MOLECULAR AND STRUCTURAL ANALYSIS OF HUMAN FACTOR X DEFICIENCY

by

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Dedication

To my wife Anna,
whose love, support and encouragement
gave me the much needed strength to complete this study.
I could not have done it without her.

Also

to my parents who taught me how to work.

No one could make a greater mistake
than he who did nothing
because he could do
only a little.

Edmund Burke
Irish orator, philosopher and politician
(1729-1797)

...application, diligence, a sense of purpose,
the power to concentrate, to persevere
and not be cast down by adversity...

Sir Peter Medawar
Nobel Laureate
From "Advice to a Young Scientist"
(1915-1987)

Abstract

Factor X (FX) is one of the vitamin K-dependent serine proteases, and is g of a catalytic serine protease domain, two EGF domains and a Gla domain. It is crucial for coagulation, however its role in disease has not been fully characterised. This thesis describes the phenotypic and genotypic analysis of kindred with FX deficiency. Because FX deficiency is rare in the general population, patient samples were drawn from across the world. Laboratory assays for FX and direct sequencing of the gene were performed on samples from ten kindred.

A total of ten mutations were identified, nine of which were novel and summarised as follows. One mutation was identified in two unrelated families (Asp373Asn). Compound heterozygous mutations were identified in two kindred (Glu19Val and IVS5+3 A-G in one kindred; Cys132Stop and Arg273Trp in the other). Two other mutations were identified in the catalytic domain (Gly222Asp and Pro382Leu), one in the EGF-1 domain (Glu51Lys), and one in the signal peptide (Ala-26Asp). No mutation was identified in one kindred.

Molecular modelling of mutations in terms of the available crystal structure of factor Xa was performed in order to correlate genotype with phenotype. Explanations were proposed in terms of the perturbation of the structure itself or its biochemical function. For Glu51Lys and Arg273Trp, it was predicted that ligand binding would be disrupted. In the case of Asp373Asn, it was proposed that perturbation of the FX sodium-binding site would impair its enzymatic function. It was predicted that the Gly222Asp and Pro382Leu mutations would disrupt protein folding.

In order to show that selected known mutations are causative for FX deficiency, recombinant FX with the Arg-1Thr, Glu51Lys, Arg273Trp and Asp373Asn substitutions was expressed. Their expression was successful. In preliminary work with these, it was not possible to recapitulate the *in vivo* data for these expressed proteins.

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Deam, S., Uprichard, J., Eaton, J.T., Perkins, S.J. & Dolan, G. Factor X Leicester: Ile411Phe associated with a low antigen level and a disproportionately low functional activity of factor X. *J Thromb Haemost,* (2003) **1,** 603-605.

Deam, S., Uprichard, J., Eaton, J.T., Perkins, S.J., Dasani, H., Hanley, J.P. & Dolan, G. Two new factor X mutations associated with low activity and low antigen levels. *Thromb Haemost,* (in press).

Abstracts

Uprichard, J., Perkins, S.J., Peyvandi, F. & Perry, D.J. (2001) Molecular modelling of mutations in human factor X. *Blood Coagulation and Fibrinolysis,* **12,** A10.

Uprichard, J., Menegatti, M., Peyvandi, F., Riddell, A.F., Perry, D.J. (2002) *In vitro* expression analysis of a naturally occurring mutation (Arg -1 Thr) in the factor X gene. *Blood,* **100,** 93b, 3847.

Uprichard, J., Perkins, S.J., Peyvandi, F. & Perry, D.J. (2002) A genetic analysis of patients with factor X deficiency from Iran. *Haemophilia,* **8,** 506.

Deam, S., Uprichard, J., Dasani, H., Lewis, S., Perkins, S.J., Hanley, J.P. & Dolan, G. (2003) Factor X Cardiff and Factor X Newcastle: Two new factor X mutations associated with low activity and low antigen levels. *J Thromb Haemost,* supplement **1,** P1190.

Kalsheker, S., Alsonso, P., Uprichard, J., Eaton, J.T., Perkins, S.J., Dasani, H., Hanley, J.P. & Dolan, G. (2004) Experience with Factor X mutation detection. *Haemophilia,* **10 (Suppl. 3),** 15 PO 18.

Abbreviations and Nomenclature

Throughout this work the residue sequence numbering of FX starts from the amino-terminus of the mature protein (alanine +1). This is 40 residues downstream from the methionine encoded by the initiation codon. The start methionine is numbered –40 (Leytus *et al* 1986). The nucleotide sequence and residue numbering for the coding region are shown in **appendix 2**. When comparisons with other proteins are needed, residues in the catalytic domain are numbered with the prefix c (e.g. c227) in parentheses. This number refers to the equivalent residue in the chymotrypsin protease domain.

A		adenine
C		cytosine
G		guanine
T		thymine
Ala	A	alanine
Arg	R	arginine
Asn	N	asparagine
Asp	D	aspartic acid
Cys	C	cysteine
Gln	Q	glutamine
Glu	E	glutamic acid
Gly	G	glycine
His	H	histidine
Ile	I	isoleucine
Leu	L	leucine
Lys	K	lysine
Met	M	methionine
Phe	F	phenylalanine
Pro	P	proline
Ser	S	serine
Thr	T	threonine
Trp	W	tryptophan
Tyr	Y	tyrosine

Val	V	valine
Å		angstrom
APTT		activated partial thromboplastin time
BHK		baby hamster kidney (cells)
bp		base pairs
BSA		bovine serum albumin
C		coulomb
cm		centimetre
Ca^{2+}		calcium ions
$CaCl_2$		calcium chloride
$CaPO_4$		calcium phosphate
cAMP		cyclic 3',5'-adenosine monophosphate
cDNA		complementary deoxyribonucleic acid
dL		decilitre
DNA		deoxyribonucleic acid
dNTP		deoxynucleotide triphosphate
DTT		dithiothreitol
ECL		enhanced chemiluminescence
EDTA		disodium ethylene diamine tetraacetic acid
EGF		epidermal growth factor
ELISA		enzyme linked immunosorbent assay
F10		factor X gene
FBS		foetal bovine serum
FFP		fresh frozen plasma
FP		forward primer
fs		femtosecond
FX		factor X
FXa		factor Xa
FX:Ag		factor X antigen assay
FX:APTT		activated partial thromboplastin time factor X assay
FX:chromogenic		chromogenic factor X assay
FX:PT		prothrombin time-based factor X assay
FX:RVV		Russell viper venom-based factor X assay
g		gram

g	acceleration of gravity
Gla	γ-carboxyglutamic acid
GP	glycoprotein
GTP	guanosine triphosphate
HEK	human embryo kidney (cells)
HRP	horseradish peroxidase
Hya	β-hydroxyaspartic acid
iu	international units
K	kelvin
KCl	potassium chloride
kDa	kiloDalton
L	litre
LC	light chain
μF	microfarad
μg	microgram
μl	microlitre
m	metre
M	mole
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$,	magnesium sulphate
ml	millilitre
mm	millimetre
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
NaH_2PO_4	sodium dihydrogen orthophosphate
NH_4Cl	ammonium chloride
Na_2HPO_4	disodium hydrogen orthophosphate
NMD	nonsense mediated messenger ribonucleic acid decay
OBS	Owren's buffered saline
PCC	prothrombin complex concentrate
PDB	protein data bank

PIVKA	protein in vitamin K absence
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PT	prothrombin time
RNA	ribonucleic acid
RP	reverse primer
rpm	revolutions per minute
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
RVV	Russell viper venom
SCR	structurally conserved region
TAFI	thrombin-activated fibrinolytic inhibitor
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TF	tissue factor
TFPI	tissue factor pathway inhibitor
U	unit
V	volt
vWF	von Willebrand factor
W	watt

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1 Introduction to Haemostasis and Factor X

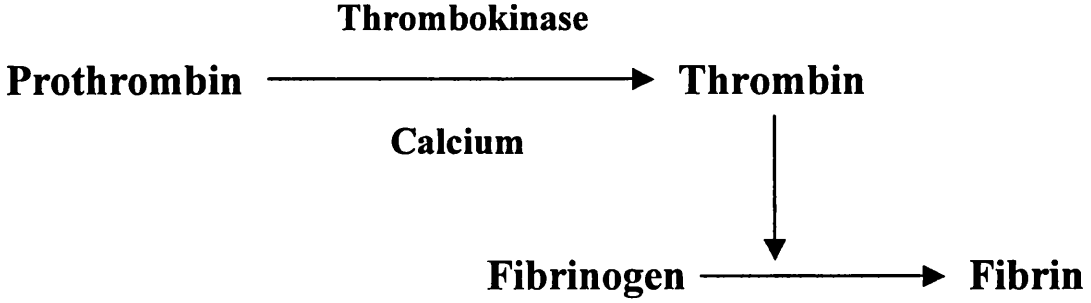
Factor X (FX) deficiency is a rare autosomally inherited bleeding disorder with an incidence of 1:1,000,000 in the general population. It is usual for the bleeding tendency to correlate with the FX level and patients with a severe FX deficiency tend to be the most seriously affected among patients with rare coagulation defects (Peyvandi and Mannucci 1999). FX is a vitamin K-dependent glycoprotein and is the zymogen of the serine protease FXa. It occupies a unique position in the coagulation cascade as the first enzyme in the common pathway of thrombus formation.

1.1 Historical Perspective of Haemostasis

The ancient Greeks, including Hippocrates (c.460-380 BCE) and Aristotle (384-322 BCE) were aware that when blood leaves the human body, it usually clots within a few minutes. They were also aware of various bleeding tendencies. However, they did not make the connection between blood coagulability and haemostasis. This association was made by the Jews in the 2nd century CE and recorded in the Babylonian Talmud. Rabbi Nattan described the situation of a mother whose first two sons had both died following ritual circumcision. The reason for their deaths was recognised as a "deficiency of the blood". He recommended that the third son should not be circumcised (Tractate Shabbat 134a). It was also recognised that the bleeding diathesis was the result of a problem inherited from the mother.

In modern times, the first account of haemophilia was written by John Conrad Otto (1774-1844), a physician from Philadelphia. He clearly recognised the three cardinal features of haemophilia A (factor VIII deficiency): that it is an inherited tendency, that it affects males and that affected individuals bleed (Otto 1803).

In 1905, Paul Morawitz formulated his four-factor hypothesis to explain the basis of coagulation:



In the presence of calcium and thrombokinase (later known as thromboplastin), prothrombin is converted to thrombin. Fibrinogen is converted to fibrin in the presence of thrombin. Fibrin causes blood to coagulate (Morawitz 1905).

From the early 1940s, it was realised that the Morawitz hypothesis fell short of the whole story. Indeed, Paul Owren studied a female who was thought to have haemophilia, a condition which was believed to be limited to males. He showed that her blood was missing a substance which was different to that which caused classical haemophilia. He named the discovered substance "accelerin" – it is now known as factor V (Owren 1947).

Following Owren's discovery, the remaining coagulation factors were discovered during the late 1940s and 1950s. The last factor to be described was FX. The natural anticoagulants were not identified until later.

1.2 An Overview of Haemostasis

The constant flow of blood through the circulation is fundamental to the maintenance of life and well-being of any organism. Haemostasis is an intricate mechanism which is designed to maintain the fluid state of blood under physiological conditions. Following vascular injury the haemostatic system reacts to stem excess bleeding at the point of injury. It must perform this physiological function whilst maintaining the circulation of blood to the vital tissues. Thrombosis, however, is a pathological process which may occur if the haemostatic stimulus is unregulated. This occurs if the inhibitory pathways are impaired or if the natural anticoagulant mechanism is overcome by the procoagulant stimulus.

In evolutionary terms, death from haemorrhage was not uncommon, especially during childbirth, so a fast and efficient way to stop blood-loss was necessary to avoid extinction. The advances of modern medicine are such that patients are more likely to survive the haemostatic challenges of major surgery, but they remain vulnerable to over-activity of the system and so to thrombosis. From this it can be seen that any haemostatic system must have the seemingly contradictory properties of anticoagulation, procoagulation and fibrinolysis. Derangement of any portion of this intricate process can produce an imbalance, which results in a haemorrhagic or thrombotic disorder.

The haemostatic system is complex. In order to maintain the flow of blood, there must be integration between organ, tissue and cellular processes. Disruption to the circulation alters physical factors associated with normal blood flow. This leads to a series of cellular and biochemical responses which result in the formation of a platelet plug and subsequent stabilization of the plug by a fibrin mesh (see **figure 1.1**).

1.2.1 Endothelium

The whole of the circulatory system is lined by a single layer of cells, the endothelium. In the adult human, the endothelial surface is composed of approximately 10^{13} cells which cover a surface area of approximately 7 m^2 and have a mass of about 720 g (Augustin *et al* 1994). Approximately 80% of these cells (and so 80% of the surface exposed to blood) are in the capillaries. The relatively large endothelial surface area to blood volume ratio in these vessels (up to 5000 cm^2/ml) allows for the efficient exchange of nutrients and hormones (Bush and Owen 1982). In contrast, arteries and veins contain a greater quantity of blood, but are covered by a smaller area of endothelium (10 cm^2/ml). Therefore the cells lining these larger vessels contribute far fewer plasma factors to the circulation than those in the capillaries.

Shear stresses caused by blood flow modulate endothelial gene expression, as well as the activity of certain endothelial enzymes (Topper and Gimbrone 1999). Under resting conditions, endothelial cell turnover is low, but at sites of haemodynamic injury, proliferation is increased.

The endothelium functions as a gateway between the blood and the tissues. It acts to regulate the extravasation of hormones, solutes and fluid. If the barrier function of the endothelium does not perform appropriately, then leakage of macromolecules may occur, causing oedema formation and exposure of the interstitium to high concentrations of plasma constituents (van Hinsbergh 1997). Vascular leakage may be desirable for recruiting plasma proteins (for example complement factors during infection), but it has the potential to be life-threatening if it occurs in the lungs or pericardium. Vessel permeability is increased by vasodilatation, by induction of severe thrombocytopenia and by high doses of heparin. The spontaneous bleeding observed with a low platelet count, or after heparin infusion may actually be induced

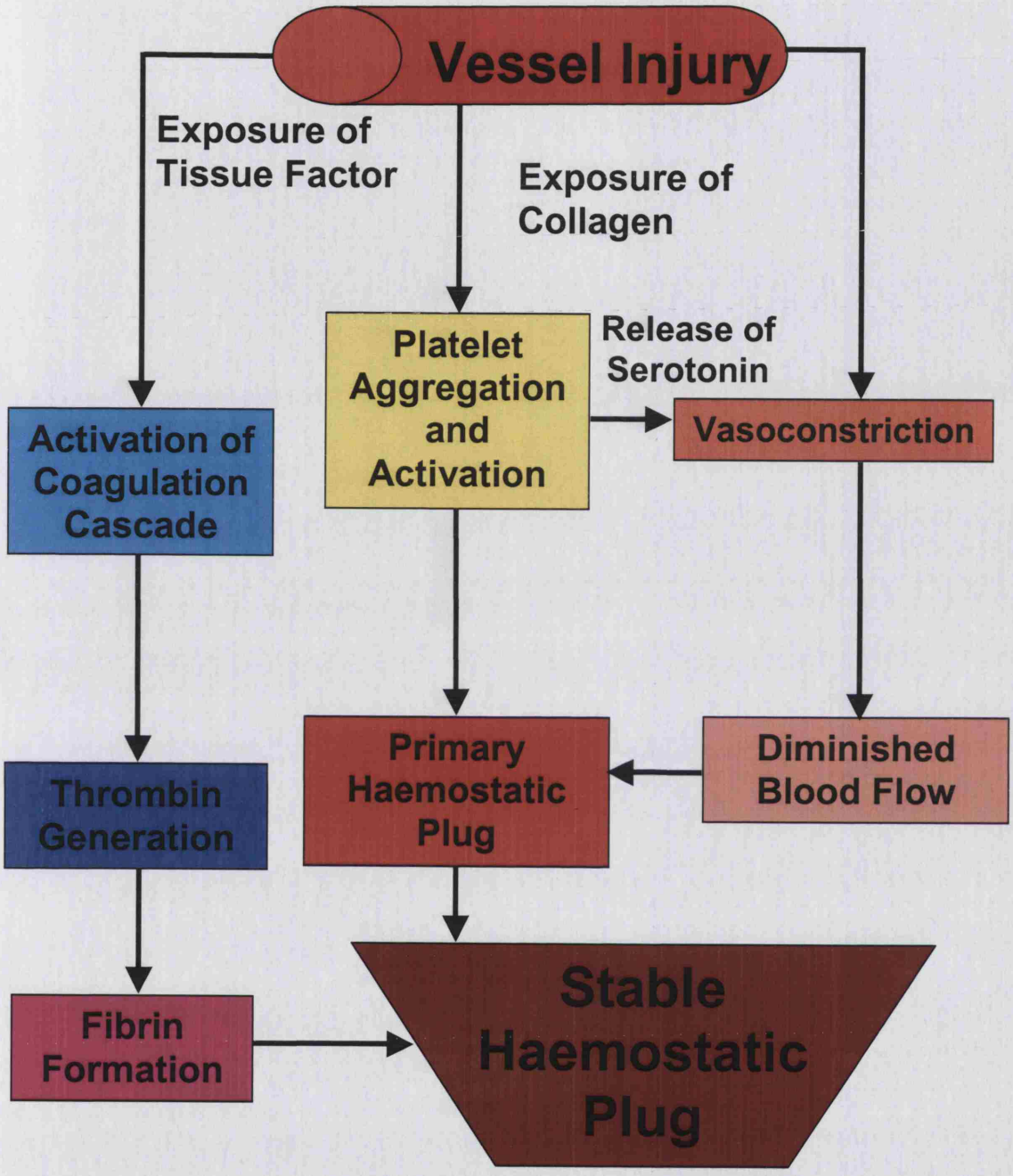


Figure 1.1. A diagrammatic representation of the events leading to the formation of the stable haemostatic plug.

by the increased vessel permeability (Minning *et al* 2002). This may be why the bleeding of idiopathic thrombocytopenic purpura often responds to treatment with systemic steroids even before a detectable rise in the platelet count (Kitchens 1980).

The endothelium also helps to maintain blood flow and prevent thrombus formation (Cines *et al* 1998; van Hinsbergh 1997). Endothelial cells are negatively charged and so are likely to repel the negatively charged platelets. This helps to limit the intravascular extension of the haemostatic reaction. Thrombomodulin and heparan sulphate are two thrombin inhibitors which are synthesized and expressed on the endothelial surface (Teien *et al* 1976). Heparan sulphate is a glycosaminoglycan which activates antithrombin and so catalyses the inhibition of thrombin and factor Xa (FXa). Thrombomodulin binds to thrombin. This leads to an inhibition of fibrinogen cleavage by thrombin and reduced activation of platelets, as well as coagulation factors Va and VIIIa.

The endothelium is capable of modulating fibrinolysis by synthesizing and secreting tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and plasminogen activator inhibitors (Minning *et al* 2002).

Thrombin and other factors (such as adrenaline and trauma) stimulate synthesis of the prostaglandin PGI_2 by endothelial cells (MacIntyre *et al* 1978). Other agonists (such as histamine, ATP, bradykinin and acetylcholine) stimulate endothelial cell guanylate cyclase, which raises the levels of intracellular cGMP (cyclic guanosine monophosphate) and results in the synthesis of nitric oxide (NO). Both PGI_2 and NO inhibit platelet aggregation and regulate vessel wall tone (Warner *et al* 1989). Stimulated endothelial cells can also synthesize endothelins, a group of peptides which have counter-regulatory properties, including vasoconstriction (MacCumber *et al* 1989).

In the process of inflammation, endothelial cells can be activated by inflammatory mediators (Etzioni *et al* 1999). This activation causes the transcription of new genes. This results in control of the recruitment of leucocytes to regions of tissue damage where they are needed. They are involved in tissue repair after injury by aiding angiogenesis.

Endothelial injury is thought to be an early event in the pathogenesis of an atherosclerotic plaque (Ross 1999). Many factors contribute to this impairment of endothelial function, including homocysteinaemia, oxidized lipoproteins, the products of smoking, bacterial toxins and advanced glycosylation products (van

Hinsbergh 2001). These factors contribute to thrombosis either by interfering with the endothelial anti-thrombotic defence system or by exposing the subendothelium to the circulating blood. Synthesis of tissue factor (TF) and PAI-1 (plasminogen activator inhibitor 1) is induced, von Willebrand Factor (vWF) is released and the concentration of thrombomodulin on the cell surface is reduced by cytokines. Thrombin which is bound to thrombomodulin is more efficient at activation of TAFI (thrombin-activated fibrinolytic inhibitor) (Nesheim *et al* 1997). This means that the newly-formed haemostatic plug is protected from premature break-down.

1.2.2 Platelets

When a blood vessel wall has been breached, platelets will adhere to the compromised portion. Following this, platelets spread along the exposed subendothelial surface, secrete stored platelet constituents and form large platelet aggregates (Ruggeri *et al* 1999). In addition to this, platelets become available for use as phospholipid membranes for the binding of clotting factors. This accelerates the process of coagulation and the formation of the fibrin network that reinforces the friable platelet plug. Subsequent to this, the platelet-fibrin clot retracts into a smaller volume.

Platelets are not attracted to normal endothelium, but to disrupted vessel wall. The endothelial disruption exposes subendothelial binding sites for vWF, fibrinogen and fibronectin (Ruggeri *et al* 1999). It is likely that these adhesive proteins aid the formation of a bridge between platelets and the subendothelial connective tissue. In Bernard-Soulier disease, in which patients lack the platelet glycoprotein (GP) complex GPIb-IX-V and in von Willebrand's disease, in which vWF is dysfunctional or reduced, platelet adherence is defective and a bleeding tendency results. Other events necessary for platelet adhesion include the interaction of collagen with the platelet GPIa-IIa receptor (Staatz *et al* 1989) and activation of intracellular signaling pathways by platelet GPVI (Howard 1980).

Once a layer of platelets has adhered to the subendothelium, further platelets (which have been delivered by the flowing blood) adhere to the first layer and then to each other. Eventually a mass of aggregated platelets is formed. A critical event in platelet aggregation and activation is a change in platelet GPIIb-IIIa so that it has an increased affinity and avidity for ligands such as fibrinogen and vWF (Shattil *et al*

1998). In turn, this results in conformational changes in fibrinogen, with the expression of receptor-induced binding sites (Ugarova *et al* 1993). Fibrinogen is possibly the most important protein in aggregation because of its divalent structure. This allows it the potential to form a bridge between platelets, thereby mediating aggregation (Minning *et al* 2002). VWF and collagen are able to interact with resting platelets, however fibrinogen can only form its high affinity bond with GPIIb-IIIa on activated platelets (Bennett and Vilaire 1979). In Glanzmann's thrombasthenia, a congenital disorder in which GPIIb-IIIa is deficient, the associated defect in fibrinogen binding results in a bleeding tendency (Nurden and Caen 1979). In congenital afibrinogenaemia, the overall coagulation defect is due in part to an abnormality of platelet aggregation.

Platelet agonists are substances which have the ability to induce platelet aggregation and granule secretion. They include adenosine diphosphate, collagen, arachidonic acid and adrenaline. The reaction of platelet with agonist results in disappearance of the band of microtubules that maintains the platelet's shape, centralization of storage granules and the formation of pseudopodia.

The platelet surface has specific receptors for these agonists (Colman 1990). Some of the agonist-receptor complexes exert their effects via G proteins, which hydrolyse guanosine triphosphate (GTP). Others are coupled to ion-permeable channels in the platelet membrane and influence ion flux (most importantly the inward flow of Ca^{2+}). Other receptors are linked to protein tyrosine kinases. Stimulatory agonists lead to the activation of phospholipase C, which eventually leads to the mobilization of Ca^{2+} and an increase in the cytoplasmic concentration of calcium. Many platelet activation processes are calcium-dependent, including phosphorylation of the light chain of myosin by a specific kinase enzyme and liberation of arachidonic acid from membrane phospholipids by the enzyme phospholipase A_2 (Adelstein and Conti 1975; Pickett *et al* 1976).

Platelets contain many organelles (including mitochondria, lysosomes, peroxisomes, glycogen granules, dense granules and α-granules). α-Granules are the principal secretory granule and the most numerous. Platelet aggregation results in centralization of the granules and fusion of the granular envelope with the intracellular canaliculi followed by external secretion of the granule contents (Fukami *et al* 2001).

Upon activation, platelets expose receptors for specific clotting factors, particularly factor Va. This may either be bound from plasma or secreted by the platelet. This receptor then functions as a focus for the *prothrombinase* complex when FXa and phospholipids from activated platelets come together to convert prothrombin to thrombin.

Platelet activation is regulated by several substances, of which the most important is cAMP (cyclic 3',5'-adenosine monophosphate) (Haslam *et al* 1978). cAMP inhibits platelet aggregation, secretion, shape change and adhesion.

1.2.3 Coagulation

When the endothelial lining is damaged and the subendothelium becomes exposed to the blood flow, the coagulation cascade is initiated (Butenas and Mann 2002). This is a series of steps in which the zymogens of serine proteases are transformed into active enzymes. These enzymes act to convert procofactors into cofactors. The complex of non-enzymatic cofactor, vitamin K-dependent serine protease and substrate on a phospholipid membrane results in maximum efficiency and velocity of the molecular reactions when Ca^{2+} are present (Mann *et al* 1990). The nature of the cascade is such that the product of each reaction serves as the enzyme in the following reaction. This amplifies the overall velocity of the reaction. The cascade ends when thrombin converts soluble fibrinogen into the insoluble polymer fibrin and the clot is formed.

According to convention, the coagulation system is divided into two arms, the "intrinsic pathway" (so called, because coagulation is initiated by components from within the vascular system) and the "extrinsic pathway" (so called, because of the need for the protein cofactor TF to initiate coagulation). These two pathways converge on the "final common pathway". This is useful for *in vitro* laboratory tests, but is of little relevance physiologically because the extrinsic pathway can activate the intrinsic pathway *in vivo*.

1.2.3.1 Extrinsic Pathway

Under normal circumstances, coagulation does not occur in the bloodstream. Initiation of the cascade depends on exposure of the blood to components that are not

present physiologically. These coagulation activators are revealed following endothelial injury. The critical component is TF which is a membrane receptor for factor VII (Rao *et al* 1996). TF is expressed by most cells that do not normally come into contact with the blood.

The principal plasma component of the extrinsic pathway is factor VII. This is one of the six vitamin K-dependent coagulation proteins (the others are prothrombin, factor IX, factor X, Protein C and Protein S). All these proteins have a number of γ-carboxyglutamic acid (Gla) residues near their amino-terminus. The hepatocyte requires vitamin K for the post-translational modification of Glutamic acid to Gla. Gla residues are required for calcium binding. The two carboxyl groups of a Gla residue bind one calcium ion. This serves as a bridge for protein binding to the phospholipid surface.

TF can bind to both factor VII and the activated factor VIIa. In normal people, the active form is present in the circulation at approximately 1% of the total concentration of factor VII (Morrissey *et al* 1993). Under normal circumstances, the factor VIIa is degraded by proteolysis, however the exposure of TF results in the binding of both factor VII and factor VIIa. Only the TF-VIIa complexes are active enzymatically. In fact, the TF-VIIa complexes "autoactivate" the TF-VII complexes (Neuenschwander *et al* 1993) in a positive feedback loop.

The TF-VIIa complex (the *extrinsic Xase complex*) has two principal substrates, the vitamin K-dependent proteins factor IX and FX. Cleavage of these zymogens by TF-VIIa on a phospholipid membrane results in the active serine proteases factor IXa and FXa (Bom *et al* 1990). These enzymes remain attached to the membrane via their Gla residues, which facilitates reactions further down the cascade.

A variety of inflammatory stimuli, including bacterial endotoxin and cytokines are known to promote the expression of TF on the surface of cells such as monocytes. This monocyte-derived TF has been shown to cause synovitis (Bokarewa *et al* 2002). This indicates that TF itself is pro-inflammatory.

1.2.3.2 Intrinsic Pathway

The initiation of the coagulation system *in vitro* via the intrinsic pathway is mediated by a group of proteins known as the "contact system". This group consists of factor XII, prekallikrein and High Molecular Weight Kininogen. It is known as the contact

system, because activation occurs upon contact with negatively charged surfaces such as kaolin and dextran sulphate. The effect of the contact system is to activate factor XI. The activated factor XIa then activates factor IX to IXa. Factor IXa and its cofactor, factor VIIIa, assemble on a phospholipid surface and together with Ca^{2+} form the *intrinsic Xase complex* which activates FX.

The relevance of the contact system to *in vivo* coagulation is questionable. Only a deficiency of factor XI is associated with clinical bleeding. Physiologically, coagulation is mediated by the *extrinsic Xase complex*. The FXa which it produces generates picomolar concentrations of thrombin. Thrombin has a number of functions, which include partial activation of platelets and cleavage of the pro-cofactors factor V and factor VIII, to generate the active cofactors Va and VIIIa respectively (Lawson *et al* 1994). It can also activate factor XI to XIa. The *intrinsic Xase complex*, which is formed when VIIIa and IXa combine, activates FX at a 50-100 fold higher rate than the *extrinsic Xase complex* (Lawson and Mann 1991).

1.2.3.3 Common Pathway

FXa catalyses the formation of thrombin from prothrombin. Working alone, the reaction is slow. However, when all four components of the "*prothrombinase complex*" (FXa, factor Va, phospholipids and Ca^{2+}) are combined, the rate of reaction is increased 280,000 fold (Furie and Furie 1988).The thrombin formed creates a positive feedback loop by activation of factor XI (Gailani and Broze 1991) and further activation of platelets, factor V and factor VIII (Pieters *et al* 1989). Thrombin also cleaves fibrinogen (Mosesson 1992) and factor XIII (Naski *et al* 1991) to form the insoluble cross-linked fibrin clot (Brummel *et al* 1999).

1.2.4 Regulation of Coagulation

The limitation of the coagulation cascade to haemostasis and the avoidance of pathological thrombosis is achieved by several mechanisms. These include coagulation factor dilution by control of blood flow, fibrinolysis and negative feedback by thrombin itself. Inadequately attached platelets are washed away by the flow of blood. Anticoagulant proteins bind to clotting factors and decrease their coagulant potential.

Antithrombin (AT) is a serine protease inhibitor (serpin) that can inhibit many of the serine proteases involved in the coagulation cascade (Olson *et al* 1993). It primarily neutralises thrombin and FXa, but also has effects on factor IXa. The naturally occurring polymer, heparin (which is related to the heparan sulphate on the endothelial surface) potentiates the effect of AT. Heparin binds to a basic group in AT and increases its rate of inactivation of thrombin. However, thrombin is no longer susceptible to the action of AT once it is bound to fibrin (Hogg and Jackson 1989).

Heparin cofactor II is another serpin. It inactivates thrombin in the presence of heparin or dermatan sulphate but is a relatively ineffective inhibitor of FXa (Tollefsen *et al* 1982).

Tissue factor pathway inhibitor (TFPI) inhibits the action of the TF-VIIa-Xa complex. TFPI is produced by endothelial cells. One domain of the protein binds to and inhibits TF-VIIa and another domain binds to FXa (Broze *et al* 1988). For efficient inhibition of TF-VIIa, TFPI needs to bind FXa (Rapaport 1991).

Thrombin is not only a procoagulant molecule, but also has inhibitory roles. As mentioned earlier, thrombomodulin, which is expressed on endothelial cells, inhibits the ability of thrombin to cleave fibrinogen and activate factor V, factor VIII and platelets. Thrombin also activates Protein C, which inactivates factors Va and VIIIa. This is enhanced by thrombomodulin. Activation of Protein C is enhanced 20-fold when Protein C is bound to the endothelial cell Protein C receptor (EPCR) (Taylor *et al* 2001). Activated Protein C retains its ability to bind EPCR and this complex is involved in cell-signalling mechanisms down-regulating inflammatory cytokine production (Riewald *et al* 2002). Protein C also enhances fibrinolysis, probably by binding an inhibitor of the plasminogen activators (Esmon *et al* 1982). Protein C activity is enhanced by its cofactor, Protein S, and is regulated by the Protein C inhibitor, plasminogen activator inhibitor-3, and α_1-proteinase inhibitor (Heeb *et al* 1987; Walker 1981). Protein S is controlled by the complement component C4b –binding protein. C4b forms a complex with Protein S, which prevents its action as a coagulation cofactor (Dahlback 1986).

The net effect of thrombomodulin binding to thrombin is the loss of the procoagulant function of thrombin and the inhibition of thrombogenesis. Thrombomodulin is expressed on endothelial cells, so following endothelial disruption the thrombomodulin which is exposed serves to limit the clot extension.

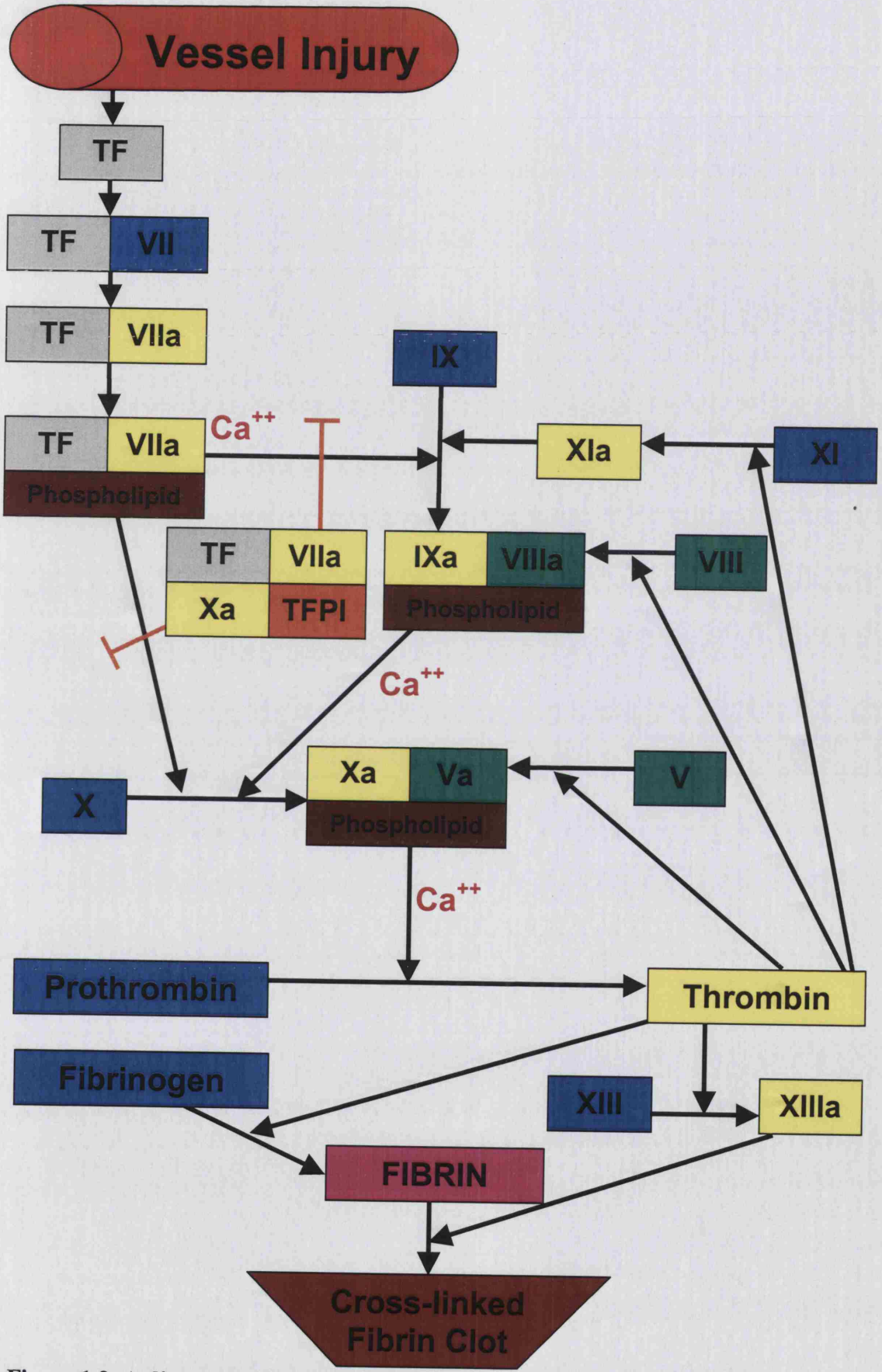


Figure 1.2. A diagrammatic representation of the generation of a fibrin clot. Co-factors are shown in green. Zymogens are in blue, whilst their activated factors are in yellow. Inhibitors and their inhibiting reactions are shown in orange.

1.2.5 Fibrin Formation

Fibrin is formed from its precursor, the 340 kDa glycoprotein fibrinogen. Fibrinogen is found in a relatively high concentration in plasma and also in platelet granules. Fibrinogen interacts with other proteins, such as factor XIII, fibronectin, α_2-antiplasmin, plasminogen and plasminogen activator (Doolittle *et al* 1978). Thrombin binds to fibrinogen and releases fibrinopeptides A and B. This results in fibrin monomer and then polymer formation (Blomback 1996). The fibrin polymer is extended by the addition of monomer molecules.

Fibrin is susceptible to degradation by plasmin. This degradation is impeded by factor XIIIa which catalyses the formation of covalent cross-links between glutamine and lysine residues in fibrin. Factor XIIIa also links α_2-antiplasmin to fibrin and this reduces susceptibility to fibrinolysis (Colman *et al* 2001). Eventually, many fibrin polymers are linked together and form a random network or mesh.

The fibrin mesh binds platelets together and interacts with proteins (such as thrombospondin, fibronectin and platelet fibrinogen) to increase the adhesion of the mesh to the vessel wall (Blomback 1996).

1.2.6 Fibrinolysis

Fibrinolysis is the mechanism by which clot formation is limited and so vessel recanalisation and endothelial cell regrowth can occur. There is a cascade mechanism which is similar to that of clotting factor activation. It involves zymogen to enzyme conversion, a feedback system and inhibitor interaction. The inactive precursor protein is plasminogen. Initially, platelets and endothelial cells release inhibitors of plasminogen activation, such as α_2-antiplasmin, which allow fibrin formation (Plow and Collen 1981). Later, endothelial cells release tissue plasminogen activator (tPA). Both tPA and prourokinase can convert plasminogen to the active enzyme, plasmin, which breaks down fibrin (Lijnen and Collen 1982).

Plasmin exerts a positive feedback on the system by cleavage of an activation peptide from Glu-plasminogen to create Lys-plasminogen. This makes it more susceptible to surface binding and activation by plasminogen activators. Plasminogen is also rendered more reactive when it is bound to fibrin (Colman *et al* 2001).

The small amount of plasminogen that is bound to fibrin is sufficient to influence fibrinolysis. However, fibrin also binds α_2-antiplasmin, which is covalently attached

by factor XIIIa (Collen 1980). The relative balance of the profibrinolytic plasminogen and plasminogen activators on one hand and the antifibrinolytic α_2-antiplasmin inhibitor molecules on the other influence the timing and efficiency of clot breakdown.

During clot dissolution, fibrin degradation products are released into the circulation. These serve as markers of ongoing fibrinolysis and hence prior thrombus formation.

Thrombin-activated fibrinolysis inhibitor (TAFI) is part of an important connection between the coagulation and fibrinolytic systems. TAFI is activated by the thrombin-thrombomodulin complex. It cleaves C-terminal lysine residues from fibrin. This prevents the binding of plasminogen, plasmin and tPA and so inhibits fibrinolysis. Thrombin-thrombomodulin also activates Protein C, which leads to the inactivation of factors Va and VIIIa and so to the arrest of further clot development.

1.3 Historical Perspective of FX

FX was identified in the 1950s by two independent groups. In 1956, a bleeding tendency was reported in a 22 year-old woman (Miss Prower) (Telfer *et al* 1956). She had an abnormal thromboplastin generation test and a prolonged prothrombin time; this was correctable by the addition of plasma from patients taking phenindione. One year later, abnormal coagulation parameters were described in a 36 year-old man (Mr Rufus Stuart) (Hougie *et al* 1957). At first, it was thought that the defect was due to factor VII deficiency, but direct mixing of his plasma with that of a known factor VII deficient patient led to a mutual correction of the prolonged prothrombin times; therefore the samples were not lacking the same clotting factor. Hougie was also able to show that there was a prolongation of the partial thromboplastin time, an abnormal thromboplastin generation test and a prolonged Russell Viper Venom ("Stypven") time. It was shown that patients with true factor VII deficiency have normal thromboplastin generation tests and normal partial thromboplastin times, whereas patients with FX deficiency (or Stuart-Prower factor as it became known) do not.

In 1954, a factor deficiency in patients receiving coumarins was reported, this factor was distinct from factors VII and IX (Duckert *et al* 1954). It was named FX, before the Roman numeral nomenclature was established. FX was given its official nomenclature by the "International Committee on the Nomenclature of Blood

Clotting Factors", which eventually became the International Society of Thrombosis and Haemostasis (Wright 1962).

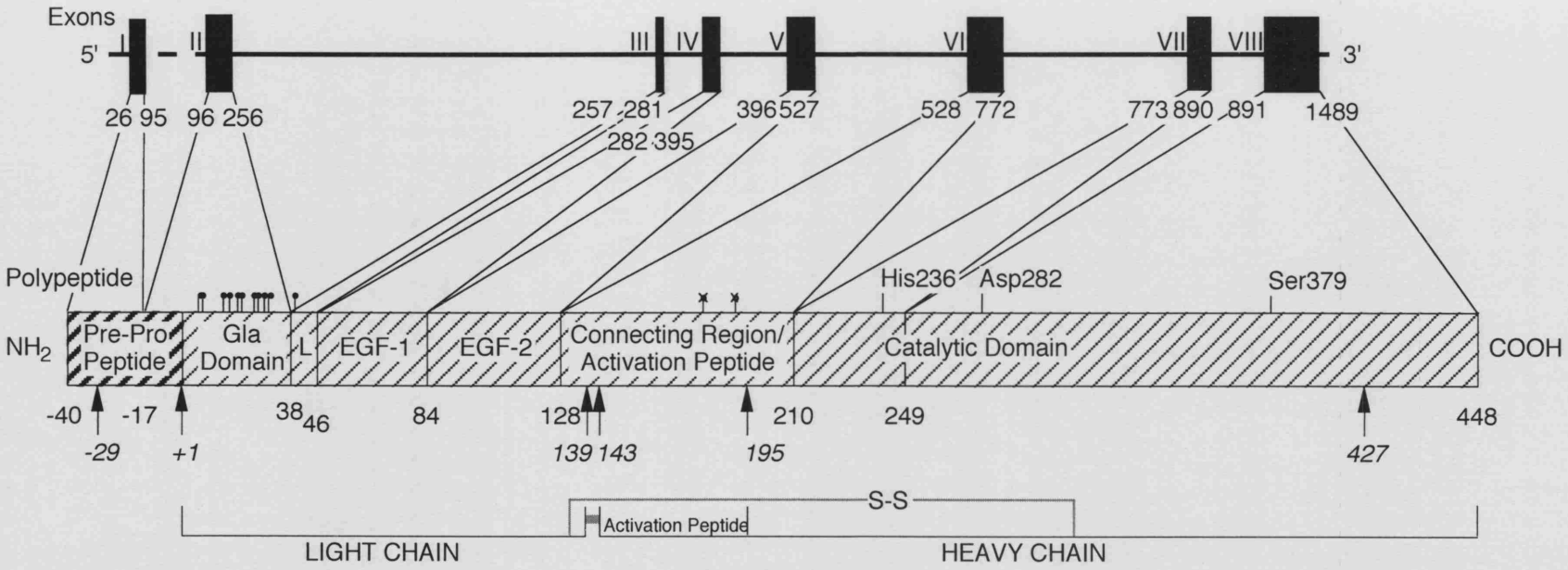
1.4 The Factor X Gene

The human FX gene (*F10*) is 22kb long and is located on the long arm of chromosome 13, at 13q34-ter (Pfeiffer *et al* 1982), just 2.8kb downstream of the factor VII gene (Miao *et al* 1992). The genes for the vitamin K-dependent proteins are homologous, both in structure and organisation, which suggests that their evolution is from a common ancestral gene (Patthy 1985). The coding sequence of each of the vitamin K-dependent proteins is divided into eight exons, each of which encodes a specific domain within the protein (Furie and Furie 1988). Seven introns divide the coding sequence at essentially identical locations to that of some of the other vitamin K-dependent proteins (Hertzberg 1994). Exon I encodes the signal peptide. Exon II codes for the propeptide and γ-carboxyglutamic acid-rich (Gla) domain. Exon III encodes a short linking segment of aromatic amino acids known as the "aromatic stack". Exons IV and V code for two regions which are homologous to epidermal growth factor (EGF). Exon VI encodes the activation region, which is at the amino-terminus of the heavy chain. Exons VII and VIII code for the two serine protease (SP) subdomains which contain the residues of the catalytic site (His236, Asp282 and Ser379). (See **figure 1.3**)

The intron-exon splice junctions follow the 'GT-AG' rule (Breathnach *et al* 1978). The *F10* cDNA consists of 1474 bp (120 bp coding for the 40 amino acid pre-pro-leader sequence, 1344 bp encoding the 488 amino acids of the mature protein and an unusually short 3' untranslated region of 10 bp preceding the poly(A) tail) (Fung *et al* 1985; Leytus *et al* 1984). The poly-adenylation signal (ATTAAA) is located 15 nucleotides upstream of the poly(A) tail of the *F10* mRNA (Fung *et al* 1985). The nucleotide sequence and residue numbering for the coding region is shown in **appendix 2.**

A TATA box usually heralds the start (a few bases downstream) of transcription, but no such sequence has been identified at the 5' end of *F10*. In keeping with other TATA-less promoters, multiple transcription initiation sites have been detected (Swick *et al* 1989). Most of these are clustered in a segment 13 to 33bp from the first

Figure 1.3 Human *F10* gene



Organization of the human *F10* gene and its encoded polypeptide. The upper part of the figure represents the *F10* gene with the eight exons shown by filled boxes. The positions of the first and last nucleotides of each exon are shown (standard nucleotides numbering is used (Leytus *et al* 1986)). The lower part of the figure shows the polypeptide structure and the various functional domains encoded by specific exons. Darker hatching indicates the pre-propeptide and lighter hatching the mature protein. Codons initiating each exon are shown (residue +1 is the first amino acid of the mature protein). Residue -17 indicates the site of probable cleavage by the signal peptidase. The light chain is encoded by residues +1 to 139 and the heavy chain by residues 143 to 448. The connecting tripeptide (RKR) is located between residues 140 and 142 and the activation peptide resides within the heavy chain at residues 143 to 195. Activation of FX occurs through cleavage at Arg194/Ile195. Residue 427 shows the site of cleavage of factor X that generates factor Xaβ. His236, Asp282 and Ser379 are the residues which constitute the catalytic triad. (🡅)the position of the 11 Gla residues; (🡇) the position of the two glycosylation sites. (Adapted from (Perry 1997))

ATG codon (Huang *et al* 1992). Huang and colleagues have also shown that the critical promoter elements lie somewhere between codons -209 and -108.

A number of polymorphisms within *F10* have been identified (de Visser *et al* 2001; Hassan *et al* 1988; Hay *et al* 1986; Jaye *et al* 1985; Millar *et al* 2000; Tuddenham and Cooper 1994; Wallmark *et al* 1991). Polymorphisms in the genes for Protein C (Spek *et al* 1995), PAI-1 (Dawson *et al* 1991), β-fibrinogen (Thomas *et al* 1991) and factor VII (Green *et al* 1991) have all been shown to affect plasma protein levels, however this is not the case for FX (de Visser *et al* 2001).

An unusual sequence variant has been identified close to the carboxy-terminal end of exon VII. FX clones have been isolated that lack a 9-bp sequence which encodes a Lys-Val-Arg tripeptide (residues 245-247) (Kaul *et al* 1986; Leytus *et al* 1984) but which was present in clones isolated by other groups (Fung *et al* 1985; Leytus *et al* 1986). At first, this was considered to be a cloning artefact, but Tuddenham & Cooper have postulated that the deletion may be due to the formation of a 'semi-cryptic' splice site, which results in alternative processing (Tuddenham and Cooper 1994).

1.5 Factor X Protein Structure

FX synthesis occurs in the liver. It is then secreted into the plasma and circulates as a two-chain molecule at a concentration of 10 μg/ml (Bajaj and Mann 1973). Rat FX is synthesised as a single-chain form, but is converted to the two-chain molecule after secretion (Graves *et al* 1982). The human hepatoma cell line, HepG2, secretes single-chain FX (Fair and Bahnak 1984). Post-secretion proteolytic cleavage of human FX to the two-chain form occurs in the Golgi apparatus (Stanton and Wallin 1992).

FX is synthesised with a 40-residue pre-pro-sequence. This contains the hydrophobic signal sequence that targets the protein for secretion (residues –37 to –22) (Leytus *et al* 1986). The pre-pro-sequence is homologous to that of other vitamin K-dependent proteins (Ichinose *et al* 1990). The pre-propeptide is cleaved in a two-step process. A signal peptidase cleaves between Ser-18 and Leu-17 and then the propeptide is removed by a second peptidase enzyme between –1Arg and +1Ala.

Post-translational modification occurs by γ-carboxylation of the 11 Glutamic acid residues in the Gla domain and also by glycosylation and β-hydroxylation before FX is fully functional. The two glycosylation sites in FX are located at residues Asn 181 and Asn 191 in the heavy chain and are, therefore, absent in FXa. Sinha and colleagues have used the production of recombinant FX mutants to demonstrate that activation of FX is inhibited if glycosylation of the two Asn residues does not occur (Sinha and Wolf 1993). β-Hydroxylation occurs at Asp63 in the first EGF domain, resulting in β-hydroxyaspartic acid (Hya) (McMullen *et al* 1983). The biological function of Hya in coagulation proteins is unknown, although it has been shown that it is not necessary for Ca^{2+} interactions (Nelson *et al* 1991).

The mature two-chain form of FX has a molecular weight of 59 kDa (Fair and Bahnak 1984). It consists of a light chain of 139 amino acids (17 kDa) and a heavy chain of 346 residues (42 kDa) (Di Scipio *et al* 1977). The two chains are linked by a disulphide bond between residues Cys89 and Cys124 and by an Arg-Lys-Arg (RKR) tripeptide (Perry 1997). The light chain contains the Gla domain, aromatic stack and two epidermal growth factor (EGF)-like domains. After the Gla domain, the next segment of protein (residues 38 to 46) has a high proportion of aromatic amino acids (Phe40, Trp41 and Tyr44) and therefore is known as the 'aromatic stack'. Residues 46 to 84 and 84 to 128 constitute the two EGF domains. Each EGF domain consists of three conserved Gly residues and six Cys residues, which form three structurally unique disulphide linkages. The EGF domains are thought to be important not only in binding Ca^{2+} but also in maintaining the correct conformation of FXa. The heavy chain contains the catalytic serine-protease domain and a 52 amino-acid activation peptide (residues 143 to 195) which is released on conversion of FX to FXa (Perry 1997). The catalytic domain (residues 210 to 448) contains the catalytic site (His236, Asp282 and Ser379).

Members of the serine protease family share considerable sequence homology. This suggests that they are derived from a single ancestral gene (Patthy 1985). The structural similarity of factors VII, IX, X and Protein C, both in exon organization and amino acid sequence, suggests a relatively recent evolutionary divergence.

1.6 Activation of FX

The active form (FXa) is produced by cleavage of the Arg194-Ile195 peptide bond in the heavy chain of the zymogen FX. Cleavage releases the 52-residue activation peptide. There are assays available for this activation peptide (Philippou *et al* 1995). FXa is then converted to FXaβ by hydrolysis of an Arg-Gly peptide bond at the carboxy-terminus (Fujikawa *et al* 1975). Several proteases can activate FX. Physiologically this occurs via the intrinsic pathway (by factor IXa) or via the extrinsic pathway (by factor VIIa) (Fujikawa *et al* 1974). *In vitro* activation can also occur with the addition of Russell Viper Venom, a metalloproteinase isolated from the venom of the snake *Vipera russelli* (Kisiel *et al* 1976).

1.6.1 Extrinsic pathway activation of FX

Activation via the extrinsic pathway occurs upon exposure of TF to plasma. FX is converted to Xa and factor IX to IXa by the *extrinsic Xase* complex in the presence of Ca^{2+} and a suitable phospholipid membrane (Rao and Rapaport 1988). The conversion of factor IX to IXa by VIIa-bound TF is 3-4 times more potent than activation of FX to FXa, which suggests that this may be physiologically more important (Komiyama *et al* 1990).

It has been found that many different cell types (including endothelial cells, fibroblasts, monocytes-macrophages and tumour cells) can provide an appropriate phospholipid membrane. The formation of the quaternary complex of factor VIIa with TF and phospholipid is responsible for a 15 million-fold increase in the catalytic efficiency of FX activation (Bom and Bertina 1990). The generated FXa becomes part of a positive feedback loop, increasing the conversion of factor VII to VIIa (Radcliffe and Nemerson 1975).

1.6.2 Intrinsic pathway activation of FX

FX activation via the intrinsic pathway occurs with the interaction of the *intrinsic Xase* complex, Ca^{2+} and acidic phospholipid surfaces (van Dieijen *et al* 1981). Factor IXa binds to platelets with a five-fold greater affinity if in the presence of factor VIII and FX (Ahmad *et al* 1989). The maximal velocity (Vmax) of the reaction is

increased about 200,000-fold and the Km is reduced from 300 μM to 60 nM by the presence of the complete FX activating complex (van Dieijen *et al* 1981).

1.6.3 Russell viper venom activation of FX

Activation of FX *in vitro* can occur by the direct action of a metalloproteinase found in the venom of *Vipera russelli*. The action of the metalloproteinase requires divalent cations (Amphlett *et al* 1982). Russell Viper Venom (RVV) has also been shown to activate Protein C and factor IX (Jackson 1984).

1.6.4 Other activators

Other activators of FX have been reported. These include a cysteine proteinase (named cancer procoagulant) which is associated with malignant disease (Gordon and Mielicki 1997), as well as CD11b (a monocyte surface marker) (Parratt and Hunt 1998) and enzymes from the venoms of other snakes (Yamada *et al* 1997). Trypsin has been shown to activate FX (Jackson 1984).

1.7 FXa and its Substrates

1.7.1 Prothrombin.

Physiologically, FXa is the most important activator of prothrombin. Factor Va binds to the phospholipid membrane and then acts as a co-factor by forming a receptor for FXa at the membrane surface in a 1:1 stoichiometry of high affinity (Nesheim *et al* 1981). FXa binds to the phospholipid membrane via its light chain, but to factor Va using its heavy chain. Prothrombin interacts with factor Va through the heavy chain of factor Va; this is independent of Ca^{2+}. Prothrombin then binds to the phospholipid via the Gla residues and the formation of Ca^{2+} bridges. Finally, prothrombin is cleaved, generating thrombin.

The phospholipid membrane increases the local concentration of coagulation proteins at sites of thrombus formation, whilst factor Va increases the catalytic efficiency of FXa (Mann *et al* 1990).

1.7.2 Factor V, factor VII and factor VIII.

FXa can cleave both factors V and VIII, to produce factors Va and VIIIa. Factor VII is hydrolysed by FXa to the two-chain factor VIIa, which has 85 times the coagulant activity of the single-chain factor VII. From this, it can be seen that factors VII and X form a positive feedback loop (Radcliffe and Nemerson 1975).

1.8 Regulation of FXa Activity

1.8.1 Antithrombin.

FXa forms a stable, inactive complex with antithrombin. The complex is removed from the circulation by the liver. The principle action of low molecular weight (short-chain) heparins is to increase the anti-Xa activity of antithrombin. Antithrombin blocks the activation of TF:VIIa, and so regulates the extrinsic pathway (Jesty *et al* 1996).

1.8.2 Tissue factor pathway inhibitor (TFPI).

TFPI is the major inhibitor of the extrinsic pathway (Rapaport 1991). FXa binds to TFPI in a 1:1 ratio to form a complex, which then binds to TF:VIIa to form a quaternary complex. This quaternary complex lacks any activity as a catalyst of the extrinsic pathway. Another serine protease inhibitor (TFPI-2) has been discovered (Sprecher *et al* 1994). It is homologous to TFPI, although it inhibits TF poorly and, as yet, has no defined physiological function (Herman *et al* 2001).

1.8.3 Factor VIII and FXa.

FXa has been found to inactivate factor VIII (Eaton *et al* 1986), and so takes part in its own negative feedback loop.

1.9 Other Actions of FX

It has been demonstrated that FX takes part in fibrinolysis as well as involvement in both the extrinsic and intrinsic coagulation pathways (Pryzdial *et al* 1999). FX

undergoes proteolysis by plasmin, which leads to the expression of a plasminogen binding site on FX. This gives FX the ability to accelerate the rate of plasmin generation by tissue plasminogen activator 30-fold.

1.10 Factor X deficiency

1.10.1 Clinical features of FX deficiency

Patients with FX deficiency may present with a bleeding tendency at any age, although the more severely affected ones (FX activity <1 iu/dL; reference range 50-150 iu/dL) present early in life, with for example, umbilical stump bleeding. Umbilical stump bleeding was thought to be characteristic of factor XIII deficiency. However it may occur in up to 30% of patients with FX deficiency. Haemarthroses, gastrointestinal bleeding and central nervous system haemorrhage have also been reported (**figure 1.4**). Less severely affected patients may bleed only after haemostatic challenge (e.g. trauma or surgery). Some cases are identified incidentally during routine screening or family studies. Patients who are only mildly affected may experience easy bruising or menorrhagia.

1.10.2 Diagnosis of FX deficiency

Following the finding of both a prolonged PT (prothrombin time) and APTT (activated partial thromboplastin time), a deficiency of one of the factors in the common pathway is suspected. The diagnosis of FX deficiency is confirmed by measuring plasma FX assay levels. There are five different assays for measuring FX levels: a PT-based assay, in which FX is activated via the extrinsic pathway, an APTT-based assay, in which FX is activated via the intrinsic pathway, RVV-based assay in which FX is activated with RVV, a chromogenic assay and an immunological assay. Most cases of FX deficiency are characterized by a prolongation in PT, APTT and RVV clotting times, along with low chromogenic assay and antigen levels. Therefore, the United Kingdom Haemophilia Centre Doctors' Organization guidelines for the management of rare bleeding disorders recommend that one-stage PT or APTT-based assays are usually sufficient for the diagnosis of a FX deficiency (Bolton-Maggs *et al* 2004). However, a number of mutations have been described which have one or more normal assay results

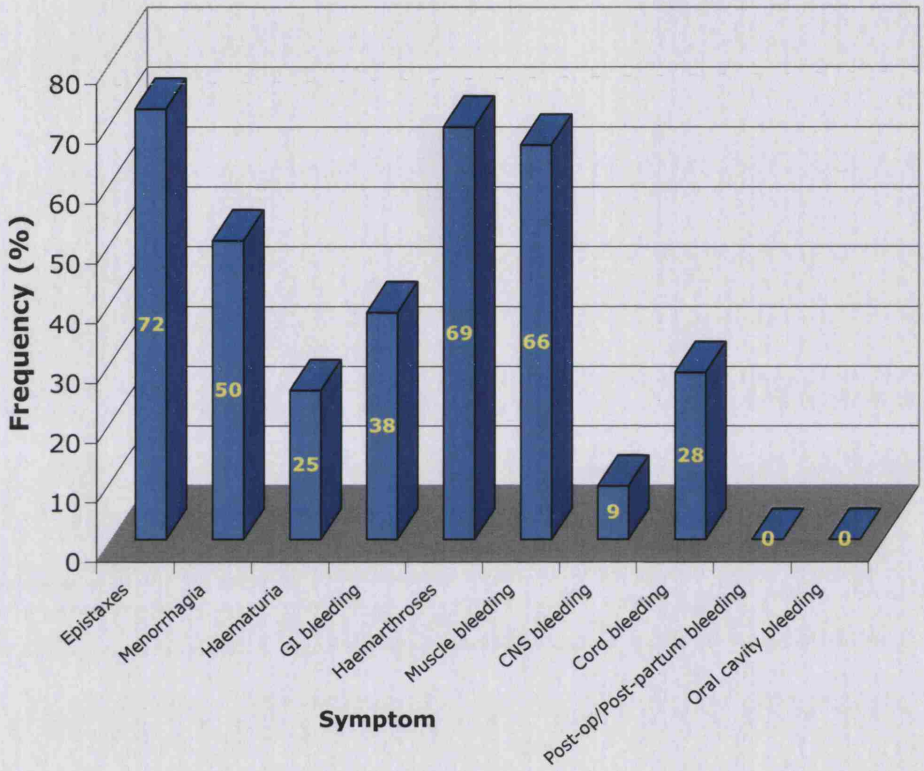


Figure 1.4 Frequency of bleeding symptoms in 32 patients with FX deficiency (<1 iu/dL). (Adapted from (Peyvandi *et al* 1998)). Post-operative and post-partum haemorrhage rates are zero because all patients in this survey received prophylactic factor replacement before haemostatic challenges.

