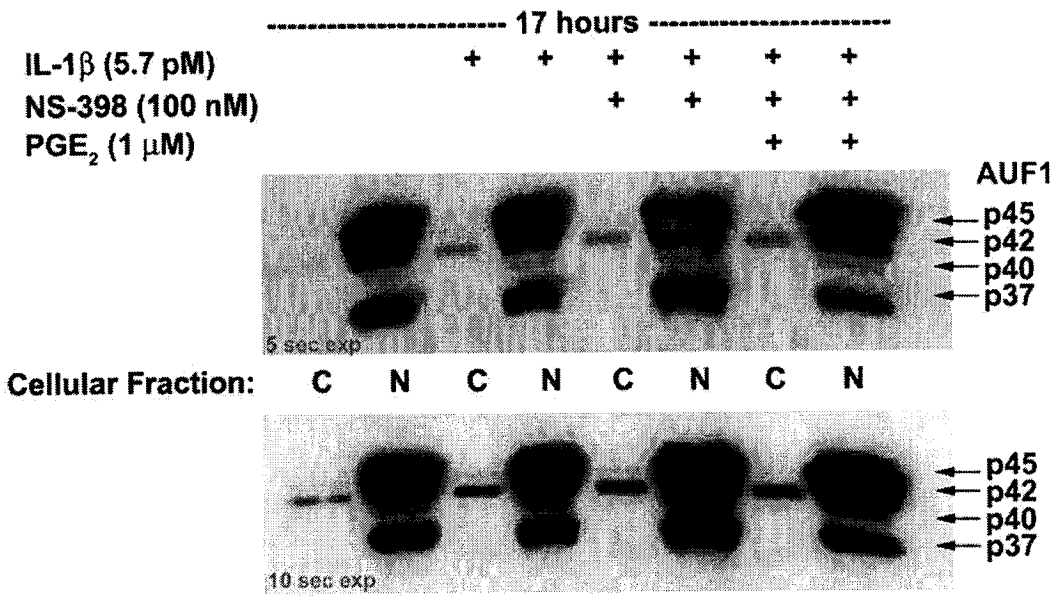
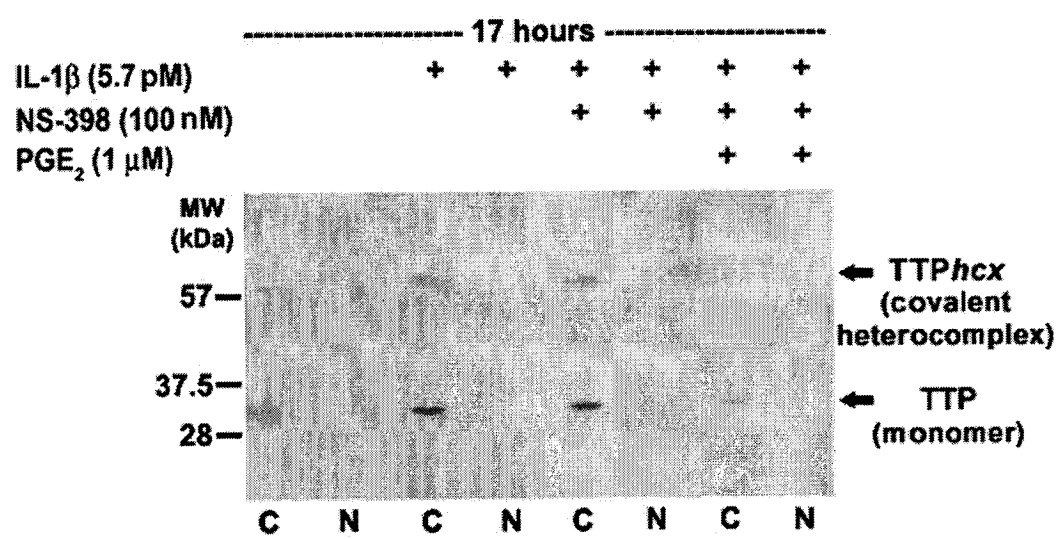


Figure 2: Effects of transiently transfected HuR (*B*), AUF1 (*C*) and TTP (*D*) on COX 3 UTR-Luc reporter activity in HSFs. HSFs were transiently co-transfected with a COX-2 3 UTR reporter plasmid (illustrated in *A*) with or without an expression plasmid for one of the AUBPs. Cells were lysed 48 h post-transfection and luciferase activity was measured immediately with a luminometer. Luciferase activity values (RLUs) for each transfection were normalized to β-gal activity (expressed from a co-transfected plasmid) and total cell protein. The resulting normalized values were expressed as a percentage change relative to controls. (n = 4 determinations).

A


17 hours
IL-1β (5.7 pM)
NS-398 (100 nM)
PGE2 (1 μM)
AUF1
p45
p42
p40
p37
5 sec exp
Cellular Fraction: C N C N C N C N
p45
p42
p40
p37
10 sec exp

B


17 hours
IL-1β (5.7 pM)
NS-398 (100 nM)
PGE2 (1 μM)
MW (kDa)
57
37.5
28
TTPhcx (covalent heterocomplex)
TTP (monomer)
C N C N C N C N

C

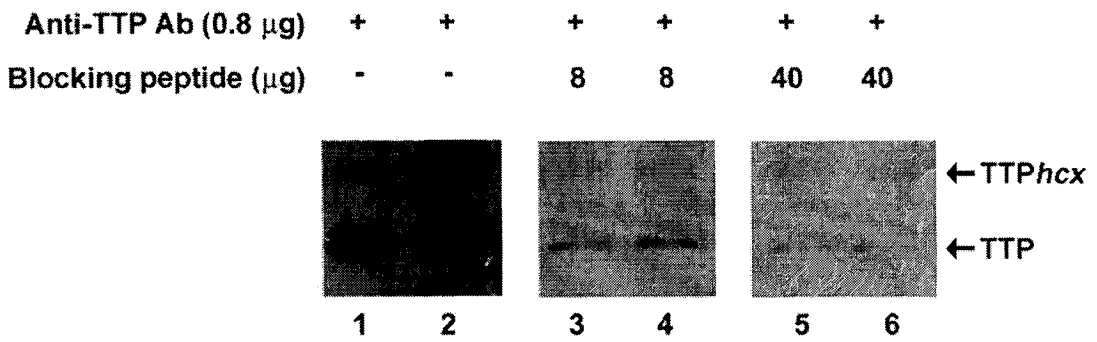


Figure 3: PGE_2-mediated modulation of p37AUF1 (*A*) and TTP (*B*) protein synthesis in HSFs. Confluent HSFs were preincubated for 3 h in DMEM supplemented with 1% FCS plus antibiotics at 37°C in order to ensure synchrony and quiescence. Cells were then treated with 5.7 pM of rhIL-1β for 17 h with or without NS-398 (100 nM) in the absence or presence of PGE_2 (1 µM). Monolayers were extracted for nuclear (N) and cytosolic (C) proteins. Fifty ug and 25 µg of each protein fraction were used for AUF1 (*A*) and TTP (*B*) analysis, respectively, by Western blotting (as described in "Materials and methods"). In (*A*), the top and bottom images correspond to 5 s and 10 s exposures, respectively, of the same immunoblot. TTP antibody specificity was assayed in (*C*) by Western blotting in the absence (lanes 1-2) or in the presence of excess TTP blocking peptide (lanes 3-6). Western blotting was performed using two different protein extracts from untreated HSF cultures (*extract 1*: lanes 1, 3, 5; *extract 2*: lanes 2, 4, 6).

