2014

Model Systems to Explore CPB2 Pre-mRNA Splicing

Christina Marisa Rizzo

University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation
Rizzo, Christina Marisa, "Model Systems to Explore CPB2 Pre-mRNA Splicing" (2014). Electronic Theses and Dissertations. 5176.
https://scholar.uwindsor.ca/etd/5176

This online database contains the full-text of PhD dissertations and Masters' theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email (scholarship@uwindsor.ca) or by telephone at 519-253-3000 ext. 3208.
Model Systems to Explore \textit{CPB2} Pre-mRNA Splicing

By

CHRISTINA M. RIZZO

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2014

© 2014 Christina M. Rizzo
Model Systems to Explore *CPB2* Pre-mRNA Splicing

By

CHRISTINA M. RIZZO

APPROVED BY:

A. Hubberstey  
Department of Biological Sciences

S. Ananvoranich  
Department of Chemistry and Biochemistry

M. Koschinsky  
Department of Chemistry and Biochemistry

M. Boffa, Advisor  
Department of Chemistry and Biochemistry

September 10, 2014
Declaration of Originality

I hereby certify that I am sole author of this thesis and that no part of this thesis has been published or submitted for publication.

I certify that, to the best of my knowledge, my thesis does not infringe upon anyone’s copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis and have included copies of such copyright clearances to my appendix.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.
Abstract

Thrombin Activatable Fibrinolysis Inhibitor (TAFI), encoded by CPB2, cleaves carboxyl-terminal lysine residues from partially degraded fibrin, thus assisting in the attenuation of fibrinolysis. The finding of several new alternatively spliced (AS) CPB2 variants within numerous cell types suggests that AS is an important mechanism regulating CPB2 gene expression. This thesis investigated the extent of AS events occurring in various vascular and immune cells, and identified proteins translated from AS CPB2 mRNA. To determine whether a minigene approach could be used to model the cell-specific AS pattern that occurs endogenously, two minigene constructs were created. Real time RT-PCR analysis revealed that the pattern and extent of AS varies in a cell-specific manner. However, the minigenes were found to be unable to recapitulate the cell-specific splicing pattern of CPB2 that occurs endogenously. Metabolic labeling in conjunction with immunoprecipitation resulted in the detection of the Δ7 TAFI variant retained within HepG2 cells.
I am among those who think that science has great beauty.

A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena, which impress him like a fairy tale.

- Marie Curie
For Giuseppe and Thérèse Rizzo
Acknowledgements

I would like to thank my supervisors Dr. Michael Boffa and Dr. Marlys Koschinsky firstly for allowing me to work in their laboratories on this very exciting research project. I have attained a new appreciation for the field of science, as well as gained many invaluable life lessons during my time here in the lab. Thank you again for all the helpful suggestions and counsel during the course of this research project.

I’d also like to thank my committee members Dr. Andrew Hubberstey and Dr. Sirinart Ananvoranich for their valuable insight and advice during this research project. Also, special thanks to Dr. Sirinart Ananvoranich for teaching me the difficult task of writing a grant proposal, an invaluable lesson that I will never forget. Also, for lending an ear and smile during the many trying days that are the life of a research scientist.

I’d also like to thank all my lab members for their continual support and motivation during this thesis. Thank you for all the memories and fun times. You all made the lab an enjoyable experience for me, one that I will never forget.

I’d like to thank Tanya, Zainab, Dragana, Jen, Branna, Anastassia, Tazeen, Justin, and all the past Boffa lab members. I’d also like to thank the members of the Koschinsky lab group: Corey, Rocco, Jackson, and Sera. Thank you everyone for all the helpful suggestions, as well as the daily entertainment in the lab.

I’d also like to thank my boyfriend Aaron for always believing in me and for his unwavering support over the last few years.

Lastly, I would like to thank my parents Giuseppe and Thérèse Rizzo for always pushing me to go farther in school and to follow my dreams wherever they may lead me.
Table of Contents

Declaration of Originality iii
Abstract iv
Dedication vi
Acknowledgments vii
List of Abbreviations xi
List of Figures xvi
List of Tables xviii

Chapter 1: Introduction

1.1 Coagulation, Fibrinolysis, and TAFI as a Molecular Link 1
1.2 CPB2, The Gene Encoding TAFI 5
1.3 TAFI Protein 9
1.4 Case Studies: Imbalances in TAFI and the Risk of Atherothrombotic Disease 12
1.5 TAFI and the Regulation of Inflammation 24
1.6 Platelet Pool of TAFI 28
1.7 Extra-Hepatic TAFI 29
1.8 The Discovery of TAFI in the Brain and Alternative Splicing of TAFI 31
1.9 Rationale, Hypothesis, and Thesis Objectives 37
Chapter 2: Materials and Methods

2.1 Minigene1 and Minigene2 Rationale 39

2.2 Construction of Plasmids 45

2.2.A. MiniGene1 Construction 45

2.2.B. MiniGene2 Construction 49

2.2.C. Full Length TAFI, and Alternatively Spliced TAFI Variants 51

2.3 Cell Culture 60

2.4 Transfection of Expression Plasmids for RT-PCR and qRT-PCR 61

2.4.A. Transfection of Minigene1-4A 61

2.4.B. Transfection of Minigene2-4A 62

2.5 RNA Extraction and Subsequent DNase Treatment of RNA 62

2.6 Reverse Transcriptase-Polymerase Chain Reaction 63

2.7 Real-Time Quantitative RT-PCR 63

2.8 Metabolic Labeling of Cells 65

2.8.A. Steady-State Labeling 65

2.8.B. Pulse-Chase Labeling 67

Chapter 3: Results

3.1 Identification of Minigene $CPB2$ mRNA in Several Cell Types Using RT-PCR 69

3.2 Quantification of Endogenous and Minigene $CPB2$ mRNA in Several Cell Types via Real Time RT-PCR 71

3.3 Identification of Full Length and Alternatively Spliced TAFI Variants via Metabolic Labeling 84
# Chapter 4: Discussion

4.1 Alternative Splicing and its Role in the Regulation of \textit{CPB2} Gene Expression  
4.2 Identification of Minigene \textit{CPB2} mRNA in Several Cell Types via RT-PCR  
4.3 Quantification of Endogenous and Minigene \textit{CPB2} mRNA in Several Cell Types via Real Time RT-PCR  
4.4 Identification of Full Length and Alternatively Spliced TAFI Variants via Metabolic Labeling  
4.5 Conclusion  

| References | 110 |

| VITA AUCTORIS | 120 |
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-UTR</td>
<td>3’-Untranslated Region</td>
</tr>
<tr>
<td>5'-UTR</td>
<td>5’-Untranslated Region</td>
</tr>
<tr>
<td>α-2-AP</td>
<td>α-2-Antiplasmin</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>APR</td>
<td>Acute Phase Response</td>
</tr>
<tr>
<td>AS</td>
<td>Alternative Splicing</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>β-APP</td>
<td>Beta-Amyloid Precursor Protein</td>
</tr>
<tr>
<td>BME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby Hamster Kidney Cells</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
</tr>
<tr>
<td>CCAAT/EBP</td>
<td>CCAAT/-Enhancer Binding Protein</td>
</tr>
<tr>
<td>CE</td>
<td>Coronary Event</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>CPB2</td>
<td>Carboxypeptidase B2</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold Cycle</td>
</tr>
<tr>
<td>CVF</td>
<td>Cobra Venom Factor</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated Intravascular Coagulation</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep Vein Thrombosis</td>
</tr>
<tr>
<td>ε-ACA</td>
<td>Epsilon Aminocaproic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ESEs</td>
<td>Exon Splicing Enhancers</td>
</tr>
<tr>
<td>ESSs</td>
<td>Exon Splicing Silencers</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxyfluorescein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDPs</td>
<td>Fibrin Degradation Products</td>
</tr>
<tr>
<td>Fibrin’</td>
<td>Partially Degraded Fibrin</td>
</tr>
<tr>
<td>FL</td>
<td>Full Length</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GEMSA</td>
<td>2-guanidinoethylmercaptosuccinic acid</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid Response Element</td>
</tr>
<tr>
<td>HBCPB</td>
<td>Human Brain Carboxypeptidase B</td>
</tr>
<tr>
<td>Hek293</td>
<td>Human Embryonic Kidney Cells</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatocellular Carcinoma Cells</td>
</tr>
<tr>
<td>HNF-1</td>
<td>Hepatic Nuclear Factor-1</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogeneous Nuclear Ribonucleoprotein</td>
</tr>
<tr>
<td>HPCPB</td>
<td>Human Plasma Carboxypeptidase B</td>
</tr>
<tr>
<td>HUGO</td>
<td>Human Genome Organization</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin -1β</td>
</tr>
<tr>
<td>Il-6</td>
<td>Interleukin -6</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl Thiogalactoside</td>
</tr>
<tr>
<td>ISEs</td>
<td>Intron Splicing Enhancers</td>
</tr>
<tr>
<td>ISSs</td>
<td>Intron Splicing Silencers</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LCI</td>
<td>Leech Carboxypeptidase Inhibitor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium</td>
</tr>
<tr>
<td>MERGEPTA</td>
<td>DL-2-mercaptomethyl-3-guaninoethyl-thio-propanoic acid</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple Organ Dysfunction Syndrome</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>NCS</td>
<td>Newborn Calf Serum</td>
</tr>
<tr>
<td>NFQ-MGB</td>
<td>Nonfluorescent Quencher - Minor Groove Binder</td>
</tr>
<tr>
<td>NF-Y</td>
<td>Nuclear Factor-Y</td>
</tr>
<tr>
<td>NRT</td>
<td>No Reverse Transcriptase</td>
</tr>
<tr>
<td>NTC</td>
<td>No Template Control</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral Arterial Disease</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PBMNC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
</tbody>
</table>
pCMV  Cytomegalovirus Promoter
PIPES  1,4-Piperazinediethanesulfonic acid
PMA  Phorbol Myristate Acetate
PTCI  Potato Carboxypeptidase Inhibitor
RIPA Buffer  Radioimmunoprecipitation assay buffer
RRM  RNA-Recognition Motif
RS Domain  Arginine/Serine Domain
RT-PCR  Reverse Transcriptase-Polymerase Chain Reaction
SDS-PAGE  Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM  Standard Error of the Mean
SR Protein  Serine/Arginine Protein
SNPs  Single Nucleotide Polymorphisms
snRNA  Small Nuclear RNA
TAFI  Thrombin-Activable Fibrinolysis Inhibitor
TAFIa  Activated Thrombin-Activable Fibrinolysis Inhibitor
TAFIai  TAFIa inactivated
TAFI-AP  TAFI-Activation Peptide
TF  Tissue Factor
TIA  Transient Ischemic Attack
TNFα  Tumor Necrosis Factor alpha
t-PA  tissue-type Plasminogen Activator
TTP  Tristetrapolin
TXA2  Thromboxane-a2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Va</td>
<td>Activated Factor V</td>
</tr>
<tr>
<td>VIIa</td>
<td>Activated Factor VII</td>
</tr>
<tr>
<td>vWf</td>
<td>von Willebrand Factor</td>
</tr>
<tr>
<td>Xa</td>
<td>Activated Factor X</td>
</tr>
<tr>
<td>Xgal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside</td>
</tr>
</tbody>
</table>
List of Figures

1.1. Coagulation and Fibrinolytic Pathways with TAFI as a Molecular Link 3

2.1. Plasmid Construction of Minigene1 41
2.2. Plasmid Construction of Minigene2 42
2.3. Splice Variants of Minigene1 43
2.4. Splice Variants of Minigene2 44
2.5. Plasmid Construction of Full Length TAFI Variants 52
2.6. Plasmid Construction of Δ7 TAFI Variants 53
2.7. Plasmid Construction of Δ11 TAFI Variants 54
2.8. Plasmid Construction of Δ7+11 TAFI Variants 55

3.1. Detection of Minigene1-4A and Minigene2-4A mRNA Using EGFP Primer Set 70
3.2. Standard Curve of Full Length CPB2 Standards for Full Length Endogenous CPB2 and Full Length Minigene 1 Absolute Quantification for Real Time PCR Analysis 73
3.3. Standard Curve of Δ7 CPB2 Standards for Δ7 Endogenous CPB2 and Δ7 Minigene 1 Absolute Quantification for Real Time PCR Analysis 74
3.4. Standard Curve of Δ7+8 CPB2 Standards for Δ7+8 Endogenous CPB2 and Δ7+8 Minigene 1 Absolute Quantification for Real Time PCR Analysis 75
3.5. Standard Curve of Full Length CPB2 Standards for Full Length Endogenous CPB2 and Full Length Minigene 2 Absolute Quantification for Real Time PCR Analysis 76
3.6. Standard Curve of Δ11 CPB2 Standards for Δ11 Endogenous CPB2 and Δ11 Minigene 2 Absolute Quantification for Real Time PCR Analysis 77
3.7. Quantitative Analysis of Endogenous and Minigene1 Full Length CPB2 mRNA in Several Cell Types 78
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8</td>
<td>Quantitative Analysis of Endogenous and Minigene1 $\Delta 7 \ CPB2$ mRNA in Several Cell Types</td>
<td>79</td>
</tr>
<tr>
<td>3.9</td>
<td>Quantitative Analysis of Endogenous and Minigene1 $\Delta 7+8 \ CPB2$ mRNA in Several Cell Types</td>
<td>80</td>
</tr>
<tr>
<td>3.10</td>
<td>Quantitative Analysis of Endogenous and Minigene2 Full Length $CPB2$ mRNA in Several Cell Types</td>
<td>81</td>
</tr>
<tr>
<td>3.11</td>
<td>Quantitative Analysis of Endogenous and Minigene2 $\Delta 11 \ CPB2$ mRNA in Several Cell Types</td>
<td>82</td>
</tr>
<tr>
<td>3.12</td>
<td>Identification of Full Length and Alternatively Spliced TAFI Variants in HepG2 Cells Using Steady State Metabolic Labeling and Immunoprecipitation</td>
<td>87</td>
</tr>
<tr>
<td>3.13</td>
<td>Identification of Full Length and Alternatively Spliced TAFI Variants in BHK Cells Using Steady State Metabolic Labeling and Immunoprecipitation</td>
<td>88</td>
</tr>
<tr>
<td>3.14</td>
<td>Identification of Full Length TAFI in HepG2 Cells Using Pulse Chase Metabolic Labeling and Immunoprecipitation</td>
<td>89</td>
</tr>
<tr>
<td>3.15</td>
<td>Identification of $\Delta 7$ TAFI in HepG2 Cells Using Pulse Chase Metabolic Labeling and Immunoprecipitation in HepG2 Cells</td>
<td>90</td>
</tr>
</tbody>
</table>
## List of Tables

2.1. Primer Sequences Used to Construct Minigene1

2.2. Primer Sequences Used to Construct Minigene2

2.3. Primer Sequences Used to Construct the Alternatively Spliced TAFI Variants

2.4. Probe and Primer Sequences Used For Real-Time RT – PCR

3.1. Summary Tables of Real Time PCR Analysis of Full Length *CPB2* and Alternatively Spliced Minigene *CPB2* Variants
Chapter 1: Introduction

1.1 Coagulation, Fibrinolysis and TAFI as a Molecular Link

Maintenance of the fluidity of blood within the vasculature is a key factor required for survival by multicellular organisms (1). Trauma induces the hemostatic response to reduce the probability of death by hemorrhaging (2). The first step in the hemostatic response is the formation of the platelet plug, via platelet adhesion, activation and aggregation (2). Following the initial platelet plug formation, the coagulation pathway is activated and results in the production of fibrin, which helps to maintain the structure and integrity of the blood clot (1). Subsequently, the fibrinolytic pathway is initiated to remove the clot after the damage at the vessel wall has been repaired (1). The balance between the deposition and the removal of fibrin is crucial to the maintenance of hemostasis within the organism (1). Imbalances in the functioning of either pathway can lead to various pathological disorders, such as an increased risk of thrombosis or bleeding (1). Thus, precise regulation of these pathways as well as communication between them is key for survival.

Endothelial cells play an important role in separating the tissues from the dynamic environment occurring within the bloodstream (3). Platelets are cell fragments that are produced by megakaryocytes of the bone marrow and then released into the bloodstream (3). There, they play an important role in surveying the endothelium for lesions (3). Under normal circumstances, circulating platelets do not adhere to the endothelial cells of the vasculature (3). However, when a breach in the integrity of the vasculature ensues,
three important successive events must occur before the coagulation cascade is initiated, which include: platelet adhesion, platelet activation and platelet aggregation (4).

Trauma induced rupture of the endothelium results in the exposure of sub-endothelium collagen (2). Neighboring intact endothelial cells trigger the release of von Willebrand factor (vWf), a multimeric glycoprotein that attaches to the collagen via a glycoprotein anchor (2). Circulating platelets are attracted to the collagen-vWf complex, and bind to it through glycoprotein Ib on the surface of platelet membranes (2). Platelet adhesion results in the activation of the platelets and subsequent release of the contents of their intracellular granules (2). Release of Adenosine Diphosphate (ADP) causes the platelets to undergo a morphological change in shape, as well as release of Thromboxane A₂, (TXA₂) (2). TXA₂ leads to the increased recruitment of circulating platelets to the site of injury, and subsequent adherence and activation of these platelets to form the primary hemostatic structure, the platelet plug (2). Activated platelets also release large stores of intracellular calcium, which are required for several reactions in the coagulation cascade (2). Lastly, activated platelets undergo a conformational rearrangement of their surface membrane phospholipids (4). Phosphatidylserine is exposed on the platelet surface, and helps facilitate the binding of coagulation cascade cofactors, Factor V, and Factor VIII to the platelet surfaces (4).

Once the initial platelet plug is formed, the coagulation cascade (Figure 1.1) is activated when an endothelial cell membrane bound protein, Tissue Factor (TF), is exposed to flowing blood (5). TF binds Factor VII within the plasma, and activates it to Factor VIIa (5). When TF concentrations are high, this complex (TF-VIIa) has the ability
Figure 1.1 Coagulation and Fibrinolytic Pathways with TAFI as a Molecular Link

**A.** The coagulation cascade is activated upon injury to the endothelial vessel wall, which results in the exposure of Tissue Factor (TF) to the bloodstream. Circulating Factor VII will bind TF to become activated Factor VII, VIIa. On the surface of activated platelets, Factor X is activated (Xa), which will go on to activate the zymogen prothrombin to thrombin. Thrombin will convert soluble fibrinogen to insoluble fibrin, which will help strengthen the initial blood clot formed by the aggregation of platelets at the site of vessel injury.

**B.** Plasmin-mediated fibrinolysis is induced following the activation of plasminogen to plasmin via tissue-type plasminogen activator (tPA). Plasmin cleavage of the thrombus results in clot lysis and subsequent fibrin degradation products (FDPs). Plasminogen activator inhibitor 1 (PAI-1) inhibits the activation of plasminogen to plasmin by inhibiting tPA. α-2-Antiplasmin (α-2-AP) binds free circulating plasmin and inactivates it, thus inhibiting clot lysis. Thrombin Activatable Fibrinolysis Inhibitor (TAFI) cleaves carboxyl-terminal lysine residues on partially degraded fibrin, thus inhibiting the binding of tPA and plasminogen to the fibrin surface for plasminogen activation to plasmin.
to activate Factor X to Factor Xa on the surfaces of activated platelets (5). In the presence of calcium and negatively charged phospholipids (such as on the surface of activated platelets), Factor Xa, along with its cofactor Factor Va, results in the formation of thrombin from the inactive zymogen, prothrombin. Thrombin activation is a key step in the coagulation cascade, and results in the conversion of soluble fibrinogen into insoluble fibrin. Insoluble fibrin strands helps to stabilize the initial blood clot formed by the aggregation of platelets at the site of vessel injury.

Once fibrin has been produced, the fibrinolytic pathway is initiated (6) (Figure 1.1). Plasmin is a serine protease capable of cleaving fibrin strands into fibrin degradation products (FDPs). Plasmin is formed from cleavage of the zymogen plasminogen by tissue-type plasminogen activator (t-PA). In the absence of fibrin, t-PA is an extremely inefficient activator of plasminogen. Fibrin acts a cofactor to bind both of these components to stimulate plasminogen activation, hence localizing biologically significant plasminogen activation to where it is required (7). Plasmin activity is countered by the presence of fast-acting serpin inhibitors, principally antiplasmin.