((De Stefano *et al* 1988; Uprichard and Perry 2002) and references within). Consequently, caution must be exercised when diagnosing FX deficiency using only one assay; all five assays are required for detailed protein characterization (Perry 1997).

It is important to note that levels of most of the coagulation proteins (including FX) are low at birth. If a deficiency is suspected then age-related ranges for FX are essential (Williams *et al* 2002).

There are rare causes of acquired FX deficiency and these are addressed in **section 1.10.4.**

1.10.3 Classification of FX deficiency

The classification of FX deficiency is based on the results of laboratory assays (both immunological and functional). The underlying molecular defects are heterogeneous, so their phenotypic profiles differ markedly. In the Type I deficiency state, both functional activity and antigen level are decreased, as in the case of Stuart (Hougie *et al* 1957). However, in the case of Prower, the antigenic level is near normal while the activity is reduced (Fair *et al* 1979); this is known as Type II deficiency and indicates the presence of a dysfunctional FX.

A more complex classification of FX deficiency involving the results of functional (RVV, PT, APTT) and immunological assays has been devised (Fair and Edgington 1985). Their system shows the diverse nature of FX deficiency, but is rather cumbersome to use, see **table 1.** It is likely that a simpler classification system will be devised once more is known about the mutations at the molecular level.

1.10.4 Acquired FX deficiency

1 *Liver disease/ vitamin K deficiency.* Lack of vitamin K (from malabsorption or coumarin anticoagulants) will lead to deficiency in FX. Hepatocellular damage (including hepatocellular carcinoma (van der Walt *et al* 1977)) is also a cause of acquired FX deficiency. However, in both these cases there are usually deficiencies of other clotting factors.

2 *Amyloidosis.* The first description of an association between Primary (AL) amyloidosis and FX deficiency was in 1962 (Korsan-Bengsten et al 1962). Since

		FX Activity Assays		
Class	**FX:Ag**	**FX:PT**	**FX:APTT**	**FX:RVV**
1	S	N	N	N
3a	S	Ab	N	N
3b	Ab	Ab	N	N
5	Ab	Ab	Ab	N
6	S	N	Ab	Ab
7	Ab	Ab	N	Ab
8a	S	Ab	Ab	Ab
8c	N	Ab	Ab	Ab
8f	N	Ab*	Ab*	Ab

Table 1 Classification of hereditary FX deficiency

Classification based on the antigen concentrations and activity assays of abnormal FX molecules (Adapted from (Fair and Edgington 1985)).

FX:Ag, Antigen assay. FX:PT, prothrombin-time based assay. FX:APTT, activated partial thromboplastin time-based assay. FX:RVV, Russell Viper Venom-based assay.

N, normal (within 2SD of the mean). Ab abnormal (less than 2SD of the mean). S, severe (Ag levels less than 10% of the mean). (* extremely low assay results).

then, there have been numerous other reports. FX deficiency is the commonest coagulation factor deficiency in AL amyloidosis, occurring in 8.7% of patients (Choufani et al 2001). It rarely occurs in secondary (AA) amyloidosis (Glenner 1977). Usually there is a small reduction in FX antigen level, but a greater decrease in the FX activity (Fair et al 1979). FX adsorbs onto amyloid fibrils and so has a shortened half-life in plasma (Furie et al 1981). Therapeutic options include replacement by prothrombin complex concentrates or plasma, although this is often ineffective due to rapid removal of FX from the circulation (Duncan et al 1999). Recently recombinant factor VIIa has been used successfully (Boggio and Green 2001). Treating the amyloidosis with cytotoxic chemotherapy has been shown to improve FX levels (Camoriano et al 1987). Splenectomy may lead to a normalization of FX levels, presumably due to removal of amyloid fibrils in the spleen (Greipp et al 1979; Rosenstein et al 1983). Plasma exchange has also been reported to return FX levels to normal (Beardell et al 1997).

3. *Myeloma.* There is a single case-report of myeloma associated with FX deficiency (Schwarzinger *et al* 1992). The FX levels normalized with a concomitant complete remission of the myeloma. However, eventually the levels fell; this was associated with a relapse of myeloma and development of systemic amyloidosis. The mechanism is unclear, although it is thought that the excess light chains produced by the myeloma clone bind the circulating FX.
4. *Tumours.* Acquired FX deficiency has been reported in association with various tumours. These include spindle cell thymoma (Nora *et al* 1985), renal/adrenal carcinoma (Stefanini and Wiggishoff 1966), gastric carcinoma (Korte and Flury 1992), acute nonlymphoblastic leukaemia (Caimi *et al* 1991; Pabinger *et al* 1991) and acute myeloid leukaemia treated with amsacrine (Carter and Winfield 1988).
5. *Infections.* Infection with *Mycoplasma pneumoniae* has been associated with a transient FX deficiency (Peuscher *et al* 1979). The FX levels returned to normal 20 days after the patient was admitted and no other cause for the deficiency could be found. Acute upper respiratory tract infections preceding FX deficiency have been reported (Mulhare *et al* 1991). More recently, two cases of transient FX deficiency and Lupus Anticoagulant were associated with lower respiratory tract infections (Ashrani *et al* 2003). In both cases, no causative organism was identified.

Two cases of leprosy associated with acquired inhibitors to FX have been described (Ness *et al* 1980). The mechanism by which infection leads to a reduction in FX level is unknown, although the infectious agent and FX may share similar epitopes and so are removed from the circulation by the host's immune system.

6. *Drugs.* Treatment with sodium valproate has been associated with transient, acquired FX deficiency (Gallais *et al* 1996).
7. *Acquired inhibitors to FX.* There are a few reports of such inhibitors, in association with upper respiratory tract infection (Mulhare *et al* 1991), burns (Matsunaga and Shafer 1996), leprosy (Ness *et al* 1980) and exposure to topical thrombin (Israels and Leaker 1997). Inhibitors to FX have also been described with no known precipitating factor (Lankiewicz and Bell 1992).

1.10.5 Treatment of FX deficiency

The need for replacement therapy is guided by the particular haemorrhagic episode. The biological half-life of FX is 20-40 hours (Roberts *et al* 1965), so an adequate level can be built up with repeated infusions. Factor levels of 10-20 iu/dL are generally sufficient for haemostasis, even in the immediate post-operative period (Knight *et al* 1985). As yet, no purified FX concentrate is commercially available. Blood (Girolami *et al* 1970a), fresh frozen plasma (FFP) (Girolami *et al* 1970b), intermediate-purity factor IX concentrates (prothrombin complex concentrates - PCCs) (Lechler 1999) and recombinant factor VIIa (Boggio and Green 2001) have all been used. Fibrin glue may be effective in facilitating local haemostasis. Tranexamic acid is particularly useful in cases of mucosal bleeding, when it can be administered as a mouthwash (10 ml of a 5% solution three times a day). It can also be ingested at a dose of 15 mg/kg three times a day; this is of particular benefit for women with menorrhagia when taken during the menstrual period (Bolton-Maggs *et al* 2004). Plasma is usually given in a loading dose of 20 ml/kg, followed by 3-6 ml/kg twice daily (Perry 1997), aiming to keep trough levels above the 10-20 iu/dL needed for haemostasis.

Intermediate-purity factor IX concentrates contain significant amounts of FX (~1 unit of FX per unit of factor IX). However, there is a significant risk of thrombosis with PCCs which is probably related to the presence of activated coagulation factors;

they should be used with caution, especially in patients with concomitant liver disease, large haematomas and antithrombin deficiency (Kohler 1999). Tranexamic acid should not be used concurrently with PCC because of the risk of thrombosis. Concentrates are heat-treated, which significantly reduces the risk of viral transmission. Dose requirements are based on the finding that 1 iu of FX per kg bodyweight raises the plasma FX level by 1.5% of normal (Bolton-Maggs *et al* 2004).

PCCs have been used as regular prophylaxis in patients with severe FX deficiency, both as secondary prophylaxis (Kouides and Kulzer 2001), and as primary prophylaxis (McMahon *et al* 2002). In the case of secondary prophylaxis, 30 units/kg of concentrate was administered twice weekly. At a 12-month follow-up, no significant bleeding episodes had occurred. In McMahon's paper, four cases of severe FX deficiency in which primary prophylaxis with PCCs was undertaken are described. They had all presented within 24-72 hours of birth with life-threatening bleeds. Prophylaxis has changed the course of their disease and avoided joint damage (McMahon *et al* 2002).

Recombinant factor VIIa has been used to treat amyloid-associated FX deficiency (Boggio and Green 2001). There are some data indicating that adequate levels of FX appear to be important for the action of recombinant factor VIIa (Allen *et al* 2000). It may be that recombinant factor VIIa is not effective in cases of severe FX deficiency.

Steroids, intravenous immunoglobulin and plasmapheresis have been used in patients with acquired FX deficiency in the presence of an inhibitor (Smith *et al* 1998).

Guidelines for the management for patients with FX deficiency from the United Kingdom Haemophilia Centre Doctors' Organisation have recently been published (Bolton-Maggs *et al* 2004).

1.10.6 Surgery in cases of FX deficiency

The use of FFP and PCCs has allowed surgery to be performed successfully in individuals with severe FX deficiency. FX levels of 35 iu/dL have been used prior to surgery and levels maintained above 20 iu/dL in the post-operative period. This

avoids significant haemorrhage (Knight *et al* 1985). More recently it has been suggested that a FX level of 5 iu/dL is sufficient to maintain haemostasis (McMahon *et al* 2002).

1.10.7 FX deficiency in Pregnancy

Pregnancy in women with congenital deficiencies of coagulation factors is often associated with adverse outcomes, including spontaneous abortions, placental abruptions and premature births (Kumar and Mehta 1994). The levels of FX increase during pregnancy (Condie 1976), but women with severe FX deficiency and a history of adverse outcome in pregnancy may benefit from aggressive replacement therapy (Rezig *et al* 2002). However, the potential for thrombosis associated with replacement therapy must be carefully evaluated.

1.10.8 Neonatal FX deficiency

Diagnosis of FX deficiency in the neonate is difficult, due to low physiological levels of almost all coagulation factors (Williams *et al* 2002). Where FX deficiency is suspected, it may be beneficial to screen the parents to establish a diagnosis.

In cases where both parents are known to have FX deficiency, it is important to manage the pregnancy and delivery proactively. Ideally this should be in an obstetric unit with experience in bleeding disorders. An umbilical cord blood sample should be obtained at delivery for a FX assay of the neonate.

Cranial ultrasound may be necessary in severely affected children because of the increased risk of intracranial haemorrhage. Prophylaxis during the neonatal period may also be necessary.

1.11 Aims

FX occupies a unique position in the coagulation cascade as the first enzyme in the common pathway of thrombus formation. Despite its fundamental importance to coagulation, there is much that we do not understand about it. For example, why do some individuals suffer haemorrhagic symptoms despite having FX activity levels that are above the haemostatic threshold of 10-20 iu/dL? Why do different mutations result in altered functional activity? Why is FX deficiency so rare? Is a complete absence of FX incompatible with life?

The aims of this research which are addressed in this thesis are:

1. Confirmation of the diagnosis of FX deficiency by laboratory assays performed locally.
2. Identification of mutations in the *F10* genes of these individuals.
3. Structural analysis of novel mutations to correlate genotype with phenotype.
4. *in vitro* expression of novel mutations and subsequent biochemical characterization of the recombinant proteins.

2 Standard Materials and Methods

2.1 Patient Material

Patients studied were registered at Haemophilia Centres from around the world. All patients had been previously diagnosed with FX deficiency. The patient samples were collected with informed consent. Plasma samples were frozen and maintained at -80°C. Assays were performed within six months of venepuncture.

2.2 Reagents

All reagents for this research were purchased from Sigma-Aldrich, Poole, UK unless otherwise indicated in this or successive chapters.

2.2.1 Reagents for ELISA

Coating solution: 0.015 M $NaHCO_3$ and 0.035 M $NaHCO_3$, pH 9.6.
High salt phosphate wash buffer: 0.49 M NaCl, 7.5 mM $Na_2HPO_4 \cdot 12H_2O$, 2.5 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4.
Dilution buffer: 0.49 M NaCl, 7.5 mM $Na_2HPO_4 \cdot 12H_2O$, 2.5 mM $NaH_2PO_4 \cdot 2H_2O$, 3% (w/v) Polyethylene Glycol 8000, pH 7.4).
Substrate buffer: 66 mM $Na_2HPO_4 \cdot 12H_2O$, 0.347 M Citric acid·H_2O, pH 5.0.

2.2.2 Reagents for Coagulation assays

Owren's barbiturate buffer: 5.88 g sodium diethylbarbiturate, 7.34 g NaCl, 21.5 ml 1 M HCl. Made up to 1 L with H_2O (pH 7.35).
Owren's buffered saline: Owren's barbiturate buffer diluted 1:5 with 0.9% NaCl.

2.2.3 Reagents for DNA extraction

Cell lysis buffer: 0.32 M sucrose, 1% (v/v) Triton X-100, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5.
TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
Nuclear lysis buffer 0.32 M Lithium Acetate, 2% (w/v) Lithium Dodecyl Sulphate, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

2.2.4 Reagents for RNA extraction

10× NH_4Cl solution: 8.29 g NaCl, 1 g $KHCO_3$, 0.02 g Disodium EDTA. Made up to 100 ml with H_2O.

2.2.5 Reagents for PCR

NH_4 buffer: 160 mM $(NH_4)_2SO_4$, 670 mM Tris-Cl (pH 8.8), 0.1% Tween-20.

KCl buffer: 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-Cl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton® X-100, 0.01% (w/v) bovine serum albumin.

TBE (10×): 0.89M Tris borate, 0.02M EDTA, pH 8.3.

Loading buffer (6×): 40% Sucrose (w/v), 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol.

LB medium: 1% Tryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v), pH 7.0.

2.2.6 Restriction Enzyme digestion

10× *Nru* I buffer: 100 mM KCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, pH7.7.

1× T4 DNA Ligase buffer: 50 mM Tris-HCl (pH7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin.

1× NEB buffer 1: 10 mM Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9.

1× NEB buffer 2: 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, pH 7.9.

1× NEB buffer 3: 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, pH 7.9.

1× NEB buffer 4: 20 mM Tris-acetate, 10 mM $(CH_3O_2)_2Mg$, 50 mM $KC_2H_3O_2$, 1 mM dithiothreitol, pH 7.9.

10× buffer K: 1M KCl, 100 mM $MgCl_2$, 10 mM dithiothreitol, 200 mM Tris-HCl (pH 8.5).

2.2.7 Western Blotting

Running buffer: 1 M 3-(N-Morpholino)propanesulfonic acid, 1 M Tris base, 69.3 mM Sodium Dodecyl Sulphate, 20.5 mM EDTA.

Sample buffer: 1.17 M Sucrose, 563 mM Tris Base, 423 mM Tris HCl, 278 mM Sodium Dodecyl Sulphate, 2.05 mM EDTA, 0.7 mM Phenol Red, 0.88 mM Serva Blue G250.

Transfer buffer: 500 mM Bicine, 500 mM Bis-Tris, 20.5 mM EDTA.

Wash buffer: 1×TBS with 0.2% Tween (v/v).

Ponceau S stain: 0.1% Ponceau S (w/v) in 5% acetic acid (v/v), Sigma-Aldrich.

Blocking solution: 5% Polyvinyl pyrrolidone (w/v) in 1×TBS, with 2% neonatal calf serum (v/v).

2.3 Immunological Assays

FX antigen levels were measured using an in-house ELISA. Plastic 96-well microtitre plates (Life Technologies Ltd, Paisley, Scotland) were coated with a rabbit anti-human FX polyclonal antibody (Dako Ltd, Ely, UK) diluted 1:1000 with ELISA dilution buffer. The plate was sealed with an adhesive plastic cover (BIS Ltd., Lancs., UK) and left overnight at 4°C. After removal of the coating solution, plates were washed five times with a high salt phosphate wash buffer using a plate washer (MRW Microplate Washer, Dynex Technologies GmbH, Denkendorf, Germany). A standard curve (125 to 6.25 iu/dL) was constructed with serial dilutions of pooled normal plasma in a dilution buffer. The plate was loaded with 100 µl aliquots of the diluted plasma samples and controls. It was then sealed and incubated for 1 hour on a plate shaker (Amersham Biosciences, Buckinghamshire) at 400 oscillations per minute at room temperature. The plates were then washed five times with the same phosphate wash buffer and each well was loaded with 100 µl 1:3000 dilution of horseradish peroxidase (HRP) conjugated rabbit anti-human FX polyclonal antibody (Dako Ltd, Ely, UK). Again the plate was sealed and incubated for 1 hour at room temperature on the plate shaker. After a final washing step, the enzymatic activity was detected by adding 100 µl of the substrate solution (substrate buffer, with the addition of 67% (w/v) ortho-phenylene-diamine (Sigma Chemical, Poole, UK) and 0.05% (w/v) 30% H_2O_2) to each well. This reaction was stopped with 100 µl 2 M H_2SO_4 per well and the optical density was determined at 492 nm using a "MRX Microtiter Plate Reader" (Dynex Technologies GmbH, Denkendorf, Germany). The plate reader software calibrated a standard FX curve from the standard dilutions and then calculated the mean sample and control results from the curve.

Results from this in-house enzyme immunoassay have been compared with those from a commercial enzyme immunoassay (Dako Ltd, Ely, UK) using the same coating and labelling antibodies. The commercial assay was found to give the same FX:Ag values in each patient plasma tested. The FX:Ag values were calculated using as reference plasma pooled from 20 normal individuals. "Trol P" (Dade Behring, Marburg, Germany) was used along with the reference plasma pool for quality control.

The historical reference range of 50-150 iu/dl was used for the immunological and functional FX assays in this thesis. The range was not established locally.

2.4 Functional Assays

2.4.1 Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) assays

These assays measure the ability of FX in the sample to clot FX deficient plasma when the sample is activated by the *extrinsic Xase* complex (FX:PT) or the *intrinsic Xase* complex (FX:APTT).

Both assays were performed on an ACL3000 automated analyser (Instrumentation Laboratory, Milan, Italy). Samples were diluted 1:10, 1:20 and 1:40 in Owren's Buffered Saline. For the PT assay, 100 µl of diluted sample was combined with 100 µl FX deficient plasma at 37°C. 200 µl of a mixture containing Rabbit Brain Thromboplastin and $CaCl_2$ (Instrumentation Laboratory, Milan, Italy) was added and the time to coagulation measured. For the APTT assay, 100 µl of diluted sample was combined with 100 µl FX deficient plasma. 100 µl of reconstituted APTT Lyophilised Silica (Instrumentation Laboratory, Milan, Italy) was added and this was incubated for five minutes at 37°C. After the addition of 100 µl 25 mM $CaCl_2$, the time to coagulation was measured.

2.4.2 Russell viper venom (RVV) assay

A semi-automated one-stage Russell viper venom (RVV) assay (Diagen Ltd, Thame, UK) was performed using a KC10 coagulometer (Amelung, Lehbrinksweg, Germany). The assay measures the ability of FX in the sample to clot FX deficient plasma when the sample is activated by RVV. Again, samples were diluted 1:10,

1:20 and 1:40 in Owren's Buffered Saline. 100 µl of diluted plasma sample was added to 100 µl of FX deficient plasma. 100 µl of a platelet substitute/RVV mixture was added and after 30 seconds 100 µl 25 mM $CaCl_2$ was added to achieve clot formation.

2.4.3 Chromogenic assays.

In a chromogenic assay, the function of an enzyme (in this case FX) is measured by its ability to cleave a chromophore from an artificial (chromogenic) substrate. The released chromophore gives rise to a colour change which can be followed spectrophotometrically.

RVV 0.087 mg/ml was mixed with an equal volume of 0.1 mol/L $CaCl_2$. 50 µl of the diluted sample was added to a microtitre plate and incubated at 37°C for 3-4 minutes. 50 µl of the chromogenic substrate S2765 (Chromogenix - Instrumentation Laboratory, Milan, Italy) was added to the sample and mixed. Within 30 seconds, 50 µl of the RVV/$CaCl_2$ was added and the mixture incubated at 37°C for 3 minutes. After this time, the reaction was stopped by the addition of 50 µl 20% acetic acid. The absorbance was read at 405 nm.

FX:C levels were calculated using as a reference the same pooled plasma used for the FX:Ag assays. Reference plasma was assigned an arbitrary FX:C value of 100%.

2.5 Mutation Analysis

2.5.1 Extraction of Genomic DNA

Blood was collected by venepuncture into tubes containing ethylene diamine tetraacetic acid (EDTA) to a final concentration of 1.6 mg EDTA per ml whole blood. Buffy coats were obtained from 20 ml of whole blood prepared by centrifugation at 1500 *g* for 20 minutes and decanted into 50 ml centrifuge tubes. 50 ml of cold cell lysis buffer was added and the mixture incubated on ice for 20 minutes. A pellet of cells was formed by centrifugation at 1000 *g* for 20 minutes. The supernatant was discarded and the pellet resuspended in 1 ml TE. 2 ml nuclear lysis buffer was added and the mixture was gently rotated on a mechanical rotator (approximately 30 rpm) for 20 minutes. 1 ml of a 25:24:1 mixture of phenol: chloroform: isoamyl alcohol was added and the sample mixed. Centrifugation at

1000 *g* for 5 minutes was performed to separate the phases. The upper, aqueous, phase was removed to a clean tube using a wide-bore pipette. The sample was mixed with 1 ml chloroform and centrifuged as before. The upper, aqueous phase was removed to another clean tube and 2.5 volumes 100% ethanol added. The tube was slowly inverted to precipitate the DNA. The DNA was collected onto a sealed glass Pasteur pipette and resuspended in 100 µl TE pH8. The DNA samples were kept at 4°C for 72 hours to allow the DNA to dissolve.

2.5.2 Total Leucocyte Preparation

Buffy coat was prepared from whole blood as described earlier (**section 2.5.1**). This was added to 20 ml 1× NH_4Cl solution and incubated on ice for 20-30 minutes to allow red cell lysis to occur. Following centrifugation at 1000 *g*, the supernatant was removed and the pellet washed in 20 ml PBS.

2.5.3 Isolation of RNA

Cells were prepared as above and after a final spin at 1000 *g*, the pellet was resuspended in 1 ml TRIzol® reagent (Invitrogen). The use of TRIzol® (a monophasic solution of phenol and guanidine isothiocyanate) is based on the method of RNA extraction of (Chomczynski and Sacchi 1987).

200 µl chloroform was added to the suspension. This mixture was incubated at room temperature for 3 minutes before centrifugation at 12000 *g* for 15 minutes. The top (aqueous) layer was transferred to a clean tube and 500 µl isopropanol added. This was incubated at –70°C overnight before centrifugation at 12000 *g* for 15 minutes. The pellet was washed with 70% ethanol and spun at 12000 *g* for 5 minutes. After removal of the supernatant, the pellet was air-dried and dissolved in 50 µl DEPC-treated water (0.1% diethylpyrocarbonate). The optical density was measured at absorbances of 260 and 280 nm to confirm the purity of the preparation. (The 260/280 ratio should be between 1.7 and 2.1.)

2.5.4 Oligonucleotides

Oligonucleotides for PCR and for sequencing were designed using Oligo4 software program (Molecular Biology Insights, Cascade, CO, USA) and synthesized by

MWG-Biotech (Ebersberg, Germany). All primers were designed specifically for this work, except where references are mentioned.

2.5.5 DNA Polymerase Chain Reaction

Amplification reactions were carried out in 100 µl volumes and comprised 200 ng of DNA, 100 pmoles of each oligonucleotide primer, 200 µmoles of each dNTP, 1.5 mM $MgCl_2$, 1× NH_4 buffer and 2.5 units of *Taq* polymerase (all from Bioline, London, UK). A number of experiments were performed to optimise the PCR parameters. The "Hot-Start" technique was used, in which the polymerase enzyme was not added until the reaction mixture reached 94°C. Reactions were denatured at 94°C for 5 min and then the cycles of amplification were performed. On the last cycle, the extension time was increased to 10 min. For several of the exons, a "Touchdown" technique for annealing was used. In this, the annealing temperature was reduced by 0.5°C each cycle for the first ten amplification cycles. After this, further cycles of PCR were carried out, with annealing at the lowest temperature reached (5°C lower than the start temperature).
Each PCR experiment included a negative control containing no template.

Sequences for the forward (FP) and reverse (RP) primers and the cycling parameters are shown in **table 2.**

2.5.6 Sequence Analysis

PCR products were purified (Qiagen Purification Kit, Milan, Italy) and sequenced directly using an ABI Prism310 genetic analyser automated sequencer (PE Applied Biosystems, Milan, Italy). BigDye™ terminator v1.1 was used according to the manufacturer's protocol. Sequencing was performed with the same primers used for the amplification reactions. In the case of exon 8, an additional primer situated in the middle of the exon was also used for sequencing. This additional primer has the following 5'→3' sequence: CAGAAGACGGGGATTGTGAG.

Table 2 PCR parameters for amplification of *F10* exons.

Exon	Primer Sequence 5'→3'	Denaturing	Annealing	Extension	Cycles	Fragment size (bp)
PROM	FP: TCTCTTCTGGAAAAATGATGGGG RP: CTGAGCAGGACGAGGTGCAGT*	95°C x 30s	62°C x 30s	72°C x 30s	35	415
1	FP: GACAACAGCCATCCCAGCTGGGGCG° RP: CTGCCGCCCCTCCAAGCTTCCTGGCGCTGC°	94°C x 30s	64°C x 20s	72°C x 20s	35	220
2	FP: GTGACCAGAGCTTTTAACCC RP: CTATCCCCAGCCCTTACCGT	94°C x 30s	63°C x 30s Touchdown	72°C x 40s	10 + 30	235
3	FP: GTTATTGGTATAAAATGTCTCTG RP: ATGAGAACAAAAGCAGAAAGGA	94°C x 30s	49°C x 20s	72°C x 20s	35	188
4	FP: TGATGCCGGAAACAGCTTGCAG RP: YGCCACTCTTCAGGGGCATCTG	94°C x 30s	63°C x 30s Touchdown	72°C x 40s	10 + 30	220
5	FP: TGACAGGCAAGTGGATGTAGCTG RP: CAGTCCTGTCCTCTTGGTGTCAC	94°C x 30s	65°C x 30s Touchdown	72°C x 30s	10 + 30	353
6	FP: TCTCTGACTCTTCTCCCTCA RP: GGATTGCTATTCCAAGTGTC	94°C x 30s	58°C x 20s	72°C x 20s	40	416
7	FP: AGTCAGGCAACACCTGTCCACCTG RP: TGAAAAGCAGACAGTGACGGTGC	94°C x 30s	63°C x 30s Touchdown	72°C x 30s	40	234
8	FP: AACGGATGTGCGAGAGCATGTC RP: GAAAGGAATGCCCATGGCAGTC	94°C x 30s	62°C x 40s Touchdown	72°C x 40s	10 + 28	878

*(Miyata *et al* 1998); °(Reddy *et al* 1989)

2.5.7 cDNA preparation

2 μg RNA was added to 200 units RNAsin and the volume adjusted to 20 μl with DEPC water. This mixture was incubated at 65°C for five minutes and placed on ice for five minutes.

Reverse transcription reactions were carried out in 50 μl volumes and comprised 0.5 mM dNTPs (Amersham), 200 units RNAsin (Promega), 400 units Murine Maloney Leukaemia Virus reverse transcriptase enzyme (Invitrogen), 100 mM dithiothreitol (Invitrogen), 30 pmoles "Down Outer" primer, 20 μl RNA (2 μg prepared as above) and 1×cDNA buffer (Invitrogen). The samples were incubated at 37°C for one hour and then heated to 65°C for ten minutes before cooling on ice.

2.5.8 First-round PCR

The first set "Outer" primers were designed using Oligo4 program. The nested, "Inner" primers started ten bases into the outer primer sequence.

Table 3 Primers for investigation of cDNA of patient B:II:2

Reaction	Primer	Sequence (5' to 3')	PCR product length
First Round	Upper Outer	GCACCTCGTCCTGCTCAGTG	
	Down Outer	ACAGTCCCCGTTGTCCAGGC	394 bp
Second Round	Upper Inner	CTGCTCAGTGCCTCCCTGGC	
	Down Inner	TTGTCCAGGCTGCAGAGCTT	374 bp

Amplification reactions were performed in 30 μl volumes and comprised 3μl of each "Outer" primer (10 mM), 3 μl 10× PCR buffer, 3 μl 2 mM dNTPs and 2 units *Taq* polymerase, with 5 μl cDNA template.

Samples were denatured at 94°C for 5 min before 35 cycles of amplification were performed. The cycling parameters consisted of denaturing at 94°C for 30 seconds, annealing at 58°C for 30 seconds, followed by extension at 72°C for 30 seconds. On the last cycle, the extension time was increased to 10 min.

2.5.9 Second-round "Nested" PCR

30 µl amplification reactions were performed using 5 µl of the product from the first reaction as DNA template, 3 µl of each "Inner" primer (10 mM), 3 µl 10× PCR buffer, 3 µl 2 mM dNTPs and 2 units *Taq* polymerase. Cycling parameters were as for the first round, except that the annealing temperature was 50°C for all 35 cycles.

2.5.10 Restriction Enzyme Digestion

Digestion reactions were carried out in 20 µl volumes and comprised 5 U enzyme, 2 µl 10× digestion buffer and 300 µg DNA. For *Apa* I digests, Buffer A (Promega, UK) was used. For *Bsm* I digests, buffer H (New England Biolabs, Beverly, MA, USA) was used. For *Bgl* I digests, buffer H (New England Biolabs, Beverly, MA, USA) was used. All digestions were performed for four hours at 37°C, except for *Bsm* I digests which were performed at 55°C.

2.6 Agarose Gel Electrophoresis

Analytical grade agarose (Promega, UK) was dissolved in 0.5× TBE buffer to the appropriate concentration and heated to boiling for two minutes in a 750 W microwave oven. The molten agarose was allowed to cool to approximately 50°C with stirring, before the addition of Ethidium Bromide to a final concentration of 0.5 µg/ml. The gel was then poured into a horizontal casting tray with a comb of the desired size in place and allowed to set (approximately 90 minutes at room temperature). The comb and tray sealers were removed and the set gel (including casting tray) was submerged in an electrophoresis tank containing 0.5× TBE. Samples were combined with 5× loading buffer (Bioline, UK) and loaded into wells, with an appropriate size marker in one well. The electrophoresis was carried out at approximately 10 V/cm.

2.7 Protein Assay

Total protein estimation was performed using the Bio-Rad Protein Assay (Bio-Rad, Hemel Hempstead, UK). This is a dye-binding assay based on the differential colour

change of a dye (Coomassie Brilliant Blue) in response to various concentrations of protein.

A standard curve (1 to 20 μg/ml) was constructed with serial dilutions of a protein standard (bovine serum albumin). The dye reagent was added to the standards and unknowns. The absorbance was read at 595 nm. The absorbance was plotted against the protein concentration and the unknowns were read from the graph.

3 Introduction and Methods for Molecular Modelling

3.1 X Ray Crystallography

Protein X-ray crystallography provides detailed information on the three-dimensional structure of proteins to approximately 2-3 Å resolution. Since the elucidation of the structure of haemoglobin (Perutz 1951), the structures of over 25,000 proteins have been determined by this method. It requires the protein to be crystallised, which places the protein molecules into a regular lattice formation. This crystal lattice diffracts a beam of X-rays produced by accelerating electrons against a copper target. While most X-rays pass though the crystal directly, some are diffracted and can be detected (for example by photographic film). The atomic arrangement of the crystal determines several features of the scattered waveform. The amplitude of the wave is proportional to the number of electrons in the atom. As the scattered waves recombine, they either reinforce each other (if they are in phase), or they cancel each other out (if they are out of phase). The major requirement for this technique is the determination of phases, from which an electron density map at atomic resolution can be determined (Blundell and Johnson 1986). The technique assumes that the structure of the protein under crystallisation conditions is the same as that in its natural environment.

3.2 Molecular Modelling

It is assumed that mutations in genes occur at random sites. Providing transcription and translation can occur, a mutant protein will be produced. If the mutant protein is able to fold and function satisfactorily, then the mutation can be accepted: the organism can either be neutrally accumulated into the population or actively selected for. However, if the mutation destabilises protein-folding, or affects a catalytically active residue then the mutation may be selected against. Knowledge of the tertiary structure and modes of interaction of the protein can help the prediction of what might happen if a particular mutation occurs.

When the structure of a protein has been elucidated by crystallography, the atomic coordinates of the protein can be entered into the Protein Data Bank (PDB), which is kept at the Brookhaven National Laboratory, Upton, New York. The coordinates can be retrieved (in the form of PDB codes) and used for the manipulations of molecular

modelling. Various suites of programs are available to achieve this. In the course of this work, the suite INSIGHT II 98.0 (Biosym/MSI, San Diego, USA) was used. This enables the protein model to be visualised.

3.2.1 Solvent Accessibility Analyses

The exterior portion of a protein that is in contact with solvent molecules is referred to as the accessible surface area. The solvent is generally described as the centre of a probe as it moves across the surface of a molecule and is in van der Waals contact with the molecule. The probe is approximated to a sphere of radius 1.4 Å which represents a water molecule. The accessibility of atoms or groups of atoms within a protein crystal structure to solvent molecules has been assessed using an algorithm (Lee and Richards 1971). This showed that about 40-50% of the surface area of a typical globular protein is occupied by non-polar atoms. Relative accessibility is calculated by dividing the residue solvent accessibility by the value for maximal accessibility for that residue type. These values are used to train a neural network system using amino acid substitution profiles derived from multiple sequence alignments. The predicted solvent accessibility (a value of 0-9) is the square root of the relative accessibility (Rost and Sander 1994).

For this study, the COMPARER program (Sali and Blundell 1990) was used to assess the solvent contact areas of known crystal structures. COMPARER uses an algorithm (Richmond and Richards 1978) with side-chain accessibility calculations and normalisation.

Patterns of solvent accessibility can give clues to secondary structure as well as the tertiary structure of the protein, such as the occurrence of buried residues in parallel and anti-parallel β-sheets. In a solvent-exposed anti-parallel β-sheet, a pattern of buried-exposed residues alternates every two residues. Buried residues are positioned at the centre and exposed residues at the ends of the β-strands in parallel β-sheets as these structural elements tend to be buried (Richardson and Richardson 1989). Solvent-exposed α-helices are more likely to exhibit a pattern of alternating two exposed and two buried residues per helical turn.

3.2.2 Secondary Structure Analyses

The DSSP (Define Secondary Structure of Proteins) program uses the atomic coordinates of a protein from the PDB to identify secondary structure in protein models according to unambiguous definitions of secondary structure classes (Kabsch and Sander 1983). DSSP implements a secondary structure algorithm that recognises seven classes of secondary structure. Secondary structure assignment is based on mainchain hydrogen-bonding patterns. The electrostatic interaction energy between the C=O of residue *i* and the N-H of residue *j* is defined as a hydrogen bond if the binding energy is less than –0.5 kcal $mole^{-1}$. An isolated turn, "T", is defined as a hydrogen bond between residue *i* and residue *i+n*, where *n* =3, 4 or 5. Two or more consecutive turns are known as helices: a 3_{10} helix, "G", for three turns, an α-helix, "H", for four turns and a π-helix, "I", for five turns. An isolated bridge formed by two non-consecutive residues is termed "B". A ladder is a set of consecutive bridges. A set of ladders connected by shared residues form extended β-strands "E". The program also defines regions of curvature in the mainchain as "S" bends, providing that the angle between the vector joining the C^{α} carbon atoms of residues *i* and *i*+2 and the vector joining the C^{α} carbon atoms of residues *i* and *i*-2 is greater than 70°.

3.2.3 Molecular Simulation Modelling

DISCOVER_3 is a molecular simulation program within INSIGHT II, which can perform many routines, including energy minimisation and dynamic simulation. It allows prediction of structure and energy properties of biological systems.

Simulated annealing is a method of optimisation of a molecular model. The annealing process is analogous to physical annealing in which a material is heated to a high temperature and then slowly cooled. When in the liquid state, molecules are constantly moving and so are able to visit different configurations, or microstates (Bailer-Jones and Bailer-Jones 2002), which correspond to different energies of the material. If the temperature is reduced slowly, then the molecules can move between many microstates and reach a thermal equilibrium. The material will converge towards the lowest energy state.

However, if the material is cooled rapidly ("quenching"), then the molecules have little opportunity to visit different configurations. The end-point will not be far from

the start-point. If the start-point is at a high temperature, then it is likely that the final configuration will be a high-energy state. In simulated annealing, this is analogous to allowing too few iterative cycles before lowering the temperature. The molecules are forced to find a local energy minimum which is close to their start-point, without exploring far. They are less likely to find the global minimum.

Annealing provides a framework in which to avoid local minima of energy states in order to reach the global minimum.

Energy minimisation is used to decide if a particular model is conformationally acceptable. It is assumed that wildtype proteins are in an acceptable conformation and so at low energy states. However, if models are built "*in silico*", then it is likely that the bond angles and perhaps the bond lengths will not be at their equilibrium values and inevitably there will be strain. A molecule with a strained structure will have a high energy and so will be unstable. Energy minimisation relaxes any strain which has been introduced into the model during manipulation and in the process finds alternative conformations with less strain. In essence, this involves moving the position of each atom, re-calculating the total energy and then relocating (if necessary) the atom to the lowest energy point. The advantage of energy minimisation over simulated annealing is that user-defined sets of residues can be selected for minimisation, whereas simulated annealing involves the whole molecule.

The simplest (and slowest) method of energy minimisation is one in which each atom is moved along the x, y and z axes in both directions. The total energy is calculated for each position and the lowest energy position is selected. Two faster minimisation algorithms that are frequently used in molecular modelling are the "steepest descent" and "conjugate gradient" methods. These gradually change the coordinates of the atoms as they move the system closer to the point of minimum energy. The minimisation program runs a number of iterative cycles, normally 200-300. If too few iterations are run, then the stereochemistry of the molecule may not be modified sufficiently to reach the bottom of the global energy well (i.e. zero gradient); the molecule may settle in a local energy well (shallow local wells are known as saddles) (Leach 1996).

The steepest descent method moves in the direction parallel to the net force. The conjugate gradient method produces a set of directions which does not show the

oscillatory behaviour of the steepest descent method. In the steepest descent method, both the gradients and direction of successive steps are orthogonal. In conjugate gradients (the one used by DISCOVER_3), the gradients at each point are orthogonal but the directions are conjugate (Leach 1996).

The stability of a protein is strongly influenced by the exclusion of solvent from the non-polar sidechains of the hydrophobic core. Mutations in solvent-inaccessible residues are generally found at a lower rate than those on the surface (Hubbard and Blundell 1987). It is presumed that proteins are intolerant of mutations in their core and are unable to fold correctly. The accepted mutations for buried residues prefer a small volume change (Overington *et al* 1990). Protein stability is also dependent upon the formation of hydrogen bonds between residues. Polar residues which are linked by hydrogen-bonds can be buried and these are usually highly conserved (Bajaj and Blundell 1984). It is also known that most amino acids have a significant preference for certain secondary structures. For example, residues with bulky side-chains or large aromatic rings favour β-sheets (Levitt 1978).

3.2.4 Homology modelling

Homology modelling offers the best means of obtaining atomic coordinates for a protein in the absence of X-ray crystallographic or nuclear magnetic resonance data (Lattman 1995). The technique is based on the assumption that protein domains which appear to share a common ancestral gene will adopt a common protein fold structure. Homology models are not as accurate as experimentally determined structures; in particular surface loops can be less accurate. However in many cases homology modelling is able to provide representations of the protein structure that are suitable for interpretation and prediction of functionality.

The HOMOLOGY program within INSIGHT 98 uses an homologous protein structure as a template upon which to build an atomic coordinate model for the unknown structure. The crystal structure of the template is imported and the sequence to be modelled is aligned to it.

Structurally conserved regions (SCRs) are defined in the protein. SCRs are usually α-helices and β-sheets which have been identified in the template structure by DSSP. Following alignment for the amino acid sequences of the template and the target,

SCR definitions are assigned to the target sequence. The atomic coordinates of the template SCRs are then copied to the model. Where differences occur between the sidechain atoms of the two proteins, the coordinates are modelled from a library of amino acid structures.

The protein loops that join the SCRs are then modelled. Hydrogen bonding constraints restrict the conformations of SCRs, however loops are not usually restricted in this way and so often display a higher degree of sequence (and structure) divergence. As a consequence, loops are more difficult to model. Protein loops from the template are used but this is only possible when the length of the loop in the template and target is identical. Again, when differences occur between the sidechain atoms, the coordinates are modelled from a library of amino acid structures.

When there is a difference between loop lengths, it is necessary to perform a search for loops from compatible loop structures. HOMOLOGY searches the PDB for loops which have the correct length and are most likely to satisfy the geometric constraints required to join the two SCRs. The database calculates the distance between the model residues either side of the loop and this is used to search the database. Usually the program will suggest several alternative loops. Each of these alternatives can be tested in the model. Selection of a particular loop is governed by a number of considerations, including torsion angles and distances at the points that the loop joins the SCRs. The coordinates of a searched loop are then copied to the model, except when differences occur between the sidechain atoms and then the coordinates are modelled from library structures.

Splicing loops from one set of PDB coordinates into another may produce a model with structural artefacts. In particular peptide bonds may be strained and the imported loops may not be in optimal conformations. The model will need refining. A process known as *splice repair* is carried out. This is an energy minimisation process designed specifically to minimise the energy of the peptide bonds at the junctions created by introducing regions from different structures.

3.3 Interpretation of the Effect of Missense Mutations

The prediction of the functional effect of missense mutations is facilitated by the availability of crystal structures, sequence alignments and molecular graphics modelling programs (Jenkins *et al* 1998). Relevant protein sequences can be

identified from the SWISSPROT and TREMBL databases using PSI-BLAST (Altschul *et al* 1997). Seven partial crystal structures of FXa, lacking the Gla domain, have been solved and refined at 2.2Å (Brandstetter *et al* 1996; Kamata *et al* 1998; Maignan *et al* 2000; Padmanabhan *et al* 1993).

3.3.1 Methods

The crystal coordinates of FXa were obtained from the Protein Data Bank (PDB) (codes 1ezq, 1f0r, 1f0s, 1fax, 1hcg, 1xka, 1xkb) (Brandstetter *et al* 1996; Kamata *et al* 1998; Maignan *et al* 2000; Padmanabhan *et al* 1993). Protein structures were visualised using INSIGHT II on Silicon Graphics INDY workstations, using Crystal Eyes stereo glasses for detailed inspections. **Figure 3.1** shows a graphical representation of the crystal structure of FXa. Sites of known mutations, sites of mutations described in this thesis and the catalytic triad are highlighted.

The quantitative assignment of the experimentally-observed mainchain secondary structure of each protein was made using the DSSP program (Kabsch and Sander 1983), where **figure 3.2** shows the α-helix and β-strand contents (denoted H and E respectively) and other types of secondary structures including loops and turns (T, S, B and G). The quantitative assignment of solvent-exposed residue sidechains was made using the COMPARER program by the Lee and Richards method using a water molecule radius of 1.4 Å (Lee and Richards 1971; Sali and Blundell 1990). Sidechains were defined as solvent exposed if their accessibilities ranged between 20-100% (values 2-9 in **figure 3.2**) and buried if these ranged between 0-19% (values of 0 and 1 in **figure 3.2**). A more detailed version of **figure 3.2** can be found in **appendix 1**. This version includes the primary amino acid sequence of FX and the standard chymotrypsin sequence numbering used for the catalytic domain of trypsin-like serine proteases. Also shown are the mutations which have been reported so far, the mutations described in this thesis, as well as the DSSP and COMPARER outputs of all seven available crystal structures and the consensus values from these programs.

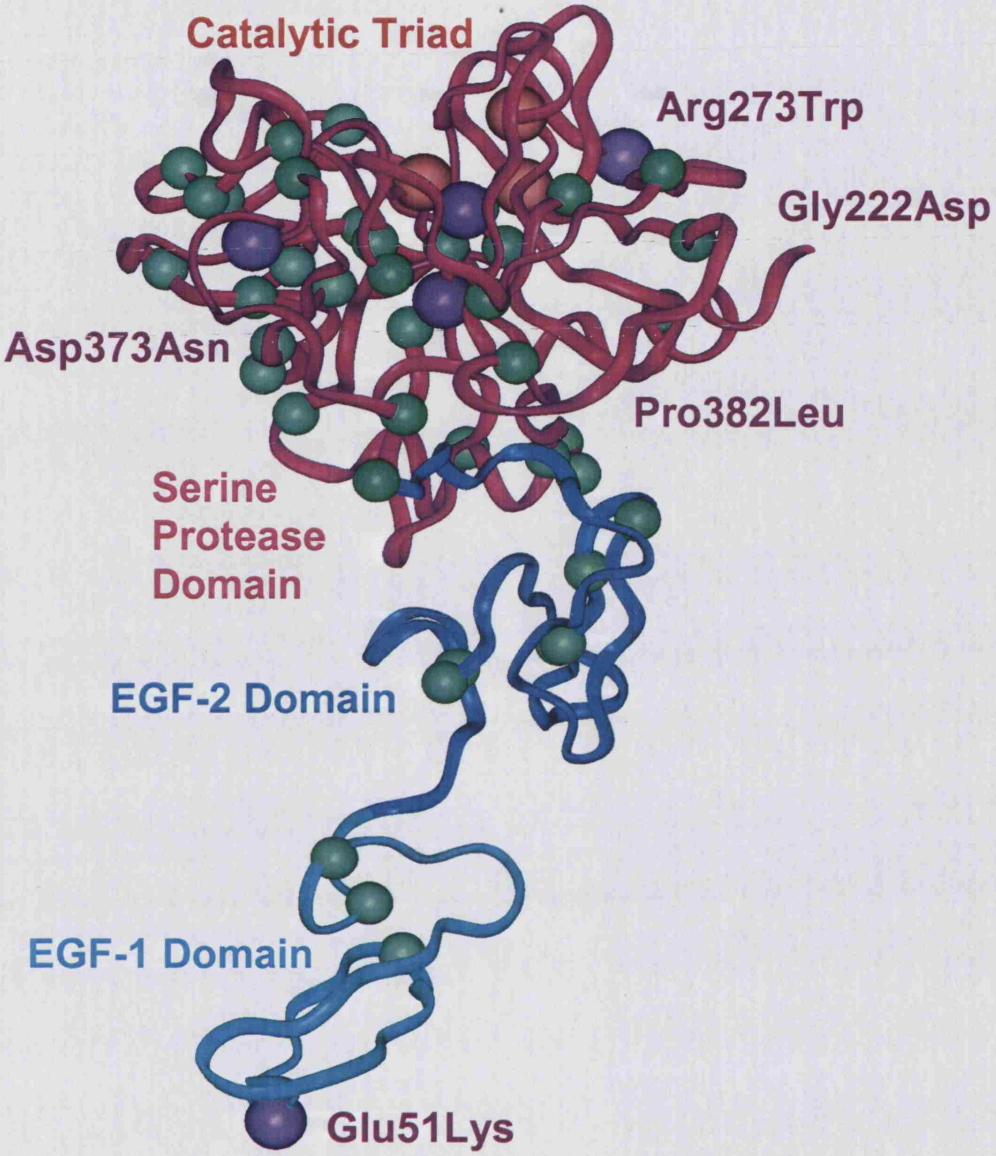


Figure 3.1. Ribbon view of the crystal structure of FX (PDB code 1xka). The α-carbon atoms of the 5 missense mutations modelled in this thesis are represented by purple spheres. The previously-identified missense mutations (see **Figure 3.2**) are shown in green. The catalytic triad is shown in red. The domains in FX are identified as follows: EGF-1, turquoise; EGF-2, blue; serine protease, magenta.

3.3.2 Interpretation of Type I and Type II Mutations

As discussed in **section 1.10.3**, mutations in enzymes are often classified as Type I or Type II. In type I mutations, the plasma antigen level is low. This is either due to a problem in the cytoplasmic secretory pathway of the protein or due to rapid degradation by plasma proteases. If the molecule is not secreted in normal amounts, then this may be related to a problem with protein folding. Inspection of the crystal structure of the molecule and in particular the region surrounding the mutation site may reveal why the mutant protein is unable to fold correctly. Several type I mutations in the coagulation *factor VII* gene and their interpretation by molecular modelling have been reported (Peyvandi *et al* 2000a).

In contrast, type II mutations result in normal antigen levels, but reduced activity assays. This implies that protein secretion is unaffected, but that the mutation has occurred in an enzymatically important residue. Inspection of the crystal structure may reveal that the mutated residue is in close proximity to the catalytic triad or a metal ion binding site. For example, a mutation in the Ca^{2+} binding site of FX has recently been described (Girolami *et al* 2004).

Figure 3.2 Protein sequence and structure analysis of human FX.

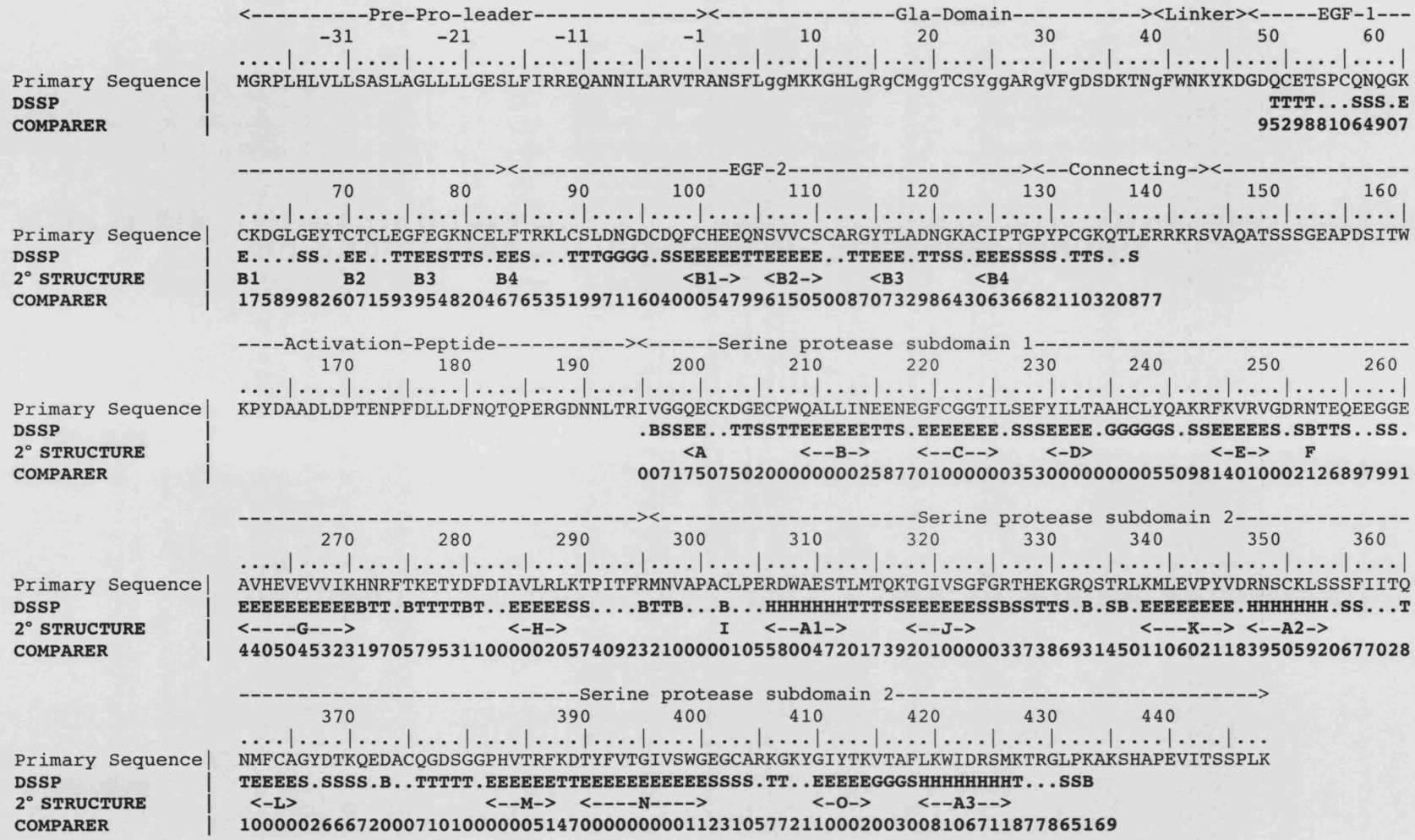


Figure 3.2 Protein sequence and structure analysis of human FX

The Gla, EGF and catalytic serine protease domain assignments are shown above the sequence numbering for FX (SWISSPROT code P00742). In the Gla domain, Gla residues are identified by g. The DSSP output shows the consensus secondary structures from seven crystal structures of FX when these correspond to experimentally observed structures. No output is shown if no structures were present. The β-strands in the two EGF domains (denoted by **E** in the DSSP output) are each labelled **B1-B4**, and those in the serine protease subdomains are labelled **A-O**. The α-helices (denoted by **H**) in the serine protease subdomains are labelled **A1-A3**. The COMPARER output shows a value (0-9) that represents the consensus solvent accessibility of each sidechain in FX. Exposed residues are identified by values of 2-9, and buried residues by values of 0 or 1.

4 Analysis of Mutations Outside the Catalytic Domain of FX

4.1 Introduction

To date, 62 point mutations have been reported in the *F10* gene (Arnold *et al* 2003; Au *et al* 2004; Girolami *et al* 2004; Menegatti *et al* 2004; Peyvandi *et al* 2002; Pinotti *et al* 2002; Pinotti *et al* 2004; Uprichard and Perry 2002). Most of these result in a type I deficiency, with about a third producing a type II deficiency (Uprichard and Perry 2002). This classification of enzymatic mutations and its relevance to molecular modelling has been discussed in **sections 1.10.3 and 3.3.2.**

The prevalence of heterozygous FX deficiency is about 1:500, but most cases are clinically asymptomatic (Graham *et al* 1957). However, some heterozygotes do have a significant bleeding tendency. It is possible that this may be due to either insufficient enzymatic activity by wildtype FX or inhibition of one of the reactions in the coagulation pathway by a mutant protein. It is possible that a dysfunctional FXa molecule may compete with the normal FXa for binding sites on factor Va to form the *prothrombinase* complex. The effect may be to reduce the formation of the active *prothrombinase* complex.

FX mutations are thought to be rare because of the central role of FX in the coagulation cascade. $FX^{-/-}$ knockout mice have been shown to present a lethal phenotype with death from intraabdominal, subcutaneous, or intracranial bleeding. This is consistent with the hypothesis that a complete absence of FX is fatal (Dewerchin *et al* 2000).

As discussed earlier, patients with FX deficiency may present with a bleeding tendency at any age, although the more severely affected ones (FX activity <1 iu/dL) present early in life, with umbilical-stump bleeding, for example. Haemarthroses, severe post-operative haemorrhage and central nervous system haemorrhage have also been reported. Less severely affected patients may bleed only after haemostatic challenge (e.g. trauma or surgery). Some cases are identified incidentally during routine screening or family studies. Patients who are only mildly affected may experience easy bruising or menorrhagia.

Congenital deficiencies of coagulation factors usually arise from missense mutations. In the case of FX, the majority of mutations are missense, with ten deletions and

splice-site mutations also reported (Millar *et al* 2000; Peyvandi *et al* 2002; Uprichard and Perry 2002).

Nonsense mutations occur when the nucleotide substitution produces a stop codon, or when a nucleotide deletion results in a frame-shift and a stop codon further downstream. This will produce a truncated protein and is almost always clinically apparent. Until recently, the only nonsense mutations reported in *F10* had been heterozygous. This would support the hypothesis that absence of FX is incompatible with life. However, there is a single report of a homozygous nonsense mutation (Zivelin *et al* 2003). This was identified in three patients, all of whom had a FX activity level of <1 iu/dL. Two of the three patients are maintained on prophylactic replacement therapy and the third was treated "on demand" 2-3 times per month.

Larger, heterozygous, deletions have been reported. These all leave at least one partially-functional copy of the gene to produce FX protein.

FX operates enzymatically as a serine protease. Mutations affecting this action are likely to be found in the catalytic domain of the protein. The remainder of the protein is predominantly involved in ligand binding and protein stability. Most of the mutations discussed in this chapter (outside the catalytic domain) do not result in a residue substitution in the mature protein, but are either at splice-sites or in the signal peptide.

The *in vitro* expression work performed on the Glu51Lys mutation identified in kindred D is described in **chapter 9**.

4.2 Kindred Studied

The studies on five kindred are reported in this chapter. The individual samples originated from many countries across the world and unfortunately were not always accompanied by bleeding histories or detailed pedigrees. All laboratory testing, except where indicated, was performed at the Royal Free Haemophilia Centre.

4.3 Kindred A (Ala-26Asp mutation)

4.3.1 History

This kindred is large and there is a great deal of consanguinity: in the extended family, four brothers married four sisters. The section of the family discussed in this

thesis live in the Midlands, UK and are registered with the haemophilia centre in Birmingham. Many of the children are severely affected with FX deficiency and require regular treatment with PCCs. The pedigree is shown in **figure 4.1**.

4.3.2 Phenotype assay results

FX assays were carried out on plasma as previously described in **sections 2.3 and 2.4**. Results are shown in **table 4**.

Table 4 Phenotypic assay results for kindred A

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
A:III:1	ND	ND	ND	ND	ND
A:III:2	60	62	56	54	52
A:IV:1	1.7	<1	<1	<1	<1
A:IV:2	ND	0.00*	ND	ND	ND
A:IV:4	ND	0.02*	ND	ND	ND

All units are iu/dL, with the reference ranges 50-150 iu/dL. ND – No data. *assays not performed at Royal Free Haemophilia Centre.