(Figure 3*A*, *top*, *lane 6*). Addition of a pathophysiological concentration of exogenous PGE_2 (i.e., 1 μM) to the culture medium along with NS-398 and rhIL-1β reversed this nuclear upregulation of p37AUF1 and reduced its level to sub-control quantities **(Figure 3*A*, *top*, compare *lanes 2* and *8*)**. A similar, although more modest, effect was noted with cytosolic p45AUF1 **(Figure 3*A*, *bottom*, compare *lanes 5* and *7*)**. It is important to consider that the effect on p45AUF1 is not necessarily due to changes in protein synthesis, but may also be explained by PGE_2-enhanced nuclear retention or import of a minor amount of p45AUF1. However, this claim cannot be confirmed by the obtained data; given the large amount of nuclear p45AUF1, an extremely sensitive technique would be required to confirm this apparently infinitesimal shift in its subcellular localization. Nonetheless, this possibility cannot be excluded. The subcellular distribution of all other AUF1isoforms was unresponsive to PGE_2.

The same "standard" experimental protocol described previously (see **Figure 3** caption for details) was used to analyze PGE_2's effects on the level and localization of TTP protein in HSFs. rhIL-1β alone or with NS-398 upregulated the protein levels of both the TTP monomer (lower band) and a ~60 kDa species that was recognized by the anti-TTP antibody **(Figure 3*B*)**. The exact composition of this higher molecular weight protein (hereafter referred to as TTP*hcx*) is unknown, although it likely consists of a heterocomplex comprising a TTP monomer covalently associated with another protein. The specificity of the TTP*hcx* band was demonstrated by the linear decrease in TTP*hcx* band intensity following preabsorbtion of the anti-TTP antibody with increasing amounts of TTP blocking peptide **(Figure 3*C*)**. Exogenous PGE_2 almost completely abolished the presence of TTP*hcx*, whereas it only modestly reduced the levels of the TTP monomer. PGE_2 had no discernable effects on either TTP monomer or TTP*hcx* subcellular localization after 17 hours of cell stimulation **(Figure 3*B*)**.

TTP is encoded by the immediate-early response gene *ZFP36*, which is rapidly induced in fibroblasts by mitogens and growth factors (Taylor, Thompson *et al.* 1996). Given the long incubation period for the previously-described experiment **(Figure 3*B*)**, it was possible that we failed to observe early-onset effects of PGE_2 on TTP protein expression. Consequently, we conducted a short-range time course study on the influence(s) of PGE_2 on TTP protein synthesis **(Figure 4)**. The resulting Western

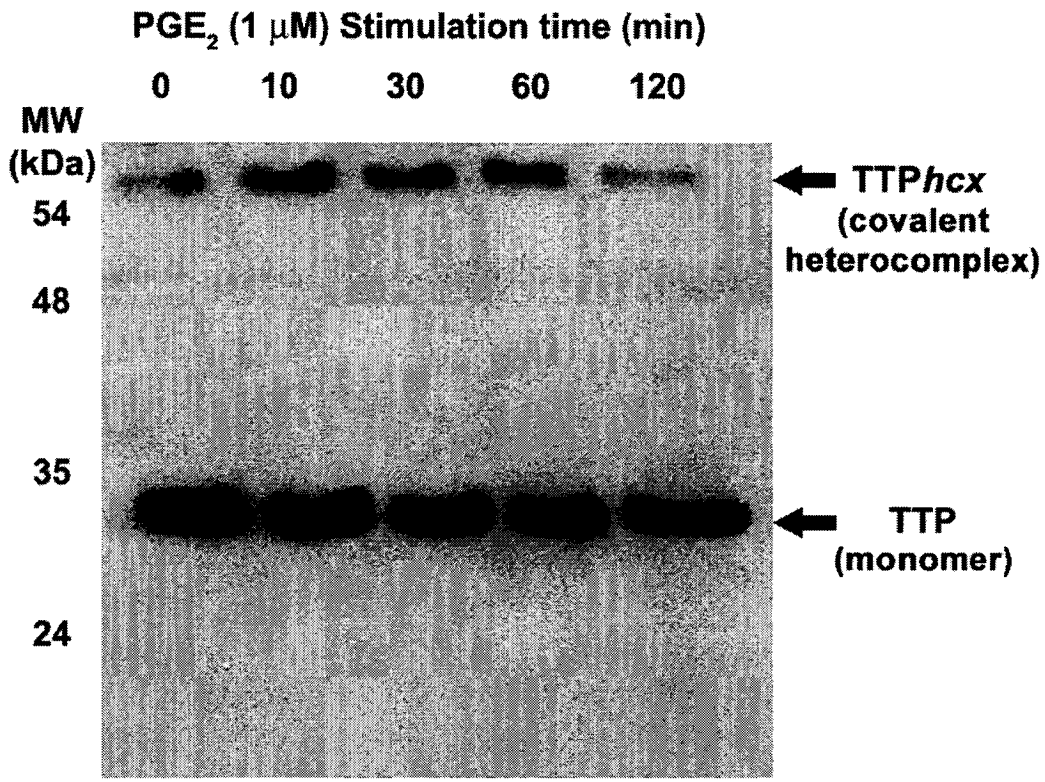


Figure 4: Time course analysis of PGE_2-mediated changes in HSF TTP expression. Cultured confluent HSFs (3 x 10^6 cells in 4-well cluster plates) were rendered synchronous and quiescent by serum deprivation for 3 h. Cells were then treated for varying times (0 –120 min) with 1 μM PGE_2. Fifty μg of total cell extracts (obtained by hot 1XSDS cell lysis) were analyzed for TTP protein by Western blotting (as described in "Materials and methods").

blot data, obtained using total cell protein extracts, demonstrated a quick (i.e., within 10 minutes) upregulation of TTP*hcx*, which remained stable for 1 hour and subsequently declined and returned to normal by 2 hours. TTP monomer levels remained unchanged throughout the 2-hour stimulation period relative to untreated cells.

To verify for transcriptional elements of PGE_2-mediated TTP expression, TTP mRNA levels in cells treated as per the standard experimental protocol were analyzed at various times by Northern blot **(Figure 5)**. TTP mRNA was detectable in quiescent unstimulated HSFs **(Figure 5, *lanes 1*)**. Treatment with rhIL-1β alone induced a dramatic increase in TTP transcript relative to unstimulated cells after only 20 minutes **(Figure 5, compare *lanes 1* and *2*)**. These rapid effects were not mediated by PGE_2, as shown by the unchanged transcript levels upon co-incubation with NS-398 alone **(Figure 5, *lane 3*)** or NS-398 and PGE_2 **(Figure 5, *lane 4*)**. The most important differences in TTP mRNA levels among the different treatment groups were seen after 2 hours stimulation. rhIL-1β-treated cells displayed an almost 2-fold decrease in TTP mRNA levels relative cells stimulated for 20 minutes **(Figure 5, compare *lanes 2* and *5*)**. Addition of NS-398 to the culture medium reduced rhIL-1β-induced transcript levels by one-half **(Figure 5, compare *lanes 5* and *6*)** and supplementation of PGE_2 **(Figure 5, *lanes 7*)** further decreased the amounts of TTP mRNA at 2 hours. After 16 hours, TTP mRNA levels for all treatment groups were sub-control **(Figure 5, *lanes 8-10*)**. These results therefore demonstrated no definite relationship between PGE_2 stimulation and transcriptional regulation of the TTP gene. In light of the TTP Western blot results **(Figures 3*B* and 4)**, which showed that TTP monomer levels were relatively unchanged between unstimulated and PGE_2-treated cells, it would appear as though TTP's expression is ultimately regulated at a translational level as to keep the amount of TTP protein relatively constant despite variations in the level of its mRNA. PGE_2 appeared to be more influential in regulating TTP's ability to functionally interact with other factors **(Figure 4)**.

