MOLECULAR ANALYSIS OF THE PROTHROMBIN GENE IN TWO PATIENTS

by

Leslea Marie Duke

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We accept this thesis as conforming to the required standard

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Department of Biochemistry and Molecular Biology

The University of British Columbia
2174 Health Sciences Mall
Vancouver, Canada
V6T 1Z3

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[Signature]

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ABSTRACT

A deficiency of prothrombin (FII) is an extremely rare bleeding disorder. Two patients were studied with a severe form of this disorder which is known as Hypoprothrombinemia. Each patient was identified as being the product of a consanguineous union, and therefore a single homozygous mutation would be expected. To characterize the deficiency, a clinical analysis was performed. FII antigen and activity levels were provided for each patient. FII-Vancouver was assessed at 3% FII activity and 3% FII antigen levels. FII-Utrecht exhibited similar levels of 3% for both activity and antigen. The objective of this study was to analyze the FII gene in each patient, and to identify the molecular basis for the disease.

Genomic DNA was provided for each patient and the exons of the prothrombin gene were amplified specifically by using the polymerase chain reaction. Primers were designed to include the intron/exon splice junctions and approximately 100 bp 5' to exon 1 in the amplification reactions. To facilitate directional cloning, restriction sites were incorporated into the primers. In this way, each region could then be analyzed by DNA sequencing. The sequence analyzed in each patient was then compared to the reported sequence of wild-type FII gene (Degan et al., 1987). Several base changes were observed in both patients, and all were found to be homozygous.

A single inserted 'A' was found in the region 5' to exon 1, at nucleotide position -54, in both FII-Vancouver and FII-Utrecht (numbering is according to the sequence of Degan et al., 1987). This insertion was later found in the wild-type sequence reported by Bancroft et al., (1990). It is evident therefore that this alteration is not the cause of the deficiency; however, it does provide the opportunity to focus on the promoter region as
a potential region for mutation. At nucleotide position 461, a single 'T' was deleted from the splice region 5' to exon 2. This change was not expected to interfere with the proper splicing of FII, as the deleted base occurs in a series of 3 'T' residues. The function of this base could be easily adapted by either of the flanking bases. This change was observed in both patients. A silent polymorphism resulting from the point mutation of the codon CTA -> CTG was found in exon 2 of FII-Vancouver and FII-Utrecht. The leucine residue at codon position 56 would be unaffected by this mutation. A polymorphism was found in the splice region 3' to exon 6, which results from the point mutation of G -> A, at nucleotide position 4272. This mutation occurs in both patients, and was also reported by Iwahana et al., (1992). The most significant mutation found was the result of a three bp deletion of the in-frame codon AAG, at nucleotide position 7485-7487. This mutation deletes a single lysine residue at the codon position 301. This mutation was found in FII-Vancouver, and was found to be homozygous. This deletion occurs in the activation region of FII, in what would eventually be the A-chain of thrombin (FIIa). This mutation is expected to have an effect on the 3-dimensional structure of the polypeptide. A silent polymorphism was identified in exon 10 of both patients. This point mutation resulted from the substitution of ACA -> ACC, at nucleotide position 8903. This threonine at position 388 was unaffected by this change.

The molecular basis of the deficiency in FII-Vancouver appears to be the result of a single deleted lysine residue. This mutation is expected to have global effects on the proper folding of the protein during synthesis, resulting in its degradation. A mutation resulting in the synthesis of an aberrantly folded protein is quite common among disorders where there is little or no generation of protein. The basis of the deficiency in FII-Utrecht remains
elusive. The observed changes observed in the FII gene were not expected to have a significant effect on the generation of the protein. The intron/exon junctions and splice branch points all remain intact, therefore, the disorder is not expected to be due to an error in splicing. It is possible that the deficiency is in fact, due to a defect in transcription. It is common to find mutations in the upstream promoter and enhancer regions which affect transcription such that minimal amounts of protein are generated. Further studies would be necessary to test the fidelity of the FII promoter in this patient.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Adenosine</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bis</td>
<td>N, N'-methylenebisacrylamide</td>
</tr>
<tr>
<td>bME</td>
<td>b-mercaptoethanol</td>
</tr>
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<td>bp(s)</td>
<td>base pair(s)</td>
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<td>Curie</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
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<td>diethylaminoethyl</td>
</tr>
<tr>
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</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FII</td>
<td>prothrombin (Factor II)</td>
</tr>
<tr>
<td>G</td>
<td>guanosine</td>
</tr>
<tr>
<td>Gla</td>
<td>γ-carboxyl glutamic acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>kbp(s)</td>
<td>kilo base pair(s)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>nt(s)</td>
<td>nucleotide(s)</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
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<td>polyethylene glycol</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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INTRODUCTION

1. BLOOD COAGULATION

A. Overview of Hemostasis

Hemostasis is the arrest of hemorrhage through the interplay and regulation of four systems: endothelium, platelets, coagulation, and fibrinolysis (Colman et al., 1987). Normal endothelium inhibits coagulation and adhesion of platelets on its surface by the expression of heparin-like substances, and the presence of protein complexes such as thrombin-thrombomodulin. Disruption of the vascular endothelium, due to blood vessel injury, precipitates activation of a number of systems designed to contain blood flow and repair the damaged tissue. Exposure of the subendothelium causes the accumulation and subsequent activation of platelets at the site of injury. Platelets form the initial line of defense against vascular leakage forming a platelet plug. Once activated, platelets expose receptors on their surface, such as the receptor to which fibrinogen (Fgn) binds. In larger vessels, simultaneous activation of the blood clotting cascade parallels formation of the platelet plug. Although not completely understood, the blood clotting cascade is a tightly regulated system of hemostasis (Davie et al., 1992). This cascade involves a sequential series of reactions involving the activation, by limited proteolysis, of plasma protein zymogens (Davie et al., 1979). In the final step of this cascade, prothrombin (FII) is converted to thrombin. The serine protease thrombin is then able to convert the plasma soluble fibrinogen (Fgn) to fibrin. Fibrin forms a mesh network with activated platelets at the site of injury to halt bleeding. Prior to repair of the damaged endothelium is the removal of the blood clot by a process known as fibrinolysis. Plasminogen is a key component of fibrinolysis. Both Fgn and fibrin maintain receptors for plasminogen and
plasmin. Plasminogen becomes integrated into the clot during its formation. Plasminogen is activated to plasmin which can then cleave fibrin polymers for solublization of the clot. Fibrin dissolution products then impede further clot formation in two ways: fibrin products can bind to the Fgn sites on thrombin, preventing activation of more Fgn; in addition, the split products can be inefficiently incorporated into the clot and destabilize it by impeding fibrin polymerization (Colman et al., 1987). Coordination and regulation of these four components of hemostasis maintains the integrity of the circulatory system.

B. Blood Clotting Cascade

The blood clotting cascade involves the systematic activation and amplification of a series of reactions resulting in the formation of an insoluble blood clot (Davie et al., 1991). This process is achieved by one of two separate but converging pathways as shown in Figure 1 (Davie et al., 1991, MacFarlane, 1964.; Davie and Ratnoff, 1964). The extrinsic pathway of blood coagulation is initiated by the expression of tissue factor (TF). TF is a membrane protein which is exposed when there is a blood vessel injury. TF acts as a cofactor in FVII activation; however, the mechanism involved has yet to be identified. In the presence of Ca$^{2+}$, the TF-FVIIa complex activates FIX and FX. FXa assembles on the membrane surface through the cofactor FVa, and in the presence of Ca$^{2+}$. This assembly of factors is known as the prothrombinase complex, and its function is to activate prothrombin (FII) to thrombin (FIIa). FIIa catalyses the conversion of fibrinogen (Fgn) to fibrin (Fn) by the proteolytic release of fibrinopeptides A and B. These Fn monomers spontaneously polymerize to form a network. FIIa also activates the transglutaminase FXIII to FXIIIa. The fibrin network is stabilized by
Figure 1. Diagramatic Representation of the Blood Clotting Cascade. The process of blood coagulation can be initiated by either the intrinsic or extrinsic paths. Both paths converge with the activation of FX. The major reactions of this complex and highly regulated system are depicted above. Blood clotting factors (F) are identified by Roman numerals. Activated forms of proteins can be distinguished from inactive forms by the subscript 'a'. Ca++ represents calcium ions. TF stands for tissue factor, and PL stands for phospholipids. The components of the prothrombinase complex are indicated by the box.
crosslinking of the γ-chains of Fn by this transglutaminase. This generates a strong clot which is resistant to the sheer forces imposed within the vascular system.

The intrinsic pathway originates with all of its components in the blood. Initially, FXII was thought to be the first protein to be activated in this process; however, it is now thought that the primary role of FXII may be in the inflammatory response. FXI has been shown to be activated by thrombin in the presence of a negatively charged surface (Naito and Fujikawa). FXII can then be activated by FXIa to FXIIa by simultaneous binding with high molecular weight kininogen (HMWK) to a negatively charged surface. FXIIa can upregulate the activation of FXI. FXIa activates FIX to FIXa, in the presence of calcium ions (Ca^{2+}). In a membrane bound complex with FVIIIa, FIXa causes the activation of FX. At this point in the cascade the two pathways converge into the common path as described above. The intrinsic system is probably not physiologically relevant for initiation of blood coagulation; however, it may be involved in maintenance of the cascade once it is activated by the extrinsic system.

The process of blood coagulation is a complex, highly regulated system. Activation of each of the individual components is achieved by limited proteolysis. Each individual component is regulated by a number of proteins in a feedback mechanism. In addition, the entire pathway is also globally regulated by inhibitors, anticoagulants, and by the blood clotting factors themselves. This representation, therefore, includes only the major reactions involved in the formation of a blood clot.
2. STRUCTURAL ORGANIZATION OF THE DOMAINS OF THE VITAMIN K DEPENDENT BLOOD CLOTTING PROTEINS

Prothrombin, as well as many of the blood clotting proteins, belongs to a family of proteins known as the serine proteases. This group of proteins is characterized by the catalytic triad which serves as the active site. This triad is composed of a serine, a histidine, and an aspartic acid. The serine proteases hydrolyze peptide bonds specific for each individual enzyme. Trypsin is a common serine protease that shares a high degree of sequence identity with the blood clotting serine proteases. Thrombin has the same specificity for small substrates as does trypsin, but they differ in physiological substrates. This uniqueness of substrate specificity seems to be dependent on the molecular surface surrounding the active site (Furie and Furie, 1988). Overall, the blood clotting serine proteases are twice the size of trypsin but the catalytic domains are quite similar (Furie and Furie, 1988). Differences among the enzymes are manifested predominantly by substrate specificity.