4.3.3 Genotype Analysis

DNA extraction, PCR and direct sequencing were performed as described in **section 2.5**.

Sequence analysis revealed GCT-GAT at codon –26, resulting in the substitution of Alanine by Aspartate (see **figure 4.2**). This mutation obliterated a unique *Bgl* I site in exon 1 of *F10*. Restriction fragment length analysis revealed that individuals A:III:1 and A:III:4 were heterozygous for this mutation. Individuals A:IV:1, A:IV:2 and A:IV:4 were homozygous for the mutation (see **figure 4.3**). This was confirmed by direct sequencing.

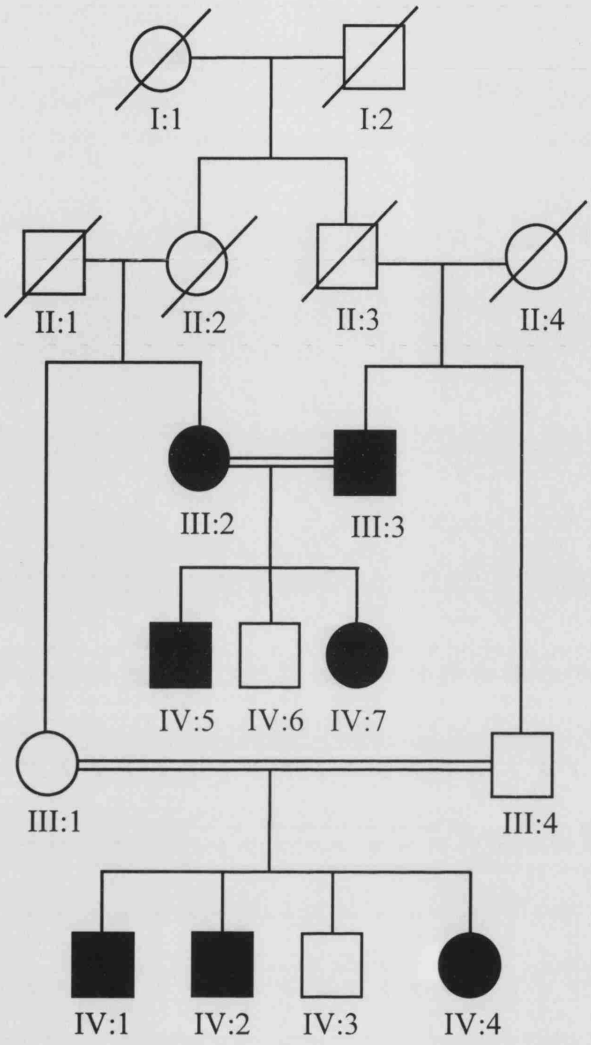


Figure 4.1 Family pedigree for kindred A

Circles represent females and squares represent males. Family members who have been shown to be clinically affected are indicated by filled black symbols. Deceased individuals are indicated by a diagonal line through their symbol. The double lines connecting individuals III:1 with III:4 and III:2 with III:3 indicate that their union is consanguineous.

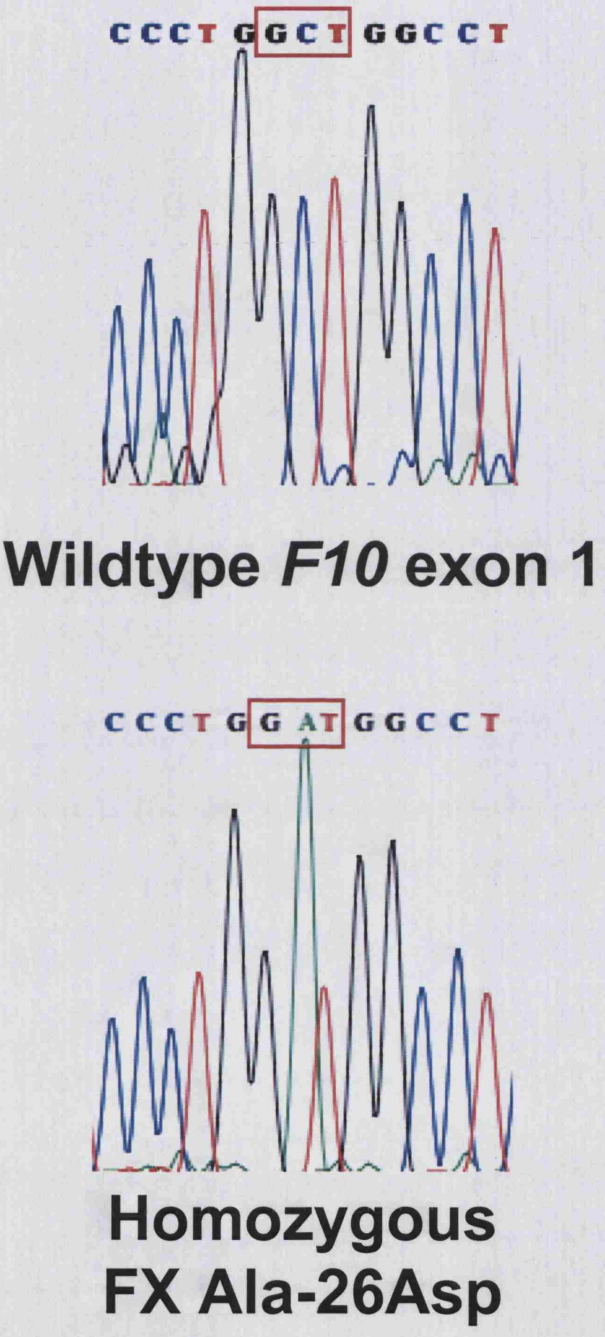


Figure 4.2. An electropherogram showing part of the sequence of exon 1 of *F10*. A GCT-GAT nucleotide substitution resulted in the missense mutation Ala-26Asp.

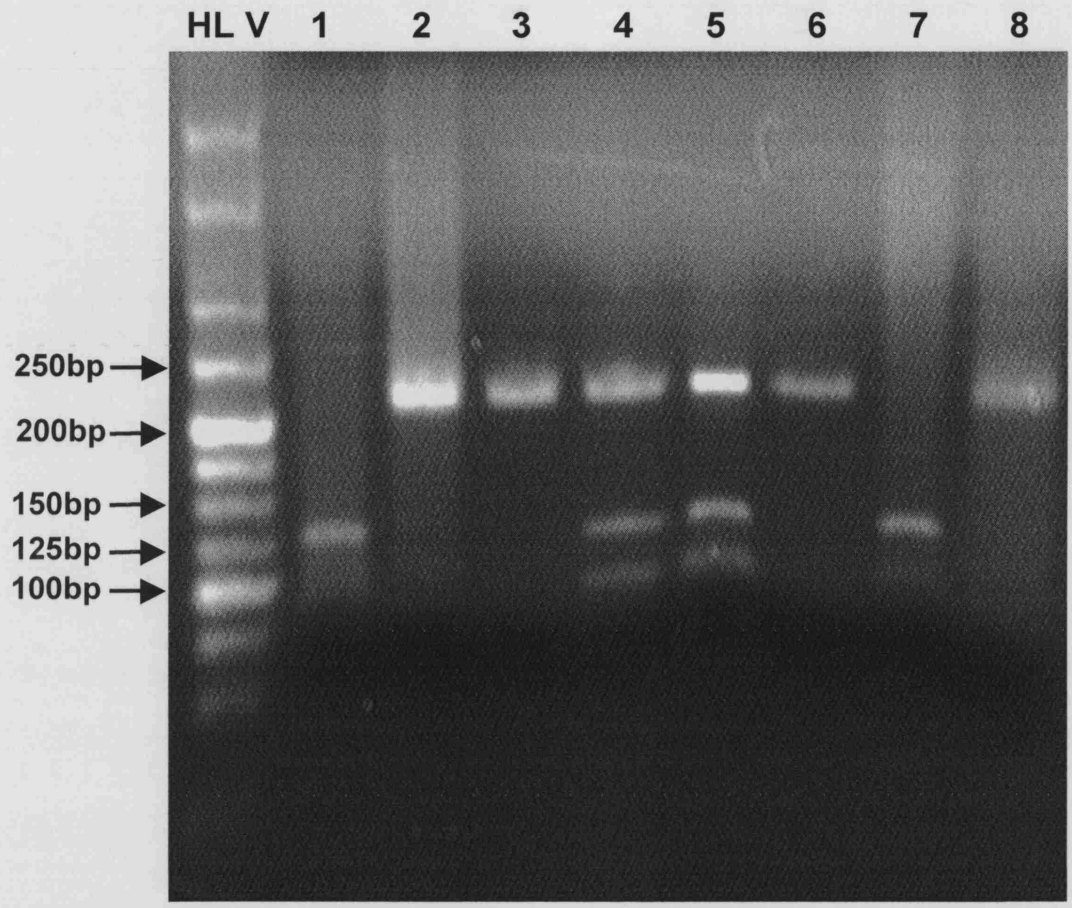


Figure 4.3 This shows a 1.5% agarose gel with the products of a *Bgl* I restriction digest of exon 1 of *F10*. The wildtype exon is cut once by *Bgl* I to create two fragments of 124 bp and 90 bp each. The GCT-GAT mutation obliterates this unique *Bgl* I site, so the homozygous mutant remains uncut at 214 bp. Lane 1 contains a wildtype control. DNA from individual A:IV:4 is in lane 2; A:IV:1 in lane 3; A:III:4 in lane 4; A:III:1 in lane 5; A:IV:2 in lane 6; wildtype control in lane 7 and wildtype control without enzyme in lane 8. HLV: Hyperladder V marker (Bioline, London, UK).

Ala-26 lies within the signal peptide of FX. Signal peptide cleavage prediction was performed, using the SignalP 3.0 program on the website of the Center for Biological Sequence Analysis at the Technical University of Denmark (www.cbs.dtu.dk).

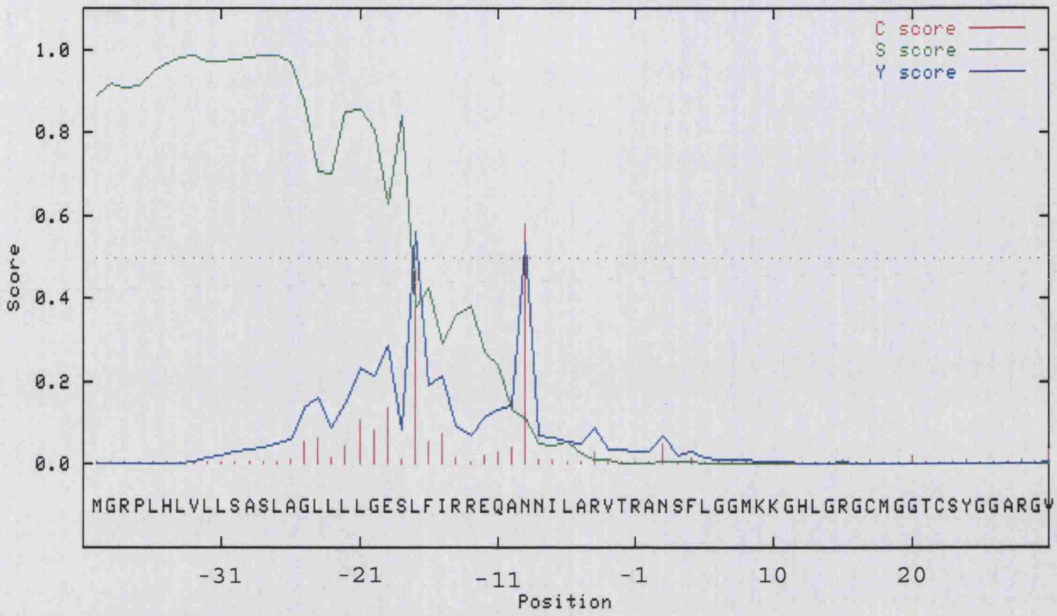
The program uses neural networks to predict the position of the signal peptidase I cleavage site. This is based on the idea that cleavage site position and amino acid composition of the signal peptide are correlated (Bendtsen *et al* 2004). There are several outputs from the network. The *S-score* is a value given to each residue in the submitted sequence. High scores indicate that a particular residue is part of the signal peptide; low scores indicate that the residue is part of the mature protein (or propeptide in the case of FX). Each position is also given a *C-score* "cleavage-site" score. The C-score is highest at the position immediately after the cleavage site and should be low at all other positions. The *Y-score* is a geometric mean between the C-score and a derivative of the S-score. The cleavage site is assigned when the Y-score is maximal (when the descent of the S-score slope is greatest and the C-score is significantly high). As with the C-score, the Y-score is highest at the position immediately after the cleavage site.

The program correctly predicted the wildtype signal peptide cleavage site (see **figure 4.4**). The substitution of Aspartate for Alanine at position –26 resulted in a minimal change to the C-score (it remained maximal at position –9). However, the position of the maximal S-score changed from –27 to –29. This affected the Y-score, moving the maximal Y-score from -17 to –9.

4.3.4 Discussion

The program predicted that the Ala-26Asp mutation will move the signal peptide cleavage site from GES-LF to EQA-NN (see **figure 4.5**). This would mean that that propeptide would be shortened by eight residues and would be predicted to be dysfunctional. If the shortened propeptide interfered with normal folding of the mature protein, then this could lead to reduced secretion of the protein, which would concur with the extremely low antigen levels detected in the homozygous patient. The heterozygous patient would have one normal allele which should produce functionally normal FX protein. This is confirmed by the phenotypic data (**table 4**) in which the heterozygote assay levels (patient A:III:2) are approximately half that of normal plasma.

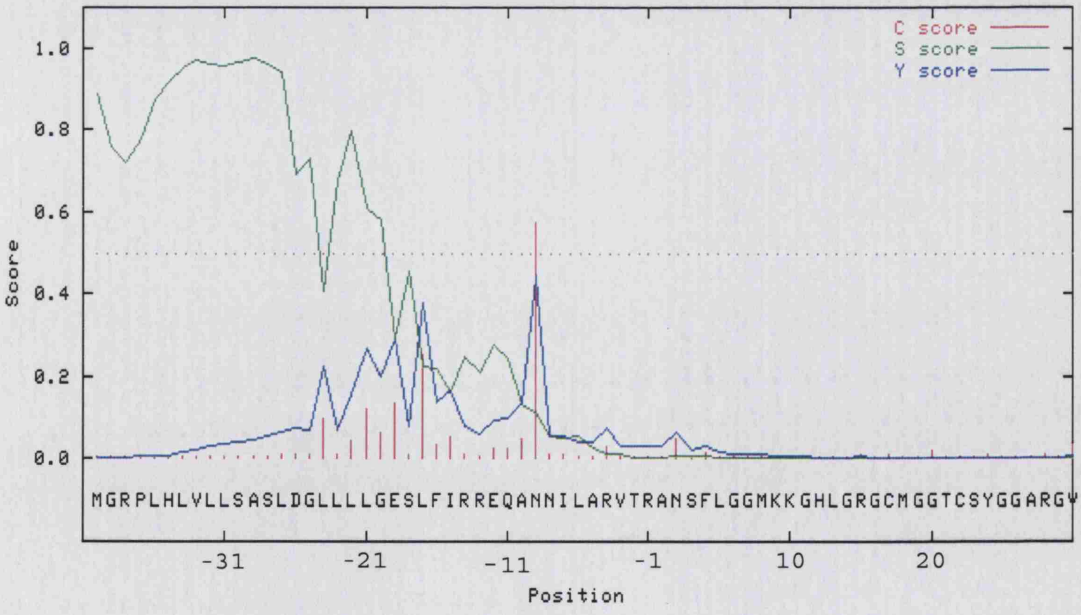
Figure 4.4
(A) Wildtype FX



Measure	Position	Value
Maximum C-score	-9	0.579
Maximum S-score	-27	0.986
Maximum Y-score	-17	0.559

Most likely cleavage site between positions –18 and –17.

(B) FX Ala-26Asp



Measure	Position	Value
Maximum C-score	-9	0.575
Maximum S-score	-29	0.973
Maximum Y-score	-9	0.443

Most likely cleavage site between positions –10 and –9.

Figure 4.4 is a graphical representation of the output from the SignalP 3.0 program.
(A) shows the C, S and Y scores for each of the 70 N-terminal residues in wildtype FX. Although the maximum C-score is at position –9 and the maximum S-score is at position –27, the overall Y-score is maximal at –17. This predicts that signal peptide cleavage will occur between positions –18 and –17.
(B) shows the C, S and Y scores for each of the 70 N-terminal residues in the Ala-26Asp mutant FX. The maximum C-score is at position –9 and the maximum S-score is at position –29. This gives an overall Y-score which is maximal at –9 and predicts that signal peptide cleavage will occur between positions –10 and –9.

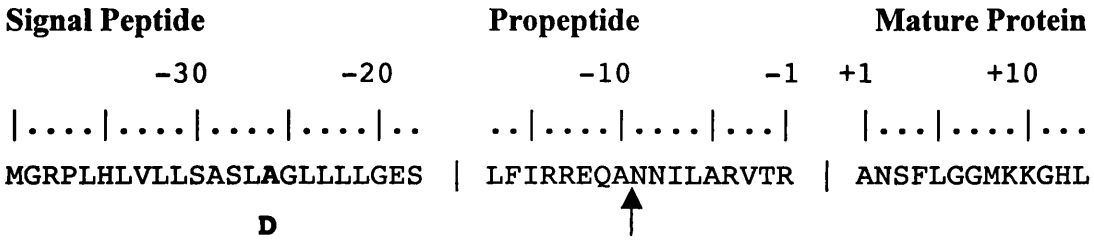


Figure 4.5 Predicted signal peptide cleavage site in the Ala-26Arg mutant.

The substitution of Aspartate for Alanine at position –26 in the signal peptide of FX is shown. The arrow indicates the predicted cleavage site after the residue is mutated.

4.4 Kindred B (IVS1-1 G-C mutation)

4.4.1 History

Patient B:II:2 was registered at the Haemophilia Centre, Royal Free Hospital, London. He was born in 1985 by spontaneous vaginal delivery. His parents are first cousins (**see figure 4.6**). At day three after birth, bleeding was noticed from the umbilical cord stump. Two days later, when the haemorrhage had failed to stop, FX deficiency was diagnosed. At the age of ten weeks, he suffered a left parietal haemorrhage. This required a left-sided craniotomy, evacuation of the clot and insertion of a ventriculo-peritoneal shunt for communicating hydrocephalus.

Since the intracerebral haemorrhage and shunt insertion, the patient has suffered from petit mal epilepsy. He has had treatment for this in the form of the anti-convulsant agents carbamazepine and sodium valproate. From the age of five he was noted to be mentally slower than the children in his peer group. He was formally diagnosed with learning difficulties shortly afterwards.

Treatment for his FX deficiency has been in the form of alternate day prophylaxis with PCC. Currently this is "HT DEFIX" (Scottish National Blood Transfusion Service). HT DEFIX is a heat-treated concentrate which contains approximately 1 iu antithrombin, 40 iu prothrombin and 40 iu FX for every 60 iu factor IX.

To date, he has serological immunity to hepatitis A and hepatitis B, following successful vaccination against these blood-borne viruses. Fortunately he remains HIV negative and Hepatitis C negative.

Since his intracerebral haemorrhage in infancy, he has not suffered a spontaneous bleed. He has, however, suffered several traumatic bleeds and so has required extra doses of PCC.

4.4.2 Phenotype Assays

FX assays were performed on the plasma as previously described in **section 2.3 and 2.4**. Results are shown in **table 5**.

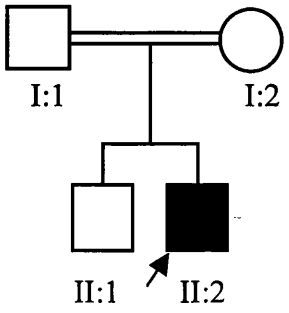


Figure 4.6 Family pedigree for kindred B

Circles represent females and squares represent males. Family members who have been shown to be clinically affected are indicated by filled black symbols. The double line connecting individuals I:1 and I:2 indicates that their union is consanguineous. The proband is indicated by the arrow.

Table 5 Phenotypic assay results for patient B:II:2

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
B:II:2	7	3	4.5	4	6.5

All units are iu/dL, with the reference ranges 50-150 iu/dL.

4.4.3 Genotype Results

DNA extraction, PCR and direct sequencing were performed as described in **section 2.5.**

Sequence analysis of the genomic DNA of the patient B:II:2 revealed a mutation at an intron-exon splice site. The patient was found to be homozygous for an AG→AC acceptor splice site defect at the –1 position in IVS1 (See **figure 4.7**).

Splice site computer analysis was performed, using the Berkley Drosophila Genome Project human database website (http://www.fruitfly.org/seq_tools/splice.html). This program uses neural networks to recognise donor and acceptor splice sites. The output for the network is a probability score that a potential splice site is the actual site (Reese *et al* 1997). The program gave a score of 94% for the correct splice site in the wildtype *F10*. The substitution of C for G would almost certainly abolish the splice-site (Breathnach *et al* 1978).
The program predicted that splicing would occur 13 bases downstream, at the next AG dinucleotide (see **figure 4.8**). This was predicted with a probability score of 94%. The resulting mRNA transcript would be 13 nucleotides shorter than that of the wildtype. This would lead to a frameshift and so result in a stop codon 21 residues downstream from Ser-18.

4.4.4 Further investigation

In order to show the consequence of the IVS1-1 G-C mutation in patient B:II:2, it would be ideal to sequence the *F10* mRNA. This is not practical; however, direct sequencing of complementary DNA (cDNA) which has been produced from mRNA is possible.
Development and differentiation in multicellular eukaryotes relies on tissue-specific regulation of gene expression. In general, genes can be classified into two groups: tissue-specific genes and housekeeping genes. Tissue-specific genes, are expressed at a specific stage of development in certain tissues. Housekeeping genes are essentially expressed in all cells (Maniatis *et al* 1987). The first group of genes encodes proteins which are involved in the functional characteristics of cells, for example. The second group encodes common structural proteins or ubiquitous enzymes.

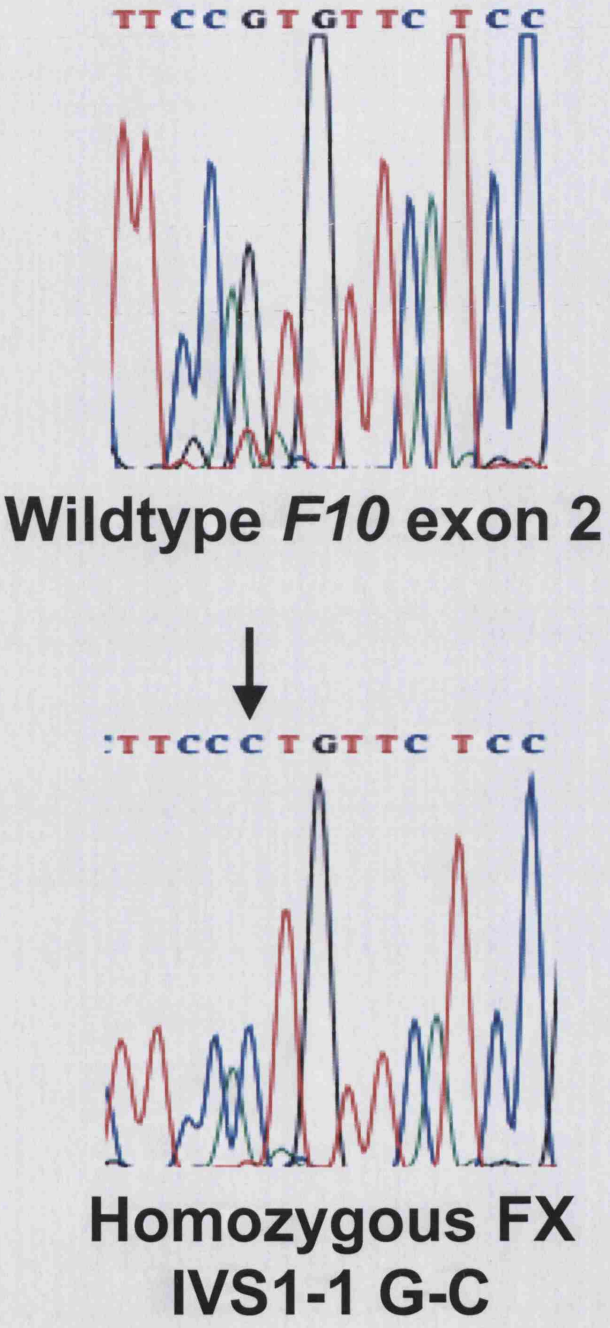


Figure 4.7

An electropherogram showing part of the *F10* genomic sequence. A single mutation was found in the acceptor splice site of exon 2. A homozygous G→C substitution is shown by the black arrow. It is noteworthy that the base-calling software has failed to recognise any of the green Adenine peaks of the electropherogram. This is not uncommon, especially when the Adenine occurs directly after a Cytosine residue.

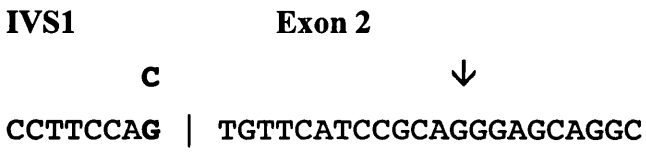


Figure 4.8 The predicted position of the activated splice site in kindred B

The single nucleotide substitution of **G→C** at position –1 of intron one (IVS1) in *F10* is shown. The arrow indicates the predicted position of the cryptic splice-site which is activated.

The protein-coding regions of most eukaryotic genes are split, giving rise to primary transcripts containing both introns and exons. The exons are spliced in the nucleus to form the mature mRNA, which then moves to the cytoplasm for translation into protein.

Northern blot analysis has shown that FX expression is restricted to hepatocytes (Bahnak *et al* 1987). In order to examine the effect of a splice site mutation, one would need to obtain hepatic tissue. Liver biopsy is not a simple procedure and it is associated with a considerable risk of morbidity and mortality, particularly in an individual with a haemorrhagic tendency. It was not appropriate to subject patient B:II:2 to this action.

Traditionally, it was believed that transcription of tissue-specific genes only occurred in the corresponding tissue. However, it has been shown that highly tissue-specific genes are expressed as spliced transcripts at low levels in non-specific tissues (Chelly *et al* 1989). The levels are as low as one copy of specific spliced RNA per 500-1000 cells. It has been suggested that the presence of these transcripts at a basal level in tissues where they are not normally active be known as "illegitimate transcription".

The discovery that such mRNAs are present in virtually all cell-types allows the possibility of amplification of cDNA fragments of the relevant sequence by PCR techniques. The low levels of mRNA (and so low levels of cDNA template for PCR) would result in a great deal of background priming from other genomic sources. In order to increase the specificity and yield of DNA, a second PCR reaction using a "nested" set of PCR amplification primers is used. These nested primers hybridise internally on the amplified DNA (Engelke *et al* 1988).

Northern blot hybridization (Kafatos *et al* 1979) takes three days to perform and is sensitive enough to detect transcripts that represent 0.01% of the mRNA population with a blot of 10 μg total mammalian RNA. The use of nested RT-PCR is more sensitive than Northern blot hybridization and can give positive results from as few as 10 cells per ml (Shimazui *et al* 2003).

In order to confirm that the methodology was robust, peripheral blood and bone marrow samples from normal controls were used. Total RNA extracted from the bone marrow and peripheral blood of individuals without FX deficiency were kindly given by Dr Letizia Foroni (Haematology department, Royal Free Hospital). Use of primers for amplification of the housekeeping gene β-actin had previously confirmed

that the RNA was of sufficient quality for successful RT-PCR. Normal liver RNA (a kind gift from Mrs Ruth Jacobs, Hepatology department, Royal Free Hospital) was used in the reverse transcription and PCR stages as a positive control.

4.4.5 Results of cDNA analysis

From gel A in **figure 4.9**, a single band of the correct size in the lane from normal liver RNA can be seen. This indicates that the cDNA preparation was successful and that *F10* cDNA can be obtained from normal liver. There are no bands in the other lanes. Gel B shows a greater relative intensity band of the correct size in the liver lane. However, the nested PCR reaction failed to amplify a fragment in the other lanes. This indicates that *F10* mRNA can neither be obtained from normal peripheral blood nor from normal bone marrow.

4.4.6 Discussion

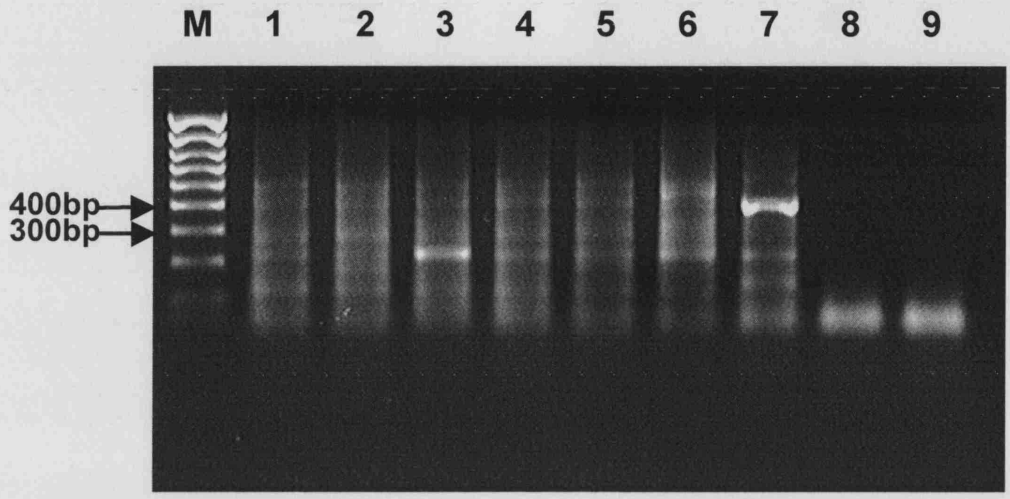
The attempt to amplify *F10* cDNA from peripheral blood and bone marrow was unsuccessful. It is unlikely that the method is not sensitive enough to detect low level transcription of *F10* in peripheral blood as each first round PCR reaction used the cDNA derived from approximately 10^5 cells as a template. According to (Shimazui *et al* 2003), this is a factor of 10^4 more than is necessary. Many other genes (including that for human coagulation factor VIII) undergo leaky transcription in peripheral blood and can be detected using this technique (Chelly *et al* 1989). It must be concluded that *F10* is an exception to this.

Other workers have attempted to examine the mRNA of coagulation factor IX in peripheral blood lymphocytes by nested RT-PCR (Green *et al* 2003). This work revealed that lymphocytes do not express the *F9* gene even at low levels. However, it was possible to amplify a transcript containing the final two exons of the *F9* gene from peripheral blood.

Even if amplification of *F10* mRNA from the RNA samples of normal individuals had been successful, then it is still be possible that the *F10* mRNA of patient B:II:2 would not be found in peripheral blood. Translation of mRNA transcripts into protein is subject to quality control procedures. This is to prevent the synthesis of potentially deleterious truncated proteins when translation is prematurely terminated

Figure 4.9

(A) First Round PCR



(B) Second Round PCR

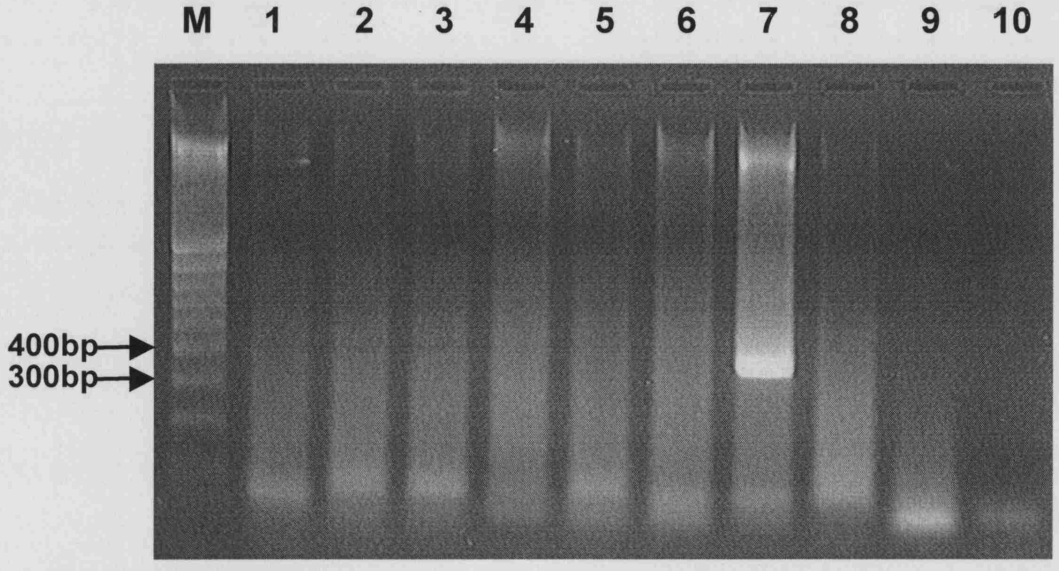


Figure 4.9

(A) 1.5% agarose gel showing the results of the first round PCR reaction. 5 µl from the total 30 µl reaction was run in each lane. Lanes 1, 2, 3 and 5 contain the products from different normal peripheral blood samples. Lanes 4 and 6 are from normal bone marrow samples. Lane 7 reaction is from normal liver RNA. Lane 8 is the negative control from the cDNA preparation. Lane 9 is the negative control for the first round reaction.

(B) 1.5% agarose gel showing the results of the second round PCR reaction. 5 µl from the total 30 µl reaction was run in each lane. Each lane contains the products of the second reaction from gel A. Lane 10 is the negative control for the second round reaction.

due to errors in gene expression. Such errors include inaccurate or inefficient splicing, aberrant transcription initiation as well as frameshift mutations. This quality control mechanism is known as nonsense-mediated mRNA decay (NMD) or mRNA surveillance (Maquat and Carmichael 2001). The premature termination of translation results in downregulation of mRNA translation by decapping (removal of the 5' cap which binds to the small subunit of the ribosome as the first step in translation) and degradation.

In mammalian cells, NMD occurs when translation terminates more than 50-55 nucleotides upstream of the nearest 3' exon-exon junction. Pre-mRNA splicing leads to the deposition of a complex of proteins 20-24 nucleotides upstream of the exon-exon junction. These proteins are part of the splicing apparatus or are involved in mRNA transport. The protein complex recruits Upf3 proteins, which usually reside in the nucleus, but are exported to the cytoplasm with the mRNA. Here, other Upf proteins are recruited and NMD is commenced. However, if translation terminates less than 50-55 nucleotides upstream of the 3'-most exon-exon junction or downstream of the junction, then NMD is not initiated and translating ribosomes are thought to remove the Upfs (Maquat 2002).

Computer analysis predicted that the frameshift resulting from the activation of the cryptic splice site in our patient would produce premature termination of translation 86 nucleotides upstream of the 3' exon-exon junction. In this case, it would be predicted that NMD would occur. This would mean that the peptide encoded by exon 2 would not be produced.

If NMD occurred in the mRNA of exon 2, then it would be predicted that the FX protein would not fold correctly and so no FX would be secreted. Phenotypic analysis of the plasma of B:II:2 showed that some FX activity is present. The most likely explanation for this is that the sample was taken towards the end of the trough period after a prophylactic dose of PCC, so the antigen and activity levels measured refer to the coagulating effect of the PCC. An alternative, but less likely, explanation is that our patient secretes some functioning FX into his plasma. Genomic sequencing revealed that he is homozygous for the acceptor splice site mutation. If he does secrete normal FX, then he must employ some mechanism whereby the mutation is corrected in a small number of hepatocytes.

It may be possible to investigate the results of a splice site mutation using "Exon trapping" (Auch and Reth 1990). In this technique, genomic DNA fragments are

cloned into plasmids or cosmids (Datson *et al* 1996). Transient expression in mammalian cells can occur, with transcription of the genomic insert and splicing of exons. RT-PCR, cDNA amplification and direct sequencing can then be performed to confirm or refute the prediction of the altered splice site.

4.5 Kindred C (Glu19Val and IVS5+3 A-G mutations)

4.5.1 History

Patient C:II:2 is registered with the Haemophilia Centre at Oxford, UK. He has a clinical history of easy bruising, although he has never suffered major bleeding. He is the only affected of five children. His mother has FX deficiency, although she is clinically unaffected. There are no data on his father nor on his three children. The pedigree is shown in **figure 4.10**.

4.5.2 Phenotype Assays

FX assays were carried out on plasma as previously described in **sections 2.3 and 2.4**. Results are shown in **table 6**.

Table 6 Phenotypic assay results for kindred C

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
C:I:2	ND	20*	ND	ND	ND
C:II:2	ND	3*	ND	ND	ND

All units are iu/dL, with the reference ranges 50-150 iu/dL. ND – No data. *assays not performed at Royal Free Haemophilia Centre.

4.5.3 Genotype Analysis

DNA extraction, PCR and direct sequencing were performed as described in **section 2.5**.

Sequence analysis revealed that patient C:II:2 is a compound heterozygote.

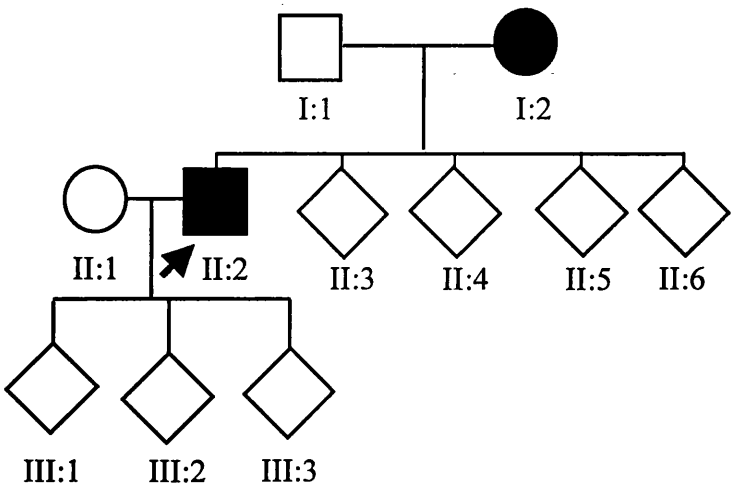


Figure 4.10 Family pedigree for kindred C

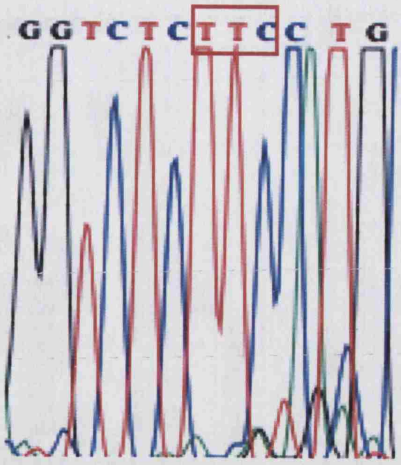
Circles represent females and squares represent males. Diamonds represent individuals of unknown gender. Family members who have been shown to be clinically affected are indicated by filled black symbols. The proband is indicated by the arrow.

There is a GAA-GTA substitution in exon 2, resulting in the missense mutation Glu19Val (see **figure 4.11**). A donor splice site mutation was also identified in intron 5 (IVS5+3 A→G). This is shown in **figure 4.12**.
No DNA was available on any other member of the family.

The Berkley Drosophila Genome Project human database website (http://www.fruitfly.org/seq_tools/splice.html) was used to predict if the A→G substitution in intron 5 would affect the position of the donor splice site. In this case, the probability score for the wildtype sequence cleaving at the correct site was 94%. The program predicted that the mutant would cleave at the same site, but with a probability score of only 84%. An alternative splice site was predicted; however the probability score was 83% and so cleavage is more likely to occur at the wildtype site. This alternative site was 109 nucleotides downstream. The splice sites are shown in **figure 4.13**.

4.5.4 Discussion

The identification of the Glu19Val mutation in exon 2 brings the total number of mutations reported in Gla residues to nine. These occur in seven of the eleven Gla residues which are situated towards the N-terminus of FX. The previous reports suggest that substitution of a Gla residue is likely to affect the calcium binding of the molecule and so its function as a coagulation protein (Rudolph *et al* 1996; Zama *et al* 1999). In some cases, the antigen level was found to be low. It has been suggested that the loss of a Gla residue results in incomplete γ-carboxylation and this leads to incomplete processing which would account for the low antigen levels (Diuguid *et al* 1989; Kim *et al* 1995).
Patient C:II:2 has a FX level by PT method of only 3%, which we have been unable to repeat. It would be predicted that he must be a compound heterozygote because if Glu19Val was his only mutation, then his FX assay levels would be half that of normal plasma. Presumably the splice site mutation does has a role to play here, in spite of the lower prediction score.
If the alternative splice site 109 nucleotides downstream is activated, then when exon 5 is translated into protein, the EGF-2 domain (which it encodes) would be abnormal. This is unlikely to produce a functional FX molecule. If the mutations lie



Wildtype *F10* exon 2
(reverse sequence)

Heterozygous FX Glu19Val
(reverse sequence)

Figure 4.11. An electropherogram showing part of the sequence of exon two of *F10*. A GAA-GTA nucleotide substitution resulted in the missense mutation Glu19Val in patient C:II:2. For reasons of clarity, the reverse sequence is shown here (TTC-TAC).

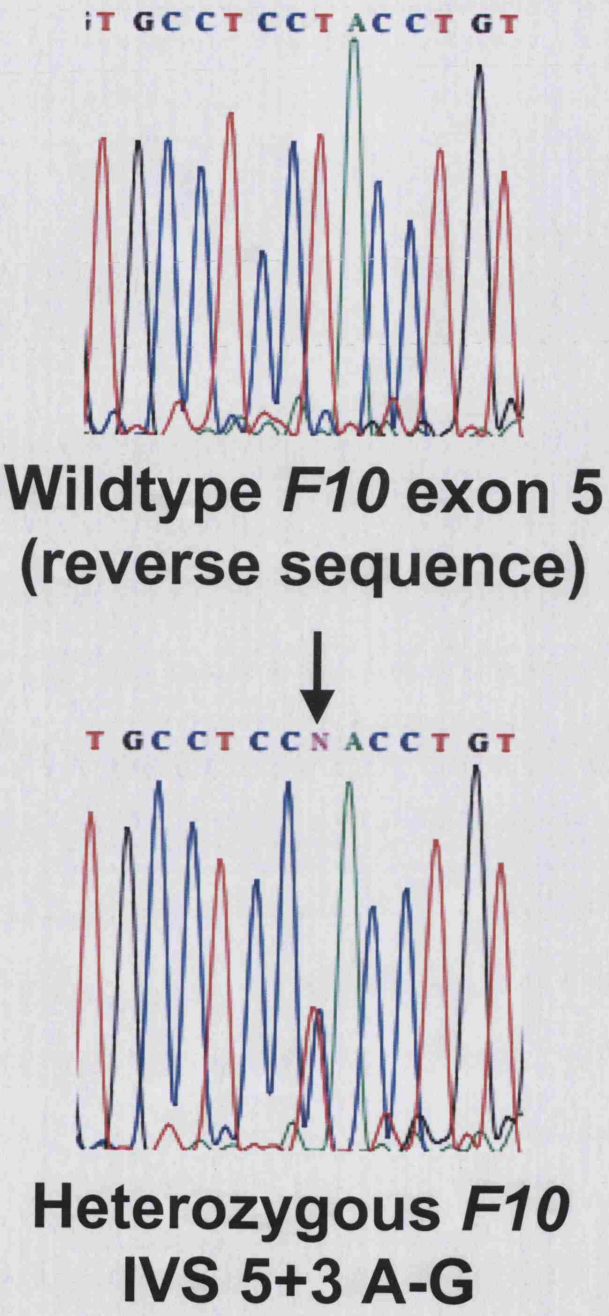


Figure 4.12. An electropherogram showing part of the sequence of exon 5 of *F10*. The position of the heterozygous nucleotide substitution of A→G at IVS 5+3 is identified by the arrow. For reasons of clarity, the reverse sequence is shown here (T→C).

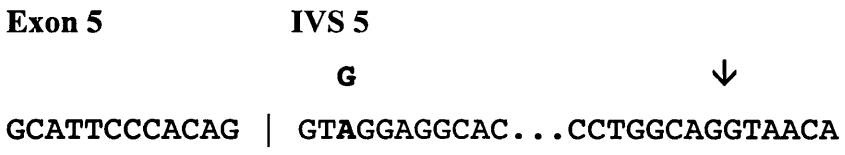


Figure 4.13 The predicted position of the alternative splice-site in kindred C

The single nucleotide substitution of **A→G** at position +3 in IVS5 in *F10* is shown. The arrow indicates the position of the alternative splice-site (with the probability score of 83%). The ellipsis represents 90 nucleotides of intronic sequence.

on separate alleles, then this could explain why his FX level is so low. If the mutations are on the same allele, then it would be predicted that the normal allele would produce functional protein and so the assay levels would be at least half that of normal plasma.

4.6 Kindred D (Glu51Lys mutation)

4.6.1 History

The proband is registered with the haematology service in Riyadh, Saudi Arabia. A pedigree was not available. The only clinical information was that the propositus had two asymptomatic parents.

4.6.2 Phenotypic Assays

FX assays were carried out on plasma as previously described in **sections 2.3 and 2.4**. Results are shown in **table 7**.

Table 7 Phenotypic assay results for kindred D

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
Propositus	84	14	90	85	98
Father	80	48	79	85	86
Mother	90	50	90	100	100

All units are iu/dL, with the reference ranges 50-150 iu/dL.

4.6.3 Genotype Analysis

DNA extraction, PCR and direct sequencing were performed as described in **section 2.5.**

Mutational analysis revealed a single missense mutation GAG-AAG corresponding to the substitution of Glutamic acid by Lysine at position 51. This is in the EGF-1 domain and is encoded by exon 4 (see **figure 4.14**).

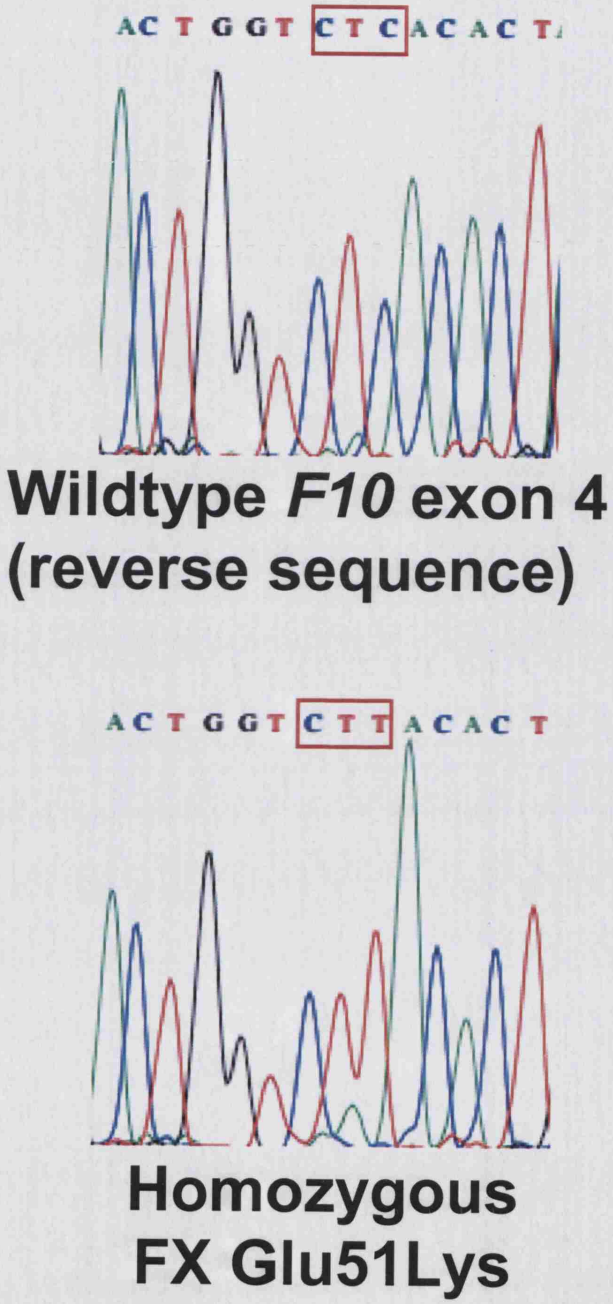


Figure 4.14. An electropherogram showing part of the sequence of exon 4 of *F10*. A GAG-AAG nucleotide substitution resulted in the missense mutation Glu51Lys in kindred D. For reasons of clarity, the reverse sequence is shown here (CTC-CTT).

4.6.4 Further Investigation.

From the laboratory data, it can be concluded that FX activity is normal when activation occurs using either RVV or the *intrinsic Xase* complex. Liberation of FXa is not affected by this mutation, as the chromogenic assay result is also within the reference range. However, activation of FX by the *extrinsic Xase* complex is affected. The prothrombin time assay is low for the propositus and borderline-low for the parents.

A possible explanation is that Glu51 is involved in the binding of the *extrinsic Xase* complex to FX. Using the computer program SURFDOCK, Norledge and co-workers predicted that the interface region of the FX EGF-1 domain for binding to the human TF:VIIa complex is centred on the region Glu51 to Gln58 (Norledge *et al* 2003). SURFDOCK is a molecular modelling program which can be used to perform protein-protein docking calculations.

In order to determine whether Glu51 binds to TF or to factor VIIa of the *extrinsic Xase* complex, prothrombin times were measured using a variety of thromboplastins. This would alter the source of TF whilst keeping the factor VIIa constant. The original FX:PT assays were performed using a mixture containing Rabbit Brain Thromboplastin and $CaCl_2$ (Instrumentation Laboratory, Milan, Italy).

4.6.4.1 Method

Prothrombin times were performed using a KC10 coagulometer (Amelung, Lehbrinksweg, Germany).

The source of bovine TF was "Two-Seven-Ten Reagent", a kind gift from Dr K. Denson (Diagnostic Reagents, Oxon, UK). This freeze-dried mixture of ox brain thromboplastin and adsorbed ox plasma was reconstituted with 5 ml of 4 mM $CaCl_2$. 250 µl of the reconstituted reagent was warmed to 37°C for two minutes and then 50 µl citrated plasma added. The time required for the formation of a clot was recorded.

The source of human TF was Innovin (Dade Behring, Marburg, Germany). This is human recombinant relipidated thromboplastin, containing $CaCl_2$. 100 µl of this was warmed to 37°C for two minutes and then 50 µl plasma added. Again, the time required for the formation of a clot was measured.

As for the original assays, the source of the rabbit TF was the IL Test PT-Fibrinogen HS Plus reagent (Instrumentation Laboratory, Milan, Italy). This contains rabbit brain thromboplastin and $CaCl_2$. 100 µl of this was warmed to 37°C for two minutes and then 50 µl plasma added. Again, the time taken for the formation of a clot was recorded.

Control samples included reference plasma pooled from 20 normal individuals. The blank sample used FX deficient plasma (Instrumentation Laboratory, Milan, Italy) and OBS dilution buffer only.

4.6.4.2 Results

Table 8 Plasma PT using thromboplastin from different sources

	Thromboplastin source		
Plasma source	**Bovine**	**Human**	**Rabbit**
Reference plasma	37.0	12.3	20.0
Propositus	22.9	22.9	NP
Mother	22.3	22.1	42.2
Father	NP	NP	NP
Blank	> 240	> 240	> 240

Clotting times are shown in seconds. NP: No plasma available.

Unfortunately not enough plasma sample was available for several of the tests.

The original assays were performed 30 months previously and although the plasma was stored at -80°C, some degradation was likely to have occurred (Woodhams *et al* 2001). This was the case here, as can be seen from the clotting times from the original assays (using rabbit thromboplastin) in **table 9**.

The clotting time for the mother's plasma diluted to 1:10 (32.5 seconds) (**table 9**) was shorter than the time for the undiluted plasma 30 months later (42.2 seconds) (**table 8**), confirming that there has been loss of coagulation activity over time.

Despite the fact that there had been degradation of the plasma over time, it can be seen from **table 8** that the use of both human and rabbit thromboplastins resulted in a prolonged prothrombin time in patients with the Glu51Lys mutation. However, the use of bovine thromboplastin results in a shortening of the prothrombin time. The

extrinsic Xase complex involves both TF and factor VIIa. When measuring a prothrombin time, factor VIIa is provided by the sample and TF by the addition of thromboplastin. The difference in the prothrombin times noted in **table 8** is due to the use of TF from different sources. This indicates that it is the TF of the *extrinsic Xase* complex and not the factor VIIa which interacts with Glu51.

Table 9 PT clotting times for dilutions of patients' plasma

	Plasma dilution		
Plasma source	**1:10**	**1:20**	**1:40**
Reference plasma	24.9	31.9	42.1
Propositus	54.6	66.8	84.9
Mother	32.5	40.8	53.4
Father	33.1	NP	54.3

Clotting times are shown in seconds. NP: No plasma available.

4.6.5 Molecular Modelling

Work using the SURFDOCK program suggests that human TF binds to the FX EGF-1 domain region Glu51 to Gln58 (Norledge *et al* 2003). From this, it is predicted that a mutation at this site would reduce the strength of binding of TF to FX and so prolong the coagulation time. This prediction is backed up by the experimental data showing that the use of both rabbit and human thromboplastins results in prolonged prothrombin times in plasma from this family.

However, the use of bovine thromboplastin resulted in a prothrombin time in the mutant plasma that was shorter than that of the reference plasma.

Glu51 interacts with the residue Asn199 which forms part of a basic patch on the surface of TF (Norledge *et al* 2003). In order to examine the differences between human, rabbit and bovine tissue factors, MEGALIGN (DNASTAR, Madison, USA) was used to align their amino acid sequences using the CLUSTAL V algorithm. This alignment is shown in **figure 4.15**. The sequence identities between the three TF sequences was found to be 69-70%.

Figure 4.15 shows that the equivalent residues to Asn199 in human TF are Lys197 in rabbit TF and Asn193 in bovine TF.

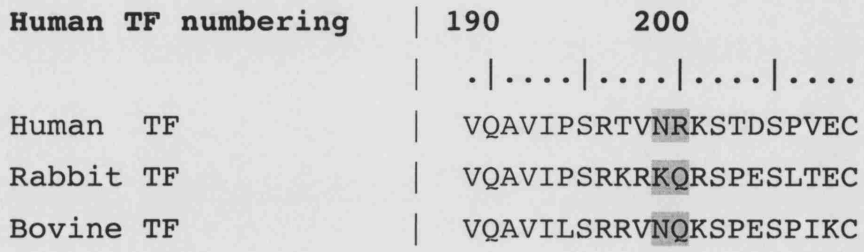


Figure 4.15 Sequence alignment of human, rabbit and bovine tissue factors

The SWISSPROT accession codes from top to bottom are P13726, P24055 and P30931. The basic patch on the surface of human TF which interacts with FX is highlighted in grey. The equivalent residues in rabbit and bovine TF are also highlighted.

The structure for the TF/factor VIIa/FXa complex was taken from the PDB code 1nl8 (Norledge *et al* 2003). The structure for rabbit TF was taken from the PDB code 1A21. The crystal structure for bovine TF has not been solved so this was built by homology modelling (see **section 3.2.4**).

The amino acid sequences of the three TF proteins are similar, so the three-dimensional structures are likely to be similar. The sequence of rabbit TF was used as the template structure in this case because the coordinates for human TF were found to be incomplete. Protein loops were imported from the PDB and *splice repair* energy minimisation was performed.

Using the 1nl8 model of the TF/factor VIIa/FXa complex, the coordinates of human TF were removed and in two separate procedures, the coordinates for rabbit TF (1A21) and the coordinates of the homology model of bovine TF were inserted. The three models (containing human TF, rabbit TF or bovine TF) were inspected to examine the differences between the interactions of the FX residue Glu51 with each TF molecule (**figure 4.16**).

Inspection of the models showed that in human TF, Asn199 is in close proximity to FX Glu51 and it is possible that the pair would form a hydrogen bond. The substitution of the acidic Glutamate by the basic Lysine would be predicted to reduce the interaction between the two molecules. This would explain why the clotting time is prolonged in the mutant compared to the wildtype.

In rabbit TF, Lys197 (the equivalent residue to Asn199 in human TF) is in close proximity to FX Glu51 and the pair would form a hydrogen bond. Again, the substitution of Glu51 by Lysine would be predicted to reduce the interaction between the two molecules and prolong the clotting time in the mutant.

Examination of the bovine TF model revealed that Asn193 is further away from FX Glu51 than the equivalent residues in human and rabbit TF. This distance is too great for a hydrogen bond. Consequently, it would be predicted that the substitution of Glu51 by Lysine would not change this interaction.

4.6.6 Expression Work

This is discussed in **chapter 9**.

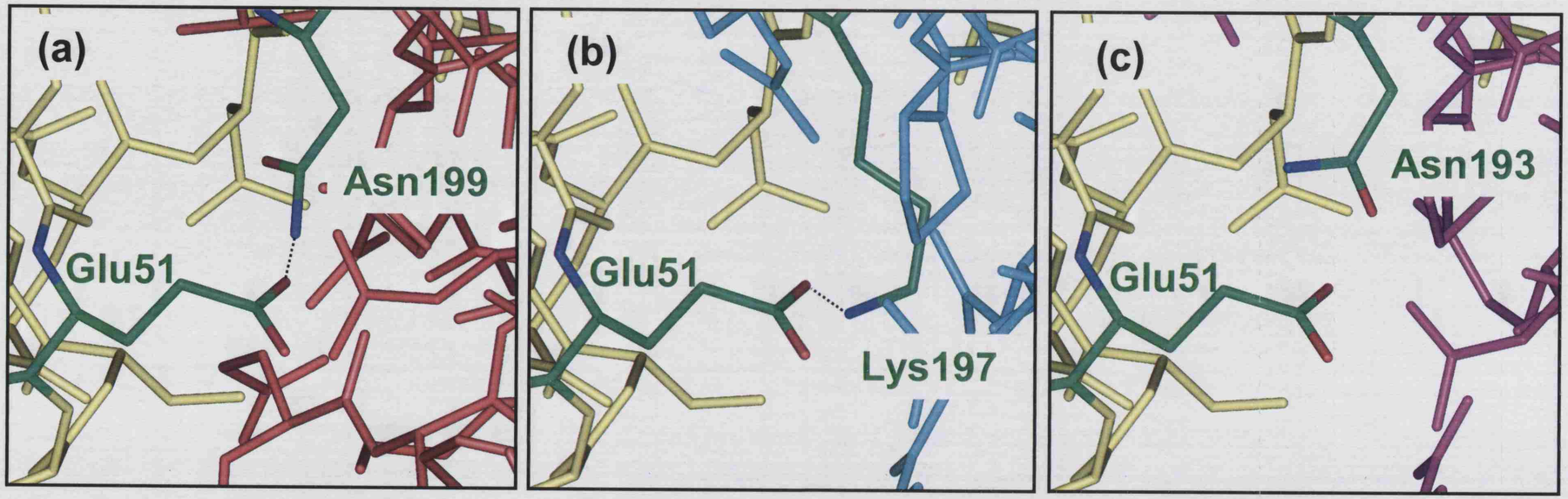


Figure 4.16 Molecular graphics views of the interaction between Glu51 and TF from three species.

Figure 4.16 Molecular graphics views of the interaction between Glu51 and TF from three species.