Inhibition of the p38 MAPK pathway has no significant influence on formation or composition of TTP*hcx*: TTP binds a family of proteins known as the 14-3-3 proteins; these factors are involved in the cytoplasmic localization and/or sequestration of a variety of binding partners (Kao *et al.* 2001; Wang and Yang 2001;

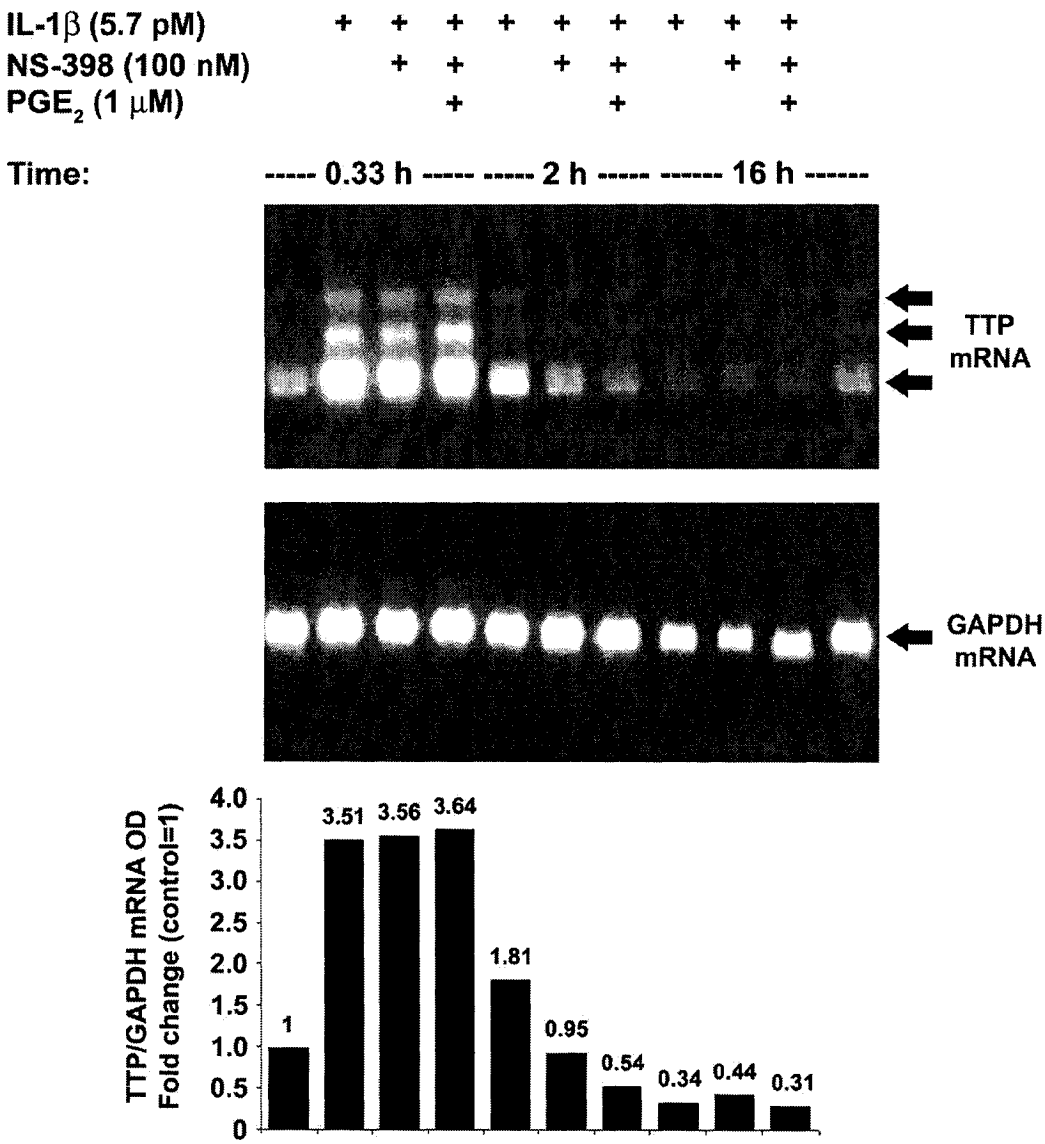


Figure 5: Quiescent and synchronous HSFs (1.2 x 10^6 cells in 6-well cluster plates) were treated with the factors indicated for 0.33, 2 or 16 h. Monolayers were then extracted for total RNA and prepared for TTP analysis by Northern blotting as explained under "Materials and Methods". (*Below*) TTP mRNA OD was quantified digitally by densitometry and normalized to the OD of GAPDH mRNA from the same sample; all normalized TTP mRNA values are expressed as change relative to control.

Kino *et al.* 2003). *In vivo* studies on murine TTP have shown that the association between TTP and 14-3-3 is dependent on the phosphorylation of TTP on serines 52 and 178 (Chrestensen *et al.* 2004). *In vitro* studies have shown that both serines are putative p38 MAPK cascade targets *in vivo*. Our TTP Western blot data consistently showed the presence of a specific, exclusively cytosolic TTP*hcx* band that was rapidly upregulated **(Figure 4)** and subsequently downregulated **(Figure 3*B*)** by PGE_2. Given these observations and the fact that PGE_2 is a potent activator of the p38 MAPK cascade in

HSFs (Faour *et al.* 2001), we asked whether the formation and/or expression of this uncharacterized complex were in any way dependent on PGE_2-mediated activation of the p38 MAPK pathway. Results from a time course study of the effects of p38 MAPK-inhibition (via pre-incubation with p38 MAPK inhibitor SB202190) in PGE_2-treated HSFs showed that the formation or composition of TTP*hcx* was not altered by shut-down of p38 MAPK signaling over a period of 2 hours **(Figure 6)**. Interestingly, it appeared that inhibition of the p38 MAPK pathway in both untreated and PGE_2-stimulated HSFs lead to a small, although noticeable, upregulation of both the TTP monomer and TTP*hcx* **(Figure 6, compare *lane 1 vs. lane 2*; *lane 3 vs. lanes 4-7*)**. The significance of this finding must be further examined.

PGE_2 stimulates TTP nuclear export in HeLa cells: It was initially reported that mitogen and growth factor stimulation of quiescent mouse fibroblasts and LPS stimulation of RAW264.7 macrophages induced rapid TTP shuttling (Taylor, Thompson *et al.* 1996). Subsequent studies in NIH 3T3, HEK293 and HeLa cell lines demonstrated that exogenous TTP was subject to both CRM1- and 14-3-3-dependent nucleocytoplasmic shuttling via site-specific association of these accessory proteins with TTP (Johnson, Stehn *et al.* 2002; Phillips *et al.* 2002). To examine if the subcellular localization of TTP was sensitive to PGE_2, HeLa cells transfected with a TTP-eGFP fusion protein were treated with 1 μM PGE_2 and immediately examined by confocal microscopy. In serum-reduced HeLa cells not yet treated with PGE_2, TTP-eGFP was principally cytoplasmic although a small amount was also present in the nucleus **(Figure 7, *0 min*)**. Addition of PGE_2 to the culture medium initiated nuclear export of TTP-