The common structural features of the blood clotting proteins can be attributed to evolutionary events in the diversification of this family of proteins. Common gene organization among the members suggests a common ancestral gene diversified by evolutionary processes such as exon swapping, gene duplications, rearrangements, and homologous crossovers. Further diversification could evolve through individual mutations. The blood clotting proteins represent a family of proteins with diversified functions, but common structural elements (Furie and Furie, 1988).

Analysis of the domains of several of the blood clotting proteins reveals extensive regions of sequence identity. Figure 2 depicts the common
Figure 2. Common Features of Blood Clotting Proteins.
Many of the structural domains of the blood clotting proteins are conserved. The similarity can often be extrapolated to function. It is believed that regions of identity evolved from a single ancestral gene. The structural regions are indicated in the key, and their function described in the text. The structural diagrams for prothrombin (Degen et al., 1983), FIX (Kurachi and Davie, 1982; Choo et al., 1982), FX (Leytus et al., 1984; Fung et al., 1985), FVII (Hagen et al., 1986), and Protein C (Foster and Davie, 1984; Beckmann et al., 1985, Long et al., 1984) taken from Furie and Furie (1988).
structural domains of many of the blood clotting proteins. The C-terminal domain is shared by all of the blood clotting factors shown. This region contains the conserved sequence Gly-Asp-Ser-Gly-Gly, which includes the active site serine (Furie and Furie, 1988). This area shares amino acid sequence identity with the pancreatic serine proteases such as trypsin and chymotrypsin (MacGillivray et al., 1988). Prothrombin, FX, FIX, and Protein C (PC), are all synthesized as preproproteins. All of these proteins have extensive sequence identity in their signal and propeptide regions. The amino termini of these proteins are characterized by the presence of Gla residues (γ-carboxyglutamic acid residues) that are involved in calcium-dependent membrane binding. Prothrombin contains two regions called kringles (Magnusson et al., 1975). This sequence involves approximately 80 amino acids, and three invariant disulfide bridges clustered in the center of the kringle. This structure is also found in FXII, plasminogen, and plasminogen activator. The exact function of these kringles has not been precisely determined; however, they are thought to be involved in protein-protein interactions and macromolecular assembly (Furie and Furie, 1988). The kringle structure is not present in FVII, FIX, FX, or PC, but in its place is an EGF-like domain. This region is composed of 53 amino acids and three disulfide bonds, and functions to bind specific cell-surface receptors. A common feature to all of FII, FVII, FX, FIX, and PC, is the aromatic amino acid stack. This region contains a conserved sequence of amino acids with aromatic side chains which interact in a ring cluster. This region is thought to play a role in membrane binding (Furie and Furie, 1988). The activation regions also share structural similarities in all of the described proteins. This subclass of proteins, therefore represents a case of evolutionary diversification of common genes to carry out unique functions.
3. PROTHROMBIN

A. Gene Structure and cDNA

Figure 3 shows a diagramatic representation of the gene, the mRNA, and the FII protein. The gene for prothrombin (FII) has been localized to chromosome 11 at position 11p11-q12 (Royle et al., 1987), and is 21kb in length (Furie et al., 1988; Degan et al., 1987). The entire prothrombin gene has been sequenced (Degan et al., 1987). The gene is composed of fourteen exons, separated by thirteen introns (Degan et al., 1987). Exons range in size from 25 to 315 bp, whereas introns range from 84 to 9447 bp. Approximately ninety percent of the gene involves intervening sequence. Of this, forty percent is composed of repetitive DNA sequences in a clustered fashion; 30 copies of Alu and 2 of the Kpn family of repeats (Degan et al., 1987; Furie et al., 1988). The sequence of the splice junctions at the 5' and 3' of intervening sequences is consistent with that found in other eukaryotic genes, in that they follow the GT-AG rule of Breathnach et al., (1978). The one exception to this is the presence of a GC rather than a GT at the 5' end of the intron spanning exons 12 and 13 (Degan et al., 1987). The 5' consensus sequences of TATA (TATAAA) within 50 bp and CCAAT sequences (5'GGCCAATCT3') within 200 bp of the transcription start site which normally direct the proper initiation of transcription by RNA polymerase are absent in prothrombin (Chow et al., 1991, Bancroft et al., 1992). A lack of a TATA sequence may correspond to the heterogeneity of start sites (Bancroft et al., 1992). Major transcription initiation sites have been mapped to 23 and 36 bp upstream of the initiator codon by Chow et al., (1991); whereas Bancroft et al., (1992)
Figure 3. Organization of the Prothrombin Gene, mRNA, and Protein.
A. The FII gene is depicted in the 5' to 3' direction (Degen and Davie, 1987). The relative size of the exons are indicated by boxes, separated by intervening sequences (introns are not drawn to scale). The FII gene is 20 kb in length. Refer to text for specific information on the FII gene. B. The mRNA for human FII is 2 kb in length. A leader sequence, shown in black, is followed by the entire coding region for FII, followed by a 97 bp non-coding region, and a 27 bp poly(A) tract (Degen et al., 1983). C. The entire FII protein structure is shown (Degen et al., 1983). Structural regions are labelled as shown, and are further described in the text.
A.

KEY:

-  SIGNAL PEPTIDE
- ● PROPEPTIDE
- § AROMATIC STACK
- Ø KRINGLE
- Y Y Y CLA DOMAIN
- □ ACTIVATION PEPTIDE
- ▲ CATALYTIC DOMAIN

B.

5' LEADER
CODING REGION
3' UTR

C.
identified the sites between -37 and -31. A weak promoter has therefore been proposed immediately upstream of the transcription start site (-1 to -435), and an enhancer has been identified between nucleotides -860 and -940. (Chow et al., 1991). Bancroft et al., (1992) identified a positive cis-acting regulatory region between nucleotides -2969 to -797 bp. A region highly similar to the HNF-1 protein binding site has been identified at -888 to -876 (Bancroft et al., 1992).

The cDNA for prothrombin is 2005 bp in length (Furie and Furie., 1988; MacGillivray and Davie, 1984; Degan et al., 1983). The cDNA encodes a leader sequence of 36 amino acids, 579 amino acids of the mature protein, a stop codon, an intervening sequence of 97 bases, and a 27 base poly(A) tail (Degan et al., 1983). The longest 5' noncoding region preceding the initiator methionine is 27 bp by MacGillivray et al. (1986); whereas Degan et al., (1989) reported it to be 26 bp.

B. Protein Structure

Plasma prothrombin is synthesized exclusively in the liver as a single chain glycoprotein, of 72 000 MW. The entire protein sequence of FII has been described (Magnusson et al., 1975; Butkowski et al., 1977; Walz et al., 1977). The nascent protein is composed of three regions; a hydrophobic pre-region of up to 34 amino acid residues, a basic pro-region of up to 9 residues, and the coding region of 579 residues. The pre-region is cleaved by a signal peptidase on the lumenal side of the endoplasmic reticulum (ER). The pro-region is partially split in the smooth ER, and completely in the Golgi (Jackson et al., 1987). The 579 residue mature protein contains 8% carbohydrate in 3 chains from asparagine residues 78, 100, and 373 (Degan and
Davie, 1987). Plasma levels of prothrombin circulate at about 100 μg/ml (Furie and Furie, 1988).

C. Gene Structure Related to Protein Structure and Function

The exons of the prothrombin gene are organized such that each encodes a particular structural domain of the protein. This structural domain can then be related to the function of that region of the molecule, with respect to the overall function of the protein.

1. Signal and Propeptide Regions

FII is synthesized with an additional 43 residues at the amino terminus encoded by exon 1. Two arginine residues precede the N-terminal alanine found in the mature protein. Typically, an Arg-Ala bond is not cleaved by a signal peptidase. This region represents a signal and a propeptide region. By analogy with FIX, cleavage by the signal peptidase is at approximately -18 (Banfield et al., 1993). The pro-region is cleaved by a proprotein peptidase to reveal the mature protein. There is a large degree of sequence identity of these regions in all of the vitamin K dependent clotting proteins. The propeptide region contains the γ-carboxylation recognition site (γ-CRS). Ten glutamic acid residues, encoded by GAG, are post-translationally modified to gamma-carboxylated glutamic acid residues (Gla) by a membrane bound vitamin K dependent carboxylase (Suttie et al., 1993). This Gla domain is thought to be involved in calcium dependent membrane binding of the protein. Two or three Gla residues bind a single calcium ion, which forms a non-covalent intramolecular bridge between the polypeptide backbone (Furie and Furie, 1988). Membrane binding is then facilitated by direct binding
through the calcium ions (Mann et al., 1982), or by exposure of a secondary binding site due to the tertiary folding of the protein (Borowski et al., 1986).

2. Aromatic Stack

Exon 3 encodes the aromatic amino acid stack. This region has a conserved sequence of Phe-Trp-X-X-Tyr. This region contains a series of hydrophobic side chains which interact in a ring cluster. This region is found on the surface of the protein and is thought to be involved in membrane binding (Park and Tulinsky, 1986; Furie and Furie, 1988).

3. Kringle Domains

In prothrombin, exons 4 through 7 encode 2 kringle domains (Magnusson et al., 1975). These structures are composed of approximately 80 amino acids. Three disulfide bonds are generated from 6 invariant cysteine residues (Magnusson et al., 1975). The sulfur atoms of the disulfide bonds are clustered in the center of the kringle (Park and Tulinsky, 1986) which is responsible for its unique tertiary structure. The exact function of kringles has not yet been positively elucidated, but it is speculated that they are involved in membrane binding as it has been shown that the second kringle of prothrombin binds FVa (Esmon and Jackson, 1974.). Kringle structures have also been observed in tissue plasminogen activator, urokinase, plasminogen, and FXII (Furie and Furie, 1988).

4. Activation Domain

Exons 8 and 9 encode the activation domain of FII, which includes the two sites of FXa cleavage. FXa cleaves FII at Arg273-Thr, and Arg322-Ile
This region also contains one of the two FIIa cleavage sites, at Arg286-Thr. This region then becomes the A chain of thrombin.