The views of (a), (b) and (c) are identical in orientation. The EGF-1 domain of FXa is depicted in yellow. Glu51 of FXa is shown in green.

a) Human TF is depicted in red, with the Asn199 of the basic patch shown in green. A hydrogen bond between the acidic Glu51 of FX and the terminal amide group of Asn199 is shown in black.

b) Rabbit TF is depicted in cyan. Lys197 (the equivalent residue to Asn199 in human TF) is shown in green. A hydrogen bond between the acidic Glu51 of FX and the basic sidechain of Lys197 is shown in black.

c) Bovine TF is depicted in magenta. Asn193 (the equivalent residue to Asn199 in human TF) is shown in green. Asn193 is not close enough to Glu51 to form a bond.

4.6.7 Discussion

The Glu51Lys mutation results in a reduced activation of FX by the *extrinsic Xase* complex. Activation by the *intrinsic Xase* complex and by RVV is normal. Glu51 is in the EGF-1 domain of FX. It is involved in the interaction of FX with a basic patch of residues on TF. Using a variety of thromboplastin sources, activation of the mutant FX using human and rabbit thromboplastins was similarly reduced compared to wildtype. However, use of bovine thromboplastin resulted in a shortened clotting time compared to wildtype.

The previously reported FX variant Arg251Trp (FX Padua) also shows impaired activation by the *extrinsic Xase* complex. This is due to disruption of a high-affinity Ca^{2+} binding site in FX (Girolami *et al* 2004). In contrast to the Glu51Lys mutation described here, the response of FX Padua to thromboplastins from various sources (rabbit brain, ox brain and human placenta) was identical.

A factor VII mutant (factor VII Padua) has been described in which there is a thromboplastin-dependent defect in factor VII activity but a normal FVII antigen. Using rabbit-derived thromboplastin, the activity level was found to be 10% of normal; however, using thromboplastin of bovine origin, the activity was normal. Human-derived thromboplastin resulted in a level of 45% of normal (Girolami *et al* 1978). Genotype analysis showed that this defect results from the substitution of Glutamine for Arginine at position 306 in factor VII. It was predicted that the mutation site is important in the interaction of rabbit TF with factor VII, but less important in the bovine TF-human factor VII interaction.

An alignment of the sequences of rabbit, human and bovine TF showed that the equivalent residues to Asn199 of the basic patch in human TF are Lys197 in rabbit TF and Asn193 in bovine TF.

Inspection of the crystal structures of human and rabbit TF and a homology model of bovine TF revealed that human Asn199 and rabbit Lys197 are in close enough proximity to Glu51 to form hydrogen bonds. However, bovine Asn193 is too great a distance from Glu51 for this to happen.

When each of the TF molecules form the *extrinsic Xase* complex with the mutant Glu51Lys molecule, the interactions between the mutant and human TF and the

mutant and rabbit TF are inhibited because of the proximity of the residues. Bovine TF is not affected in this way.

This gives a possible explanation why the PT for the mutant using bovine thromboplastin is not prolonged compared to that of wildtype. However, inspection of the molecular models did not show that the substitution of Glutamate by Lysine strengthened the interaction with bovine TF and so it does not explain why the PT is shortened in that case.

It is possible that the alignment is incorrect and that Asn193 is not the equivalent residue to human Asn199. It is also possible that the homology model is not a true representation of the protein structure of bovine TF, in which case positions of individual residues would be meaningless.

4.7 Kindred E

4.7.1 History

Patient E:V:2 presented with severe haemorrhage from the umbilical stump at the age of two weeks. By six months of age, he had suffered three episodes of gastrointestinal bleeding which required admission to hospital and infusion of plasma. He is registered with the Pediatric Hematology Unit in Ankara, Turkey. His immediate family (E:IV:4, E:IV:5 and E:V:1) are all asymptomatic. The family pedigree is shown in **figure 4.17**.

4.7.2 Phenotype Analysis

FX assays were carried out on plasma as previously described in **sections 2.3 and 2.4**. Results are shown in **table 10**.

Table 10 Phenotypic assay results for kindred E

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
E:IV:4	60	62	60	95	43
E:IV:5	72	60	50	85	56
E:V:2	<1	<1	<1	<1	<1

All units are iu/dL, with the reference ranges 50-150 iu/dL.

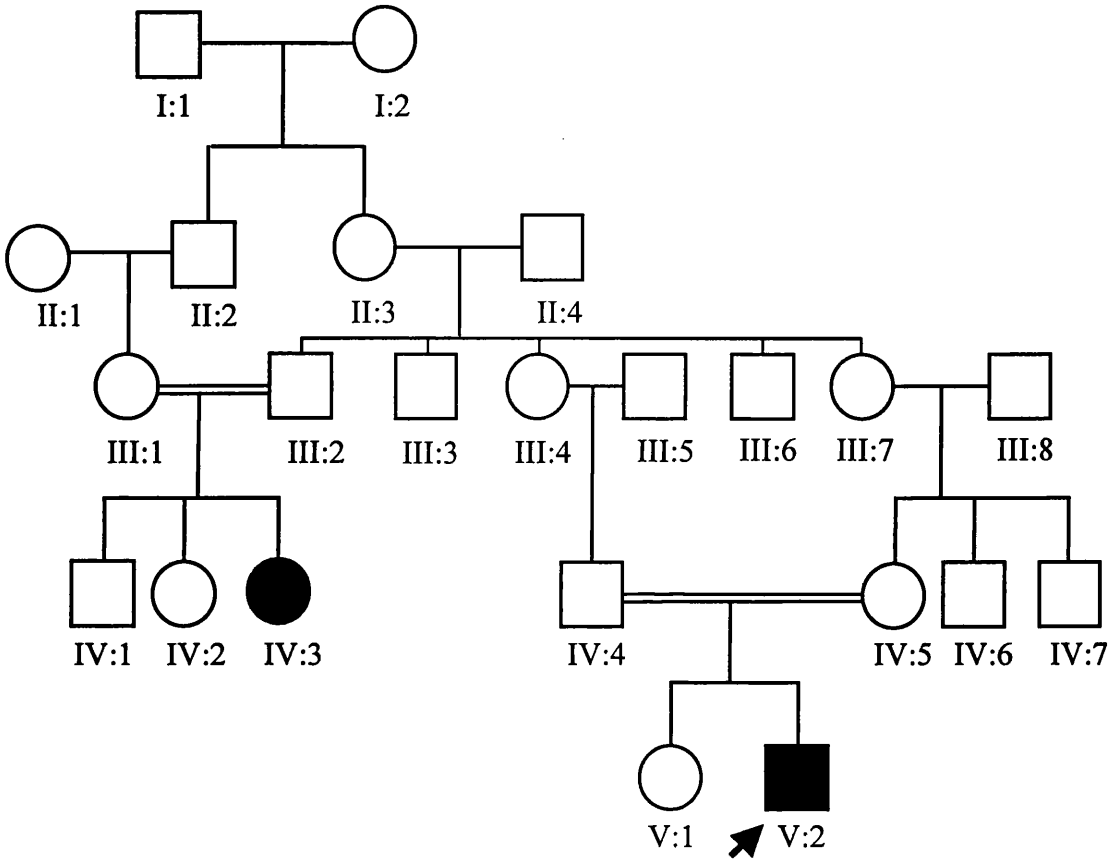


Figure 4.17 Family pedigree for kindred E

The proband is indicated by the arrow. Circles represent females and squares represent males. Family members who have been shown to be clinically affected are indicated by filled black symbols. The double lines connecting individuals III:1 with III:2 and IV:4 with IV:5 indicates that their union is consanguineous.

4.7.3 Genotype Analysis

DNA extraction, PCR and direct sequencing were performed as described in **section 2.5.**

Despite repeated sequencing of all eight exons, intron-exon splice sites and the promoter region, no mutation could be identified.

4.7.4 Discussion

No mutation was identified in this kindred. From the phenotypic data, one can speculate that the proband is homozygous for a mutation and that the parents are heterozygous for this. This would concur with their consanguineous marriage.

It is not uncommon for a causative mutation to escape detection. In a cohort of 600 patients with Haemophilia A, no mutation was identified in 11 patients (2% of the total) (Klopp *et al* 2002). It is possible that the mutation is within the gene but is intronic or affects regulatory elements upstream of the promoter. A mutation which is located deep within an intron would not be detected by analysis of exon sequences and exon boundaries. An intronic mutation in the coagulation factor VIII gene has been reported which activates a cryptic splice site (Bagnall *et al* 1999). This results in a 191 bp cryptic exon. This was not detectable by analysis of the genomic coding region, but required RT-PCR amplification of factor VIII mRNA.

It is also possible that the mutation is non-allelic. In this case, the mutation might be in a protein that interacts with the FX molecule, either during synthesis within the cell, secretion or possibly with its function in the blood.

Alternatively somatic mosaicism may have occurred during early embryogenesis. If the mutation is only present at a low level in peripheral blood cells, but affects the majority of hepatocytes, then the mutation would not be detectable by the methods described, but would still cause a haemorrhagic phenotype. However, this is unlikely to account for a severe phenotype, such as is described in patient E:V:2 because it would need to affect almost all liver cells while not being detectable in the peripheral blood.

4.8 Conclusions

FX operates enzymatically as a serine protease. Mutations affecting this action are likely to be found in the catalytic domain of the protein. The remainder of the protein is predominantly involved in ligand binding and protein stability. Most of the mutations discussed in this chapter do not result in a residue substitution in the mature protein, but are either at splice-sites or in the signal peptide.

Five kindred are discussed in this chapter. Mutations were identified in four of the kindred. The mutation in the signal peptide of kindred A resulted in a type I mutation with a severe phenotype. Splice site mutations were identified in kindred B and kindred C. Limited phenotypic data were available on kindred C but the homozygous mutation in kindred B resulted in a severe type I phenotype. Cleavage prediction programs based on neural networks predicted abnormal cleavage in kindred A and kindred B.

A single missense mutation was identified in kindred D. This produced an unusual phenotype with reduced activation of the mutant FX by the *extrinsic Xase* complex. Activation by other methods was normal. The explanation for this phenotype was aided by molecular modelling: the mutation is at the site of interaction of FX with TF.

No mutation could be identified in kindred E. This was disappointing, but is not uncommon. It is possible that the mutation has been missed or that it exists in another gene. A chaperone protein (GRP78/BiP) which escorts coagulation factor VII through the endoplasmic reticulum has been identified (Arbini *et al* 1996). So far, no chaperone proteins for FX have been identified. However, a mutation in an as yet unidentified chaperone protein for FX could result in the observed phenotype.

5 Analysis of Mutations Identified in the Catalytic Domain of FX

5.1 Introduction

35 of the 62 missense mutations that have been reported in *F10* are located within exons 7 and 8. These exons encode the serine protease domain. Exons 7 and 8 encode 253 of the 488 residues in FX, so the distribution of mutations does not appear to be biased towards the catalytic domain. Seven missense mutations were identified in the course of this work; four of them lie within the serine protease domain. This concurs with the previously reported distribution of mutations within the FX protein.

For trypsin-like serine proteases, there is a standard chymotrypsin sequence numbering, so residues within the catalytic domain are identified using FX numbering, followed by a bracketed number prefixed by a "c" that corresponds to the chymotrypsin numbering.

The *in vitro* expression work performed on the Arg273Trp and the Asp373Asn mutations is described in **chapter 9**.

5.2 Kindred F (Gly222Asp mutation)

5.2.1 History

The proband is a girl who is severely affected by FX deficiency. She presented at the age of one month with intracranial bleeding. During her first year of life, she suffered two further episodes. At the time the samples were received, she was eight years old and was having frequent intramuscular bleeds and occasional haemarthroses (approximately six episodes each year). She denied significant mucosal bleeding. She is registered with the haematology department at Gazi University in Ankara, Turkey. Her parents are first cousins; they are both asymptomatic. The pedigree is shown in **figure 5.1**.

5.2.2 Phenotype Assays

FX assays were carried out on plasma as previously described in **sections 2.3 and 2.4**. Results are shown in **table 11**.

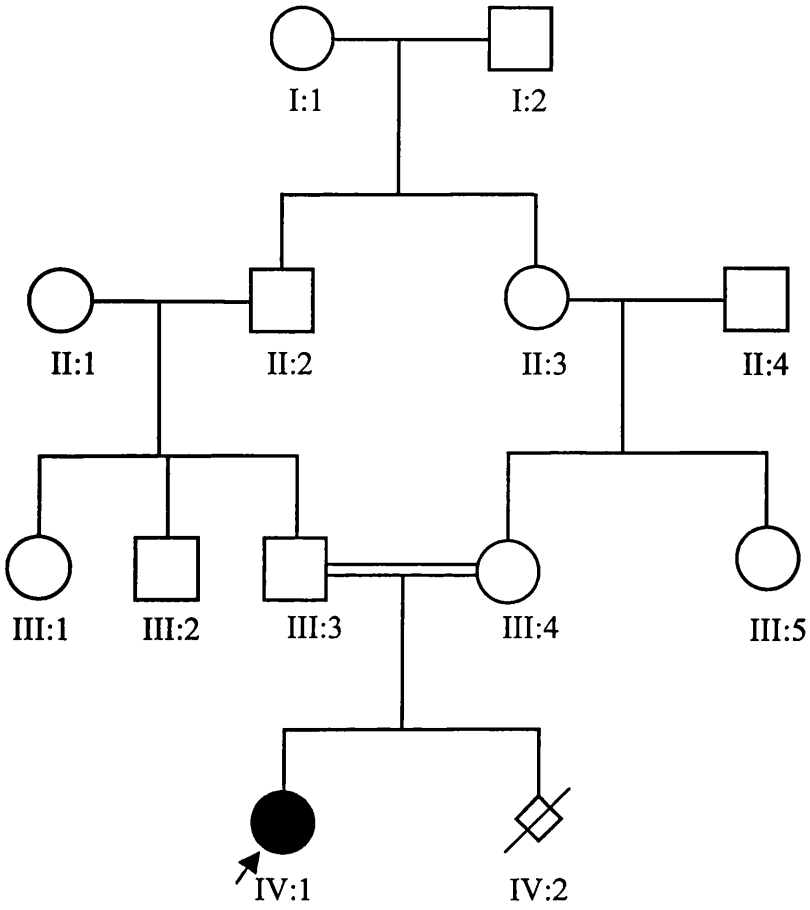


Figure 5.1 Family pedigree for kindred F

Circles represent females and squares represent males. The diamond-shape with the diagonal line represents a termination of pregnancy following prenatal diagnosis. Family members who have been shown to be clinically affected are indicated by filled black symbols. The proband is indicated by the arrow. The double lines connecting individual III:3 with III:4 indicates that their marriage is consanguineous.

Table 11 Phenotypic assay results for kindred F

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
F:III:3	ND	42*	ND	ND	ND
F:III:4	ND	48*	ND	ND	ND
F:IV:1	ND	<1*	ND	ND	ND

All units are iu/dL, with the reference ranges 50-150 iu/dL. ND – No data. *assays not performed at Royal Free Haemophilia Centre.

5.2.3 Genotype Analysis

DNA extraction, PCR and direct sequencing were performed as described in **section 2.5.**

Sequence analysis revealed GGT-GAT at codon 222, resulting in the substitution of Glycine by Aspartate (see **figure 5.2**).

5.2.4 Further data on the Gly222Asp Mutation

This mutation has also been described in two apparently unrelated individuals from Iran (Menegatti *et al* 2002). Patient 1 suffered recurrent haematomas and haemarthroses, haematuria and epistaxis. Patient 2 presented with epistaxis and gastrointestinal bleeding which required several blood transfusions. Laboratory assays of the patients' plasma are shown in **table 12** (as reported by Menegatti *et al*).

Table 12 Phenotypic data from Iranian patients with Gly222Asp mutation

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
1	10	<1	<1	<1	3
2	15	<1	<1	<1	6

Figures are expressed as % of the FX level in normal plasma (100%).

Sequence analysis showed that both of these patients were homozygous for the Gly222Asp mutation. The low antigen levels could be the result of defective folding of the FX molecule or intracellular accumulation. However, the lower activity levels indicate that any protein which is secreted is not functionally active.

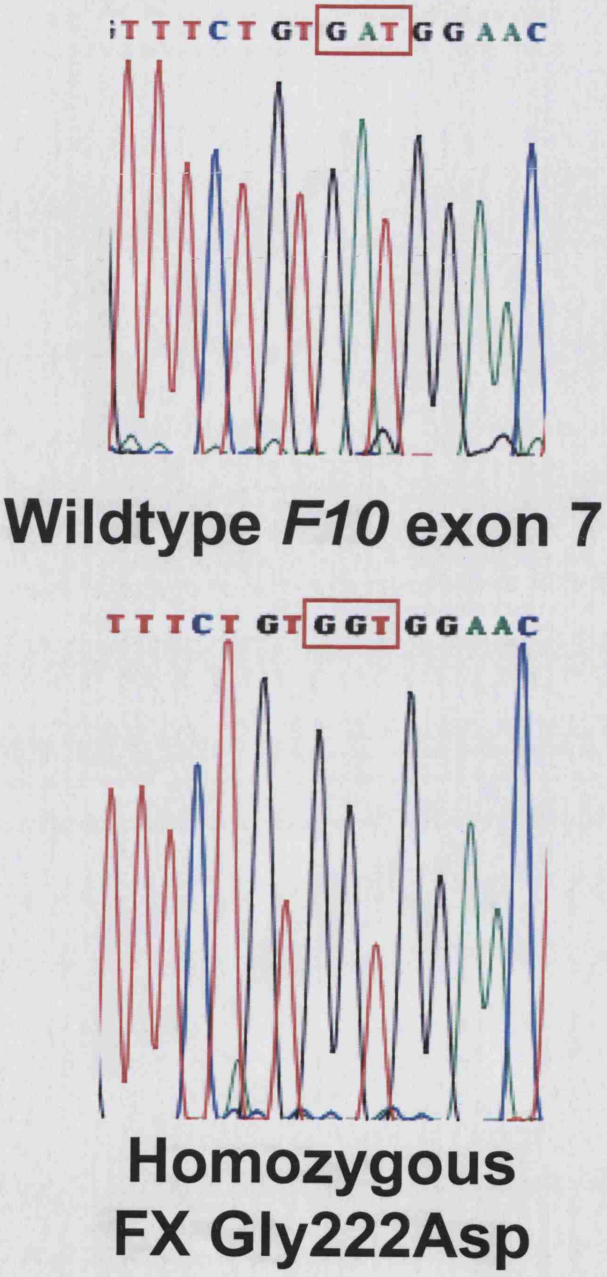


Figure 5.2 . An electropherogram showing part of the sequence of exon 7 of *F10*. A GGT-GAT nucleotide substitution resulted in the missense mutation Gly222Asp in kindred F.

5.2.5 Molecular Modelling

MEGALIGN (DNASTAR, Madison, USA) was used to perform a multiple sequence alignment using the CLUSTAL V algorithm (Higgins *et al* 1992). It was found that Gly222 (c43) is fully conserved in FX of other species. The residue at position c43 was found to be Glycine in all but five of 71 other human serine protease sequences available for comparison. An Aspartate residue does not appear at this position in any of the other homologous sequences.

The catalytic activity of FX resides in the serine protease domain, in which the active site is formed from the triad of residues, His236 (c57), Asp282 (c102) and Ser379 (c195). The Gly222Asp (c43) mutation corresponds to the replacement of a tiny fully-buried residue at the centre of the buried β-strand C (**figure 3.2**) by a larger charged residue adjacent to the catalytic triad. The insertion of a charged hydrophilic residue into the protein core will disrupt its correct folding. This would explain the low antigen levels. The proximity of this charged group to the catalytic triad (**figure 5.3**) is likely to affect the function of the protein, which may account for the observation that any protein which is secreted is not functionally active.

5.2.6 in vitro Expression Studies

In order to confirm the molecular modelling predictions, expression work was performed (Menegatti *et al* 2002). Both wildtype and Gly222Asp mutant FX cDNAs were expressed transiently in Human Embryo Kidney (HEK) 293 cells using the mammalian expression vector pCMV4 containing either FX wildtype cDNA or the Gly222Asp mutant FX cDNA. The FX coagulant activity was measured in the conditioned media. FX:Ag level was measured in both cell lysates and conditioned media of transfected cells.

The results showed that the procoagulant activity of FX wildtype was normal whereas the mutant protein had no coagulant activity. The conditioned media of the cells transfected by the mutant cDNA had only 33% of the wildtype FX:Ag level. This is concordant with the *in vivo* data. FX:Ag in the lysate from cells transfected by the mutant construct were approximately 40% higher than that of the wildtype. This indicates intracellular accumulation.

Further biochemical characterization would be required to explain why this partially secreted mutant FX protein is not functionally active.

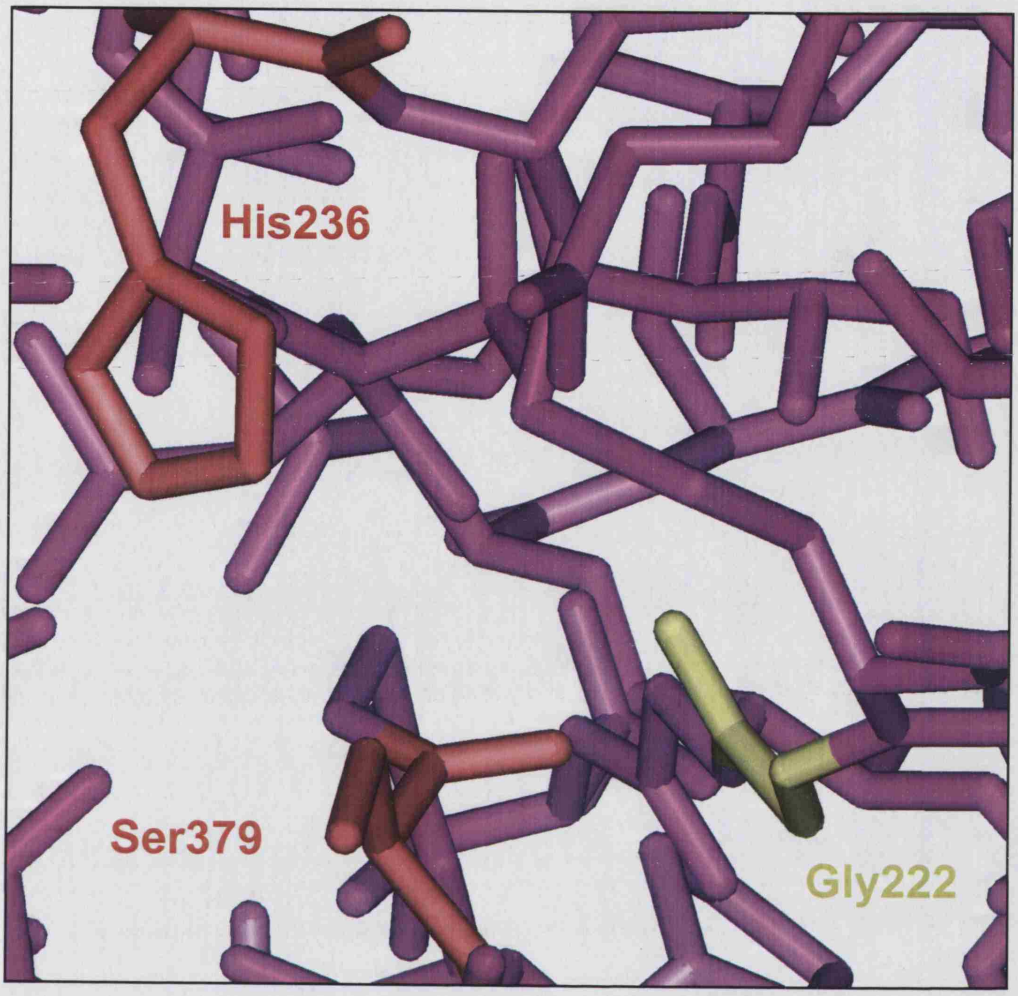


Figure 5.3. Structural analysis of the Gly222Asp (c43) substitution.

In the catalytic domain, Gly222 (yellow) is shown in close proximity to His236 (c57) and Ser379 (c195) of the Asp-Ser-His catalytic triad (red). Asp282 (c102) is above the top of the figure as viewed, and is not shown for reason of clarity.

5.2.7 Discussion

The Gly222Asp mutation produces a severe clinical bleeding phenotype in the homozygous state. The phenotypic assays show that little FX is secreted into the plasma and that any which is secreted is dysfunctional. Molecular modelling predicted that the substitution of a small neutral residue by a larger charged hydrophilic residue would perturb the catalytic triad and disrupt the normal folding of the molecule. Expression studies using mammalian cells concurred with the plasma assays confirming that Gly222Asp is the causative mutation. FX:Ag assays from the cell lysates revealed that much of the protein is retained within the cell, pointing towards a defect in the secretion pathway.

5.3 Kindred G (Pro382Leu mutation)

5.3.1 History

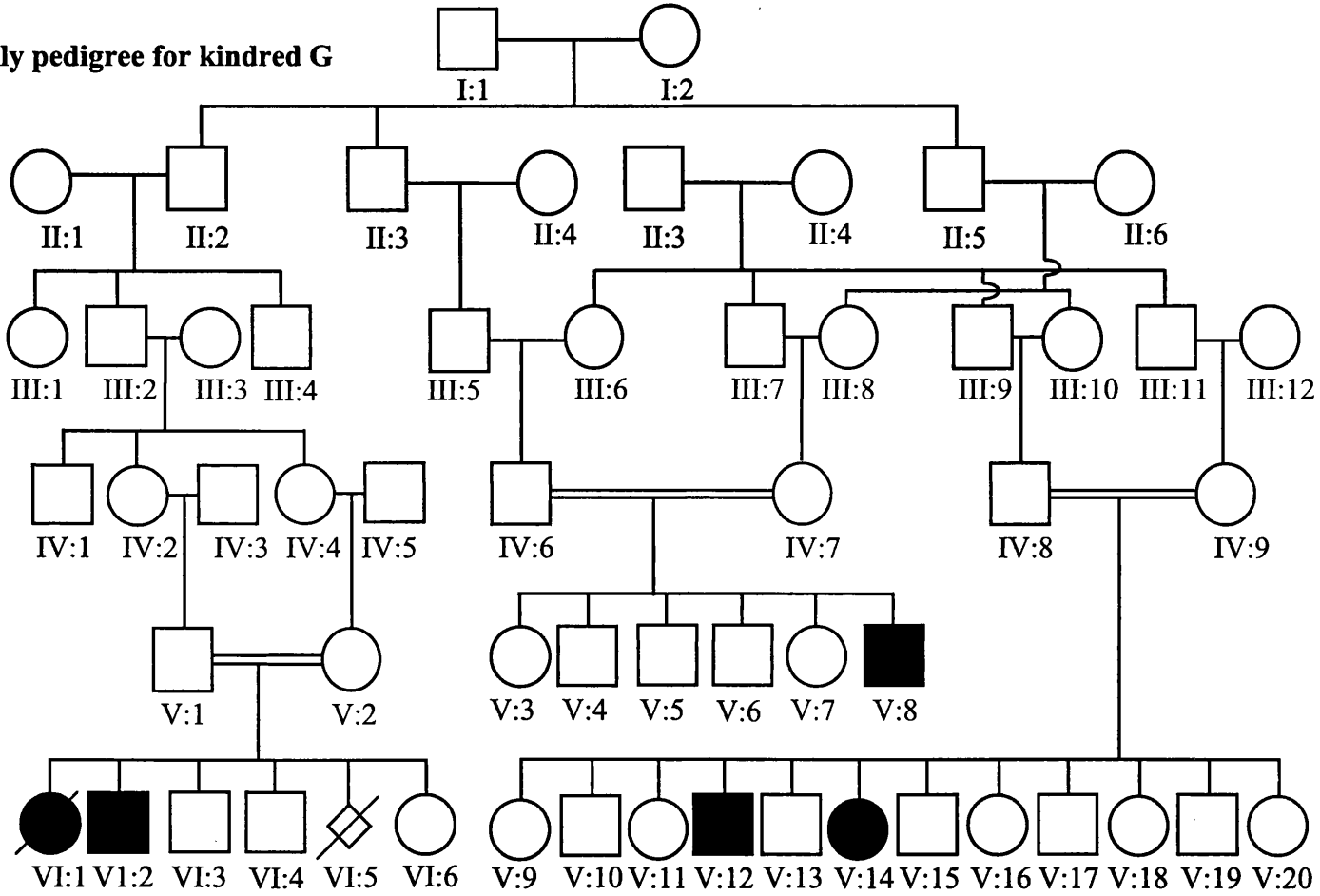
Kindred G is large. The individuals are members of a travelling community in Ireland. Consanguineous union has occurred on at least three occasions (see **figure 5.4**). The individuals discussed in this thesis are registered with the National Centre for Hereditary Coagulation Disorders in Dublin.

Patients G:V:8, G:V:12, G:V:14 and G:VI:2 receive regular prophylaxis for their FX deficiency in the form of PCCs. In the case of G:V:14 this is at a dose of 70 iu/kg twice weekly. The other three receive the same dose but only at weekly intervals. This has kept bleeding, including haemarthroses to a minimum.

5.3.2 Phenotypic assays

FX assays were carried out on plasma as previously described in **sections 2.3 and 2.4**. Results are shown in **table 13**.

Figure 5.4 Family pedigree for kindred G



Circles represent females and squares represent males. Family members who have been shown to be clinically affected are indicated by filled black symbols. Deceased individuals are indicated by a diagonal line through their symbol. The diamond indicates a stillbirth. The double lines connecting individuals V:1 with V:2, IV:6 with IV:7 and IV:8 with IV:9 indicate that their union is consanguineous.

Table 13 Phenotypic assay results for kindred G

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
G:V:3	46	56	48	43	63
G:V:4	84	100	82	80	106
G:V:5	70	60	65	52	76
G:V:6	70	46	40	30	53
G:V:7	75	62	50	50	61
G:V:8	3	1.5	2	1.5	22
G:V:9	42	47	52	50	46
G:V:11	53	65	82	75	65
G:V:12	6	7	8	6	13
G:V:13	100	100	80	100	110
G:V:14	11	11	13	10	3
G:V:16	42	60	66	55	41
G:V:17	42	52	58	60	44
G:V:18	62	46	52	59	63
G:V:20	33	45	54	50	46
G:VI:3	62	47	45	60	43
G:VI:6	48	38	40	40	42

All units are iu/dL, with the reference ranges 50-150 iu/dL.

5.3.3 Genotype Analysis

DNA extraction, PCR and direct sequencing were performed as described in **section 2.**

Sequence analysis revealed CCG-CTG at codon 382, resulting in the substitution of Proline by Leucine (see **figure 5.5**). This mutation obliterates a unique *Apa* I site in exon 8 of *F10*. Restriction fragment length analysis revealed that individuals J:V:8, J:V:12 and J:V:14 were homozygous for this mutation. Individuals J:V:3, J:V:6, J:V:9, J:V:16, J:V:18, and J:V:20 were heterozygous for the mutation (see **figure 5.6**). This was confirmed by direct sequencing.

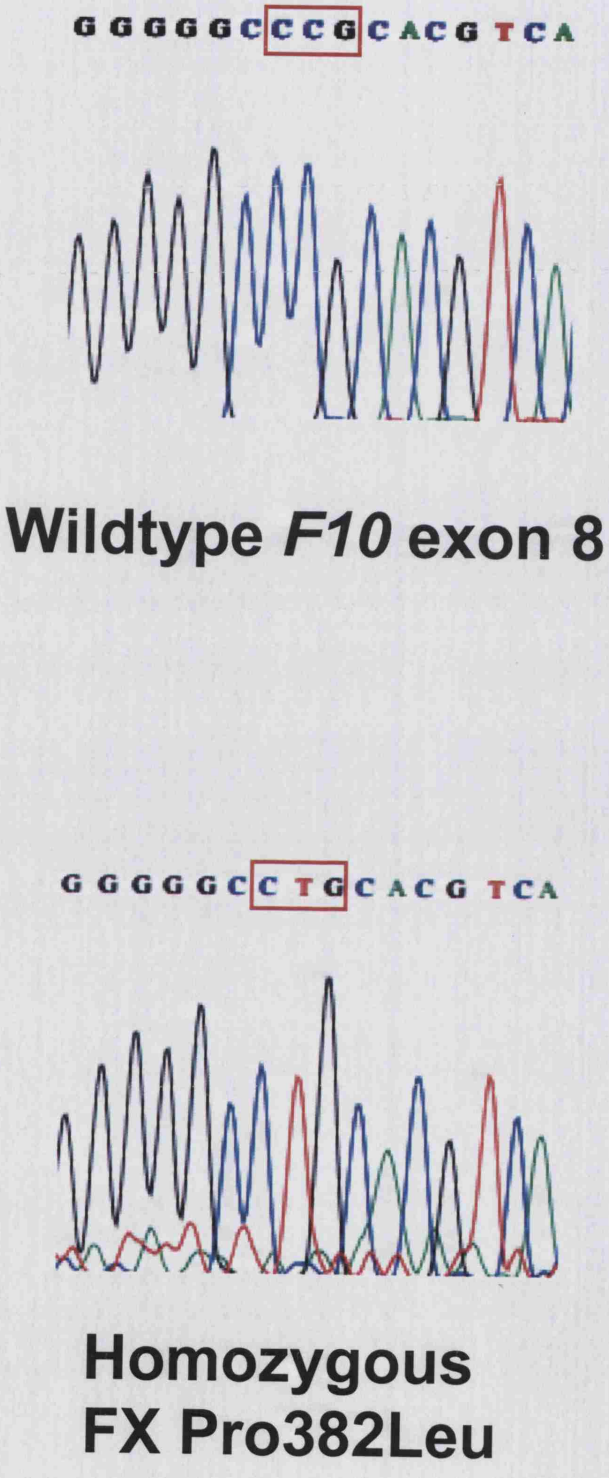


Figure 5.5. An electropherogram showing part of the sequence of exon eight of *F10*. A CCG-CTG nucleotide substitution resulted in the missense mutation Pro382Leu.

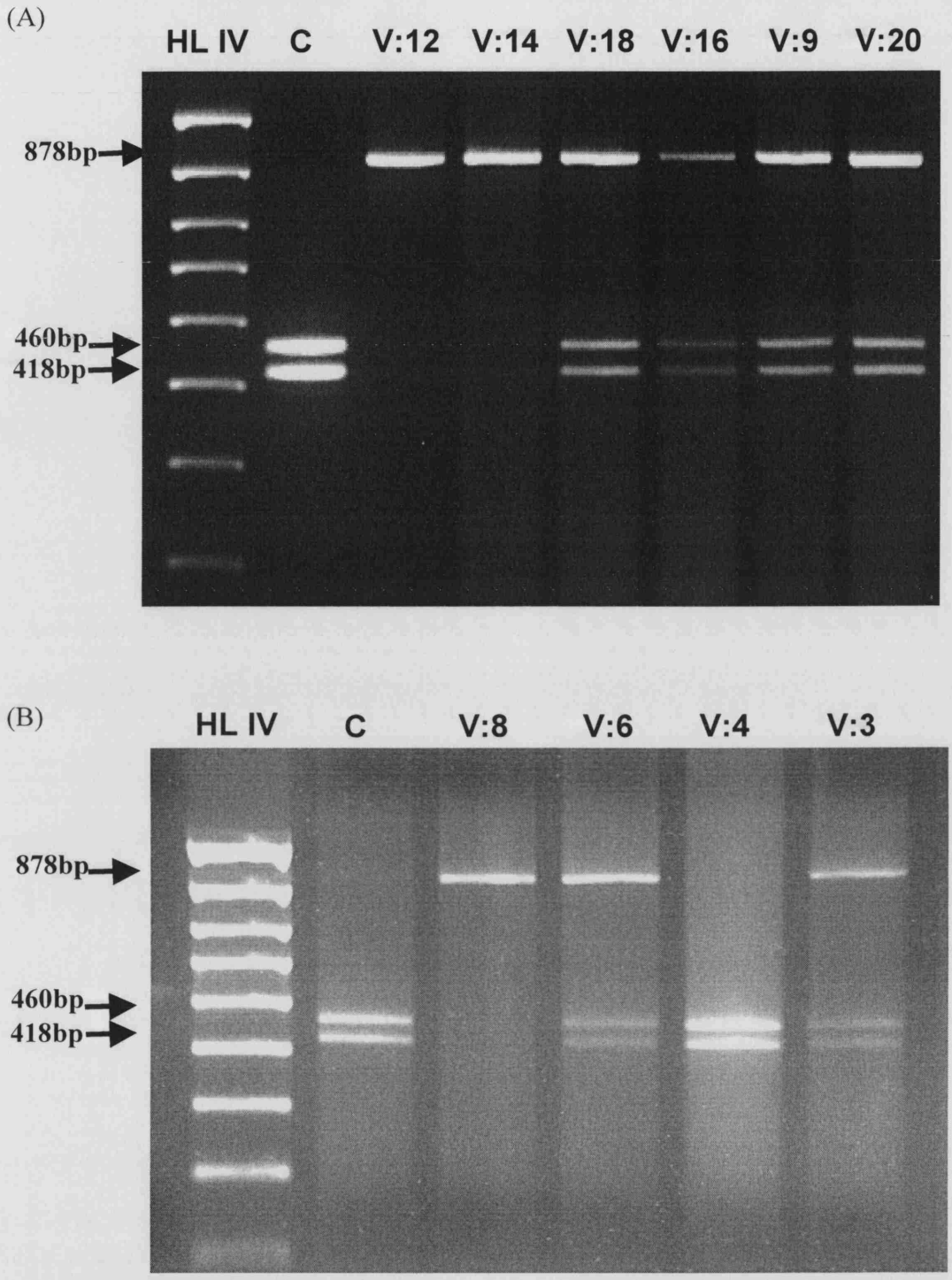


Figure 5.6. Shows two 1.5% agarose gels with the products of a restriction digest of exon 8 of *F10* using *Apa* I enzyme. The wildtype exon is cut once by *Apa* I to create two fragments of 460bp and 418bp each. The CCG-CTG mutation obliterates this unique *Apa* I site, so the homozygous mutant remains uncut at 878bp. HL IV: marker; C: wildtype control; other lanes correspond to labels in **table 13**.

5.3.4 Molecular Modelling

MEGALIGN (DNASTAR, Madison, USA) was used to perform a multiple sequence alignment using the CLUSTAL V algorithm (Higgins et al 1992). It was found that Pro382 (c198) is 100% conserved amongst 71 serine protease sequences available for comparison. Pro382 is situated in a well-conserved sequence GDSGGP that includes Ser379 (c195) of the catalytic triad. This strategic location indicates the importance of this residue in the formation of the catalytically-active folded protein structure. The serine protease fold consists of two subdomains, the first containing six β-strands B, C, D, F, G and H and the second containing six β-strands J, K, L, M, N and O (Perkins and Smith 1993). The secondary structure analysis using DSSP showed that Pro382 is situated at the end of a long loop and immediately precedes the β-strands M and N (**figure 5.7**). The solvent accessibility analysis using COMPARER showed that, in the conserved loop sequence GDSGGP, all the residue sidechains occupy buried positions (0 or 1 in **figure 5.7**), implying that the presence of all these residues is critical for correct folding immediately after synthesis.

A mutant structure containing Leu382 (c198) was built using the BIOPOLYMER module of INSIGHT II. The 1xka crystal structure of FXa (Kamata *et al* 1998) was used as the starting wildtype model. Both the mutant model and the wildtype structure were subjected to 2 × 300 rounds of global Polak conjugate gradient minimisation with the AMBER forcefield, using the DISCOVER_3 module of INSIGHT II. The models were then subjected to molecular dynamics simulation during which they were heated to 600 K over 100 fs, maintained at that temperature for a further 100 fs of simulation and then cooled over 100 fs. A further 2 × 300 rounds of energy minimisation were finally performed. Refinement was ended when no further significant change in protein conformation was observed. Proline is unique in that the end of its side-chain is covalently bound to its mainchain nitrogen atom. Since this leaves the mainchain with no amide hydrogen at this point, Proline residues cannot participate in secondary structure hydrogen bonds. In addition, the five-sided pyrrolidone ring imposes constraints on the N-C^{α} rotational angle and the available conformational space for the residue preceding proline. Since Proline residues are significant in their effect on chain conformation and protein folding, Proline residues tend to be conserved (MacArthur and Thornton 1991).

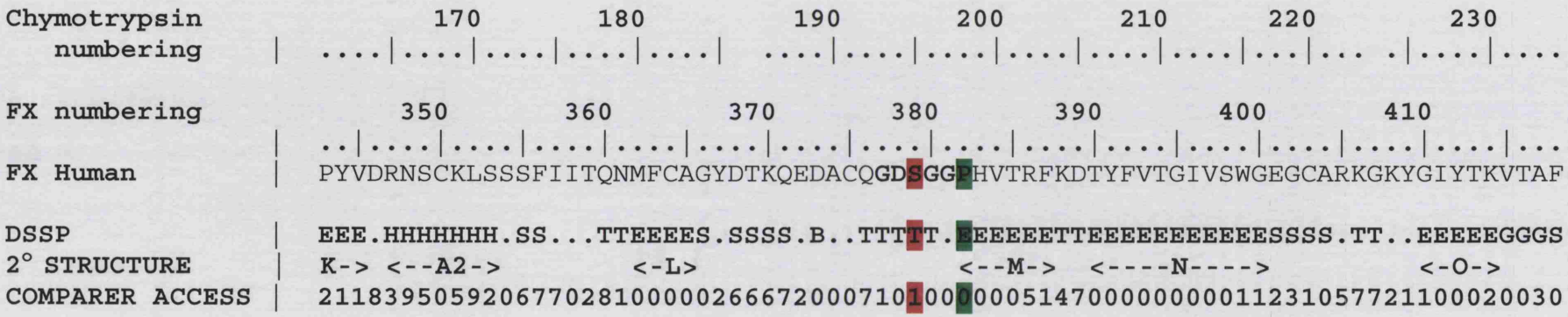


Figure 5.7 Protein sequence and structure analysis of part of the C-terminal subdomain of the serine protease domain of human FX (SWISSPROT accession code P00742). The residues of interest are colour-coded to match **figure 5.8**. The highly conserved sequence GDSGGP is shown in bold. The DSSP output shows the consensus secondary structures found in seven experimentally observed structures of FX. The β-strands K to O (denoted by E in the DSSP output) are featured here. An α-helix (denoted by H in the DSSP output) is labelled A2. The COMPARER output shows a value (0-9) that represents the consensus solvent accessibility of each sidechain in FX. Exposed residues are identified by values of 2-9, and buried residues by values of 0 or 1.

Global simulated annealing and energy minimisation calculations were carried out to determine whether the crystal structure could accommodate the Pro382Leu mutation. The structural analysis showed that the folded protein structure could accommodate the mutation without significant distortion (**figure 5.8**). In particular, the structure of the catalytic triad at His236-Asp282-Ser379 is left intact.

5.3.5 Discussion

The laboratory phenotype shows a severe reduction in both the activity and antigen concentration of FX. The homozygous patients are all receiving regular FX prophylaxis and so it is likely that the plasma assays are measuring the FX in PCC rather than the patients' native FX.

From the molecular modelling, it is concluded that the Pro382Leu mutant affects FX activity and antigen levels through an inability to form a stable folded conformation as the result of a disruption to its kinetic folding pathway caused by the absence of the Proline residue (Creighton 1993).

5.4 Kindred H (Cys132Stop and Arg273Trp mutations)

5.4.1 History

Patient H:II:4 is registered at the Royal Free Haemophilia Centre. FX deficiency was first diagnosed elsewhere at the age of 13 following excessive haemorrhage after dental extractions. At this time, family screening was performed and his younger sister (H:II:7) was also found to be FX deficient. The proband has a history of two other surgical challenges: a ganglion removal from his right wrist in 1987 and a vasectomy reversal in 2001. Both of these procedures resulted in large haematoma formation. He has never had any treatment for FX deficiency.

There is also a family history of arrythmogenic right ventricular dysplasia. His elder sister (H:II:2) died from this condition at the age of 50. No other member of the family is known to suffer from this.

Patient H:II:7 is registered with the Haemophilia Centre in Oxford. She was diagnosed shortly after her brother (H:II:4). She bled profusely following dental extractions at the age of ten. Her menarche was at the age of 12 and her period of menstruation often lasted up to four weeks. She was found to be anaemic and

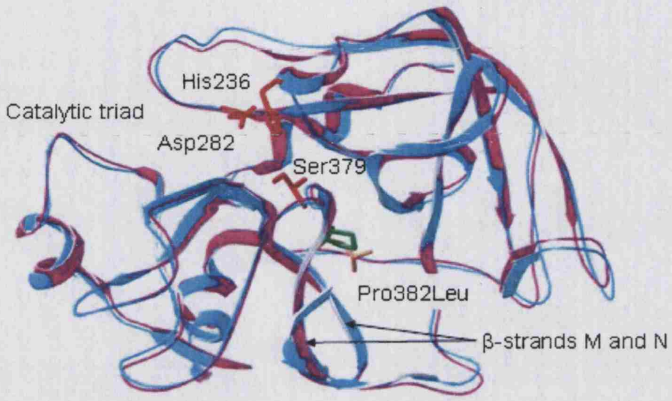


Figure 5.8 Molecular view of the Pro382Leu mutant in the FX serine protease domain.

The wildtype (blue) and the mutant (pink) FX structures are shown superimposed upon each other. For clarity, residues 319-343 have not been shown. Leucine can be easily accommodated at the Pro382 site in the model. However, the Proline introduces a kink in the mainchain structure which may be essential for the correct folding of a loop of two β-strands N and M highlighted in the figure. Failure to generate this kink could result in the misfolding of the core of the protein and explain the low antibody titre associated with the mutation. The catalytic triad is shown in red.

required blood transfusion. Tranexamic acid therapy helped reduce her blood loss and she was also prescribed the oral contraceptive pill.

At the age of 36 she underwent a hysterectomy. The procedure was covered by the use of FFP and PCC. There was no excessive bleeding and she made an uneventful recovery.

The family pedigree is shown in **figure 5.9**.

5.4.2 Phenotype assays

FX assays were carried out on plasma as previously described in **sections 2.3 and 2.4.** Results are shown in **table 14.**

Table 14 Phenotypic assay results for kindred H

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
H:II:4	30	18	5	16	40
H:II:7	22	15	3	14	40
H:III:4	74	64	82	80	88

All units are iu/dL, with the reference ranges 50-150 iu/dL.

5.4.3 Genotype analysis

DNA extraction, PCR and direct sequencing were performed as described in **section 2.5.**

Sequence analysis revealed that patients H:II:4 and H:II:7 are heterozygous for two mutations. They have a CGG-TGG substitution in exon 8, resulting in the missense mutation Arg273Trp (see **figure 5.10**). The other mutation is a TGT-TGA substitution in exon 6; this results in the nonsense mutation Cys132Stop. This is shown in **figure 5.11**. The other individuals for whom DNA was available were heterozygous for the exon 6 mutation only. This is shown in **table 15.**

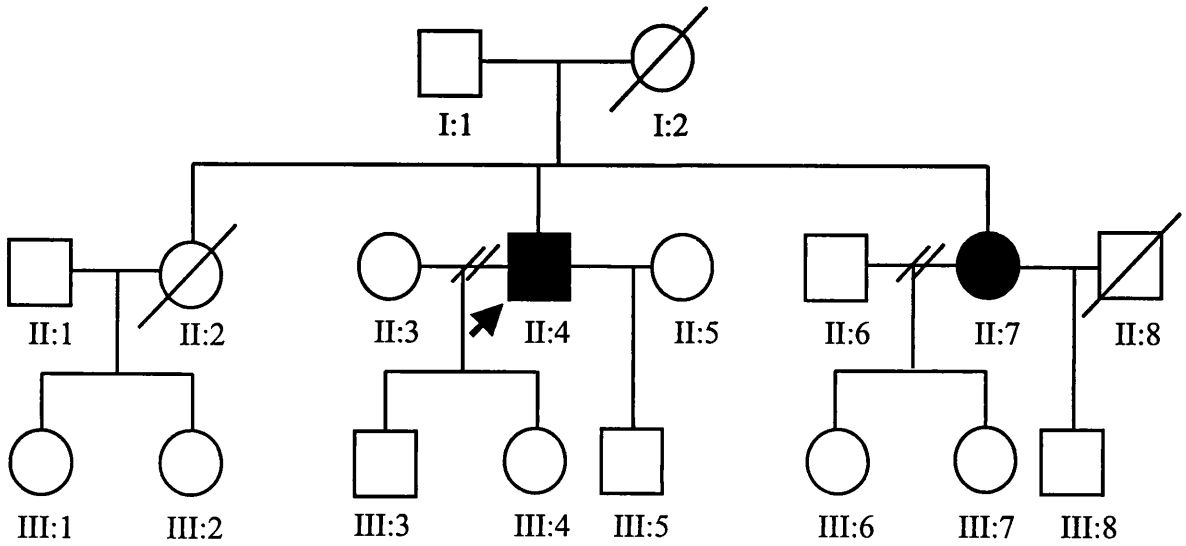


Figure 5.9 Family pedigree for kindred H

The proband is indicated by the arrow. Circles represent females and squares represent males. Family members who have been shown to be clinically affected are indicated by filled black symbols. Deceased individuals are indicated by a diagonal line through their symbol. Divorce is indicated by a double diagonal line through a union.

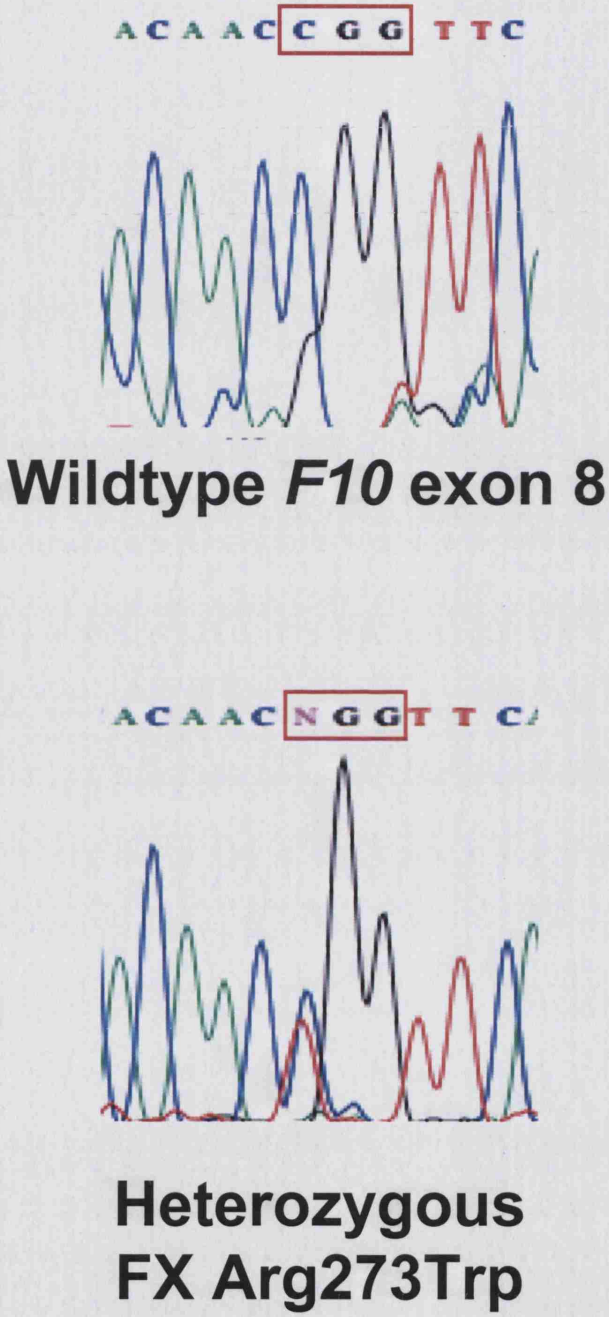


Figure 5.10. An electropherogram showing part of the sequence of exon 8 of *F10*. A CGG-TGG nucleotide substitution resulted in the missense mutation Arg273Trp.

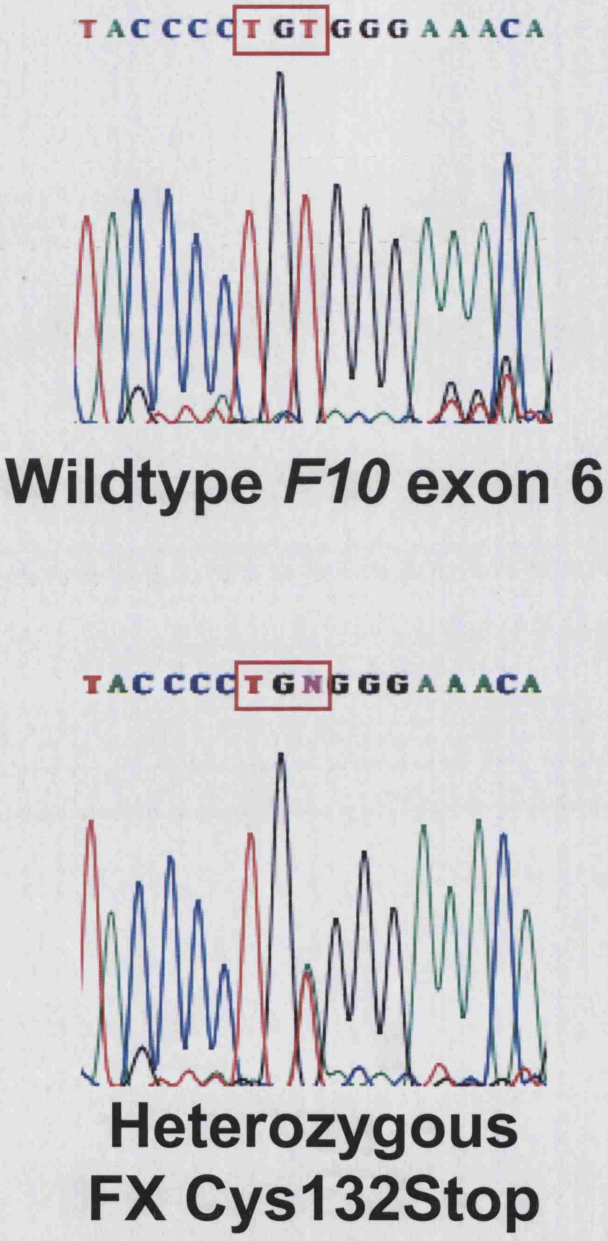


Figure 5.11. An electropherogram showing part of the sequence of exon 6 of *F10*. A TGT-TGA nucleotide substitution resulted in the nonsense mutation Cys132Stop.

Table 15 Genotypic results for kindred H

Patient	Nucleotide Change	Mutation Codon
H:I:1	TGT-TGA	Cys132Stop
H:II:4	CGG-TGG; TGT-TGA	Arg273Trp; Cys132Stop
H:II:7	CGG-TGG; TGT-TGA	Arg273Trp; Cys132Stop
H:III:3	TGT-TGA	Cys132Stop
H:III:6	TGT-TGA	Cys132Stop
H:III:7	TGT-TGA	Cys132Stop

5.4.4 Molecular modelling

From a multiple sequence alignment using the CLUSTAL V algorithm in the program MEGALIGN, it was found that Arg273 (c93) is conserved as a basic residue in all mammalian FX species available. It is not conserved in other human serine proteases. A Tryptophan does not appear in this position in any of the other homologous sequences. Arg273 has a COMPARER output score of 7, indicating that it is surface exposed. Inspection of the protein model failed to show any structural implication for this mutant. Although it is in close proximity to the catalytic triad, it would be predicted that the position of the residue at the surface would allow room for the bulkier aromatic ring of Tryptophan (see **figure 5.12**).

5.4.5 Expression Work

This is discussed in **chapter 9**.

5.4.6 Discussion

The symptomatic individuals in this family are compound heterozygotes for a missense mutation in exon 8 and a nonsense mutation in exon 6. The asymptomatic members of the family who were tested only had the exon 6 mutation. This indicates that the two mutations are on separate alleles.

A nonsense mutation would be predicted to produce a truncated protein or no protein at all. The normal allele should produce normal FX. The results from individual H:III:4 show that she is producing a normal level of functional FX.

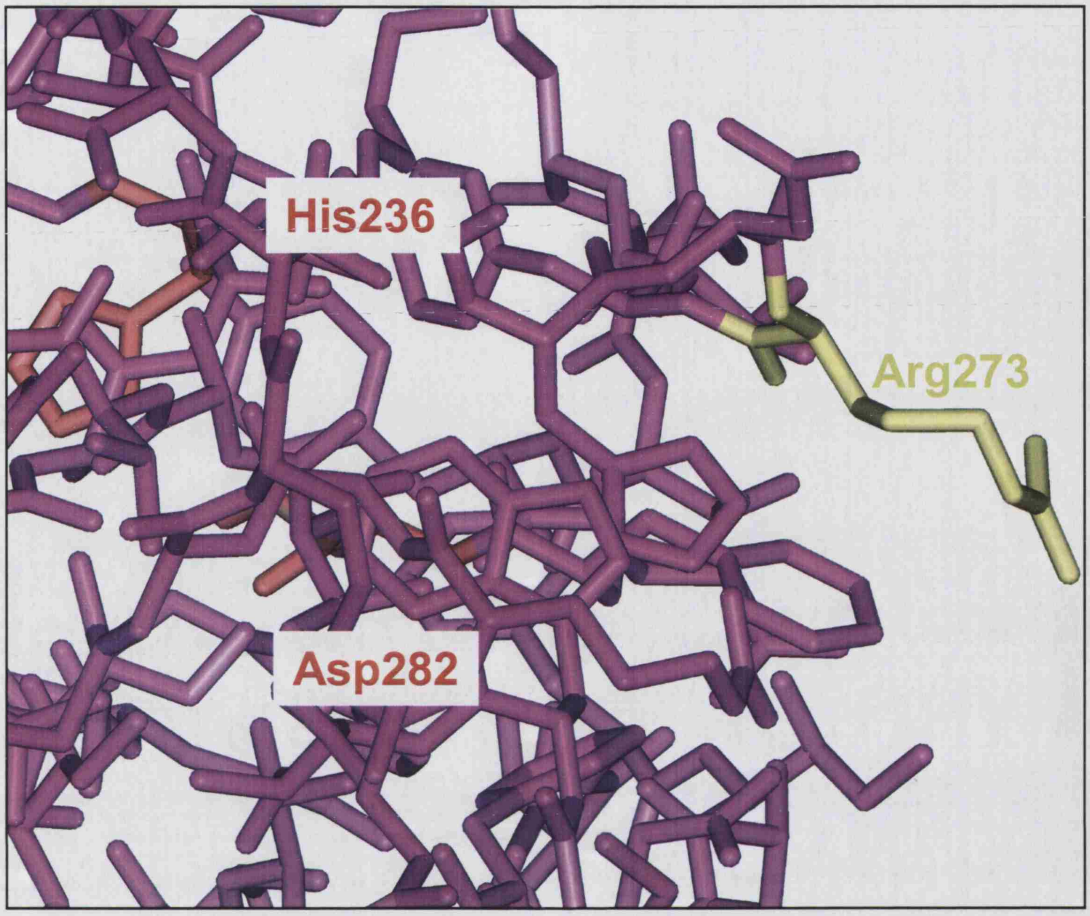


Figure 5.12 Structural analysis of the Arg273Trp (c93) substitution

In the catalytic domain, the surface residue Arg273 (yellow) is shown in close proximity to His236 (c57) and Asp282 (c102) of the Asp-Ser-His catalytic triad (red). Ser379 (c195) is to the left of the figure as viewed, and is not shown for reason of clarity.