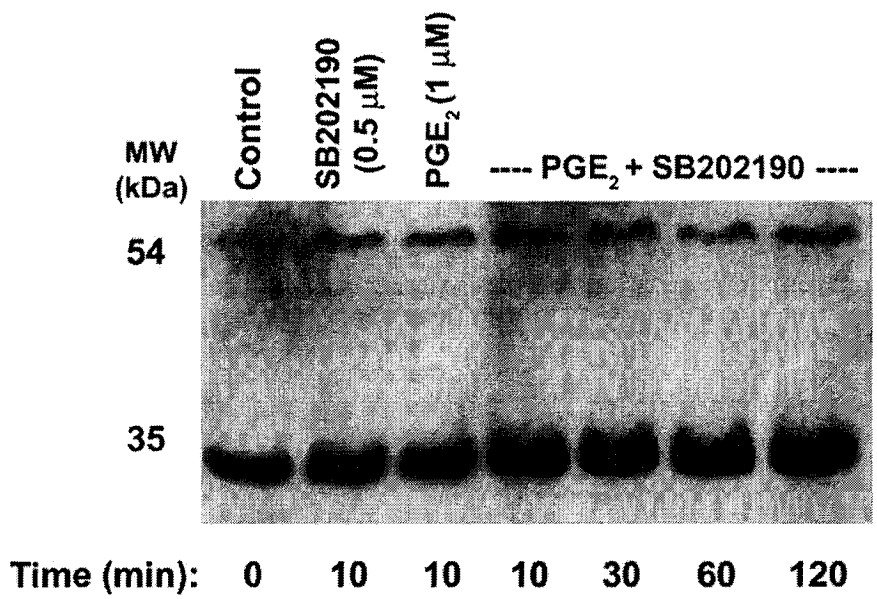


Figure 6: Effects of PGE_2 activation of the p38 MAPK on the formation of TTP*hcx*. Confluent and quiescent HSFs were incubated with either 0.5 μM SB202190, 1 μM PGE_2 or a combination of both factors for the times indicated. Cells were then lysed with hot 1XSDS and 50 μg of total cell protein was analyzed for TTP by Western blotting.

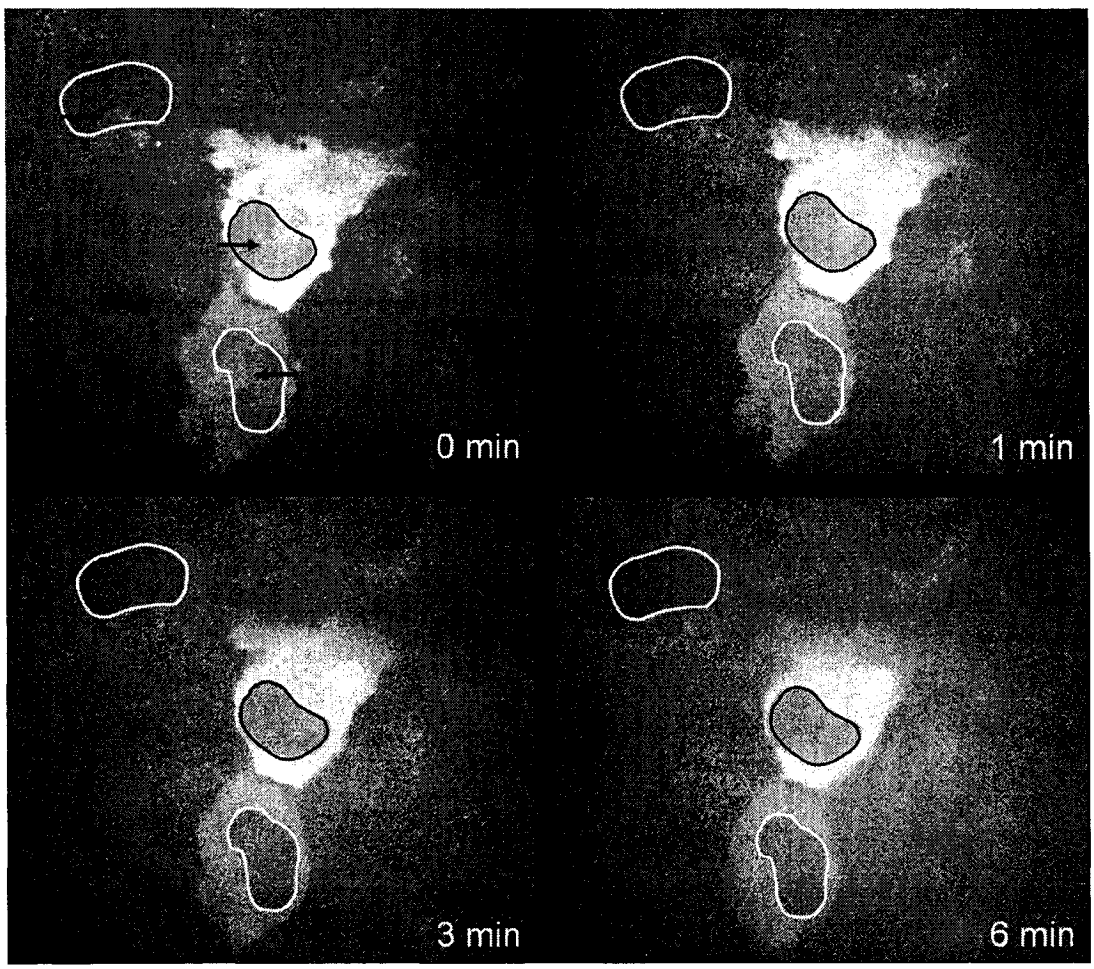


Figure 7: TTP nucleocytoplasmic shuttling in HeLa cells following PGE_2 stimulation. HeLa cells were plated in 4-chamber Lab-Tek coverglasses (50 000 cells / chamber) and transfected with 50 ng of TTP-eGFP fusion plasmid and 150 ng of pcDNA3.1 plasmid (carrier DNA). Cells were grown for an additional 24 h, after which they were rendered synchronous and quiescent (by incubation in serum-reduced medium for 3 h) and treated with 1 μM PGE_2. Following stimulation, TTP-eGFP shuttling was imaged in real-time by confocal microscopy. The microscopic images shown depict TTP-eGFP subcellular distribution at various times following stimulation (0 min = unstimulated). HeLa cell nuclei in which TTP-eGFP nuclear export is evident are outlined; nuclear pools of TTP-eGFP at 0 min are indicated by arrows.

eGFP within 1 minute of stimulation **(Figure 7, *1 min*)**; this nuclear protein export was complete within 6 minutes of stimulation **(Figure 7, *6 min*)**.

The distal COX-2 3 UTR specifically interacts with a variety of synovial fibroblast mRNA-binding proteins: It has been widely reported that the proximal COX-2 ARE, a conserved region defined by the first 116 nucleotides of the COX-2 3 UTR and containing 6 AUUUA motifs, binds a variety of AUBPs and mediates COX-2 mRNA decay and translational efficiency (Dixon *et al.* 2000; Cok *et al.* 2003, 2004). We have previously demonstrated that the distal AREs were important for the PGE_2-mediated increases in COX-2 mRNA stability and translation in HSFs (Faour *et al.* 2001). As a first effort in the clarification of the latter results, we used *in vitro* techniques to analyze whether the distal COX-2 3 UTR associated with PGE_2-regulated mRNA-binding proteins. RNA gel shift/UV crosslinking analysis of nuclear and cytosolic protein extracts from HSFs treated according to the standard experimental protocol displayed the existence of various protein:cRNA complexes **(Figure 8*B*)**. The COX-2_dist3 UTR cRNA probe **(Figure 8*A*)** was involved in the formation of at least six distinct specific protein:cRNA complexes in HSFs (indicated by arrows in **Figure 8*B*, *lanes 7-8***). Binding was specific since addition of 100-fold molar excess of non-labeled cRNA probe to binding reactions prevented protein association to the labeled probe **(Figure 8*B*, *lane 10*)**. Most of the binding species appeared to display a capacity to shuttle between the nucleus and cytoplasm of HSFs, although the highest concentration of these proteins was found in nuclear fractions. Of the complexes detected by this method, two were PGE_2-responsive (black arrows). The level of the larger PGE_2-responsive species was upregulated by the eicosanoid, as was its accumulation in the nucleus **(Figure 8*B*, compare *lanes 6* and *8*)**. The second (~60 kDa) PGE_2-regulated protein was also a shuttling species whose nuclear export was induced by PGE_2.