5. Protease Domain

This region is encoded by exons 10 through 14. The amino acids that encode this catalytic triad are encoded on separate exons in FII. Histidine is on exon 10, aspartate is on 11, and serine is found on exon 13 (Furie and Furie, 1988). Once activated by FXa, the protease region, comprising the active site catalytic triad and the substrate binding pocket, resides in the B-chain of FIIa. Crystallographic studies of chymotrypsin and trypsin revealed not only a high degree of sequence identity between the proteins, but identity which extended to the three dimensional structure and function of the polypeptide backbone. This catalytic region contains the residues like Ser-195, His 57, Asp 102, and the salt bridge like Ile 16 to Asp 194 of chymotrypsin (numbering based on chymotrypsinogen) (Magnusson et al., 1975). Models of FIIa have shown a substrate binding pocket with Asp522 at the base, to be similar to Asp189 in trypsin, and Ser189 of chymotrypsin (Magnusson et al., 1975). Entrance to the pocket is via two glycine residues (551 and 561), which is similar to chymotrypsin. It has been speculated that the different molecular surfaces surrounding the enzyme active sites may be responsible for the restricted substrate specificity of FIIa compared to trypsin and chymotrypsin (Furie and Furie, 1988).

3. PROTHROMBIN ACTIVATION AND THE PROTHROMBINASE COMPLEX

A schematic diagram of the prothrombinase complex is shown in Figure 4. The activation of the proenzyme FII involves the assembly of four
Figure 4. The Prothrombinase Complex
Schematic diagram outlining the components of the prothrombinase complex on a phospholipid surface. Clotting factors (F) are labelled by Roman numerals. Gla residues, involved in phospholipid binding, are shown on FXa and FII. Activation of FII, results in the release of the thrombin portion of the molecule (FIIa). The Pro region of FII, including the Gla residues remains associated with the membrane after cleavage.
additional components at the site of injury. The amplification process involved in the activation of various components of the blood clotting cascade, as well as the specific concentration of the required components to a localized area, facilitates the rapid activation of FII. FII is present in low amounts in plasma, but localization to the membrane allows concentration of this essential protein. The components required for FII activation are FVa, FXa, calcium, and phospholipids. The sum of these components comprise the prothrombinase complex which assembles on the activated platelet surface. FV is activated by thrombin to FVa; this protein acts as a surface receptor on platelets and phospholipids, to which FXa and FII attach. Phospholipids provide a membrane surface on which the complex can assemble. FXa and FII bind to the membrane through their γ-carboxyglutamic acid residues (Gla). This interaction is mediated by the presence of calcium ions. Calcium is thought to either directly bind the proteins through the Gla residues, or to cause a membrane binding site to be exposed through a conformational change in the protein.

Prothrombin is synthesized as a single chain plasma glycoprotein. Activation of this proenzyme involves a number of site specific cleavages. A diagram of the process of FII activation is shown in Figure 5. Based on proteolytic fragmentation, FII can be divided into 3 domains (Mann et al. 1981). The prothrombin fragment 1 region is composed of residues 1-155. The prothrombin fragment 2 region spans residues 156-273. The prethrombin region, which is the immediate precursor of thrombin, spans residues 274-581. FXa cleaves FII sequentially at two specific sites. The first cleavage occurs at Arg273-Thr, releasing fragment 1.2 (pro region comprising residues 1-273) from the prethrombin 2 (Figure 5A). A second FXa cleavage occurs at Arg322-Ile, splitting the prethrombin 2 region into the A (274-322)
Figure 5. Schematic Diagram of Products of Prothrombin Activation by FXa or FIIa.

Prothrombin has two sites that are sequentially cleaved by FXa to yield Fragment 1.2 and the two chain thrombin molecule. Cleavage at the first site yields Fragment 1.2 and Prethrombin 2 (A). Cleavage at the second site alone yields Meizothrombin (B). Thrombin has two additional cleavage sites in prothrombin. Independent of FXa, FIIa cleaves FII to yield Fragment 1, Fragment 2, and Prethrombin 2. A second cleavage by FIIa removes 13 residues from the N-terminus of Prethrombin 2, or what will finally be the A chain of thrombin.
and B (323-581) chains of α-thrombin. In the absence of the first cleavage, this product is called Meizothrombin (Figure 5B). Two additional cleavages of FII are recognized by thrombin. The first occurs between Arg155-Ser. This cleavage generates prothrombin fragment 1 and prethrombin 1. This bond is also cleaved in fragment 1.2, giving rise to fragment 1 and fragment 2. A second thrombin cleavage occurs at Arg256-Thr in both the A-chain of thrombin, and in prethrombin 2. This cleavage shortens the N-terminus of the A chain by 13 residues. The cleavage of both FXa sites and the second FIIa site generates thrombin (Figure 5C).

5. VITAMIN K DEPENDENCE OF PROTHROMBIN

Like many of the blood clotting factors, synthesis of functional prothrombin is dependent on the presence of vitamin K. Vitamin K is required by a membrane bound carboxylase, which is involved in the post-translational modification of Gla residues (Nelsestuen et al., 1974). Ten glutamic acid residues of prothrombin are modified in this way. This carboxylation is important for the calcium binding properties of the molecule. The administration of vitamin K agonists such as Dicumarol leads to abnormal FII synthesis, as well as abnormal synthesis of other vitamin K dependent proteins FIX and FX (Ferland et al., 1975). This abnormal FII is no longer able to bind calcium, and subsequently is unable to participate in coagulation, although abnormal FII can be activated by non-physiological means to generate fully functional FIIa (Ferland et al., 1975). By allowing association with the membrane during macromolecular assembly, this dependence on the Gla region serves to increase the specificity of FII activation, and limits the reactions by dissociation of the active portion of the molecule from the membrane bound region.
6. DEFICIENCIES OF PROTHROMBIN

Prothrombin deficiencies can occur in two distinct forms: Hypoprothrombinemia and Dysprothrombinemia. Both of these disorders are extremely rare in the population. The two forms of the deficiency exhibit the same clinical symptoms, but to varying degrees. Affected individuals exhibit bleeding whose severity varies with the level and activity of prothrombin. Bleeding manifestations of bruising, menorrhagia, postpartum hemorrhage, and hemorrhage following surgery or trauma are common among affected patients. Diagnosis is made on the basis of analysis of the family history, functional FII activity levels, and immunological levels. Acquired FII deficiency can therefore be distinguished from congenital FII deficiency. To eliminate the possibilities of combined defects, it is necessary to assay for deficiencies of other coagulation factors. The presence of a vitamin K deficiency, warfarin ingestion, or liver disease would result in a prothrombin deficiency, but other coagulation factor levels would be low as well. In vitamin K deficiency and warfarin ingestion, the active levels of FII would be decreased. In addition, immunological levels would be approximately 50%, as the decarboxylated form of FII is less stable (Roberts et al., 1981). Prothrombin deficiencies are generally treated by replacement therapy with plasma, or prothrombin complex concentrates.

A. Hypoprothrombinemia

Hypoprothrombinemia is classified as a true deficiency of prothrombin. It is inherited in an autosomal recessive fashion.
Heterozygotes are asymptomatic with functional FII levels of approximately 50%. Homozygotes are symptomatic, with functional FII activity levels of 2-25%. In hypoprothrombinemia, functional activity levels correlate with immunological levels of the protein.

**B. Dysprothrombinemia**

Dysprothrombinemia involves the synthesis of an abnormal FII molecule which maintains antigenicity, but lacks normal activity. The abnormal molecule is present, but has decreased or absent biological function. Again, heterozygotes are asymptomatic, with immunological levels of 100%, and activity levels of approximately 50%. Homozygotes have activity levels of less than 50%.

**C. Combined Hypo-Dysprothrombinemia**

The occurrence of a combined deficiency in a population is extremely rare. This condition occurs when one allele for prothrombin encodes a dysfunctional molecule resulting in a dysprothrombinemia, and the other allele encodes a hypoprothrombinemic condition, or absence of a molecule. Immunological levels approach 50% due to the presence of the defective FII molecule, whereas functional activity levels are found to be minimal.

**7. SCREENING TESTS FOR DISORDERS IN HEMOSTASIS**

To identify the source of a disorder in hemostasis, it is necessary to establish in which of the four areas the deficiency or dysfunction lies. This is accomplished by establishing the integrity of each of the four systems; vascular maintenance, platelet function, coagulation, and fibrinolysis (Colman et al., 1987). The bleeding time remains the most fundamental of
these tests. This measures the interaction of platelets with the vessel wall. Along with establishing the integrity of the components of the coagulation system, this tests reflects the presence or absence of sufficient platelets, and the integrity of the vascular wall with which they interact. A direct platelet count is definitive for a deficiency or dysfunction of platelets.

A general estimate of problems within the coagulation system can be established by a number of assays. The activated partial thromboplastin time (APTT) establishes the integrity of the intrinsic pathway of coagulation. This test is a measure of the contact activation of FXII, FXI, FIX, and FVIII, as a group. Less specifically this tests measures FV, FII, FX, and Fgn. Excluded is FVII which is part of the extrinsic pathway. This tests gives prolonged times relative to normal in the presence of dysfunctional or deficient coagulation factors. The prothrombin time (PT) measures the integrity of the intrinsic system, including FVII, FV, FX, FII, and Fgn. The thrombin time is a measure of clot formation, or rather, the conversion of Fgn to fibrin. Thrombin is the initiator and the formation of the clot is the endpoint.

Specific factor assays are necessary if the above tests reveal abnormalities in the system. The PT and PTT can be performed with patients' plasma, mixed with the plasma of a patient with a known deficiency of a particular protein. The deficiency is identified by correction of an abnormal PT or PTT by all but one of the plasmas with a known deficiency.

Activity of specific factors can also be established by amidolytic assays. A chromogenic substrate, specific for the factor to be tested is used. As the substrate is cleaved, a chromogen is generated whose concentration can be measured by absorbance and then related to the level of activity of the acting factor. This assay measures the activity of a particular protein, however, it is
not always indicative of the ability of the protein to function in the actual physiological system.

Various snake venoms have the ability to cleave clotting factors at the same sites as some of their physiological activators. *Echis carinatus* (EC) venom contains a protease which splits the Arg320-Ile bond of prothrombin. *Taipan* venom cleaves the same sites in prothrombin as the physiological activator FXa (Huisse et al., 1986). Activation by this means, and evaluation of the reaction products establishes the integrity of these sites in the protein. Again, this method is not indicative of the protein's ability to function in a physiological system.