From the assay levels of the compound heterozygotes (H:II:4 and H:II:7) shown in **table 14**, it can be seen that the chromogenic result is approximately normal, if one accepts that only one allele is producing FX (as the other contains a nonsense mutation). This indicates that the catalytic site is unaffected by the mutation and confirms the prediction made by molecular modelling.

The specific activity by PT assay (the ratio of activity: antigen) is approximately 0.6. However, the specific activity by APTT assay is approximately 0.2. This indicates that activation of FX by the *intrinsic pathway* complex is not as efficient as activation by the *extrinsic pathway* complex.

Chen and co-workers have shown that the calcium binding loop of FX (containing residues Arg251 (c71), Glu254 (c74), Glu256 (c76) and Glu257 (c77)) is critical for substrate recognition by the *intrinsic Xase* complex (Chen *et al* 2004).

However, the residues do not play a significant role in the interaction of FX with the *extrinsic Xase* complex. In addition, they are not involved in the catalytic function of FXa in the *prothrombinase* complex.

Arg273 is not adjacent to the calcium binding loop. However, this does not preclude the involvement of Arg273 in the interaction of FX with the *intrinsic Xase* complex. Exosites (extended binding sites) are regions on molecules which are involved in ligand binding but are remote from the active site of the ligand. Arg273 may be part of an exosite for binding of the *intrinsic Xase* complex. Substitution of Arg273 by Tryptophan may interrupt the substrate recognition and so reduce the efficiency of binding. This would account for the lower specific activity by APTT assay.

5.5 Kindred J (Asp373Asn mutation)

5.5.1 History

Patient J:III:4 suffered life-long easy bruising and epistaxis. He bled excessively after a dental extraction at the age of 13 years. This required hospital admission and surgical intervention. He denied spontaneous haemorrhage.

His mother (J:II:2) gave a history of menorrhagia and easy bruising. She had suffered post-partum haemorrhages and had bled for three days after a dental extraction. She denied spontaneous bleeds.

His sister (J:III:3) died after falling from a swing at the age of three years. She suffered a fatal cerebral haemorrhage. The bleeding was thought to be out of

proportion to the force of the fall. No data are available on whether she had FX deficiency, however this is likely.

His half-sister (J:III:1) complained of menorrhagia and bleeding in early pregnancy. She also suffered from easy bruising and bleeding after mild trauma. She bled excessively following an operation for the removal of an in-growing toenail.

No historical data are available on any other family members.

The family pedigree is shown in **figure 5.13**.

5.5.2 Phenotypic assays

FX assays were carried out on plasma as previously described in **sections 2.3 and 2.4**. Results are shown in **table 16**.

Table 16 Phenotypic assay results for kindred J

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
J:II:2	130	55	48	60	76
J:III:1	135	50	22	50	61
J:III:4	ND	39	ND	ND	ND

All units are iu/dL, with the reference ranges 50-150 iu/dL. ND – No data.

5.5.3 Genotype Analysis

DNA extraction, PCR and direct sequencing were performed as described in **section 2.5**.

Sequence analysis revealed a single missense mutation GAT-AAT at position 373, corresponding to the substitution of Aspartic acid by Asparagine. This residue is in the catalytic domain and is encoded by exon 8 (see **figure 5.14**). This mutation creates a unique *Bsm* I site in that exon. Restriction fragment length analysis revealed that individuals J:II:2, J:III:1 and J:III:4 were heterozygous for the mutation (see **figure 5.15**). This was confirmed by direct sequencing.

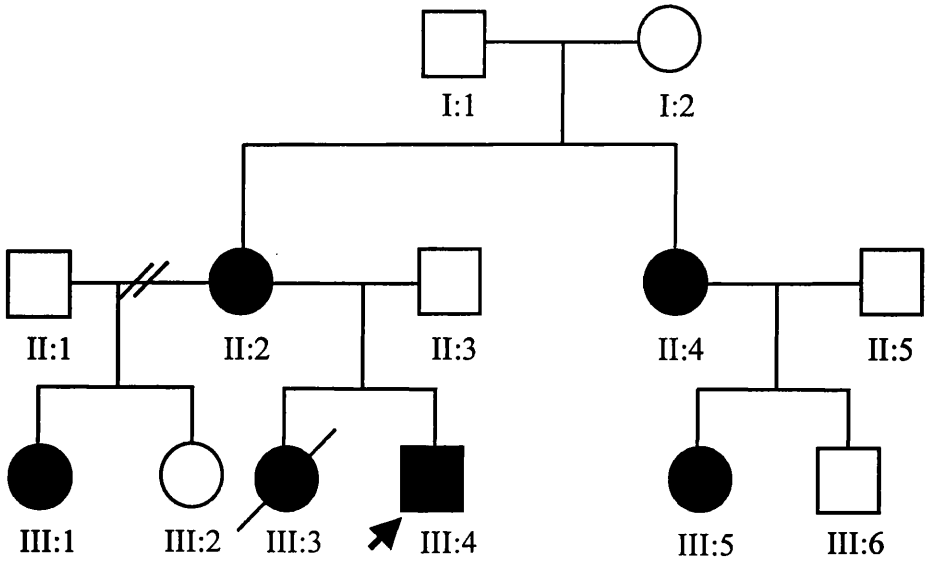


Figure 5.13 Family pedigree for kindred J

The proband is indicated by the arrow. Circles represent females and squares represent males. Family members who have been shown to be clinically affected are indicated by filled black symbols. Deceased individuals are indicated by a diagonal line through their symbol. Divorce is indicated by a double diagonal line through a union.

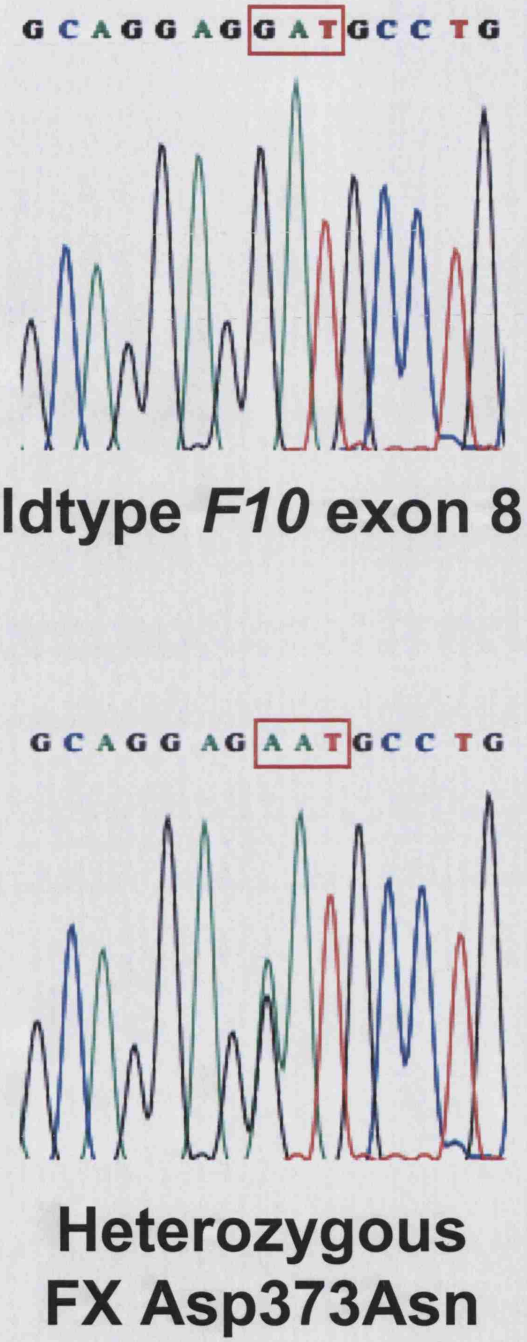


Figure 5.14. An electropherogram showing part of the sequence of exon 8 of *F10*. A GAT-AAT nucleotide substitution resulted in the missense mutation Asp373Asn.

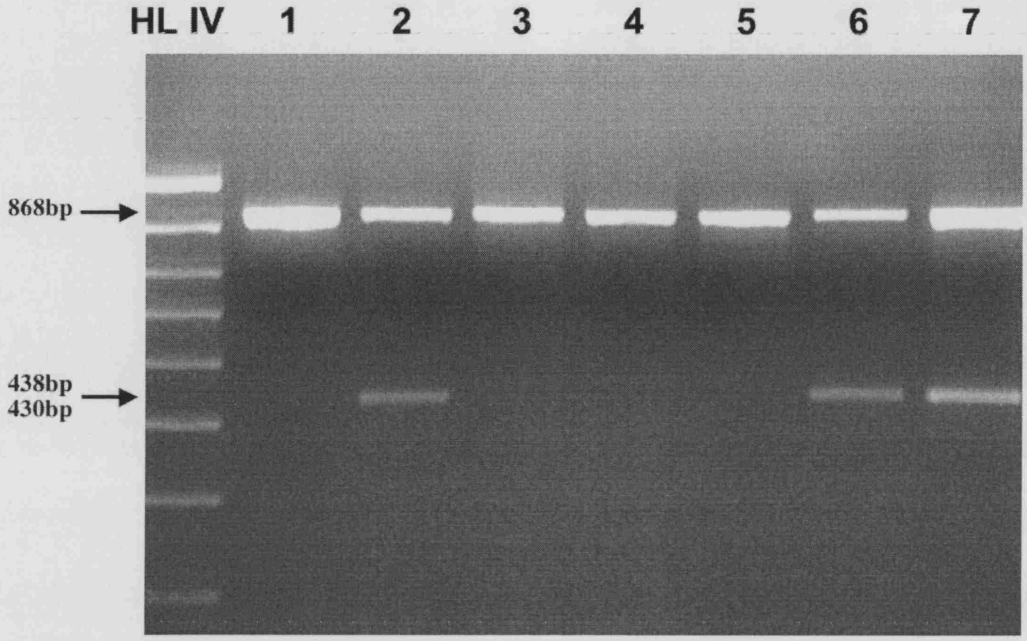


Figure 5.15. Shows a 1.5% agarose gel with the products of a restriction digest of exon 8 of *F10* using *Bsm* I enzyme. The wildtype exon is cut once by *Bsm* I to create two fragments of 868 bp and 10 bp each. The GAT-AAT mutation creates an additional *Bsm* I site, so digestion would create three fragments of 438 bp, 430 bp and 10 bp. The 438 bp and 430 bp fragments can not be resolved on a 1.5% gel and the 10 bp fragment is too small to be visualised. The effect is that digestion of the wildtype exon appears to produce a single band of 868 bp. The homozygous mutant would appear to produce a single band of approximately 430 bp and the heterozygote would appear to produce both the 868 bp band and the 430 bp band.

From this gel it can be seen that lanes 2, 6 and 7 (patients J:II:2, J:III:1 and J:III:4 respectively) are heterozygous for the mutation. Lanes 1, 3, 4 and 5 contain wildtype controls.

5.5.4 Molecular modelling

Asp373 (c189) is a fully-buried residue in the catalytic domain of FX. From a MEGALIGN multiple sequence alignment of serine proteases, c189 is 100% conserved in FX from all available mammalian species and in all human vitamin K-dependent coagulation proteins. However, it is not conserved in other human serine proteases.

Inspection of the crystal structure revealed that Asp373 is involved in the sodium-binding site of FX (**figure 5.16**). The sodium ion is secured by ionic contacts with residues in the two sodium-binding loops (Arg405-Tyr409 (c222-c225) and Ala365-Asp373 (c183-c189)). The loss of the acidic Asp373 residue would be predicted to disrupt the balance of charges in the sodium-binding pocket. This would reduce the capacity of the pocket to bind sodium and the ion would be destabilised. The mutation would not be predicted to affect protein folding.

5.5.5 Expression work

This is discussed in **chapter 9**.

5.5.6 Discussion

Laboratory analysis of the available samples showed that the Asp373Asn mutation produces a type II phenotype. Molecular modelling has aided the explanation of the phenotype. Sodium binding by FXa is necessary for efficient prothrombin activation (Rezaie and He 2000). Destabilisation of the sodium ion would be predicted to render the molecule inactive, but leave protein folding unaffected, resulting in a normal antigen level. In the cases of J:I:2 and J:II:1, genotype analysis revealed that they are heterozygous for the mutation in which case they would be predicted to have one normal FX allele producing fully-functional FX protein. This would account for the observed laboratory phenotype.

5.6 Kindred K (Asp373Asn mutation)

5.6.1 History

Patient K:II:1 is registered with the Haemophilia Centre at the Royal Free Hospital. She gave a history of excessive bleeding following dental extractions at the age of

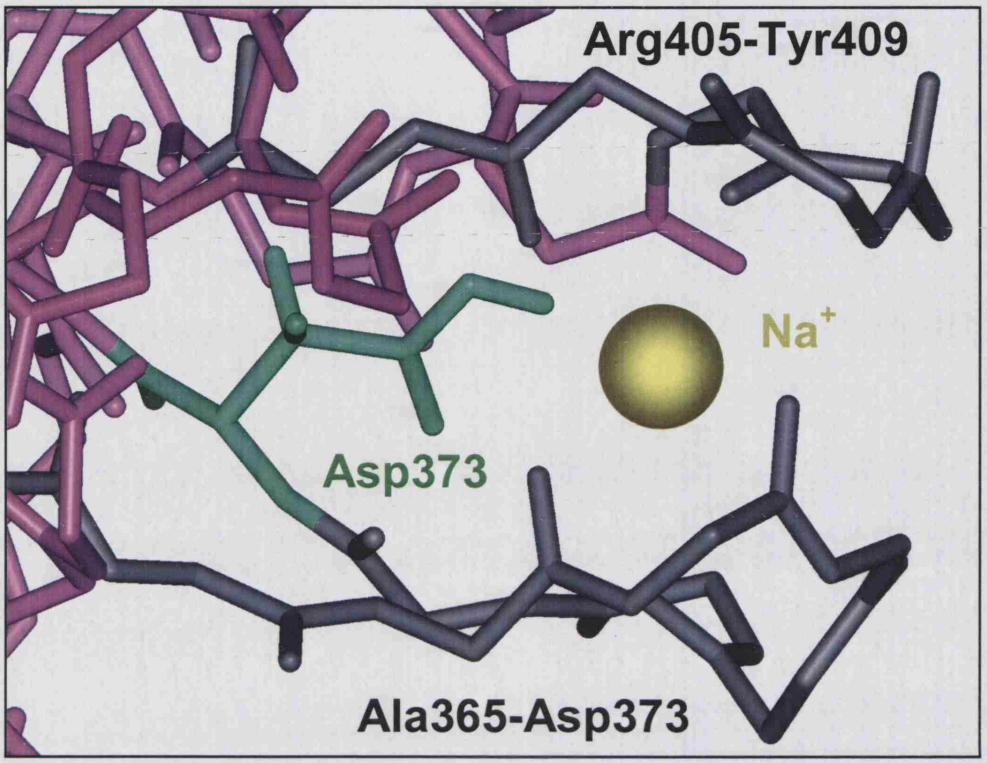


Figure 5.16 Structural analysis of the Asp373Asn mutation.

The green residue represents Asp373 (c189) with the hydrogen atoms added to the sidechain. The sodium ion is shown in yellow. The two sodium-binding loops are shown in grey (Arg405-Tyr409 (c222-c225) and Ala365-Asp373 (c183-c189)).

eight. She also complained of menorrhagia. Following the birth of her second daughter (K:III:2), she suffered a post-partum haemorrhage of 1.5 L. She was treated for this with a transfusion of four units of red cells. However, she did not receive specific FX replacement therapy. Her youngest sister (K:II:4) suffered a secondary haemorrhage after tonsillectomy, but did not require blood products. Her eldest daughter (K:III:1) has FX deficiency, but so far she is asymptomatic. She has not received a surgical challenge.

No historical data are not available on patients K:I:1 and K:II:3.

The family pedigree is shown in **figure 5.17**.

5.6.2 Phenotype Assays

FX assays were carried out on plasma as previously described in **sections 2.3 and 2.4**. Results are shown in **table 17**.

Table 17 Phenotypic assay results for kindred K

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
K:II:1	ND	45	ND	ND	ND
K:III:1	ND	39	ND	ND	ND

All units are iu/dL, with the reference ranges 50-150 iu/dL. ND – No data.

5.6.3 Genotype Analysis

DNA extraction, PCR and sequencing were performed as described in **section 2.5.**

DNA was only available on K:II:I. Sequence analysis revealed that this individual was heterozygous for a single missense mutation GAT-AAT at position 373, corresponding to the substitution of Aspartic acid by Asparagine. This mutation was also identified in kindred J (**section 5.5**).

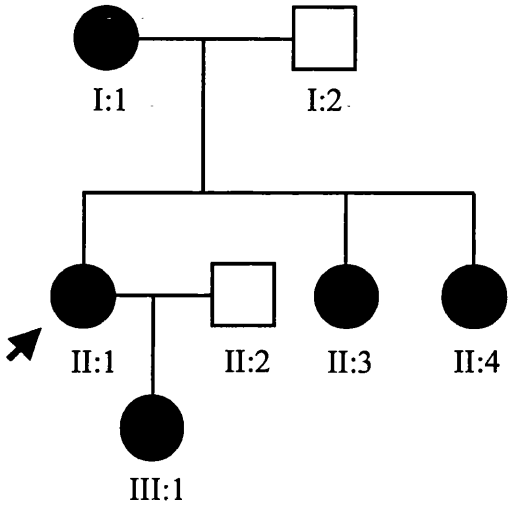


Figure 5.17 Family pedigree for kindred K

The proband is indicated by the arrow. Circles represent females and squares represent males. Family members who have been shown to be clinically affected are indicated by filled black symbols.

5.7 Conclusions

FX is the predominant physiological activator of prothrombin. This function is performed by the serine protease domain and in particular the catalytic triad (His236-Asp282-Ser379). Mutations in the serine protease domain may disrupt protein folding and so impair secretion from the cell. This would result in a type I mutation. Other mutations may disrupt the catalytic triad but allow the protein to assemble unimpeded. This would be predicted to cause a type II phenotype.

Catalytic domain mutations were identified in five kindred. The mutations identified in kindred F and kindred G have been identified in other kindred (Deam *et al* in press; Peyvandi *et al* 2002). It is possible that the individuals with the same mutations are all members of the same kindred, but that the relationships are unknown. It is also possible that these areas are mutation hot-spots. It is most likely that mutations have occurred in the same residues by chance.

Members of kindred H are compound heterozygotes for a missense mutation and a nonsense mutation. The nonsense mutation would account in part for the low assay levels and the missense mutation for the remainder of the laboratory phenotype. Genotype studies on DNA from several generations of the family revealed that the mutations occur on different alleles.

All the patients with the Asp373Asn mutation (kindred J and kindred K) on whom phenotypic data are available have a strong bleeding history, except for K:III:1. However, they all have activity assay levels that are above the haemostatic threshold of 10-20 iu/dL (Knight *et al* 1985). It is possible that the predicted loss of sodium-binding in half the FX molecules inhibits one of the reactions in the coagulation pathway and impairs haemostasis further.

6 Analysis of Mutations Identified by Collaborative Groups

6.1 Introduction

A novel FX Ile411Phe mutation (FX Leicester) was identified in an Asian patient by a collaborative group at the University of Nottingham (Deam *et al* 2003). The molecular interpretation of this mutation was uniquely facilitated by the occurrence of a Phe562 residue in thrombin at the equivalent position to that of Ile411 in FXa. Crystal structures are available for both proteins.

Two further mutations in *F10* have been identified by the Nottingham group: Pro382Leu (FX Cardiff) and Phe356Cys (FX Newcastle). Molecular dynamics studies on the Pro382Leu mutant have already been described as this mutation was identified in kindred G (see **section 5.3**). Molecular modelling, including energy minimisation and simulated annealing was performed in order to help explain the laboratory phenotype of the Phe356Cys mutation.

From 15 Iranian patients in whom the genotypic analysis has been completed, nine different homozygous candidate mutations were identified, of which eight were novel (Peyvandi *et al* 2002). Two of the novel mutations were in splice-sites and one was in exon 2, which encodes the propeptide. Molecular modelling of one of the other mutations has already been discussed (see **section 5.2**) as this mutation was identified in kindred F (Gly222Asp). Molecular modelling of the four remaining mutations is described below. This work was performed in collaboration with a group from the Angelo Bianchi Bonomi Haemophilia Centre in Milan.

6.2 FX Leicester

6.2.1 History

The proband is a patient of Gujarati Indian extraction whose parents are first cousins. She was treated initially for severe menorrhagia with FFP. Other haemorrhagic symptoms included spontaneous haematomas and haemarthroses. She completed three successful pregnancies under prophylactic coagulation factor replacement therapy.

6.2.2 Phenotype and Genotype Results

The FX activity level was <1 iu/dl and her antigen level was 8 iu/dl. All other members of the patient's family had either normal or intermediate levels of FX activity. No other member of this family has any history of a bleeding disorder.
The only mutation found on sequencing of the *F10* gene was a homozygous single point mutation ATC-TTC in exon 8 causing the substitution of Phenylalanine for Isoleucine at amino acid position 411 (chymotrypsin numbering c227). DNA from the patient's mother, brother and one of her sons was available for genotyping and they were found to be heterozygous for this amino acid substitution.

6.2.3 Modelling methods

MEGALIGN (DNASTAR, Madison, USA) was used to align the sequences of FX from a total of seven mammalian species and for 74 human serine proteases using the CLUSTAL V algorithm (Higgins *et al* 1992). The crystal structure for FXa was taken from the PDB code 1xka (Kamata *et al* 1998) and that for prothrombin was taken from the PDB code 1ppb (Bode *et al* 1989). The 1xka crystal structure of FXa was used as a model for the subsequent energy minimisation of the Ile411Phe (c227) mutant form of FXa. Phe411 (c227) was built using the BIOPOLYMER builder module of INSIGHT II before subjecting both the mutant model and wildtype structures to 300 rounds of global Polak conjugate gradient minimisation with the AMBER forcefield, using the DISCOVER_3 module of INSIGHT II. Further rounds of localised energy minimisation were carried out over the two regions of the protein that showed the most change in the first two rounds of refinement. These were Phe356-Asn361 (c173-c179) and Ser398-Ile422 (c213-c238). Refinement was ended when no further significant change in protein conformation was observed.

6.2.4 Modelling Results

Figure 6.1 shows the multiple sequence alignment of serine proteases. The residue c227 is Isoleucine or Valine in all but one of these 81 sequences. Both Isoleucine and Valine are branched aliphatic hydrophobic residues. The sole exception was Phe562 (c227) in human prothrombin at this position. The occurrence of Phe562 in prothrombin is unexpected as prothrombin is a fully active serine protease. Accordingly the crystal structures of FXa and thrombin were examined. The

secondary structure analysis using DSSP showed that Ile411 occurred in β-strand O (**figure 6.1**). The solvent accessibility analysis using COMPARER showed that this is completely buried within the protein core (**figure 6.1**).

Inspection of the environment surrounding the mutation site in FXa and thrombin showed that Ile411/Phe562 (c227) are adjacent to a conserved Trp399/Trp550 (c215) residue in identical conformations. Trp399/Trp550 is adjacent to the active site groove that separates the N-terminal and C-terminal subdomains of the serine protease domain (**figure 6.2**). Trp339/Trp550 is also adjacent to the catalytic triad in the serine protease domain (not shown). On the opposite side of Ile411/Phe562, a conserved disulphide bridge (Cys350-Cys364 in FXa; Cys496-Cys510 in thrombin; Cys168-Cys182 in chymotrypsin) links the α-helix A2 with the β-strand L. The disulphide bridge is different in FXa and thrombin in that it points towards Ile411 in FXa, but points away from Phe562 in thrombin. This reorientation allows room for the bulkier aromatic ring of Phe562 in thrombin. **Figure 6.2** also shows that a major determinant of the Cys-Cys disulphide bridge conformation is its packing against a surface loop of residues Tyr367-Gln371 in FXa (c185-c187) between β-strands L and M.

The successful incorporation of Phe562 (c227) in thrombin but not in FXa was explained by the different sizes of proximate surface loops in the two sequences. The Tyr367-Gln371 loop (c185-c187) in FXa is three residues shorter than the corresponding loop Tyr513-Arg520 (c185-c187) in thrombin (asterisked and greyed in **figure 6.1**). This shortening is retained in all seven mammalian FX sequences (**figure 6.1**). None of the 73 other human serine protease sequences show the longer loop seen in thrombin. The closest ones are found in factor B of complement and motopsin, both being one residue shorter. It was concluded that the longer surface loop of thrombin is the major determinant of the successful incorporation of a Phenylalanine residue at position c227 in thrombin.

Control calculations using energy minimisation confirmed that FX is unable to accommodate a Phe411 (c227) residue within its protein core. A global minimisation

Figure 6.1

Sequence alignment of part of the serine proteinase domains in human FX and prothrombin, and in FX from seven different species.

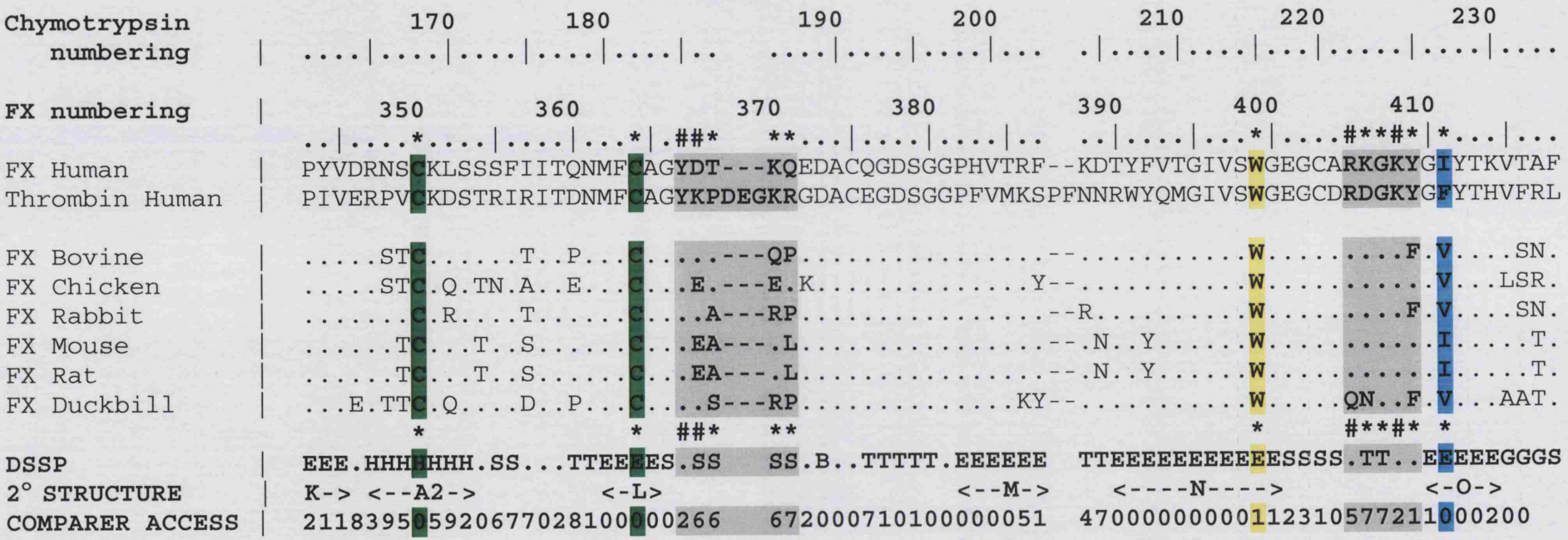


Figure 6.1 Sequence alignment of part of the C-terminal subdomain of the serine protease domains in human FX and prothrombin, and in FX from seven different species. Both the chymotrypsin and FX sequence numberings are shown. The accession codes from top to bottom are P00742, P00734, P00743, P25155 and P019045 (SWISSPROT), and Q99L32, Q63109 and Q9GMD9 (TREMBL). The residues of interest are asterisked or hatched above and below the sequences in the region preceding the Ile411Phe (c227) mutation, and are colour-coded to match **figure 6.2 and figure 6.3**. The four sodium ion binding residues are denoted by hatches. The DSSP output is shown. The β-strands K to O (denoted by E in the DSSP output) are featured here. An α-helix (denoted by H in the DSSP output) is labelled A2. The COMPARER output is also shown.

Figure 6.2 Molecular graphics of Ile411 in FXa and Phe562 in thrombin

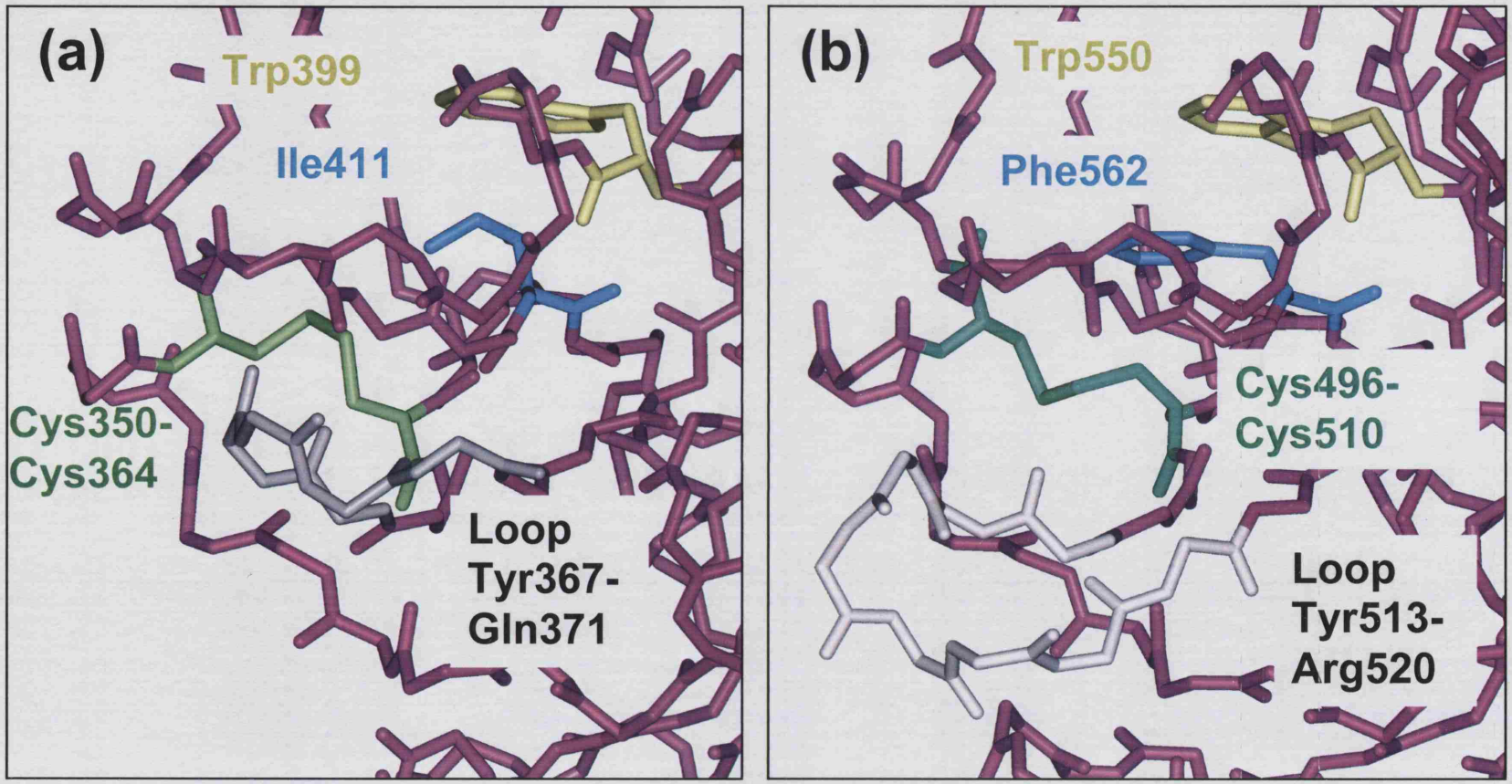


Figure 6.2 Molecular graphics views of Ile411 (c227) in human FXa and its analogue Phe562 (c227) in human thrombin.

The views of (a) and (b) are identical in orientation. The catalytic triad in the active site is positioned immediately above Trp399 (c215) in the view of this picture.

(a) The serine protease domain of FXa is depicted with Trp399 (c215) in yellow, Ile411 (c227) in blue, the Cys350-Cys364 (c168-c182) disulphide bridge in green, and the four-residue surface loop Tyr367-Gln371 (c185-c187) in grey. Ile411 is fully buried and is packed against the Cys350-Cys364 disulphide bridge. The conformation of the sidechains of Cys350 and Cys364 is twisted towards that of Ile411 for reason of the short Tyr367-Gln371 loop.

(b) The equivalent view of the serine protease domain of thrombin is shown here. Note that the conformation of the sidechains of Cys496 and Cys510 (c168 and c182) is now twisted in the opposite direction to that in FXa, and this permits Phe562 (c227) to be packed within the protein core. The larger eight-residue loop enables the Cys496-Cys510 disulphide bridge to adopt a different conformation here compared to FXa.

was carried out in which Ile411 in FXa was converted to Phenylalanine, and also for each of the wildtype FXa and thrombin crystal structures. The three corresponding localised minimisations were also carried out as a check. In these, only the conformations of two surface loops Phe356-Asn361 (c174-c179) and Ser398-Ile422 (c213-c238) were allowed to vary, as these had changed the most in the global minimisations. **Figure 6.3** shows that the incorporation of Phe411 in FX resulted in a significant structural rearrangement in the Ala365-Asp373 loop. This was not seen in the two minimised native structures. This protein loop includes two of the four residues, Tyr367 and Asp368 (c185, c185A), whose mainchain carbonyl oxygen atoms form ligands of a site for a sodium ion in FX. The minimisations show that not only is the protein structure perturbed by the Ile411Phe mutation, but also that the strength of sodium binding to FX is probably affected.

It is proposed that the effect of the Ile411Phe mutation in FX is caused by the inability of the protein structure to incorporate a bulkier hydrophobic residue at this position, resulting in protein degradation, and the disruption of adjacent functionally important binding sites, hence resulting in reduced activity. Both these effects are consistent with the observed phenotype of this mutation.

6.3 FX Newcastle

6.3.1 History

The index case was a child who was diagnosed with FX deficiency following a routine PT and APTT screen on admission for suspected meningitis. He denied spontaneous haemorrhage and had not been exposed to any surgical challenge. There was no family history of bleeding.

6.3.2 Phenotype and Genotype Results

The FX activity of the proband by both assay methods was found to be 3 iu/dl. The antigen level was 25 iu/dl.

Genetic analysis revealed that the child was heterozygous for a TTC to TGC change in exon 8 resulting in the substitution Phe356Cys in the catalytic domain. He was

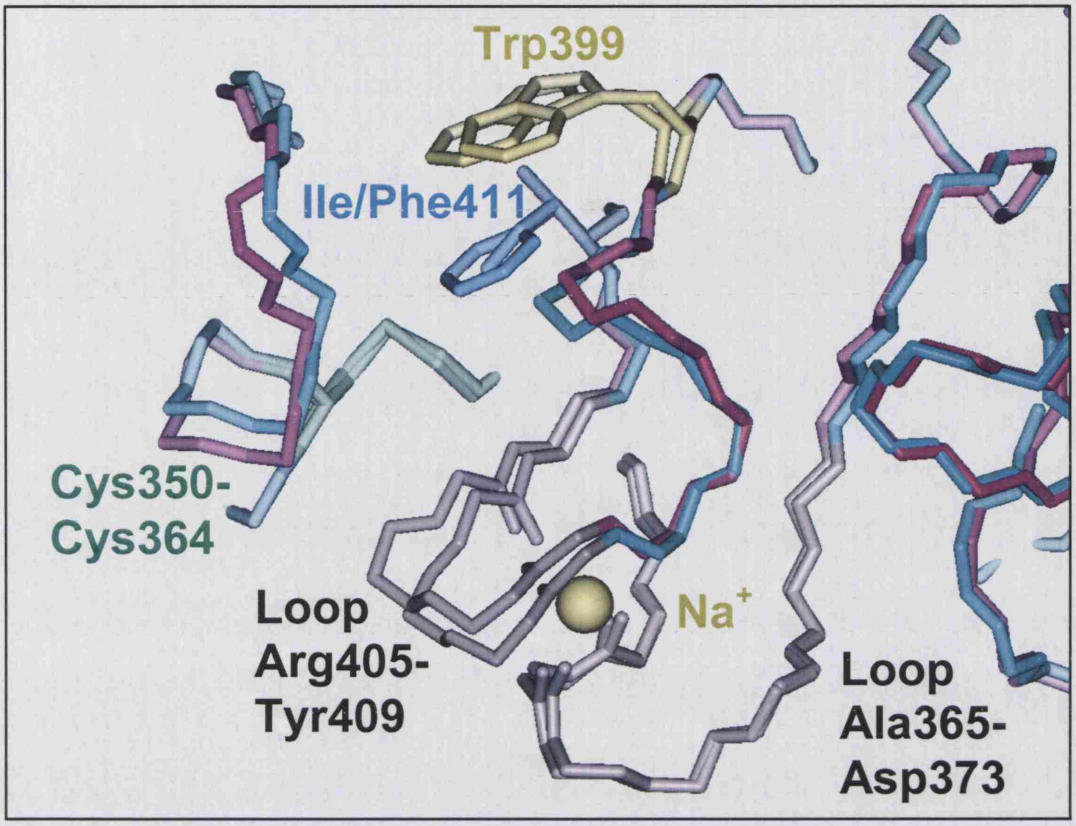


Figure 6.3 Outcome of energy minimisation analyses for wildtype FXa and its Ile411Phe mutant.

The two fully-minimised structures were superimposed using all the backbone atoms in order to highlight the conformational differences between the two structures. The mainchain trace views without carbonyl atoms of the two structures are shown (wildtype: blue; mutant: purple). The residue colouring from **figure 6.2** is used again here. The inclusion of the second sodium-binding loop Arg405-Tyr409 (c222-c225) is shown in grey and the sodium ion is shown in yellow, together with the four carbonyl oxygen atoms of residues 367, 368, 405 and 408 (c185, c185A, c222 and c224) that form ionic contacts with the sodium ion.

found to be a compound heterozygote for this mutation and Glu7Gly which has been previously described as St. Louis II (Rudolph *et al* 1996).

Expression studies in HEK 293 cells have revealed that the Glu7Gly mutation causes reduced FX activity due to reduced calcium binding as the result of a dysfunctional Gla domain (Rudolph *et al* 1996). The antigen level for St. Louis II was previously described as being in the normal range, indicating that Phe356Cys must be an antigen negative mutation.

6.3.3 Structural Analysis

A multiple sequence alignment of human FX and other mammalian FX, and human FX and human serine proteases showed that Phe356 (c174) is fully conserved in FX of other species, but not in the other human serine proteases. A Cys residue does not appear at this position in any of the other homologous sequences. The sidechain of Phe356 is solvent exposed on a surface loop near the active site of FX (**figure 6.4**). It is located close to Cys350 and Cys364 (c168 and c182), which form a disulphide bridge in FX (**figure 6.5**). It was postulated that the replacement of Phe356 with Cys356 would provide a third adjacent Cys residue that would compete effectively with either Cys350 or Cys364 to form one of two alternative disulphide bridges. If either of these could be formed, it would disrupt the correct kinetic folding pathway of the mutant FX and result in the observed reduced antigen level.

Molecular dynamics simulations were performed to test this hypothesis. Two mutant models with either the Cys356-Cys350 or Cys356-Cys364 bridges were created using the BIOPOLYMER module of INSIGHT II, and a third model with the native bridge Cys350-Cys364 in the presence of the Phe356Cys mutation was made as a control. The 1xka crystal structure of FXa (Kamata *et al* 1998) was used as the starting wildtype model.

Both mutant models and wildtype structures were subjected to 2 × 300 rounds of global Polak conjugate gradient minimisation with the AMBER forcefield, using the DISCOVER_3 module of INSIGHT II. The models were then subjected to molecular dynamics simulation during which they were heated to 600 K over 100 fs, maintained at that temperature for a further 100 fs of simulation and then cooled over 100 fs. A further 2 × 300 rounds of energy minimisation were finally performed.

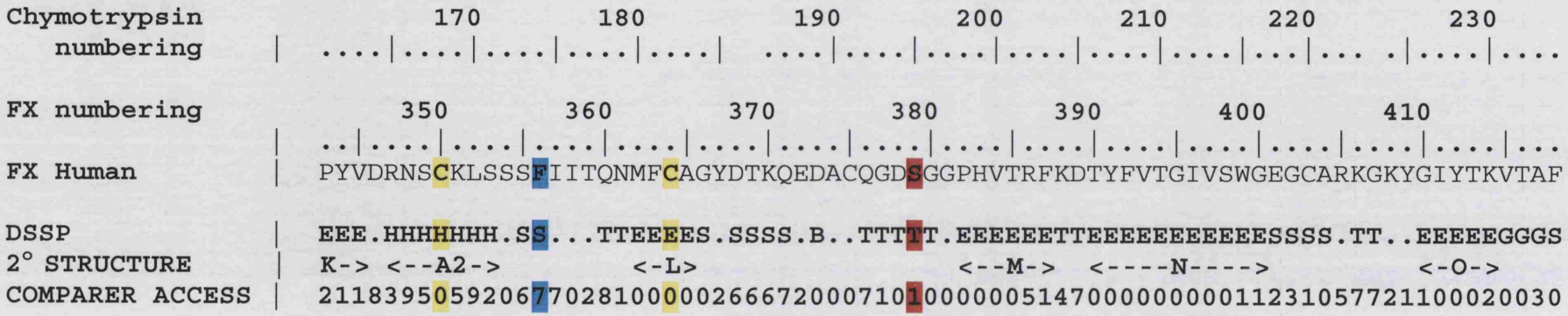


Figure 6.4. Protein sequence and structure analysis of part of the C-terminal subdomain of the serine protease domain of human FX (SWISSPROT accession code P00742). The residues of interest are colour-coded to match **figure 6.5**. The DSSP output shows the consensus secondary structures found in seven experimentally observed structures of FX. The β-strands K to O (denoted by E in the DSSP output) are featured here. An α-helix (denoted by H in the DSSP output) is labelled A2. The COMPARER output shows a value (0-9) that represents the consensus solvent accessibility of each sidechain in FX. Exposed residues are identified by values of 2-9, and buried residues by values of 0 or 1.

Refinement was ended when no further significant change in protein conformation was observed.

This revealed that either of the two alternate disulphide-bridged models could be accommodated by minor local conformational changes within the acceptable error of the FX crystal structure (**figure 6.5**). Although Phe356 is located near the active site cleft between the two subdomains of the serine protease fold, molecular dynamics studies showed that it is unlikely that the catalytic activity of FX would be affected by the Phe356Cys mutation. Accordingly, it is concluded that the kinetics of the folding pathway for FX during synthesis are affected by the possibility of forming any one of three disulphide bridges. The formation of either of the two inappropriate disulphide bridges is presumed to prevent the mutant FX from folding correctly into its mature wildtype conformation (Creighton 1993), and this would be consistent with the reduction in antigen level observed in the compound heterozygote.

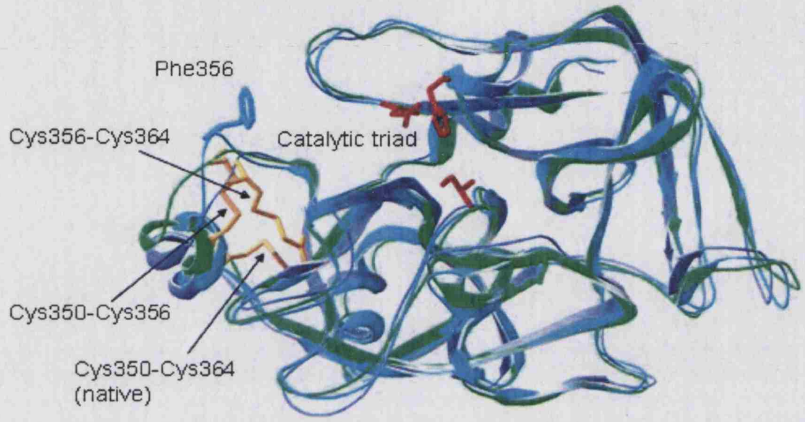


Figure 6.5 Molecular views of the Phe356Cys mutant in the FX serine protease domain.

Superimposition of the wild-type disulphide bridge Cys350-Cys364 (pale blue) and two disulphide bond variants that are possible in the Phe356Cys mutant, namely Cys356-Cys364 (purple) and Cys350-Cys356 (green). In each variant, the catalytic triad of the active site is unaltered, and the exposed surface loop containing the mutation can accommodate the disulphide bond pattern (in yellow) by altering the conformation of the backbone. The catalytic triad is shown in red.

6.4 Mutations in Iranian Patients

Patients from four kindred with FX deficiency were identified. Patient E presented with severe bleeding after circumcision, recurrent haemarthroses, haematomas, haematuria and mucosal bleeding. This patient required frequent replacement therapy with FFP, PCC and red cells. No history was available on patients F and G.

Two siblings (H1 and H2) presented with recurrent haematomas, haemarthroses, epistaxes and oral bleeding. They were not born from a consanguineous relationship. Both patients came from a small village of an Iranian Turkish ethnic group.

6.4.1 Phenotype Assays

Table 18 Phenotypic assay results for Iranian patients

Patient	FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
E	8	<1	2	1.5	22
F	28	25	30	28	37
G	14	9	12	3.3	20
H1	4	<1	<1	35	9
H2	3	<1	<1	35	8

Figures are expressed as % of the FX level in normal plasma (100%).

6.4.2 Genotype Results

Table 19 Genotypic results for Iranian patients

Patient	Nucleotide Change	Mutation Codon
E	GGC-GAC	Gly78Asp
F	TGT-TAT	Cys81Tyr
G	TGT-TAT	Cys81Tyr
H1	GGG-AGG; GAC-GAA	Gly94Arg; Asp95Glu
H2	GGG-AGG; GAC-GAA	Gly94Arg; Asp95Glu

6.4.3 Molecular Modelling

MEGALIGN (DNASTAR, Madison, USA) was used to perform a multiple sequence alignment using the CLUSTAL V algorithm (Higgins *et al* 1992). It was found that Gly78 and Cys81 are 100% conserved in human vitamin K-dependent proteins and in all mammalian FX sequences available. From the COMPARER output (**figure 3.2**), Gly78 is a surface-exposed residue, whereas Cys81 is fully-buried. This is shown in **figure 6.6(a)**. The replacement of the small Gly residue by a bulkier charged residue may prevent the EGF-1 domain from folding correctly. The loss of one of the six Cys residues will prevent the correct formation of the three disulphide bridges that are essential for the folding of the EGF-1 domain.

In the EGF-2 domain, the simultaneous replacement of Gly94Arg and Asp95Glu occurs in a pocket containing three other acidic groups and three basic groups from the EGF-1, EGF-2 and catalytic domains (**figure 6.6(b)**). The introduction of an extra charged residue in this region is likely to affect the correct folding of FX, and in turn its secretion.

6.4.4 Discussion

In two patients from the same family (H1 and H2), a measurable coagulant activity by the RVV assay was found, which was in contrast to the undetectable FX:C activity measured by either a PT or APTT-based assay; the chromogenic and antigenic assays were also reduced. A mild reduction in coagulation activity by RVV assay compared with lower activities by PT, APTT and chromogenic assays has been reported previously (Simioni *et al* 2001b). However, in that report, the antigen level was normal.

A possible explanation for the incongruent RVV assay level is that there was some cross-reaction: RVV may have recognised another substrate in the sample. It is known that RVV can also activate protein C and factor IX (Jackson 1984).

Expression analysis would be required in order to verify the effect of this mutation.

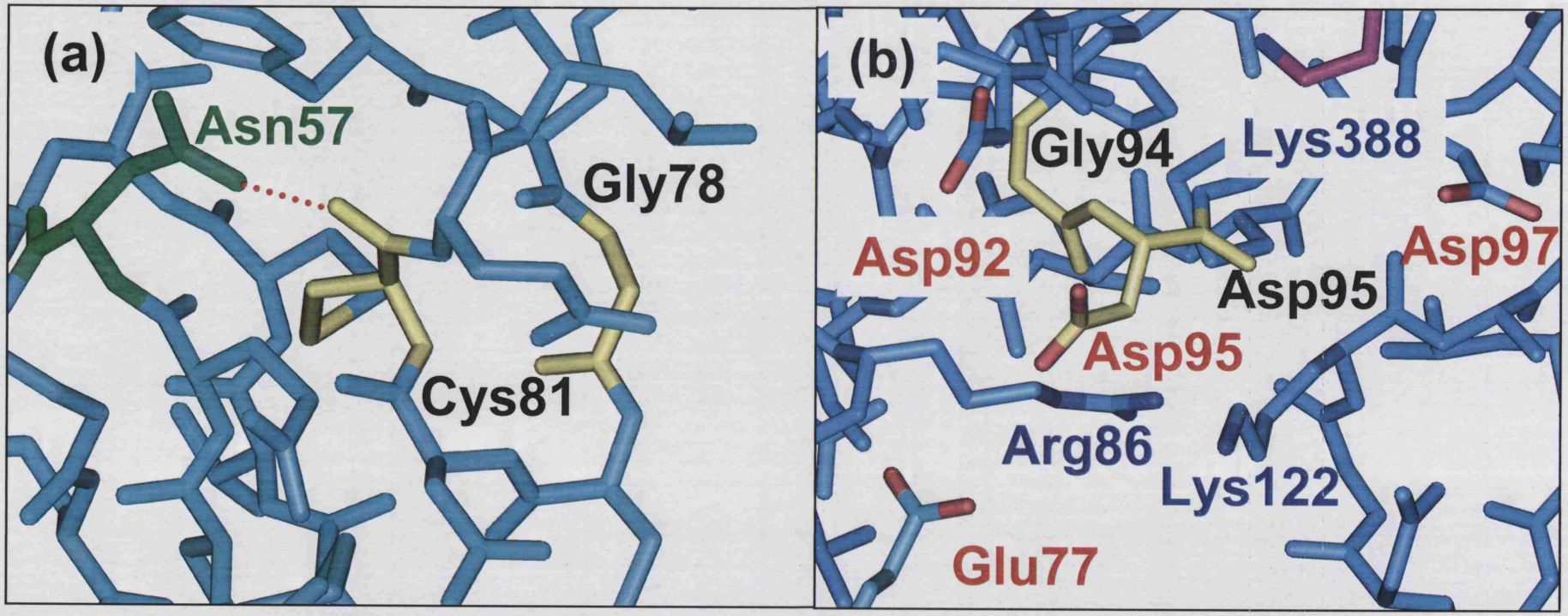


Figure 6.6. Structural analysis of the amino acid substitutions in the EGF domains of the Iranian patients.

(a) The EGF-1 domain is depicted with Cys81 and Gly78 in yellow. A hydrogen bond between the sidechain of Asn57 (green) and the mainchain oxygen of Cys81 is shown in red. The sidechain of Gly78 is surface-exposed, while that of Cys81 is buried.

(b) The EGF-2 domain is depicted, showing the double mutation residues at Gly94 and Asp95. The charged groups from the adjacent acidic residues Asp92, Asp97 (red) and Glu77 (cyan) and the adjacent basic residues Arg86, Lys122 (blue) and Lys388 (magenta) are shown.

7 Introduction and Methods for *in vitro* Expression

The introduction of exogenous DNA into cultured cells is a powerful tool for the molecular biologist. In particular, the use of eukaryotic expression vectors and mammalian cells has been essential to our understanding of the functional significance of genes and the development of gene therapy strategies. Vector systems facilitate the introduction of specific genes into cells and so allow transcription and subsequent translation of the genes into proteins. The use of mammalian cells allows the appropriate post-translational modifications to be performed. Therefore the recombinant protein produced should reflect the molecular structure and biological function of the native protein. The impact of specific mutations on protein function can be characterised.

The vectors used for transfection can be divided into two categories: viral vectors and plasmid vectors. Viral vectors are essentially inactivated viruses into which genes are cloned. Plasmid vectors include prokaryotic, eukaryotic and viral sequences. The prokaryotic elements facilitate bacterial propagation and so maintenance of the plasmid itself. The viral and eukaryotic sequences comprise transcription and translation enhancers as well as sequences encoding selectable markers.

Expression systems can be defined as transient or stable. In transient systems, the gene product can be analysed 12 to 72 hours after transfection. After this time, there is a rapid deterioration in expression of the transgene because of cell death or loss of the plasmid vector. Stable (permanent) expression systems can produce large quantities of protein. The vector needs to contain a marker which will allow chronic selection of the cells which contain the transgene of interest. Typically the vector used in such a transfection will carry a gene essential for the survival of a particular cell line that is defective in that gene or void of the gene altogether. Commonly used markers confer resistance to antibiotics such as neomycin or zeocin.

Several methods are available for the delivery of DNA into cells. Naked plasmid vectors are transfected at low efficiency. The efficiency is greatly improved by the use of transfection vehicles, which can be either chemical (such as phosphate precipitation, cationic polymers, liposomes and molecular conjugates) or physical (electroporation, biolistic and microinjection). The choice of vehicle depends on the

cell type and desired transfection efficiency. Best results are achieved when the cells are transfected in log phase. This is vital for stable transfection, but less important for transient transfection. Supercoiled, uncut DNA is optimal for transient expression, while linear DNA (which tends to be more recombinogenic) is preferred in stable systems. Viral expression vectors do not require a delivery vehicle for DNA transfer. Viruses infect cells as part of their natural biological role and so deliver their DNA without assistance.

Calcium phosphate precipitation has been successfully used to transfect various types of mammalian cells. The efficiency of transfection varies with the cell type, but in stable systems, $CaPO_4$ produces an efficiency of 10^{-3} to 10^{-5} (Colosimo *et al* 2000). The basic technique involves slowly mixing a solution of $CaCl_2$ and DNA with a phosphate buffer solution (Chen and Okayama 1987). This results in the formation of a precipitate containing DNA and $CaPO_4$. When the suspension is dropped onto a monolayer of cells, the precipitate adheres to the cell surface. Following incubation under conditions that encourage pinocytosis, the precipitate is removed and further incubation occurs in fresh culture medium.

Transfection using liposomes occurs in a similar manner. Plasmid DNA is complexed with a liposome suspension in serum-free medium. The liposome/DNA complex is added directly to the monolayer of cells. Following an incubation period of a few hours, the medium is replaced by fresh (serum-containing) medium.

Electroporation is one of the simplest and most widely used physical methods. The technique makes use of the fact that the cell membrane acts as an electrical capacitor, so current is unable to pass (except though ion channels). When the membrane is exposed to a pulsed electrical field, a reversible increase in the permeability of the cell membrane results in the formation of pores which are large enough for macromolecules to enter or leave the cell. The re-closing of the membrane pores occurs naturally. During the time that the pores are open, nucleic acid can enter the cell and eventually the nucleus (Potter *et al* 1984).

Two basic categories of commercial device are available to perform electroporation. There are square wave generators and capacitor discharge systems. The latter charges an internal capacitor to a predetermined voltage and then discharges the exponentially decaying current pulse though the cell-DNA suspension. The current pulse is a function of the initial voltage, the capacitance and the resistance of the system (including the sample). The voltage and capacitance can be altered to effect

different decay times and hence pulse durations. Square wave generators control both the voltage and duration of the pulse with solid-state switching mechanisms. They are generally more expensive, but can be more effective for cells that are difficult to transfect.

Optimisation of the electroporation parameters should be performed for each cell line. However, maximum efficiency and cell survival with mammalian cells tends to occur with low voltage (about 1000 V) and high capacitance (about 500 μF) regimens. As with other transfection methods, gene transfer is maximised when cells are proliferating. Ideally, electroporation should involve mid-log phase cells in a suspension of 10^6 to 10^8 cells/ml. For transient transfection, the DNA concentration should be in the range of 10-40 μg/ml.

Initial work by other groups on the expression of FX was performed using Baby Hamster Kidney cells (BHK cells). This cell line was used because it was known to efficiently express coagulation factor VII, which is homologous to FX (Nakagaki *et al* 1991). However, it was found that the clotting activity of the expressed FX in the culture media was approximately half that of plasma-derived FX. Further work demonstrated that the FX from BHK cells reacted poorly with a calcium-dependent monoclonal antibody directed against the Gla domain. This suggested that γ-carboxylation of FX by BHK cells is incomplete. However, when expressed in HEK cells, FX is fully functional compared to plasma-derived FX (Rudolph *et al* 1997).

7.1 Parental plasmid.

The expression vector pCMV4 containing the 3' untranslated region and entire coding sequence of the *F10* gene was a generous gift from Dr Rodney Camire (Children's Hospital of Philadelphia, PA, USA). In this vector (F10-1R-2), the codon corresponding to the residue at the -2 position in the FX propeptide has been changed from ACG(Thr) to AGG(Arg) in order to increase the efficiency of cleavage of the propeptide when expressed in HEK 293 cells (Rudolph *et al* 1997).

7.2 Vectors

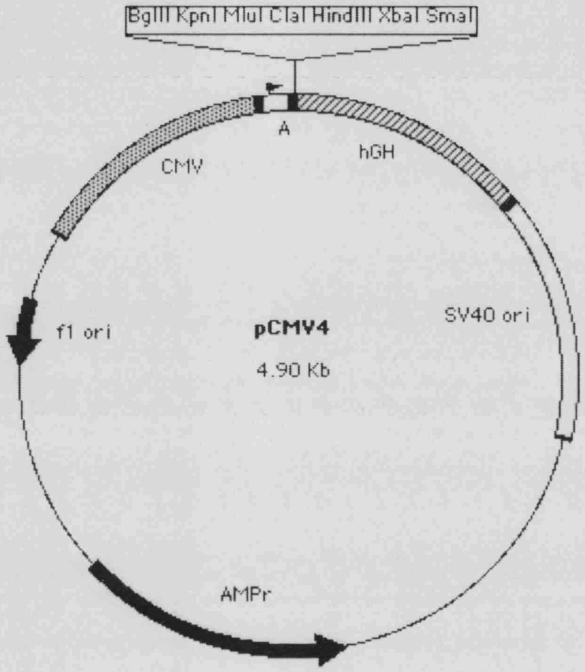


Figure 7.1 Plasmid pCMV4.

The vector pCMV4 represents the starting expression vector. It contains the immediate early promoter region of the human cytomegalovirus (CMV), a DNA copy of a segment of the Alfalfa mosaic virus 4 RNA that contains a translational augmenter (A), a polylinker containing unique sites for seven restriction enzymes, 3' splice and polyadenylation signals from the human growth hormone gene (hGH) and the SV40 origin of DNA replication (SV40ori). The plasmid also contains a bacteriophage f1 origin of DNA replication (f1ori) and an *Escherichia coli* gene encoding ampicillin-resistance (AMPr) (Andersson *et al* 1989).