As an alternative, non-radioactive, *in vitro* method to detect COX-2 distal 3 UTR binding factors, subtractive affinity chromatography with poly(U) beads was performed. In this technique, HSFs were treated according to the standard experimental protocol (see **Figure 3** caption for details) and total protein extracts from these cells were incubated with poly-uridylate coated sepharose beads. The poly(U)-associated proteins were

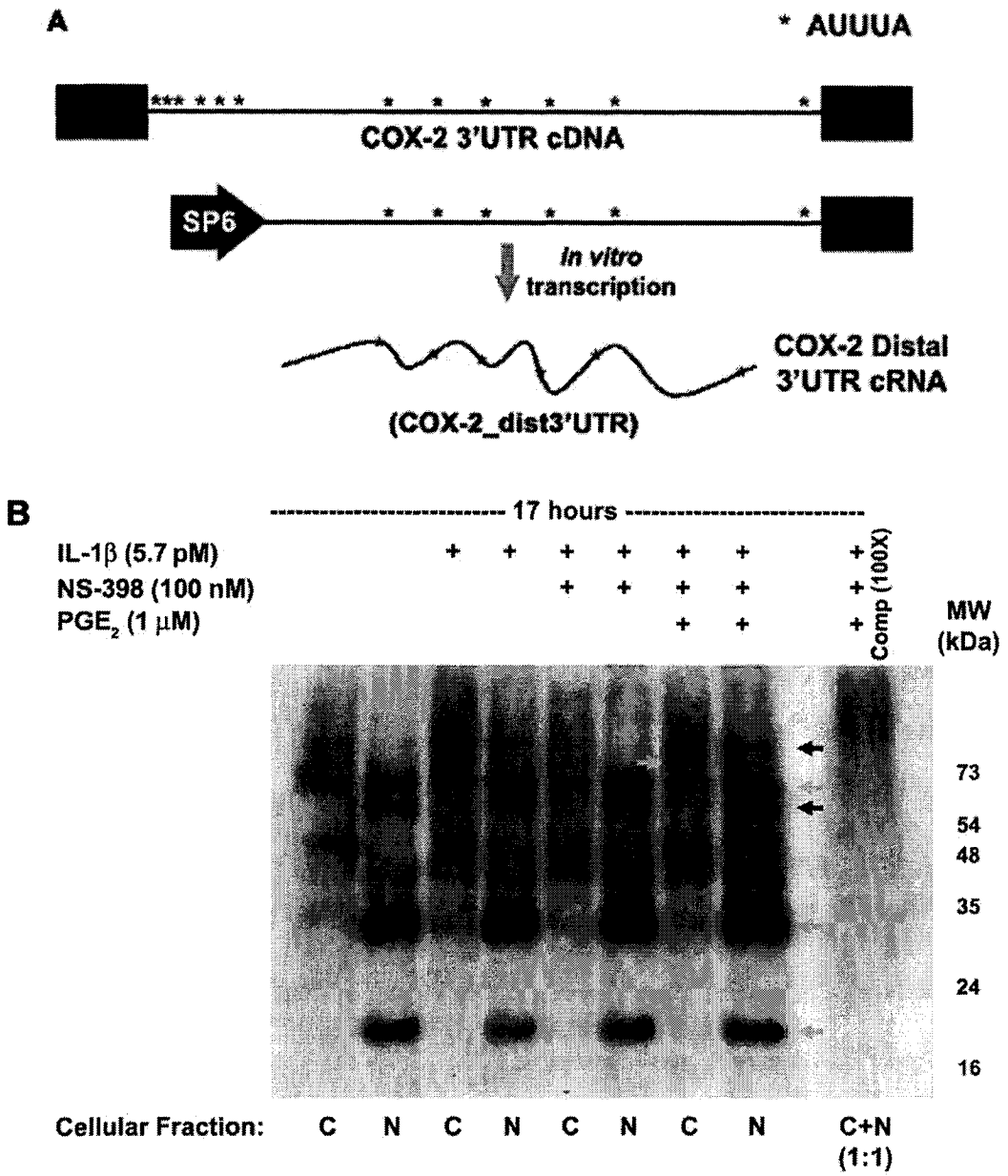


Figure 8: Analysis of PGE_2-modulated COX-2 distal 3 UTR binding proteins by REMSA. (*A*) Procedural outline for the synthesis of the COX-2_dist3 UTR cRNA probe (refer to "Materials and Methods" for details). (*B*) Quiescent and synchronous HSFs were treated according to the standard experimental protocol (as described in caption to **Figure 3**). Cells were then extracted for nuclear (N) and cytosolic (C) proteins and prepared for REMSA/UV crosslinking analysis exactly as described under "Materials and Methods". Arrows indicate specific COX-2_dist3 UTR:protein complexes; black arrows highlight those complexes that are PGE_2-regulated.

subsequently analyzed by SDS-PAGE and silver staining. Poly(U) RNA has been reported to bind most AUBPs and other mRNA-binding proteins with high affinity (Chen *et al.* 2001) and thus, this strategy allowed us to obtain a broad picture of PGE_2-regulated mRNA-binding proteins. As demonstrated **(Figure 9)**, a wide variety of mRNA-binding factors were resolved, a greater part of which were upregulated following treatment with rhIL-1β. Among these factors was one that was visibly dowregulated by PGE_2-treatment (black arrow in **Figure 9**; **compare *lanes 2-5***). In order to determine if this protein could bind specifically to the COX-2 distal 3 UTR, a competition reaction consisting of (rhIL-1β+NS-398+PGE_2)-treated extracts in the presence of both poly(U) beads and competitor (i.e., unlabeled COX-2_dist3 UTR cRNA) was conducted.. Differences in the visualized binding species between standard **(Figure 9, *lanes 2-5*)** and competition **(Figure 9, *lane 6*)** reactions were identified as COX-2 distal 3 UTR-specific binding proteins. As shown by the bracketed bands in **Figure 9**, at least ten discernable species, including the PGE_2-regulated factor, were competed away by the COX-2_dist3 UTR probe. Note that the large, intensely stained spot detected in the competition reaction lane likely corresponded to a contaminating protein (i.e., keratin). Hence, this assay enabled us to identify an additional PGE_2-regulated protein that could form a specific complex with the COX-2 distal 3 UTR. Importantly, it appeared (by molecular weight comparison) that the COX-2 distal 3 UTR binding proteins isolated by subtractive poly(U) affinity chromatography were also visible on the REMSA/UV crosslinking gel. The reproducibility of our results by two different techniques demonstrated without a doubt that the COX-2 distal 3 UTR is a binding target for various HSF proteins, some of which appear to be constitutively expressed and others that are regulated.