Specific factor antibodies facilitate the detection of levels of clotting factors present in individuals with a potential deficiency. Coagulation factor levels are measured against levels established from a pooled population and subsequent deficiencies are determined.

8. PREVIOUSLY REPORTED PROTHROMBIN DEFICIENCIES

A deficiency of II can be classified into one of two forms. A complete deficiency of the protein is classified as a hypoprothrombinemic condition. Synthesis of an abnormal protein on the other hand, results in a dysprothrombinemic condition. Each respective disorder may be present in a hetero or homozygous state. A variety of complex combinations are then possible. A complex heterozygous situation may result where one allele encodes a hypoprothrombinemic condition, and the other allele encodes a dysprothrombinemic condition. Two different dysprothrombins can occur in a single patient. It is also possible to see a double heterozygous condition arising from the presence of two different hypoprothrombins; however, there
have been no reported cases as such. The resulting clinical symptoms have been discussed previously.

The incidence of a prothrombin deficiency is quite rare in a population. There are 15 reported cases of dysprothrombinemias, 4 of which are complex heterozygotes for 2 different dysfunctional II molecules. Girolami (1971) summarized 21 known cases of congenital hypoprothrombinemias, of which over 50% are consanguineous. Five cases exist as complex heterozygotes for dysprothrombinemia as well as hypoprothrombinemia. Since then, two more cases of homozygous congenital hypoprothrombinemia have been reported (Montgomery et al., 1978; Baudo et al., 1972). There has also been one documented case of a deficiency of II, which also included a deficiency of FVII, FIX, and FX. Although this condition resulted in a deficiency of II, it is neither a case of hypo- nor dysprothrombinemia, but rather a defect in either the γ-carboxylation mechanism within the hepatocyte, or a faulty vitamin K transport (Chung et al., 1979). This case does not represent a vitamin K deficiency, however, as administration of vitamin K does not alter the condition. There have been several reports of II deficiency resulting from a vitamin K deficiency, however, this does not involve hypo- or dysprothrombinemia as a sole cause. In order to establish a true FII disorder, it is necessary to eliminate a vitamin K deficiency as a potential cause. A summary of reported prothrombin variants, and proposed sites of mutations is included in Table 1.

Studying cases of reported FII deficiencies is useful for providing information on the structural and functional aspects of the prothrombin molecule. The analysis of previously reported defects may provide insight into the cause of the deficiencies reported in this study.
### TABLE 1: SUMMARY OF REPORTED PROTHROMBIN DEFICIENCIES

<table>
<thead>
<tr>
<th>FII VARIANT</th>
<th>LOCATION OF MUTATION</th>
<th>CONDITION</th>
<th>GENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poissy</td>
<td>N/C</td>
<td>Dys-II</td>
<td>Homozygous</td>
</tr>
<tr>
<td>Clamart</td>
<td>Activation Region</td>
<td>Dys-II</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>Denver</td>
<td>N/C</td>
<td>Dys-II</td>
<td>Homozygous</td>
</tr>
<tr>
<td>Brussels</td>
<td>Propeptide Region</td>
<td>Dys-II</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>Madrid</td>
<td>Activation Region</td>
<td>DYS-II</td>
<td>Homozygous</td>
</tr>
<tr>
<td>Gainesville</td>
<td>N/C</td>
<td>Dys-II</td>
<td></td>
</tr>
<tr>
<td>Houston</td>
<td>N/C</td>
<td>Dys-II</td>
<td></td>
</tr>
<tr>
<td>Cardeza</td>
<td>Activation Region</td>
<td>Dys-II</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>Barcelona</td>
<td>Activation Region</td>
<td>Dys-II</td>
<td>Homozygous</td>
</tr>
<tr>
<td>Mollese</td>
<td>Activation Region</td>
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<td>Compound Heterozygous</td>
</tr>
<tr>
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<td>N/C</td>
<td>Dys-II (2)</td>
<td>Compound Heterozygous</td>
</tr>
<tr>
<td>Perija</td>
<td>N/C</td>
<td>Dys-II</td>
<td></td>
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<tr>
<td>Salakta</td>
<td>Substrate Binding Region</td>
<td>Dys-II</td>
<td></td>
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<tr>
<td>Himi</td>
<td>Protease Region</td>
<td>Dys-II (2)</td>
<td>Compound Heterozygous</td>
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<td>Fibrinogen Recognition</td>
<td>Dys-II (2)</td>
<td>Compound Heterozygous</td>
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<tr>
<td>Tokushima</td>
<td>Protease Domain</td>
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<td></td>
<td>Kringle Domain</td>
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<td></td>
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<tr>
<td>Mexico City</td>
<td>Fragment 2 Region</td>
<td>Dys/Hypo-II (2)</td>
<td>Compound Heterozygous</td>
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<td>Habana</td>
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</tr>
<tr>
<td></td>
<td>Fragment 1 Region</td>
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<td></td>
</tr>
</tbody>
</table>

N/C = not characterized  
Dys = Dysprothrombinemia  
Hypo = Hypoprothrombinemia
A. Hypoprothrombinemias

Girolami et al., reported 3 cases of congenital hypoprothrombinemia, of which 2 were related. All 3 individuals were homozygous for the condition, with FII activity levels of 9-16% of normal. None of these FII variants have been characterized as yet.

B. Dysprothrombinemias

The presence of an abnormal molecule circulating in the blood has facilitated studies on structure and function, as the abnormal molecule can be isolated and characterized. Many of the dysprothrombinemias have been characterized at the protein level, and a few at the molecular level. Identification of the defect in these naturally occurring variants has allowed the identification of structural features critical for the activation process, assembly of the prothrombinase complex, and substrate recognition.

Of the prothrombin variants that have been identified, FII-Brussels, FII-Mexico City, and FII-San Juan have mutations in the propeptide region of the molecule (Kahn et al., 1974; Valls-de-Ruiz et al., 1987; Roberts et al., 1987). FII-San Juan and FII-Mexico City are examples of complex heterozygotes and will be described in a later section.

FII-Brussels has not been sufficiently characterized. This variant was found to have half the normal level of functional prothrombin by coagulation assays, but normal levels by immunological assays. Based on abnormal electrophoresis patterns using antibodies specific for this region, the defect was thought to be contained in the pro region of FII. This patient exhibited hemorrhagic symptoms, such that the mutation causing the variant FII would be expected to be significant.
Four of the dysprothrombinemias contain mutations in the activation region of the protein. FII-Clamart and FII-Cardeza are heterozygous for the disorder (Huisse et al., 1986; Shapiro et al., 1969); FII-Madrid and FII-Barcelona are homozygous (Diuguid et al., 1989; Rabiet et al., 1986). FII-Cardeza has prothrombin activity of 50% and antigen levels of 100% of normal. The defect in this prothrombin has been localized to the prethrombin 2 region of the protein. FII-Cardeza was shown to be unable to bind FXa, and subsequently, the Arg320-Ile bond cannot be cleaved (Shapiro et al., 1969).

FII-Clamart appears to have a defect in the FXa cleavage of the Arg320-Ile bond of prothrombin. This bond is normally cleaved by *Echis carinatus* (EC) venom. This would indicate that the mutation is probably not at this specific site, but rather a site nearby which causes changes in secondary structure, altering the accessibility of this bond by FXa, but not by E.C. venom. The cleavage at Arg271 seems affected at least in a secondary way as this bond cleavage is delayed relative to normal (Huisse et al., 1986).

FII-Barcelona exhibited severe hemorrhagic symptoms exemplified by low coagulant activity. FII protein levels were found to be normal. This protein was found to have a defect in activation, indicated by the absence of 1 of the 2 FXa cleavages. Amino acid analysis revealed a mutation of Arg271 to Cys. In addition, the active site was titrated in order to see what forms were present. It was shown that the second FXa cleavage is necessary for the appearance of the active site. The first FXa cleavage (to remove the pro-region) is not essential for thrombin-like activity on small substrates, but is necessary for clotting activity (Rabiet et al., 1986).

FII-Madrid was shown to have normal antigenic levels, but low coagulant activity. This patient was found to have a severe hemorrhagic
symptoms. The mutation was determined to be in the FXa-catalyzed cleavage site between the profragment and thrombin regions as found in FII-Barcelona (Diuguid et al., 1989).

A case of dysprothrombinemia has been reported where the suspected mutation resides in the substrate binding region. FII-Salakta was reported to have 100% FII antigenicity, but only 15-18% coagulant activity. The FXa cleavage was found to be normal, but thrombin activity was abnormal. Interactions of FII-Salakta, with compounds that bind to the primary binding site were found to be abnormal. Peptide sequence analysis revealed a substitution of Glu466 with Ala. This mutation was thought to change the conformation around the substrate binding site containing Trp468, which is in a unique surface loop on the molecule. This amino acid change was speculated to cause an alteration in this loop which in turn would reduce the affinity for antithrombin III and fibrinogen (Miyata et al., 1992).

A number of dysprothrombinemias have been reported but their defects have yet to be identified. FII-Gainsville was discovered in a pair of identical twins who exhibited FII activity levels of 23-25% of normal (Smith et al., 1981). FII-Houston exhibited half normal antigenic levels of FII, and minimal FII activity. Crossed immunoelectrophoresis may indicate the presence of two forms of dysfunctional FII, although this has yet to be confirmed (Weinger et al., 1980). FII-Perija, as well as FII-Denver, and FII-Poissey, are all examples of homozygous dysprothrombinemia (Ruiz-Saez et al., 1986; Montgomery et al., 1980; Tapon-Bretaudiere et al., 1983). All three cases exhibit severe hemorrhagic symptoms, corresponding to minimal coagulant levels. These variants have been studied by immunological and biological assays only.
There are at least four reported cases of compound heterozygotes of dysprothrombinemia: FII-Padua, FII-Himi, FII-Quick, and FII San Juan (Girolami et al., 1974; Morishita et al., 1992; Henriksen et al., 1986; Roberts et al., 1987).

FII-Padua represents an interesting case where FII activity levels are decreased in one and two stage assays, but normal by staphylococagulase assays. The parents of this individual are not consanguineous. It is therefore suspected that the structural abnormality in this dysprothrombinemia may consist of two separate populations of abnormal FII, or a single population that is altered in such a way that it is half active and half inactive (Girolami et al., 1974).