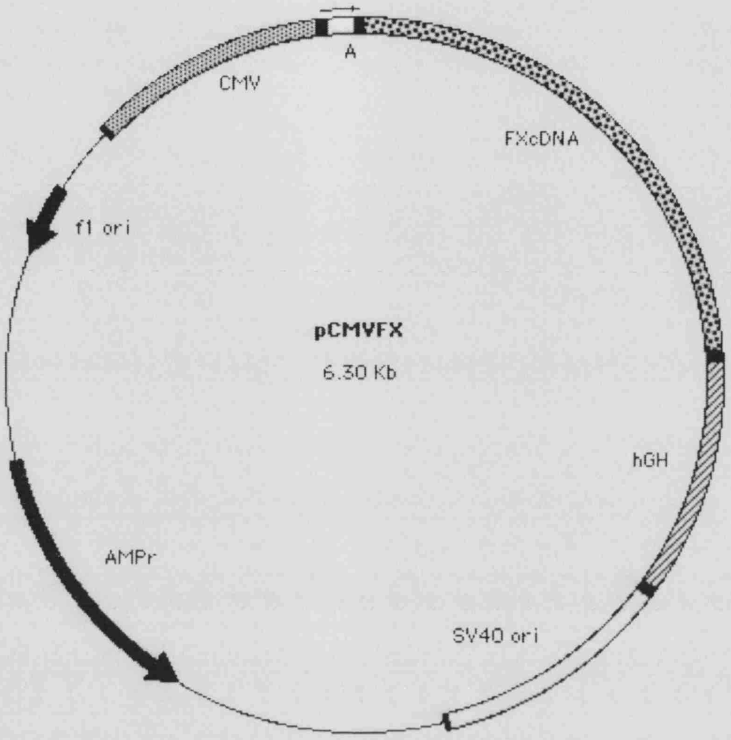


Figure 7.2 Plasmid pCMV4 F10-1R-2R

Plasmid pCMV4 F10-1R-2R contains the 1.4 kb cDNA for FX cloned into the *Bgl* II and *Hin*d III sites of the polylinker region of pCMV4. This was a generous gift from Dr Rodney Camire of the Children's Hospital, Philadelphia, USA. The mutant plasmids were created using pCMV4 F10-1R-2R as a template.

7.3 Site-Directed Mutagenesis

Plasmids encoding the desired mutations were created by site-directed mutagenesis. This is an invaluable technique for studying protein structure-function relationships and for investigating naturally-occurring mutations. Mutagenesis was performed using a QuickchangeTM kit (Stratagene La Jolla, CA, USA). This makes use of *Pfu* DNA polymerase, which has a six-fold lower error rate in DNA synthesis than conventional thermostable polymerase enzymes such as *Taq*. *Pfu* polymerase extends two mutant oligonucleotide primers which are complementary to opposite DNA strands of the plasmid. Thermocycling produces copies of the plasmid, incorporating the mutation into one of the strands. The parental (non-mutated) plasmid strands, have been isolated from *Escherichia coli* culture and so are dam methylated. The endonuclease *Dpn* I is specific for methylated DNA and so is used to digest the parental DNA template. This step leaves the mutated DNA intact which can be used to transform the bacteria and so produce suitable quantities of the desired plasmid DNA. The presence of the desired mutations and the absence of other mutations were verified by direct sequencing.

7.3.1 PCR

Reactions were carried out in 50 µl volumes and comprised 50 ng of pCMV4 F10-1R-2R DNA, 125 ng of each oligonucleotide primer, 1× KCl reaction buffer, 12.5 mM of each dNTP and 2.5 units of *Pfu* polymerase (all from Stratagene La Jolla, CA, USA). 30 µl mineral oil was added to the top of each reaction mixture.
Reactions were denatured at 95°C for 30 seconds and then 15 cycles of amplification were performed. During each cycle, the samples were denatured at 95°C for 30 seconds, followed by 60 seconds at 55°C for annealing and then 13 minutes at 74°C to allow extension. Following temperature cycling, the reactions were placed on ice for two minutes.

7.3.2 Digestion of products

Using a fine pipette tip, 10 units of *Dpn* I were added directly to each amplification reaction, underneath the layer of mineral oil. The reactions were mixed by pipetting

the solution up and down. The reaction tubes were spun briefly in a microcentrifuge and then incubated at 37°C for one hour.

7.3.3 Transformation of Escherichia coli cells

A 50 µl aliquot (for each sample reaction) of competent XL1-Blue *Escherichia coli* cells (Stratagene, La Jolla, CA, USA), which are tetracycline resistant, was thawed on ice. 1 µl of the *Dpn* I digest DNA, containing an ampicillin resistance gene, was mixed in to the aliquot. The cells were incubated on ice for 30 minutes. Heat-shock transformation was performed by incubation at 42°C for 45 seconds and replacement on ice for two minutes. 500 µl Luria-Bertani (LB) broth was added to the mixture, before incubation at 37°C for 60 minutes whilst agitating at 250 oscillations per minute. Using a sterile glass spreader, 200 µl of the transformed cells were spread onto pre-warmed LB agar plates which contained 50 µg ampicillin and 50 µg tetracycline per ml of agar (to select for transformed bacteria). After spreading, the plates were allowed 10 minutes to absorb the liquid, then they were inverted and incubated at 37°C overnight.

7.3.4 Cloning

Following the overnight incubation, a starter culture consisting of 2 ml LB broth with 50 µg ampicillin and 50 µg tetracycline per ml of broth was inoculated with a single colony from the plate. The culture was incubated at 37°C for three hours at 250 oscillations per minute. After this time, the starter culture was diluted into 1 L selective LB broth. This was incubated at 37°C overnight at 250 oscillations per minute.

7.3.5 Plasmid Purification

The plasmid DNA was recovered using a QIAGEN maxiprep kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany. The DNA pellet was air-dried for 10 minutes and redissolved in 500 µl TE buffer, pH 8.0.

7.4 Production of Plasmids for Stable Expression

For stable expression, the FX cDNA from pCMV4 F10-1R-2R was ligated into the stable expression vector pCDNA4/TO (Invitrogen, Paisley, UK). This plasmid was used because unlike pCMV4, it contains a gene encoding resistance to the antibiotic zeocin which can be used for selection. Digestion reactions were performed in 30 µl reaction volumes using five units of *Sna* BI, 20 units of *Xba* I, 1 µl plasmid DNA and 1× NEB buffer 4 (New England Biolabs, Beverly, MA, USA). The reactions were incubated at 37°C overnight. Plasmids pCMV4 F10-1R-2R and pCDNA4/T0 DNA were digested in this way.

The products were visualised by running all 30 µl on a 1% agarose gel. Each lane contained two bands, the plasmid (4.5 or 5.0 kb) and the insert (1.4kb). The bands containing the pCDNA4/TO plasmid and the FX cDNA were cut out of the gel and purified using a Qiagen gel purification kit (Qiagen GmbH, Hilden, Germany).

Ligations were performed in 25 µl reactions as follows: 1 µl purified pCDNA4/TO DNA, 15 µl purified FX cDNA, 80 units T4 DNA Ligase (New England Biolabs, Beverly, MA, USA) and 1× NEB ligation buffer. A control reaction, containing no FX cDNA was also performed. The reactions were incubated at room temperature for four hours.

A 50 µl aliquot of competent XL1-Blue *Escherichia coli* cells (Stratagene, La Jolla, CA, USA) for the ligation reaction and control was transformed as before. The transformed cells were spread onto pre-warmed LB agar plates which contained 50 µg ampicillin per ml of agar. The plates were incubated at 37°C overnight.

The following morning, colonies were picked from the plate using a sterile orange stick. PCR reactions were performed to check that the colonies contained the FX cDNA inserts. The inside of a PCR tube was touched by the orange stick and the reaction mix made up to 100 µl with 1× NH_4 buffer, 1.5 mM $MgCl_2$, 100 pmoles of each "FX cDNA Inner" oligonucleotide primer, 10 mM of each dNTP and 2.5 units of *Taq* polymerase (all from Bioline, London, UK). Primer sequences are shown in **table 20**.

Table 20 Primer sequences for FX cDNA

Primer	Sequence (5' to 3')
FX cDNA Up Inner	CTGCTCAGTGCCTCCCTGGC
FX cDNA Down Inner	TTGTCCAGGCTGCAGAGCTT

Of the colonies which amplified the FX cDNA, one colony from each plate was cloned and the plasmid purified as above.

Restriction digests were performed to ensure that the recovered DNA was the correct product. Double digest reactions were performed in 30 µl reaction volumes using five units of *Sna* BI, 20 units of *Xba* I, 1 µl plasmid DNA and 1× NEB buffer 4 (New England Biolabs, Beverly, MA, USA). The reactions were incubated at 37°C overnight and the products visualised by running an aliquot on a 1% agarose gel.

7.5 Mammalian Cell Culture

Mammalian cells were maintained in a humidified incubator (LEEC, Nottingham, UK) at 37°C and at 5% CO_2. All procedures involving live cells were performed in a Class II Microflow Biological Safety Cabinet (MDH, Andover, Hampshire, UK) using aseptic techniques and following local biological safety requirements.

The HEK 293 cell line was purchased from ATCC. Manassas, VA, USA.

7.5.1 Cell Culture Materials

All culture media and supplements were obtained from Invitrogen (Paisley, UK), unless otherwise stated and all tissue culture flasks and plates were obtained from Nunc (Fisher Scientific UK, Loughborough, UK).

The culture medium consisted of Dulbecco's Modified Eagles Medium enriched with F12 nutrients, 2 mM/L L-Glutamine, 10% foetal bovine serum (FBS), 500 µg/ml Streptomycin, 500 units/ml Penicillin G and 6 µg/L vitamin K (Roche diagnostics, Lewes, UK).

Serum-free culture medium consisted of Dulbecco's Modified Eagles Medium enriched with F12 nutrients, 2 mM/L L-Glutamine, 500 µg/ml Streptomycin, 500

units/ml Penicillin G, 10 mg/L Insulin-Transferrin-Sodium Selenite supplement (Roche diagnostics, Lewes, UK) and 6 μg/L vitamin K (Roche diagnostics, Lewes, UK).

7.5.2 Cell Culture Maintenance

Growth medium was replaced every 48 hours. 20 ml media was used in each 80 cm^2 culture flask and each 144×21 mm plate. 2 ml media was used in each well of a six-well plate.

Passage of the cell line was carried out when the cell density was approximately 90% confluent. The medium was aspirated and then 1-2 ml trypsin-EDTA (pre-warmed to 37°C) was added to the flask. This was swirled over the cells for two seconds and then promptly removed. A further 1-2 ml pre-warmed trypsin-EDTA was then added and the cells incubated at room temperature for two minutes. At this time, the cells detached from the flask surface and culture media containing FBS was added to inactivate the trypsin. The cells were gently resuspended in 20 ml medium per flask/plate and then seeded into pre-warmed flasks/plates.

7.5.3 Transfection

Three different methods of transfection were used for transient expression of recombinant FX in aliquots of mammalian cells: electroporation, liposome-mediated and calcium phosphate-mediated.

Electroporation was carried out in 4 mm eukaryotic cell cuvettes, with an EasyJect Plus electroporator (Equibio, Ashford, UK), using 280 V against 1500 C. Cells from 60 mm plates were transfected by electroporation with 30 μg of plasmid.

Liposome-mediated transfection was performed using DMRIE-C reagent (Invitrogen, Paisley, Scotland) according to the manufacturer's instructions. The procedure was optimised for HEK293 cells. DMRIE-C is a 1:1 liposome formulation of the cationic lipid DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) and cholesterol. It interacts spontaneously with DNA to form a lipid-DNA complex.

Calcium phosphate precipitation was carried out using the CalPhos™ Mammalian Transfection Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol.

72 hours following transient transfection, the supernatants (and lysates when examined) were harvested. FX:Ag assays were performed on both the supernatants and the lysates. FX:C assays were carried out on the supernatants only.

For stable transfection, DMRIE-C reagent (Invitrogen, Paisley, Scotland) was used. The antibiotic zeocin was used to select transfected cells. A "kill curve" was performed to ascertain the ideal concentration of the selection antibiotic (Zeocin). This is the dose of antibiotic at which untransfected cells would die, but transfected cells would survive. HEK 293 cells were plated and grown to 25% confluence in 16 plates. Eight concentrations of Zeocin (ranging from 0 to 1000 μg/ml culture medium) were tested in duplicate. The Zeocin-containing medium was replaced every third day and the proportion of surviving cells was observed over time. It was found that 400 μg/ml was the suitable Zeocin concentration and this was used in all subsequent selection media.

On the day following stable transfection, the culture medium was replaced with the selection medium. This contained 400 μg of Zeocin per ml of culture medium. The growth medium was changed every 48 hours to encourage death of untransfected cells.

After three weeks, there was little further cell death and macroscopically visible colonies could be detected on the plate surface. Colonies were picked as follows: medium was removed from the plates. The cells were washed with PBS. Individual colonies were harvested by dipping a sterile swab into the trypsin-EDTA solution and dabbing the swab onto the colony. The swab was then rotated onto the bottom of a 24 well plate. Each well contained 1 ml of FX medium. Approximately 20 colonies were harvested for each FX mutant.

When the cells in the wells appeared to be at 50% confluence, the medium was changed. When the cells were at 90% confluence, 100 μl of the medium from each well was aspirated and a FX ELISA was performed (see **section 2.3**). The cells from the wells which produced the greatest FX antigen level were trypsinized as before and seeded into culture plates.

7.5.4 Storage of cell lines

When the plates were at 90% confluence, they were trypsinized as before. The cells were resuspended in 5 ml of FX medium before centrifugation at 300 *g* for five minutes at room temperature. The supernatant was removed and the pellet re-suspended in 1 ml of freezing mixture, consisting of 10% Dimethyl Sulfoxide and 10% FBS in the regular FX medium. The cell suspension was frozen in a cryo-chamber by 1°C per minute to -80°C and then moved to a vapour-phase liquid nitrogen canister.

7.6 Western Blotting

7.6.1 Electrophoresis and Transfer

Polyacrylamide gel electrophoresis was performed using an Xcell II™ Mini-Cell (Novex, San Diego, CA, USA). 4-12% Tris-Glycine gels (Invitrogen, Paisley, UK) were rinsed with ultrapure water and then 1× running buffer. 13 µl sample was added to 5 µl 4× sample buffer and 2 µl 0.02M dithiothreitol. The mixture was incubated at 80°C for 10 minutes and then loaded into the wells. Electrophoresis was carried out at a constant 200 V in 1× running buffer.

After 45 minutes, the gel was removed and the proteins transferred to nitrocellulose membrane (Hybond ECL, Amersham Biosciences). This was carried out using an Xcell II™ Blot Module in transfer buffer (Novex, San Diego, CA, USA). Transfer occurred using a constant 25 V for 90 minutes.

7.6.2 Reversible Staining of Proteins

Following transfer, blots were stained for five minutes with Ponceau S reversible total protein stain to confirm successful transfer of proteins. The blot was rinsed in wash buffer to remove excess stain. De-staining was performed by the addition of neonatal calf serum to the wash buffer to take up the Ponceau S.

7.6.3 Antibody Binding

Blots were blocked by agitating in the blocking solution for 60 minutes. The blots were then washed in wash buffer. The primary antibody (see **table 21**) was diluted in

blocking solution with 0.1% sodium azide and the blots were incubated in this mixture overnight. The following day, the blots were washed by agitation for 20 minutes in wash buffer. For the antibodies which were not HRP conjugated, it was necessary to use a secondary, goat anti-mouse antibody (Dako Ltd, Ely, UK) which was HRP conjugated to allow detection of antibody binding. This secondary antibody was diluted 1:2000 with blocking solution. The blots were incubated in the mixture for three hours before washing as before.

Table 21 Antibodies for use in Western blots

Antibody	Monoclonal/ Polyclonal	Dilution	Source
Rabbit anti-human HRP conjugated	Polyclonal	1:2000	Dako Ltd, Ely, UK
Mouse anti-human FX light chain	Monoclonal	1:1000	Enzyme research Laboratories, Swansea, UK
Mouse anti-human FX heavy chain	Monoclonal	1:500	Enzyme research Laboratories, Swansea, UK

7.6.4 Immunodetection Using Enhanced Chemiluminescence (ECL)

This was performed according to the manufacturer's protocol. Blots were laid protein side up on a single layer of Saran™ wrap. 750 µl ECL reagent 1 (Amersham Biosciences, Bucks, UK) was added to 750 µl ECL reagent 2 and the mixture applied to the protein side of the blot. After incubating for 60 seconds, the excess reagent was drained off and the blots wrapped in a fresh piece of Saran™ wrap. Radiographic film was exposed to the blots in a light-proof cassette. Films were developed using an automated Fuji RGII X-ray film processor (Fuji Photo Film Co. London, UK) and Photosol developing reagents (Photosol, Essex, UK).

8 Analysis of FX Arg-1Thr Mutation

8.1 Introduction

The propeptides of vitamin K-dependent proteins are important for directing intracellular post-translational modification, which is essential for correct function. The propeptides of these proteins contains a γ-carboxylation recognition site that directly binds the vitamin K-dependent γ-glutamyl carboxylase (Jorgensen *et al* 1987). The light chain of FX contains 11 Glutamic acid residues that are γ-carboxylated to the γ-carboxyglutamic acid (Gla) residues. The Gla residues allow Ca^{2+} dependent binding of FX to negatively charged phospholipid membranes (Stenflo and Suttie 1977). This is necessary for the normal coagulant activity of the protein (Mann *et al* 1992). There are seven glycoproteins that require vitamin K for their biosynthesis (the others are factors II, VII, IX, Protein C, Protein S and Protein Z). γ-Carboxylation is dependent upon adequate levels of vitamin K and its absence, or inhibition by coumarins, results in the production of dysfunctional molecules termed PIVKAs (Proteins in Vitamin K Absence) (Hemker *et al* 1968).

The mutation FX Arg-1Thr was identified in an Iranian patient by Dr Dale Owens at the Royal Free Hospital (Owens *et al* 1998). The patient suffered a severe clinical phenotype (including recurrent haemarthroses and haematomas) and required frequent treatment. The mutation results in a type II laboratory phenotype, but with an incongruent chromogenic assay result. Laboratory assays for this Iranian patient (also performed by Dr Owens) are shown in **table 22**.

Table 22 Phenotype assays for patient with Arg-1Thr mutation

FX:Ag	FX:PT	FX:APTT	FX:RVV	FX:Chromogenic
66	<1	<1	<1	65

Figures are expressed as % of the FX level in normal plasma (100%).

8.2 Propeptide Cleavage

The Arg-1Thr mutation occurs in the P1 site (carboxy-terminal residue) of FX propeptide. The removal of propeptides and so the conversion of precursors of peptide hormones, neuropeptides and many other proteins into their biologically

active forms is performed by the members of the proprotein convertase enzyme family. The first such enzyme to be described was the product of the *KEX2* gene (Kex2p) in the yeast *Saccharomyces cerevisiae* (Fuller *et al* 1988). Since then, several eukaryotic endoproteases homologous to Kex2p have been reported, some having isoforms generated via alternative splicing. These enzymes include Furin (Roebroek *et al* 1986) (also known as PACE (Wise *et al* 1990)), PC2 (Smeekens and Steiner 1990), PC1 (Smeekens and Steiner 1990) (also known as PC3 (Nakayama *et al* 1991)), PACE4 (Kiefer *et al* 1991), PC4 (Nakayama *et al* 1992), PC5 (Lusson *et al* 1993) (also known as PC6 (Nakagawa *et al* 1993)), PC7 (Seidah *et al* 1996) (also known as LPC (Meerabux *et al* 1996), PC8 (Bruzzaniti *et al* 1996) and SPC7 (Constam *et al* 1996)).

Conformation-specific antibodies raised against the Ca^{2+}-induced Gla domain in plasma prothrombin have been used to follow the prothrombin precursor through its secretory pathway in the Sprague-Dawley rat liver cell (Wallin *et al* 1993). Prothrombin precursors in which the propeptide remains attached do not exhibit the Ca^{2+}-induced conformational change. These species are dysfunctional. This suggests that a bleeding disorder may result if the propeptide remains attached. It has also been shown that in terms of intracellular processing, γ-carboxylation occurs before propeptide cleavage (Stanton *et al* 1991). It may be that the presence of the propeptide protects against intracellular activation of vitamin K-dependent coagulation proteins.

Several naturally occurring substitutions for P1Arg have been described in other vitamin K-dependent proteins. These are summarised in **table 23**. No naturally occurring mutations in the P1 position of factor VII have yet been reported. However, the *in vitro* expression of the synthetic factor VII mutant Arg-1Ser has been described (Busby *et al* 1988).
In FX, the substitution of Arg by Thr at P1 position would be predicted to obliterate the cleavage-site motif that Furin-like proteases recognise. Cleavage at this site is likely to be impaired if it happens at all. The protease may recognise another site, in which case the mature protein will be of a different length to that of wildtype FX or the enzyme may not cleave at all, in which case the mature protein will circulate with the propeptide attached.

Table 23 Mutations in P1Arg residue of vitamin K-dependent proteins

Protein	Mutation	Ag	Chromogenic	Clotting	N-terminus	γ-carboxylation	Reference
Factor IX Cambridge	Arg-1Ser	Normal	ND	Reduced	Circulates with propeptide	Impaired	(Diuguid *et al* 1989)
Protein S	Arg-1His	Normal	ND	Borderline low	Cleavage unimpaired	ND	(Gandrille *et al* 1995)
Prothrombin	Arg-1Gln	Reduced	Reduced	Reduced	ND	ND	(Akhavan *et al* 2000)
Protein C Padua 2	Arg-1Cys	Normal	Normal	Reduced	ND	Impaired	(Girolami *et al* 1993) (Gaussem *et al* 1994)
Protein C Osaka 10	Arg-1Ser	Normal	Normal	Reduced	Circulates with Ser-1 residue	Normal	(Miyata *et al* 1995)
Protein C	Arg-1His	Normal	Normal	Reduced	Circulates with His-1 residue	Normal	(Lind *et al* 1997)
Protein C Padua 3	Arg-1Leu	Normal	Normal	Reduced	60% of protein circulates with propeptide	ND	(Simioni *et al* 2001a)
Factor VII (*in vitro* only)	Arg-1Ser	Reduced	ND	Reduced	Circulates with propeptide	Impaired	(Busby *et al* 1988)

ND – No Data

8.3 Plasmid Mutagenesis

In order to confirm or refute the prediction that the cleavage-site would be obliterated by the mutation, a series of expression constructs was employed. The expression vector pCMV4 containing the 3' untranslated region and entire coding sequence of the *F10* gene was used as the starting vector for transient expression work (see **section 7.1**). In this vector (F10-1R-2R), the codon corresponding to the residue at the -2 position in the FX propeptide had been changed from ACG(Thr) to AGG(Arg) in order to increase the efficiency of cleavage of the propeptide when expressed in HEK 293 cells (Rudolph *et al* 1997).

Three further plasmids were created, by the introduction of mutations into exon 2 (which encodes the propeptide and Gla domains). Site-directed mutagenesis was performed using a Quickchange™ kit (Stratagene La Jolla, CA). A silent mutation in Leu-6 (CTG to CTC) was introduced to create an *Nru* I restriction site in all three mutant plasmids. In the first plasmid (F10-1T-2R), AGG to ACG at codon -1 resulted in Arg to Thr. In the second plasmid (F10-1T-2T), both AGG (Arg) to ACG (Thr) at the -1 position and AGG (Arg) to ACG (Thr) at the -2 position resulted in a *F10* cDNA which matched that of our patient. The mutation in the final plasmid (F10-1R-2T) (AGG(Arg) to ACG(Thr) at the -2 position) resulted in the reversion of the F10-1R-2R plasmid to the cDNA of the wildtype *F10*. The presence of the desired mutations and the absence of other mutations were verified by restriction enzyme digestion and by direct sequencing.

The sequences of the forward (FP) and reverse (RP) mutagenesis primers used are shown in **table 24**.

In effect, there are two "wildtype" plasmids and two mutants. The F10-1T-2T plasmid is the Arg-1Thr mutant of the true wildtype (F10-1R-2T). The F10-1T-2R plasmid is the Arg-1Thr mutant of the FX "wildtype" (F10-1R-2R) which has been shown to allow correct cleavage of the propeptide when expressed in HEK 293 cells (Rudolph *et al* 1997). The amino acid sequence around the mutation site for each plasmid are shown in **figure 8.1**.

Plasmid	Primer	Primer Sequence (5' to 3')
F10-1T-2R	FP	ATCCTCGCGAGGGTCAGGACGGCCAATTCCT
(-1Arg, -2Thr)	RP	AGGAATTGGCCGTCCTGACCCTCGCGAGGAT
F10-1T-2T	FP	ATCCTCGCGAGGGTCACGACGGCCAATTCCT
(-1Thr, -2Thr)	RP	AGGAATTGGCCGTCGTGACCCTCGCGAGGAT
F10-1R-2T	FP	ATCCTCGCGAGGGTCACGACGGCCAATTCCT
(-1Arg, -2Thr)	RP	AGGAATTGGCCCTCGTGACCCTCGCGAGGAT

Table 24 Mutagenesis primers for Arg-1Thr mutant

```
Residue numbering:      -4  -1 +1
F10-1R-2R: LFIRREQANNILARVRR-ANSFLEEMKKGHLERECMEETC
F10-1T-2R: LFIRREQANNILARVRT-ANSFLEEMKKGHLERECMEETC
F10-1R-2T: LFIRREQANNILARVTR-ANSFLEEMKKGHLERECMEETC
F10-1T-2T: LFIRREQANNILARVTT-ANSFLEEMKKGHLERECMEETC
```

Figure 8.1 Residue sequence around the P1 site for each of the four plasmids

The +1 residue of the mature protein is highlighted in pink, immediately C-terminal to the propeptide cleavage site. The two residues of the propeptide endoprotease recognition site which were mutated in the course of this work are shown in grey (-1 and -2).

8.4 Cleavage Prediction

Propeptide cleavage prediction was performed, using the ProP v.1.0b ProPeptide Cleavage Site Prediction program on the website of the Center for Biological Sequence Analysis at the Technical University of Denmark (www.cbs.dtu.dk).

Prediction is based on neural networks (Duckert *et al* 2004). The program uses two different types of network: a furin-specific network based on experimental results derived from the literature, and a general network, trained on data from the SWISSPROT protein database. The method predicts cleavage sites in independent sequences with a sensitivity of 95% for the furin neural network and 62% for the general network.

The output for the program is a score for each of the Arginine and Lysine residues of the input sequence. If the score is >0.5 the residue is predicted to be followed by a propeptide cleavage site; the higher the score the more confident the prediction. The output is represented graphically: the prediction score for each Arginine and Lysine is plotted against the residue position in the sequence.

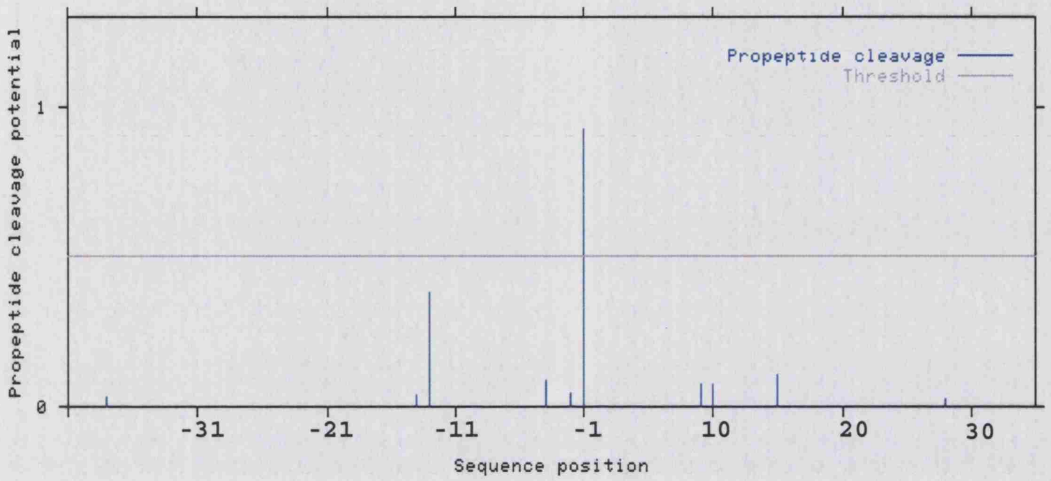
The program was run using all four *F10* sequences (F10-1R-2R, F10-1T-2R, F10-1T-2T and F10-1R-2T). The results are shown in **figures 8.2** and **8.3**.

The program correctly predicted the *in vivo* propeptide cleavage site in the wildtype F10-1R-2T sequence (between Arg-1 and Ala+1). The prediction score for this cleavage site is 0.901. From *in vitro* expression work it was found that the efficiency of cleavage was improved by substituting Thr-2 by Arg-2 (Rudolph *et al* 1997). When this modified sequence (F10-1R-2R) was used in the prediction program, the maximum score was again at the *in vivo* cleavage site, but the score was higher (0.924). This indicates that the likelihood of cleavage occurring at this point is increased by the residue substitution at the P2 position. In both of these sequences, the residue with the second highest score is Arg-13. However the score at the –13 position is 0.381 in both cases and does not reach the threshold of 0.5.

When the P1 residue is mutated from Arginine to Threonine, the program predicts that the cleavage site at –1/+1 is abolished (this can be seen in both F10-1T-2T and F10-1T-2R). This leaves the highest score at Arg-13. However, the score at Arg-13 remains below the threshold at 0.381.

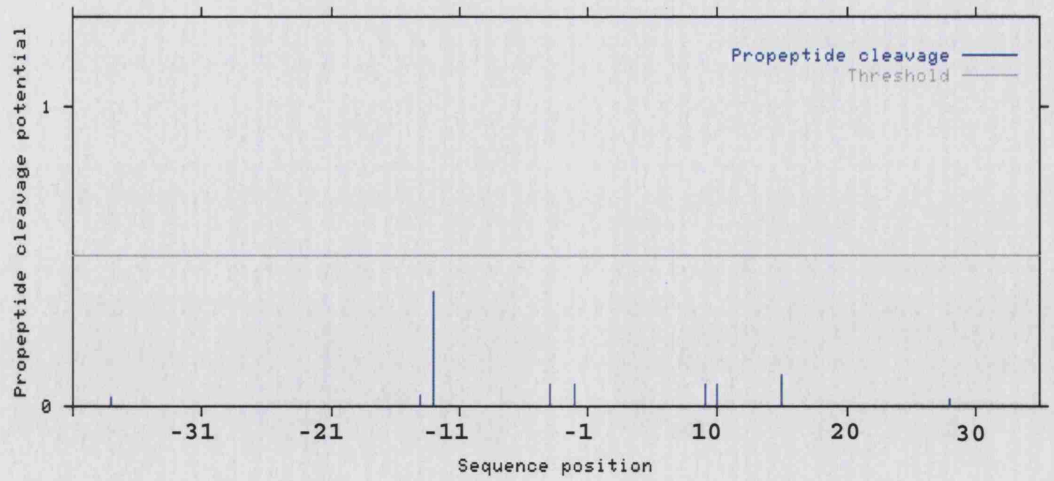
Figure 8.2

(A) F10-1R-2R



Position	-38	-14	-13	-4	-2	-1	9	10	15	28
Score	0.03	0.039	0.381	0.087	0.040	0.924	0.074	0.071	0.105	0.026

(B) F10-1T-2R



Position	-38	-14	-13	-4	-2	-1	9	10	15	28
Score	0.03	0.039	0.381	0.071	0.071	0	0.074	0.071	0.105	0.026

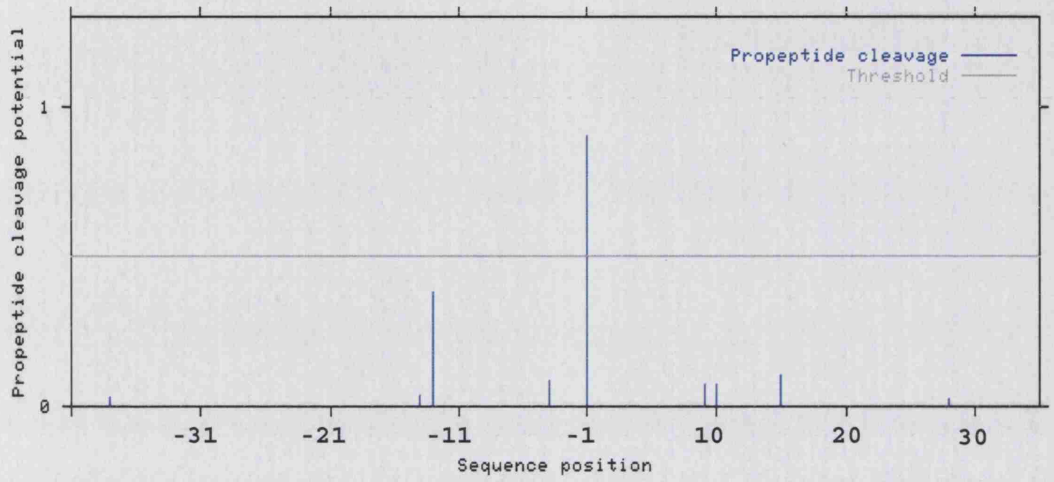
Figure 8.2 is a graphical representation of the output from the ProP v.1.0b program for F10-1R-2R and F10-1T-2R

(A) Shows the prediction score for each Arginine and Lysine residue in the F10-1R-2R wildtype sequence. The highest score (and the only score above the threshold of 0.5) occurs at the –1 position.

(B) Shows the prediction score for each Arginine and Lysine residue in the F10-1T-2R mutant sequence. No score reaches the threshold value of 0.5.

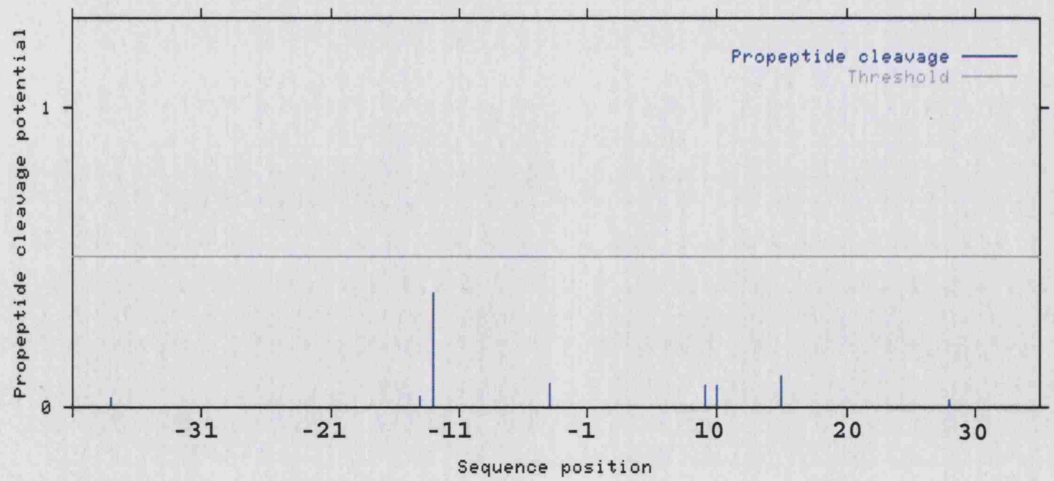
Figure 8.3

(A) F10-1R-2T



Position	-38	-14	-13	-4	-2	-1	9	10	15	28
Score	0.03	0.039	0.381	0.087	0	0.901	0.074	0.071	0.105	0.026

(B) F10-1T-2T



Position	-38	-14	-13	-4	-2	-1	9	10	15	28
Score	0.03	0.039	0.381	0.078	0	0	0.074	0.071	0.105	0.026

Figure 8.3 is a graphical representation of the output from the ProP v.1.0b program for F10-1R-2T and F10-1T-2T

(A) Shows the prediction score for each Arginine and Lysine residue in the F10-1R-2T wildtype sequence. The highest score (and the only score above the threshold of 0.5) occurs at the –1 position.

(B) Shows the prediction score for each Arginine and Lysine residue in the F10-1T-2T mutant sequence. No score reaches the threshold value of 0.5.

The program predicts that the Arg-1Thr mutant would abolish propeptide cleavage in FX. This would mean that the mature protein would be predicted to circulate with the propeptide attached.

8.5 *In vitro* Expression

In order to confirm the cleavage predictions, transient transfection of HEK 293 cells was performed (see **chapter 7**).

72 hours following transfection, both the supernatants and lysates were harvested. FX:Ag assays were performed on both the supernatants and the lysates. FX:C assays were carried out on the supernatants only. The results of these assays are shown in **table 25**. Results for each mutant plasmid are expressed as a percentage of the assay values obtained for the wildtype plasmid and are normalized for cell confluence.

Table 25 Assay results from transfection of cells with four plasmids

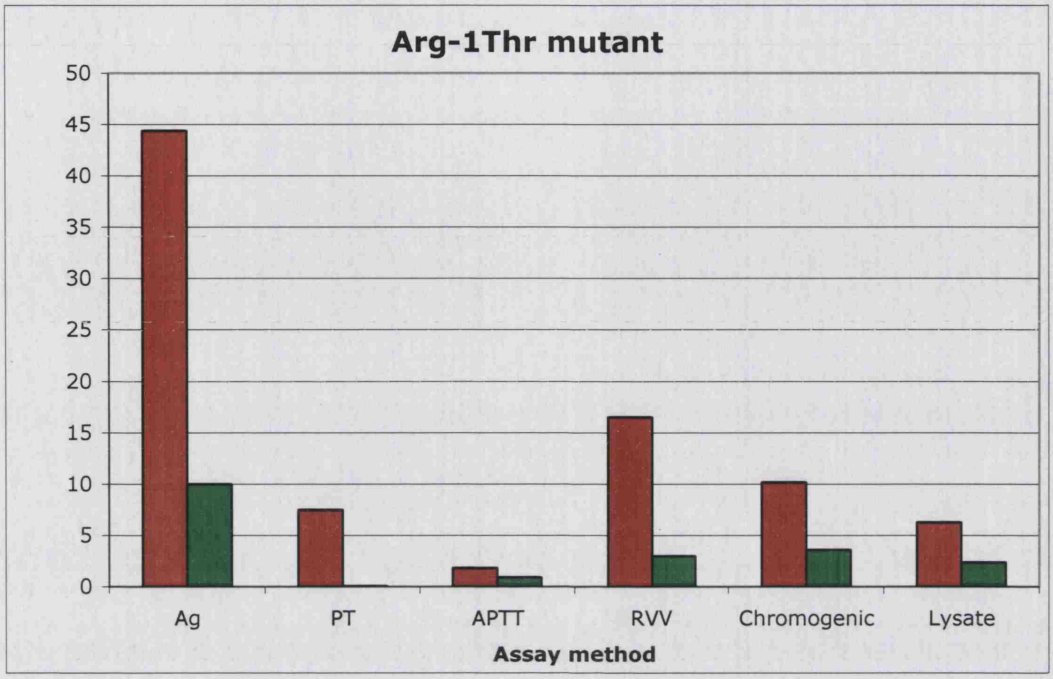
Plasmid	Ag	PT	APTT	RVV	Chromogenic	Lysate
F10-1R-2R	100	100	100	100	100	100
F10-1T-2R	23	<0.5	50	18	35	38
F10-1R-2T	100	100	100	100	100	100
F10-1T-2T	142	<0.5	70	46	76	107

Figures are expressed as % of the FX level from transfection of the wildtype plasmid (100%).

From **table 25**, it can be seen that each plasmid resulted in the secretion of some FX into the supernatant. The F10-1T-2R mutant is expressed at lower levels than the wildtype construct F10-1R-2R. This does not concur with the *in vivo* data, in which protein secretion is normal. It is possible that unlike native hepatocytes, the HEK cells are not able to secrete FX proteins with propeptides attached.

From the graphical representation of the expression by cells transfected with wildtype and mutant plasmids (**figure 8.4**), it can be seen that in the cases of both F10-1R-2T and F10-1T-2T, protein is successfully expressed. There is no evidence of intracellular accumulation as the lysate values are approximately 10%. However,

(a)



(b)

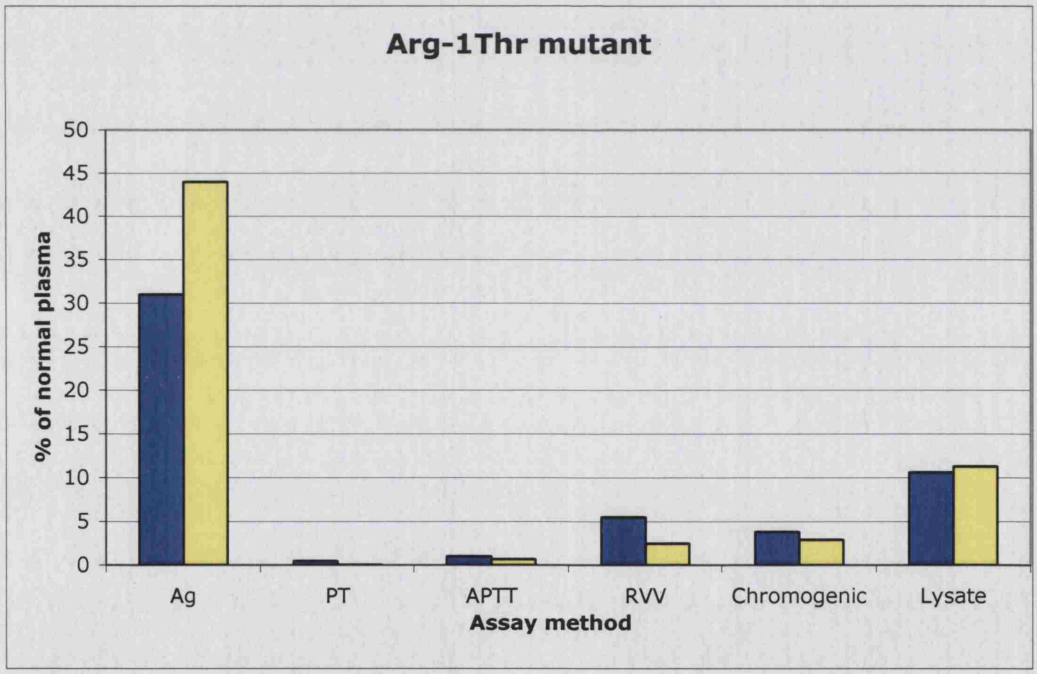


Figure 8.4 Assay results from transfection with four plasmids

(a) Expresssion levels in the supernatant from cells transfected with F10-1R-2R (red) and F10-1T-2R (green).

(b) Expresssion levels in the supernatant from cells transfected with F10-1R-2T (blue) and F10-1T-2T (yellow).

neither protein is functionally active.

The activity assay levels for F10-1R-2T are lower than those of the wildtype plasmid (F10-1R-2R). This confirms previous work demonstrating the importance of the replacement of the Thr-2 residue by Arginine (Rudolph *et al* 1997).

The dysfunctional protein could be explained by poor post-translational modification *in vitro*. Another possibility is that the recombinant protein has been partially degraded in the cell supernatant. The FX:Ag ELISA uses a polyclonal antibody to FX. If part of the recombinant protein remains intact then it will be recognised by the antibody and will register in the FX:Ag assay.

8.5.1 Western Blotting

In order to confirm that the protein detected in the FX:Ag assays was FX protein of the correct molecular size, Western blotting was performed. Aliquots of the supernatants from the culture plates of cells transfected with each of the plasmids pCMV4 F10-1R-2R, pCMV4 F10-1T-2R, pCMV4 F10-1T-2T and pCMV4 F10-1R-2T were prepared (see **section 7.6**) and run on a 4-12% polyacrylamide gel. A control lane containing culture medium (including 10% FBS) was also run.

Following electroblotting, the nitrocellulose membrane was stained with Ponceau S to confirm protein transfer (see **figure 8.5**).

Figure 8.5 shows that there is one protein band of approximately 60 kDa visible in all five lanes. This corresponds to the size of FX (59 kDa), but is also the size of human albumin (56 kDa). It is likely that the band contains mostly albumin as each of the samples contains 10% FBS.

After de-staining, the nitrocellulose membrane was incubated with the polyclonal rabbit anti-human FX antibody. Imaging was performed using the ECL system and radiographic film. **Figure 8.6(a)** shows the final radiograph.

A Bio-Rad protein assay was performed on the supernatants (see **section 2.7**). The results are shown in **table 26**. The FX:Ag results from the ELISA tests on the supernatants are also shown.

Table 26 shows that FX is approximately 0.1% of the total protein in the supernatant.

Table 26 Total protein assay results on cell supernatants

Plasmid	Total Protein (mg/ml)	FX:Ag (μg/ml)
F10-1R-2R	3.6	3
F10-1R-2T	3.6	3.1
F10-1T-2R	3.6	1
F10-1T-2T	3.4	4.4

The Western blot (**figure 8.6(a)**) confirms the presence of FX in the supernatants from all four transfections. The control lane contained no FX. The dithiothreitol reduced the bonds linking the two chains of FX, so the heavy chain (42 kDa) and the light chain (17 kDa) could be visualized separately. The presence of the individual chains was confirmed by subsequent blots using monoclonal antibodies to the heavy chain and light chain in separate experiments (not shown).

No FX antigen could be detected in lane 5 (negative control containing culture medium with 10% FBS). The bands in lanes 1-4 that correspond to the heavy chain of FX are of equal size (approximately 42 kDa). However, the bands of lower molecular weight (approximately 20 kDa) are not. Lane one has two bands, both of which run faster than the band in lane two. The band in lane three appears to run at the same speed as the slower of the bands in lane one. There are at least two bands in lane four. One appears to run at the same speed as the band in lane two and the other is slower.

Figure 8.6(b) is a schematic representation of the light chain isoforms of the FX proteins expressed in the cell supernatants. Amongst the four sets of transfections, there appear to be four isoforms. Using the nomenclature from (Himmelspach *et al* 2000) to relate isoform to electrophoretic mobility, lane 1 contains LC3 and LC4; lane 2 contains LC2; lane 3 contains LC3; lane 4 contains LC1 and LC2.

If the substitutions of the P1 and P2 residues resulted in altered propeptide cleavage, then the resultant mature single-chain FX molecules should be of varying molecular mass. In order to establish this, a non-reducing SDS-PAGE gel was run and a Western Blot performed. Species of differing molecular mass could not be resolved (not shown).

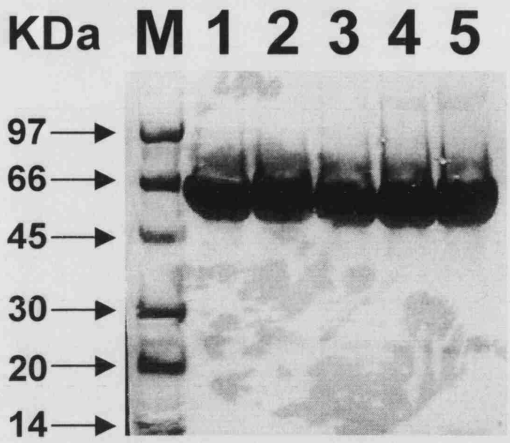


Figure 8.5: Ponceau S stain of the nitrocellulose membrane confirming transfer of protein from the polyacrylamide gel. Lane M: protein marker. Lane 1: F10-1R-2R. Lane 2: F10-1R-2T. Lane 3: F10-1T-2R. Lane 4: F10-1T-2T. Lane 5: negative control.

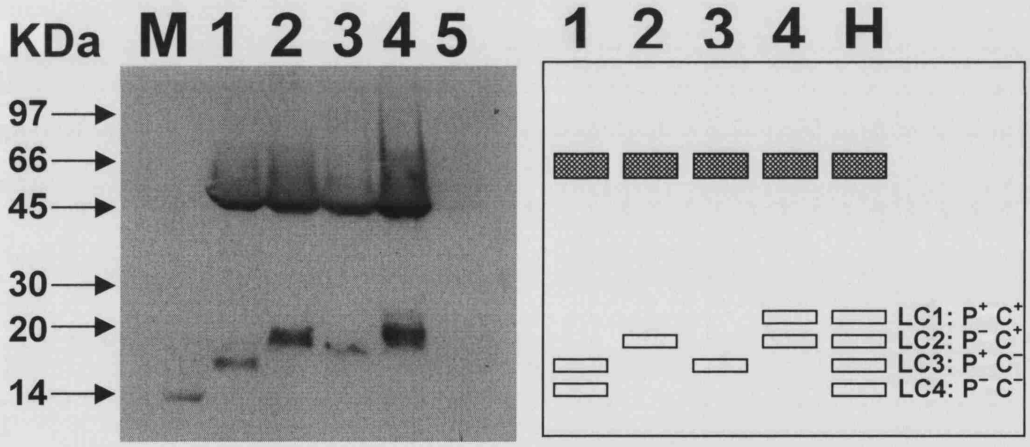


Figure 8.6.

(a) Radiograph showing a Western Blot of the supernatants from the transient transfection of the four plasmids, using a polyclonal antibody to FX. Lane M: protein marker. Lane 1: F10-1R-2R. Lane 2: F10-1R-2T. Lane 3: F10-1T-2R. Lane 4: F10-1T-2T. Lane 5 negative control.

(b) A schematic representation on the right shows the heavy chains (shaded) and light chain isoforms of recombinant FX. Lane H shows the heavy chain and all four light chain isoforms (LC1 to LC4) discussed by (Himmelspach *et al* 2000). P^+, propeptide-containing; P^-, propeptide-free; C^+, fully-carboxylated; C^-, under-carboxylated.

8.5.2 Production of Recombinant Proteins for Biochemical Analysis

In order to explore N-terminal amino acid sequencing and γ-carboxylation analysis of the recombinant proteins, a relatively large quantity of sufficiently pure protein would need to be produced. Although this is possible by repeated transient transfections, stable expression systems provide a more efficient way to provide the desired amount. Stable transfection vectors were created as discussed in **section 7.4**. Stable transfection was achieved using DMRIE-C reagent according to the manufacturer's instructions. 2 μg plasmid DNA and 2 ml culture medium were used in each 35 mm plate. Following selection, macroscopically visible colonies were picked, expanded and assayed by FX ELISA. The best performing colonies were seeded into 60 mm plates.

8.5.2.1 FX assays

Activity and antigen assays were performed on the supernatants from the stable cell lines. The results are shown in **table 27**.

Table 27 Phenotypic assay results on supernatants from stable cell lines

Plasmid	FX:PT	FX:Ag	PT:Ag ratio
F10-1R-2R	100, 97	105, 98	0.95, 0.99
F10-1R-2T	5, 4	73, 140	0.07, 0.02
F10-1T-2R	<1, <1	107, 75	<0.01, <0.01
F10-1T-2T	<1, <1	80, 77	<0.01, <0.01

Figures are expressed as % of the FX level in normal plasma (100%). Experiments were performed in duplicate. The duplicate results are shown separated by commas.

From **table 27** it can be seen that the PT activity assay for the two Arg-1Thr mutants is <1% of normal plasma. This concurs with the *in vivo* assay results for the patient (see **table 22**). The specific activity for the wildtype plasmid F10-1R-2R is approximately 1. This concurs with the *in vivo* data for normal individuals. The specific activity for the F10-1R-2T plasmid is much reduced compared to that for plasmid F10-1R-2R. It is known that there is reduced efficiency of propeptide processing with F10-1R-2T. The presence of the propeptide still attached to the

mature protein may affect the coagulation activity of the protein, but allow normal secretion and so a normal antigen level. This would reduce the specific activity.

8.5.2.2 Purification of conditioned media

Initial experiments were performed in the process of purification of recombinant FX. Stably transfected cells were seeded into four 500 cm^2 triple flasks. Each flask contained 100 ml medium. When the cells reached 70% confluence, the culture medium was changed to serum-free medium. The media in all four flasks was changed each day for four days. (In total 1.6 L was collected.)

The initial stages of the purification protocol of (Larson *et al* 1998) were followed for the F10-1R-2R wildtype. The collected media were centrifuged and passed through a 0.2 μm filter to discard any cell debris. EDTA was added to a concentration of 5 mM, Soybean trypsin inhibitor was added to a concentration of 10 μg/ml and 100 μl of a protease inhibitor cocktail (both from Sigma-Aldrich, Poole, UK) was also added. The medium was diluted 1:1 with 20 mM Tris (pH 7.2) to a final NaCl concentration of 60 mM. 10 ml of equilibrated Q-sepharose beads (Amersham Biosciences, Uppasala, Sweden) were added to the diluted medium and stirred at 4°C for 30 minutes. The beads were loaded onto a chromatography column and washed with ten column volumes of 20 mM Tris (pH 7.2)/60 mM NaCl. Ten fractions of 10 ml each were eluted with 20 mM Tris (pH 7.2)/700 mM NaCl. FX:PT assays were performed on the fractions. Fractions containing FX were pooled and dialysed with a 1 mM phosphate buffer (pH 6.8).

An immunoaffinity column was prepared according to the manufacturer's instructions. 1 ml (8.4 mg) of rabbit anti-human FX polyclonal antibody (Dako Ltd, Ely, UK) was dialysed against the column coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH8.3) to remove NaN_3. After dialysis the antibody was coupled to a HiTrap™ NHS-activated column (Amersham Biosciences, Uppasala, Sweden) according to the manufacturer's recommendations.

The pooled fractions were applied to the column and the column washed with 20 mM Tris (pH 7.2)/60 mM NaCl. Ten fractions of 2.5 ml were eluted with 20 mM Tris (pH 7.2), 60 mM NaCl, 8 mM Na_2EDTA.

At this stage a chromogenic assay for FX was performed on the samples of the flow-through, the wash and the eluate. This revealed that virtually all the FX was to be found in the flow-through and the wash.

A vial of PCC containing 200 iu of FX was loaded onto the column to test whether the column was able to bind FX at all. This was washed and eluted in ten fractions as before. Again, a chromogenic assay showed that almost all the FX was to be found in the flow-through and the wash. This indicated that FX had not bound to the column.

It was postulated that this was either because ligand coupling had not occurred (the antibody had not bound to the column) or that antibody binding had been successful but that FX had not bound to the antibody.

If ligand coupling had not occurred, then antibody would still be present in the flow-through sample following the ligand coupling. To investigate this, a protein estimation assay was performed on this sample. This showed a protein concentration below the level of sensitivity of the assay, excluding the possibility that the antibody had not coupled.

Further confirmation that the antibody had bound to the column was provided by a Western Blot. 10 μl of sepharose slurry from the immunoaffinity column was added to 5 μl 4× sample buffer and 2 μl 0.02 M dithiothreitol. The mixture was incubated at 80°C for 10 minutes. This was also performed with a 1:10 dilution of the slurry and with a murine IgG standard (Sigma-Aldrich, Poole, UK). Electrophoresis and protein transfer was carried out as previously described (**7.6.1**).

The nitrocellulose membrane was stained with Ponceau S and is shown in **figure 8.7**. A schematic is shown to the right for clarity. The figure shows the heavy and light chains of the immunoglobulins in all three lanes, indicating that ligand coupling had been successful.

The PCC used to test the binding capability of the column contains excipients including NaCl and NaH_2PO_4. It is possible that the presence of these excipients may not be conducive to the antibody-antigen interaction in the column. Consequently, PCC was dialysed with the wash buffer (20 mM Tris (pH 7.2)/60 mM NaCl). Following dialysis, the PCC was loaded onto the column, before washing and elution steps as before. Chromogenic assays were performed on the dialysed PCC, flow-through, wash and eluates. Again, the FX did not bind to the column.

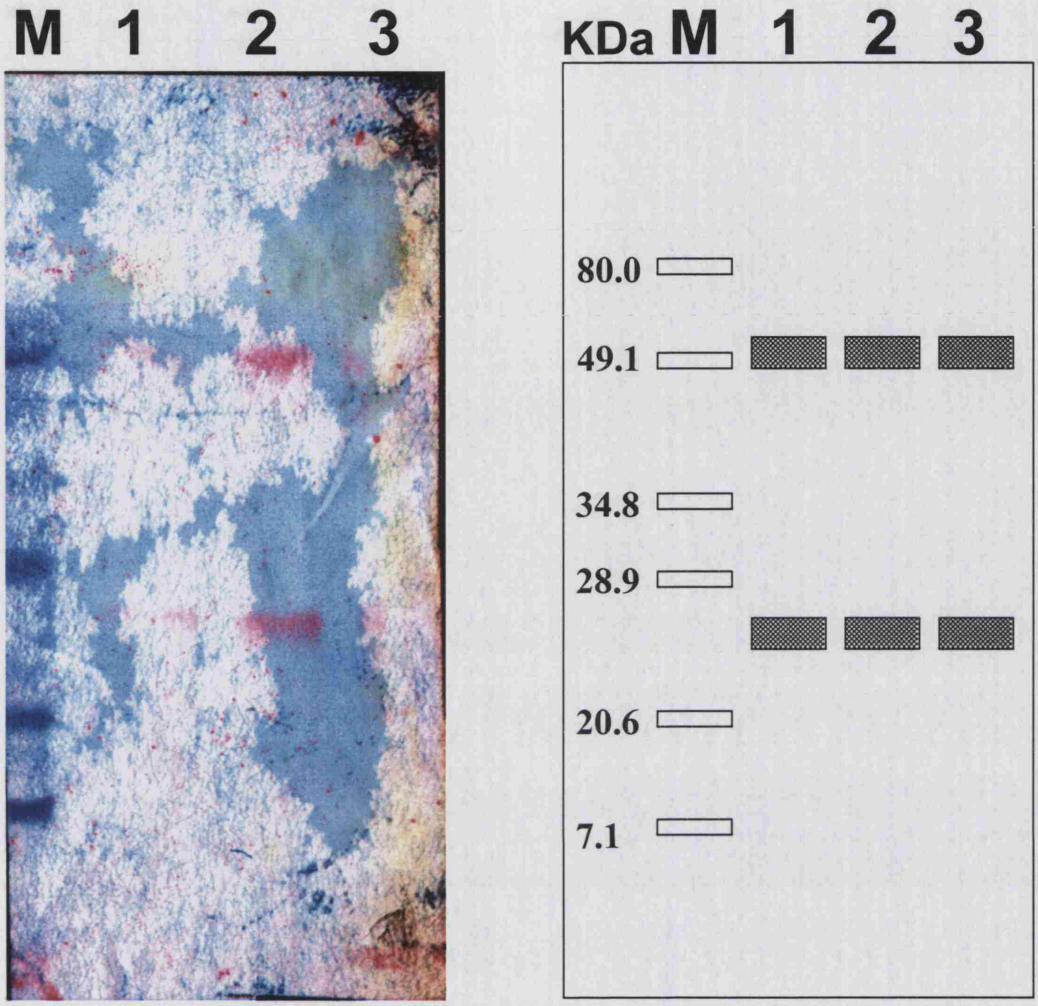


Figure 8.7. Ponceau S stain of a nitrocellulose membrane confirming transfer of protein from the polyacrylamide gel is shown on the left. Two bands (pink) are visible in each lane, representing the heavy and light chains of the immunoglobulin molecules. Lane 1 contains murine IgG; lane 2 contains the Sepharose slurry from the immunoaffinity column; lane 3 contains a 1:10 dilution of the Sepharose slurry. M is the protein marker 85059 (Bio-Rad, Hemel Hempstead, UK).
This is shown in schematic form on the right.

It was proposed that not enough time had been allowed for the antibody to bind the antigen. In order to explore this, 5 ml PCC was loaded onto the column and left in place with the column upright at 4°C overnight. Washing and elution was performed as before. Chromogenic assay results showed that all FX was in the flow and the wash, confirming that antigen had remained unbound to the column.
The final step in the purification protocol is hydroxylapatite chromatography. This was not attempted as the immunoaffinity had been unsuccessful.