III) DISCUSSION

COX-2 is a fundamental regulator of cellular homeostasis whose importance to normal cell function is highlighted by the wide range of pathologies in which its expression is deregulated (Minghetti 2004; Zha *et al.* 2004). Aberrant COX-2 expression in synovial membranes has been linked to the etiology and progression of OA and RA (Martel-Pelletier *et al.* 1999). We have previously shown that in primary HSFs, COX-2 expression was dramatically upregulated through PGE_2-mediated post-transcriptional

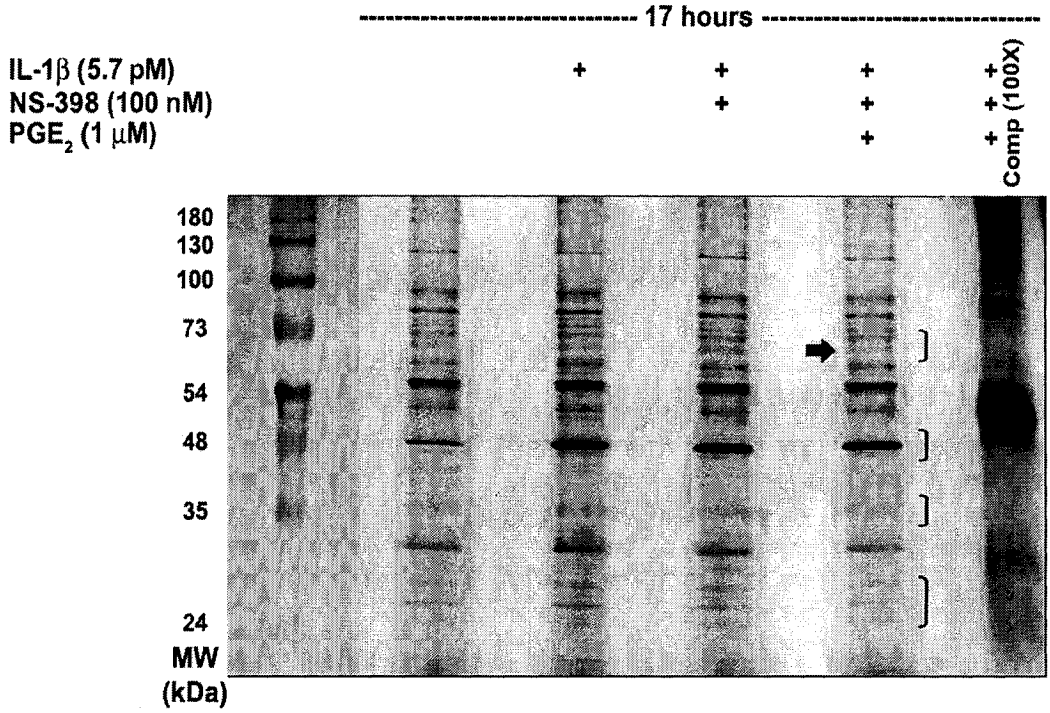


Figure 9: Analysis of PGE_2-modulated COX-2 distal 3 UTR binding proteins by subtractive affinity chromatography. HSFs treated as per the standard experimental protocol were extracted for total cellular protein and prepared for subtractive affinity chromatography with Sepharose-coupled poly(U) RNA as described under "Materials and Methods". Brackets are next to the subset of isolated proteins that specifically bind the COX-2_dist3 UTR probe; the black arrow highlights a COX-2_dist3 UTR binding protein that is modulated by PGE_2.

mechanisms targeting COX-2 s ARE-rich 3 UTR (Faour *et al.* 2001). It was hypothesized that PGE_2 likely acted by modulating the expression and/or activity of mRNA-binding proteins capable of affecting COX-2 mRNA stability and/or translation. The results from this study supported our hypothesis by showing that PGE_2 modulated the expression, subcellular localization or activity of two prominent AUBPs and three uncharacterized COX-2 distal 3 UTR binding factors.

We first analyzed AUF1, HuR and TTP expression and subcellular localization in quiescent, unstimulated primary HSFs. A review of the literature revealed that our study was the first to document primary HSF expression of these AUBPs at the molecular level. Our data demonstrated that all factors except AUF1 isoform p37 displayed both nuclear and cytosolic localization. HuR and AUF1 isoforms were largely nuclear, whereas TTP was mostly cytosolic **(Figure 1)**.

Transient transfection experiments showed that the COX-2 3 UTR conferred post-transcriptional regulation to a reporter mRNA by p37AUF1 and TTP, two AUBPs that were responsive to PGE_2 **(Figures 2 and 3)**. Although we did not directly study the binding of these factors to the COX-2 3 UTR, both factors have been demonstrated to associate specifically with the COX-2 proximal ARE (Lasa *et al.* 2000; Sully *et al.* 2004); distal COX-2 3 UTR-binding of TTP has also been reported (Sawaoka *et al.* 2003). Despite these findings, the exact function of these factors in COX-2 post-transcriptional regulation is unclear. In a recent study by Sully *et al.* (2004), the authors claimed that these factors were not implicated in this process. This conclusion contrasted the findings from our transfection assays. Multiple reasons for this difference can be suggested. First, the study by Sully *et al.* (2004) was conducted using extracts from transformed cell lines (i.e., HeLa, RAW264.7), which reportedly display altered post-transcriptional regulatory mechanisms relative to normal primary human cells (as used in our study) (Audic and Hartley 2004). Furthermore, the study by Sully *et al.* (2004) analyzed the binding properties of the proximal ARE in isolation and independently of distal AREs. The proximal ARE has been the focus of most studies on COX-2 post-transcriptional regulation given reports showing that it consists of the minimal destabilizing element in the COX-2 3 UTR (Dixon *et al.* 2000; Cok *et al.* 2004). The use of this region in isolation is problematic in that the intramolecular folding (i.e., secondary structure) of the

entire COX-2 3 UTR is not conserved. The potential importance of 3 UTR secondary structure in enhancing mRNA turnover has been previously reported (Henics *et al.* 1994). Thus, it is possible that formation of a specific mRNA secondary structure leads to the recruitment of accessory proteins required for activation of COX-2 3 UTR-bound AUBPs (or other factors). Alternately, it is possible that mRNA folding leads to the juxtapostion of otherwise distant accessory factors that subsequently activates AUBPs. In order to account for these possibilities, our studies were conducted using a reporter mRNA containing both proximal and distal AREs and is a potential reason why TTP and AUF1 influenced COX-2 3 UTR reporter activity in our system.

The lack of HuR's effect on reporter activity was somewhat surprising. Relative to the other AUBPs, HuR has been the most widely studied in terms of COX-2 expression and shown to affect COX-2 post-transcriptional regulation (Sengupta *et al.* 2003), particularly within the context of various cancers (Dixon *et al.* 2001; Denkert, Weichert, Pest *et al.* 2004; Denkert, Weichert, Winzer *et al.* 2004). The failure to observe these effects in HSFs could have been a cell-specific phenomenon, but most likely due to differences in cell context. It follows that most of the studies analyzing HuR's influence on COX-2 regulation were conducted in transformed cells, both primary and cell lines. As noted previously, cellular transformations are known to provoke mechanistic changes in mRNA post-transcriptional regulation (Audic and Hartley 2004) and as a result, such mechanisms would not be detected in normal cells. It is also possible that AUF1 competitively prevented HuR from interacting with the COX-2 3 UTR, as is the case *in vivo* with cyclin D1 and p21 mRNAs (Lal *et al.* 2004). Further studies will be required to confirm the existence of such antagonistic activity in HSFs.

Our results also illustrated that PGE_2 influenced AUF1 protein expression in an isoform-selective manner **(Figure 3*A*)**. PGE_2 acted to maintain normal (i.e., control) p37AUF1 levels in an inflammatory-like state *in vitro*, as demonstrated by elevation of p37AUF1 protein levels upon removal of PGE_2 in rhIL-1β-treated HSFs. This finding was indicative of a homeostatic role for PGE_2 during inflammatory conditions *in vivo*. The isoform-specificity of PGE_2's effect was particularly interesting, especially when considered in the context of Raineri *et al.*'s (2004) recent report revealing that the relative levels of individual isoforms, rather than the absolute amount of AUF1, determined

AUF1 s net effect on ARE-mRNA stability. Hence, the PGE_2-mediated changes in p37- and (arguably) p42AUF1 expression possibly consists of a specific mechanism by which the prostaglandin fine-tunes AUF1 s influence on COX-2 post-transcriptional regulation in various cellular states. This report would also help explain the counterintuitive experimental data that showed the PGE_2-mediated suppression of a factor (i.e., p37AUF1) that positively influenced the stability and/or translation of a luciferase-COX-2 3 UTR reporter **(Figure 2*C*)**. Raineri *et al.*'s conclusions would imply that the changes in one AUF1 isoform are insufficient to draw inferences about the effects on the stability of an ARE-rich transcript. By the same argument, the minor PGE_2-dependent changes in p45AUF1, whose overexpression failed to appreciably influence the activity of the COX-2 3 UTR reporter, were not necessarily insignificant and must be analyzed within the context of other AUF1 isoforms.