FII-Himi was identified as a complex heterozygote for two dysfunctional prothrombin molecules. FII antigen levels were normal, but FII activity levels were only 10% of normal. Two point mutations were identified in the thrombin portion of the molecule. A mutation of a 'T' to a 'C' at nucleotide position 8751, results in the substitution of a Thr for Met at codon 337 (Thrombin Himi I). A second mutation of a 'G' to an 'A' at nucleotide position 8904 encodes His instead of Arg at codon 388 (Thrombin Himi II). It was established that Himi I was inherited from the father and Himi II from the mother (Girolami et al., 1974).

FII-Quick, upon activation, reveals two dysthrombins. The mutation involved in Quick I was identified as a substitution of Arg382 with Cys. Arg382 has been shown to be critical in determining thrombin specificity for fibrinogen. Quick II was found to have some unusual properties. Quick II was unable to clot fibrinogen, nor could it bind artificial substrates or competitive inhibitors of thrombin. Quick II was identified as a substitution of Gly558 by valine. This glycine residue has been shown to be highly
conserved in the chymotrypsin family of serine proteases where it forms part
of the substrate binding pocket. This alteration provides evidence that the
not only does this glycine influence primary substrate specificity, but provides
evidence that the catalytic activity of the serine proteases are influenced by
structural changes within the primary binding pocket (Henriksen et al., 1986).

FII-San Juan is a compound heterozygote, with FII activity levels of
20% and FII antigen levels of 93% of normal. The two forms of dysfunctional
FII in this patient have not been thoroughly characterized. Roberts et al.,
(1987) have summarized was has been discovered so far. The mutation
involved in San Juan I appears to be a defect in the activation region. The
mutation associated with San Juan II appears to involve the gamma-
carboxylation region of prothrombin fragment 1.

C. Compound Heterozygotes for Dysprothrombinemia and
Hypoprothrombinemia

The presence of both conditions involving a deficiency in prothrombin
in a single patient results in a compound heterozygous condition. This
occurs when an allele for dysprothrombinemia is inherited from one parent,
and an allele for hypoprothrombinemia is inherited from the other. Patients
with this condition exhibit severe hemorrhagic symptoms. FII antigen levels
are usually less than 50%, and FII activity levels are minimal. There have
been five reported cases of compound heterozygotes for Dys-
Hypoprothrombinemia: FII-Molise, FII-Tokushima, FII-Mexico City, FII-
Habana, and FII-Metz (Girolami et al., 1978; Iwahana et al., 1992; Valls-de-Ruiz
et al., 1987; Rubio et al., 1983; Rabiet et al., 1979).

FII-Molise has 50% FII antigen, and 10% FII activity. The cause of
either of these conditions is uncertain. It is thought that the mutation resides
in the FXa sensitive region (Girolami et al., 1978).
FII-Tokushima has been characterized on the molecular level. The mutation involving the dysprothrombinemia was identified as a point mutation of a 'T' to a 'C' at nucleotide position 9490. This results in a substitution of Arg418 with tryptophan. The hypoprothrombinemic condition resulted from a single base insertion in exon 6. A 'T' was inserted at nucleotide position 4177. This change alters the sequence from codon 114, resulting in a premature stop at codon 174 in exon 7. This stop occurs in the kringle 2 domain, preceding the thrombin portion of the molecule. The dysprothrombinemic allele was inherited from the mother whereas the hypoprothrombinemic allele was inherited from the father (Iwahana et al., 1992).

FII-Mexico City has been studied at the protein level. The dysfunctional prothrombin molecule is suspected to have a mutation in fragment 2. The cause of the hypoprothrombinemic condition remains uncertain; however, this is an acquired mutation in the patient as neither parent was found to be heterozygous for the hypoprothrombinemic condition (Valls-de-Ruiz et al., 1987).

FII-Habana has been identified clinically as a complex heterozygote for both types of prothrombin deficiency; however, no characterization of either condition has been reported. The mother was found to be heterozygous for the dysprothrombinemia, and the father was heterozygous for the hypoprothrombinemic condition (Rubio et al., 1983).

FII-Metz has been studied on the level of the protein. The dysprothrombin was isolated and its activity towards natural and synthetic substrates was evaluated. Interactions of FII substrates and inhibitors with FII-Metz indicates that the structural defect involving the dysprothrombinemia resides in the thrombin portion of the molecule, more
specifically in the catalytic site, and not the specific interaction with substrate. The identity of the hypoprothrombinemic condition is undetermined (Rabiet et al., 1979).

9. OBJECTIVES OF THIS STUDY

Two patients were presented with a severe bleeding disorder. Screening assays for hemostatic disorders were employed, as well as specific factor activity and antigen assays. The patients were identified as having severe hypoprothrombinemia. As this disorder is extremely rare in the population, it presented a unique opportunity to examine the molecular basis of this disease. DNA samples from the afflicted individuals were provided. Our goal was to identify causative mutations in the prothrombin gene of these patients, and speculate on the effects that these mutations would have caused on the protein. The strategy employed involved the use of molecular biology techniques such as the Polymerase Chain Reaction (PCR), cloning, and DNA sequencing in an attempt to elucidate discrepancies between the normal prothrombin gene, and that present in the hypoprothrombinemic patients. This study may facilitate not only an understanding of the structure and folding properties of the prothrombin molecule, but may provide insight into the transcriptional processes of regulation.
MATERIALS AND METHODS

1. REAGENTS

Yeast extract, bacto-agar, and bacto-tryptone were purchased from Difco Laboratories. Agarose, acrylamide, bisacrylamide, urea, ammonium persulfate, and TEMED were purchased from Bio-Rad Laboratories. Phenol was purchased from British Drug Houses Ltd. (BDH). DTT, EtBr, βME, DMSO, and RNase A were purchased from Sigma Chemical Co. α-[thio-35S]-dATP was purchased from New England Nuclear. XGAL and IPTG were purchased from 5 Prime -> 3Prime Inc. Deoxy and dideoxy-ribonucleotides were obtained from Pharmacia. All other reagents were of reagent grade or higher, and were obtained from Bio-Rad, BDH, Pharmacia or Sigma.

Restriction endonucleases, T4 DNA Ligase, and T4 DNA Polymerase were purchased from Bethesda Research Laboratories (BRL), Boehringer Mannheim, or Pharmacia. Sequenase version 2.0 was purchased from United States Biochemical. Klenow-large fragment was obtained from BRL. Recombinant Taq polymerase (Amplitaq) was obtained from Perkin Elmer Cetus. Kodak X-Omat and Kodak XAR film was used for autoradiography.

2. STRAINS, VECTORS, AND MEDIA

A. Vectors

PCR amplified regions of the prothrombin gene were ligated into the polycloning site of the vector Bluescript, obtained from Stratagene. This phagemid is 2.9 kb in length, and contains the Escherichia coli (E. coli) origin of replication, a gene for ampicillin resistance, the f1 intragenic region, and part of the lac Z gene for α-complementation with the lac Z gene of E. coli.
B. Bacterial Strains

The hosts for transformations were the *E. coli* strain DH5αF'. The genotype of these cells are: F*endA1, hsdR17( rK- mK + ), supE44, thi -1, recA1, gyrA96, relA1, 080d, lacZD15, D(lacZYAargF)U169, Maniatis et al., (1989). The blue/white color selection was used for screening colonies on plates. Single stranded DNA was generated by addition of the helper phage M13 vcs. This helper phage conveys kanamycin resistance to its *E. coli* host.

C. Media

Bacterial hosts were grown on LB plates (5g bacto-yeast extract, 10g NaCl, 10g bacto-tryptone, pH to 7.5, 15g agar, adjust volume to 1 litre, autoclave to sterilize) supplemented with 50 μg/ml ampicillin, 25μg/ml IPTG, and 50μg/ml XGAL. IPTG and XGAL were made up in dimethylformamide.

3. OLIGONUCLEOTIDES

Oligonucleotides were designed for use in the Polymerase Chain Reaction (PCR), in the amplification of specific exons of the prothrombin gene. A summary of the oligonucleotide primers is shown in Table 2. Primers were designed to flank the target region, the forward primer was designed to be complementary to the (-) strand, and the reverse primer to be complementary to the (+) strand. Primers were designed to include the splice junctions of each exon. In the case of exon 1, the primer also included the first 100 bases 5' to the exon for amplification. Oligonucleotides were
### TABLE 2. PCR PRIMERS AND CLONING STRATEGY

<table>
<thead>
<tr>
<th>EXON</th>
<th>LENGTH (bp)</th>
<th>PRIMERS</th>
<th>TARGET</th>
<th>PRIMER SEQUENCE 5'-3'</th>
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<th>RESTRICTION</th>
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<td>Pst 1</td>
</tr>
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</table>

TA= annealing temperature
synthesized on an Applied Biosystems 391 DNA Synthesizer, and were purified by reverse-phase chromatography on Sep-Pak C18 cartridges as described by Atkinson and Smith (1984). The resulting primers were resuspended in distilled water (dH2O), and stored at -20°C.

4. Genomic DNA

DNA samples from hypoprothrombinemic patients were supplied for the purposes of this investigation. Prothrombin Utrecht, was kindly donated by Dr. H.K. Nieuwenhuis. Prothrombin activity and antigen levels were determined to be 3% of normal in both cases. Prothrombin Vancouver was donated by Dr. Ka-Wah Chan. Activity and antigen levels in this case were each found to be 3% of normal.

5. ISOLATION OF DNA

A. Plasmid DNA

Plasmid DNA was obtained by a modification of the Alkaline Lysis procedure of Maniatis et al., (1989). LB (5ml), containing ampicillin (50 μg/ml) was inoculated with a single colony of bacteria and grown at 37°C overnight. The culture was centrifuged at 3000 rpm for 15 min in a benchtop centrifuge. The cell pellet was resuspended in 200 μl of Glucose Buffer (50mM glucose, 25mM Tris-HCL pH 8.0, 10 mM EDTA). Suspensions were incubated at room temperature for 5 min, following addition of 400 μl Alkaline-SDS Buffer (0.2 N NaOH, 1% SDS). Cellular debris and genomic DNA was removed by the addition of 30 μl of 3M NaOAc pH 5.2, incubation at 4°C, and centrifugation in a microfuge for 10 min at room temperature. To 750 μl of the supernatant, 450 μl of cold isopropanol was added, and incubated at 4°C for 5 min. The plasmid DNA
was precipitated by centrifugation in a microfuge for 5 min at room
temperature. The resulting pellet was resuspended in 200 µl dH2O, followed
by a short centrifugation to remove any particulate matter. The DNA was
reprecipitated by addition of 100 µl of 7.5 M NaOAc pH 7.0, and 1 ml 95%
ethanol. After centrifugation for 15 min at room temperature, the pellet was
washed with 70% ethanol, dried, and finally resuspended in 35 µl dH2O.