Since plasmin cleaves polypeptides after basic residues, limited cleavage of fibrin results in the formation of a form of fibrin containing carboxyl-terminal lysine residues (fibrin’) (Figure 1.1). These residues serve as binding sites for plasminogen and t-PA, thereby accelerating the rate of plasmin formation and leading to positive feedback in the fibrinolytic cascade (7). The carboxyl-terminal lysine residues also promote another positive feedback step in fibrinolysis, the plasmin-mediated conversion of native Glu-plasminogen to Lys-plasminogen, which binds fibrin more avidly and which is a better substrate for t-PA (7). The carboxyl-terminal lysines also bind to plasmin and hence
protect the enzyme from consumption by antiplasmin (7). Altogether, the various positive feedback loops in the fibrinolytic cascade result in increased production of plasmin, ultimately leading to dissolution of the fibrin clot (8).

A key regulator and intermediary between the coagulation and fibrinolytic pathways is Thrombin-Activable Fibrinolysis Inhibitor, TAFI. Activated TAFI (TAFIa) cleaves carboxyl-terminal lysine and arginine residues from fibrin’, which results in decreased binding sites available for plasminogen and t-PA to bind to the fibrin’ surface (9) (Figure 1.1). This results in the inhibition of positive feedback in the fibrinolytic cascade with reduced plasmin formation, thus attenuating the lysis of the blood clot. Therefore, TAFI is a direct link between the coagulation and fibrinolytic pathways, and performs a key regulatory function in the removal of the fibrin clot (10).

1.2 CPB2, The Gene Encoding TAFI

The human gene encoding TAFI is CPB2 (Carboxypeptidase B2 by the Human Genome Organization (HUGO)), and is located on chromosome 13 (13q14.11) (11). The gene spans 48 kb of genomic DNA, and has 11 exons and 10 introns (12). The position of the exon/intron boundaries established in CPB2 were found to be the same as in the genes that TAFI is evolutionarily related to, such as the pancreatic and mast cell carboxypeptidases (13).

Characterization of the 5’ flanking region of the CPB2 gene resulted in identification of the promoter, which lacks a consensus TATA sequence (12). Accordingly, several
transcription initiation sites were identified in liver, which is the site of expression of
*CPB2* leading to secretion of TAFI into plasma. Based on which transcription initiation
start site is used, various *CPB2* transcripts exist with 5’ untranslated regions (5’-UTR)
ranging from 9 to 46 bps in length (12). Through successive deletions of the human
*CPB2* promoter, a 70 bp sequence was identified between -141 and -72 that is required
for the liver specific expression of *CPB2* (12). Deletion of this sequence within the *CPB2*
promoter was found to eliminate the expression of a reporter gene driven by the *CPB2*
promoter in Hepatocellular carcinoma cells (HepG2) (12).

Our lab has extensively studied the proximal region of the *CPB2* promoter
between nucleotides -424 and +16. Multiple liver transcription factor binding sites were
discovered using *in vitro* DNaseI footprinting, and are designated Site A through Site J,
with Site A proximal to the initiator methionine sequence (14). Analysis of these sites
revealed that CCAAT/-enhancer binding protein (CCAAT/EBP) binds at Site A, Nuclear
Factor –Y (NF-Y) binds at Site B, and Glucocorticoid Receptor (GR) binds the
Glucocorticoid Response Element (GRE) at Site C (15, 14, 16). Furthermore, Hepatic
Nuclear Factor-1 (HNF-1) was also found to bind in the region midway between Site B
and Site C (14). All of these transcription factor binding sites are important for the liver
specific expression of *CPB2*, with the exception of the GRE, which mediates increased
*CPB2* transcription in response to the synthetic glucocorticoid dexamethasone (16).

To date, there are 19 single nucleotide polymorphisms (SNPs) documented within
*CPB2* (1). There are ten SNPs located within the 5’ flanking region, six within the coding
region and three within the 3’ flanking region (1). With respect to the SNPs found within
the 5’-UTR, none have been shown to have a significant effect on the activity of the
Of the six within the coding region, only two result in an amino acid substitution. At nucleotide position +505, an adenine substitution for a guanine results in an alanine substitution for a threonine at amino acid position 147 (18). At nucleotide position +1040, a cytosine substitution for a thymine results in a threonine substitution for an isoleucine at amino acid position 325 (19). The amino acid substitution at position 147 was found not to have an effect on TAFI activation, TAFIa stability or antifibrinolytic activity of TAFI (18). However, the Ile substitution at 325 resulted in a two-fold more stable form of TAFIa at 37°C than the variant containing Thr. The Ile325 variant thus has a greater antifibrinolytic effect (20).

Examination of the 3′-untranslated region (3′-UTR) of the CPB2 mRNA resulted in the discovery of three different polyadenylation sites, which are located at +1677, +1710 and +1836, yielding 3′-UTR’s of the following lengths: 390, 423, and 549 nucleotides, respectively (12, 21). Our laboratory has shown that the length of the 3′-UTR determines the stability of the mRNA transcript, with the stability of the transcript decreasing as the length of the 3′-UTR increases (21). The half-lives of the CPB2 transcripts, from shortest to longest, were determined to be: 5.1 hours, 3.3 hours, and 2.6 hours (21). Furthermore, we have also demonstrated that the rate of usage of each polyadenylation site can be modified through the treatment with various cytokines (21). Treatment of HepG2 cells with interleukins 1β (IL-1β) and interleukin 6 (IL-6) in combination results in a 2-fold decline in the stability of the transcript, as well as a 60% reduction in the mRNA abundance (16). With respect to usage of each polyadenylation site without cytokine treatment, it was found that the first polyadenylation site was determined to be used 28 times more frequently than the last polyadenylation site, and the
second polyadenylation site was utilized three times more frequently than the last polyadenylation site (21). In addition, it was also noted that under cytokine treatment, the longest transcript, which normally accounts for less than 1% of the total polyA forms, became the dominant transcript under these conditions (21). Therefore, the decrease in mRNA abundance that is observed with this treatment is attributable to a shift in use of polyadenylation sites, which selects for the most unstable transcript, as well as specific destabilization (2-fold decrease in half-life) of the longest and most unstable transcript (21).

One very well established class of instability elements within the 3’-UTR of an mRNA transcript is the A/U rich elements. A/U rich elements are generally 50 to 150 base pairs in length, are rich in U nucleotides, and can either contain multiple copies of the pentameric sequence, AUUUA, or the nonameric sequence, UUAUUUAAUU. The TAFI 3’-UTR has been found to contain one pentameric sequence, AUUUA, at nucleotide +1325, and is fairly A/U rich in content as well (21).

In a subsequent study conducted by our laboratory, cis-acting elements were found in the 3’-UTR of CPB2 mRNA (22). One stability and three instability elements were identified. In the segment common to all three transcripts lies the stability element and also one of the three instability elements. The second instability element lies between the first and the second polyadenylation site, and the third instability element lies between the second and the third polyadenylation site. Furthermore, gel mobility shift assays and bioinformatic analysis revealed that tristetrapolin (TTP) is a trans-acting factor protein that could bind at nucleotides +1668 to +1677 in the 3’-UTR of CPB2 mRNA. Tristetrapolin is a zinc finger RNA binding protein capable of destabilizing
mRNA transcripts by encouraging deadenylation of the polyA tail of the transcript (23). An RNA radiolabeled probe that contained a mutated TTP binding site resulted in increased stability of a β-globin CPB2 fusion transcript. In addition, knockdown of TTP in the presence of a β-globin CPB2 fusion transcript with an intact TTP binding site also resulted in increased stability of a β-globin CPB2 transcript (22). Together these results indicate that TTP is an important trans-acting factor protein that can negatively regulate CPB2 mRNA transcript stability (22).

1.3 TAFI Protein

Four different research groups independently discovered TAFI between 1989 and 1995; hence it is also known as procarboxypeptidase U (due to its intrinsic instability) (24), procarboxypeptidase R (because it appeared to prefer carboxyl-terminal arginine residues) (25), plasma procarboxypeptidase B (due to its similarity to pancreatic carboxypeptidase B) (26), and TAFI (because it could inhibit fibrinolysis after activation by thrombin) (27).

The plasma pool of TAFI is synthesized within the liver as a pre-pro-peptide of 423 amino acids (26). Upon secretion from the cell, the amino-terminal signal peptide of 22 amino acids is cleaved off to form the mature TAFI zymogen, which is 401 amino acids in length (26). The mature TAFI protein consists of two domains: an activation peptide (92 amino acids in length) and a catalytic domain (309 amino acids in length). Although the predicted molecular weight of TAFI is 46 kDa, TAFI has four sites for addition of N-
linked glycans and therefore migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at ~ 60 kDa (28, 26). The four glycosylation sites are located within the activation domain (Asn$_{22}$, Asn$_{51}$, Asn$_{63}$, Asn$_{86}$), and are always glycosylated (27).

TAFI can be activated through proteolytic cleavage at Arg$_{92}$, by thrombin, plasmin, or thrombin in complex with thrombomodulin (28, 26, 29). Activation of TAFI results in the release of the activation peptide from TAFI to form the active enzyme, TAFIa, which consists of 309 amino acids and migrates on SDS-PAGE at approximately 35 kDa (30). Thrombin and plasmin are relatively weak activators of TAFI, requiring large amounts of each enzyme, as well as long incubation periods with TAFI (30). However, activation of TAFI by thrombin in complex with the endothelial cell receptor thrombomodulin, results in over a 1000-fold increase in the rate of TAFI activation (29). Thus, thrombin in complex with thrombomodulin is thought to be the main physiological activator of TAFI (29). It is also important to note that glycosaminoglycans can accelerate TAFI activation by plasmin up to 20-fold (31).

TAFIa has basic carboxypeptidase activity: it cleaves carboxyl-terminal lysines and arginines from TAFIa substrates such as partially-degraded fibrin. The ability of TAFIa to attenuate clot lysis occurs through a threshold-dependent mechanism (32, 33). If the TAFIa concentration remains above its threshold it can effectively slow down the conversion of plasminogen to plasmin, and thus increase clot lysis time. However, as soon as the TAFIa concentration falls below its threshold, fibrinolysis rate is accelerated. The TAFIa threshold value is dependent on the following factors: rate at which TAFI is
converted to TAFIa, the intrinsic instability of TAFIa, concentration of plasmin activators (t-PA), and plasmin inhibitors (antiplasmin).

Currently, there is no known physiological inhibitor of TAFIa in plasma (34). However, TAFIa activity can be regulated through its own intrinsic instability (35). The half-life of TAFIa is 8 (Thr325) or 15 (Ile325) minutes at 37°C, 45 minutes at 30°C, and several hours at 22°C (30). The intrinsic instability of TAFIa is mediated through a conformational change, which causes the protein to unfold (36). It has been demonstrated that the antifibrinolytic potency of TAFIa is directly related to its half-life (35) indicating that its conformational instability is the physiological mechanism by which TAFIa activity is controlled. Upon unfolding, several previously cryptic cleavage sites become available for proteolytic cleavage by thrombin at Arg\textsuperscript{302}, or by plasmin at Lys\textsuperscript{327} or Arg\textsuperscript{330} (37). Although the spontaneous unfolding of TAFIa is not thought to be reversible, these cleavages would certainly inactivate TAFIa. Moreover, there is evidence that plasmin can cleave TAFIa even in the absence of the conformational change (36).

Due to the zinc ion present in the active site of TAFIa, TAFIa is also sensitive to inactivation by chelating agents, such as o-phenanthroline, and ethylenediaminetetraacetic acid (EDTA) (38). TAFIa can also be inhibited by small synthetic inhibitors such as the lysine analogue epsilon aminocaproic acid (\(\varepsilon\)-ACA) (28, 30) and the arginine analogues 2-guanidinoethylmercapto succinate (GEMSA) (30) and DL-2-mercaptopentethyl-3-guaninoethyl-thio-propanoic acid (MERGEPTA), reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol (BME), and naturally occurring inhibitors in the potato (potato tuber carboxypeptidase inhibitor; PTCI) (39) and leech Hirudo medicinalis (leech carboxypeptidase inhibitor; LCI) (40).
1.4 Case Studies: Imbalances in TAFI and the Risk of Atherothrombotic Disease

Normally, a fine balance exists between the coagulation and fibrinolytic systems within the body. Changes within either cascade can result in an increased tendency to bleed or an increased susceptibility to ongoing thrombotic events (1). The formation of excessive thrombi can cause heart attacks or strokes. TAFI plays an important regulatory role in maintaining the balance between these two systems, and thus may serve as an important risk factor in the development of atherothrombotic disease. Currently, the role of TAFI in atherothrombotic diseases is unclear. However, it is postulated that elevated levels of TAFI may be linked to an increased risk for atherothrombotic disease.

Various assays have been developed in order to measure the different forms of TAFI. Due to the potential role of TAFI as a risk factor in atherothrombotic disease, several studies have measured the plasma TAFI concentration in healthy individuals in order to determine a baseline reference value. From these studies, it was found that the plasma TAFI concentration within the human population falls within the range of 73 – 275 nM (41, 42, 43). Thus, the plasma TAFI antigen levels in healthy individuals display a large amount of variability between individuals (42). Even within different ethnic groups, there are significant differences in the plasma TAFI levels observed. For instance, TAFI antigen levels were found to be significantly lower in a black African male group in comparison to age-matched Caucasian males (44).

Hence, many studies have been aimed at deducing the factors that contribute to the great diversity observed in plasma TAFI antigen levels in the human population. Besides environmental factors, heritable genetic factors have been an important area of
study. Polymorphisms within the 5'-UTR were shown not to affect \textit{CPB2} promoter activity and thus had no direct effect on \textit{CPB2} gene expression (13). Also, polymorphisms within the 3'-UTR could affect \textit{CPB2} mRNA transcript stability and thus mRNA abundance (13). Each of the three polymorphisms in this region were discovered to influence \textit{CPB2} mRNA stability in a manner partially in agreement with the effects of these polymorphisms on plasma concentrations of TAFI (17). Amino acid substitutions within the coding region could result in altered rates of TAFI protein synthesis or TAFI protein secretion from the cell (13), although this has yet to be documented directly. Thus, the \textit{CPB2} genotype can affect the plasma TAFI antigen levels one possesses, and studies have investigated how both of these factors contribute to an individual’s risk of developing atherothrombotic disease.

In a study conducted by Juhan-Vague and coworkers (45), the relationship between plasma TAFI antigen levels and the presence of traditional cardiovascular risk factors was examined. Six hundred twenty six patients participating in primary prevention of coronary heart disease (CHD) at a metabolic ward were assessed for family history of heart disease, smoking use, drug use, alcohol consumption, hormonal therapy, body mass index, waist-to-hip circumference ratio, plasma lipids, diabetes, blood pressure, and physical activity level. In this study, they found TAFI antigen levels were associated with age in women, but not in men. Also, traditional cardiovascular risk factors were only able to explain a small proportion of the variability in plasma TAFI levels observed in this study group. Waist-to-hip circumference ratio accounted for 2% of the variability in plasma TAFI antigen levels in men, and 3% of the variability in plasma TAFI antigen levels in females. Thus, from this study it was concluded that traditional
cardiovascular risk factors, which can be indicative of environmental influences on an individual, play only a minor role in determining plasma TAFI antigen levels and that genetic influences may be involved.

In a study by Henry and coworkers (46), seven novel SNPs were discovered within the \textit{CPB2} gene, five in the 5’ flanking region: -2599 G/C, -2345 2G/1G, -1690 G/A, -1102 T/G and -438 A/G, and two in the 3’ flanking region: +1542 C/G and +1583 A/T, by thorough screening of 40 healthy individuals. All of the SNPs identified were found to be in strong linkage disequilibrium with each other, as well as with the SNP in the coding region, Ala147Thr, previously identified by Zhao and coworkers in 1998. Furthermore, all of the SNPs were found to be correlated with plasma TAFI antigen levels, and overall, were responsible for 20-52% of the variability in plasma TAFI antigen levels. This was the first study to show that genetic polymorphisms within the \textit{CPB2} gene could influence plasma TAFI antigen levels, which was previously postulated by Juhan-Vague in 2000 (45).

Moreover, another study by Franco and coworkers (47) also investigated the 5’ flanking region of the \textit{CPB2} gene for SNPs and their relationship to plasma TAFI antigen levels. By sequencing the 5’ flanking region, they found seven SNPs: three that were previously characterized by Henry and coworkers (46) (-1690 G/A, -1102 T/G and -438 A/G) and four novel SNPs (-1925 T/C, -1053 T/C, -530 C/T and -152 A/G). They found that -152 A/G, -530 C/T, -1925 T/C were in strong linkage disequilibrium with each other, and that -438 A/G, -1053 T/C, -1102 T/G, and -1690 AA were in strong linkage disequilibrium with each other. Five of the seven SNPs were found to be in close proximity to several established transcription factor-binding sites within the 5’ flanking region.
region of \textit{CPB2}. Thus, it was postulated that the SNPs might influence plasma TAFI antigen levels through transcription regulation processes. With respect to plasma TAFI antigen levels, -438 GG/ -1053 CC/ -1102 GG/ -1690AA homozygotes were found to have the highest plasma TAFI antigen levels versus heterozygotes (-438 AG/ -1053TC/ -1102 TG/ -1690 GA), and homozygotes (-438 AA/ -1053TT/ -1102TT/ -1690GG), the latter of whom had the lowest plasma TAFI antigen levels. These findings further supported the study by Henry and coworkers (46), who established that plasma TAFI antigen levels were, at least in part, under genetic control.

Furthermore, Brouwers and coworkers (19), identified a novel SNP within exon 9 of the coding region, +1040 C/T, which results in a threonine for isoleucine amino acid substitution at position 325 of the protein. Genotype and allele frequency analysis of 152 healthy blood donors determined that the +1040 CC (325Thr/Thr) genotype correlated with the highest plasma TAFI antigen levels while the +1040 TT (325Ile/Ile) genotype correlated with the lowest. Further characterization led to the discovery that the Ile325 TAFIa isoform had a half life of 16 minutes, in comparison to wild type TAFIa, which has a half life of 8 minutes (48). Thus, this SNP results in a TAFIa isoform that is twice as stable at body temperature, and therefore has the potential for greater antifibrinolytic activity. However, the +1040 TT genotype encoding the more stable Ile325 variant is also associated with the lowest plasma TAFI antigen concentrations, which may cancel out the effect of stability on antifibrinolytic potential. With respect to the other SNP-encoded amino acid substitution within the coding region, Ala147Thr, functional analysis determined that this amino acid substitution was found not to affect the ability of the TAFI isoform to become activated, or function as a basic carboxypeptidase B (18).
In a later study by Morange and coworkers (49) the association between \textit{CPB2} gene polymorphisms and risk of CHD was investigated. A total of six of the previously characterized SNPs were examined, two within the 5’-UTR (-2599 G/C and -438 A/G), two within the coding region (Ala147Thr and Thr325Ile), and two within the 3’-UTR (+1542 G/C and +1583 A/T), for their correlation to plasma TAFI antigen levels. It was discovered that 20% of the variability in plasma TAFI concentration within the population is due to SNPs within the \textit{CPB2} gene. This further supports the earlier study by Henry and coworkers (46), who found that SNPs within the \textit{CPB2} gene could explain between 20-52% of the variation in plasma TAFI concentration. However, none of the SNPs investigated were found to contribute to a greater risk of CHD. An important implication from this study is that only a fraction of the variation in plasma TAFI concentrations can be attributed to genetic factors; thus, the remainder of the variation arises from non-genetic factors such as regulation of \textit{CPB2} gene expression by external stimuli.

In a study conducted by van Tilburg and coworkers (50) the connection between elevated plasma TAFI antigen levels and a first incidence of deep vein thrombosis (DVT) was investigated in a large population-based control study, with 474 age and sex matched subjects. The effect of sex, age, and oral contraceptive use on plasma TAFI concentration levels was examined, as well as the relationship between elevated TAFI levels and other coagulation factors. From this study, it was determined that 14% of patients with a first deep vein thrombotic event had TAFI antigen levels greater than the 90th percentile, in comparison to controls, which only 9% had TAFI antigen levels greater than the 90th percentile. In men, there was no correlation found between elevated TAFI levels and risk
for thrombosis. However, in women, an age-dependent increase was observed.

Furthermore, elevated plasma TAFI levels were also observed amongst women between the ages 31-50 who used oral contraceptives, in comparison to those who did not. With regard to other coagulation factors, patients with TAFI levels above the 90th percentile were also found to have elevated thrombin levels, which is a known activator of TAFI. From this study, it was concluded that elevated TAFI antigen levels above the 90th percentile increased the risk for a first deep vein thrombotic event by two-fold.

In a second study conducted by Eichinger and coworkers (51) the association between elevated TAFI levels and risk for recurring venous thromboembolism was investigated. Six hundred patients with a first spontaneous venous thromboembolism were entered into this study. Plasma TAFI antigen levels were measured via the ImuClone TAFI enzyme linked immunosorbent assay (ELISA). In this study, they found that patients who had TAFI levels above the 75th percentile had a 14.5% probability of recurrence of venous thromboembolism, in comparison to 6.8% found in patients with TAFI levels below the 75th percentile. From this study, it was concluded that high TAFI levels increased the risk of recurrent venous thromboembolism nearly two-fold as well.