As already mentioned, PGE_2's effects on p45AUF1 may have been explained by enhanced nuclear import or retention. Interestingly, p45AUF1 displayed the ability to associate with the transport receptor Trn1 *in vitro* (Siomi *et al.* 1997), which, in conjunction with other transport receptors, may potentially mediate AUF1 shuttling *in vivo* (Sarkar *et al.* 2003). In addition, AUF1 isoforms have been shown to directly associate in an mRNA-independent fashion so that the larger AUF1 isoforms (which contain a C-terminal sequence facilitating nuclear export but lack a nuclear import signal) can be re-localized to the nucleus by associating and co-shuttling with a smaller AUF1 isoform (which have a nuclear import (or retention) signal) (Sarkar *et al.* 2003). As suggested by Sarkar *et al.* (2003), this arrangement of signals may be a possible mechanism to assure co-shuttling of a subset of AUF1 proteins that interact in a heterocomplex. Given the lack of a nuclear import signal in p45AUF1, the only way that PGE_2 could have influenced p45AUF1 s subcellular localization was by modifying its interaction with Trn1, the smaller AUF1 isoforms (i.e., p37- or p40AUF1) or with other non-shuttling proteins shown to influence p45AUF1 s nucleocytoplasmic distribution (e.g., scaffold attachment factor B (Arao *et al.* 2000)). Hence, in addition to the isoform-specific effects on AUF1 protein expression, modulation of isoform subcellular localization may be another mechanism by which PGE_2 regulates AUF1 s activity.

The TTP Western blot data was intriguing in that it revealed the existence of two anti-TTP antibody specific bands; the lower band corresponded to the TTP monomer (based on the reported molecular weight, 34 kDa), while the composition of higher molecular weight species (TTP*hcx*) was speculative. The specificity of this band was confirmed by preabsorbing a constant amount of TTP antibody with increasing concentrations of blocking peptide. TTP*hcx* was competed in a linear fashion as the concentration of the blocking peptide was increased **(Figure 3*C*)**. Interestingly, PGE_2 appeared to specifically modify the levels of this larger species and not the amount of TTP monomer after both short- and long time periods **(Figure 3*B* and 4)**. PGE_2-stimulation of HSFs led to increased levels of TTP*hcx* within 10 minutes; elevated levels persisted for 1 hour, declined to control levels by 2 hours and dropped to sub-control amounts after 17 hours of stimulation. It was first believed that this species was an unidentified TTP isoform resulting from alternative splicing and/or usage of an alternative translational start or stop site. However, 5 - and 3 -rapid amplification of cDNA ends (RACE) results did not confirm this hypothesis (data not shown). Subsequent data strongly favored the possibility that TTP*hcx* consisted of a covalent TTP monomer:14-3-3 monomer heterocomplex. This association had been described by Johnson, Stehn *et al.* (2002) as a mechanism by which TTP is localized to the cytoplasm. Although we did not determine the exact composition of TTP*hcx*, the latter hypothesis was supported experimentally by the complex's approximate molecular weight on SDS gel, which corresponded to that expected for a TTP:14-3-3 heterodimer (i.e., ~61.5-63 kDa). Furthermore, TTP*hcx* was found exclusively in the cytoplasm of HSFs, whereas the monomer was found in both the nucleus and cytoplasm **(Figure 1*C*)**. This finding was in accordance with the function of the 14-3-3 proteins in cytoplasmic localization and sequestration of binding partners (Kao *et al.* 2001; Wang and Yang 2001; Kino *et al.* 2003). However, our demonstration that TTP*hcx* was insensitive to p38 MAPK inhibition in PGE_2-treated HSFs challenged our hypothesis about its composition **(Figure 6)**. As previously reported, the interaction between TTP and 14-3-3 proteins is dependent on TTP's phosphorylation on serines 52 and 178, supposedly by the p38 MAPK/MAPKAPK-2 pathway (Chrestensen *et al.* 2004; Stoecklin *et al.* 2004). We previously established that PGE_2 is a potent p38 MAPK activator in HSFs (Faour *et al.*

2001); it was thus deduced, in the context of our hypothesis, that the observed PGE_2-mediated changes in TTP*hcx* occurred via activation of the p38 MAPK cascade and would be eliminated following its blockade. Preliminary data (not shown) suggests that TTP is serine-phosphorylated in quiescent, unstimulated HSFs, thereby opening up the possibility that assembly of a putative TTP:14-3-3 dimer is regulated through yet unreported effects of PGE_2 on 14-3-3 proteins. Until further characterization of this complex, our concept regarding its structure cannot be discounted.

In addition to the above-mentioned effects, we showed that PGE_2 enhanced the nuclear export of a TTP-eGFP fusion protein in HeLa cells. These cells were used mainly due to their reported lack of endogenous TTP expression (Sully *et al.* 2004), which eliminated potential experimental artifacts related to the sequestration or modification of essential shuttling factors by endogenous TTP. This decision was also based on the availability of information on TTP-eGFP shuttling in HeLa cells. Our data revealed that TTP-eGFP was predominantly cytosolic in quiescent, serum-reduced cells, as previously reported by others (Phillips *et al.* 2002), and rapidly exported from the nucleus upon PGE_2-stimulation **(Figure 7)**. Assuming that TTP*hcx* is a TTP:14-3-3 complex, then the rapid onset of both PGE_2-mediated TTP*hcx* assembly **(Figure 4)** and TTP-eGFP shuttling pointed to mechanism whereby PGE_2 induces TTP nuclear export through favoring its association with 14-3-3 proteins. In corroboration of this model and our hypothesis on TTP*hcx*'s composition, Johnson, Stehn *et al.* (2002) showed that the same TTP fusion protein underwent 14-3-3-mediated shuttling in HeLa cells.

Overall, despite our uncertainties about TTP*hcx*'s composition, our data strongly suggest that PGE_2 modulates TTP exclusively at the level of its activity and subcellular localization by regulating its interaction with accessory proteins. PGE_2 had no influence on either TTP mRNA expression **(Figure 5)** or protein synthesis, although TTP (monomer) synthesis was apparently regulated at the level of translation by PGE_2-independent mechanisms **(compare Figures 4 and 5)**. These observations kept with the emerging theme of PGE_2 as a regulator of AUBP activity (and localization) through its influence on AUBP:accessory protein interactions. Of notable importance, TTP is also involved in translational arrest, as suggested by its association to stress granules (Stoecklin *et al.* 2004). In view of this finding, its primarily cytosolic localization and our

observation that TTP had the most profound influence on COX-2 3 UTR reporter activity, it is likely that this factor may account for PGE_2's effects on both COX-2 mRNA stability and translation.

As a second approach in the study of PGE_2-mediated effectors and mechanisms in COX-2 mRNA post-transcriptional regulation, we sought to analyze the protein binding profile of the COX-2 3 UTR in HSFs. A majority of similar studies focused on the COX-2 proximal 3 UTR for reasons already mentioned. However, based on previous findings (Faour *et al.* 2001), we were interested in analyzing the binding characteristics of the distal COX-2 3 UTR, which contains six non-overlapping AUUUA motifs **(Figure 8*A*)** and binding sites for HuR and TTP (Sawaoka *et al.* 2003; Sengupta *et al.* 2003). Our REMSA/UV crosslinking assay and subtractive affinity chromatography results led to the identification of at least six distinct and specific cRNA:protein complexes, three of which were modulated by PGE_2 **(Figure 8*B* and 9)**. Interestingly, one of the complexes identified by REMSA/UV crosslinking analysis was similar in size to TTP*hcx* (i.e., ~60 kDa) and behaved in a similar fashion in response to PGE_2 (i.e., exported from the nucleus). The relative novelty of our study and lack of previous data in this field made it difficult to hypothesize about the identity of the factors isolated. Hence, the exact identification of the cRNA-binding proteins is pending on the use of more sophisticated proteomic techniques, namely two-dimensional electrophoresis, matrix-assisted laser desorption/ionization (MALDI) and liquid chromatograph/mass spectrometry/mass spectrometry (LC/MS/MS).