B. Isolation of Single Stranded DNA.

After a 4 hour incubation of a culture as described above, 200 µl of M13
vcs helper phage were added to each culture. After a further hour at 37°C,
kanamycin was added to a final concentration of 50 µg/ml and the culture
was left to incubate overnight. Following a 15 min centrifugation at 3000
rpm, the supernatant was incubated on ice for 30 min, after the addition of 1
ml 50% PEG: 7.5 M NaOAc pH 7.0 (1:1, v/v). The suspension was
centrifuged at 3000 rpm for 15 min at 4°C. The resulting pellet was
resuspended in 300µl Tris-EDTA pH 8.0. The DNA was purified by extraction
with an equal volume of phenol:chloroform (1:1, v/v). DNA was
precipitated by addition of 30 µl 3 M NaOAc pH 7.0, and 1 ml 95% ethanol,
with centrifugation for 15 min in a microfuge. The pellet was washed with
70% ethanol, dried, and resuspended in 25 µl dH2O.

6. Gel Electrophoresis

A. Agarose Gel Electrophoresis.

DNA fragments, in the form of plasmid DNA, plasmid inserts, or PCR
fragments were separated according to size by electrophoresis in agarose gels.
The running buffer was 1X TAE (32 mM Tris, 16 mM NaOAc, 0.8 mM EDTA,
pH 7.2). EtBr was included in the gels at a concentration of 1µg/ml, and DNA
was visualized by examination under ultraviolet light. DNA samples were prepared in 1X Loading Dye (3% ficoll, 0.02% xylene cyanol, 0.02% bromophenol blue), and electrophoresed at 5-10 volts/cm, until an adequate separation of the DNA had been achieved.

B. Denaturing Polyacrylamide Gels.

Sequence analysis of radiolabelled DNA fragments was facilitated by electrophoresis of the DNA on 6% polyacrylamide gels at 60 watts constant power for 2 to 6 hours. The gels were prepared by addition of 8.3 M urea and an appropriate volume of bis:acrylamide (38%:2%, w/v), made up in 1X TBE. The running buffer was also 1X TBE (50 mM Tris base, 50 mM boric acid, 1 mM EDTA). Polymerization of the gel was initiated by addition of 0.066% (w/v) ammonium persulfate and 0.024% TEMED. DNA was visualized by autoradiography by drying the gel on 3MM paper in a heated Bio-Rad gel drier for 1 hour, followed by exposure of the gel to Kodax X-Ray film overnight.

7. POLYMERASE CHAIN REACTION.

The PCR was used for amplification of target sequences of DNA for subsequent cloning. A summary of the PCR and cloning strategy is shown in Table 2. A schematic diagram of the oligonucleotide primers, and their position relative to the exons of FII is shown in Figure 6. The method is described by Saiki et al (1985), and was carried out by the use of a Perkin Elmer Cetus DNA Thermocycler. Reactions contained a total volume of 50μl. Several different PCR buffers were used to maximize amplification of various fragments. Buffer A (67 mM Tris pH 8.8, 16.6 mM (NH₄)₂SO₄, 10 mM 2-mercaptoethanol, 1.0 mM MgSO₄), Buffer B (10 mM Tris pH 8.5, 0.05%
Figure 6. **PCR Strategy for the Amplification of the Exons of Prothrombin**

This schematic diagram illustrates the orientation of PCR primers with respect to the exons of human prothrombin. The boxes symbolize the relative size of each exon, with the lines between representing introns. Forward and reverse primers are indicated by their orientation and number. Primer 29 (hPT29) is shown as a nested primer between primers 26 and 27 for exon 7.
Tween-20, 0.05% Nonidet P-40, 1.0 mM MgCl₂, and the standard buffer recommended by Perkin Elmer Cetus (500 mM KCl, 100 mM Tris-HCL pH 8.3, 15 mM MgCl₂, 0.1% gelatin (w/v)) were all employed. Reactions contained 2µl genomic DNA (100 ng/µl), 5 µl 10X PCR buffer, 1 µl dNTP (10mM of each of dATP, dCTP, dGTP, dTTP), 1 µl forward primer (100ng/µl), 1 µl reverse primer (100ng/µl), and 1 µl Taq polymerase (5 units/µl), with the volume made up to 50 µl with dH₂O. PCR reactions varied but followed the general format of denaturation at 94°C for 15 to 30 sec, annealing at 50-56°C for 15 to 30 sec, and extension at 72°C for 30 to 60 sec, for a period of 25-30 cycles.

Annealing temperatures were initially estimated by the equation:

\[ TA = \left[ 4°C(GC) + 2°C(AT) \right] - 5°C \]

TA represents the final annealing temperature for a particular primer. The optimal annealing temperature was estimated based on the TA values from each set of primers.

8. DNA SUBCLONING

A. Purification of DNA Fragments

PCR reactions containing a single product (evaluated by electrophoresis on an agarose gel) were precipitated by addition of 0.5X volume 7.5M NaOAc pH 7.0, and 2X volume 95% ethanol. After centrifugation, the pellet was washed with 70% ethanol, dried, and resuspended in 20 µl dH₂O. A single PCR product was rescued from a non-specific amplification reaction by gel elution. The PCR reaction was made up in loading dye, and electrophoresed on a 1% agarose gel. The desired product was excised from the gel by removing the block of agarose which contained it. The block was subjected to electrophoresis within an dialysis bag with 300 µl TE pH 8.0 for 10 min at 90 volts. The DNA, having electrophoresed out of the gel and into solution was removed to an eppendorf tube where it was precipitated as described as above.
B. Restriction Digestion and Blunt-ending of Purified PCR Products

Fragments were digested with the appropriate restriction enzymes (as directed by the manufacturer) according to the strategy for directional cloning as outlined in Table 2. The digested product was precipitated as described above, and finally resuspended in 10 μl dH2O. In some cases, where PCR products were not directionally cloned, a blunt-ending strategy was employed. The enzyme used to do this was the Klenow fragment of *E. coli* DNA Polymerase 1 (Maniatis et al., 1989). Klenow fragment (1 μl of 5 units/μl), 3 μl 10X Klenow buffer (70 mM Tris pH 7.4, 500 mM NaCl, 70 mM MgCl2), and 20 μl of purified PCR DNA were incubated at 37°C for 5 min. After 1 μl of 1.25 mM dNTP (1.25 mM with respect to each nucleotide) was added, and the solution was incubated for 25 min at 37°C. The enzyme was heat inactivated for 10 min at 68°C. The product was precipitated as described above, then resuspended in 10 μl dH2O.

C. Vectors Used for Ligations

The vector Bluescript (Stratagene) was used for all ligations. Bluescript (BS) was cut with the appropriate enzymes for either directional, or blunt-end cloning. The cut vector was ethanol precipitated and resuspended in dH2O. The quantity of vector was estimated by electrophoresis on an agarose gel. Vectors containing both orientations of the polycloning site (BSKS/BSSK) were used for ligations. Incorporation of the PCR insert was evaluated by blue/white colour selection of resulting transformed colonies.
D. Ligation of PCR Fragments into Bluescript Vectors

Ligations were carried out using an appropriate ratio of 4:1 insert:vector (as judged by electrophoresis on an agarose gel). Reactions typically contained 1 μl of linearized vector, 3.0 μl 5X Ligase Buffer (50 mM Tris pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT 5%PEG 8000 (w/v)), 1 μl T4 ligase (5 units/μl), and the DNA insert made up to a volume of 15 μl with dH₂O. Ligations were carried out for 3 to 12 hours at room temperature.

9. TRANSFORMATION OF RECOMBINANT BLUESCRIPT INTO E COLI

Competent bacteria were obtained by growing a 50 ml culture of DH5αF' to an OD₆₀₀ of 0.5-0.6. Centrifugation of the culture at 5000Xg for 10 min resulted in a bacterial pellet which could be resuspended in ice cold 50 mM CaCl₂. Incubation on ice for 30 min followed by centrifugation generated a second bacterial pellet which was resuspended in 4 ml ice cold 50 mM CaCl₂, 20% glycerol, and stored at -70°C. Cells were thawed immediately prior to use. The bacterial strain DH5αF' was used such that the generation of single stranded DNA from M13 infected cells was an option.

Ligations were used to transform bacteria by addition of 1-5 μl of the ligation mixture to 50 μl competent cells, followed by incubation on ice for 30 min. The cells were heat shocked at 42°C for 2 min, 1 ml of LB was added to each reaction, and the reactions were incubated at 37°C with shaking for 20 min. Transformed bacteria were selected by plating 50-100 μl of the bacterial culture on LB agar plates supplemented with 100 μg/ml ampicillin, 25 μg/ml IPTG, and 50 μg/ml XGAL. Plates were incubated at 37°C overnight. Colonies containing inserts were chosen for further DNA isolations.
10. DNA SEQUENCE ANALYSIS

A. Single Stranded DNA Sequencing

DNA sequence was determined by the chain termination method (Sanger et al., 1977). Single stranded DNA was sequenced with T7 DNA Polymerase (Sequenase) using solutions described by the Sequenase protocol (3rd ed.) Single stranded template (7 µl) was mixed with 2 µl 5X Annealing Buffer (200 mM Tris pH 7.5, 100 mM MgCl₂, 250 mM NaCl), and 1 µl of sequencing primer (100 ng/µl) (see Table 3). The reactions are incubated at 68°C for 10 min, and slowly cooled to room temperature over a period of at least twenty minutes. To each sequencing reaction, the following was added at room temperature: 1 µl 100 mM DTT, 0.5 µl [α-35S]dATP (3000 Ci/mmol), 2.5 µl Labeling Mix (1.5 µM dCTP, 1.5 µMdTTP, 1.5 µM dGTP), and 2 µl T7 Polymerase (diluted 1:15 with Enzyme Dilution Buffer (10 mM Tris-HCL pH 7.5, 5 mM DTT, 0.5 mg/ml BSA)). The labeling reactions proceeded for 1.5-2 min before 3.5 µl of the reaction mix was transferred to 2.5 µl of each termination mix (80 µM with respect to each of ddATP, ddCTP, ddGTP, ddTTP, and 50 mM NaCl) pre-warmed to 42°C. Stop buffer dye (98% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) was added after a total of 5 min incubation at 42°C. Samples were boiled for 3 min, before being loaded (2 µl) on a 6% polyacrylamide sequencing gel. Samples were stored at -20°C.
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<td>Vector</td>
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<tr>
<td>K2</td>
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B. Double Stranded DNA Sequencing

Double stranded DNA sequencing was carried out as described above with the addition of a denaturation step. 15 µl DNA was added to 9.6 µl dH₂O, 3 µl 2N NaOH, and 2.4 µl 2.5 mM EDTA. Incubation at 37°C for 25 min was followed by ethanol precipitation by the addition of 0.1X volume 3 M NaOAc pH 5.2 and 2X volume cold 95% ethanol. The DNA was centrifuged in a microfuge, washed with 70% ethanol, and dried. The pellet was resuspended in 7 µl dH₂O, and sequencing was carried on as above.
RESULTS AND DISCUSSION

1. SUMMARY OF CLINICAL STATUS OF PATIENTS

DNA samples were provided from two patients for the purposes of this study. Both patients exhibited severe bleeding problems. Patients were found to have severe hypoprothrombinemia based on a clinical analysis. Prothrombin activity and antigen levels were analyzed for each individual. FII-Vancouver activity and antigen levels were both assessed at 3% of normal. FII-Utrecht had 3% activity and antigen levels. Plasma samples were not made available for this study. In addition, family studies confirmed that each patient was the result of a consanguineous union.