Furthermore, in a study conducted by Santamaria and coworkers (52) high functional TAFIa levels were correlated with an increased risk for ischemic stroke. In this study, functional TAFIa activity was examined in 114 patients who had experienced at least one ischemic stroke event in comparison to age-and sex-matched controls with no history of arterial disease. Blood was taken from subjects approximately one month after the initial stroke event, and TAFIa activity was measured after activation by thrombin-thrombomodulin in the presence of a synthetic substrate, hippuryl-arginine. It was found
that TAFIa levels were significantly higher in patients with ischemic stroke, in comparison to controls. Sex or age of the subject were both found not to be significant factors with respect to variation in TAFIa levels and risk of ischemic stroke. However, it was found that patients with a family history of arterial disease did have higher TAFIa levels in comparison to subjects without a family history. Overall, from this study, it was concluded that increased levels of functional TAFIa in subjects with a history of arterial disease increases the risk of ischemic stroke by six-fold.

In a second study conducted by Leebeek and coworkers (53) the association between elevated functional TAFI levels and incidence of a first only ischemic stroke event was investigated. In this study, 124 patients with a recent first ischemic stroke event were compared to age- and sex-matched controls. Blood was taken from patients within two weeks of the initial stroke event, and risk factors, such as smoking, hypertension, diabetes, hypercholesteremia, use of oral contraceptives, positive family history and previous DVT were assessed. Functional TAFIa levels were measured via a functional assay, the clot lysis assay (54). In this study, they found that patients with ischemic stroke had prolonged clot lysis times, which is indicative of elevated TAFIa levels. In addition, factors found not to affect TAFIa levels in ischemic stroke patients included gender, diabetes, and smoking. However, hypertension, hypercholesteremia, and age (>45 years) were found to be present in patients with ischemic stroke and increased TAFIa levels. Moreover, in a three-month follow-up, 36 patients out of the 124 were randomly selected, and TAFIa levels were re-measured. TAFIa levels were found to be consistently high in this subset, and thus it was concluded that increases in TAFIa levels
are not due to an acute phase response. From this study, it was also concluded that increased TAFIa levels are correlated with an elevated risk of ischemic stroke.

In a third study conducted by Ladenvall and coworkers (55), they examined the association between TAFI and ischemic stroke in more depth. Due to the diverse nature of stroke, and the various stroke subtypes, they employed a large population study including 600 white age-and sex-matched individuals, with one of five possible stroke sub-type classifications. Blood was taken from individuals at two time points: once within ten days of the initial stroke event and once at a three-month follow-up. Intact plasma TAFI levels and released TAFI activation peptide (TAFI-AP) levels were measured via sandwich ELISAs (56). In this study, they found that ischemic stroke patients of all subtypes had elevated intact TAFI and released TAFI-AP levels in comparison to controls. Furthermore, it was discovered that released TAFI-AP levels were higher at the three-month follow up in comparison to the initial measure. This study further supported the previous study completed by Leebeek and coworkers (53) that showed that elevated TAFI levels are associated with ischemic stroke, but this occurrence is not due to an acute phase response.

In addition, in a study conducted by de Bruijne and coworkers (57), TAFI levels and their connection to arterial thrombosis were evaluated. 327 patients (ages 18-45 for males or 18-55 for females) with a single CHD or ischemic stroke event were compared to 332 healthy controls. Only relatively young subjects were included since they would be expected to have minimal atherosclerosis, which would allow the researchers to investigate other possible risk factors contributing to cardiovascular disease development such as thrombophilia. Also, patients were only included if the event had occurred at least
one month prior to the study to exclude the possibility of elevated TAFI levels arising from an acute phase response. Intact plasma TAFI, released TAFI-AP, and inactive TAFIa (TAFIai) were all measured using three different sandwich ELISAs (56); TAFIa activity was also measured via a clot lysis assay. It was found that TAFIai levels were higher in both patient groups (i.e. CHD and ischemic stroke patients), in comparison to healthy controls. However, intact plasma TAFI, released TAFI-AP levels, and TAFIa activity were found to be similar between patients and controls, which is in contrast to the previous study, conducted by Ladenvall and coworkers (55), who reported elevated released TAFI-AP levels in patients with ischemic stroke. However, with respect to the CHD subgroup only, plasma levels of intact TAFI were also found to be elevated in comparison to controls. Furthermore, this study also investigated the role of SNPs within the CPB2 gene on plasma TAFI concentration and its association with arterial thrombosis. They found that individuals who are homozygous for the Ile325 isoform of TAFI had lower TAFI levels and a reduced risk for arterial thrombosis. Thus, from this study, it was concluded that young CHD and ischemic stroke patients had elevated TAFIai levels, and that individuals homozygous for the Ile325Ile SNP had a protective advantage against arterial thrombosis.

In a study conducted by Santamaria and coworkers (58), risk of acute coronary artery disease (CAD) was examined in 174 acute CAD patients in comparison to 211 controls with respect to TAFIa activity. They found that TAFIa activity levels were higher in patients with CAD compared to controls, and that individuals with high TAFIa levels had a four-fold increased risk of developing CAD compared to healthy controls. Interestingly, they also found no correlation between TAFIa levels and traditional
cardiovascular risk factors, as was previously reported by Juhan-Vague, and coworkers (45). However, in a later study by Meltzer and coworkers (59), TAFIa activity levels were examined for correlation to risk of myocardial infarction (MI). TAFIa levels were measured in 554 men with a first incidence of MI in comparison to 643 healthy controls. They found that individuals in the first quartile (i.e. having the lowest TAFIa levels) had a 3.4-fold increased risk of MI in comparison to individuals in the third quartile, who had a 2.4-fold increased risk of MI. This result is in contrast to the previous study by Santamaria and coworkers (58) who found a four-fold increased risk of CAD in individuals who had elevated concentrations of TAFIa.

In a later study conducted by de Bruijne and coworkers (60), the role of TAFI in young patients with peripheral arterial disease (PAD) was assessed. Forty-seven patients aged 21-55 with a first indication of PAD and 141 healthy controls were included in this study. Intact plasma TAFI, released TAFI-AP and TAFIai were measured in patients and controls using sandwich ELISAs (56). In addition, functional TAFI, TAFIa was measured via clot lysis assay (54). Intact plasma TAFI antigen levels were found to be significantly higher in patients with PAD in comparison to controls. Risk of PAD was found to increase by 18% in PAD patients with every 10% increase in intact plasma TAFI antigen levels. Released TAFI-AP and TAFIai were found not to be significantly different with respect to patients and controls. Also, levels of TAFIa were found to be marginally higher in patients with PAD, although this difference was not statistically significant. From this study, it was concluded that high intact TAFI plasma antigen levels are associated with increased risk of developing PAD.
In a study conducted by Jood and coworkers (61), the association between TAFI levels and risk of a future atherothrombotic event and/or death was investigated. Intact TAFI plasma levels and released TAFI-AP were measured in 517 patients three months after they experienced an acute ischemic stroke via sandwich ELISA (56). The survival rate of the patients two years after recruitment into the study was then analyzed. Composite endpoints for patients were defined as death, fatal or non-fatal recurrent stroke, transient ischemic attack (TIA), and coronary event (CE). At follow-up, 37 composite endpoints had occurred and it was found that patients who had experienced a composite endpoint had elevated released TAFI-AP levels. However, no statistically significant correlation was found between intact plasma TAFI levels and patients who developed a composite endpoint. Furthermore, the released TAFI-AP/intact TAFI ratio was assessed for its potential to predict the risk of developing a future composite endpoint. Unfortunately, no correlation was observed between the released TAFI-AP/intact TAFI ratio and composite endpoint. Thus, from this study it was shown that increased levels of released TAFI-AP but not intact TAFI correlated to an increased risk for a future atherothrombotic event and/or death in ischemic stroke survivors.

Taken together, the studies surrounding the question of whether or not TAFI is an important risk factor for atherothrombotic disease are somewhat contradictory. Some important factors to consider when analyzing results from prior clinical studies include: the method used to measure TAFI, which TAFI isoform was measured, and whether the study took into account CPB2 genotype. One of the main methods used to measure TAFI levels in the population are ELISAs (62). ELISAs utilize TAFI-specific antibodies in order to capture and measure TAFI antigen levels in a plasma sample. However, in a
study conducted by Gils and coworkers (63), 27 monoclonal antibodies were tested for their reactivity to the different forms of TAFI (intact TAFI, released TAFI-AP, and TAFIai). It was found that all the antibodies tested had different reactivities towards the different forms of TAFI. Some of the antibodies only reacted with the inactive zymogen, while others reacted with the inactive zymogen, activated enzyme peptide, as well as the inactivated enzyme forms. In addition, they also found that some of the commercially available ELISA kits had a partial dependence on the Thr/Ile325 isoform, with some kits having only 44% of the response to the Ile325 variant compared to the Thr325 variant (63). Therefore, conclusions made from studies in the past, which used these ELISAs to measure plasma TAFI antigen levels, should be re-evaluated (19, 45-46, 63-66).

Overall, there seems to be a clear relationship between increased TAFI levels and increased risk of venous thrombosis (50), recurrent venous thrombosis (51), and ischemic stroke (52, 53, 61). However, the data from studies pertaining to CHD have resulted in more inconsistent results (58, 59). A possible explanation for the differences in the uniformity of the results from the CHD studies in comparison to the venous thrombosis and ischemic stroke studies could be due to the differences in the structure of the vasculature in which the clot is formed. For example, veins are large blood vessels, which carry deoxygenated blood from the extremities back to the heart and lungs under low-pressure conditions (67). In contrast to the arteries, which carry oxygenated blood from the heart to the extremities under high-pressure conditions (67).

Furthermore, the characteristics of the clots that form within each area are also markedly different. In large arteries such as the coronary and carotid arteries, thrombi form usually because of rupture of atherosclerotic plaque and are typically platelet-rich
and fibrin-poor. Alternatively, in the venous system where atherosclerosis does not occur and thrombi most commonly form due to stasis, red blood cell- and fibrin-rich clots tend to form on undamaged endothelial walls (67). Hence, due to the differences in the environment of the vasculature, as well as the components and the structure of the actual clots in each environment, the relationship between TAFI and risk of developing atherothrombotic disease becomes quite complex.

Although the role of TAFI in regulating fibrinolysis has been extensively documented, proving its status as a risk factor for atherothrombotic disease has proven difficult due to the results of several conflicting studies. In addition, even though the relationship between elevated TAFI levels and risk of venous thrombosis and ischemic stroke has been more direct, the mechanism by which elevated TAFI levels contributes to venous thrombosis and ischemic stroke are still unknown. However, it is postulated that increased TAFIa levels results in a decrease in fibrinolysis, which results in a clot that is less likely to be lysed. Ultimately, while variation in plasma TAFI concentrations and functional TAFI levels due to genetic and non-genetic factors appears to correlate with vascular risk, more studies are required in order to clarify the role TAFI plays in the development of atherothrombotic disease.

1.5 TAFI and the Regulation of Inflammation

Although the primary function of TAFI was originally demonstrated to be inhibition of fibrinolysis through cleavage of carboxyl terminal lysine and arginine
residues from partially-degraded fibrin (41), many studies in the years following its discovery have uncovered evidence that TAFI also plays a role in the modulation of inflammation and the acute phase response. \( CPB2 \) mRNA levels and TAFI protein levels were first shown to be elevated in mice following treatment with lipopolysaccharide (LPS) (68). LPS is a major component of the cell membrane of gram-negative bacteria, and can induce an inflammatory response by activating the complement pathway (69).

Besides fibrin, TAFIa has also been shown to be able to cleave bradykinin (70), the complement anaphylatoxins, C3a and C5a (71), thrombin cleaved osteopontin, OPN (72), and plasmin cleaved chemerin (73). TAFIa cleavage of bradykinin, C3a and C5a, and thrombin cleaved OPN all result in inactivation of these pro-inflammatory mediators. In contrast, TAFIa cleavage of chemerin results in an increased activity of this anti-inflammatory mediator. Thus, the effect that TAFIa has shown to possess towards a diverse array of inflammatory related substrates demonstrates TAFI’s role as an anti-inflammatory mediator of the inflammatory response.

In assessing the role that established acute phase mediators had on \( CPB2 \) gene expression, it was uncovered that treatment of HepG2 cells with IL-1\( \beta \) and IL-6 in combination resulted in a 60% decrease in \( CPB2 \) mRNA abundance (16). However, no effect was observed on \( CPB2 \) promoter activity. Furthermore, treatment of HepG2 cells with a synthetic glucocorticoid, dexamethasone resulted in a 2-fold increase in \( CPB2 \) mRNA abundance. Further analysis led to the discovery of a glucocorticoid responsive element (GRE), at position -92 to -78 within the human \( CPB2 \) promoter. Glucocorticoids are hormones capable of down-regulating the acute phase response (APR) of the immune system (74). Glucocorticoids can reduce the APR by inhibiting the expression of many
pro-inflammatory cytokines, such as IL-1β, IL-6, and Tumor Necrosis Factor alpha (TNFα). Furthermore, they can also reduce the activation of the pro-inflammatory transcription factor NFκB by inducing the transcription of the NFκB inhibitor, IκB (74). Thus, glucocorticoids can increase plasma TAFI concentration levels during the APR of an inflammatory episode by reducing the expression of IL-1β, IL-6, and TNFα, which are known to down-regulate CPB2 mRNA abundance.

The ability of TAFI to regulate inflammation within a hyper-inflammatory system was studied by Asai and coworkers (69). With the knowledge that LPS treatment of mice causes an up-regulation of CPB2 mRNA and TAFI protein levels, Asai and coworkers (64), created TAFI deficient mice to assess TAFI’s role in a hyper-inflammatory state. Mice were treated with cobra venom factor (CVF), which is known to activate the complement cascade of the immune system. Surprisingly, there was no difference in survival between TAFI deficient, heterozygous, or wild-type mice. However, when the mice were pretreated with a sub-lethal dose of LPS prior to CVF treatment, 60% of TAFI deficient mice died, compared to no death amongst the heterozygous or wild-type mice. During an inflammatory response, complement activation leads to an increase in plasma C5a, and LPS can induce the up-regulation of the C5aR. TAFIa has been previously shown to be able to cleave C5a, and thus down regulate its pro-inflammatory effects. TAFI deficient mice could not overcome the hyper inflammatory state induced by the presence of excessive C5a, and thus mortality resulted. This study effectively demonstrated the important role TAFI plays in inflammation in mice.

Studies in humans have provided contradictory results in comparison to results obtained using mouse models. In a study conducted by Watanabe and coworkers (75), 36
patients with disseminated intravascular coagulation (DIC) sepsis induced multiple organ
dysfunction syndrome (MODS), were found to have significantly lower TAFI antigen
and functional TAFIa levels in comparison to healthy controls. The results obtained from
this clinical study were further supported by a later study conducted by Zeerleder and
coworkers (76), who also investigated the association between TAFI antigen levels and
TAFIa activity in patients with severe sepsis. This study also reported significantly
decreased levels of TAFI and TAFIa in patients with severe sepsis, in comparison to
healthy controls.

A possible explanation for the discrepancy in TAFI levels between the two
species during an inflammatory episode came from a study conducted by Garand and
coworkers (77). In this study, they investigated the effect of another known acute phase
mediator TNFα and its effect on the expression of CPB2 in an inflammatory system.
Treatment of primary mouse hepatocytes with TNFα for 12-48 hours resulted in a two-
fold increase of CPB2 mRNA, a 2.7 fold increase in TAFI protein levels after 48 hours of
treatment with TNFα, and an increase in mouse CPB2 promoter activity by 1.5 fold.
Further analysis of the 5’ flanking region resulted in the discovery of a NFκB site within
the mouse promoter region that is not conserved in humans. Hence, in mice undergoing
an inflammatory response, activation of TNFα results in the translocation of the NFκB
transcription factor to the nucleus where it binds CPB2, and up-regulates its expression.

Overall, the precise role of TAFI in inflammation is still emerging. The ability of
TAFI to modulate the inflammatory response through inactivation of various pro-
flammatory cytokines and activation of anti-inflammatory cytokines demonstrates the
diverse mechanisms by which TAFI can affect the APR. Also, glucocorticoid
enhancement of plasma TAFI concentrations during an inflammatory state suggests that TAFI is required during a host’s immune system attack in order to reduce the likelihood of developing sepsis (16). Sepsis is a state characterized by extreme stimulation of a host’s immune system, and often is accompanied by the induction of a pro-coagulant state in the host (76). Thus, TAFI serves as an important molecular link between the coagulation cascade and the inflammatory system.

1.6 Platelet Pool of TAFI

Mosnier and coworkers (78) discovered a second pool of TAFI in the alpha granules of platelets. CPB2 mRNA was detected within the well-characterized Dami megakaryocytic cell line. The detection of CPB2 mRNA within this cell line suggests that these cells are capable of producing their own endogenous TAFI source in contrast to uptake of TAFI via endocytosis from the cell’s surroundings. Platelet TAFI was reported to be smaller (~ 50 kDa) than plasma TAFI (~60 kDa), which was due to differential glycosylation patterns. Removal of the glycosaminoglycans from both forms of TAFI resulted in the expected migration of hypo-glycosylated TAFI at a MW of ~ 47 kDa on SDS-PAGE (78). Platelet TAFI is similar functionally to plasma TAFI in that it is effectively activated by thrombin in complex with thrombomodulin, and is unstable at 37°C (78). Activated platelets can release platelet TAFI to the site of injury, which can also contribute to the antifibrinolytic effect of TAFI (78).
In a latter study conducted by Schadinger and coworkers (79), however, it was found that there is no difference in the electrophoretic mobility of platelet-derived TAFI in comparison to plasma-derived TAFI. In this study, both sources of TAFI were found to migrate on SDS-PAGE at approximately 58 kDa, thus they are not as drastically different in size as was formerly reported (78). This study was also the first to show that platelet-derived TAFI could function independently of plasma-derived TAFI to attenuate the lysis of a platelet-rich thrombus (79). Platelet-rich thrombi are characteristically more resilient to fibrinolysis than clots consisting of fibrin only (80). Thus, the secretion of TAFI from the alpha granules of platelets during a thrombotic event advocates for a fibrinolytic regulatory role of platelet derived TAFI independent of plasma derived TAFI. One such role might be that platelet-derived TAFI acts from within the thrombus to prevent the breakdown of the clot, while plasma-derived TAFI performs its antifibrinolytic functions from the exterior of the clot (79). Thus, it is evident that activation of platelet-derived TAFI at a site of vessel injury could have a substantial effect on the fibrinolytic cascade.

1.7 Extra-Hepatic TAFI

The plasma pool of TAFI arising from expression of \textit{CPB2} in the liver contributes the majority of the TAFI circulating within the vasculature. In addition to the discovery of a second pool of TAFI located within the alpha granules of platelets, further investigation of TAFI within other tissues led to the finding of \textit{CPB2} mRNA within adipose tissue (81), as well as human umbilical vein endothelial cells (HUVECs) (81) and human
peripheral blood mononuclear cells (PBMNC) using RT-PCR. However, no associated TAFI protein was identified in these studies.

Since these initial discoveries, our laboratory has addressed the question of other extra-hepatic TAFI sites in a more systematic and rigorous fashion. A study by Lin and coworkers (82) led to the discovery of CPB2 mRNA within the leukemic monocyte cell line THP-1 and re-confirmed the expression of CPB2 mRNA within PBMNCs. In addition, low TAFI protein levels were also identified from the medium of THP-1 cells differentiated into macrophages using a highly sensitive assay for TAFIa detection (82). Furthermore, CPB2 mRNA expression was also re-confirmed in Dami cells, which is a well-characterized megakaryocytic cell line (82).

Next, the researchers were interested in the association between stage of differentiation and the level of CPB2 mRNA levels. Dami cells were treated with Phorbol Myristate Acetate (PMA), which caused the cells to differentiate further along their megakaryocytic cell lineage. CPB2 mRNA levels detected in Dami cells after differentiation were found to be 4.5 fold higher than in un-differentiated Dami cells. Hence, stage of differentiation was found to be an important factor in determining CPB2 mRNA abundance levels in these cells (82). Moreover, low TAFI protein levels were also identified in the medium of differentiated Dami cells by western blot analysis. By using a highly quantitative TAFIa assay, it was shown that the concentration of TAFI within the medium of differentiated Dami cells is about one seventh the concentration of TAFI within the medium of HepG2 cells. (82).