8.6 Discussion

Arg-1 and other amino acids around the cleavage site are conserved in vitamin K-dependent glycoproteins (Stanley *et al* 1999). A sequence alignment of these proteins is shown in **figure 8.8**. Originally it was thought that cleavage was carried out by Furin, which recognises the motif Arg^{P4}-X^{P3}-Lys/Arg^{P2}-Arg^{P1}, where X is any residue and cleavage occurs after the carboxy-terminal Arg (Hosaka *et al* 1991). However, more recently it has been demonstrated that FX propeptide removal occurred in Furin-deficient cells and so must be mediated by some other process or by one of the other family members (Himmelspach *et al* 2000).
Work by Bristol *et al* showed the importance of the residues around the cleavage site in factor IX for the activity of the peptidase enzyme. He concluded that a basic residue is mandatory at the P1 position and that Arginine is preferred (Bristol *et al* 1993). A basic residue is preferred at position P2, although he noted that there is cleavage of the wildtype FX propeptide which has Threonine at P2 (see **figure 8.8**).
This work was followed by Rudolph *et al* who found that stable *in vitro* cell lines failed to remove the entire propeptide from the secreted recombinant FX protein. Cleavage was most efficient when the P1 residue is Arginine, P2 residue is Lysine or Arginine, and P4 residue is Arginine (Rudolph *et al* 1997).

Himmelspach and co-workers looked at the effect of modulation of γ-carboxylation and/or Furin-mediated proteolytic processing on *in vitro* expression of FX (Himmelspach *et al* 2000). A combination of conditions including the presence of vitamin K, the presence of warfarin and the presence of recombinant Furin in the culture medium of cells expressing FX were used. It was concluded that four isoforms of the FX light chain exist (which were named LC1 to LC4). These

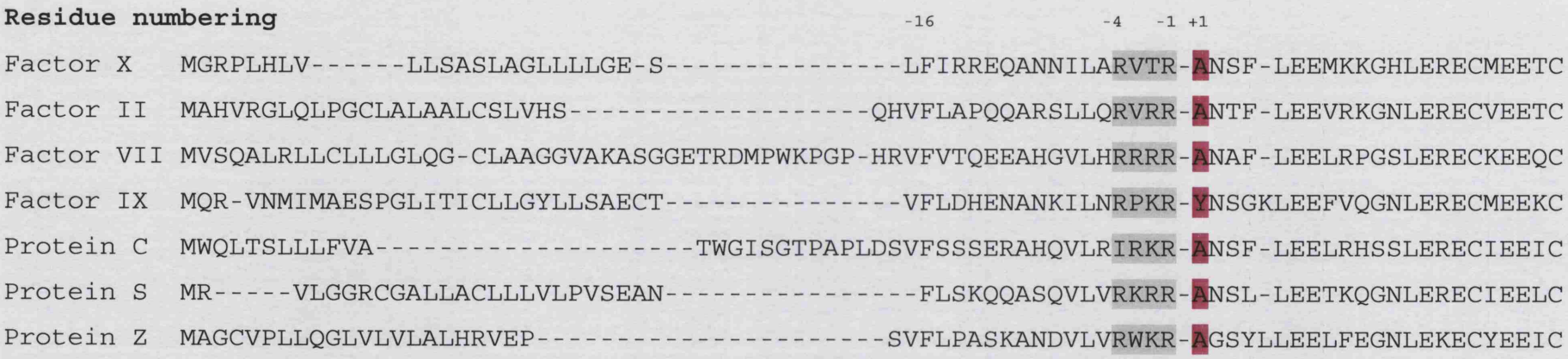


Figure 8.8. Sequence alignment of vitamin K-dependent glycoproteins

Parts of the N-termini of human vitamin K-dependent proteins are shown. The conventional numbering is adhered to: the mature protein starts at +1 and the propeptide is N-terminal to this. The SWISSPROT accession codes from top to bottom are P00742, P00734, P08709, P00740 , P04070, P07225 and P22891. The +1 residue of the mature protein is highlighted in pink, immediately C-terminal to the propeptide cleavage site. The four residues of the propeptide endoprotease recognition site are shown in grey (-1 to -4). This demonstrates that in the cases of both FX and Protein C, cleavage of the propeptide from the mature protein occurs despite the lack of the complete Arg-X-Lys/Arg-Arg motif.

isoforms migrate at varying speeds in a polyacrylamide gel, with LC1 migrating slowest and LC4 migrating quickest. Characterization of the light chain isoforms by N-terminal protein sequencing and expression in media containing vitamin K or containing warfarin revealed that LC1 represented fully-carboxylated but propeptide-containing molecules; LC2 represented fully-carboxylated and fully-processed molecules; LC3 represented under-carboxylated and propeptide-containing molecules; LC4 represented under-carboxylated but propeptide-free molecules. Only supernatants containing LC2 exhibited detectable functional activity, and plasma-derived FX light chain electrophoretic migration corresponded to that of LC2; both of these observations indicate that LC2 is the physiologically relevant light chain molecule.

From **figure 8.6**, lanes 1 and 2 contain the wildtype FX molecules and so would be predicted to contain isoforms which are fully-processed. According to Himmelspach and co-workers, these should be LC2 and LC4. Lanes 3 and 4 contain the Arg-1Thr mutants in which it is predicted that propeptide cleavage has been abolished. According to Himmelspach, LC 1 and LC3 are propeptide-containing isoforms.
If the expression system was perfect, it would be expected that F10-1R-2R (lane 1) would produce only propeptide-free isoforms. F10-1T-2R (lane 3) and F10-1T-2T (lane 4) should contain only propeptide-containing isoforms. It is known that F10-1R-2T (lane 2) is likely to suffer from inefficient processing, so a mixture of propeptide-free and propeptide-containing isoforms would be expected.
An abnormal propeptide may interfere with γ-carboxylation and so result in production of isoform LC3 and/or LC4. However it would be predicted that F10-1R-2R (lane 1) would be carboxylated normally and so should produce LC2 isoform only. With the known inefficiency of propeptide processing associated with Thr-2, F10-1R-2T (lane 2) may not produce fully-carboxylated light chains.

Several mutations at the P1 site of the vitamin K-dependent coagulation proteins have been described in the other proteins, but Arg-1Thr is the first to be described in FX. This mutation has an unusual laboratory phenotype, with low coagulation assay levels, but a chromogenic assay which is commensurate with the normal antigen level. The coagulation assay levels are all <1% of normal. The patient suffered a severe bleeding phenotype which is concordant with this.

A possible explanation for the laboratory phenotype of the mutant is as follows:
If the propeptide cleavage site is obliterated, then the mature protein may circulate with the propeptide attached. This would impair the Ca^{2+}-dependent folding of the Gla domain. If the Gla domain is unable to bind to the phospholipid surface, then this would account for the reduced coagulant properties of the mutant. The chromogenic assay measures the liberation of FXa upon cleavage of the Arg194-Ile195 bond. This should not be affected by an abnormal conformation in the Gla domain.
Alternatively, a mutation in the propeptide may impair γ-carboxylation of the Gla residues, because it is known that the propeptide contains the recognition site for the γ-carboxylase enzyme. Impaired γ-carboxylation would not affect the liberation of FXa and so the chromogenic assay would be unaffected. Direct γ-carboxylation analysis of the proteins is possible (Camire *et al* 2000) and should give information on the number of carboxylated Glu residues.
However, the second hypothesis is unlikely to be true because it is known that warfarin acts by inhibiting the action of the enzyme vitamin K epoxide reductase. This means that γ-carboxylation of the Gla residues is impaired and PIVKAs are produced. PIVKAs have low coagulation assays. However, the chromogenic FX assay can be used to monitor patients on warfarin therapy (Erskine *et al* 1982). If the second hypothesis was correct, then the chromogenic assay would be unaffected by coumarin drugs and so would not be a sensitive assay for monitoring oral anticoagulant treatment.
In order to determine whether reduced γ-carboxylation and/or incorrect propeptide processing accounted for the laboratory phenotype, *in vitro* expression was performed. Wildtype and mutant recombinant proteins were produced by HEK 293 cells.
The results were disappointing. Recombinant FX proteins which mimicked the assay results of the wildtype and the Arg-1Thr mutant were not obtained. Post-translational modification takes many forms in FX: Asparagine-linked glycosylation at Asn181 and Asn191, Aspartate hydroxylation at Asp63, removal of the signal peptide for translocation of polypeptide into endoplasmic reticulum, removal of the propeptide (which directs vitamin K-dependent γ-carboxylation) and γ-carboxylation of 11 Glutamate residues to Gla. Failure of any of these mechanisms could result in a dysfunctional protein.

9 *In vitro* Expression of Other Recombinant FX Mutant Proteins

9.1 Introduction

Production of recombinant mutant proteins can be used to provide confirmation that a mutation which has been identified in a particular gene is causative. Provided that no other mutations occur in either the native gene or the plasmid used *in vitro*, then recapitulation of the *in vivo* data effectively confirms that the identified mutation is causative. If enough recombinant protein is produced and purified, then biochemical characterization of the mutant can be performed.

9.2 Mutations Selected for Expression

The mutation identified in kindred D (Glu51Lys) was selected for stable transfection. This mutation presents an unusual laboratory phenotype: all assay results are within the reference range except for the FX:PT assay. This lead to the hypothesis that the site of the mutation is involved in the binding of the *extrinsic Xase* complex to FX. Molecular modelling predicted that this is indeed the case. Stable expression of the mutant protein in cell culture was performed in an attempt to carry out biochemical characterization of the mutant.

The residue substitution identified in kindred J and kindred K (Asp373Asn) produces a type II laboratory phenotype. Molecular modelling predicted that Asp373 is an important residue in the sodium-binding pocket of FX. A mutation here would be predicted to disrupt sodium-binding and produce a dysfunctional molecule. Transient expression of the mutant protein in cell culture was performed in order to recapitulate the *in vivo* data.

Genotype analysis of DNA from members of kindred H revealed two mutations in the *F10* gene: a heterozygous nonsense mutation in exon 6 and a heterozygous missense mutation in exon 8. The nonsense mutation would be predicted to produce a truncated protein or no protein at all. It would be predicted that all the FX protein (and activity) would be the result of translation of the other allele (without the stop codon). From inspection of the crystal structure of the molecule, the missense

mutation (Arg273Trp) in exon 8 does not appear to have any structural implications for the protein. The laboratory assays revealed that the specific activity by APTT assay was much lower than that by PT assay. It was concluded that Arg273 is involved in the interaction of the *intrinsic Xase* complex with FX. Transient expression of the mutant protein in cell culture was performed in order to confirm the disparity between the specific activities.

9.3 Plasmid Mutagenesis

The vector pCDNA4/TO F10-1R-2R was used as the starting vector for stable expression of the mutation found in kindred A (Glu51Lys). The vector pCMV4 F10-1R-2R was used as the starting vector for transient expression of the mutations found in kindred C (Arg273Trp), kindred G (Asp373Asn) and kindred H (Asp373Asn). The sequences of the forward (FP) and reverse (RP) mutagenesis primers for creation of the three mutant plasmids are shown in **table 28.**

Table 28 Mutagenesis primers for E51K, R273W and D373N

Plasmid	Primer	Primer Sequence (5' to 3')
F10_E51K	FP	GATGGCGACCAGTGTAAGACTAGTCCTTGCCAG
	RP	CTGGCAAGGACTAGTCTTACACTGGTCGCCATC
F10_R273W	FP	GTGGTCATCAAGCACAACTGGTTCACAAAGGAGACC
	RP	CACCAGTAGTTCGTGTTGACCAAGTGTTTCCTCTGG
F10_D373N	FP	GACACCAAGCAGGAGAATGCCTGCCAGGGG
	RP	CTGTGGTTCGTCCTCGGACGGACGGTCCCC

Mutagenesis was performed as described in **section 7.3.** The presence of the desired mutations and the absence of other mutations were verified by direct sequencing.

9.4 Transfection

Both transient and stable transfection was carried out using DMRIE-C reagent (Invitrogen, Paisley, Scotland) according to the manufacturer's protocol. For each 35 mm plate, 2 μg plasmid DNA and 5 μl DMRIE-C were used. Zeocin was used to

select cells transfected with pCDNA4/TO F10_E51K and resistant colonies were picked approximately three weeks after transfection.

9.5 Harvesting of Supernatants

Supernatants were harvested in four aliquots of 400 µl. These were centrifuged at 13000 rpm and any cell debris was discarded. These were snap-frozen and FX assays were performed within a week.

9.6 Results

9.6.1 Glu51Lys mutation

Positive ELISA results on the conditioned media revealed that the cells had been successfully transfected and were producing recombinant FX protein. No FX antigen could be detected in the supernatant from the mock transfection. However this "blank" medium was able to clot FX deficient plasma in both APTT and PT based FX assays. The results are shown in **table 29**.

Table 29 Coagulation assay results for Glu51Lys mutation

Well	FX:PT	FX:APTT
Mock	9.5	6.5
Mean E51K	13.5	10.5
SD E51K	0.7	3.2

Figures are expressed as % of the FX level in normal plasma (100%).

Table 29 shows that the background FX activity level was high.

In order to show the effect that FBS had on the FX levels in the media, assays were performed on the supernatants of cells which were grown in both serum-free and serum-containing media. HEK 293 cells which had been stably transfected with the plasmid pCDNA4/TO F10-1R-2R were seeded into 35mm plates. Once the cells were 80% confluent, fresh media was added (either serum-containing or serum-free).

48 hours later, the supernatants were harvested and the FX assays performed. The results are shown graphically in **figure 9.1**.

The graph shows that the specific activities for serum-free media are approximately half those of the serum-containing media. This is true for all assay methods apart from FX:RVV. This demonstrates that the FBS-containing medium has intrinsic coagulant activity in FX clotting assays. For a true reflection of the coagulant activity of any recombinant FX protein produced by transfection, the assays should be performed on supernatants from cells grown in serum-free media.

Following these results, further work was performed with cells transfected with plasmid pCDNA4/TO F10_E51K. Again, transfected cells were seeded into 35mm plates. Once the cells were 80% confluent, the media were changed to serum-free media. 48 hours later, the supernatants were harvested and the FX assays performed. The results are shown graphically in **figure 9.2**.

The specific activities by FX:PT are low. These data concur with the *in vivo* results from the plasma of the propositus of kindred D. However, all the other specific activities are also low. The RVV assay result is higher than the others, but this concurs with the data on the plasmid pCDNA4/TO F10-1R-2R shown in **figure 9.1**. The cells have produced FX protein, but it is dysfunctional.

9.6.2 Arg273Trp mutation and Asp373Asn mutation

Transient transfection was carried out with the mutagenised plasmids. In order to recapitulate the *in vivo* data for the heterozygous patients, aliquots of cells were transfected with a 50:50 mixture of DNA from plasmid pCMV4 F10-1R-2R and from the mutagenised plasmid, as described by (Hilbert *et al* 2002). Separate transfections were also performed with wildtype and homozygous mutant plasmid DNA. The cells were grown to 80% confluence and then the serum-containing media was replaced with serum-free media. The supernatants were harvested 48 hours later. All plates contained FX protein, as measured by ELISA (concentrations varying between 2.5-5 μg/ml). However, all activity assay results were <1% of the plasma concentration, except for FX:RVV of the supernatants from the homozygous wildtype plasmid pCMV4 F10-1R-2R. These supernatants gave a mean specific activity result for FX:RVV of 0.08 with a standard deviation of 0.02. This indicates that the cells have produced recombinant FX protein, but that it is dysfunctional.

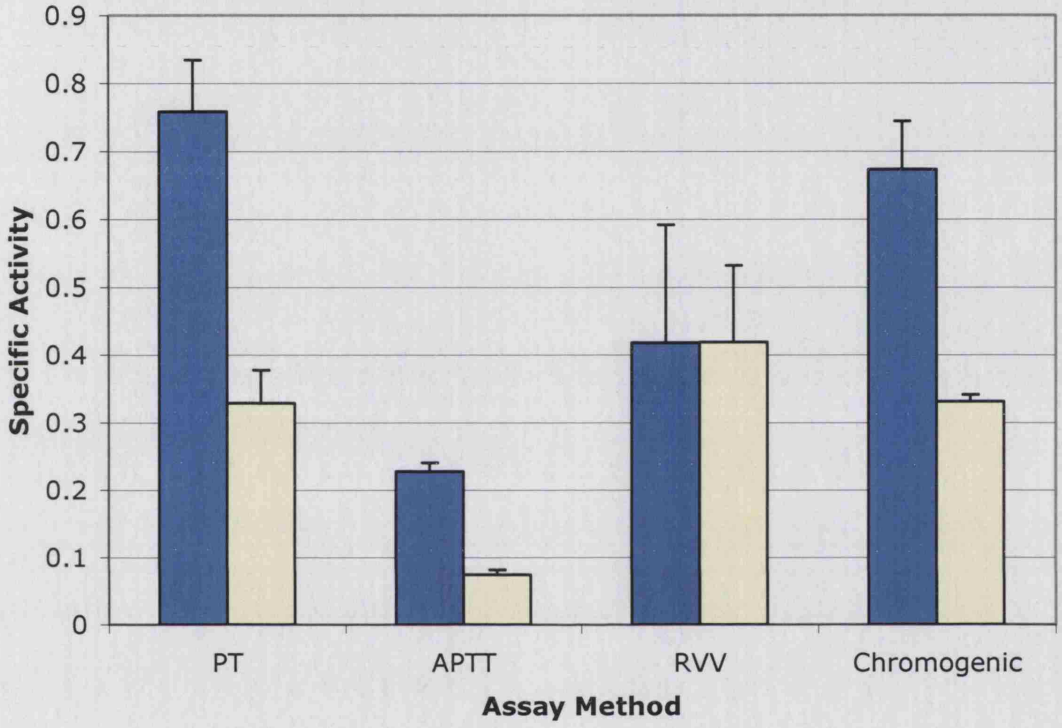


Figure 9.1. Specific activities of the supernatants of cells transfected with plasmid pCDNA4/TO F10-1R-2R. Supernatants from cells maintained in serum-containing media are shown in blue. Supernatants from cells maintained in serum-free media are shown in yellow. Assays were performed on the conditioned media from six separate tissue culture dishes. Error bars indicate standard deviation from the mean.

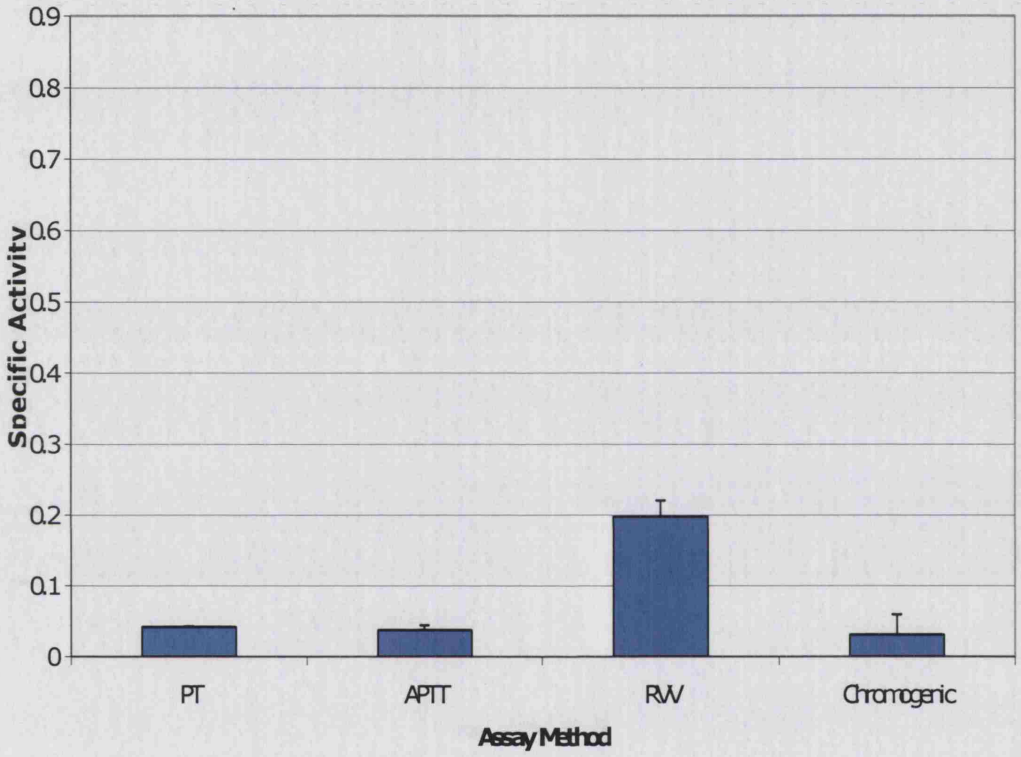


Figure 9.2. Specific activities of the serum-free supernatants of cells transfected with plasmid pCDNA4/TO E51K. Assays were performed on the conditioned media from six separate tissue culture dishes. Error bars indicate standard deviation from the mean.

9.7 Discussion

No expression system is perfect and it is known that the specific activities for recombinant proteins are often lower than those of their naturally-occurring counterparts. This is thought to be related to problems in post-translational modification of the recombinant proteins in cell culture. For example, γ-carboxylation, propeptide cleavage or processing from the single-chain molecule to the two-chain species may be impaired.

Initial work on the expression of FX was performed using BHK cells (Rudolph *et al* 1997). It had been previously shown that BHK cells efficiently express the homologous coagulation protein, factor VII (Nakagaki *et al* 1991). However, the specific activity of BHK-derived recombinant FX by coagulant assay was found to be approximately half that of plasma-derived FX. This was found to be due to poor γ-carboxylation of FX by BHK cells. When FX was expressed in HEK 293 cells, it was found to be fully functional (Rudolph *et al* 1997). Assuming that the cell line used for the work in this thesis is HEK 293, then there should be no reason why the recombinant FX produced would be dysfunctional.

Sub-optimal purity of plasmid DNA may lead to poor transfection. However the ratio of the Optical Density measured at 260nm and 280nm was between 1.8 and 1.95, indicating a sufficiently high degree of purity.

The transfection parameters were not optimised for each experiment: 2 μg plasmid DNA and 5 μl DMRIE-C was used for each 35mm plate. This may result in reduced efficiency of transfection, but should not affect post-translational modification of the recombinant protein.

If there was another mutation in the coding region of FX in the plasmid, then this could result in dysfunctional protein. This was excluded by direct sequencing of the plasmid.

To check whether post-translational modification was normal, the recombinant protein could be assayed for γ-carboxylation, glycosylation and hydroxylation. Western blots could be run to detect whether the single-chain form of FX has been processed to the two-chain form. N-terminal amino acid sequencing could be performed to detect if cleavage of the propeptide has occurred.

10 Conclusions

10.1 Diagnosis and Classification of FX deficiency Based on Phenotype Assays

Family histories and pedigrees are important when investigating possible bleeding diatheses. For example, Haemophilia A and B are inherited in an X-linked fashion, von Willebrand Disease can be inherited in either an autosomal dominant or recessive manner. Traditionally, the other inherited coagulation disorders (including FX deficiency) are described as showing autosomal recessive inheritance. Individuals who are heterozygous for FX deficiency are usually clinically asymptomatic (Graham *et al* 1957). However, in the course of this work several heterozygotes were found to have a significant bleeding tendency (for example individuals J:II:2, J:III:1, J:III:4 and K:II:1). This does not concur with a recessive pattern of inheritance in which an abnormal gene from each parent is required to cause the disease and people with only one abnormal gene do not exhibit the disease. It is not known why these heterozygotes present a bleeding phenotype. They all have FX levels which are above the suggested haemostatic threshold (as do individuals K:III:1, H:II:4 and the propositus in kindred D). Previously it was believed that factor levels of 10-20 iu/dL were sufficient for haemostasis (Knight *et al* 1985). More recently it has been suggested that a FX level of 5 iu/dl is sufficient to maintain haemostasis (McMahon *et al* 2002).

It is possible that the mutant FX protein inhibits one of the reactions in the coagulation pathway or that the wildtype allele does not result in sufficient FX enzymatic activity.

A reference range of normal values implies that affected individuals should fall outwith the range. The use of the historical range for FX (50-150 iu/dl) has usually not been a problem as most clinical bleeders have had low levels of FX (<10 iu/dl for example). However the examples of individuals J:II:2 and K:II:1, in whom the levels are only marginally below the lower limit of normal, show that this is not always the case. It is likely that the historical range is not valid and this underlines the importance of establishing a local reference range for the population studied.

Mutations in enzymes are often classified as type I or type II (as discussed in **section 1.10.3**). If the antigen level of a protein/coagulation factor can be measured by a suitable immunological assay and the activity by a suitable functional assay, then this can be a useful way to distinguish mutations which produce a dysfunctional molecule from those which result in a molecule which is not secreted.

Immunological assays rely on an antibody binding to the antigen (for example FX). The epitope recognised by a monoclonal antibody to FX may be perturbed by a particular mutation, so the assay may not be sensitive to the FX protein in the sample. This would underestimate the antigen concentration in the sample and may result in the incorrect classification of a type II mutation as a type I mutation. In the course of this work on FX, this problem was circumvented by the use of an in-house ELISA using a polyclonal antibody.

The work in this thesis demonstrates that the type I and type II classification system is of limited value in defining the phenotype of FX deficiency. There are four functional assays for FX. If all four assays are concordant, then it would be possible to categorise mutations as type I or type II. However in the cases of several mutations described in **chapters 4, 5 and 8**, there is a disparity between the functional assays. A more complex classification system has been devised in which FX mutants are divided into 12 classes and subclasses (Fair and Edgington 1985). However it is not simple to use and it is likely that an easier system will be proposed when more is known about the molecular basis of individual mutations.

In the case of the Arg-1Thr mutation described in **chapter 8**, the assay results indicate a classical type II mutation with normal FX:Ag and low clotting assay results. However, the chromogenic assay result is concordant with FX:Ag. This demonstrates the importance of not using a chromogenic assay as the sole assay for FX deficient patients. Had the chromogenic assay been used alone, then the diagnosis of FX deficiency would have been missed.

The Glu51Lys mutation described in **section 4.6** also demonstrates discrepancy. All five assay results fall within the reference range except for FX:PT. If the PT-based assay had not been performed then FX deficiency would not have been diagnosed. Furthermore, the work in **section 4.6.4** shows that the use of different thromboplastins can have a profound effect on the PT. Those performing routine testing should be aware of this. This has been demonstrated previously in a factor

VII variant (Girolami *et al* 1978); however there is no description in the literature of this occurring with a FX mutant.

Patients J:I:2 and J:II:1 are heterozygous for the Asp373Asn mutation (**section 5.5**). All their assay levels are within the historical reference ranges apart from FX:APTT. The diagnosis of FX deficiency would have been missed had the APTT-based assay not been performed.

The Arg273Trp mutation described in **section 5.4** results in discrepant laboratory assays. Although all five assay results fall below their reference ranges, the specific activity by APTT assay is much lower than that by FX:PT or FX:RVV. The severity of the FX deficiency would not have been appreciated if the FX:APTT had not been performed.

A telephone survey of the 25 comprehensive care haemophilia centres in England, Wales and Scotland conducted for this thesis revealed that only two centres routinely perform both FX:PT and FX:APTT in the diagnosis of FX deficiency. 19 centres use a single assay alone (either FX:PT, FX:APTT or FX:RVV). The remaining four centres routinely carry out either FX:PT or FX:APTT but would perform an additional assay if it was deemed necessary by a clinician. From the disparity in the levels demonstrated by the work in this thesis, a FX assay by one method alone is not sufficient for diagnosis.

10.2 Mutational Analysis

62 point mutations in *F10* have been described in the literature at the present time (October 2004). In the course of this work, genotype analysis was performed on samples from ten kindred with FX deficiency. A total of ten mutations were identified, nine of which were novel.

It is assumed that mutations in genes occur at random sites. If all mutations are clinically apparent, then the identified point mutations in any gene should be evenly spaced throughout the coding region. In general this is true for *F10* (for example 35 of the 62 missense mutations that have been reported in *F10* are located within exons 7 and 8. These exons encode 253 of the 488 residues in FX). However, so far 12 mutations have been described in exon 2 which encodes only 54 residues. Of the ten Gla residues encoded by exon 2, mutations have been reported in seven of them.

The dinucleotide CpG is a "hotspot" for mutation in the human genome as a result of methylation-mediated deamination (for example when Cytosine is methylated at 5' position, it has the propensity to undergo spontaneous deamination to Thymidine) (Cooper and Krawczak 1989). The CpG dinucleotide only occurs at the site of three of the Gla residues (Glu14, Glu25 and Glu29). Three mutations have been reported in these residues: Glu14Gly, Glu14Lys and Glu25Lys. In both cases of the Gla to Lysine substitution, Cytosine has indeed mutated to Thymidine. However, this only represents two mutations of ten so far described in the Gla residues (including the Glu19Val mutation identified in the course of the work for this thesis). It seems unlikely that Glutamic acid residues are particularly prone to mutation, but a more plausible explanation is that a mutation in a Gla residue has a high propensity to affect the coagulation function of the protein and so cause a bleeding tendency, whereas a mutation elsewhere may be clinically silent.

The Asp373Asn (GAT-AAT) mutation was identified in kindred J and kindred K. As far as could be ascertained, these families are unrelated. This mutation does not occur at the site of a CpG dinucleotide. It is most likely that the occurrence of the same nucleotide substitution in two kindred was by chance.

Mutations at the P1 site of other vitamin K-dependent proteins have been reported (see **table 23**). However Arg-1Thr is the first P1 mutation identified in FX. It is predicted that the mutation results in altered propeptide cleavage. In addition, γ-carboxylation may be affected.

FX-/- knockout mice have been shown to present a lethal phenotype with death from intraabdominal, subcutaneous, or intracranial bleeding. No homozygous deletions or insertions have been reported in FX, unlike the case of FVII in which a 2 bp deletion has been described (Peyvandi *et al* 2000b). These are consistent with the hypothesis that a complete absence of FX is fatal (Dewerchin *et al* 2000). However, there is a single report of a homozygous nonsense mutation (Zivelin *et al* 2003). It is unclear why this mutation does not present a lethal phenotype. It is possible that there is an *in vivo* correction mechanism which results in the production of enough FX to prevent embryonic lethality. Transcriptional errors restoring the reading frame and/or ribosomal slippage during translation have resulted in partial correction of the molecular defect in hypobetalipoproteinaemia and Haemophilia A (Linton *et al* 1992; Young *et al* 1997). A second mutation within the gene might exist to correct a frameshift resulting from a deletion or insertion, but this could not correct a nonsense

mutation. An alternative mechanism may exist to correct the clinical phenotype. This might involve the activation of coagulation through a novel serine protease but at present this is speculation.

The mutations identified in the course of this work are unlikely to be polymorphisms. Most have not been identified before and none are listed in any online polymorphism databases.

10.3 Structure/Function Correlation

Prediction of the effect that the mutation would have on the phenotype of the protein was performed. Molecular modelling aids this prediction when the crystal structure of the protein has been solved. The published crystal structures of the human proteins FXa, TF, factor VIIa and thrombin as well as the structure of rabbit TF were available in the PDB. Five of the mutations identified in the course of this work fall within the available crystal structure of FXa. Energy minimisation, simulated annealing and homology modelling were performed to predict the effect of the mutations on the structure and function of the molecule. Explanations in terms of the perturbation of the structure itself or its biochemical function were proposed in all cases.

It was proposed that the Glu51Lys mutation disrupted the interaction of FX with TF of the *extrinsic Xase* complex (**section 4.6**). Gly222Asp mutation was predicted to disrupt the correct folding of the molecule as the protein core would be unlikely to tolerate a charged hydrophilic residue (**section 5.2**). The explanation for the phenotype of the Arg273Trp mutant rested on the observed disparity between the specific activity by FX:APTT compared with that by FX:PT and FX:APTT. It was proposed that this mutation impaired the binding of the *intrinsic Xase* complex to FX (**section 5.4**). In the case of the Asp373Asn mutant, it was proposed that perturbation of the sodium-binding site would impair the enzymatic function of the molecule and result in a dysfunctional protein (**section 5.5**). It was predicted that the Pro382Leu mutation would disrupt protein folding because the lack of the Proline residue would impair the kinetic folding pathway of the molecule (**section 5.3**).

For the mutations in splice sites (IVS5+3 A-G and IVS1-1 G-C), in the propeptide (Arg-1Thr) and in the signal peptide (Ala-26Asp), internet-based prediction programs were used to predict alterations in the cleavage sites.

10.4 In vitro expression

Attempts to establish a causal link between the nucleotide substitution identified and the FX phenotype were made by *in vitro* expression of recombinant mutants in cell culture. The results of this expression work were disappointing. Recapitulation of the *in vivo* assay results with the conditioned media containing recombinant FX proteins was not possible. FX protein was produced following transfection of mammalian cells, but the protein was dysfunctional. The cell line HEK293 was chosen because previous work had shown it to be capable of producing fully-functional FX protein (Rudolph *et al* 1997).

Dysfunctional protein may be the result of poor post-translational modification. It is possible that the cells were not derived from HEK293, but from another cell line which cannot modify recombinant FX satisfactorily. Alternatively the cells may have lost their ability to perform one or more of the various modifications (such as γ-carboxylation, β-hydroxylation and removal of the signal-peptide and propeptide). It is known that cells derived from immortalised cell lines are prone to become "tired" and perform less well after approximately 50 generations of passage. The cells used for these experiments were not used beyond 20 passages so this explanation is unlikely.

It was also disappointing that recombinant FX could not be purified from conditioned media despite the use of a published protocol (Larson *et al* 1998). It was shown that the FX antibody had bound to the immunoaffinity column, but FX protein would not bind to the column. The FX:Ag assay (ELISA) uses the same antibody to bind FX on a microtitre plate, so antibody: antigen binding should not be problematic. It is possible that the particular column used was not optimal for FX antibody: antigen interactions.

FX assays on conditioned media should reflect the FX assays performed on plasma. The FX:RVV assays in this work generally gave higher results than the other functional assays. FX:RVV is usually thought of as specific for FX, however RVV has also been shown to activate Protein C and factor IX (Jackson 1984). It was

postulated that the FBS in the conditioned media could contain a non-FX substrate for activation by RVV. This was discounted by results demonstrating that the specific activity by FX:RVV did not change whether the media collected were serum-free or serum-containing (see **figure 9.1**).

10.5 Conclusion

Patients who are severely deficient in FX (with a reduced procoagulant activity and antigen level) are severe bleeders; they require FX replacement therapy. Prenatal diagnosis should be considered in pregnancies from families with at least one member affected by this coagulation disorder; knowledge of the underlying mutation is essential to achieve this goal. Molecular modelling is an invaluable tool for correlating structure with function when mutations have been identified at the molecular level.

References

Adelstein, R.S. & Conti, M.A. (1975) Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity. *Nature,* **256,** 597-598.

Ahmad, S.S., Rawala-Sheikh, R. & Walsh, P.N. (1989) Comparative interactions of factor IX and factor IXa with human platelets. *Journal of Biological Chemistry,* **264,** 3244-3251.

Akhavan, S., Mannucci, P.M., Lak, M., Mancuso, G., Mazzucconi, M.G., Rocino, A., Jenkins, P.V. & Perkins, S.J. (2000) Identification and three-dimensional structural analysis of nine novel mutations in patients with prothrombin deficiency. *Thrombosis and Haemostasis,* **84,** 989-997.

Allen, G.A., Monroe, D.M., 3rd, Roberts, H.R. & Hoffman, M. (2000) The effect of factor X level on thrombin generation and the procoagulant effect of activated factor VII in a cell-based model of coagulation. *Blood Coagulation and Fibrinolysis,* **11 Suppl 1,** S3-7.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* **25,** 3389-3402.

Amphlett, G.W., Byrne, R. & Castellino, F.J. (1982) Cation binding properties of the multiple subforms of RVV-X, the coagulant protein from Vipera russelli. *Biochemistry,* **21,** 125-132.

Andersson, S., Davis, D.L., Dahlback, H., Jornvall, H. & Russell, D.W. (1989) Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *Journal of Biological Chemistry,* **264,** 8222-8229.

Arbini, A.A., Mannucci, M. & Bauer, K.A. (1996) A Thr359Met mutation in factor VII of a patient with a hereditary deficiency causes defective secretion of the molecule. *Blood,* **87,** 5085-5094.

Arnold, A.L., Mitchell, M.J., Cutler, J.A. & Savidge, G.F. (2003) Identification of four novel mutations in patients with FX deficiency, implications for future mutation screening. *British Journal of Haematology,* **121 (s1),** 21.

Ashrani, A.A., Aysola, A., Al-Khatib, H., Nichols, W.L. & Key, N.S. (2003) Lupus anticoagulant associated with transient severe factor X deficiency: a report of two patients presenting with major bleeding complications. *British Journal of Haematology,* **121,** 639-642.

Au, W.Y., Lam, C.C., Cheung, W.C. & Kwong, Y.L. (2004) Two novel factor X gene mutations in a Chinese family with factor X deficiency. *Annals of Hematology,* **83,** 304-306.

Auch, D. & Reth, M. (1990) Exon trap cloning: using PCR to rapidly detect and clone exons from genomic DNA fragments. *Nucleic Acids Res,* **18,** 6743-6744.

Augustin, H.G., Kozian, D.H. & Johnson, R.C. (1994) Differentiation of endothelial cells: analysis of the constitutive and activated endothelial cell phenotypes. *Bioessays,* **16,** 901-906.

Bagnall, R.D., Waseem, N.H., Green, P.M., Colvin, B., Lee, C. & Giannelli, F. (1999) Creation of a novel donor splice site in intron 1 of the factor VIII gene leads to activation of a 191 bp cryptic exon in two haemophilia A patients. *British Journal of Haematology,* **107,** 766-771.

Bahnak, B.R., Howk, R., Morrissey, J.H., Ricca, G.A., Edgington, T.S., Jaye, M.C., Drohan, W.W. & Fair, D.S. (1987) Steady state levels of factor X mRNA in liver and Hep G2 cells. *Blood,* **69,** 224-230.

Bailer-Jones, D.M. & Bailer-Jones, C.A. (2002) Modelling data: Analogies in neural networks, simulated annealing and genetic algorithms. In: *Model-Based Reasoning:Science, Technology, Values* (ed. by L. Magnani & N. Nersessian). Kluwer Academic/Plenum Publishers, New York.

Bajaj, M. & Blundell, T. (1984) Evolution and the tertiary structure of proteins. *Annu Rev Biophys Bioeng,* **13,** 453-492.

Bajaj, S.P. & Mann, K.G. (1973) Simultaneous purification of bovine prothrombin and factor X. Activation of prothrombin by trypsin-activated factor X. *Journal of Biological Chemistry,* **248,** 7729-7741.

Beardell, F.V., Varma, M. & Martinez, J. (1997) Normalization of plasma factor X levels in amyloidosis after plasma exchange. *American Journal of Hematology,* **54,** 68-71.

Bendtsen, J.D., Nielsen, H., von Heijne, G. & Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol,* **340,** 783-795.

Bennett, J.S. & Vilaire, G. (1979) Exposure of platelet fibrinogen receptors by ADP and epinephrine. *Journal of Clinical Investigation,* **64,** 1393-1401.

Blomback, B. (1996) Fibrinogen and fibrin--proteins with complex roles in hemostasis and thrombosis. *Thrombosis Research,* **83,** 1-75.

Blundell, T.L. & Johnson, L.N. (1986) *Protein Crystallography.* Academic Press, London.

Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S.R. & Hofsteenge, J. (1989) The refined 1.9 A crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *Embo J,* **8,** 3467-3475.

Boggio, L. & Green, D. (2001) Recombinant human factor VIIa in the management of amyloid-associated factor X deficiency. *British Journal of Haematology,* **112,** 1074-1075.

Bokarewa, M.I., Morrissey, J.H. & Tarkowski, A. (2002) Tissue factor as a proinflammatory agent. *Arthritis Res,* **4,** 190-195.

Bolton-Maggs, P.H., Perry, D.J., Chalmers, E.A., Parapia, L.A., Wilde, J.T., Williams, M.D., Collins, P.W., Kitchen, S., Dolan, G. & Mumford, A.D. (2004) The rare coagulation disorders - review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' Organisation. *Haemophilia,* **10,** 593-628.

Bom, V.J. & Bertina, R.M. (1990) The contributions of Ca2+, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII. *Biochemical Journal,* **265,** 327-336.

Bom, V.J., Reinalda-Poot, J.H., Cupers, R. & Bertina, R.M. (1990) Extrinsic activation of human blood coagulation factors IX and X. *Thrombosis and Haemostasis,* **63,** 224-230.

Brandstetter, H., Kuhne, A., Bode, W., Huber, R., von der Saal, W., Wirthensohn, K. & Engh, R.A. (1996) X-ray structure of active site-inhibited clotting factor Xa. Implications for drug design and substrate recognition. *Journal of Biological Chemistry,* **271,** 29988-29992.

Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. & Chambon, P. (1978) Ovalbumin gene: evidence for a leader sequence in mRNA and DNA

sequences at the exon-intron boundaries. *Proceedings of the National Academy of Sciences of the United States of America,* **75,** 4853-4857.
Bristol, J.A., Furie, B.C. & Furie, B. (1993) Propeptide processing during factor IX biosynthesis. Effect of point mutations adjacent to the propeptide cleavage site. *Journal of Biological Chemistry,* **268,** 7577-7584.
Broze, G.J., Jr., Warren, L.A., Novotny, W.F., Higuchi, D.A., Girard, J.J. & Miletich, J.P. (1988) The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action. *Blood,* **71,** 335-343.
Brummel, K.E., Butenas, S. & Mann, K.G. (1999) An integrated study of fibrinogen during blood coagulation. *Journal of Biological Chemistry,* **274,** 22862-22870.
Bruzzaniti, A., Goodge, K., Jay, P., Taviaux, S.A., Lam, M.H., Berta, P., Martin, T.J., Moseley, J.M. & Gillespie, M.T. (1996) PC8 [corrected], a new member of the convertase family. *Biochemical Journal,* **314 (Pt 3),** 727-731.
Busby, S.J., K.L., B., L.M., H., J.E., G. & A.A., K. (1988) Alteration of Propeptide Sequence Impairs Biological Activity of Human Factor VII. In: *Current Advances in Vitamin K Research* (ed. by J.W. Suttie), pp. 173-181. Elsevier Science Publishing Co., New York.
Bush, P.C. & Owen, W.G. (1982) Interactions of thrombin with endothelium. In: *Pathophysiology of the endothelial cell* (ed. by H.L. Nosse & H.J. Vogel), pp. 97-101. Academic Press, New York.
Butenas, S. & Mann, K.G. (2002) Blood coagulation. *Biochemistry,* **67,** 3-12.
Caimi, M.T., Redaelli, R., Cattaneo, D., Nosari, A.M., Baudo, F. & de Cataldo, F. (1991) Acquired selective factor X deficiency in acute nonlymphocytic leukemia. *American Journal of Hematology,* **36,** 65-66.
Camire, R.M., Larson, P.J., Stafford, D.W. & High, K.A. (2000) Enhanced gamma-carboxylation of recombinant factor X using a chimeric construct containing the prothrombin propeptide. *Biochemistry,* **39,** 14322-14329.
Camoriano, J.K., Greipp, P.R., Bayer, G.K. & Bowie, E.J. (1987) Resolution of acquired factor X deficiency and amyloidosis with melphalan and prednisone therapy. *New England Journal of Medicine,* **316,** 1133-1135.
Carter, C. & Winfield, D.A. (1988) Factor X deficiency during treatment of relapsed acute myeloid leukaemia with amsacrine. *Clinical and Laboratory Haematology,* **10,** 225-228.
Chelly, J., Concordet, J.P., Kaplan, J.C. & Kahn, A. (1989) Illegitimate transcription: transcription of any gene in any cell type. *Proceedings of the National Academy of Sciences of the United States of America,* **86,** 2617-2621.
Chen, C. & Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Molecular and Cellular Biology,* **7,** 2745-2752.
Chen, L., Manithody, C., Yang, L. & Rezaie, A.R. (2004) Zymogenic and enzymatic properties of the 70-80 loop mutants of factor X/Xa. *Protein Science,* **13,** 431-442.
Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry,* **162,** 156-159.
Choufani, E.B., Sanchorawala, V., Ernst, T., Quillen, K., Skinner, M., Wright, D.G. & Seldin, D.C. (2001) Acquired factor X deficiency in patients with amyloid light-chain amyloidosis: incidence, bleeding manifestations, and response to high- dose chemotherapy. *Blood,* **97,** 1885-1887.

Cines, D.B., Pollak, E.S., Buck, C.A., Loscalzo, J., Zimmerman, G.A., McEver, R.P., Pober, J.S., Wick, T.M., Konkle, B.A., Schwartz, B.S., Barnathan, E.S., McCrae, K.R., Hug, B.A., Schmidt, A.M. & Stern, D.M. (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood,* **91,** 3527-3561.

Collen, D. (1980) On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. *Thrombosis and Haemostasis,* **43,** 77-89.

Colman, R.W. (1990) Platelet receptors. *Hematology and Oncology Clinics of North America,* **4,** 27-42.

Colman, R.W., Clowes, A.W., George, J.N., Hirsch, J. & Marder, V.J. (2001) Overview of Hemostasis. In: *Hemostasis and Thrombosis: Basic Principles and Clinical Practice* (ed. by R.W. Colman, J. Hirsch, V.J. Marder, A.W. Clowes & J.N. George), pp. 3-16. Lippincott Williams and Wilkins, Philadelphia.

Colosimo, A., Goncz, K.K., Holmes, A.R., Kunzelmann, K., Novelli, G., Malone, R.W., Bennett, M.J. & Gruenert, D.C. (2000) Transfer and expression of foreign genes in mammalian cells. *Biotechniques,* **29,** 314-318, 320-312, 324 passim.

Condie, R.G. (1976) A serial study of coagulation factors XII, XI and X in plasma in normal pregnancy and in pregnancy complicated by pre-eclampsia. *British Journal of Obstetrics and Gynaecology,* **83,** 636-639.

Constam, D.B., Calfon, M. & Robertson, E.J. (1996) SPC4, SPC6, and the novel protease SPC7 are coexpressed with bone morphogenetic proteins at distinct sites during embryogenesis. *Journal of Cell Biology,* **134,** 181-191.

Cooper, D.N. & Krawczak, M. (1989) Cytosine methylation and the fate of CpG dinucleotides in vertebrate genomes. *Human Genetics,* **83,** 181-188.

Creighton, T.E. (1993) *Proteins: Structures and Molecular Properties.* W.H. Freeman, New York.

Dahlback, B. (1986) Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. *Journal of Biological Chemistry,* **261,** 12022-12027.

Datson, N.A., van de Vosse, E., Dauwerse, H.G., Bout, M., van Ommen, G.J. & den Dunnen, J.T. (1996) Scanning for genes in large genomic regions: cosmid-based exon trapping of multiple exons in a single product. *Nucleic Acids Res,* **24,** 1105-1111.

Dawson, S., Hamsten, A., Wiman, B., Henney, A. & Humphries, S. (1991) Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity. *Arteriosclerosis, Thrombosis, and Vascular Biology,* **11,** 183-190.

De Stefano, V., Leone, G., Ferrelli, R., Hassan, H.J., Macioce, G. & Bizzi, B. (1988) Factor X Roma: a congenital factor X variant defective at different degrees in the intrinsic and the extrinsic activation. *British Journal of Haematology,* **69,** 387-391.

de Visser, M.C., Poort, S.R., Vos, H.L., Rosendaal, F.R. & Bertina, R.M. (2001) Factor X levels, polymorphisms in the promoter region of factor X, and the risk of venous thrombosis. *Thrombosis and Haemostasis,* **85,** 1011-1017.

Deam, S., Uprichard, J., Eaton, J.T., Perkins, S.J., Dasani, H., Hanley, J.P. & Dolan, G. (in press) Two new factor X mutations associated with low activity and low antigen levels. *Thrombosis and Haemostasis.*

Deam, S., Uprichard, J., Eaton, J.T., Perkins, S.J. & Dolan, G. (2003) Factor X Leicester: Ile411Phe associated with a low antigen level and a disproportionately low functional activity of factor X. *J Thromb Haemost,* **1,** 603-605.

Dewerchin, M., Liang, Z., Moons, L., Carmeliet, P., Castellino, F.J., Collen, D. & Rosen, E.D. (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. *Thrombosis and Haemostasis,* **83,** 185-190.

Di Scipio, R.G., Hermodson, M.A., Yates, S.G. & Davie, E.W. (1977) A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S. *Biochemistry,* **16,** 698-706.

Diuguid, D.L., Rabiet, M.J., Furie, B.C. & Furie, B. (1989) Molecular defects of factor IX Chicago-2 (Arg 145----His) and prothrombin Madrid (Arg 271----cys): arginine mutations that preclude zymogen activation. *Blood,* **74,** 193-200.

Doolittle, R.F., Goldbaum, D.M. & Doolittle, L.R. (1978) Designation of sequences involved in the "coiled-coil" interdomainal connections in fibrinogen: constructions of an atomic scale model. *Journal of Molecular Biology,* **120,** 311-325.

Duckert, F., Fluckiger, P. & Koller, F. (1954) Le role de facteur X dans la formation de la thromboplastine sanguine. *Revue d'Hematologie.,* **9,** 489-492.

Duckert, P., Brunak, S. & Blom, N. (2004) Prediction of proprotein convertase cleavage sites. *Protein Eng Des Sel,* **17,** 107-112.

Duncan, E.M., Cole, J., Clarkson, A.R. & Lloyd, J.V. (1999) Poor recovery and short survival of infused factor X in a case of acquired factor X deficiency and amyloidosis. *Thrombosis and Haemostasis,* **82,** 1375-1376.

Eaton, D., Rodriguez, H. & Vehar, G.A. (1986) Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. *Biochemistry,* **25,** 505-512.

Engelke, D.R., Hoener, P.A. & Collins, F.S. (1988) Direct sequencing of enzymatically amplified human genomic DNA. *Proceedings of the National Academy of Sciences of the United States of America,* **85,** 544-548.

Erskine, J.G., Walker, I.D. & Davidson, J.F. (1982) Maintenance control of oral anticoagulant therapy by an automated chromogenic substrate assay of factor X. *Clin Lab Haematol,* **4,** 179-186.

Esmon, N.L., Owen, W.G. & Esmon, C.T. (1982) Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. *Journal of Biological Chemistry,* **257,** 859-864.

Etzioni, A., Doerschuk, C.M. & Harlan, J.M. (1999) Of man and mouse: leukocyte and endothelial adhesion molecule deficiencies. *Blood,* **94,** 3281-3288.

Fair, D.S. & Bahnak, B.R. (1984) Human hepatoma cells secrete single chain factor X, prothrombin, and antithrombin III. *Blood,* **64,** 194-204.

Fair, D.S. & Edgington, T.S. (1985) Heterogeneity of hereditary and acquired factor X deficiencies by combined immunochemical and functional analyses. *British Journal of Haematology,* **59,** 235-248.

Fair, D.S., Plow, E.F. & Edgington, T.S. (1979) Combined functional and immunochemical analysis of normal and abnormal human factor X. *Journal of Clinical Investigation,* **64,** 884-894.

Fujikawa, K., Coan, M.H., Legaz, M.E. & Davie, E.W. (1974) The mechanism of activation of bovine factor X (Stuart factor) by intrinsic and extrinsic pathways. *Biochemistry,* **13,** 5290-5299.

Fujikawa, K., Titani, K. & Davie, E.W. (1975) Activation of bovine factor X (Stuart factor): conversion of factor Xaalpha to factor Xabeta. *Proceedings of the National Academy of Sciences of the United States of America,* **72,** 3359-3363.

Fukami, M.H., Holmsen, H., Kowalska, M.A. & Niewiarowski, S. (2001) Platelet Secretion. In: *Hemostasis and Thrombosis: Basic Principles and Clinical Practice* (ed. by R.W. Colman, J. Hirsch, V.J. Marder, A.W. Clowes & J.N. George), pp. 561-573. Lippincott Williams and Wilkins, Philadelphia.

Fuller, R.S., Sterne, R.E. & Thorner, J. (1988) Enzymes required for yeast prohormone processing. *Annu Rev Physiol,* **50,** 345-362.

Fung, M.R., Hay, C.W. & MacGillivray, R.T. (1985) Characterization of an almost full-length cDNA coding for human blood coagulation factor X. *Proceedings of the National Academy of Sciences of the United States of America,* **82,** 3591-3595.

Furie, B. & Furie, B.C. (1988) The molecular basis of blood coagulation. *Cell,* **53,** 505-518.

Furie, B., Voo, L., McAdam, K.P. & Furie, B.C. (1981) Mechanism of factor X deficiency in systemic amyloidosis. *New England Journal of Medicine,* **304,** 827-830.

Gailani, D. & Broze, G.J., Jr. (1991) Factor XI activation in a revised model of blood coagulation. *Science,* **253,** 909-912.

Gallais, V., Bredoux, H., le Roux, G. & Laroche, L. (1996) Acquired and transient factor X deficiency associated with sodium valproate treatment. *European Journal of Haematology,* **57,** 330.

Gandrille, S., Borgel, D., Eschwege-Gufflet, V., Aillaud, M., Dreyfus, M., Matheron, C., Gaussem, P., Abgrall, J.F., Jude, B., Sie, P. & et al. (1995) Identification of 15 different candidate causal point mutations and three polymorphisms in 19 patients with protein S deficiency using a scanning method for the analysis of the protein S active gene. *Blood,* **85,** 130-138.

Gaussem, P., Gandrille, S., Duchemin, J., Emmerich, J., Alhenc-Gelas, M., Aillaud, M.F. & Aiach, M. (1994) Influence of six mutations of the protein C gene on the Gla domain conformation and calcium affinity. *Thrombosis and Haemostasis,* **71,** 748-754.

Girolami, A., Fabris, F., Dal Bo Zanon, R., Ghiotto, G. & Burul, A. (1978) Factor VII Padua: a congenital coagulation disorder due to an abnormal factor VII with a peculiar activation pattern. *Journal of Laboratory and Clinical Medicine,* **91,** 387-395.

Girolami, A., Molaro, G., Calligaris, A. & De Luca, G. (1970a) Severe congenital factor X deficiency in 5-month-old child. *Thrombosis et Diathesis Haemorrhagica,* **24,** 175-184.

Girolami, A., Molaro, G., Lazzarin, M., Scarpa, R. & Brunetti, A. (1970b) A "new" congenital haemorrhagic condition due to the presence of an abnormal factor X (factor X Friuli): study of a large kindred. *British Journal of Haematology,* **19,** 179-192.

Girolami, A., Simioni, P., Girolami, B., Marchiori, A., Millar, D.S., Bignell, P., Kakkar, V.V. & Cooper, D.N. (1993) A novel dysfunctional protein C (protein C Padua 2) associated with a thrombotic tendency: substitution of

Cys for Arg-1 results in a strongly reduced affinity for binding of Ca++. *British Journal of Haematology,* **85,** 521-527.
Girolami, A., Vianello, F., Cabrio, L. & Lombardi, A.M. (2004) A new mutation (Arg251Trp) in the Ca2+ binding site of factor X protease domain appears to be responsible for the defect in the extrinsic pathway activation of factor X Padua. *Clin Appl Thromb Hemost,* **10,** 5-8.
Glenner, G.G. (1977) Factor X deficiency and systemic amyloidosis. *New England Journal of Medicine,* **297,** 108-109.
Gordon, S.G. & Mielicki, W.P. (1997) Cancer procoagulant: a factor X activator, tumor marker and growth factor from malignant tissue. *Blood Coagulation and Fibrinolysis,* **8,** 73-86.
Graham, J.B., Barrow, E.M. & Hougie, C. (1957) Genetic aspects of a 'new' hemorrhagic state. *Journal of Clinical Investigation,* **36,** 497-503.
Graves, C.B., Munns, T.W., Willingham, A.K. & Strauss, A.W. (1982) Rat factor X is synthesized as a single chain precursor inducible by prothrombin fragments. *Journal of Biological Chemistry,* **257,** 13108-13113.
Green, F., Kelleher, C., Wilkes, H., Temple, A., Meade, T. & Humphries, S. (1991) A common genetic polymorphism associated with lower coagulation factor VII levels in healthy individuals. *Arteriosclerosis, Thrombosis, and Vascular Biology,* **11,** 540-546.
Green, P.M., Rowley, G. & Giannelli, F. (2003) Unusual expression of the F9 gene in peripheral lymphocytes hinders investigation of F9 mRNA in hemophilia B patients. *J Thromb Haemost,* **1,** 2675-2676.
Greipp, P.R., Kyle, R.A. & Bowie, E.J. (1979) Factor X deficiency in primary amyloidosis: resolution after splenectomy. *New England Journal of Medicine,* **301,** 1050-1051.
Haslam, R.J., Davidson, M.M., Fox, J.E. & Lynham, J.A. (1978) Cyclic nucleotides in platelet function. *Thrombosis and Haemostasis,* **40,** 232-240.
Hassan, H.J., Guerriero, R., Chelucci, C., Leonardi, A., Mattia, G., Leone, G., Mariani, G., Mannucci, P.M. & Peschle, C. (1988) Multiple polymorphic sites in factor X locus. *Blood,* **71,** 1353-1356.
Hay, C.W., Robertson, K.A., Fung, M.R. & MacGillivray, R.T. (1986) RFLPs for PstI and EcoRI in the human blood clotting factor X gene. *Nucleic Acids Research,* **14,** 5118.
Heeb, M.J., Espana, F., Geiger, M., Collen, D., Stump, D.C. & Griffin, J.H. (1987) Immunological identity of heparin-dependent plasma and urinary protein C inhibitor and plasminogen activator inhibitor-3. *Journal of Biological Chemistry,* **262,** 15813-15816.
Hemker, H.C., Veltkamp, J.J. & Loeliger, E.A. (1968) Kinetic aspects of the interaction of blood clotting enzymes. 3. Demonstration of an inhibitor of prothrombin conversion in vitamin K deficiency. *Thrombosis et Diathesis Haemorrhagica,* **19,** 346-363.
Herman, M.P., Sukhova, G.K., Kisiel, W., Foster, D., Kehry, M.R., Libby, P. & Schonbeck, U. (2001) Tissue factor pathway inhibitor-2 is a novel inhibitor of matrix metalloproteinases with implications for atherosclerosis. *Journal of Clinical Investigation,* **107,** 1117-1126.
Hertzberg, M. (1994) Biochemistry of factor X. *Blood Reviews,* **8,** 56-62.
Higgins, D.G., Bleasby, A.J. & Fuchs, R. (1992) CLUSTAL V: improved software for multiple sequence alignment. *Comput Appl Biosci,* **8,** 189-191.

Hilbert, L., Fressinaud, E., Ribba, A.S., Meyer, D. & Mazurier, C. (2002) Identification of a new type 2M von Willebrand disease mutation also at position 1324 of von Willebrand factor. *Thrombosis and Haemostasis,* **87,** 635-640.

Himmelspach, M., Pfleiderer, M., Fischer, B.E., Plaimauer, B., Antoine, G., Falkner, F.G., Dorner, F. & Schlokat, U. (2000) Recombinant human factor X: high yield expression and the role of furin in proteolytic maturation in vivo and in vitro. *Thrombosis Research,* **97,** 51-67.

Hogg, P.J. & Jackson, C.M. (1989) Fibrin monomer protects thrombin from inactivation by heparin-antithrombin III: implications for heparin efficacy. *Proceedings of the National Academy of Sciences of the United States of America,* **86,** 3619-3623.