2. PCR AMPLIFICATION OF THE PROTHROMBIN GENE IN EACH PATIENT

All of the exons, including the 5' and 3' splice consensus regions were successfully amplified by using the PCR. As all fragments were amplified and were of the expected size, it can be assumed that there were no gross deletions or insertions within the exons. Exons were usually amplified in pairs, depending on their size and distance apart (see Figure 6). Restriction sites were incorporated into each primer to allow for directional cloning of inserts (refer Table 2).

Exons 1 and 2, as a single fragment, were extremely difficult to amplify. Even at minimum annealing temperatures, several different products were obtained. Isolation of the correct region was finally achieved by extraction from an agarose gel, of the region that contained DNA of the expected size.
This sample was then used as a template for a second PCR reaction. This allowed for the specific amplification of the correct region.

In addition, exon 7 was a difficult piece to amplify. The first round of the PCR resulted in a smear of several products on an agarose gel. To maximize the generation of a specific product, a second PCR reaction was performed using the first PCR product as a template. A third primer was designed such that it would anneal inside the region spanned by the first set of primers (nested primer), within the region spanning exon 7 (refer Figure 6). The third primer (annealing 3' to exon 7), was used in conjunction with the forward primer (annealing 5' to exon 7) used in the first round of the PCR. In this way, specific amplification of the exon VII target region was maximized.

Exons 8 and 9 amplified as a single product quite successfully, but were extremely difficult to clone. Several attempts at directional cloning were unsuccessful. The piece was finally cloned by using a blunt-end strategy. This strategy was successful, but was of low efficiency. Several ligation reactions were necessary in order to isolate a sufficient number of transformants for sequencing.

Exon 12 was another fragment which did not amplify with great efficiency; however, cloning of this piece was very efficient.

3. DNA SEQUENCE ANALYSIS OF THE PROTHROMBIN GENE IN HYPOPROTHROMBINEMIC PATIENTS

The exons of the prothrombin gene, including the 5' and 3' splice junctions, were sequenced in each patient. In addition, the first 100 bp 5' to exon 1, including the putative promoter region was sequenced. Sequence analysis performed for each patient was compared to that of wild-type
prothrombin (Degan et al., 1987). Several unique polymorphisms, as well as potentially significant base changes were identified in both patients. The results are summarized in Table 4.

A. Sequence Changes Observed in FII-Utrecht

An insertion of a single 'A' was observed in the putative promoter region 54 bp 5' to the transcription start site in exon 1. Nucleotide numbering is according to the wildtype FII sequence of Degan et al., (1987). In the splice consensus region 5' to exon 2, there was a deletion of a 'T' at nucleotide position 461. Autoradiograms showing both of these changes are shown in Figure 7. A silent leucine polymorphism, resulting from a single base change from CTA->CTG was identified in exon 2 at position 554. No changes were observed in exons 3, 4, or 5, including their corresponding splice regions. A single base change of a 'G' to an 'A' was observed in the consensus splice region 3' to exon 6 at position 4272. No changes were found in exons 7 through 9. A silent threonine polymorphism was observed in exon 10. This results from a single base change from ACA to ACC at position 8903. Sequence analysis of exons 11 through 14 revealed no diversions from the published wildtype sequence. Compared to the normal prothrombin gene, all mutations found in this patient were homozygous. These results are summarized in Table 4. Autoradiograms of the described sequences are shown in Figures 7, 8, and 10.

B. Sequence Changes Observed in FII-Vancouver

An insertion of a single 'A' was observed 54 bp 5' to exon 1 as in the above patient. This insertion occurs within the putative promoter region of the II gene. Similarly, a deletion of a 'T' was observed in the 5' splice
## TABLE 4. SUMMARY OF SEQUENCE ANALYSIS ON PROTHROMBIN DEFICIENT PATIENTS

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<th>NUCLEOTIDE (bp*)</th>
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<th>COMMENTS</th>
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<td>-54</td>
<td>Inserted A</td>
<td>putative promoter region</td>
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<td>461</td>
<td>Deleted T</td>
<td>splice region 5' to exon 2</td>
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<td></td>
<td>554</td>
<td>A-&gt; G substitution</td>
<td>Leucine polymorphism in exon 2</td>
</tr>
<tr>
<td></td>
<td>4272</td>
<td>G-&gt; A substitution</td>
<td>polymorphism in splice region 3' to exon 6</td>
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<td></td>
<td>8903</td>
<td>A-&gt; C substitution</td>
<td>Threonine polymorphism in exon 10</td>
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<tr>
<td></td>
<td>7485-7487</td>
<td>3 bp (AAG) deletion</td>
<td>Lysine deletion in exon 9</td>
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</table>

| II-Vancouver | -54              | Inserted A    | putative promoter region                |
|             | 461              | Deleted T     | splice region 5' to exon 2              |
|             | 554              | A-> G substitution | Leucine polymorphism in exon 2 |
|             | 4272             | G-> A substitution | polymorphism in splice region 3' to exon 6 |
|             | 7485-7487        | 3 bp (AAG) deletion | Lysine deletion in exon 9 |
|             | 8903             | A-> C substitution | Threonine polymorphism in exon 10 |

consensus region of exon 2. A single base change of a 'G' to an 'A' was observed in the splice consensus sequence 3' to exon 6, again as in the previous patient. A 3 bp deletion of a AAG codon was observed in exon 9 at position 7485 to 7487. This change deletes a single lysine residue. A silent threonine polymorphism was observed in exon 10, resulting from a single base substitution of A -> C at position 8903. No other changes were observed in the exons or corresponding splice regions of the FII gene in this patient. All observed mutations, and polymorphisms were found to be homozygous. These results are summarized in Table 4. Autoradiograms are shown in Figures 7-10.
Figure 7. Sequence Changes Involving an Inserted 'A' in the Putative Promoter Region and a Deleted 'T' in the Splice Region 5' to Exon 2.

Sequence analysis using the chain termination method revealed two changes observed in both FII-Vancouver and FII-Utrecht. A single 'A' residue was observed 54 bp 5' to the transition start site of exon 1 (numbering according to Degen et al., 1987) on the sense strand. This change is found in the sequence reported by Bancroft et al., 1990. In the splice consensus region 5' to exon 2, a single 'T' has been deleted. This deletion occurs at nucleotide position 461 on the sense strand. Base changes are indicated with an arrow.
Figure 8. Autoradiograms of Polymorphisms in Exon 2 and the Splice Region 3' to Exon 6.

Sequence analysis revealed a new silent Leucine polymorphism in exon 2. This sequence change occurs at nucleotide position 554 on the sense strand, and involves a single base change in the codon CTA to CTG. The autoradiogram is of the anti-sense strand. A second polymorphism was found at nucleotide position 4272, involving a single base change of a 'G' to an 'A'. This polymorphism was previously reported by Iwahana et al., 1992. The sequence shown here is of the anti-sense strand and therefore shows the corresponding 'T'. Both of these changes were observed in FII-Vancouver and FII-Utrecht.
Figure 9. *Deletion of the Codon 'AAG' in Exon 9 of FII-Vancouver.*
Sequence analysis revealed the deletion of a 3 bp stretch, involving a single in-frame codon 'AAG'. This sequence change occurs at nucleotide position 7485 to 7487, and involves the deletion of a single lysine residue. Autoradiograms of the anti-sense strand of this mutation, as well as the normal wild-type sequence are indicated with an arrow.
Figure 10. Sequence Analysis of Threonine Polymorphism in Exon 10.
Sequence analysis revealed a single base change of an 'A' to a 'C' at nucleotide position 8903 on the sense strand. This silent Threonine polymorphism was found in both FII-Vancouver and FII-Utrecht. The single base change is indicated with an arrow.
1. NATURALLY OCCURRING MUTATIONS FOR STRUCTURAL AND FUNCTIONAL ANALYSIS OF PROTEINS

The natural occurrence of individuals with FII deficiencies has facilitated studies on the structure and function of the FII molecule. Mutations which result in a hypoprothrombinemic condition allow analysis of structure and folding properties of the molecule. Generally, where little or no protein is being synthesized, the disruption tends to be in the transcription process, and therefore in the promoter or enhancer regions. Alternatively, a mutation within the signal peptide may prevent the protein from being exported from the cell. Improper folding of the protein, based on a change in the primary structure, may result in the protein being recognized and hydrolyzed within the cell. Mutations which result in a dysprothrombinemic condition, are much more useful for studying functional aspects of the protein. Identification of the structural defect that results in decreased or eliminated function can provide insight into the mechanism of action of the protein.

2. SEQUENCE ANALYSIS OF PROTHROMBIN MUTATIONS

Upon sequence analysis of the prothrombin gene in both FII-Vancouver and FII-Utrecht, a number of deviations were observed when compared to the wild-type sequence. A number of silent polymorphisms were evident, as well as potentially significant mutations within the prothrombin gene. The wild-type sequence used for comparison, and relative numbering of bases is that of Degan et al., (1987).
A. Inserted 'A' in Putative Promoter Region

The first change was observed in what would be the putative promoter region in both FII-Vancouver, and FII-Utrecht. At 54 bp before the transcription start site (tss), there is an insertion of an additional base 'A'.