The discovery of CPB2 mRNA within the THP-1 monocytic cell line and PBMNC is very intriguing because monocytes and macrophages are very important cells with
respect to the inflammatory response. Upon initiation of an immune response, circulating monocytes enter an infected tissue, where they differentiate into macrophages. Macrophages can engulf pathogens, cellular debris, and can support an inflammatory response. TAFIa has been previously shown to be able to cleave pro-inflammatory cytokines, such as C3a and C5a of the complement system to inactivate them (71, 72), which further supports the idea that TAFI has a role in the modulation of the inflammatory response. The extra-hepatic pool of TAFI within monocytes and macrophages suggests that TAFI may have other functions besides its ability to attenuate fibrinolysis including, but not limited to, effects on inflammation.

1.8 Discovery of TAFI in the Brain and Alternative Splicing of TAFI

In a study by Matsumoto and coworkers (83), a novel alternatively-spliced form of TAFI was discovered. Through northern blot analysis of RNA from seven different tissues, the expression of this form of TAFI was determined to be brain (hippocampus)-specific, and thus it was named Human Brain Carboxypeptidase B (HBCPB). In a later study by the same group, they were able to detect HBCPB in human serum and cerebrospinal fluid via western blot analysis (84).

From molecular sequence analysis, they discovered that HBCPB was highly related to CPB2, which is synthesized in the liver (83). However, HBCPB was found to have two major sequence differences with respect to CPB2. The first major difference in HBCPB is the deletion of exon 7, which results in a deletion of 37 amino acids within the region
spanning amino acids 198-234 of the mature protein. The second major structural
difference in HBCPB is due to a partial deletion in exon 11 of 52 base pairs between
nucleotides 1190-1241, which results in a deletion of 18 amino acids, and an addition of a
novel 14 amino acid residue carboxyl terminus. The exon 7 deletion results in an in-
frame deletion. However, the partial deletion in exon 11 results in an out of frame
deletion, and the use of new stop codon for translation. Outside of these changes, the
nucleotide sequences were identical between liver and brain transcripts, suggesting that
they arose from alternative splicing of the same primary transcript.

HBCPB was identified as a pre-pro-peptide of 40 kDa that is expressed in a
variety of healthy, human brain structures based on immunohistochemical analysis (85).
By using a novel anti-C14 antibody, which specifically recognizes the unique C-terminus
of HBCPB, they were able to show that HBCPB is expressed to a greater extent within
the hippocampus, amygdala, basal nucleus of Meynert, lateral geniculate body, and large
pyramidal neurons of the cerebral neo-cortex (85). In comparison to brain tissues from
patients with Alzheimer’s disease, a large reduction in HBCPB was most striking within
the hippocampus and amygdala, which are two structures that are known to be affected
by Alzheimer’s disease (85).

In vitro proteolytic analysis of HBCPB determined that the active form of
HBCPB, which is 30 kDa, could cleave β-amyloid precursor protein (β-APP) and its
derivative, Aβ1-42, into 10 to 12 kDa cleavage products (83, 86). Impaired processing of
β-APP results in amyloid plaque formation and aggregation within the brain (87).
Increased plaque formation interrupts signal transduction pathways between healthy
neurons, and can cause neuronal death over time. Neuronal death following increases in
plaque formation within the brain is a well-known characteristic in the pathology of Alzheimer’s disease. Alzheimer’s disease is a neurodegenerative disorder of the brain, which eventually results in dementia due to the progressive death of neurons over time (87). Thus, the discovery of a novel protease which can cleave β-APP into a non-amyloidogenic variant may prove significant for the development of a treatment for patients with Alzheimer’s disease.

A subsequent study by Cagliani and coworkers (88) investigated the regions between exon 6 and exon 7 (region one), and exon 9 to the 3’-UTR (region two) of CPB2 to re-confirm the alternative splicing events previously documented by Matsumoto and coworkers (83). First, they assessed the rate of nucleotide diversity between Europeans (United Kingdom), East Asians and Yorubans (West Africans) for region one and region two. It was found that there is a high degree of nucleotide diversity in region one across all three ethnic groups, which is indicative of balancing selection. Region two showed no significant difference in any ethnic group, and was concluded to be functioning under neutral evolution.

In addition, they were also able to demonstrate that the SNP located within exon 6 (+505A/G), which has previously been shown not to affect TAFI stability or enzyme activity (18), was preferentially associated with skipping of exon 7. Although the function of the variant lacking the residues encoded by exon 7 is currently unknown, it was suggested by the authors that balancing selection helps to maintain the alternatively spliced product within the population due to heterozygote advantage. The authors were also able to detect the wild-type and exon 7-lacking mRNA in HepG2 cells and in two different liver specimens by RT-PCR. However, amplicons indicative of the exon 11
alternative splicing event were not observed. The authors suggested that the reason why they were unable to detect the exon 11 alternative splicing event is either because of its brain tissue specificity or because its occurrence in the liver arises under different parameters than the ones they had investigated.

A study conducted by Lin and coworkers (89) set out to confirm these previous reports of alternatively spliced $CPB2$ mRNA in the brain and the liver, as well as to investigate the extent of splicing that occurs within tissues and cell types that are central to vascular biology. In addition, the ability of TAFI protein translated from the alternatively spliced $CPB2$ mRNA to function like TAFIa was also assessed. RNA from various human tissue samples was extracted and RT-PCR experiments were performed with various TAFI primer sets designed to identify the various alternatively spliced $CPB2$ mRNA variants. RT-PCR analysis revealed that alternatively spliced TAFI variants were found in all tissues tested, which included brain (cerebellum and cortex), testis, and liver. One of the novel and interesting findings from this study was the presence of full length $CPB2$ cDNA within the cerebellum and the cortex. This was the first report of full length $CPB2$ cDNA within the brain. mRNA missing exon 7 was also found in the cerebellum, liver and testis, but not in the cortex. mRNA containing alternatively spliced exon 11 was also identified in the cortex, cerebellum and testis, but not in the liver. The presence of exon 7-lacking mRNA within the liver and the absence of exon 11 alternative splicing from the liver were both in accordance with the previous report by Cagliani and coworkers (88).

Lin and coworkers (89) also used the same TAFI primer sets to analyze RNA extracted from platelets, HepG2 cells, and SH-SY5Y (neuroblastoma) cells. In SH-
SY5Y cells, the exon 7-lacking and exon 11 alternatively spliced variants were actually found to be the major transcripts present. As with the HepG2 cells, three cDNA species were amplified with a primer set flanking exon 7: two of the bands were identified as full length and exon 7-lacking CPB2 cDNA while the third represented a novel alternatively spliced variant lacking exon 7 and exon 8. This species was also detected in HepG2 cells, cerebellum, and testis. The skipping of both exon 7 and exon 8 results in a frameshift and premature termination 70 base pairs into exon 9. Thus, like HBCPB this variant has a novel carboxyl-terminus although it is 23 amino acids in length in comparison to the 14 amino acid unique C-terminus of HBCPB and is missing even more of the amino acid residues present in full-length TAFI (89).

In a prior study by Lin and coworkers (82), while trying to detect full length CPB2 mRNA within extra hepatic sites using RT-PCR, they noticed recurring bands of smaller sizes along with their expected full-length amplicons. With the new knowledge of alternative splicing events of CPB2 mRNA occurring within the brain and liver, they set out to determine if those bands they had seen previously in the megakaryocyte cell line (Dami) and monocytoid cell line (THP-1) likewise represented alternative splicing events. From the RT-PCR results, exon 7 skipping was also detected in differentiated Dami cells and THP-1 derived macrophages. In addition, exon 7 and 8 skipping was detectable in differentiated Dami cells, but to a lesser degree (89).

Lin and coworkers (89) also assessed the ability of alternatively spliced TAFI to encode a functional TAFI protein. Expression plasmids containing the full length (wt) and alternatively spliced variants (known as Δ7, Δ11, and Δ7+11) were constructed and stably transfected into the Baby Hamster Kidney (BHK) cell line, which does not
endogenously express TAFI. From western blot analysis using a polyclonal anti-TAFI antibody, full-length TAFI (60 kDa) was detected in conditioned medium of cells transfected with full-length TAFI expression plasmid while no immuno-reactive bands were detected in medium harvested from cells transfected with any of the other expression plasmids. Interestingly, however, within lysates immuno-reactive bands between of the appropriate sizes (40-48 kDa) in size were detected for all the expression plasmids. These likely represent immature, incompletely glycosylated forms of the recombinant proteins present in the endoplasmic reticulum (ER). Only the cells transfected with the full-length TAFI expression plasmid, however, showed the mature and fully glycosylated 60 kDa form within the lysates.

Western blot analysis showed that the full-length TAFI present in either the conditioned medium or the lysates could be activated by thrombin-thrombomodulin. In parallel experiments performed in transfected HepG2 cells, only full-length TAFI was detected in the medium. TAFIa activity was readily detected in thrombin-thrombomodulin-treated medium or lysates from BHK cells transfected with the full-length TAFI expression plasmid. However, no TAFIa activity was detected in medium or lysates of BHK cells transfected with alternatively-spliced TAFI variants.

Thus, the authors concluded that alternatively spliced TAFI is not secreted from the cell, and cannot be activated by thrombin-thrombomodulin. It is possible that the missing amino acid residues prevent the protein from being properly folded and thus released from the endoplasmic reticulum into the secretory pathway. The findings suggest the possibility of an intracellular role for TAFI variants arising from alternative splicing.
or that alternative splicing performs a regulatory function to limit the expression and secretion of TAFI.

1.9 Rationale, Hypothesis, and Thesis Objectives

Within the human population, there is considerable variation in the plasma TAFI antigen level, which ranges anywhere from 73 to 275 nm (41, 42). Higher levels of plasma TAFI or TAFIa have been associated with an increased risk for various vascular pathological disorders including venous thrombosis (50), recurrent venous thromboembolism (51), ischemic stroke (52, 53, 55) and CAD (58). Genetic studies (46, 49) have shown that only 15-25% of this variation is attributable to SNPs. Thus, the majority of the factors that contribute to the regulation of CPB2 gene expression remain largely unknown. Evidence of extra-hepatic TAFI expression, and alternative splicing of CPB2 mRNA within these sites, suggests that TAFI may have other functions in addition to its role in fibrinolysis.

The hypothesis of this study is that the extent of alternative splicing of CPB2 mRNA within a cell type is regulated in a cell-specific manner, and that the alternatively spliced CPB2 mRNA encodes TAFI variants with distinct functions.

The objectives of this research project are:

1.) To determine quantitatively the extent of alternative splicing within various vascular and inflammatory cell types, and whether a minigene approach can be
used to recapitulate the alternative splicing pattern previously documented in literature for further study.

2.) To identify proteins within HepG2 cells that are translated from alternatively spliced TAFI variants.
Chapter 2: Materials and Methods

2.1 MiniGene1 and Minigene2 Rationale

To study the extent of alternative splicing of CPB2 in various vascular and immune cell types, two minigene constructs were designed (Figure 2.1.A and Figure 2.2). Minigene1 was constructed to include two constitutively expressed exons (exon 6 and exon 9 of CPB2) that surround two alternatively spliced exons (exon 7 and exon 8 of CPB2) (Figure 2.1.A). Minigene2 was constructed to include one constitutively expressed exon (exon 10) and one alternatively spliced exon (exon 11) (Figure 2.2). Minigene1 can be alternatively spliced to produce three variants (Figure 2.3): Full length Minigene1, which contains exons 6, 7, 8, and 9 of CPB2 (Figure 2.3.A); Δ7 Minigene1, which contains exons 6, 8, and 9 of CPB2 (Figure 2.3.B), and Δ7+8 Minigene1, which contains exons 6 and 9 of CPB2 (Figure 2.3.C). Minigene2 can be alternatively spliced to produce two variants (Figure 2.4): Full length Minigene2, which contains exons 10, and the full length version of exon 11 of CPB2 (Figure 2.4.A); and Δ11 Minigene2, which contains exons 10, and the alternatively spliced version of exon 11 of CPB2 (Figure 2.4.B).

On either end of the CPB2 DNA fragments, two irrelevant short sequences of EGFP were added to distinguish between minigene CPB2 gene expression and endogenous CPB2 gene expression (Figure 2.1.A and Figure 2.2). To ensure that each minigene undergoes splicing, approximately 300 bp of intronic region was included before and after each alternatively spliced exon of interest. 300 bp of intronic region was...
chosen because the primary regulatory elements for alternative splicing events are usually located within 200-300 bp upstream and downstream of the exon of interest (97). Both minigenes were constructed in a plasmid that contains a strong constitutive promoter (from cytomegalovirus) to ensure that transcription from the minigenes are initiated without the need for an inducer molecule.
A.

Figure 2.1. Plasmid Construction of Minigene1.

Two versions of minigene1 were constructed, which differ only in the promoter that drives their expression in their respective plasmids. Version 1 of Minigene1 was constructed with a pCMV promoter in the pcDNA4/myc-his-A plasmid, and was the primary minigene1 used in the following experiments throughout this thesis. Version 2 of Minigene1 which contains a pTET promoter in the pcDNA4/myc-his-B plasmid, was produced for future work. The location and type of restriction enzymes used to construct the minigenes are indicated by the black arrows. In addition, exons are indicated by the boxes, and the introns are indicated by the broken dashed lines. Also, the sizes of the respective exonic and intronic regions included in the constructs are also indicated.
Minigene2 was constructed with a pCMV promoter in the pcDNA4/myc-his-A plasmid, and was the primary minigene2 used in the following experiments throughout this thesis. The location and type of restriction enzymes used to construct the minigenes are indicated by the black arrows. In addition, exons are indicated by the boxes, and the introns are indicated by the broken dashed lines. Also, the sizes of the respective exonic and intronic regions included in the constructs are also indicated.
The following are schematic images indicating the resulting transcripts that could arise from alternative splicing of the pCMV-minigene1-4A plasmid. A. Full Length (FL) Minigene1, which contains all exons. B. $\Delta 7$ Minigene1, which is representative of exon 7 skipping only. C. $\Delta 7+8$ Minigene1, which is representative of both exon 7 and exon 8 skipping. Also shown is the polyA tail that would be present on the following transcripts.
Figure 2.4. Splice Variants of Minigene2

The following are schematic images indicating the resulting transcripts that could arise from alternative splicing of the pCMV-minigene2-4A plasmid. A. Full Length (FL) Minigene2, which contains exon 10 and the full length version of exon 11. B. Δ11 Minigene2, which contains exon 10 and the alternatively spliced version of exon 11. Also shown is the polyA tail that would be present on the following transcripts.
2.2 A Minigene1 Construction

Primer sets were designed to amplify two irrelevant sequences within Enhanced Green Fluorescent Protein (EGFP), using pEGFP-C3 as a template to perform PCR. In addition, primer sets were designed to amplify sequences surrounding each exon of CPB2, as well as intronic sequences approximately 300 base pairs upstream and downstream of each exon. Genomic DNA (gDNA) was extracted from THP-1 monocytes, using QIAamp DNA Blood Midi kit (Qiagen), and used as a template to perform PCR for the CPB2 sequences. See Table 2.1 for primer sequences, engineered restriction sites, expected amplicon sizes, and annealing temperatures. PCR amplification of the desired sequence was carried out with the appropriate primer pairs, using Taq DNA Polymerase (GoTaq Green Master Mix - Promega). In a 96-well optical plate (BioRad), 12.5 µL of 2× GoTaq Green Master Mix (final concentration 1×), was added to 150 ng of gDNA template and 2.5 µL of each forward and reverse primers (final concentration 1 µM). Thermocycling conditions were carried out as follows: pre-denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at a primer pair-specific temperature (See Table 2.1) for 30 seconds, extension at 72°C for 2 minutes, and a final extension at 72°C for 5 minutes.

PCR products were resolved on a 1% agarose gel to separate the amplified products. Bands of the correct size were excised from the gel and isolated using a Gene Clean III kit (MP Biomedicals). The PCR products were digested with the appropriate restriction enzymes corresponding to the flanking engineered cut sites for 1 hour at 37°C. In parallel, the pBluescript SK II + vector (Stratagene) was digested with EcoRV for 1
Table 2.1. Primer Sequences Used to Construct Minigene1

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’ → 3’)</th>
<th>Expected Size</th>
<th>Restriction Site</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ EGFP – 5’ Primer</td>
<td>ATG GAT CCG CAA GGG CGA GGA GCT GTT CAC CGG G</td>
<td>160 bps</td>
<td>BamHI</td>
<td>69°C</td>
</tr>
<tr>
<td>5’ EGFP – 3’ Primer</td>
<td>ATG AAT TCG GCC AGG GCA CGG GCA GCT TGC CGG TG</td>
<td></td>
<td>EcoRI</td>
<td></td>
</tr>
<tr>
<td>Region 6 – 5’ Primer</td>
<td>ATG AAT TCG TTT CTG GAA AAG AAC AAG CAG CC</td>
<td>630 bps</td>
<td>EcoRI</td>
<td>60°C</td>
</tr>
<tr>
<td>Region 6 – 3’ Primer</td>
<td>ATC TGC AGC ATA CAG TTT GTC TTA TAT ATG GC</td>
<td></td>
<td>PstI</td>
<td></td>
</tr>
<tr>
<td>Region 7 – 5’ Primer</td>
<td>ATC TGC AGC TGC CTT CAT (\text{TAA CTA GGA ATA CTG GAC CAC GTG TAA GGC})</td>
<td>660 bps</td>
<td>PstI</td>
<td>65°C</td>
</tr>
<tr>
<td>Region 7 – 3’ Primer</td>
<td>ATG ATA TCA TGC ATA CTG AAA TAT TTA TTG ATG AAG TGA TAT GGT ACC</td>
<td></td>
<td>EcoRV</td>
<td></td>
</tr>
<tr>
<td>Region 8 – 5’ Primer</td>
<td>ATG ATA TCC GGA TAG CAA TAG GGA CCG TGC</td>
<td>680 bps</td>
<td>EcoRV</td>
<td>62°C</td>
</tr>
<tr>
<td>Region 8 – 3’ Primer</td>
<td>ATG CGG CCG CTG CAA CAC AGC AAG ACT CTG</td>
<td></td>
<td>NotI</td>
<td></td>
</tr>
<tr>
<td>Region 9 – 5’ Primer</td>
<td>ATG CGG CCG CGG GAC AGG GGG AGA AGT GTT GGA AGG CAG CC</td>
<td>800 bps</td>
<td>NotI</td>
<td>72°C</td>
</tr>
<tr>
<td>Region 9 – 3’ Primer</td>
<td>ATC TCG AGC AGT TCC TCA TGG TCT TTT CTT TTA CTT C GT GTA TAG</td>
<td></td>
<td>XhoI</td>
<td></td>
</tr>
<tr>
<td>3’ EGFP – 5’ Primer</td>
<td>ATC TCG AGC CTG ACC TAC GGC GTG CAG TGC TCC AG</td>
<td>168 bps</td>
<td>XhoI</td>
<td>70°C</td>
</tr>
<tr>
<td>3’ EGFP – 3’ Primer</td>
<td>ATT CTA GAC GCC CTC GAA CT T CAC CTC GGC GCG G</td>
<td></td>
<td>XbaI</td>
<td></td>
</tr>
</tbody>
</table>
hour at 37°C. All digests were subsequently resolved on a 1% agarose gel. Correctly digested fragments were excised from the agarose gel, and isolated using Gene Clean III kit (MP Biomedicals). The ligation reaction was performed using 6.5 µL PCR product “insert,” 0.5 µL of pBluescript “vector,” 1 µL of T4 DNA Ligase (Invitrogen), and 2 µL of T4 DNA Ligase Reaction Buffer (Invitrogen) overnight at 16°C within a mini-thermocycler (MJ Research PTC-150 MiniCycler).

The DNA in the ligations was then ethanol-precipitated and dissolved in MilliQ-H₂O before introduction into electrocompetent MAX Efficiency DH5α competent cells (Invitrogen) via electroporation. Cells were allowed to recover for 40 minutes in Luria broth (LB) at 37°C before being plated onto LB-ampicillin (100 µg/mL) (Fisher) agar plates with 1 mg of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal) (Bioshop), and 2 mg of isopropyl thiogalactoside (IPTG) (Sigma) for colony color selection. After overnight growth, colonies were picked and grown overnight in LB broth with 100 µg/mL of ampicillin (Fisher) for selection. The next day, plasmid DNA was isolated and digested with the appropriate restriction enzymes. Samples that yielded the correct band sizes were sent for sequencing (Robarts Research Institute DNA Sequencing Facility). DNA from clones of the correct sequences was prepared using a Plasmid Plus Midi Prep kit (Qiagen), and digested with the appropriate restriction enzymes to re-verify their identity.