IV) Conclusions and Research Prospectives

In summary, our study has led to the identification of potential factors and mechanisms, both known and unknown, by which the COX-2 product PGE_2 regulates COX-2 mRNA stability and translation in HSFs. Our global experimental approach has elucidated the broad range of mechanisms used by this factor in the fine-tuning of post-transcriptional gene regulation. The study is important in that it is the first to shed light on the latter post-transcriptional mechanisms in primary cells within a pathologically relevant context. In addition, this study clearly showed that PGE_2 is more than simply a

metabolic product, but instead a potent pleiotropic secondary messenger in cytokine cell-signaling capable of mediating various levels of gene expression.

This project has opened many research avenues and left many unanswered questions that must be addressed in further studies. First, intracellular binding of TTP and AUF1 to the COX-2 distal 3 UTR in HSFs must be confirmed, likely through overexpression of these proteins followed by their immunoprecipitation and reverse-transcription of the bound ribonucleotide. Secondly, a COX-2 Northern blot experiment in TTP- and AUF1-overexpressing, actinomycin D-treated HSFs would be required to confirm the observed effects of these factors on mRNA stability. Thirdly, the existence of a mutual competition between AUF1 and HuR binding to the COX-2 3 UTR must be considred as an explanation for the inability of HuR to affect COX-2 3 UTR repoter activity. A mutual competition assay would consist of COX-2 mRNA and protein analysis in HSFs co-transfected with varying levels of one factor (i.e., AUF-1) and constant levels of the second (i.e., HuR). Fourthly, the ability of PGE_2 to induce TTP shuttling will have to be shown in HSFs by confocal microscopy. Lastly, the composition of TTP*hcx* and the identity of the unknown COX-2 distal 3 UTR binding factors must be determined; two-dimensional electrophoresis, MALDI and LC/MS/MS will serve in this process.

PART G:
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PART H:
Appendix

Centre universitaire de santé McGill
McGill University Health Centre

Internal Permit #: 5-0050-05

Permit Holder: Giovani .John Di Battista **Office:** M11.22

Department: RVH - Medicine **Telephone:** 34401

(A) Location

Room	Classification
M11.01*	Storage only
M11.25	Counter room
M11.27*	Basic

Rooms identified by an asterix (*) must have a door warning sign

(B) Authorized Activity

Isotope	Max Manipulated (MBq)	Max Purchase (MBq)
P32	1.85	9.25

(C) Authorised Users

Last Name	First Name	P32	P33	S35	H3	C14	Fe59	I125	Ca45	Co57	Cr51	Na22	Rb86
Di Battista	Giovani (john)												
Faour	Wissam	✔											
He	Qing Wen	✔											
Mancini	Arturo	✔											

(D) Conditions

The persons listed in section (C) are authorised to use the designated radioisotopes. The radioisotopes and their respective activities listed in section (B) can only be used in the laboratories listed in section (A) in accordance with the conditions listed in section (D). Importation, storage, manipulation and disposition of radioactive material must be performed in conformity with our CNSC licence, with Federal regulations and with the MUHC Radiation Safety Policies and Procedures. A copy of the CNSC consolidated licence is available from the Radiation Protection Service (ext. 43866).

Approved by:____ Date issued: Friday, June 11, 2004

Expiration date: Feb 28, 2006

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

Projects involving potentially biohazardous materials should not be commenced without approval from the Environmental Safety Office. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: Giovanni (John) A. Di Battista PHONE: 842-1231, xt. 34401
DEPARTMENT: Medicine FAX: 289-8542
ADDRESS: RVH, 687 Pine Avenue W., Rm M11.22 E-MAIL: john.dibattista@mcgill.ca

PROJECT TITLE: Prostaglandins: Mediators of cytokine cell signaling through post-transcriptional and translational regulatory mechanisms

2. EMERGENCY: Person(s) designated to handle emergencies
Name: Giovanni (John) A. Di Battista Phone No: work: 842-1231, 34401 home: 514-426-9358
Name: Qing Wen He Phone No: work: 842-1231, 36276 home: 514-278-8899

3. FUNDING SOURCE OR AGENCY (specify): CIHR
Grant No.: MOP-11557 Beginning date: 04/2002 End date: 03/2005

4. Indicate if this is
☑ Renewal: procedures previously approved without alterations.
Approval End Date: March 31, 2005
☐ New funding source: project previously reviewed and approved under an application to another agency.
Agency: _____ Approval End Date: _____
☐ New project: project not previously reviewed.
☐ Approved project: change in biohazardous materials or procedures.
☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: The Environmental Safety Office approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in Health Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☐ 1 ☑ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: ___ date: 2 (day) 04 (month) 2004 (year)

Approved by: Environmental Safety Office: ___ date: 07 (day) 04 (month) 2004 (year)

Expiry: 31 (day) 03 (month) 2005 (year)

*as defined in the "McGill Laboratory Biosafety Manual"

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Wissam H. Faour	Molecular Biology				PhD	
Arturo Mancini	Anatomy and Cell Biology				MSc	
Stefania Ciummo	Micro & Immunology			BSc		
Dr. Qing Wen He	Medicine	X				

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Dr. John A. Di Battista Phone No: work: 842-1231, ext 34401 home: 514-426-9358

Name: Dr.Q.W. He Phone No: work: 842-1231, ext 36276 home: 514-278-8899

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

Bacteria: DH5α for cloning purposes; Risk Group 2

DNA plasmids: For expression and subcloning purposes; Risk Group 2

Human cells lines and primary cultures; Risk Group 2

ii) the procedures involving biohazards

1) We use Class II biological safety cabinets that are new and have been tested within the last few months.

2) Centrifugation is carried out in a closed and aerosol safety heads.

3) Vacuums lines are isolated with bio-filters.

4) Lab coats and gloves are worn at all times in the area of confinement and nowhere else.

5) Only sterile disposal consumables are used.

6) All consumables are bagged and autoclaved in a laboratory autoclave and placed in leak proof container for transport and incineration. Consumables from cell cultures are bagged and placed in a safety container.

7) All bacterial cultures are stored (if necessary) in a biosafety container at –80C. Cells are stored at –170C.

8) Laboratory (ies) has a biosafety designation on the door with the level of confinement.

9) Hospital cleaning staff may clean only floors.

iii) the protocol for decontaminating spills

Spills are wiped immediately by a gowned and gloved individual using Biohazard wipers that essentially are cloths soaked in a powerful disinfectant. The cloths are then placed in autoclave bag for sterilization and put into a biohazard container for pick-up and disposal. The area where the spill occurred is thoroughly disinfected. The spill is logged, lab workers alerted and particulars described.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes, they are standard protocols described and sanctioned by the CIHR and the NIH.

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

All manipulations are done in a designated Class IIA biosafety cabinet with air recirculation and HEPA-filter filtration or in a Class I safety hood, which given the air flow minimize aerosol formation. Solutions are used at room temperature and aerosol resistant caps and bottles are routinely used. Pipetting is performed along the sides of vessels or below liquid levels. Minimal volume use is encouraged.

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
RVH, Medical Wing	M.11.27&37	Thermo-Forma	Class IIA/B3	100095	15-03-04