Although this base is absent in the sequence reported by Degan et al., it is present in the 5' sequence reported by Bancroft et al., (1990). With this information, it appears obvious that this base has little effect on the hypoprothrombinemic patients condition; however, it opens up a tremendous area of importance of study: the transcriptional control region. Typically, a condition which results in a lack of protein synthesis tends to be due to a disruption in one of three areas: the transcription process, inefficient secretion of the produced protein, or improper folding. If in fact a more obvious mutation is not present in the exons, the promoter region remains an important region of study and will be discussed in a later section.

B. A Deletion of a 'T' in the Splice Region 5' to Exon 2

A change was observed in the prothrombin gene of both patients compared to that of wild-type FII. At nucleotide position 461, a single base 'T' has been deleted. This change is expected to have negligible effects on the patients condition based on two observations. Initially, this deletion occurs in a sequence involving three 'T' bases, or more specifically, the sequence 5'CCTTTACAG3'. Even if this 'T' was important for splicing, either of the flanking 'T' bases could easily compensate. Secondly, the sequence of the splice regions all observe the GT-AG rule of Breathnach et al., (1978), with the exception of the splice region immediately 3' to exon 12. This region contains a GC rather than a GT. Studies in vitro have shown that genes containing
mutations in the GT sequence of splice regions are still functional, however, intermediates accumulate (Padgett et al., 1986). If this deleted 'T' caused an alteration in splicing, you would still expect to see some normally spliced product, due to the other two 'T' bases, which would produce normal FII, presumably at levels higher than that observed with either of these patients. In addition, there would be the accumulation of incorrectly spliced mRNA within the cell. The second observation could only be confirmed by doing some form of immunocytochemistry, in order to follow the mRNA through the cell. With this information, it would seem that this sequence change is simply a polymorphism, with no significant effect on generation of FII in either of these patients.

C. Leucine Polymorphism in Exon 2

A substitution of a G to an A, occurs at nucleotide position 554. This mutation is a silent polymorphism of Leu56 in exon 2. This is a single base change in the third position of the codon CTA. Because this change does not cause an alteration in the amino acid, it therefore has no effect on generation of protein. This polymorphism was observed in both FII-Vancouver, and FII-Utrecht.

D. Base Substitution in Splice Region 3' to Exon 6.

A single base change of a 'G' to an 'A' was observed in the consensus splice region 3' to exon 6 at position 4272. This was a homozygous mutation which was observed in both FII-Vancouver and FII-Utrecht. This is not a novel polymorphism as it was also reported by Iwahana et al., (1992).
E. Lysine Deletion in Exon 9 of FII-Vancouver

A 3 bp deletion of the sequence AAG was observed in exon 9 of the prothrombin gene in FII-Vancouver. This is an in-frame deletion which results in the deletion of the single amino acid Lys301. Although this mutation does not cause a global disruption in the amino acid sequence of FII, it is expected to have a serious effect on the protein. As mentioned previously, mutations which cause little or no protein synthesis are expected in one of three areas: the signal sequence, a mutation causing an alteration in folding, or in the absence of a more obvious mutation in an exon, a mutation involving transcriptional regulation. It would appear that this deletion would cause a structural change in the protein on the tertiary level. A deletion of an amino acid in one area, can cause global changes in the folding of a protein.

F. Threonine Polymorphism in Exon 10

A silent polymorphism was observed in exon 10 of Thr389. This is the result of a substitution of an 'A' with a 'C', in the codon ACA. Again, this silent polymorphism was observed in both patients.

3. PROPOSED EFFECTS OF OBSERVED MUTATIONS ON PROTHROMBIN

A. FII-Vancouver

It would seem that the most obvious explanation for a hypoprothrombinemic condition in this patient, would be the result of the deleted lysine residue in exon 9. The addition sequence changes described above are not expected to have a significant effect on the protein, and are therefore considered to be polymorphisms of the prothrombin gene. This
mutation at the nucleotide level is expected to cause global changes in structure of the protein, such that it is misfolded within the cell. A misfolded protein is quickly degraded by the cell as a defense against accumulation of aberrant proteins. Although this seems the most obvious explanation for the deficiency in this patient, it could be confirmed by one of two methods. It would be interesting to observe the aberrant protein as it passes through the cell. This could indeed be accomplished by immunocytochemistry. Fluorescent antibodies that are specific for FII can be used to follow the transport of recombinant FII-Vancouver through the cell. In this way, it would be obvious if the protein was being mis-shuttled in the cell, or trapped within any particular region. It would also be interesting to test the fidelity of the promoter region of FII-Vancouver to eliminate the possibility of a defect in transcriptional regulation, however, this will be discussed further with respect to FII-Utrecht.

B. FII-Utrecht

FII-Utrecht presents an interesting case for a prothrombin deficiency. Analysis of the exons and the intron-exon splice regions revealed no significant changes which could be considered causative for this condition. Because this condition generates very little protein, it may be expected that the mutation lies somewhere in transcriptional regulation. There were no changes in the signal sequence, or any that would cause aberrant folding; therefore, it could be that the mutation lies in the upstream promoter or enhancer regions. It is possible that there are global changes within the introns such as the deletions of important regulatory regions that we are unaware of, or the generation of a cryptic splice site within an intron. Although the presence of a cryptic splice site would cause a severe disruption
in the protein, it would not account for all of the mRNA generated. The fidelity of the intron-exon splice regions would allow for generation of at least some properly spliced mRNA, and we would therefore expect to see protein levels that are moderately higher than those observed in this patient. The sequence changes observed in both this patient and in the FII-Vancouver patient, were not expected to have a serious effect on the protein, and were therefore considered to be insignificant.

Very little is known about the transcriptional regulation of liver specific genes. In addition, precious little is known about the regulatory regions in prothrombin. It is speculated that the unidirectional transcription of the prothrombin gene is regulated by a proximal promoter, and regulated by more distal enhancer elements (Chow et al., 1991). It is possible, therefore, that the mutation causing a FII deficiency in FII-Utrecht is the result of a disruption in transcription. There are such reported cases of deficiencies in different proteins. Hemophilia B Leyden was identified by point mutations in the 5' flanking region of FIX at positions -20, -6, or +13, relative to the transcription start site. The mutation at +13 has been shown to affect C/EBP binding activity, and is therefore expected to play a role in expression (Crossley and Brownlee, 1990.)

DNase footprint analysis has shown that a number of liver specific proteins bind to the upstream regulatory sequence URS of human prothrombin (Chow et al. 1991). A consensus sequence for binding the transcription factor HNF-1 has been identified, as well as a GC rich region flanking the site. Mutations in the (URS) of prothrombin could potentially have one of at least three effects. The mutation could disrupt the binding of a positively acting transcription factor. The mutation could introduce a binding site for a factor that would not normally bind, which could
competitively inhibit the binding of FII-specific factors. Alternatively, the mutation could cause a change in the secondary structure of the gene, by altering the three dimensional stereospecific interactions of concurrently bound transcription factors. All of these changes would disrupt the positive effects of the enhancer, and subsequently, the promoter would produce only minimal transcripts. This would be in accordance with the minimal protein levels observed in this patient.

The existence of a GC rich region in the promoter region could potentially be a hot spot for mutations in this gene. Recurrent mutations in haemophilia A as well as the predominance of reported mutations in arginine residues (due to the GC dinucleotides in this codon) of prothrombin are indicative of this phenomenon. Deamination of methylated cytosine to thymine results in CpG -> TpG.

4. FUTURE STUDIES

There is a great deal about the hemostatic process that is poorly understood including the complex regulation of the blood clotting cascade that allows enhancement and attenuation of its response, and the specific mechanisms of activation of the individual proteins. Many of the strategies employed to elucidate the structural and functional relationships of the individual proteins have involved the use of artificial systems. X ray crystallographic data obtained from one protein is extrapolated to model a similar protein or protein region. Many proteins are studied by examining the properties of degraded, or chemically modified forms. Although this method has its uses, it is limited by the extent of the modification, and allows only partially characterization of the degraded forms. The incidence of naturally occurring mutants within a population offers a tremendous
opportunity to study protein function. Determination of the defect facilitates identification of structural features which may be involved in the activation process, assembly, or substrate recognition.

Recombinant DNA technology has greatly facilitated the study of altered forms of proteins. When recovery of a mutant protein is hampered by insufficient plasma levels of the protein, the altered protein can be generated in the laboratory. Recombinant FII has been produced in mammalian tissue culture using baby hamster kidney (BHK) cells (Le Bonniec et al, 1991), and Chinese hamster ovary (CHO) cells (Jorgensen et al., 1986). Insertion of the identified mutation can be accomplished by site-directed mutagenesis using the PCR.

The expression of mutant FII in cases where there is little protein synthesized in vivo is not a useful approach in terms of functional studies. Alternatively, these cases present a unique opportunity to study transcriptional regulation and or properties of folding and transport within the cell. Since little protein is expressed naturally, it would be difficult to produce the same recombinant protein in tissue culture. Degradation would probably occur before the protein could be secreted. Mutations found in the exons, or corresponding splice regions, such as FII-Vancouver, would be expected to cause a disruption in folding of the subsequent protein. It would be interesting to follow such a protein through the cell using immunofluorescent antibodies to see if the protein, if not immediately degraded, is shuttled to a different part, or trapped in a certain region of the cell. Mutations found in promoter or enhancer regions, allow a study of transcriptional regulation of prothrombin. Very little is known about of the liver specific expression of FII, or of any of the liver specific genes. A strategy to study cis-acting regulatory elements can be facilitated by expression of
putative promoter and enhancer regions, ligated to reporter genes such as chloramphenicol acetyltransferase (CAT), or human growth hormone (HGH). Comparison of the relative expression levels of FII-deficient promoters relative to normal FII may elucidate functional regions for transcription. This study could facilitate identification of binding proteins which may be essential for adequate expression of this protein, which in turn may be extrapolated to other liver specific proteins.

There are also some obvious clinical benefits to studying FII mutants. Diagnosis for inherited defects of prothrombin can be facilitated using oligonucleotide primers as probes. Oligonucleotides can be designed carrying an observed mutation, which may be prevalent in the population. By altering hybridization and washing conditions, a normal FII allele can be distinguished from the mutant in a Southern blot analysis. This procedure would be useful for carrier detection and pre-natal diagnosis.
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