This general cloning method was used to produce each individual fragment for the minigene within the pBluescript SK II + vector. Once all clones had been successfully constructed within pBluescript SK II +, they were sequentially inserted into the pcDNA4/myc-his-A vector, which has a strong cytomegalovirus promoter (pCMV). This
was accomplished via a series of three part and two part ligations. To insert 5’-EGFP and region-6 into pcDNA4/myc-his-A, a three-part ligation was employed. pcDNA4/myc-his-A (0.5 µg) was digested with *Bam*HI and *Pst*I, 5 µg of 5’EGFP-pBlue was digested with *Bam*HI and *Eco*RI, and 2 µg of region 6-pBlue were digested with *Eco*RI and *Pst*I for 1 hour at 37°C. DNA fragments were each resolved on agarose gels and isolated as described above in 8 µL of TE Buffer (10 mM Tris, 1 mM EDTA, pH 8). The ligation reaction was performed with: 5 µL of 5’EGFP, 1.5 µL of region 6, 0.5 µL of pcDNA4/myc-his-A, 1 µL of T4 DNA Ligase (Invitrogen), and 2 µL of T4 DNA Ligase Reaction Buffer (Invitrogen) overnight at 16°C within a mini-thermocycler (MJ Research PTC-150 MiniCycler). Ligation reactions were precipitated and transformed into electrocompetent *E. coli* as described above and correctly assembled clones identified by restriction digestion and DNA sequencing as described above.

The rest of the inserts for minigene1 were introduced into this first intermediate construct in two part ligations, using similar approaches. The final minigene construct in pcDNA4/myc-his-A was subjected to various double digestion reactions to ensure all of the inserts were present as well as complete sequencing of the entire insert to ensure the fidelity of the sequence.

In addition, a second version of the minigene1 construct was created within a modified pcDNA4/myc-his-B vector with an inducible pTET promoter. The complete minigene1-4A plasmid was digested with *Bam*HI and *Xba*I, and 0.5 µg of pTET-pcDNA4/myc-his-B was also digested with *Bam*HI and *Xba*I. The ligation reaction was performed using 6.5 µL insert, 0.5 µL of vector, 1 µL of T4 DNA Ligase (Invitrogen) and
2 µL of T4 DNA Ligase Reaction Buffer (Invitrogen) overnight at 16°C. Correctly assembled clones were isolated and verified as described above.

2.2.B. MiniGene2 Construction

Primer sets were designed to amplify sequences surrounding exon ten of CPB2 and 300 base pairs of intronic sequence downstream of exon 10, exon 11 of CPB2, and 300 base pairs of intronic sequence upstream of exon 11 in CPB2. gDNA was extracted from THP-1 monocytes using QIAamp DNA Blood Midi kit (Qiagen) and used as a template to perform PCR for the CPB2 sequences. See Table 2.2 for primer sequences, engineered restriction sites, expected amplicon sizes and annealing temperatures. PCR amplification of the desired sequence was carried out with the appropriate primer pairs using Q5 High-Fidelity DNA Polymerase (NEB).

Reactions were assembled in a 0.6-ml Eppendorf tube (Sarstedt) and contained 10 µL of 5× Q5 Reaction Buffer (final concentration 1×), 1 µL of dNTPs (final concentration 0.2 mM of each), 10 µL of 5× Q5 High GC Enhancer (final concentration 1×), 250 ng of gDNA template, 0.5 µL of Q5 High – Fidelity DNA Polymerase (final concentration 0.02 U/µL) and 2.5 µL of the forward and reverse primers (final concentration 0.5 µM). Thermocycling conditions were carried out as follows for both region-10 and region-11: predenaturation at 98°C for 30 seconds, 35 cycles of denaturation at 98°C for 10 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 20 seconds, and a final extension at 72°C for 5 minutes.
Table 2.2. Primer Sequences Used to Construct Minigene2

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’ → 3’)</th>
<th>Expected Size</th>
<th>Restriction Site</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region 10 – 5’ Primer</td>
<td>ATG AAT TCT CTC TAG TAG CCA GTG AAG C</td>
<td>564 bps</td>
<td>EcoRI</td>
<td>57°C</td>
</tr>
<tr>
<td>Region 10 – 3’ Primer</td>
<td>ATC TGC AGG TCA CAA GTT TTG TGT GTC</td>
<td></td>
<td>PstI</td>
<td></td>
</tr>
<tr>
<td>Region 11 – 5’ Primer</td>
<td>ATC TGC AGG TAG ACA AAT GAG G</td>
<td>677 bps</td>
<td>PstI</td>
<td>57°C</td>
</tr>
<tr>
<td>Region 11 – 3’ Primer</td>
<td>ATC TCG AGT TAA ACA TTC CTA ATG ACA TGC C</td>
<td></td>
<td>XhoI</td>
<td></td>
</tr>
</tbody>
</table>
PCR products were resolved on 1% agarose gels and isolated as described above in Section 2.1.A. The isolated PCR products were digested with the appropriate flanking engineered cut sites for 1 hour at 37°C. In addition, 0.5 µg of minigene1-4A was digested with EcoRI and XhoI for 1 hour at 37°C to remove the CPB2 region within minigene1-4A, so that the plasmid retained only the two EGFP inserts at each end. The ligation reaction was assembled containing 0.5 µL of EGFPminigene1-4A, 3.2 µL of region-10 digested PCR product, 3.2 µL of region-11 digested PCR product, 0.2 µL of T4 DNA Ligase (Invitrogen), 2 µL of T4 DNA Ligase Reaction Buffer (Invitrogen), and 0.9 µL of H₂O. After transformation of electrocompetent E. coli, correctly assembled clones were identified and verified as described above in Section 2.2.A.

2.2.C. Expression Plasmids for Full Length TAFI and Alternatively Spliced TAFI Variants

See Figures 2.5 - 2.8 for a schematic diagram of all the Full length and alternatively spliced TAFI variant expression plasmids constructed and used throughout this study. Primer sets were designed to amplify sequences surrounding the full length TAFI sequence, as well as each alternatively spliced TAFI variant. In addition, novel restriction enzyme sites were designed at each end of the fragment for sticky ended insertion into the plasmid of interest. Previous plasmids containing the sequences of interest in a pNUT plasmid (89) were used as templates for each construct. See Table 2.3 for primer sequences, engineered restriction cut sites, expected amplicon sizes, and annealing
Full length TAFI was cloned into three different expression plasmids: A. Full Length TAFI + pCMV-pBluescript SK II +, B. Full Length TAFI + pTET-pcDNA4/myc-his-B, and C. Full Length TAFI + pCMV-pcDNA4/myc-his-A. Version A. and Version B. of the Full Length TAFI variants were intermediates synthesized during the production of the final product, Version C. The Full Length TAFI + pCMV-pcDNA4/myc-his-A version was the plasmid used for experiments throughout this thesis. The location and type of restriction enzymes used to construct the Full Length TAFI variants are indicated by the black arrows. Also, the sizes of the respective insert and plasmid regions included in the constructs are also indicated. The plasmids that contain a myc 6x His tag are also indicated.
Δ7 TAFI was cloned into three different expression plasmids: A. Δ7 TAFI + pCMV-pBluescript SK II +, B. Δ7 TAFI + pTET-pcDNA4/myc-his-B, and C. Δ7 TAFI + pCMV-pcDNA4/myc-his-A. Version A. and Version B. of the Δ7 TAFI variants were intermediates synthesized during the production of the final product, Version C. The Δ7 TAFI + pCMV-pcDNA4/myc-his-A version was the plasmid used for experiments throughout this thesis. The location and type of restriction enzymes used to construct the Δ7 TAFI variants are indicated by the black arrows. Also, the sizes of the respective insert and plasmid regions included in the constructs are also indicated. The plasmids that contain a myc 6x His tag are also indicated.

Figure 2.6. Plasmid Construction of Δ7 TAFI Variants
Figure 2.7. Plasmid Construction of Δ11 TAFI Variants

Δ11 TAFI was cloned into three different expression plasmids: A. Δ11 TAFI + pCMV-pBluescript SK II +, B. Δ11 TAFI + pTET-pcDNA4/myc-his-B, and C. Δ11 TAFI + pCMV-pcDNA4/myc-his-A. Version A. and Version B. of the Δ11 TAFI variants were intermediates synthesized during the production of the final product, Version C. The Δ11 TAFI + pCMV-pcDNA4/myc-his-A version was the plasmid used for experiments throughout this thesis. The location and type of restriction enzymes used to construct the Δ11 TAFI variants are indicated by the black arrows. Also, the sizes of the respective insert and plasmid regions included in the constructs are also indicated. The plasmids that contain a myc 6x His tag are also indicated.
Δ7+11 TAFI was cloned into three different expression plasmids: A. Δ7+11 TAFI + pCMV-pBluescript SK II +. B. Δ7+11 TAFI + pTET-pcDNA4/myc-his-B, and C. Δ7+11 TAFI + pCMV-pcDNA4A/myc-his-A. Version A. and Version B. of the Δ7+11 TAFI variants were intermediates synthesized during the production of the final product, Version C. The Δ7+11 TAFI + pCMV-pcDNA4/myc-his-A version was the plasmid used for experiments throughout this thesis. The location and type of restriction enzymes used to construct the Δ7+11 TAFI variants are indicated by the black arrows. Also, the sizes of the respective insert and plasmid regions included in the constructs are also indicated. The plasmids that contain a myc 6x His tag are also indicated.

Figure 2.8. Plasmid Construction of Δ7+11 TAFI Variants
Table 2.3. Primer Sequences Used to Construct the Alternatively Spliced TAFI Variants

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5' → 3')</th>
<th>Expected Size</th>
<th>Restriction Site</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAFI – 5’ Primer</td>
<td>AAA CTG CAG TTG GGA TGA AGC TTT GCA</td>
<td>FL = 1296 bps, Δ7 = 1185 bps, Δ11 = 1244 bps, Δ7+11 = 1133 bps</td>
<td>PstI</td>
<td>FL (Pfu) 60°C, Δ7 (Pfu) 60°C, Δ7+11 (Taq) 60°C</td>
</tr>
<tr>
<td>TAFI – 3’ Primer</td>
<td>GGG TCT AGA AAC AAT CCT AAT GAC AT</td>
<td></td>
<td>XbaI</td>
<td></td>
</tr>
<tr>
<td>TAFI MUT – 5’ Primer</td>
<td>ATG TTT CTA GAG GGC CC(G)C GGT TCG AAC AAA AACT</td>
<td>G IS DELETED</td>
<td></td>
<td>67°C (all)</td>
</tr>
<tr>
<td>TAFI MUT – 3’ Primer</td>
<td>AGT TTT TGT TCG AAC CGG GGC CCT CTA GAA ACAT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
temperatures. PCR amplification of full length TAFI and the Δ7 TAFI sequences were carried out with the appropriate primer pair, using *Pfu* Turbo DNA Polymerase (Agilent). The reaction contained 2.5 µL of 10× Cloned *Pfu* DNA Polymerase Reaction Buffer (final concentration 1×), 0.2 µL of dNTPs (final concentration 0.2 mM of each), 50 ng of plasmid template (full length TAFI-pNUT or Δ7 TAFI-pNUT), 0.5 µL of each forward and reverse primer (final concentration 50 ng/µL) and 0.5 µL of *Pfu* Turbo DNA Polymerase (2.5 U/µL). Thermocycling conditions were carried out as follows for both full length TAFI and Δ7 TAFI: predenaturation at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute for 30 cycles, and a final extension at 72°C for 10 minutes.

PCR products were resolved on and isolated from 1% agarose gel and then digested with *Pst*I and *Xba*I restriction enzymes for 1 hour at 37°C. In addition, 0.5 µg of pBluescript SK II + was digested with *Pst*I and *Xba*I for 1 hour at 37°C. The ligation reaction was assembled from the gel-purified fragments: 6.5 µL of PCR amplified digested product, 0.5 µL of digested pBluescript SK II + vector, 1 µL of T4 DNA Ligase (Invitrogen), and 2 µL of T4 DNA Ligase Reaction Buffer (Invitrogen). Correctly assembled clones were identified and verified as described above in Section 2.2.A.

PCR amplification of Δ7+11 TAFI sequence was carried out with the appropriate primer pair using *Taq* Polymerase (GoTaq Green Master Mix Promega) in a reaction containing 12.5 µL of 2× GoTaq Green Master Mix (final concentration 1×), 150 ng of gDNA template, and 2.5 µL of each forward and reverse primers (final concentration 1 µM). Thermocycling conditions were carried out as follows: predenaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30
seconds, and extension at 72°C for 2 minutes for 40 cycles, and final extension at 72°C for 5 minutes. PCR products were inserted into pBluescript SK+ using PstI and XbaI sites for the full length and Δ7 inserts described above.

To construct the Δ11 TAFI-pBlue plasmid, the full length segment was substituted for the corresponding Δ7-containing segment in Δ7+11-pBlue using unique BglII and SphI restriction sites that flank these segments.

Once all clones had been successfully constructed within pBluescript SK II +, each cDNA segment was then inserted into a modified pcDNA4/myc-his-B vector with an inducible pTET promoter (89). One microgram of each pBluescript SK II + plasmid containing either FL TAFI, Δ7 TAFI, Δ11 TAFI, or Δ7+11 TAFI cDNA was digested with PstI and XbaI to release the cDNA inserts, and 0.5 µg of pTET-pcDNA4/myc-his-B was also digested with PstI and XbaI at 37°C for 1 hour. The fragments were isolated from agarose gel slices and ligation reactions assembled using 6.5 µL insert, 0.5 µL of vector, 1 µL of T4 DNA Ligase (Invitrogen), and 2 µL of T4 DNA Ligase Reaction Buffer. Ligation conditions, bacterial transformation, and identification of correct clones were performed as described above in Section 2.2.A.

Since the original primers for these constructs were designed for the pcDNA4/myc-his-A plasmid, and the pcDNA4/myc-his-B plasmid has an extra restriction cut site within its multiple cloning site that is not conserved in the pcDNA4/myc-his-A plasmid; insertion of the TAFI inserts into pTET-pcDNA4/myc-his-B vector resulted in a frameshift that caused the sequence to be out of phase with the myc-6× his tag within the pTET-pcDNA4/myc-his-B plasmid as confirmed by sequencing analysis. Thus, mutagenesis of all the pTET-pcDNA4/myc-his-B constructs
containing FL TAFI, Δ7 TAFI, Δ11 TAFI, and Δ7+11 TAFI, had to be performed. The mutagenesis primers used for all constructs can be found in Table 2.3 and were designed using Agilent software. Mutagenesis was carried out using a Quick-Change II-E Site-Directed Mutagenesis Kit (Agilent), which contains a PfuUltra High Fidelity DNA Polymerase. In 96-well optical plates (BioRad), 5 µL of 10× Reaction Buffer (final concentration 1×), 1 µL of dNTPs (final concentration 0.02 mM), were added to 50 ng of DNA template and 5 µL of each forward and reverse primers (final concentration 25 ng/µL). Thermal cycling conditions used for all reactions were: predenaturation at 95°C for 1 minute, 19 cycles of denaturation at 95°C for 50 seconds, annealing at 67°C for 50 seconds and extension at 68°C for 8 minutes, and then final extension at 68°C for 30 seconds. Following PCR, samples were digested with 1 µL of DpnI for 3 hours at 37°C to digest the parental DNA. Following digestion, samples were precipitated with 3M sodium acetate and 3 volumes of ethanol. Pellets were dissolved in MilliQ-H2O before electroporation into electrocompetent MAX Efficiency DH5α competent cells (Invitrogen). The presence of the correct mutations was verified by DNA sequence analysis.

In addition, a third version of the full length and alternatively spliced constructs was created within the pcDNA4/myc-his-A vector, which has a strong cytomegalovirus promoter (pCMV). To insert each fragment into pcDNA4/myc-his-A, 1 µg of each in-frame pTET-pcDNA4-myc-his-B plasmid containing either FL TAFI, Δ7 TAFI, Δ11 TAFI, or Δ7+11 TAFI was digested with PstI and XbaI and the inserts ligated into pcDNA4/myc-his-A digested with the same enzymes. Transformation, plasmid isolation and verification of clones as was described above in Section 2.2.A.
2.3 Cell Culture

All cells were sustained in a humidified incubator at 37°C and 95% air, 5% CO₂ atmosphere. HepG2 (human hepatocellular carcinoma) cells (American Type Culture Collection (ATCC)) were cultured in 100 mm tissue culture plates in minimal essential medium (MEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (ATCC), and 1% antibiotic-antimycotic (10 units/mL penicillin G sodium, 10 ug/mL streptomycin sulfate, and 25 ng/mL amphotericin B) (Gibco).

Baby Hamster Kidney (BHK) cells were cultured in 100 mm tissue culture plates in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco), supplemented with 10% newborn calf serum (NCS) (Gibco), and 1% antibiotic-antimycotic (Gibco).

Human Embryonic Kidney (Hek293) cells were cultured in 100 mm tissue culture plates in minimal essential medium (MEM) (Gibco), supplemented with 5% fetal bovine serum (FBS) (Gibco), and 1% antibiotic-antimycotic (Gibco).

SH-SY5Y (neuroblastoma cells) cells were generously provided by Dr. Siyaram Pandey (Dept, of Chemistry & Biochemistry, University of Windsor). SH-SY5Y cells were cultured in 100 mm tissue culture plates in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco), and 1% antibiotic-antimycotic (Gibco).

THP-1 (human acute monocytic leukemia) cells were cultured in 100 mm tissue culture plates with RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% antibiotic-antimycotic (Gibco), 1% Sodium Pyruvate (Gibco), and 0.075mM of 2-mercaptoethanol (Sigma).
To differentiate THP-1 cells into a macrophage-like phenotype, Phorbol Myristate Acetate (PMA) (Sigma) was added to the cells at a final concentration of 0.1 μM, and the cells incubated for 72 hours.

### 2.4 Transfection of Expression Plasmids for RT-PCR and qRT-PCR

#### 2.4.A. Transfection of Minigene1-4A

HepG2 and Hek293 cells were transfected in 100 mm tissue culture plates, at approximately 60% confluency, with 5 μg of minigene1-pcDNA4A with MegaTran 1.0 according to the manufacturer’s protocol.

SH-SY5Y cells were transfected in 100 mm tissue culture plates, at approximately 70% confluency, with 6 μg of minigene1-pcDNA4A, 18 μL of Lipofectamine 2000 (Invitrogen), 6 μL of Plus reagent in 1 mL of Opti-MEM, as per the manufacturer’s protocol.

THP-1 cells were transfected in 60 mm tissue culture plates, at approximately 4 million cells/plate, with 8 μg of DNA, 18.6 μL of Lipofectamine 2000 (Invitrogen), 13.3 μL of Plus Reagent in 500 μL of Opti-MEM, as per the manufacturer’s protocol.
2.4.B. Transfection of Minigene2-4A

HepG2, Hek293, and BHK cells were transfected in 100 mm tissue culture plates, at approximately 60% confluency, with 5 μg of minigene2-pcDNA4A vector with MegaTran 1.0 according to the manufacturer’s protocol.

SH-SY5Y cells were transfected in 100 mm tissue culture plates, at approximately 70% confluency, with 6 μg of minigene2-pcDNA4A, 18 μL of Lipofectamine 2000 (Invitrogen), 6 μL Plus reagent in 1 mL of Opti-MEM, as per the manufacturer’s protocol.

2.5 RNA Extraction and Subsequent DNase Treatment of RNA

Total RNA was extracted from cells using Isol-RNA Lysis Reagent (5Prime), according to the manufacturer’s protocol. RNA was DNase treated using RQ1 RNase-free DNase (Promega) for 2 hours at 37°C. Following DNase treatment, the RNA samples were phenol/chloroform extracted, precipitated, and re-suspended in DEPC (Sigma)-treated H₂O. RNA concentrations were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific).
2.6 Reverse Transcriptase-Polymerase Chain Reaction

RT-PCR was carried out using a OneStep RT-PCR Kit (Qiagen), following the manufacturer’s protocol. 1 µg of RNA was used for all RT-PCR experiments. The primer sequences that were used can be found in Table 2.1. Thermocycling conditions used for all reactions were: reverse transcription at 50°C for 30 minutes, activation of Taq polymerase at 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for ten minutes. The RT-PCR products were resolved on 1% agarose gels containing 0.5 µg/mL ethidium bromide. Also, a no template control (NTC) was run as a negative control, to indicate reagent or cross contamination of experimental samples.