Hosaka, M., Nagahama, M., Kim, W.S., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K. & Nakayama, K. (1991) Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. *Journal of Biological Chemistry,* **266,** 12127-12130.

Hougie, C., Barrow, H.M. & Graham, J.B. (1957) Stuart Clotting defect. Segregation of a hereditary hemorrhagic state from the heterozygous heretofore called 'stable factor' (SPCA, proconvertin, factor VII) deficiency. *Journal of Clinical Investigation,* **36,** 485-493.

Howard, J.B. (1980) Methylamine reaction and denaturation-dependent fragmentation of complement component 3. Comparison with alpha2-macroglobulin. *Journal of Biological Chemistry,* **255,** 7082-7084.

Huang, M.N., Hung, H.L., Stanfield-Oakley, S.A. & High, K.A. (1992) Characterization of the human blood coagulation factor X promoter. *Journal of Biological Chemistry,* **267,** 15440-15446.

Hubbard, T.J. & Blundell, T.L. (1987) Comparison of solvent-inaccessible cores of homologous proteins: definitions useful for protein modelling. *Protein Eng,* **1,** 159-171.

Ichinose, A., Takeya, H., Espling, E., Iwanaga, S., Kisiel, W. & Davie, E.W. (1990) Amino acid sequence of human protein Z, a vitamin K-dependent plasma glycoprotein. *Biochemical and Biophysical Research Communications,* **172,** 1139-1144.

Israels, S.J. & Leaker, M.T. (1997) Acquired inhibitors to factors V and X after exposure to topical thrombin: interference with monitoring of low molecular weight heparin and warfarin. *Journal of Pediatrics,* **131,** 480-483.

Jackson, C.M. (1984) Factor X. *Progress in Hemostasis and Thrombosis,* **7,** 55-109.

Jaye, M., Ricca, G., Kaplan, R., Howk, R., Mudd, R., Ngo, K.Y., Fair, D.S. & Drohan, W. (1985) Polymorphism associated with the human coagulation factor X (F10) gene. *Nucleic Acids Research,* **13,** 8286.

Jenkins, P.V., Pasi, K.J. & Perkins, S.J. (1998) Molecular modeling of ligand and mutation sites of the type A domains of human von Willebrand factor and their relevance to von Willebrand's disease. *Blood,* **91,** 2032-2044.

Jesty, J., Lorenz, A., Rodriguez, J. & Wun, T.C. (1996) Initiation of the tissue factor pathway of coagulation in the presence of heparin: control by antithrombin III and tissue factor pathway inhibitor. *Blood,* **87,** 2301-2307.

Jorgensen, M.J., Cantor, A.B., Furie, B.C., Brown, C.L., Shoemaker, C.B. & Furie, B. (1987) Recognition site directing vitamin K-dependent gamma-carboxylation resides on the propeptide of factor IX. *Cell,* **48,** 185-191.

Kabsch, W. & Sander, C. (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers,* **22,** 2577-2637.

Kafatos, F.C., Jones, C.W. & Efstratiadis, A. (1979) Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucleic Acids Res,* **7,** 1541-1552.

Kamata, K., Kawamoto, H., Honma, T., Iwama, T. & Kim, S.H. (1998) Structural basis for chemical inhibition of human blood coagulation factor Xa. *Proceedings of the National Academy of Sciences of the United States of America,* **95,** 6630-6635.

Kaul, R.K., Hildebrand, B., Roberts, S. & Jagadeeswaran, P. (1986) Isolation and characterization of human blood-coagulation factor X cDNA. *Gene,* **41,** 311-314.

Kiefer, M.C., Tucker, J.E., Joh, R., Landsberg, K.E., Saltman, D. & Barr, P.J. (1991) Identification of a second human subtilisin-like protease gene in the fes/fps region of chromosome 15. *DNA and Cell Biology,* **10,** 757-769.

Kim, D.J., Thompson, A.R. & James, H.L. (1995) Factor X Ketchikan: a variant molecule in which Gly replaces a Gla residue at position 14 in the light chain. *Human Genetics,* **95,** 212-214.

Kisiel, W., Hermodson, M.A. & Davie, E.W. (1976) Factor X activating enzyme from Russell's viper venom: isolation and characterization. *Biochemistry,* **15,** 4901-4906.

Kitchens, C.S. (1980) The anatomic basis of purpura. *Progress in Hemostasis and Thrombosis,* **5,** 211-244.

Klopp, N., Oldenburg, J., Uen, C., Schneppenheim, R. & Graw, J. (2002) 11 hemophilia A patients without mutations in the factor VIII encoding gene. *Thrombosis and Haemostasis,* **88,** 357-360.

Knight, R.D., Barr, C.F. & Alving, B.M. (1985) Replacement therapy for congenital Factor X deficiency. *Transfusion,* **25,** 78-80.

Kohler, M. (1999) Thrombogenicity of prothrombin complex concentrates. *Thrombosis Research,* **95,** S13-17.

Komiyama, Y., Pedersen, A.H. & Kisiel, W. (1990) Proteolytic activation of human factors IX and X by recombinant human factor VIIa: effects of calcium, phospholipids, and tissue factor. *Biochemistry,* **29,** 9418-9425.

Korsan-Bengsten, L., Hjort, P.F. & Ygge, J. (1962) Acquired factor X deficiency in a patient with amyloidosis. *Thrombosis et Diathesis Haemorrhagica,* **7,** 558-566.

Korte, W. & Flury, R. (1992) Acquired factor X deficiency and disseminated intravascular coagulation in a case of metastasizing carcinoma of the stomach and its course under chemotherapy. *Annals of Hematology,* **64,** 152-154.

Kouides, P.A. & Kulzer, L. (2001) Prophylactic treatment of severe factor X deficiency with prothrombin complex concentrate. *Haemophilia,* **7,** 220-223.

Kumar, M. & Mehta, P. (1994) Congenital coagulopathies and pregnancy: report of four pregnancies in a factor X-deficient woman. *American Journal of Hematology,* **46,** 241-244.

Lankiewicz, M.W. & Bell, W.R. (1992) A unique circulating inhibitor with specificity for coagulation factor X. *American Journal of Medicine,* **93,** 343-346.

Larson, P.J., Camire, R.M., Wong, D., Fasano, N.C., Monroe, D.M., Tracy, P.B. & High, K.A. (1998) Structure/function analyses of recombinant variants of

human factor Xa: factor Xa incorporation into prothrombinase on the thrombin-activated platelet surface is not mimicked by synthetic phospholipid vesicles. *Biochemistry,* **37,** 5029-5038.

Lattman, E.E. (1995) Protein structure prediction: a special issue. *Proteins,* **23,** i.

Lawson, J.H., Kalafatis, M., Stram, S. & Mann, K.G. (1994) A model for the tissue factor pathway to thrombin. I. An empirical study. *Journal of Biological Chemistry,* **269,** 23357-23366.

Lawson, J.H. & Mann, K.G. (1991) Cooperative activation of human factor IX by the human extrinsic pathway of blood coagulation. *Journal of Biological Chemistry,* **266,** 11317-11327.

Leach, A.R. (1996) *Molecular Modelling. Principles andn Applications.* Longman, Harlow.

Lechler, E. (1999) Use of prothrombin complex concentrates for prophylaxis and treatment of bleeding episodes in patients with hereditary deficiency of prothrombin, factor VII, factor X, protein C protein S, or protein Z. *Thrombosis Research,* **95,** S39-50.

Lee, B. & Richards, F.M. (1971) The interpretation of protein structures: estimation of static accessibility. *Journal of Molecular Biology,* **55,** 379-400.

Levitt, M. (1978) Conformational preferences of amino acids in globular proteins. *Biochemistry,* **17,** 4277-4285.

Leytus, S.P., Chung, D.W., Kisiel, W., Kurachi, K. & Davie, E.W. (1984) Characterization of a cDNA coding for human factor X. *Proceedings of the National Academy of Sciences of the United States of America,* **81,** 3699-3702.

Leytus, S.P., Foster, D.C., Kurachi, K. & Davie, E.W. (1986) Gene for human factor X: a blood coagulation factor whose gene organization is essentially identical with that of factor IX and protein C. *Biochemistry,* **25,** 5098-5102.

Lijnen, H.R. & Collen, D. (1982) Interaction of plasminogen activators and inhibitors with plasminogen and fibrin. *Seminars in Thrombosis and Hemostasis,* **8,** 2-10.

Lind, B., Johnsen, A.H. & Thorsen, S. (1997) Naturally occurring Arg(-1) to His mutation in human protein C leads to aberrant propeptide processing and secretion of dysfunctional protein C. *Blood,* **89,** 2807-2816.

Linton, M.F., Pierotti, V. & Young, S.G. (1992) Reading-frame restoration with an apolipoprotein B gene frameshift mutation. *Proceedings of the National Academy of Sciences of the United States of America,* **89,** 11431-11435.

Lusson, J., Vieau, D., Hamelin, J., Day, R., Chretien, M. & Seidah, N.G. (1993) cDNA structure of the mouse and rat subtilisin/kexin-like PC5: a candidate proprotein convertase expressed in endocrine and nonendocrine cells. *Proceedings of the National Academy of Sciences of the United States of America,* **90,** 6691-6695.

MacArthur, M.W. & Thornton, J.M. (1991) Influence of proline residues on protein conformation. *J Mol Biol,* **218,** 397-412.

MacCumber, M.W., Ross, C.A., Glaser, B.M. & Snyder, S.H. (1989) Endothelin: visualization of mRNAs by in situ hybridization provides evidence for local action. *Proceedings of the National Academy of Sciences of the United States of America,* **86,** 7285-7289.

MacIntyre, D.E., Pearson, J.D. & Gordon, J.L. (1978) Localisation and stimulation of prostacyclin production in vascular cells. *Nature,* **271,** 549-551.

Maignan, S., Guilloteau, J.P., Pouzieux, S., Choi-Sledeski, Y.M., Becker, M.R., Klein, S.I., Ewing, W.R., Pauls, H.W., Spada, A.P. & Mikol, V. (2000) Crystal structures of human factor Xa complexed with potent inhibitors. *Journal of Medicinal Chemistry,* **43,** 3226-3232.

Maniatis, T., Goodbourn, S. & Fischer, J.A. (1987) Regulation of inducible and tissue-specific gene expression. *Science,* **236,** 1237-1245.

Mann, K.G., Krishnaswamy, S. & Lawson, J.H. (1992) Surface-dependent hemostasis. *Seminars in Hematology,* **29,** 213-226.

Mann, K.G., Nesheim, M.E., Church, W.R., Haley, P. & Krishnaswamy, S. (1990) Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood,* **76,** 1-16.

Maquat, L.E. (2002) Nonsense-mediated mRNA decay. *Curr Biol,* **12,** R196-197.

Maquat, L.E. & Carmichael, G.G. (2001) Quality control of mRNA function. *Cell,* **104,** 173-176.

Matsunaga, A.T. & Shafer, F.E. (1996) An acquired inhibitor to factor X in a pediatric patient with extensive burns. *Journal of Pediatric Hematology/Oncology,* **18,** 223-226.

McMahon, C., Smith, J., Goonan, C., Byrne, M. & Smith, O.P. (2002) The role of primary prophylactic factor replacement therapy in children with severe factor X deficiency. *British Journal of Haematology,* **119,** 789-791.

McMullen, B.A., Fujikawa, K., Kisiel, W., Sasagawa, T., Howald, W.N., Kwa, E.Y. & Weinstein, B. (1983) Complete amino acid sequence of the light chain of human blood coagulation factor X: evidence for identification of residue 63 as beta- hydroxyaspartic acid. *Biochemistry,* **22,** 2875-2884.

Meerabux, J., Yaspo, M.L., Roebroek, A.J., Van de Ven, W.J., Lister, T.A. & Young, B.D. (1996) A new member of the proprotein convertase gene family (LPC) is located at a chromosome translocation breakpoint in lymphomas. *Cancer Research,* **56,** 448-451.

Menegatti, M., Karimi, M., Garagiola, I., Mannucci, P. & Peyvandi, F. (2004) A rare inherited coagulation disorder: Combined homozygous factor VII and factor X deficiency. *American Journal of Hematology,* **77,** 90-91.

Menegatti, M., Peyvandi, F., Bader, R. & Mannucci, P.M. (2002) In vitro expression Studies of a naturally occuring mutation on factor X (FX) gene (Gly222Asp) causing type I deficiency. *The Haematology Journal,* **3 (Suppl.1),** 97-98.

Miao, C.H., Leytus, S.P., Chung, D.W. & Davie, E.W. (1992) Liver-specific expression of the gene coding for human factor X, a blood coagulation factor. *Journal of Biological Chemistry,* **267,** 7395-7401.

Millar, D.S., Elliston, L., Deex, P., Krawczak, M., Wacey, A.I., Reynaud, J., Nieuwenhuis, H.K., Bolton-Maggs, P., Mannucci, P.M., Reverter, J.C., Cachia, P., Pasi, K.J., Layton, D.M. & Cooper, D.N. (2000) Molecular analysis of the genotype-phenotype relationship in factor X deficiency. *Human Genetics,* **106,** 249-257.

Minning, D.M., Brenner, B. & Marder, V.J. (2002) Overview of hemostasis. In: *Women's Issues in Thrombosis and Hemostasis* (ed. by B. Brenner, V.J. Marder & J. Conard), pp. 1-20. Martin Dunitz, London.

Miyata, T., Kojima, T., Suzuki, K., Umeyama, H., Yamazaki, T., Kamiya, T., Toyoda, H. & Kato, H. (1998) Factor X Nagoya 1 and Nagoya 2: a CRM- factor X deficiency and a dysfunctional CRM+ factor X deficiency characterized by substitution of Arg306 by Cys and of Gly366 by Ser, respectively. *Thrombosis and Haemostasis,* **79,** 486-490.

Miyata, T., Zheng, Y.Z., Sakata, T. & Kato, H. (1995) Protein C Osaka 10 with aberrant propeptide processing: loss of anticoagulant activity due to an amino acid substitution in the protein C precursor. *Thrombosis and Haemostasis,* **74,** 1003-1008.

Morawitz, P. (1905) Die Chemie der Blutgerinnung. *Ergebnisse der Physiologie, Biologischen Chemie und Experimentellen Pharmakologie,* 307-422.

Morrissey, J.H., Macik, B.G., Neuenschwander, P.F. & Comp, P.C. (1993) Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. *Blood,* **81,** 734-744.

Mosesson, M.W. (1992) The roles of fibrinogen and fibrin in hemostasis and thrombosis. *Seminars in Hematology,* **29,** 177-188.

Mulhare, P.E., Tracy, P.B., Golden, E.A., Branda, R.F. & Bovill, E.G. (1991) A case of acquired factor X deficiency with in vivo and in vitro evidence of inhibitor activity directed against factor X. *American Journal of Clinical Pathology,* **96,** 196-200.

Nakagaki, T., Foster, D.C., Berkner, K.L. & Kisiel, W. (1991) Initiation of the extrinsic pathway of blood coagulation: evidence for the tissue factor dependent autoactivation of human coagulation factor VII. *Biochemistry,* **30,** 10819-10824.

Nakagawa, T., Hosaka, M., Torii, S., Watanabe, T., Murakami, K. & Nakayama, K. (1993) Identification and functional expression of a new member of the mammalian Kex2-like processing endoprotease family: its striking structural similarity to PACE4. *Journal of Biochemistry,* **113,** 132-135.

Nakayama, K., Hosaka, M., Hatsuzawa, K. & Murakami, K. (1991) Cloning and functional expression of a novel endoprotease involved in prohormone processing at dibasic sites. *Journal of Biochemistry,* **109,** 803-806.

Nakayama, K., Kim, W.S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T. & Murakami, K. (1992) Identification of the fourth member of the mammalian endoprotease family homologous to the yeast Kex2 protease. Its testis-specific expression. *Journal of Biological Chemistry,* **267,** 5897-5900.

Naski, M.C., Lorand, L. & Shafer, J.A. (1991) Characterization of the kinetic pathway for fibrin promotion of alpha-thrombin-catalyzed activation of plasma factor XIII. *Biochemistry,* **30,** 934-941.

Nelson, R.M., VanDusen, W.J., Friedman, P.A. & Long, G.L. (1991) beta-Hydroxyaspartic acid and beta-hydroxyasparagine residues in recombinant human protein S are not required for anticoagulant cofactor activity or for binding to C4b-binding protein. *Journal of Biological Chemistry,* **266,** 20586-20589.

Nesheim, M., Wang, W., Boffa, M., Nagashima, M., Morser, J. & Bajzar, L. (1997) Thrombin, thrombomodulin and TAFI in the molecular link between coagulation and fibrinolysis. *Thrombosis and Haemostasis,* **78,** 386-391.

Nesheim, M.E., Kettner, C., Shaw, E. & Mann, K.G. (1981) Cofactor dependence of factor Xa incorporation into the prothrombinase complex. *Journal of Biological Chemistry,* **256,** 6537-6540.

Ness, P.M., Hymas, P.G., Gesme, D. & Perkins, H.A. (1980) An unusual factor-X inhibitor in leprosy. *American Journal of Hematology,* **8,** 397-402.

Neuenschwander, P.F., Fiore, M.M. & Morrissey, J.H. (1993) Factor VII autoactivation proceeds via interaction of distinct protease-cofactor and

zymogen-cofactor complexes. Implications of a two-dimensional enzyme kinetic mechanism. *Journal of Biological Chemistry,* **268,** 21489-21492.
Nora, R.E., Bell, W.R., Noe, D.A. & Sholar, P.W. (1985) Novel factor X deficiency. Normal partial thromboplastin time and associated spindle cell thymoma. *American Journal of Medicine,* **79,** 122-126.
Norledge, B.V., Petrovan, R.J., Ruf, W. & Olson, A.J. (2003) The tissue factor/factor VIIa/factor Xa complex: a model built by docking and site-directed mutagenesis. *Proteins,* **53,** 640-648.
Nurden, A.T. & Caen, J.P. (1979) The different glycoprotein abnormalities in thrombasthenic and Bernard-Soulier platelets. *Seminars in Hematology,* **16,** 234-250.
Olson, S.T., Bjork, I. & Shore, J.D. (1993) Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin. *Methods in Enzymology,* **222,** 525-559.
Otto, J.C. (1803) An account of an hemorrhagic disposition in certain families. *New York Medical Repository,* **vi,** 1.
Overington, J., Johnson, M.S., Sali, A. & Blundell, T.L. (1990) Tertiary structural constraints on protein evolutionary diversity: templates, key residues and structure prediction. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* **241,** 132-145.
Owens, D.L., Peyvandi, F. & Perry, D.J. (1998) Laboratory diagnosis of factor X deficiency. *Haemophilia,* **4,** 279.
Owren, P.A. (1947) The coagulation of blood. Investigations on a new clotting factor. *Acta Medica Scandinavica,* **128 Suppl 194,** 82.
Pabinger, I., Bettelheim, P., Dudczak, R., Hinterberger, W., Kyrle, P.A., Niessner, H., Schwarzinger, I., Speiser, W. & Lechner, K. (1991) Coincidence of acquired factor-X deficiency and disseminated intravascular coagulation in patients with acute nonlymphoblastic leukemia. *Annals of Hematology,* **62,** 174-179.
Padmanabhan, K., Padmanabhan, K.P., Tulinsky, A., Park, C.H., Bode, W., Huber, R., Blankenship, D.T., Cardin, A.D. & Kisiel, W. (1993) Structure of human des(1-45) factor Xa at 2.2 A resolution. *Journal of Molecular Biology,* **232,** 947-966.
Parratt, R. & Hunt, B.J. (1998) Direct activation of factor X by monocytes occurs during cardiopulmonary bypass. *British Journal of Haematology,* **101,** 40-46.
Patthy, L. (1985) Evolution of the proteases of blood coagulation and fibrinolysis by assembly from modules. *Cell,* **41,** 657-663.
Perkins, S.J. & Smith, K.F. (1993) Identity of the putative serine-proteinase fold in proteins of the complement system with nine relevant crystal structures. *Biochemical Journal,* **295 (Pt 1),** 109-114.
Perry, D.J. (1997) Factor X and its deficiency states. *Haemophilia,* **3,** 159-172.
Perutz, M.F. (1951) New X-Ray evidence on the configuratioin of polypeptide chains. *Nature,* **167,** 1053-1054.
Peuscher, F.W., van Aken, W.G., van Mourik, J.A., Swaak, A.J., Sie, L.H. & Statius van Eps, L.W. (1979) Acquired, transient factor X (Stuart factor) deficiency in patient with mycoplasma pneumonial infection. *Scandinavian Journal of Haematology,* **23,** 257-264.
Peyvandi, F., Jenkins, P.V., Mannucci, P.M., Billio, A., Zeinali, S., Perkins, S.J. & Perry, D.J. (2000a) Molecular characterisation and three-dimensional

structural analysis of mutations in 21 unrelated families with inherited factor VII deficiency. *Thrombosis and Haemostasis,* **84,** 250-257.

Peyvandi, F. & Mannucci, P.M. (1999) Rare coagulation disorders. *Thrombosis and Haemostasis,* **82,** 1207-1214.

Peyvandi, F., Mannucci, P.M., Jenkins, P.V., Lee, A., Coppola, R. & Perry, D.J. (2000b) Homozygous 2bp deletion in the human factor VII gene: a non-lethal mutation that is associated with a complete absence of circulating factor VII. *Thrombosis and Haemostasis,* **84,** 635-637.

Peyvandi, F., Menegatti, M., Santagostino, E., Akhavan, S., Uprichard, J., Perry, D.J., Perkins, S.J. & Mannucci, P.M. (2002) Gene mutations and three-dimensional structural analysis in 13 families with severe factor X deficiency. *British Journal of Haematology,* **117,** 685-692.

Pfeiffer, R.A., Ott, R., Gilgenkrantz, S. & Alexandre, P. (1982) Deficiency of coagulation factors VII and X associated with deletion of a chromosome 13 (q34). Evidence from two cases with 46,XY,t(13;Y)(q11;q34). *Human Genetics,* **62,** 358-360.

Philippou, H., Adami, A., Boisclair, M.D. & Lane, D.A. (1995) An ELISA for factor X activation peptide: application to the investigation of thrombogenesis in cardiopulmonary bypass. *British Journal of Haematology,* **90,** 432-437.

Pickett, W.C., Jesse, R.L. & Cohen, P. (1976) Initiation of phospholipase A2 activity in human platelets by the calcium ion ionophore A23187. *Biochimica et Biophysica Acta,* **486,** 209-213.

Pieters, J., Lindhout, T. & Hemker, H.C. (1989) In situ-generated thrombin is the only enzyme that effectively activates factor VIII and factor V in thromboplastin-activated plasma. *Blood,* **74,** 1021-1024.

Pinotti, M., Marchetti, G., Baroni, M., Cinotti, F., Morfini, M. & Bernardi, F. (2002) Reduced activation of the Gla19Ala FX variant via the extrinsic coagulation pathway results in symptomatic CRMred FX deficiency. *Thrombosis and Haemostasis,* **88,** 236-241.

Pinotti, M., Monti, M., Baroni, M., Marchetti, G. & Bernardi, F. (2004) Molecular characterization of factor X deficiency associated with borderline plasma factor X level. *Haematologica,* **89,** 501-502.

Plow, E.F. & Collen, D. (1981) The presence and release of alpha 2-antiplasmin from human platelets. *Blood,* **58,** 1069-1074.

Potter, H., Weir, L. & Leder, P. (1984) Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proceedings of the National Academy of Sciences of the United States of America,* **81,** 7161-7165.

Pryzdial, E.L., Lavigne, N., Dupuis, N. & Kessler, G.E. (1999) Plasmin converts factor X from coagulation zymogen to fibrinolysis cofactor. *Journal of Biological Chemistry,* **274,** 8500-8505.

Radcliffe, R. & Nemerson, Y. (1975) Activation and control of factor VII by activated factor X and thrombin. Isolation and characterization of a single chain form of factor VII. *Journal of Biological Chemistry,* **250,** 388-395.

Rao, L.V. & Rapaport, S.I. (1988) Activation of factor VII bound to tissue factor: a key early step in the tissue factor pathway of blood coagulation. *Proceedings of the National Academy of Sciences of the United States of America,* **85,** 6687-6691.

Rao, L.V., Williams, T. & Rapaport, S.I. (1996) Studies of the activation of factor VII bound to tissue factor. *Blood,* **87,** 3738-3748.

Rapaport, S.I. (1991) The extrinsic pathway inhibitor: a regulator of tissue factor-dependent blood coagulation. *Thrombosis and Haemostasis,* **66,** 6-15.
Reddy, S.V., Zhou, Z.Q., Rao, K.J., Scott, J.P., Watzke, H., High, K.A. & Jagadeeswaran, P. (1989) Molecular characterization of human factor X San Antonio. *Blood,* **74,** 1486-1490.
Reese, M.G., Eeckman, F.H., Kulp, D. & Haussler, D. (1997) Improved splice site detection in Genie. *Journal of Computational Biology,* **4,** 311-323.
Rezaie, A.R. & He, X. (2000) Sodium binding site of factor Xa: role of sodium in the prothrombinase complex. *Biochemistry,* **39,** 1817-1825.
Rezig, K., Diar, N., Benabidallah, D. & Audibert, J. (2002) [Factor X deficiency and pregnancy]. *Annales Francaises d Anesthesie et de Reanimation,* **21,** 521-524.
Richardson, J.S. & Richardson, D.C. (1989) Principles and patterns of protein conformation. In: *Prediction of Protein Structure and the Principles of Protein Conformation* (ed. by G.D. Fasman), pp. 1-98. Plenum Press, New York.
Richmond, T.J. & Richards, F.M. (1978) Packing of alpha-helices: geometrical constraints and contact areas. *Journal of Molecular Biology,* **119,** 537-555.
Riewald, M., Petrovan, R.J., Donner, A., Mueller, B.M. & Ruf, W. (2002) Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science,* **296,** 1880-1882.
Roberts, H.R., Lechler, E., Webster, W.P. & Penick, G.D. (1965) Survival of transfused factor X in patients with Stuart disease. *Thrombosis et Diathesis Haemorrhagica,* **13,** 305–313.
Roebroek, A.J., Schalken, J.A., Bussemakers, M.J., van Heerikhuizen, H., Onnekink, C., Debruyne, F.M., Bloemers, H.P. & Van de Ven, W.J. (1986) Characterization of human c-fes/fps reveals a new transcription unit (fur) in the immediately upstream region of the proto-oncogene. *Molecular Biology Reports,* **11,** 117-125.
Rosenstein, E.D., Itzkowitz, S.H., Penziner, A.S., Cohen, J.I. & Mornaghi, R.A. (1983) Resolution of factor X deficiency in primary amyloidosis following splenectomy. *Archives of Internal Medicine,* **143,** 597-599.
Ross, R. (1999) Atherosclerosis--an inflammatory disease. *New England Journal of Medicine,* **340,** 115-126.
Rost, B. & Sander, C. (1994) Conservation and prediction of solvent accessibility in protein families. *Proteins,* **20,** 216-226.
Rudolph, A.E., Mullane, M.P., Porche-Sorbet, R. & Miletich, J.P. (1997) Expression, purification, and characterization of recombinant human factor X. *Protein Expression and Purification,* **10,** 373-378.
Rudolph, A.E., Mullane, M.P., Porche-Sorbet, R., Tsuda, S. & Miletich, J.P. (1996) Factor X St. Louis II. Identification of a glycine substitution at residue 7 and characterization of the recombinant protein. *Journal of Biological Chemistry,* **271,** 28601-28606.
Ruggeri, Z.M., Dent, J.A. & Saldivar, E. (1999) Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood,* **94,** 172-178.
Sali, A. & Blundell, T.L. (1990) Definition of general topological equivalence in protein structures. A procedure involving comparison of properties and relationships through simulated annealing and dynamic programming. *Journal of Molecular Biology,* **212,** 403-428.

Schwarzinger, I., Stain-Kos, M., Bettelheim, P., Pabinger, I., Kyrle, P., Kalhs, P., Kapiotis, S., Jager, U. & Lechner, K. (1992) Recurrent, isolated factor X deficiency in myeloma: repeated normalization of factor X levels after cytostatic chemotherapy followed by late treatment failure associated with the development of systemic amyloidosis. *Thrombosis and Haemostasis,* **68,** 648-651.

Seidah, N.G., Hamelin, J., Mamarbachi, M., Dong, W., Tardos, H., Mbikay, M., Chretien, M. & Day, R. (1996) cDNA structure, tissue distribution, and chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. *Proceedings of the National Academy of Sciences of the United States of America,* **93,** 3388-3393.

Shattil, S.J., Kashiwagi, H. & Pampori, N. (1998) Integrin signaling: the platelet paradigm. *Blood,* **91,** 2645-2657.

Shimazui, T., Yoshikawa, K., Uemura, H., Kawamoto, R., Kawai, K., Uchida, K., Hirao, Y., Saga, S. & Akaza, H. (2003) Detection of cadherin-6 mRNA by nested RT-PCR as a potential marker for circulating cancer cells in renal cell carcinoma. *International Journal of Oncology,* **23,** 1049-1054.

Simioni, P., Kalafatis, M., Tormene, D., Luni, S., Zerbinati, P., Barzon, L., Palu, G. & Girolami, A. (2001a) Abnormal propeptide processing resulting in the presence of two abnormal species of protein C in plasma: characterization of the dysfunctional protein C Padua3 (protein C(R-1L/propeptide)). *Thrombosis and Haemostasis,* **86,** 1017-1022.

Simioni, P., Vianello, F., Kalafatis, M., Barzon, L., Ladogana, S., Paolucci, P., Carotenuto, M., Dal Bello, F., Palu, G. & Girolami, A. (2001b) A dysfunctional factor X (factor X San Giovanni Rotondo) present at homozygous and double heterozygous level: identification of a novel microdeletion (delC556) and missense mutation (Lys(408)-->Asn) in the factor X gene. A study of an Italian family. *Thrombosis Research,* **101,** 219-230.

Sinha, U. & Wolf, D.L. (1993) Carbohydrate residues modulate the activation of coagulation factor X. *Journal of Biological Chemistry,* **268,** 3048-3051.

Smeekens, S.P. & Steiner, D.F. (1990) Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. *Journal of Biological Chemistry,* **265,** 2997-3000.

Smith, S.V., Liles, D.K., White, G.C., 2nd & Brecher, M.E. (1998) Successful treatment of transient acquired factor X deficiency by plasmapheresis with concomitant intravenous immunoglobulin and steroid therapy. *American Journal of Hematology,* **57,** 245-252.

Spek, C.A., Koster, T., Rosendaal, F.R., Bertina, R.M. & Reitsma, P.H. (1995) Genotypic variation in the promoter region of the protein C gene is associated with plasma protein C levels and thrombotic risk. *Arteriosclerosis, Thrombosis, and Vascular Biology,* **15,** 214-218.

Sprecher, C.A., Kisiel, W., Mathewes, S. & Foster, D.C. (1994) Molecular cloning, expression, and partial characterization of a second human tissue-factor-pathway inhibitor. *Proceedings of the National Academy of Sciences of the United States of America,* **91,** 3353-3357.

Staatz, W.D., Rajpara, S.M., Wayner, E.A., Carter, W.G. & Santoro, S.A. (1989) The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg++-

dependent adhesion of platelets to collagen. *Journal of Cell Biology,* **108,** 1917-1924.
Stanley, T.B., Humphries, J., High, K.A. & Stafford, D.W. (1999) Amino acids responsible for reduced affinities of vitamin K-dependent propeptides for the carboxylase. *Biochemistry,* **38,** 15681-15687.
Stanton, C., Taylor, R. & Wallin, R. (1991) Processing of prothrombin in the secretory pathway. *Biochemical Journal,* **277 (Pt 1),** 59-65.
Stanton, C. & Wallin, R. (1992) Processing and trafficking of clotting factor X in the secretory pathway. Effects of warfarin. *Biochemical Journal,* **284,** 25-31.
Stefanini, M. & Wiggishoff, C.C. (1966) Stuart factor (factor X) deficiency associated with renal and adrenal cortical insufficiency. *Annals of Internal Medicine,* **64,** 1285-1291.
Stenflo, J. & Suttie, J.W. (1977) Vitamin K-dependent formation of gamma-carboxyglutamic acid. *Annual Review of Biochemistry,* **46,** 157-172.
Swick, A.G., Blake, M.C., Kahn, J.W. & Azizkhan, J.C. (1989) Functional analysis of GC element binding and transcription in the hamster dihydrofolate reductase gene promoter. *Nucleic Acids Research,* **17,** 9291-9304.
Taylor, F.B., Jr., Peer, G.T., Lockhart, M.S., Ferrell, G. & Esmon, C.T. (2001) Endothelial cell protein C receptor plays an important role in protein C activation in vivo. *Blood,* **97,** 1685-1688.
Teien, A.N., Abildgaard, U. & Hook, M. (1976) The anticoagulant effect of heparan sulfate and dermatan sulfate. *Thrombosis Research,* **8,** 859-867.
Telfer, T.P., Denson, K.W. & Wright, D.R. (1956) A new `coagulation' defect. *British Journal of Haematology,* **2,** 308-316.
Thomas, A.E., Green, F.R., Kelleher, C.H., Wilkes, H.C., Brennan, P.J., Meade, T.W. & Humphries, S.E. (1991) Variation in the promoter region of the beta fibrinogen gene is associated with plasma fibrinogen levels in smokers and non-smokers. *Thrombosis and Haemostasis,* **65,** 487-490.
Tollefsen, D.M., Majerus, D.W. & Blank, M.K. (1982) Heparin cofactor II. Purification and properties of a heparin-dependent inhibitor of thrombin in human plasma. *Journal of Biological Chemistry,* **257,** 2162-2169.
Topper, J.N. & Gimbrone, M.A., Jr. (1999) Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype. *Molecular Medicine Today,* **5,** 40-46.
Tractate Shabbat, 134a. Talmud Bavli.
Tuddenham, E.G.D. & Cooper, D.N. (1994) *The Molecular Genetics of Haemostasis and its Inherited Disorders.* Oxford University Press, Oxford.
Ugarova, T.P., Budzynski, A.Z., Shattil, S.J., Ruggeri, Z.M., Ginsberg, M.H. & Plow, E.F. (1993) Conformational changes in fibrinogen elicited by its interaction with platelet membrane glycoprotein GPIIb-IIIa. *Journal of Biological Chemistry,* **268,** 21080-21087.
Uprichard, J. & Perry, D.J. (2002) Factor X deficiency. *Blood Reviews,* **16,** 97-110.
van der Walt, J.A., Gomperts, E.D. & Kew, M.C. (1977) Hemostatic factors in primary hepatocellular cancer. *Cancer,* **40,** 1593-1603.
van Dieijen, G., Tans, G., Rosing, J. & Hemker, H.C. (1981) The role of phospholipid and factor VIIIa in the activation of bovine factor X. *Journal of Biological Chemistry,* **256,** 3433-3442.
van Hinsbergh, V.W. (2001) The endothelium: vascular control of haemostasis. *European Journal of Obstetrics, Gynecology, and Reproductive Biology,* **95,** 198-201.

van Hinsbergh, W.M. (1997) Endothelial permeability for macromolecules. Mechanistic aspects of pathophysiological modulation. *Arteriosclerosis, Thrombosis, and Vascular Biology,* **17,** 1018-1023.

Walker, F.J. (1981) Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. *Journal of Biological Chemistry,* **256,** 11128-11131.

Wallin, R., Stanton, C. & Hutson, S.M. (1993) Intracellular maturation of the gamma-carboxyglutamic acid (Gla) region in prothrombin coincides with release of the propeptide. *Biochemical Journal,* **291 (Pt 3),** 723-727.

Wallmark, A., Rose, V.L., Ho, C. & High, K.A. (1991) A NlaIV polymorphism within the human factor X gene. *Nucleic Acids Research,* **19,** 4022.

Warner, T.D., Mitchell, J.A., de Nucci, G. & Vane, J.R. (1989) Endothelin-1 and endothelin-3 release EDRF from isolated perfused arterial vessels of the rat and rabbit. *Journal of Cardiovascular Pharmacology,* **13 Suppl 5,** S85-88; discussion S102.

Williams, M.D., Chalmers, E.A. & Gibson, B.E. (2002) The investigation and management of neonatal haemostasis and thrombosis. *British Journal of Haematology,* **119,** 295-309.

Wise, R.J., Barr, P.J., Wong, P.A., Kiefer, M.C., Brake, A.J. & Kaufman, R.J. (1990) Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proceedings of the National Academy of Sciences of the United States of America,* **87,** 9378-9382.

Woodhams, B., Girardot, O., Blanco, M.J., Colesse, G. & Gourmelin, Y. (2001) Stability of coagulation proteins in frozen plasma. *Blood Coagulation and Fibrinolysis,* **12,** 229-236.

Wright, I.S. (1962) The nomenclature of blood clotting factors. *Thrombosis et Diathesis Haemorrhagica,* **7,** 381-382.

Yamada, D., Sekiya, F. & Morita, T. (1997) Prothrombin and factor X activator activities in the venoms of Viperidae snakes. *Toxicon,* **35,** 1581-1589.

Young, M., Inaba, H., Hoyer, L.W., Higuchi, M., Kazazian, H.H., Jr. & Antonarakis, S.E. (1997) Partial correction of a severe molecular defect in hemophilia A, because of errors during expression of the factor VIII gene. *American Journal of Human Genetics,* **60,** 565-573.

Zama, T., Murata, M., Watanabe, R., Yokoyama, K., Moriki, T., Ambo, H., Murakami, H., Kikuchi, M. & Ikeda, Y. (1999) A family with hereditary factor X deficiency with a point mutation Gla32 to Gln in the Gla domain (factor X Tokyo). *British Journal of Haematology,* **106,** 809-811.

Zivelin, A., Salomon, O., Kenet, G., Gillis, S., Varon, D., Goldschmidt, N., Kovalsky, V., Lijima, K. & Seligsohn, U. (2003) *Cys61stop is the first identified nonsense mutation in the factor X gene causing severe factor X deficiency in 3 unrelated homozygotes of Arab origin.* In: International Society of Thrombosis and Haemostasis, Vol. suppl, p. P1211, Birmingham, UK.

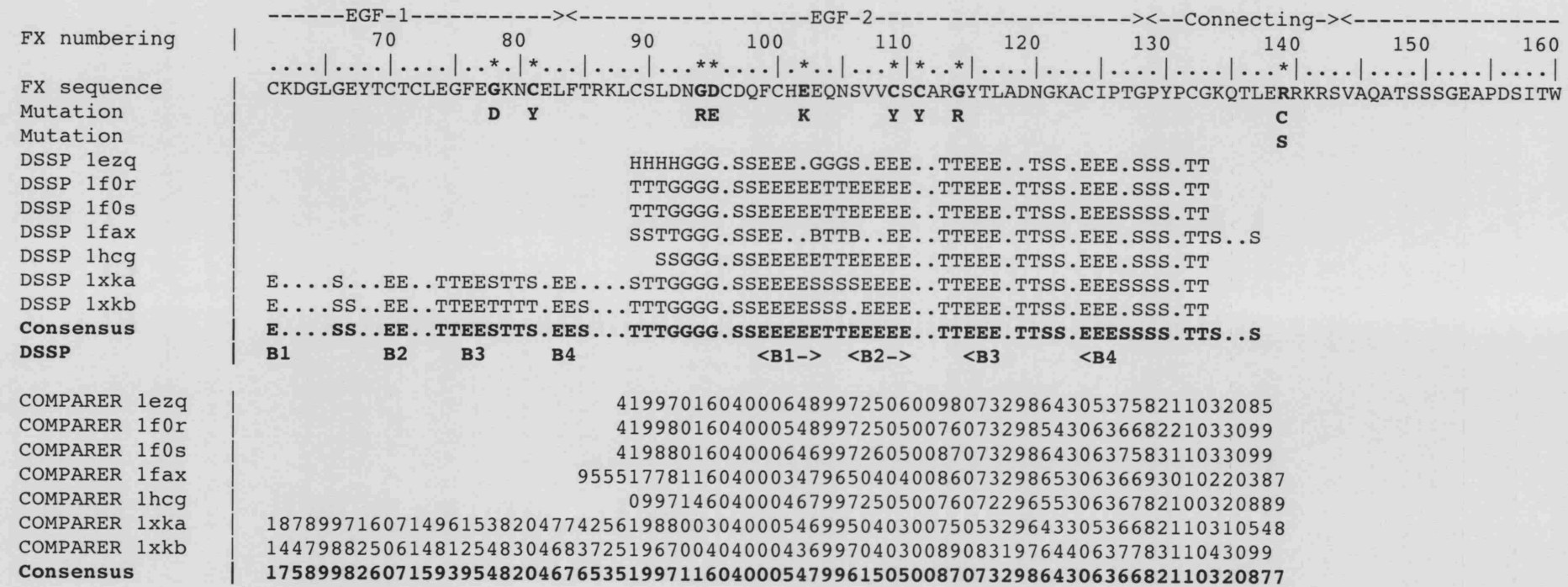
Appendix 1

Protein sequence and structure analysis of human FX

The previously known mutations are identified by asterisks (*), the mutations discussed in this thesis by dollar signs (**$**) and the catalytic triad by hatches (#). The domains are shown above the sequence-numbering for FX. The standard Chymotypsin numbering system for trypsin-like serine proteases is also shown. The consensus of the secondary structures (using DSSP program) of FX is shown. The β strands in the two EGF domains (denoted by E in the DSSP output) are each labelled B1-B4; those in the serine protease domains are labelled A-O. The α helices (denoted by H) in the serine protease domains are labelled A1-A3. The COMPARER consensus gives a value (0-9) representing the solvent accessibility of the sidechains of the FX molecule: exposed residues are assigned values of 2-9 and buried residues 0-1.

```
                  <---------Pre-Pro-leader----------------><---------------Gla-Domain------------------><-----EGF-1----
FX numbering      |          -30       -20       -10       -1        10        20        30        40        50        60
                  *....|*...|...$|....*....|....|....|..**..*.|.*..|...*|...$*....**...|**..|....|....|....|$...|.*..|
FX sequence       |MGRPLHLVLLSASLAGLLLLGESLFIRREQANNILARVTRANSFLggMKKGHLgRgCMggTCSYggARgVFgDSDKTNgFWNKYKDGDQCETSPCQNQGK
Mutation          |V     I       D     R                 MT  C   G      G    AD    KD    SQ                  K     T
Mutation          |                                                      K    V
DSSP 1xka         |                                                                                          TT...SSS.E
DSSP 1xkb         |                                                                                         TTTT....SS.E
Consensus         |                                                                                         TTTT...SSS.E

COMPARER 1xka     |                                                                                          529881054906
COMPARER 1xkb     |                                                                                         9428971064907
Consensus         |                                                                                         9529881064907
```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Mutation
D Y RE K Y Y R
Mutation
C
S
DSSP 1ezq
DSSP 1f0r
DSSP 1f0s
DSSP 1fax
DSSP 1hcg
DSSP 1xka
DSSP 1xkb
Consensus
DSSP
B1 B2 B3 B4 <B1-> <B2-> <B3 <B4
COMPARER 1ezq
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COMPARER 1f0s
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COMPARER 1hcg
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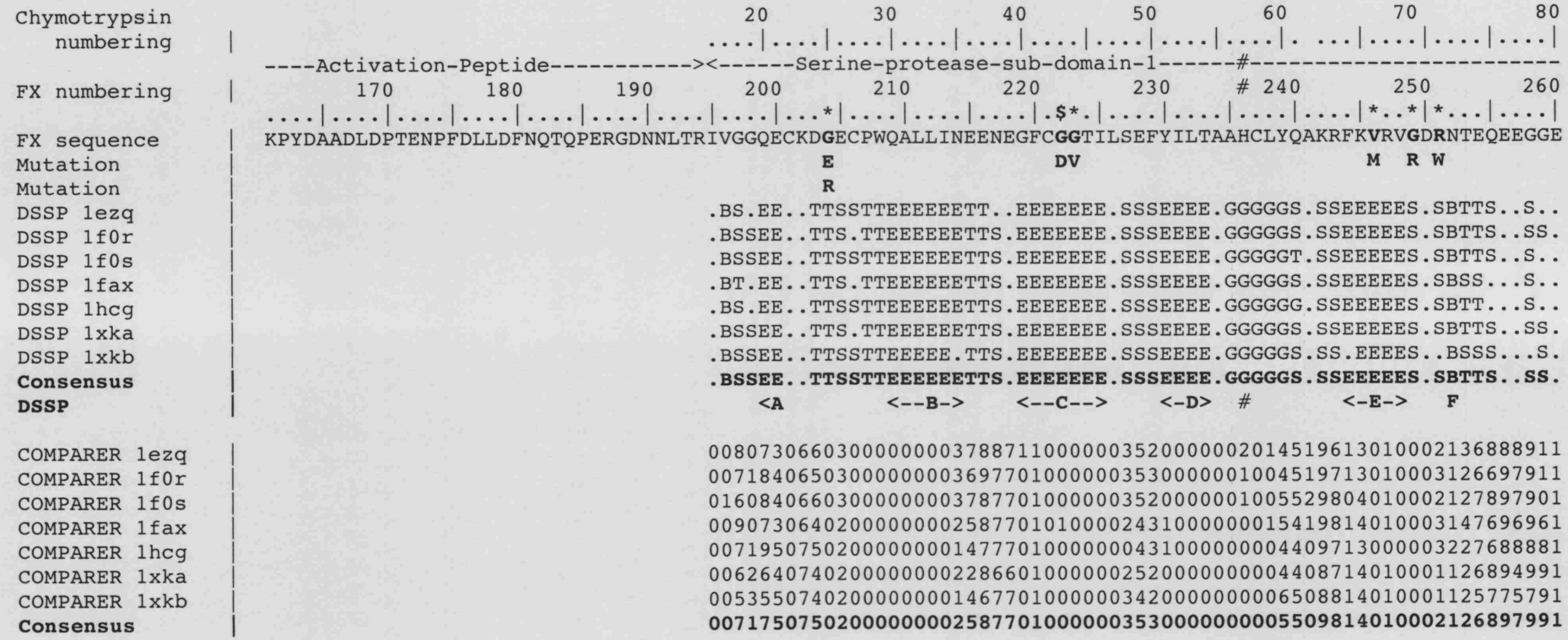
COMPARER 1xkb
Consensus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Mutation E DV M R W
Mutation R
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DSSP 1f0s .BSSEE..TTSSTTEEEEEETTS.EEEEEEE.SSSEEEE.GGGGGT.SSEEEEES.SBTTS..S..
DSSP 1fax .BT.EE..TTS.TTEEEEEETTS.EEEEEEE.SSSEEEE.GGGGGS.SSEEEEES.SBSS...S..
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DSSP 1xka .BSSEE..TTS.TTEEEEEETTS.EEEEEEE.SSSEEEE.GGGGGS.SSEEEEES.SBTTS..SS.
DSSP 1xkb .BSSEE..TTSSTTEEEEE.TTS.EEEEEEE.SSSEEEE.GGGGGS.SS.EEEES..BSSS...S.
Consensus .BSSEE..TTSSTTEEEEEETTS.EEEEEEE.SSSEEEE.GGGGGS.SSEEEEES.SBTTS..SS.
DSSP <A <--B-> <--C--> <-D> # <-E-> F
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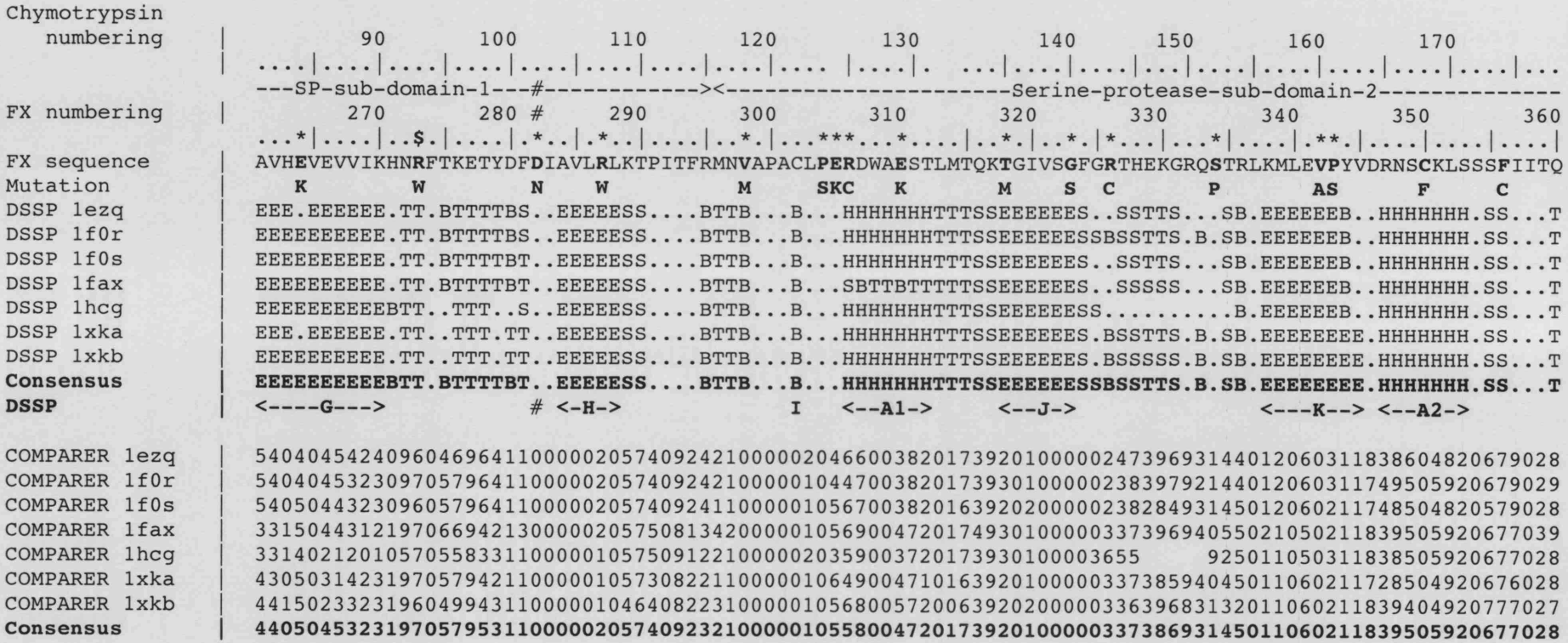
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Mutation K W N W M SKC K M S C P AS F C
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DSSP 1hcg EEEEEEEEEEBTT..TTT..S..EEEEESS....BTTB...B...HHHHHHHTTTSSEEEEEESS.....B.EEEEEEB..HHHHHHH.SS...T
DSSP 1xka EEE.EEEEEE.TT..TTT.TT..EEEEESS....BTTB...B...HHHHHHHTTTSSEEEEEES.BSSTTS.B.SB.EEEEEEEE.HHHHHHH.SS...T
DSSP 1xkb EEEEEEEEEE.TT..TTT.TT..EEEEESS....BTTB...B...HHHHHHHTTTSSEEEEEES.BSSSSS.B.SB.EEEEEEEE.HHHHHHH.SS...T
Consensus EEEEEEEEEEBTT.BTTTTBT..EEEEESS....BTTB...B...HHHHHHHTTTSSEEEEEESSBSSTTS.B.SB.EEEEEEEE.HHHHHHH.SS...T
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Chymotrypsin numbering
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Serine protease sub-domain 2
FX numbering
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DSSP 1ezq TEEEES.SSSS.B..TTTTT.EEEEEETTEEEEEEEEEE.SSSS.TT..EEEEGGGSHHHHHHHHH
DSSP 1f0r TEEEES.SSSS.B..TT.TT.EEEEEETTEEEEEEEEEE.SSSS.TT..EEEEEGGGSHHHHHHHHH
DSSP 1f0s TEEEES.SSSS.B..TT.TT.EEEEESSS.EEEEEEEEEESSSS.TT..EEEEEGGGGHHHHHHHTT
DSSP 1fax TEEEES.SSSS.B..TT.TT.EEEEEETTEEEEEEEEEE.SSSS.TT..EEEEEGGGTHHHHHHHHH
DSSP 1hcg TB..BS.SSSS.B..TTTTT.EEEEESSS.EEEEEEEEEESSSS.TT..EEEEESGGGHHHHHHHTT...SSB
DSSP 1xka TEEEES.SSSS.B..TT.TT.EEEEEETTEEEEEEEEEE.SSSS.TT..EEEEEGGGSHHHHHHHTT
DSSP 1xkb TEEEES.SSSS.B..TTTTT.EEEEEETTEEEEEEEEEEESSSS.TT..EEEEEGGGGHHHHHHHHS
Consensus TEEEES.SSSS.B..TTTTT.EEEEEETTEEEEEEEEEEESSSS.TT..EEEEEGGGSHHHHHHHHT...SSB
DSSP <-L> # <--M-> <----N----> <-O-> <--A3-->
COMPARER 1ezq 00000025667110070010000005156000000000112310577210000200301810771199
COMPARER 1f0r 00000026666110070010000005146000000000112210577211000200300710670089
COMPARER 1f0s 00000025567200060010000005147000000000112310578211000200301910671099
COMPARER 1fax 10000026667200073010000004147000000000112300576211000100200910650 2858
COMPARER 1hcg 10000026765210072020000004156010000000111710666211000100100800530 1755865169
COMPARER 1xka 10000026566100050010000004148010000000111200577211000100101710560 0747
COMPARER 1xkb 20100025566200070010000005257000000000111200577211000200400610360 1746
Consensus 10000026667200071010000005147000000000112310577211000200300810671187 7865169

Appendix 2

Nucleotide sequence for the coding region of *F10*. The residue numbering is as used most commonly in publications and throughout this thesis. The mature protein starts at +1.

```
-40                                             -30
atg ggg cgc cca ctg cac ctc gtc ctg ctc agt gcc tcc ctg gct ggc ctc ctg ctg ctc
Met gly arg pro leu his leu val leu leu ser ala ser leu ala gly leu leu leu leu
-20                                             -10
ggg gaa agt ctg ttc atc cgc agg gag cag gcc aac aac atc ctg gcg agg gtc acg agg
gly glu ser leu phe ile arg arg glu gln ala asn asn ile leu ala arg val thr arg
1                                               11
gcc aat tcc ttt ctt gaa gag atg aag aaa gga cac ctc gaa aga gag tgc atg gaa gag
ala asn ser phe leu glu glu met lys lys gly his leu glu arg glu cys met glu glu
21                                              31
acc tgc tca tac gaa gag gcc cgc gag gtc ttt gag gac agc gac aag acg aat gaa ttc
thr cys ser tyr glu glu ala arg glu val phe glu asp ser asp lys thr asn glu phe
41                                              51
tgg aat aaa tac aaa gat ggc gac cag tgt gag acc agt cct tgc cag aac cag ggc aaa
trp asn lys tyr lys asp gly asp gln cys glu thr ser pro cys gln asn gln gly lys
61                                              71
tgt aaa gac ggc ctc ggg gaa tac acc tgc acc tgt tta gaa gga ttc gaa ggc aaa aac
cys lys asp gly leu gly glu tyr thr cys thr cys leu glu gly phe glu gly lys asn
81                                              91
tgt gaa tta ttc aca cgg aag ctc tgc agc ctg gac aac ggg gac tgt gac cag ttc tgc
cys glu leu phe thr arg lys leu cys ser leu asp asn gly asp cys asp gln phe cys
101                                             111
cac gag gaa cag aac tct gtg gtg tgc tcc tgc gcc cgc ggg tac acc ctg gct gac aac
his glu glu gln asn ser val val cys ser cys ala arg gly tyr thr leu ala asp asn
121                                             131
ggc aag gcc tgc att ccc aca ggg ccc tac ccc tgt ggg aaa cag acc ctg gaa cgc agg
gly lys ala cys ile pro thr gly pro tyr pro cys gly lys gln thr leu glu arg arg
141                                             151
aag agg tca gtg gcc cag gcc acc agc agc agc ggg gag gcc cct gac agc atc aca tgg
lys arg ser val ala gln ala thr ser ser ser gly glu ala pro asp ser ile thr trp
161                                             171
aag cca tat gat gca gcc gac ctg gac ccc acc gag aac ccc ttc gac ctg ctt gac ttc
lys pro tyr asp ala ala asp leu asp pro thr glu asn pro phe asp leu leu asp phe
211                                             191
aac cag acg cag cct gag agg ggc gac aac aac ctc acc agg atc gtg gga ggc cag gaa
asn gln thr gln pro glu arg gly asp asn asn leu thr arg ile val gly gly gln glu
201                                             211
tgc aag gac ggg gag tgt ccc tgg cag gcc ctg ctc atc aat gag gaa aac gag ggt ttc
cys lys asp gly glu cys pro trp gln ala leu leu ile asn glu glu asn glu gly phe
221                                             231
tgt ggt gga act att ctg agc gag ttc tac atc cta acg gca gcc cac tgt ctc tac caa
cys gly gly thr ile leu ser glu phe tyr ile leu thr ala ala his cys leu tyr gln
241                                             251
gcc aag aga ttc aag gtg agg gta ggg gac cgg aac acg gag cag gag gag ggc ggt gag
ala lys arg phe lys val arg val gly asp arg asn thr glu gln glu glu gly gly glu
261                                             271
gcg gtg cac gag gtg gag gtg gtc atc aag cac aac cgg ttc aca aag gag acc tat gac
ala val his glu val glu val val ile lys his asn arg phe thr lys glu thr tyr asp
281                                             291
ttc gac atc gcc gtg ctc cgg ctc aag acc ccc atc acc ttc cgc atg aac gtg gcg cct
phe asp ile ala val leu arg leu lys thr pro ile thr phe arg met asn val ala pro
301                                             311
gcc tgc ctc ccc gag cgt gac tgg gcc gag tcc acg ctg atg acg cag aag acg ggg att
ala cys leu pro glu arg asp trp ala glu ser thr leu met thr gln lys thr gly ile
321                                             331
gtg agc ggc ttc ggg cgc acc cac gag aag ggc cgg cag tcc acc agg ctc aag atg ctg
val ser gly phe gly arg thr his glu lys gly arg gln ser thr arg leu lys met leu
341                                             351
gag gtg ccc tac gtg gac cgc aac agc tgc aag ctg tcc agc agc ttc atc atc acc cag
glu val pro tyr val asp arg asn ser cys lys leu ser ser ser phe ile ile thr gln
361                                             371
aac atg ttc tgt gcc ggc tac gac acc aag cag gag gat gcc tgc cag ggg gac agc ggg
asn met phe cys ala gly tyr asp thr lys gln glu asp ala cys gln gly asp ser gly
381                                             391
ggc ccg cac gtc acc cgc ttc aag gac acc tac ttc gtg aca ggc atc gtc agc tgg gga
gly pro his val thr arg phe lys asp thr tyr phe val thr gly ile val ser trp gly
401                                             411
gag ggc tgt gcc cgt aag ggg aag tac ggg atc tac acc aag gtc acc gcc ttc ctc aag
glu gly cys ala arg lys gly lys tyr gly ile tyr thr lys val thr ala phe leu lys
421                                             471
tgg atc gac agg tcc atg aaa acc agg ggc ttg ccc aag gcc aag agc cat gcc ccg gag
trp ile asp arg ser met lys thr arg gly leu pro lys ala lys ser his ala pro glu
441
gtc ata acg tcc tct cca tta aag tga gat ccc act caa aaa aaa aaa aaa aaa aaa aaa
val ile thr ser ser pro leu lys OPA asp pro thr gln lys lys lys lys lys lys lys

aaa aaa
lys lys
```