2.7 Real-Time Quantitative RT-PCR

Quantitative expression analyses of minigene and endogenous TAFI transcripts were carried out on a BioRad CFX96 Real-Time System. Probes were labeled at the 5’-end with the reporter FAM (6-carboxyfluorescein), and at the 3’ end with a nonfluorescent quencher that was bound to a minor groove binder (NFQ-MGB). The primer and probe sequences used can be found in Table 2.4. In 96-well optical plates (BioRad), 10 µL of 2× QRT-PCR Master Mix (Brilliant III Ultra-Fast QRT-PCR Master Mix - Agilent) (final concentration 1×), 0.2 µL DTT (final concentration 1 mM), and 1
### Table 2.4. Probe and Primer Sequences Used For Real-Time RT – PCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL M1 - 5’ Primer</td>
<td>GGA TTT CTA TGT TAT GCC GG</td>
</tr>
<tr>
<td>FL M1 - 3’ Primer</td>
<td>GAT TGT TCG CAT AGA AAG AAC</td>
</tr>
<tr>
<td>FL M1 Probe</td>
<td>56-FAM/CCACATTTCG/ZEN/ATTCTTTTTCC ATGAGTAGTCATAACCGTCC/3IABkFQ/</td>
</tr>
<tr>
<td>Δ7 - 5’ Primer</td>
<td>ATG GAT TGA CTG TGG AAT CC</td>
</tr>
<tr>
<td>Δ7 – 3’ Primer</td>
<td>GAT TGT TCG CAT AGA AAG AAC</td>
</tr>
<tr>
<td>Δ7 Probe</td>
<td>56-FAM/CCACATTTCG/ZEN/ATTATGGCCT ATGAACCAGCAGAAAGCAG/3IABkFQ</td>
</tr>
<tr>
<td>Δ7+8 – 5’ Primer</td>
<td>ATG GAT TGA CTG TGG AAT CC</td>
</tr>
<tr>
<td>Δ7+8 – 3’ Primer</td>
<td>TGG TTC TGA CTC AGG ATA AAG</td>
</tr>
<tr>
<td>Δ7+8 Probe</td>
<td>56-FAM/CATGAGGAA/ZEN/CTGGATGCACCT CTATGGCCTATGAACCACAAG/3IABkFQ</td>
</tr>
<tr>
<td>Δ11 – 5’ Primer</td>
<td>CTG GAG GTG GGG ACG ATT G</td>
</tr>
<tr>
<td>Δ11 – 3’ Primer</td>
<td>CAG CGG CAA AAG CTT CTC</td>
</tr>
<tr>
<td>Δ11 Probe</td>
<td>56-FAM/GTGGGTTTTG/ZEN/ATGTAACCGAA TATTTGATGCCAAATCATAG/3IABkFQ</td>
</tr>
<tr>
<td>FL M2 – 5’ Primer</td>
<td>GGG ACG ATT GGA TCT ATG ATT TGG GCA TC</td>
</tr>
<tr>
<td>FL M2 – 3’ Primer</td>
<td>TAC ATG CCA AGT TTT AGA GAC AGC GGC</td>
</tr>
<tr>
<td>FL M2 Probe</td>
<td>56-FAM/CCGGCAGCA/ZEN/AGAATCCGTATGT GCCGTATCTCGAAGTTTAATTTG/3IABkFQ</td>
</tr>
</tbody>
</table>
µL RT/RNase Block were added to total RNA, and 1 µL of forward and reverse primers and probe (final concentrations: 300 nM each primer; 200 nM probe). Thermal cycling conditions used for all reactions were: reverse transcription at 50°C for 30 minutes, activation of Taq polymerase at 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for ten minutes. A no-reverse transcriptase (NRT) control was performed as well to check for genomic or plasmid DNA contamination in the RNA samples. This control was carried out similarly to the sample reactions, except that RT/RNase Block was not included in the control. Furthermore, a no-template control (NTC) was included as well, to control for reagent or cross contamination of experimental samples.

The DNA standards used for absolute quantitation were generated by first spectrophotometrically determining the DNA concentration of the appropriate plasmid, followed by converting the DNA concentrations into the number of transcripts by using the calculated molecular mass of the DNA. The DNA standards were then serially diluted 10-fold, so that a series of standards containing $10^2$-$10^9$ copies/µL was achieved. Aliquots were stored at 4°C prior to use.

2.8 Metabolic Labeling of Cells

2.8.A. Steady-State Labeling
HepG2 and BHK cells were seeded into 60 mm tissue culture dishes and cultured to 60% confluence. They were then transiently transfected using Megatran 1.0 (DNA: Megatran 1:3) with 4 µg of each plasmid (pcDNA4A, FL TAFI-4A, Δ7-4A, Δ11-4A, Δ7+11-4A) each respectively into one 60 mm dish. Also, a non-transfected control was included as a negative control. Twenty-four hours post transfection, the cells were washed two times with 3 mL of PBS (10 mM Na₂HPO₄, 2.7 mM KCl, 137 mM NaCl, 1.76 mM KH₂PO₄, pH 7.4), fresh complete growth medium was applied to the plates and cells were allowed to recover overnight. On the following day, the medium was removed from the plates and the cells were washed two times with 3 mL of PBS; then, 2 mL of Cys/Met-free DMEM (Gibco) was added to the plates for one hour. Next, 300 µCi of [³⁵S] Cys/ [³⁵S] Met labeling mix (Perkin Elmer Life Sciences) was added to each plate, and protein labeling was allowed to occur overnight. 18 hours post labeling, both the media and lysates were harvested.

Following media collection, plates were washed two times with 5 mL of ice cold PBS. Next, 1 mL of V4 Lysis Buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate) was added to each plate, and plates were shaken at 4°C for ten minutes at high speed. The lysates were then collected and centrifuged at 16,000 × g for 5 minutes to clear the samples. Media samples were also briefly centrifuged at 700 × g to clear the samples.

Media and lysate samples were rocked overnight with 3 µg of monoclonal mouse-anti-myc antibody (Santa Cruz) at 4°C. The next day, recombinant protein G agarose (Invitrogen) (at 30 µL/mL) was added to the sample containing the antibody, and rocked at 4°C for another 2 hours. Samples were then washed one time with RIPA
(Radioimmunoprecipitation assay) buffer (50 mM Tris pH 7.4, 150 mM NaCl, 20 mM EDTA, 1% sodium deoxycholate, and 0.1% SDS), and washed two times with PBS, and finally suspended in Laemmli sample buffer. Samples were briefly centrifuged to pellet the agarose, boiled for 5 minutes, and then briefly centrifuged again before loading each sample onto a 12% polyacrylamide gel for SDS-PAGE.

Following electrophoresis, the gels were gently rocked at room temperature in fixing solution (isopropanol:H₂O:glacial acetic acid 25:65:10) for 20 minutes, and then briefly rinsed with H₂O, before gently rocking in Amplify solution (Amersham Biosciences) for 20 minutes. Next, gels were dried using a gel dryer (BioRad Model 583) on cycle 2 at 80°C for 35 minutes.

Radioactive ink was prepared by adding 50 µCi of [³⁵S] Cys/ [³⁵S] Met labeling mix (Perkin Elmer Life Sciences) to 400 µL of Super Black India ink, which was applied to the pre-labeled protein ladder using a felt-tip pen. A layer of clear plastic tape was placed on top of the ink to protect the screen.

Lastly, gels were exposed to a phosphor K screen (BioRad) at room temperature for 90 hours before screens were imaged using a BioRad Molecular Imager FX phosphorimager.

2.8.B. Pulse-Chase Labeling

HepG2 cells were seeded into 60 mm tissue culture dishes and cultured to 60% confluency. They were then transfected using Megatran 1.0 (DNA: Megatran 1:3) with 4
µg of each plasmid (FL TAFI-4A or Δ7-4A) each respectively into one 60 mm dish. Also, a non-transfected control was included as a negative control. 24 hours post transfection, the cells were washed two times with 3 mL of PBS; fresh complete growth media was applied to the plates and cells were allowed to recover overnight. On the following day, the medium was removed from the plates; the cells were washed two times with 3 mL of PBS, and then 2 mL of Cys/Met-free DMEM (Gibco) was added to the plates for 45 minutes. Next, 300 µCi of \[^{35}\text{S}\] Cys/\[^{35}\text{S}\] Met labeling mix (Perkin Elmer Life Sciences) was added to each plate, and protein labeling was allowed to occur for 1 hour. Next, the labeled media was removed from the plates, cells were washed two times with 3 mL of PBS, and then complete unlabeled growth medium was added to the plates. Both the media and lysates were harvested from plates at the following time points: 0 hr, 15 min, 30 min, 1 hr, 2 hr, 4 hr and 6 hr.

Following media collection, plates were washed two times with 5 mL of ice cold PBS. Next, 1 mL of V4 Lysis Buffer was added to each plate, and plates were shaken at 4°C for ten minutes at high speed. The lysate was then collected and centrifuged at 16,000 × g for 5 minutes to clear the sample. Media samples were also briefly centrifuged at 700 × g to clear samples. Immunoprecipitations with the anti-myc antibody, SDS-PAGE, and fluorography were carried out as described above in Section 2.8.A.
Chapter 3: Results

3.1 Identification of Minigene \textit{CPB2} mRNA in Several Cell Types Using RT-PCR

The expression of pCMV-minigene \textit{CPB2} mRNA was evaluated in several cell types via RT-PCR. The cell types that were investigated included HepG2 (human hepatocellular carcinoma) cells, BHK (baby hamster kidney) cells, Hek293 (human embryonic kidney) cells, THP-1 (human acute monocytic leukemia) cells, and SH-SY5Y (neuroblastoma) cells. The primer set used was the 5'-EGFP 5' primer, and the 3'-EGFP 3' primer (For primer sequences, see Table 2.1). The projected fragment sizes were 841 bp for full length minigene1, 730 bp for Δ7 minigene1, and 636 bp for Δ7+8 minigene1, and 597 bp for full length minigene2, and 545 bp for Δ11 minigene2. The RT-PCR results using this primer set are displayed in Figure 3.1.A for minigene1 and Figure 3.1.B for minigene2.

The anticipated fragment sizes for full length minigene1, Δ7 minigene1, and Δ7+8 minigene1 were only present in the HepG2 and Hek293 cell lines while no bands appeared for the SH-SY5Y or THP-1 cell lines (Figure 3.1.A). In addition, the NTC lane was empty; therefore, the bands present in the gel can be assumed to be \textit{bona fide} fragments, and not as a result from contamination of the reactions from the reagents or plasmid DNA. Thus, splicing of the transcripts from minigene1 occurred at the expected location in these two cell types. In the HepG2 cells, the Δ7+8 transcript appears to be more abundant than the full length, and Δ7 transcript. In the Hek293 cells, the full length transcript appears to be the dominant species present, and the splice variants appear to be
Figure 3.1. Detection of Minigene1-4A and Minigene2-4A mRNA Using EGFP Primer Set.

One Step RT-PCR was conducted on total RNA extracts from HepG2, BHK, Hek293, THP-1 and SH-SY5Y cells transfected with pCMV-minigene1-4A (A.) or pCMV-minigene2-4A (B.), using 5' EGFP 5' – Primer and 3' EGFP 3'– Primer set. The products from the RT-PCR reaction were run on a 1% agarose gel. The arrows indicate the band of the predicted alternatively spliced amplification products of each respective minigene. A No Template Control (NTC) reaction was also run that did not have an RNA template to serve as a negative control.
of approximately equal intensity (Figure 3.1.A). No bands of the correct size were detected in SH-SY5Y or differentiated THP-1 cells. It appears likely that in this experiment the minigene1 plasmid was not transfected into the cells.

The anticipated fragment size for full length minigene2 was present in all cell lines investigated (Figure 3.1.B). Once again, the NTC lane was empty. With respect to the Δ11 minigene2 version, no bands of the correct size were observed (Figure 3.1.B). In addition, two other prominent bands appeared, one at approximately 1300 bp and another at approximately 1500 bp. These bands may correspond to incompletely spliced variants of minigene2, since the minimum size of the un-spliced full length minigene2 fragment is 597 bp.

3.2 Quantification of Endogenous and Minigene CPB2 mRNA in Several Cell Types via Real Time RT-PCR

Once CPB2 mRNA corresponding to full length minigene2, as well as full length and alternatively spliced minigene1 were identified in several cell types, real time RT-PCR was performed to quantify the extent of alternative splicing within these various cell lines that occurs endogenously in comparison to that observed for transcripts expressed from the minigenes. Real time PCR is a more quantitative and sensitive method than RT-PCR, thus it was employed to detect the low copy number that is endogenously expressed by alternatively spliced CPB2 transcripts. The probe and primer sets used can be found in Table 2.4, the standard curves generated from the standards can be found in Figures 3.2-
3.6, the final results from the real time RT-PCR can be found in Figures 3.7-3.11, and a summary table of all the results can be found in Table 3.1.

With respect to endogenous and minigene1 data (Figure 3.7), endogenous full length $CPB2$ mRNA was detected in all cell types investigated, except Hek293, as was expected. In contrast, minigene1 full length $CPB2$ mRNA was detected in all cell types investigated (HepG2, Hek293, SH-SY5Y, and differentiated THP-1). With respect to $\Delta 7$ endogenous and minigene1 data (Figure 3.8), endogenous $\Delta 7$ $CPB2$ mRNA was detected in all cell types investigated, except Hek293, as was expected. In contrast, $\Delta 7$ minigene1 $CPB2$ mRNA was detected in all cell types investigated. It is also important to note that $\Delta 7$ minigene1 accounted for greater than 50% of all the transcripts detected that were alternatively spliced from minigene1 across all cell types investigated (Table 3.1). With respect to $\Delta 7+8$ endogenous and minigene1 data (Figure 3.9), endogenous $\Delta 7+8$ $CPB2$ mRNA was detected in HepG2 and differentiated THP-1 cells, but not in Hek293 (as was expected) or SH-SY5Y cells. In contrast, $\Delta 7+8$ minigene1 $CPB2$ mRNA was detected in all cell types investigated. Therefore, in all cell types examined, minigene1 was correctly spliced into full-length, and alternatively spliced $\Delta 7$ minigene1, and $\Delta 7+8$ minigene1.

With respect to endogenous and minigene2 data (Figure 3.10), endogenous full length $CPB2$ mRNA was detected in HepG2 cells and SH-SY5Y cells, but not in BHK or Hek293 cells, as was expected. In contrast, minigene2 full length $CPB2$ mRNA was detected in all cell types investigated (HepG2, BHK, Hek293 and SH-SY5Y). With respect to $\Delta 11$ endogenous and minigene2 data (Figure 3.11), endogenous $\Delta 11$ $CPB2$ mRNA was detected in HepG2 cells and also in SH-SY5Y cells, but not BHK or Hek293, as was expected. In contrast, $\Delta 11$ minigene2 $CPB2$ mRNA was detected in all cell types.
To determine the number of full length *CPB2* target transcripts present in unknown samples, standard curves were created by serially diluting full length TAFI + pBluescript SK II + plasmid 10 fold so that a series of $10^2$-$10^8$ copies/µL were achieved. These samples were used as a template and subjected to real time quantitative RT-PCR along with RNA samples extracted from HepG2, Hek293, SH-SY5Y and differentiated THP-1 cells not transfected and transfected with minigene1-4A. The standard curve shown represents the relationship between the threshold cycle (Ct) and the log amount of standard input. This primer and probe set was designed to amplify a linker region between exon 7 and exon 8 of *CPB2*. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.

**Figure 3.2. Standard Curve of Full Length *CPB2* Standards for Full Length Endogenous *CPB2* and Full Length Minigene1 Absolute Quantification for Real Time PCR Analysis.**
To determine the number of Δ7 CPB2 target transcripts present in unknown samples, standard curves were created by serially diluting Δ7 TAFI + pBluescript SK II + plasmid 10-fold so that a series of $10^1$-$10^8$ copies/μL were achieved. These samples were used as a template and subjected to real time quantitative RT-PCR along with RNA samples extracted from HepG2, Hek293, SH-SY5Y and differentiated THP-1 cells not transfected and transfected with minigene1-4A. The standard curve shown represents the relationship between the threshold cycle (Ct) and the log amount of standard input. This primer and probe set was designed to amplify the end of exon 6 in conjunction with the beginning of exon 8 of CPB2. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.

Figure 3.3. Standard Curve of Δ7 CPB2 Standards for Δ7 Endogenous CPB2 and Δ7 Minigene1 Absolute Quantification for Real Time PCR Analysis.
To determine the number of Δ7+8 CPB2 target transcripts present in unknown samples, standard curves were created by serially diluting Δ7+8 TAFI + pBluescript SK II + plasmid 10 fold so that a series of $10^1$-$10^8$ copies/μL were achieved. These samples were used as a template and subjected to real time quantitative RT-PCR along with RNA samples extracted from HepG2, Hek293, SH-SY5Y and differentiated THP-1 cells not transfected and transfected with minigene1-4A. The standard curve shown represents the relationship between the threshold cycle (Ct) and the log amount of standard input. This primer and probe set was designed to amplify the end of exon 6 in conjunction with the beginning of exon 9 of CPB2. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.

Figure 3.4. Standard Curve of Δ7+8 CPB2 Standards for Δ7+8 Endogenous CPB2 and Δ7+8 Minigene1 Absolute Quantification for Real Time PCR Analysis.
Figure 3.5. Standard Curve of Full Length CPB2 Standards for Full Length Endogenous CPB2 and Full Length Minigene2 Absolute Quantification for Real Time PCR Analysis.

To determine the number of full length CPB2 target transcripts present in unknown samples, standard curves were created by serially diluting full length TAFI + pBluescript SK II + plasmid 10 fold so that a series of 10^2-10^8 copies/μL were achieved. These samples were used as a template and subjected to real time quantitative RT-PCR along with RNA samples extracted from HepG2, BHK, Hek293, and SH-SY5Y cells not transfected and transfected with minigene2-4A. The standard curve shown represents the relationship between the threshold cycle (Ct) and the log amount of standard input. This primer and probe set was designed to amplify within the 52 bp region of exon 11 normally deleted in Δ11 TAFI of CPB2. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.
Figure 3.6. Standard Curve of Δ11 CPB2 Standards for Δ11 Endogenous CPB2 and Δ11 Minigene2 Absolute Quantification for Real Time PCR Analysis.

To determine the number of Δ11 CPB2 target transcripts present in unknown samples, standard curves were created by serially diluting Δ11 TAFI + pBluescript SK II + plasmid 10 fold so that a series of $10^1$-$10^8$ copies/μL were achieved. These samples were used as a template and subjected to real time quantitative RT-PCR along with RNA samples extracted from HepG2, BHK, Hek293, and SH-SY5Y cells not transfected and transfected with minigene2-4A. The standard curve shown represents the relationship between the threshold cycle (Ct) and the log amount of standard input. This primer and probe set was designed to amplify a region within exon 11 that conjoins the flanking regions on either side of the 52 bp region in exon 11 that is normally deleted in Δ11 TAFI of CPB2. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.
Figure 3.7. Quantitative Analysis of Endogenous and Minigene1 Full Length \textit{CPB2} mRNA in Several Cell Types

The abundance of full length endogenous and full length minigene1 \textit{CPB2} mRNA transcripts was assessed from total RNA isolated from not transfected and transfected HepG2, Hek293, SH-SY5Y and differentiated THP-1 cells with minigene1-4A using quantitative real time RT-PCR. The primer and probe set was designed to amplify a linker region between exon 7 and exon 8 of \textit{CPB2}. The quantity of unknown samples was determined via extrapolation from the full-length \textit{CPB2} minigene1 standard curve. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.
Figure 3.8. Quantitative Analysis of Endogenous and Minigene1 Δ7 CPB2 mRNA in Several Cell Types.

The abundance of Δ7 endogenous and Δ7 minigene1 CPB2 mRNA transcripts was assessed from total RNA isolated from not transfected and transfected HepG2, Hek293, SH-SY5Y and differentiated THP-1 cells with minigene1-4A using quantitative real time RT-PCR. The primer and probe set was designed to amplify the end of exon 6 in conjunction with the beginning of exon 8 of CPB2. The quantity of unknown samples was determined via extrapolation from the Δ7 CPB2 minigene1 standard curve. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.
Figure 3.9. Quantitative Analysis of Endogenous and Minigene1 Δ7+8 CPB2 mRNA in Several Cell Types.

The abundance of Δ7+8 endogenous and Δ7+8 minigene1 CPB2 mRNA transcripts was assessed from total RNA isolated from not transfected and transfected HepG2, Hek293, SH-SY5Y and differentiated THP-1 cells with minigene1-4A using quantitative real time RT-PCR. The primer and probe set was designed to amplify the end of exon 6 in conjunction with the beginning of exon 9 of CPB2. The quantity of unknown samples was determined via extrapolation from the Δ7+8 CPB2 minigene1 standard curve. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.
Figure 3.10. Quantitative Analysis of Endogenous and Minigene2 Full Length CPB2 mRNA in Several Cell Types.

The abundance of full length endogenous and full length minigene2 CPB2 mRNA transcripts was assessed from total RNA isolated from not transfected and transfected HepG2, BHK, Hek293, and SH-SY5Y cells with minigene2-4A using quantitative real time RT-PCR. The primer and probe set was designed to amplify within the 52 bp region of exon 11 normally deleted in Δ11 TAFI of CPB2. The quantity of unknown samples was determined via extrapolation from the full length CPB2 minigene2 standard curve. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.
Figure 3.11. Quantitative Analysis of Endogenous and Minigene2 Δ11 CPB2 mRNA in Several Cell Types.

The abundance of Δ11 endogenous and Δ11 minigene2 CPB2 mRNA transcripts was assessed from total RNA isolated from not transfected and transfected HepG2, BHK, Hek293, and SH-SY5Y cells with minigene2-4A using quantitative real time RT-PCR. The primer and probe set was designed to amplify a region within exon 11 that conjoins the flanking regions on either side of the 52 bp region in exon 11 that is normally deleted in Δ11 TAFI of CPB2. The quantity of unknown samples was determined via extrapolation from the Δ11 CPB2 minigene2 standard curve. Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicates.
Table 3.1. Summary Tables of Real Time PCR Analysis of Full Length \textit{CPB2} and Alternatively Spliced Minigene \textit{CPB2} Variants.

Each value was calculated from the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicate.

A.

<table>
<thead>
<tr>
<th>Sample</th>
<th>((\text{FL/Total}) %)</th>
<th>((\Delta 7/\text{Total}) %)</th>
<th>((\Delta 7+8/\text{Total}) %)</th>
<th>Sum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>88.54</td>
<td>0.22</td>
<td>11.24</td>
<td>100</td>
</tr>
<tr>
<td>HepG2 Mini1</td>
<td>33.47</td>
<td>54.73</td>
<td>11.80</td>
<td>100</td>
</tr>
<tr>
<td>Hek293</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hek293 Mini1</td>
<td>42.98</td>
<td>50.96</td>
<td>6.06</td>
<td>100</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>45.43</td>
<td>54.57</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>SH-SY5Y Mini1</td>
<td>24.76</td>
<td>53.82</td>
<td>21.42</td>
<td>100</td>
</tr>
<tr>
<td>THP-1</td>
<td>65.34</td>
<td>26.30</td>
<td>8.36</td>
<td>100</td>
</tr>
<tr>
<td>THP-1 Mini1</td>
<td>15.36</td>
<td>57.70</td>
<td>26.94</td>
<td>100</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Sample</th>
<th>((\text{FL/Total}) %)</th>
<th>((\Delta 11/\text{Total}) %)</th>
<th>Sum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>99.57</td>
<td>0.43</td>
<td>100</td>
</tr>
<tr>
<td>HepG2 Mini2</td>
<td>75.92</td>
<td>24.08</td>
<td>100</td>
</tr>
<tr>
<td>BHK</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BHK Mini2</td>
<td>77.41</td>
<td>22.59</td>
<td>100</td>
</tr>
<tr>
<td>Hek293</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hek293 Mini2</td>
<td>97.10</td>
<td>2.90</td>
<td>100</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>96.75</td>
<td>3.25</td>
<td>100</td>
</tr>
<tr>
<td>SH-SY5Y Mini2</td>
<td>19.85</td>
<td>80.15</td>
<td>100</td>
</tr>
</tbody>
</table>
Concerning the cell types that endogenously express TAFI (HepG2, SH-SY5Y, and THP-1 cells), the following trend (from most abundant to least abundant transcript number) was observed: HepG2 (full length > Δ7+8 > Δ7), SH-SY5Y (Δ7 > full length; Δ7+8 not detected and/or not expressed), differentiated THP-1 cells (full length > Δ7 > Δ7+8). With regard to minigene1 data, the following trend was observed in HepG2 and SH-SY5Y cells: (Δ7 > full length > Δ7+8), while the following trend was observed in differentiated THP-1 cells: (Δ7 > Δ7+8 > full length). With regard to minigene2 data, the following trend was observed in all cell types examined: (full length > Δ11), except in SH-SY5Y. Overall, there are considerable discrepancies in the proportions of the alternatively spliced forms when comparing the endogenous transcripts to those encoded by the minigenes (Table 3.1).

3.3 Identification of Full Length and Alternatively Spliced TAFI Variants via Metabolic Labeling

Identification of TAFI protein that is translated from alternatively spliced CPB2 mRNA was executed via metabolic labeling. First, steady state metabolic labeling was performed on HepG2 and BHK cells. Briefly, cells were transiently transfected with pcDNA4A, Full length TAFI-4A, Δ7 TAFI-4A, Δ11 TAFI-4A, or Δ7+11 TAFI-4A plasmids and labeled with ^35S-(Cys/Met); conditioned medium and lysates were prepared and immunoprecipitated with a mouse monoclonal anti-myc antibody, and the immunoprecipititates were subjected to SDS-PAGE and fluorography. As a positive control, BHK cells were transfected with the full-length-4A plasmid and
immunoprecipitations of the medium and lysate were performed with a polyclonal sheep anti-TAFI antibody. The steady state metabolic labeling results are shown in Figure 3.12 for HepG2 cells, and Figure 3.13 for BHK cells.

TAFI has an apparent MW of approximately 60 kDa on SDS-PAGE (26). However, due to the addition of the myc-6× His tag in the pcDNA4A plasmid, the MW of TAFI appears slightly larger, at approximately 63 kDa. In the HepG2 media, a distinct full-length TAFI band of approximately 63 kDa was observed only for full length TAFI (Figure 3.12.A). In the BHK media, a distinct full length TAFI band of approximately 63 kDa was only observed for the full-length TAFI sample that was immunoprecipitated with the anti-TAFI antibody, but not with the anti-myc antibody (Figure 3.13.A). No bands corresponding to protein from any of the alternatively spliced transcripts were observed in the media of HepG2 or BHK cells.

In the HepG2 lysates (Figure 3.12.B), two distinct bands appear in the full length TAFI lane, one of approximately 63 kDa which corresponds to the mature, fully-glycosylated form of TAFI, and a band of approximately 53 kDa which likely corresponds to a hypo-glycosylated form of TAFI (Figure 3.12.B). In addition, a band of approximately 51 kDa is also present that corresponds to the Δ7 TAFI variant (Figure 3.12.B). In the BHK lysates, only one distinct band appears in the full length TAFI lane (immunoprecipitated with anti-myc antibody and with the anti-TAFI antibody) of approximately 53 kDa, which corresponds to the hypo-glycosylated form of TAFI (Figure 3.13.B.). In addition, a band of approximately 51 kDa is also present that corresponds to the Δ7 TAFI variant (Figure 3.13.B).
Once alternatively spliced TAFI corresponding to the Δ7 TAFI variant was detected in HepG2 cells via steady state metabolic labeling, pulse-chase labeling was employed to determine the fate of the alternatively spliced protein. Pulse labeling of HepG2 cells was carried out similarly to steady state labeling. However, a 6-hour chase time course was used to determine the point of degradation of the alternatively spliced transcript.

In the lysates of pulse chased 35S-labeled HepG2 cells transfected with full length TAFI, only the hypo-glycosylated form of TAFI is present between 0 and 30 minutes (Figure 3.14.B). At one hour, both the hypo-glycosylated and glycosylated forms of TAFI are present in the lysate (Figure 3.14.B). In addition, in the media, there is a burst of secretion of full length TAFI after one hour, which is sustained through 6 hours (Figure 3.14.A).

In the lysates of 35S-pulse-labeled and chased HepG2 cells transfected with Δ7 TAFI, only the hypo-glycosylated form of the Δ7 TAFI variant is observed (Figure 3.15.B). This variant is present between 0 and 2 hours of chase, after which it disappears, which is indicative of its degradation in the lysate (Figure 3.15.B). The Δ7 TAFI variant is never secreted into the media like full length TAFI (Figure 3.14.A), as evidenced by the lack of bands in the media of the Δ7 TAFI variant during the 6 hour pulse chase time course (Figure 3.15.A). Moreover, a fully-glycosylated intracellular form is never observed (Figure 3.15.B).
Figure 3.12. Identification of Full Length and Alternatively Spliced TAFI Variants in HepG2 Cells Using Steady State Metabolic Labeling and Immunoprecipitation.

HepG2 cells were transiently transfected with pcDNA4A, FL-4A, Δ7-4A, Δ11-4A, and Δ7+11-4A. Also, a non-transfected plate of HepG2 cells was used as a negative control. 18 hours post transfection fresh media was added to the plates and cells were allowed to recover overnight. The following day Cys/Met-free DMEM was added to the plates for one hour, then \([^{35}\text{S}]\text{Cys/}^{[35}\text{S}]\text{Met}\) labeling mix was added to the plates and protein labeling occurred overnight. Next, both the media (A.) and lysate (B.) samples were harvested and subjected to immunoprecipitation with a monoclonal mouse-anti-myc antibody. Lastly, proteins were separated on a 12% polyacrylamide SDS-PAGE, fixed, dried and exposed to a phosphor imaging screen for 90 hours at room temperature before imaging.
Figure 3.13. Identification of Full Length and Alternatively Spliced TAFI Variants in BHK Cells Using Steady State Metabolic Labeling and Immunoprecipitation.

BHK cells were transiently transfected with pcDNA4A, FL-4A, Δ7-4A, Δ11-4A, and Δ7+11-4A. Also, a non-transfected plate of BHK cells was used as a negative control, and a second plate was transfected with FL-4A to serve as a positive control. 18 hours post transfection fresh media was added to the plates and cells were allowed to recover overnight. The following day Cys/Met-free DMEM was added to the plates for one hour, then $[^{35}\text{S}]$ Cys/$[^{35}\text{S}]$ Met labeling mix was added to the plates and protein labeling occurred overnight. Next, both the media (A.) and lysate (B.) samples were harvested and subjected to immunoprecipitation with a monoclonal mouse-anti-myc antibody. The BHK positive control used a polyclonal sheep-anti TAFI antibody for immunoprecipitation instead. Lastly, proteins were separated on a 12% polyacrylamide SDS-PAGE, fixed, dried and exposed to a phosphor imaging screen for 90 hours at room temperature before imaging.
HepG2 cells were transiently transfected with FL-4A. Also, a non-transfected plate of HepG2 cells was used as a negative control. 18 hours post transfection fresh media was added to the plates and cells were allowed to recover overnight. The following day Cys/Met-free DMEM was added to the plates for one hour, then [³⁵S] Cys/ [³⁵S] Met labeling mix was added to the plates and protein labeling occurred for one hour. Next, both the media (A.) and lysate (B.) samples were harvested at selected time points (0 hr, 15 mins, 30 mins, 1 hr, 2 hr, 4 hr and 6 hr) and subjected to immunoprecipitation with a monoclonal mouse-anti-myc antibody. Lastly, proteins were separated on a 12% polyacrylamide SDS-PAGE, fixed, dried and exposed to a phosphor imaging screen for 90 hours at room temperature before imaging.

Figure 3.14. Identification of Full Length TAFI in HepG2 Cells Using Pulse Chase Metabolic Labeling and Immunoprecipitation.
Figure 3.15. Identification of Δ7 TAFI in HepG2 Cells Using Pulse Chase Metabolic Labeling and Immunoprecipitation.

HepG2 cells were transiently transfected with Δ7-4A. Also, a non-transfected plate of HepG2 cells was used as a negative control. 18 hours post transfection fresh media was added to the plates and cells were allowed to recover overnight. The following day Cys/Met-free DMEM was added to the plates for one hour, then [35S] Cys/ [35S] Met labeling mix was added to the plates and protein labeling occurred for one hour. Next, both the media (A.) and lysate (B.) samples were harvested at selected time points (0 hr, 15 mins, 30 mins, 1 hr, 2 hr, 4 hr and 6 hr) and subjected to immunoprecipitation with a monoclonal mouse-anti-myc antibody. Lastly, proteins were separated on a 12% polyacrylamide SDS-PAGE, fixed, dried and exposed to a phosphor imaging screen for 90 hours at room temperature before imaging.
Chapter 4: Discussion

4.1 Alternative Splicing and Its Role in the Regulation of CPB2 Gene Expression

Most mammalian genes consist of several short coding exonic regions that are intervened by much larger non-coding intronic regions (91). To produce a mature mRNA species, the exons must first be recognized, followed by the removal of the intronic regions, and precise ligation of the exons together (91). Alternative splicing (AS) is a common cellular mechanism in higher eukaryotes that allows for the production of multiple mRNA isoforms from a single gene (92). This mechanism occurs in approximately 90% of all human multi-exon genes (92). Alternative splicing of premRNA transcripts takes place at a very crucial point in the flow of genetic information between transcription and translation. Alternative splicing is carried out by the spliceosome, a multi-ribonucleoprotein complex that assembles at recognized splice sites within a pre-mRNA sequence (92). The core of the spliceosome consists of five small nuclear RNAs, snRNAs (U1, U2, U4, U5, and U6 snRNA), in combination with over 150 proteins (92).

Splice sites consist of a consensus splice sequence (GU at the 5’ splice site and AG at the 3’ splice site), which are recognized by various splicing factors (92). The differential combination of competing 5’ and 3’ splice sites results in the production of alternatively spliced mature mRNA transcripts, through the inclusion/exclusion of an intron sequence, inclusion/exclusion of an exon sequence, or lengthening or shortening of an exon sequence (92). The proteins that are translated from these alternatively spliced
mRNA transcripts also have the potential to gain new protein functions, lose protein functions, or evolve into new, related protein functions. Thus, through the process of alternative splicing, the protein coding capacity of the eukaryotic genome is substantially increased without having to change the sequence of the original genomic code.

In the human population, the plasma TAFI antigen levels can vary significantly between different individuals. Currently, genetic effects on the gene encoding TAFI, CPB2, can only explain 15-25% of this differential variation in gene expression by individuals. Thus, the majority of factors that contribute to the regulation of CPB2 gene expression are still largely unknown. The main source of plasma TAFI is produced by the liver; however, recent studies have shown that TAFI is also extra-hepatically expressed in various other sites, such as the brain (cerebellum, cortex, hippocampus), testis, platelets, and immune and hematopoietic cells (89). Furthermore, the discovery of the numerous alternatively spliced TAFI variations has added a new level of complexity to the regulation of CPB2 gene expression by alternative splicing mechanisms.

The main concepts underlying the present study was that alternative splicing of CPB2 takes place by cell specific regulation processes that leads to different populations of alternatively spliced transcripts being expressed in different cell types, and that these transcripts have the potential to evolve into new, and distinct functions from full length TAFI. To study the extent of alternative splicing within various immune and vascular cell types, a minigene approach was employed. A minigene is a modified version of a naturally occurring gene that lacks one or more of its usual introns or exons. The use of minigenes as model systems to study splicing events is advantageous because it exploits the cells natural splicing and transcription machinery to investigate splicing events. Also,
the use of minigenes to study alternative splicing events has proven to be an effective strategy in the analysis of other alternatively spliced genes (93, 94, 95, 96).

In this study, we were able to successfully characterize, in a quantitative manner, the alternative splicing events occurring in various immune and vascular cell types. As was anticipated, the real time RT-PCR results revealed that alternative splicing of \( CPB2 \) is regulated in a cell-specific manner. All the cell types examined contained different populations of full length and alternatively spliced endogenous \( CPB2 \) transcripts. In addition, the abundance of each transcript varied significantly between the different cell types. We can therefore conclude that the extent of alternative splicing of \( CPB2 \) pre-mRNA occurs in a cell type-specific manner. We also found that the full spectrum of alternative splicing events could be recapitulated using the minigene approach; on the other hand, the frequency of the different splicing events differed substantially between the endogenous and minigene \( CPB2 \) transcripts. Our studies of the expression and intracellular processing of TAFI variants arising from alternative splicing confirm that these variants cannot be secreted and show differences in their intracellular trafficking kinetics.

In sum, these discoveries improve our understanding of \( CPB2 \) alternative splicing events. The knowledge gained from this study will eventually aid us in delineating the pathways by which alternative splicing events determine whether a pre-mRNA \( CPB2 \) transcript will become a full length TAFI variant involved in hemostasis or an alternatively spliced transcript, whose function remains to be discovered.
4.2 Identification of Minigene \textit{CPB2} mRNA in Several Cell Types via RT-PCR

Upon completing assembly of each minigene, they were transiently transfected into the cell types of interest to determine if mRNA that was transcribed from them could be detected. For this purpose, we employed RT-PCR using the 5’ EGFP – 5’ primer and the 3’ EGFP – 3’ primer. For minigene1, the expected amplicon lengths were: 841 bp for full length minigene1, 730 bp for Δ7 minigene1, and 636 bp for Δ7+8 minigene1; for minigene2, the expected amplicon lengths were 597 bp for full length minigene2 and 545 bp for Δ11 minigene2. The results from the RT-PCR analysis clearly reveal that minigene1 was correctly spliced into full length minigene1, Δ7 minigene1, and Δ7+8 minigene1 in HepG2 and Hek293 cells. This was evident by the detection of the expected fragment sizes for full length, Δ7, and Δ7+8 minigene1. The fact that all three variants were found in HepG2’s is in accordance with what has been previously published for this cell line (89). TAFI is not endogenously expressed in Hek293 cells, and so it is notable that the cells’ splicing machinery was able to splice minigene1 into all three variants.

No cDNA was detected for full length, or any alternatively spliced transcripts for minigene1 in SH-SY5Y cells or differentiated THP-1 cells (Figure 13.A). The fact that no bands of the correct size were detected in these cells was unanticipated. Full-length, as well as Δ7 \textit{CPB2} mRNA has been previously reported in both of these cell lines (89). In fact, Δ7 \textit{CPB2} mRNA was reported to be a dominant species present in total RNA samples from SH-SY5Y cells (89). It is evident that the cells were either not transfected or were transfected at too low an efficiency.
The results from the RT-PCR analysis also clearly reveal that minigene2 was detected in every cell type tested as full length minigene2. The fact that full length minigene2 was detected in HepG2 and SH-SY5Y cells is in accordance with what has been previously published for both of these cell lines (89). However, no cDNA was detected for Δ11 minigene2 in any cell type tested. Of all the cell types examined, the only one that endogenously expresses Δ11 is SH-SY5Y. The fact that no cDNA for Δ11 was detected in this cell line is somewhat unexpected since it has been previously reported as a dominant species present in this cell type (89). It is possible that the Δ11 variant was present at too low a proportion to be detected, although this was not borne out by our subsequent quantitative analysis (see below). Another explanation would be that the small difference in amplicon size (50 bp) could not be resolved by agarose gel electrophoresis performed under these conditions. A higher concentration of agarose, separation at a lower voltage, and loading a smaller quantity of PCR product on the gel may have revealed the presence of the Δ11 amplicon.

4.3 Quantification of Endogenous and Minigene \textit{CPB2} mRNA in Several Cell Types via Real Time RT-PCR

Since RT-PCR proved not sensitive enough to detect some of the alternatively spliced transcripts produced by the minigenes and is only semi-quantitative, we opted for a more sensitive technique, real time RT-PCR to attain a more quantitative measure of the alternative splicing events that are occurring within each cell type of interest. Real
time RT-PCR is a highly sensitive assay and can detect very low copy numbers of transcripts from a cellular heterogeneous mixture. However, since it is such a sensitive technique, it is also often susceptible to genomic DNA (gDNA) contamination during carryover in RNA extractions. To control for this possibility, no RT (NRT) controls were performed alongside samples to ensure amplification was genuinely from cDNA. In addition, no template control (NTC) reactions were also performed to ensure that the water and reagents used were free from contamination.

Previously, a study by Matsumoto and colleagues (83) reported the first incidence of an alternatively spliced brain-specific TAFI, HBCPB (aka Δ7+11). They originally detected the alternatively spliced variant in the brain (hippocampus), and also later reported its existence in human serum and cerebrospinal fluid (84). These studies were performed with the use of an antibody raised against the novel 14-residue carboxyl terminus that is encoded as a result of the Δ11 alternative splicing event. In addition, a second study performed by Cagliani and colleagues (88), reported a second finding of an alternatively spliced CPB2 mRNA which only lacked exon 7 (Δ7), and was identified in HepG2 cells and liver samples. They also reported that they did not detect any Δ11 alternatively spliced species within the liver (88). In a later study by Lin and colleagues (89), the pattern of alternatively spliced CPB2 transcripts was semi-quantitatively investigated in various tissues, and vascular and immune cells. From this study, a third novel CPB2 mRNA transcript was discovered that lacked exon 7 and exon 8 (Δ7+8) in HepG2 cells.

In this study, the results from the real time RT-PCR analysis confirmed our original hypothesis that alternative splicing of CPB2 is regulated in a cell specific
manner. We were able to show that there are several alternatively spliced variants in every cell type examined along with full length CPB2, and the abundance of every transcript varies between cell types. This is the first study to quantitatively demonstrate the pattern of alternatively spliced CPB2 transcripts within various vascular and immune cells.

In HepG2 cells, the full length CPB2 transcript is in far larger amounts than the alternatively spliced variants, of which, Δ7, Δ7+8, and Δ11 were present. The expression of Δ7 and Δ7+8 CPB2 transcripts within HepG2s is in accordance with what has been previously published for this cell line (88, 89). However, this is the first report of Δ11 CPB2 being expressed in HepG2 cells. This finding contrasts previous reports (88, 89) that did not detect Δ11 CPB2 within HepG2 cells. A possible reason why it was not previously detected could be due to the fact that only RT-PCR analysis of HepG2s for the Δ11 variant was performed, and this may not have been sensitive enough to detect its low copy number. Note that less that 1% of the total HepG2 transcripts contain the Δ11 splicing event (Table 3.1). Another possibility may be that the expression of the Δ11 variant only takes place under certain, as-yet undefined, environmental conditions (such as a different source of serum), which took place during this study.

In SH-SY5Y cells, the Δ7 CPB2 transcript is more abundant than the full length and Δ11 CPB2 transcripts. The expression of full length, Δ7 and Δ11 CPB2 transcripts in these cells (89) was confirmed in this study. No Δ7+8 CPB2 mRNA was detected in SH-SY5Ys in this study. In differentiated THP-1 cells, the full length CPB2 transcript is more abundant than the Δ7 and Δ7+8 CPB2 transcripts. The expression of full length and Δ7 CPB2 transcripts within differentiated THP-1 cells (89) was confirmed in this study.
Expression of the Δ7+8 CPB2 variant in differentiated THP-1 cells was also confirmed. CPB2 mRNA corresponding to the Δ11 variant was not investigated in differentiated THP-1 cells in this study.

What the current study crucially provides that distinguishes it from our previous work is quantitative information about the relative abundance of the various alternatively spliced forms of the CPB2 transcript. From these findings, it is clear that alternative splicing leading to variant forms of the TAFI protein is much more prevalent in non-hepatic cells than it is in HepG2 cells. These findings may reflect the importance of putatively novel functions of the TAFI variants in the non-hepatic cell types. Alternatively, it may reflect the considerably lower basal expression of the CPB2 mRNA in these cell types, such that a baseline frequency of alternative splicing makes these events more prevalent on a relative scale. Importantly, we previously demonstrated (89) that the alternatively spliced CPB2 transcripts are not subject to nonsense-mediated decay, bolstering the idea that the transcripts encode functional proteins.

The results from the real time RT-PCR analysis for minigene1 demonstrated that it does not recapitulate the frequency pattern of alternative splicing in any of the cell types tested. As previously stated, the pattern of alternative splicing is regulated in a cell specific manner and the even the abundance of each transcript varies between cell types. However, with respect to minigene1, the frequency of the different splicing events does not vary much between cell types. Minigene1 was preferentially spliced to produce the Δ7 minigene1 variant, followed by full length, then the Δ7+8 minigene1 variant within every cell type tested, except differentiated THP-1 (Δ7 minigene1 was followed by the Δ7+8 minigene1 variant, then full length minigene1). With respect to minigene2, there is
a different trend produced than what was observed for minigene1 alternative splicing events. In every cell type tested, full length minigene2 was much more abundant than Δ11 minigene2, except for in SH-SY5Y cells. Nonetheless, with either minigene, there is very poor concordance in alternative splicing frequencies between the endogenous and minigene-encoded transcripts.

From this study, we have gained a quantitative understanding of the cell specific alternative splicing regulation of CPB2 in various immune and vascular cell types. This knowledge will assist in the designing of future experiments to determine what cell specific factors are regulating the alternative splicing pattern of CPB2, as well as hopefully deduce the mechanism by which alternative splicing takes place in these cells. The data also provides an impetus to examine what function, if any, is possessed by the TAFI variants arising from alternative splicing.

Future work should be aimed at deducing the cis-acting elements within the pre-mRNA of CPB2 that stimulate the alternative splicing pathway instead of the constitutive pathway of splicing, and the trans-acting factors that initiate this as well. There are several cis-acting regulatory splicing sequences that must be recognized by the splicing machinery in order for splicing to occur correctly. These cis-splicing regulatory sequences can be located within the exon or intron sequence of the pre-mRNA. Some examples of universal regulatory splicing sequences include: the 5’ and 3’ splice sites which define the beginning and the end of the intron sequence, the intron branch point sequence, and a polypyrimidine tract located upstream of the 3’ splice site (91). Furthermore, there are several cis-acting regulatory sequences within the exon and intron sequences, which can alter the path and rate of alternative splicing when bound by their
appropriate trans-acting factor proteins. Some examples of these cis-acting regulatory sequences include: exon splicing enhancers (ESEs), exon splicing silencers (ESSs), intron splicing enhancers (ISEs), and intron splicing silencers (ISSs) (91).

Enhancer and silencer sequences within the pre-mRNA that are bound by their trans-acting factor proteins have the ability to alter splice site selection by selecting for or repressing a specific splice site (98). Enhancer sequences within exons are often bound by proteins part of the Serine/Arginine-rich (SR) splicing factor protein family (98). These proteins contain two specific domains, which are important to their function as splicing factors. The first domain is an RNA-Recognition-Motif (RRM), which is required for binding to the cis-regulatory sequence on the pre-mRNA target (98). The second is an RS domain, which is located at its carboxyl terminal, and is rich in arginine and serine dipeptides (98). The RS domain of SR proteins is important for protein-protein interactions during splicing (98). SR proteins that are bound to their respective ESE sequences can promote the recognition of an exon by the splicing machinery, and can also recruit further splicing machinery to the splice site (98).

Silencer sequences within introns are often bound by proteins part of the heterogeneous nuclear ribonucleoprotein (hnRNP) family (98). HnRNPs also have RRMs to bind to target pre-mRNAs; however, they use arginine/glycine rich domains instead of RS domains to interact with other proteins during splicing (98). HnRNPs can promote exon skipping by binding to the pre-mRNA sequence and causing the mRNA structure to change in a way that isolates the silenced exon, so that it is skipped by the splicing machinery (98). Another method by which hnRNPs can silence an exon is by binding to a high affinity ESS sequence on a pre-mRNA and inducing recruitment of additional
hnRNPs to bind to the region (98). Excessive binding of hnRNPs can block the binding of SR proteins to ESE regions, and can even displace previously bound SR proteins to ESE sequences (98).

Through bioinformatic analysis of the CPB2 gene, possible cis-acting regulatory sequences of alternative splicing can be identified. Once the location of these sequences are known, mutations can be inserted into the minigenes at these locations to study their effects on the alternative splicing pattern of CPB2. Furthermore, analysis of potential trans-acting factor proteins can also be investigated. Potential trans-acting regulatory proteins can be assessed for their ability to modulate the alternative splicing pattern of CPB2 by cloning them into expression vectors and overexpressing them within the cell, followed by monitoring their effect on the type of alternatively spliced transcripts produced, as well as their abundance levels within each cell type. The specific population and abundance of SR proteins and hnRNPs within a particular cell type could determine the output of CPB2 splicing events as well. By identifying the cis-acting regulatory sequences and their associated trans-acting factor proteins within the pre-mRNA of CPB2, the mechanism by which alternative splicing of CPB2 takes place within each cell type can start to emerge and be understood.

Furthermore, alternative splicing can also be regulated in a tissue specific (99), developmental stage specific (100), or even by environmental factors such as hormones, for example estrogen (101). Previous studies have demonstrated that CPB2 expression can also be regulated by the presence of specific inflammatory cytokines (IL-1β and IL-6) (16), as well as by the particular stage of differentiation of THP-1 and Dami cells (82). Thus, future experiments should be aimed at investigating the possible effects that these
various alternative stimuli can induce on the alternative splicing pathway of \textit{CPB2} in order to further our understanding of the mechanism by which alternative splicing of \textit{CPB2} takes place.

Although there are various published examples of minigenes that are capable of reproducing the alternative splicing pattern created by the natural gene (93, 94, 95, 96), the minigenes constructed within this study were not successful in this aim quantitatively. In this study, minigene1 was preferentially spliced to produce Δ7 minigene1 variant instead of the full length minigene1 variant in every cell type examined. The preferential skipping of exon 7 in minigene1 could be due to missing \textit{cis}-regulatory sequences within the intronic region between exon 6 and exon 7 of minigene1 that occur naturally in the full length transcript. Since the minigene only contains a small portion of the intronic region that innately resides between the exons of \textit{CPB2}, this could result in an altered ratio of enhancer to silencer sequences present in the minigene pre-mRNA transcript, which favors silencing and exclusion of exon 7 that does not normally occur during the processing of the full length transcript.

Moreover, the preferential skipping of exon 7 in minigene1 could be attributed to the production of an altered secondary structure by the pre-mRNA transcript of minigene1 that differs from the secondary structure created by the full length transcript. This change in RNA secondary structure could attract hnRNP splicing regulatory proteins to bind to ISS sequences within the pre-mRNA of minigene1 and cause a splice site that is normally recognized by the spliceosome to be looped out, resulting in increased preferential skipping of exon 7 instead of its inclusion in the mature mRNA transcript.
Modified minigenes that contain additional intronic sequences may alleviate the non-representative splicing pattern seen in the existing minigenes.

A final possibility is that the level of expression of the transcripts from the minigenes, which was much higher than for the endogenous gene, resulted in aberrant usage of splice donor and acceptor sites; some factors required for inclusion of all the exons in this transcript may be limiting. The level of expression of the minigene transcripts could be tuned by utilizing a less powerful promoter, or by using a doxycycline-inducible promoter and titrating the concentration of doxycycline in the medium.

Most pre-mRNA transcripts contain multiple ESE, ESS, ISS, ISE sequences, which are bound by unique combinations of splicing regulatory proteins, such as SR proteins and hnRNPs. Thus, the mature mRNA that results from alternative splicing events is the product of the combination of various cis-acting regulatory sequences that induce positive and negative effects on a pre-mRNA transcript through the binding of splicing regulatory proteins, particularly SR proteins and hnRNPs. The change in the presence or absence of any of these factors can alter the decision by the spliceosome to include or exclude any portion of the pre-mRNA transcript in the mature mRNA transcript in a cell specific manner.
4.4 Identification of Full Length and Alternatively Spliced TAFI Variants via Metabolic Labeling

To detect full length and alternatively spliced TAFI protein, metabolic labeling was employed. Full length TAFI (~ 63 kDa) was successfully identified in the medium of transfected HepG2 and BHK cells via metabolic labeling. This result was anticipated due to the fact that the liver is the main source of plasma TAFI (26). In addition, while BHK cells do not endogenously express TAFI, the cells are readily transfected and have been used for heterologous expression of recombinant TAFI (30).

Full length TAFI immunoprecipitated with the monoclonal anti-myc antibody was not detected in the medium of BHK cells, although it was detected in the medium of transfected HepG2 by this method; moreover, full length TAFI immunoprecipitated with the polyclonal anti-TAFI antibody was also detected in the medium of BHK cells. A possible explanation for this occurrence could be that 90 hours of exposure was not long enough to detect the TAFI present in that sample. It is also possible that there was a technical issue with the immunoprecipitation of this sample. Also, no alternatively spliced TAFI variants were detected in the medium of either cell line, as expected based on previous results (89). It remains possible that the lack of detection of these variants in the BHK media was due to the same technical issue that may have affected the full-length variant. It must be noted, however, that no mature Δ7 variant was detected in the lysates of the BHK cells.

Full length TAFI in its glycosylated mature, secretable and hypoglycosylated immature forms were also detected in the lysates of both HepG2 and BHK cell lines. In
addition, Δ7 TAFI, slightly smaller in MW than the hypoglycosylated immature full length TAFI form, was also detected in the lysates of HepG2 cells for the first time. Crucially, fully glycosylated Δ7 could not be detected. The Δ11 TAFI and Δ7+11 TAFI variants were not detected at all in the lysates of HepG2 cells, in contrast to our previous findings (89). Clearly, additional optimization is required to detect these variants, which should be present as a result of overexpression of a cDNA specifically encoding them. Although mRNA encoding the Δ11 and Δ7+11 TAFI variants has not been detected previously in the liver, it has been detected in the hippocampus region of the brain, human serum and cerebrospinal fluid (83, 84). Future experiments should be repeated in the SH-SY5Y cell line in which endogenous mRNA encoding these variants is likely to be expressed.

Pulse-chase experiments clearly showed the appearance of the mature, fully glycosylated form of TAFI in the lysates coincident with appearance of secreted TAFI in the medium. For the Δ7 variant, by contrast, there was no band of the expected size for the mature, fully glycosylated form either in the lysates or the medium. These findings are in support of our hypothesis (89) that the variants arising from alternative splicing are retained in the endoplasmic reticulum (ER) as incompletely glycosylated species and thus cannot proceed through the secretory pathway. We suspect that some aspect of the folding process is aberrant when the sequences encoded by the alternatively spliced exons are missing such that processing of nascent glycans necessary for ER exit does not occur.

Currently, the function of the alternatively spliced TAFI variants is unknown. Previous studies have shown that these variants cannot be activated by thrombin-thrombomodulin, the main physiological activator of TAFI, and thus do not possess
TAFIa carboxypeptidase activity (89, 29). In addition, they are not secreted from the cell and therefore, they cannot participate in hemostasis (89). From these observations, one could speculate that the production of alternatively spliced transcripts may be a process that the cell undergoes in order to down regulate the amount of fully functional TAFI present in circulation at any given time. Certainly, our quantitative experiments show that exon 7 is skipped in a considerable proportion of the CPB2 transcripts in non-hepatic cell types which, in addition to the lower amount of CPB2 mRNA present in these cells, is in keeping with the lower levels at which they express TAFI protein.

Alternatively, the production of alternatively spliced transcripts could also result in related TAFI proteins that have alternate functions within the cell. Support for this premise can be found in the study by Matsumoto and colleagues, where HBCPB (i.e. Δ7+11 TAFI) was reported to have endopeptidase activity towards β-APP and Aβ1-42 (83). Since the endopeptidase activity of only the Δ7+11 TAFI variant was examined in this prior study, it would be advantageous to test the other alternatively spliced TAFI variants for endopeptidase activity towards β-APP and Aβ1-42 to see if they also share this alternate function. It remains to be conclusively demonstrated that HBCPB directly acts as a peptidase; there is no sequence similarity between TAFI and endopeptidases, and the Δ11 variant even lacks a Glu residue that is absolutely required for carboxypeptidase activity (89). The availability of the myc-tagged TAFI variants will allow this question to be addressed definitively.

Further evidence supporting the notion that the alternatively spliced transcripts may have an alternate intracellular role comes from examining the crystal structure of the TAFI protein produced by the alternative splicing events. For example, alternative
splicing of exon 7 results in the loss of 37 amino acids, and deletion of a β-strand and parts of 2 α-helices encoded by exon 7 (102). Also, alternative splicing that leads to the deletion of the 52 bp within exon 11 results in the loss of a flexible loop that attaches a β-sheet to an α-helix segment (102). These deletions, alone or jointly, would produce a protein with a significantly altered 3-D structure, which would thus also affect its functional properties. Due to the substantial amount of deletions within the amino acid sequence, the protein produced by the alternative splicing events is likely to be misfolded and thus retained within the ER. This alternative localization pattern for the alternatively spliced TAFI proteins could potentially result in a new function for these proteins within the endoplasmic reticulum. For example, they could function as molecular chaperones assisting in protein folding in the ER.

To investigate the possibility that alternatively spliced TAFI variants can act as molecular chaperones within the endoplasmic reticulum, affinity chromatography could be used to capture alternatively spliced TAFI variants in complex with an interacting protein via the His tag on the recombinant TAFI proteins. To determine the identity of the proteins, mass spectrometry could be employed. By determining the molecules that the alternatively spliced TAFI proteins interact with, their biological function within the cell can begin to be determined. From these findings, future experiments can be designed to advance the understanding of the mechanisms by which these proteins interact. A tempting hypothesis is that TAFI variants are not themselves the endopeptidase that processed β-APP and/or Aβ1-42, but rather act as chaperones or cofactors for the bona fide endopeptidases that do perform these functions.
4.5 Conclusion

In conclusion, this study quantitatively determined the extent of \textit{CPB2} pre-mRNA alternative splicing events occurring in various vascular and immune cells via real time RT-PCR analysis. The minigene constructs produced in this study could be spliced into the full length and alternatively spliced \textit{CPB2} transcripts predicted as demonstrated by the RT-PCR and real time RT-PCR analysis. However, neither one was successful in recapitulating, in a quantitative sense, the alternative splicing pattern of \textit{CPB2} by the cell specific regulated mechanism that occurs endogenously. Future work should be aimed at deducing the \textit{cis}-acting regulatory sequences within \textit{CPB2} pre-mRNA and the splicing regulatory proteins that govern the splicing behaviour of \textit{CPB2} in different cell types. In addition, future experiments using different environmental stimuli to assess their effect on the alternative splicing pattern of \textit{CPB2} will also provide insight into the mechanism by which alternative splicing is initiated to regulate \textit{CPB2} gene expression under different environmental conditions. Overall, this information will give us a better understanding of the unique sequences and splicing regulatory proteins involved in alternative splicing of \textit{CPB2}, as well as demonstrate the specific environmental stimuli that induce the alternative splicing pathway of \textit{CPB2} during different cellular conditions.

This study also identified protein corresponding to the \textit{Δ7 TAFI} variant within the lysates of HepG2 cells via metabolic labeling. Since the alternatively spliced \textit{Δ7 TAFI} variant was not secreted from the cell, this reconfirms the notion that alternatively spliced TAFI variants do not participate in hemostasis. Currently, the biological function of the alternatively spliced transcripts remains unknown. One prior study demonstrated that the
Δ7+11 TAFI variant possessed endopeptidase activity towards β-APP. Future experiments should be aimed at verifying this functional property of the Δ7+11 TAFI variant, as well as test the ability of the other alternatively spliced TAFI variants for endopeptidase activity towards β-APP. In addition, future work aimed at deducing the interacting partners of these alternatively spliced intracellular TAFI variants will give us insight into the unique roles that these TAFI variants possess within each cell type. Overall, the knowledge gained from these findings will help us achieve a greater understanding of the functional and regulatory significance of the alternative splicing mechanisms that govern CPB2 gene expression.
References


25. Campbell, W., Okada, H. An arginine specific carboxypeptidase generated in blood during coagulation or inflammation which is unrelated to carboxypeptidase N or its subunits. *Biochemical and Biophysical Research Communications*, 1989; 162: 933-939.


33. Walker, J.B., Bajzar, L. The intrinsic threshold of the fibrinolytic system is modulated by basic carboxypeptidases, but the magnitude of the antifibrinolytic effect of activated thrombin-activable fibrinolysis inhibitor is masked by its instability. *Journal of Biological Chemistry*, 2004; 279: 27896-27904.


35. Schneider, M., Boffa, M.B., Stewart, R., Rahman, M., Koschinsky, M., Nesheim, M.E. Two naturally occurring variants of TAFI (Thr-325 and Ile-325) differ substantially with respect to thermal stability and antifibrinolytic activity of the enzyme. *Journal of Biological Chemistry*, 2002; 277: 1021-1030.


48. Stewart, R.J., Schneider, M.S., Manuel, R.P., Boffa, M.B., Nesheim, M.E. A naturally occurring TAFI variant with Ile at position 325 is more potent at suppressing plasminogen activation than wild type TAFI. Blood; 2001: 98: 254A-255A.


68. Sato, T., Miwa, T., Akatsu, H., Matsukawa, N., Obata, K., Okada, N., Campbell, W., Okada, H. Pro-carboxypeptidase R is an acute phase protein in the mouse, whereas carboxypeptidase N is not. Journal of Immunology, 2000; 165: 1053-1058.

70. Shinohara, T., Sakurada, C., Suzuki, T., Takeuchi, O., Campbell, W., Ikeda, S., Okada, N., Okada, H. Pro-carboxypeptidase R cleaves bradykinin following activation. *International Archives of Allergy and Applied Immunology*, 1994; 103: 400-404.


VITA AUCTORIS

NAME: Christina M. Rizzo

PLACE OF BIRTH: Windsor, ON

YEAR OF BIRTH: 1988

EDUCATION: University of Windsor,
B.Sc. in Biological Sciences,
Windsor, ON, 2011

University of Windsor,
M.Sc. in Chemistry and Biochemistry,
Windsor, ON, 2014